CHAPTER 4

Microbial community in anaerobic co-digestion of pig manure with Napier grass in channel digester-upflow anaerobic sludge blanket (CD-UASB) and completely stirred tank reactor (CSTR)

4.1 Introduction

Anaerobic digestion is a biological process which is widely used to treat wastewater containing high concentrated organic compounds (Lee *et al*., 2008). This process does not only help to reduce pollution of water from organic wastes, but also result in a biogas production in the final process. Methane within the biogas can be used as an alternative energy to generate electricity, heat or fuel for vehicles (Ziemiński and Frąc, 2012). The anaerobic digestion is multi-steps process performed by various groups of microorganisms. The first step, complex organic compounds are hydrolyzed to simple compounds by hydrolytic bacteria. The second step, acidogenic and acetogenic bacteria convert these products to acetate, hydrogen (H_2) and carbon dioxide (CO_2). Finally, these compounds are converted to methane and CO_2 by methanogenic bacteria in the last step (Gerardi, 2003).

Recently, the energy demand of pig farms in Thailand has been increased due to development of automatic feeding system in pig farms. One interesting strategy for improving biogas production is co-digestion, which treats at least two substrates within the same digester. The benefits of co-digestion include dilution of potential toxic compounds, improve balance of nutrients, synergistic effects of microorganisms, increase load of biodegradable organic matter and better biogas yield (Callaghan *et al*., 2002; Macias-Corral *et al*., 2008; Cuetos *et al*., 2011). Moreover, it decreases ammonium inhibition which may occur in the anaerobic digestion of manure alone (Campos *et al*., 1999). Thus, the addition of available co-substrates in the vicinity of pig farm is of interest. Pig manure is an ideal feedstock for biogas production. It is a

plentiful source of organic matter and has high buffering capacity which can protect the digestion process due to build-up of volatile fatty acids (VFAs) and consequential drop in the pH of system. Napier grass (*Pennisetum purpureum*) is one of the most popular material used for biogas production due to it easily grown and can be harvest many times during the year (Strezov *et al*., 2008; Xie *et al*., 2011). However, it has low nitrogen and high carbon content. Thus, this material may be suitable for co-digestion with manure (Callaghana *et al*., 2002).

The performance of anaerobic digestion depends on many factors such feedstock characteristics, reactor design and operation conditions which related to microbial community within the reactor (Akarsubasi *et al*., 2005; Pholchan *et al*., 2010). Therefore, successful biogas production is based on stable, adaptation and activity of microbial community which depends on environmental conditions in the reactor (Gerardi, 2003). In order to control and improve the reactor performance, the knowledge of microbial community involved in the process is important. However, the information of microbial community structure in anaerobic co-digestion is still lacking.

Nowadays, molecular techniques such as denaturing gradient gel electrophoresis (DGGE) has been frequently used to investigate and monitor change of microbial community in environment samples (Muyzer and Smalla, 1998) including activated sludge in wastewater treatment (Lee *et al*., 2008). This technique demonstrates overall picture and ultimately lead to understanding of complex microbial community in anaerobic co-digestion. In this study, microbial community (archaea and bacteria) of anaerobic co-digestion of Napier grass with pig manure in CD-UASB and CSTR under different hydraulic retention time (HRT) of 10, 20 and 30 days were investigated during operation by DGGE method.

4.2 Materials and Methods

4.2.1 Sludge sampling and DNA extraction

Sludge samples were collected at different time (see Chapter 3) from laboratory scale CD-UASB and CSTR with working volume of 1000 L and 500 L, respectively. Pig manure and Napier grass in the ratio of 70:30 base on volatile solid (VS) were fed into 3 reactors with different HRT of 10, 20 and 30 days (Rerkkriangkrai *et al*., 2009;

Muenjee, 2010). The sludge samples were stored at -20 °C until use. Total genomic DNA was extracted as described in Chapter 3.

4.2.2 PCR amplification

The variable V3-V5 region of bacterial 16S rDNA was amplified by PCR using the universal bacterial primers 357f-GC and 907rM (Muyzer *et al*., 2004) which attached a GC-clamp at 5' end of the forward primer. The primer details, reaction mixtures and cycle conditions were described in Chapter 3.

For analysis of archaeal population, the variable V3 region of 16S rDNA was amplified using 2 sets of universal archaeal primer. The primers PRE46f and PREA1100r (Øvreås *et al*., 1997) were used in first round and these PCR products were then used as template in a second round of PCR amplification using the primers PARCH340f-GC and PARCH519r (Øvreås *et al*., 1997) which attached a GC-clamp at 5' end of forward primer. The primer details, reaction mixtures and cycle conditions were described in Chapter 3. The size and concentration of PCR products were estimated by electrophoresis in a 1.4% (w/v) agarose gel followed by ethidium bromide (EtBr) staining and visualized under UV light.

4.2.3 DGGE analysis

DGGE analysis of the PCR products was performed using Dcode^{TM} Universal Mutation Detection System (Bio-Rad, USA) according to Muyzer *et al*. (1993). 8% and 6% (w/v) polyacrylamide gel (40% acrylamide/bis solution, 37:1) with a denaturing gradient of 20-45% and 30-55% (100% denaturant contain 7M urea and 40% (v/v) formamide) were used for archaea and bacteria, respectively. Electrophoresis was performed in 1X TAE buffer at 130 Volts for 5 hours at 60 ºC. After electrophoresis, the gels were stained in EtBr for 20 min and visualized under UV light.

Prominent DGGE bands were excised from the DGGE gel with sterile blade and suspended in 30 µl of sterile deionized water at 4 ºC overnight to diffuse DNA. The 2 µl of the eluted DNA was re-amplified by PCR with the same primers without GC-clamp and the same condition as described in Chapter 3. The PCR products were then purified with GF-1 Ambiclean Kit (Vivantis, Malaysia) and ligated into the pGEM-T Easy vector (Promega, USA) according to the manufacturer's instructions. Plasmid was

purified with PureYield™ Plasmid Miniprep System (Promega, USA) according to the manufacturer's instructions and sent for sequencing at 1st BASE, Malaysia.

4.2.4 Statistical analysis of DGGE profile

To analyses microbial community structure in each gel, the banding pattern was converted as a binary matrix base on the presence (1) or absence (0) of each band. Jaccard coefficients of similarity were calculated for the similarities of a binary banding matrix, which were clustered by unweighted pair group method with arithmetic mean (UPGMA) algorithm with 1000 bootstrap replicates to obtain confidence estimates. These calculations were performed using FreeTree (Hampl *et al*., 2001) and the resultant tree displayed using TreeView (Page, 1996).

4.2.5 Establishment of archaeal 16S rDNA library

Archaeal clone library was generated from PCR amplified 16S rDNA using archaeal primer set PRE46f and PREA1100r under condition as described above. The PCR products were then purified with illustra™ GFX™ PCR DNA and Gel Band Purification Kits (GE Healthcare, UK). The PCR product was cloned into pGEM-T Easy vector (Promega, USA) and checked insert using the same method as describe in Chapter 3.

4.2.6 DNA sequencing and phylogenetic analysis

The 16S rDNA inserts were sequenced using the M13 primer by 1st BASE, Malaysia. All sequences were checked for chimeric molecules using Bellerophon program (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) and sequences determined to be chimera were removed from further analysis. Sequences were compared with related available sequences in the GenBank (http://blast.ncbi.nlm. nih.gov/Blast.cgi) and EzTaxon (http://www.ezbiocloud.net/eztaxon) databases. The sequences were then aligned using Clustral X and neighbor-joining tree was constructed from the aligned sequences using the molecular evolutionary genetics analysis program version 4.0 (MEGA 4.0).

4.3 Results and discussion

4.3.1 Microbial community

Microbial communities (bacteria and archaea) were monitored during operation of CD-UASB and CSTRs fed with Napier grass and pig manure in ratio 30:70 based on

VS under different HRT of 10, 20 and 30 days by DGGE technique. Moreover, similarity coefficient (Jaccard's index) was used to compare the bacterial DGGE profiles during operation of system.

1) Bacterial population

1.1) Bacterial population profile

The DGGE profiles of bacteria in CD-UASB reactor under different HRT were shown in Figure 4.1 (a, c and e). The result of HRT 10 days found that bands 3, 6, 7 and 8 were found at the beginning and middle of operation. Until day 45 onward, bacterial profiles was shifted to another pattern, bands 9, 10, 11 and 12 were found, and stable until the end of operation. Bands stably detected throughout the process were bands 1, 4, 5, S1, S2, S3, S4, S5 and S6, although some bands were not very intense, whereas bands S1, S2, S3, S4, S5 and S6 were also found in seed. Considering these bands, S4 were the most dominant band as shown in Figure 4.1a. The resulting profile of bacteria in CD-UASB reactor at HRT 20 days was shown in Figured 4.1c. Bands 14, 15, S7, S8, S9 and S10 in the beginning and middle of operation until day 45, these bands were faint and bacterial profile was shifted to another pattern and stable until the end of operation. New bands 16, 17 and 18 were found in this period. Bands 13, S1, S2, S3, S4 and S5 were stably detected throughout the process; especially S4 was the most dominant band. Bands S1, S2, S3, S4, S5, S7, S8, S9 and S10 were also found in seed. The resulting profile of bacteria in CD-UASB reactor at HRT 30 days was shown in Figured 4.1e. This profile shows bands 19, 20, 21, 22 and 23 in the beginning and middle of operation until day 45, bacterial profile was shifted to another pattern and stable until the end of operation. Bands 24, 25, 26 and 27 were dominant during this period. Bands S4, S5 and S6, that found in seed were observed throughout the process, especially S4 was the most dominant band. Moreover, band S5 was more intense during day 45-66.

The generated dendrogram from DGGE profiles of CD-UASB reactor at all HRT 10, 20 and 30 days exhibited two different clusters as shown in Figure 4.1(b, d and f). The first cluster contained DGGE profile of seed and days 3-36. The second cluster contained DGGE profile of days 45-66. These results indicated that bacterial population

structure in the beginning and middle of operation was rather stable and similar to seed. After day 45, bacterial population structure was shifted to another pattern and stable.

The resulting profiles of bacteria in CSTR at different HRT were shown in Figure 4.2 (a, c and e). The results of HRT 10 days showed that bands 1, 2, 3, 4, 5 and 6 were found in the beginning and middle of operation. Bands 7, 8 and 9 were found in the middle to the end of operation. Since day 24 onward, new bands for example bands 10, 11, 12, 13, 14 and 15 were appeared. Moreover, the bands from seed were also found during operation such as bands S4 and S5, especially band S4 which appeared as dominant band as shown in Figure 4.2a. The bacterial DGGE band pattern in CSTR at HRT 20 days was showed in Figure 4.2c. Bands 16, 17, 18 and 19 appeared in the beginning and middle of operation until day 36, bacterial profile was shifted to another pattern and stable until the end of operation. New bands, for example bands 20-27 were found. In addition, bacteria in seed such as bands S2, S4 and S5 were also found, especially band S4 which appeared as dominant band. The bacterial DGGE band pattern in CSTR at HRT 30 days was showed in Figure 4.2e. Bands 28-33 were found during the initial and middle of operation. Since day 36 onward, bacterial profile was shifted to another pattern and stable until the end of operation for example bands 34-39 were found in this period. In addition, bands S2, S4 and S5 in the seed were also found, especially band S4 which appeared as dominant band.

The generated dendrogram from DGGE profiles of CSTR at all HRT 10, 20 and 30 days exhibited two different clusters as shown in Figure 4.2(b, d and f). The first cluster contained DGGE profile of seed and days 3-22 for HRT 10 days, and days 3-24 for HRT 20 and 30 days. The second cluster contained DGGE profile of days 24-62 for HRT 10 days and day 36-62 for HRT 20 and 30 days. These results indicated that bacterial population structure in the beginning was similar to seed. After days 24 and 36, structure of bacterial population was shifted to another pattern and stable.

The DGGE profiles and dendrograms from the profile during operation of both reactor types showed different in bacterial pattern (Figure 4.1 and 4.2). However, the change of bacterial population structure in both reactor types was clustered in two distinct groups. Bacterial population structure at the initial and middle stage of CD-UASB reactor was more stable and similar to seed than CSTR. This is because the seed

was collected from CD-UASB reactor which could result in the bacterial population in CD-UASB adapted to the system better than CSTR. After that, bacterial population of all reactors shifted to another pattern and stable in the final stage of operation (since day 45 for CD-UASB, day 24 for CSTR at HRT 10 days and day 36 for CSTR at HRT 20 and 30 days). Some bands appeared and some bands became more intense in this stage of operation suggesting that these bands may play an important role in the optimal functioning of the reactor. From the report of Rerkkriangkrai *et al.* (2009) and Muenjee (2010), the methane production reached the steady state on day 31 and 41 for CD-UASB and CSTR, respectively. This indicated that bacterial population was also stable during the steady state of methane production. This observation pointed to a close relationship between bacterial population and methane production. Many previous reported found that the pattern of the bacterial population changed very little during steady operation time and similar to those of bioreactor performance (Lee *et al*., 2008; Li *et al*., 2010). LaPara *et al*. (2002) also found that functionally stable wastewater treatment bioreactors have stable microbial community structure. When consider the steady period of system it was found that bacterial pattern in CSTR reached its stable before CD-UASB reactor. This indicated that mixing affected on the bacterial population. The mixing improved contact between the organic matter and microorganisms and also affected distribution of microorganisms (Anderson *et al*., 2003). These results also showed that bacteria in original seed, especially band S4 was the most dominant at all HRT for both CD-UASB and CSTR, can adapt to new condition and play crucial role in the digestion. In addition, the bacterial profiles of each HRT also contained many small DNA bands, which are difficult to detect indicated that the bacterial population in anaerobic digestion is relatively complex and high diversity.

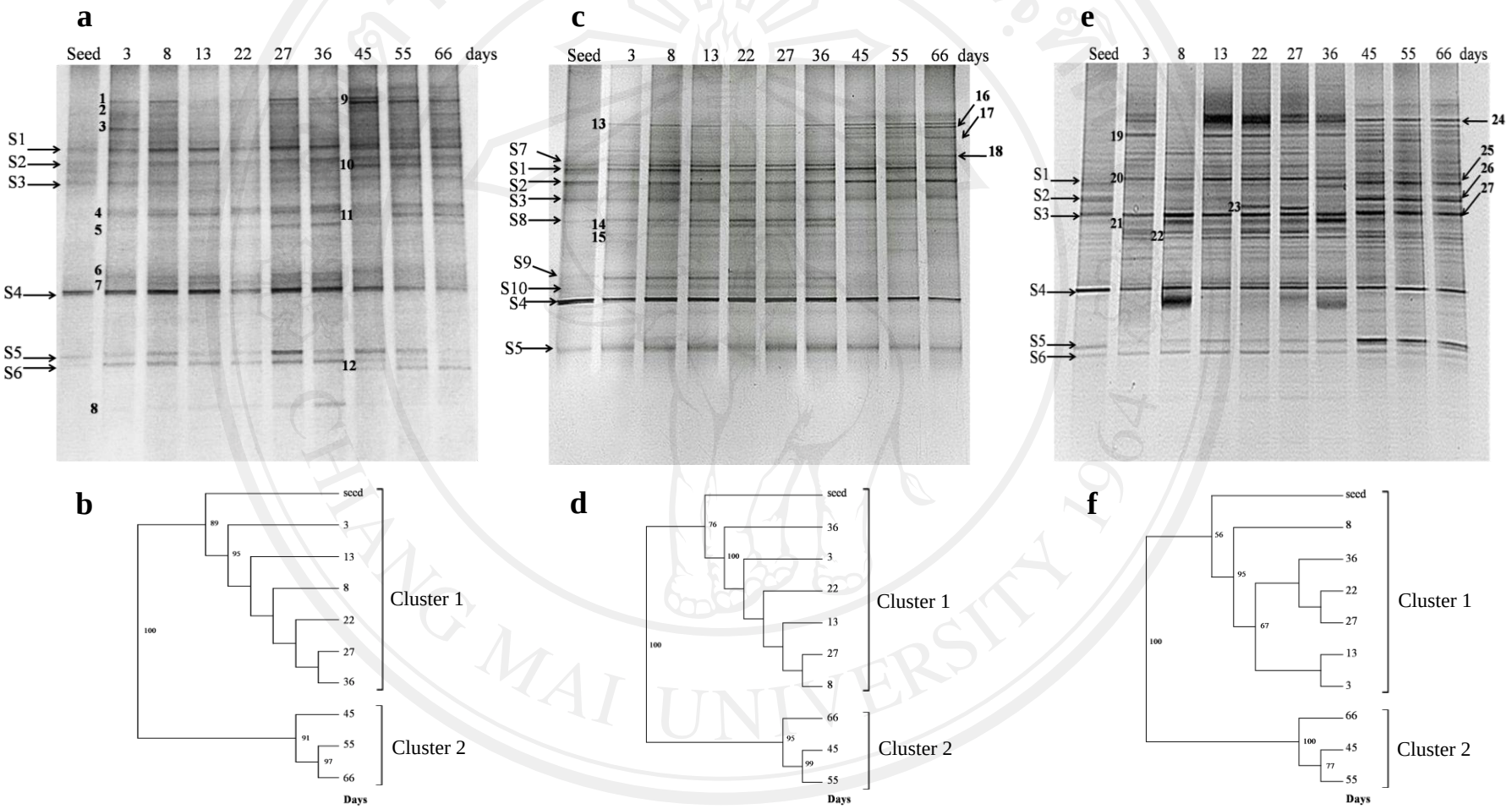

Figure 4.1 DGGE profiles and dendrograms (UPGMA clustering) of bacterial 16S rDNA fragments amplified from sludge in CD-UASB reactor co-digested with Napier grass under different HRT; HRT 10 days (a, b), HRT 20 days (c, d) and HRT 30 days (e, f).

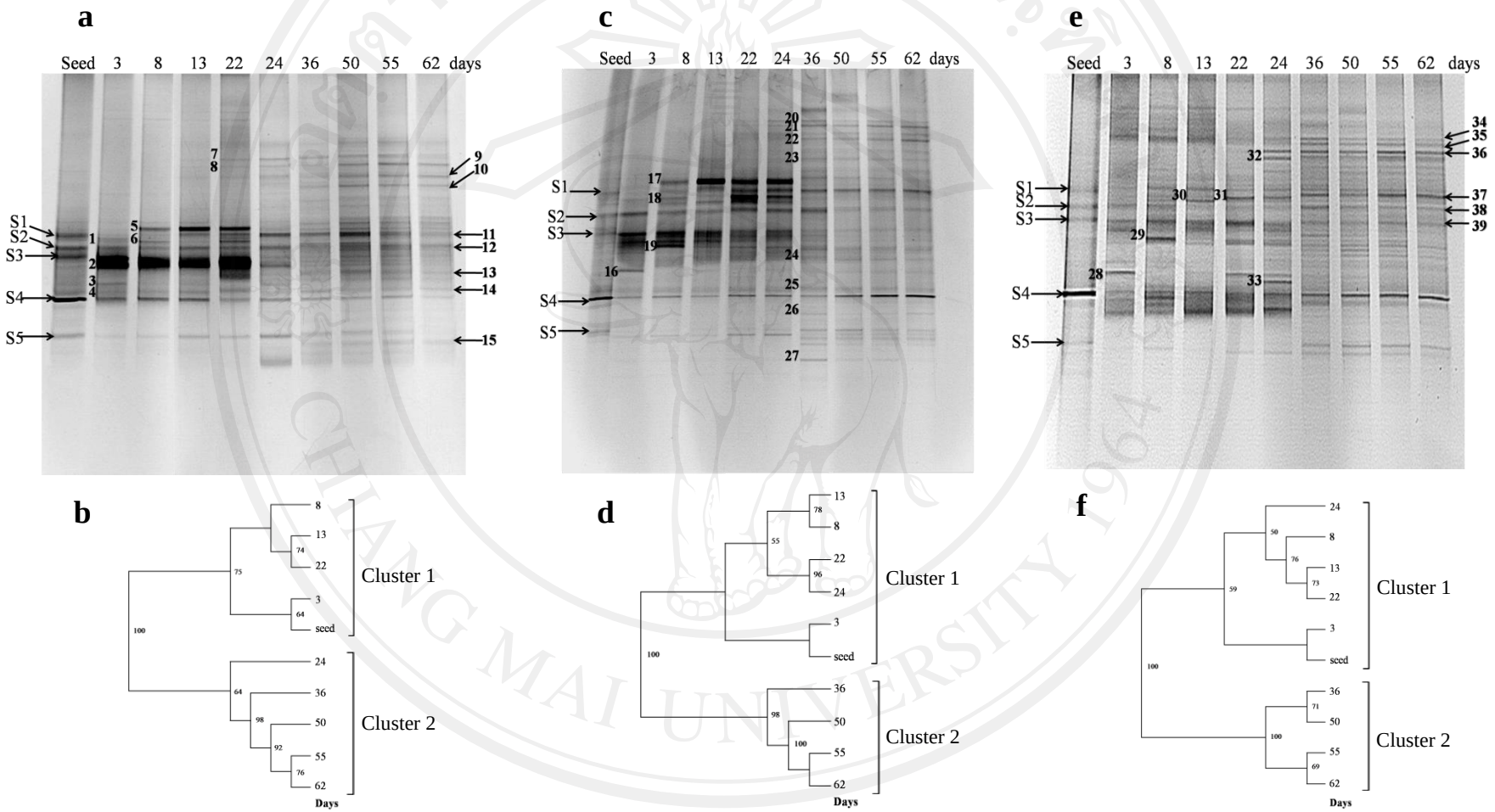

Figure 4.2 DGGE profiles and dendrograms (UPGMA clustering) of bacterial 16S rDNA fragments amplified from sludge in CSTR co-digested with Napier grass under different HRT; HRT 10 days (a, b), HRT 20 days (c, d) and HRT 30 days (e, f).

1.2) Comparison of bacterial population structure between reactors

The cluster analysis base on Jaccard similarity was used to compare the bacterial DGGE profiles at steady state of system. The generated dendrogram exhibited two different clusters as shown in Figure 4.3. The first cluster contained bacterial DGGE profiles of CD-UASB and CSTR at HRT 20 and 30 days. The second cluster contained bacterial DGGE profile of CD-UASB and CSTR at HRT 10 days. These results indicated that structure of bacterial population of both reactor types at HRT 20 and 30 days was similar and different from HRT 10 days. Structure of bacterial population was similar at long HRT. These results demonstrated that HRT affected structure of bacterial population involved in anaerobic digestion of Napier grass and pig manure. Reduction in HRT may have resulted in enrichment of microbial species outcompeting for essential resources in the reactor, whereas species with less competitive are removed from reactor (Wang *et al*., 2009).

Comparison between structure of bacterial population and average methane yields at steady state of system showed that bacterial population in CD-UASB reactor was correlated to methane yield. HRT 20 and 30 days gave similar values and higher than HRT 10 days (0.229, 0.334 and 0.360 m^3/kg VS_{add} for HRT 10, 20 and 30 days) (Rerkkriangkrai *et al*., 2009). This was because grass contains high amount of hard degradable components such as cellulose and lignin, thus bacteria within reactors need longer time for hydrolysis of organic substances while those of CSTR was 0.629, 0.372 and 0.637 m^3/kg VS_{add}. Methane yield at HRT 10 and 30 days was similar and higher than at HRT 20 days. The DGGE profiles of bacterial population structure at HRT 20 days during steady state of system were stable with similar structure to HRT 30 days. This indicated that the low amount of methane yield at HRT 20 days may not be a result of bacterial population but may be an effect from problem of system. This finding was in consistent with the reported of Muenjee (2010) which found biogas leak from a reactor at HRT 20 days in certain day of operation resulted in average methane yields lower than actual value and also lower than HRT 10 and 30 days.

The results of CSTR showed that methane yield between HRT 10 and 30 days were similar although the bacterial population was different. This indicated that mixing affected gas production by increased intimate contact between microorganisms and organic substances (Anderson *et al*., 2003).

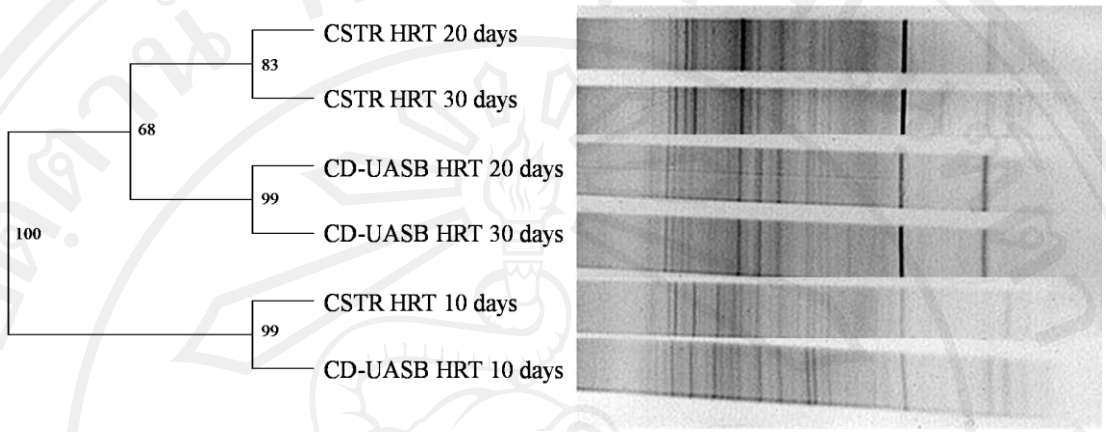

Figure 4.3 Cluster analysis of bacterial banding patterns at steady state from reactors co-digested with Napier grass under different HRT. The UPGMA algorithm was used to cluster pattern based on Jaccard similarity coefficient. Bootstrap values (>50%) based on 1000 replicates are given at each node.

1.3) Identification of dominant bands from DGGE profiles

Identification of dominant bands in DGGE profiles from CD-UASB (Figure 4.4) and CSTR (Figure 4.5) was performed with HRT 30 days due to this HRT gave high methane yields and with consistency in organic compound removal than other HRT (Rerkkriangkrai *et al*., 2009; Muenjee, 2010). The dominant bands from each profile were visually detected and excised from the gel for subsequent sequence analyses in order to determine the composition of dominant bacterial population. However, minor bands with low intensity were difficult to excise, therefore were excluded from our study. The dominant 16S rDNA sequences were compared with available sequences in the GenBank database and compared with cultured species in the EzTaxon database. The phylogenetic affiliations of bacterial sequences were presented in Table 4.1. Their phylogenetic relationships were presented in Figure 4.6 and 4.7.

Looking at the bacterial DGGE profile of CD-UASB reactor (Figure 4.4), three bands namely bands B1, B2 and B3 were dominant in initial and middle periods of

operation. The sequence of band B1 was closely related to uncultured bacterium clone 4C1_cons with 99% similarity and distantly related with cultured species of *Desulfovibrio simplex* with 83% similarity. *Desulfovibrio* is proteolytic bacteria which can produce acetate and succinate during protein degradation (Hassan and Nelson, 2012). Deublein and Steinhauser (2011) reported that the number of this bacteria decrease at the end of fermentation. Band B2 shared 99% similarity with *Clostridium* sp. *Clostridium* species are known to have ability to degrade a wide variety of organic matters i.e. protein, carbohydrate and lipid. They can also hydrolyze cellulose, hemi-cellulose and pectin to produce VFAs, alcohol, CO_2 and H_2 (Anderson *et al*., 2003). They were normally found in anaerobic digestion sludge. Band B3 was closely related to uncultured unclassified bacterium clone QEDQ1BD11 with 95% similarity and related with cultured species *Simplicispira metamorphum* with 94% similarity. *Simplicispira* is a member of family *Comamonadaceae* which have ability to degrad protein (Deublein and Steinhauser, 2011). Band B4 existed throughout the study period and became more intense during steady state. This band was closely related to uncultured Bacteroidetes bacterium clone QEDR1CA07 with 92% similarity and showed 86% similarity with cultured specie *Natroflexus pectinivorans*, which produces acetate and succinate during carbohydrate fermentation. It can also hydrolyze and utilize pectin, xylan and starch as growth substrate (Sorokin *et al*., 2011). When the reactor reach steady state period, three intense bands (B5, B6 and B7) were appeared. Band B5 was similar (99%) to uncultured bacterium clone R2B11L and closely related with cultured species of *Cloacamonas acidaminovorans* with 92% similarity. It is a member of a new bacterial division which found in an anaerobic digester of a municipal wastewater treatment plant and has ability to ferment amino acids and sugars (Pelletire *et al*., 2008). Band B6 was similar to uncultured Bacteroidetes bacterium clone QEDN11AC08 with 99% similarity. Band B7 showed 94% similarity to uncultured unclassified bacterium clone QEDR1BG07. These two bands were closely related to sugar fermenting *Prolixibacter bellariivorans* with 87% and 84% similarity, respectively. Bands S4 and S5 which found in seed, survived throughout the operation, whereas S5 increased its intensity in steady state period. Band S4 was closely related to uncultured bacterium clone PB 94 with 99% similarity and closely related with cultured species of *Thermovenabulum ferriorganovorum* (class *Clostridia*) with 84% similarity.

Band S5 presented 98% similarity to uncultured bacterium clone BUT1_OTUB16 which similar (86%) to cultured species of *Dehalogenimonas lykanthroporepellens*, within phylum *Chloroflexi*. Several studies showed the potential role of *Chloroflexi* in the polysaccharide and glucose degradation within anaerobic sludge digester (Ariesyady *et al*., 2007; Danon *et al*., 2008; Rivière *et al*., 2009). In addition, *Chloroflexi* was reported as core microorganism involved in anaerobic digestion of sludge (Rivière *et al*., 2009).

For the bacterial DGGE profile of CSTR (Figure 4.5), bands B9 and B10 appeared in day 3 and 8, respectively and faded out, then more intense again in day 22 and 24. These two bands belonged to class *Betaproteobacteria*. Band B9 was closely related to *Azonexus* sp. with 99% similarity. This genus has been found in a biogas digester utilizing marine energy crop, which was high in polysaccharide (Pope *et al*., 2013). Band B10 was closely related (99%) to *Janthinobacterium lividum*. This genus has been reported to involve in the fermentation of starch (Akutsu *et al*., 2008). Band B11 was prominent in day 13. This band was closely related to uncultured bacterium clone SAMBR300bact5 with 99% similarity and related to saccharolytic *Spirocheata caldaria* with 87% similarity. *Spirocheta* species were frequently and normally found in sludge (Narihiro and Sekiguchi, 2007). They can ferment carbohydrates with formation of acetate, ethanol, CO_2 and H_2 (Delbès *et al*., 2000; Leschine *et al*., 2006). Band B12 was found in day 22 and 24. It was closely related (99%) to *Acinetobacter* sp. This genus has been found in a full-scale anaerobic bioreactor treating high concentration of organic cassava wastewater (Gao *et al*., 2012). *Acinetobacter* sp. was potential cellulose and lignin utilizer (Ku *et al*., 2000; Gao *et al*., 2012). Bands B13 and B14 were found in day 24 and then disappeared. Band 13 was closely related (99%) to *Pseudomonas* sp. This genus has ability to degrade cellulose, amino acid and sugar to produce acetate (Anderson *et al*., 2003; Insam *et al*., 2010). Band 14 was closely related to uncultured bacterium clone M02-1-Bac-H11_27F_1_H11_081, 250 m with 99% similarity and related to cultured species of *Azonexus hydrophila* with 97% similarity. When the reactor reached steady state, bands B15, B16, B17 and B18 appeared as dominant bands. Band 15 showed 100% similarity to *Pseudomonas* sp. Band 16 was 99% similar to uncultured bacterium clone B1-15 and closely related to cultured *Cytophaga* sp. with 89% similarity. *Cytophaga* can produce acetate, succinate,

propionate, lactate, formate, CO_2 and H_2 from glucose fermentation (Suzuki and Genus, 2010). Cirne *et al*. (2007) reported that *Cytophaga* participated in the degradation of beet and have ability to degrade macromolecule such as cellulose. Band 17 was closely related to uncultured Bacteroidetes bacterium clone 207 with 99% similarity and related to cultured species of *Prolixibacter bellariivorans* with 85% similarity. Band 18 was related to uncultured bacterium clone 13 with 99% similarity and 90% similarity to *Cloacamonas acidaminovorans*. Moreover, bands S4 and S5 which found in seed, existed throughout the operation and increased their intensity during steady state period. Band S4 was closely related to uncultured bacterium clone PB 94 with 99% similarity and closely related with cultured species of *Thermovenabulum ferriorganovorum* (class *Clostridia*) with 83% similarity. Band S5 presented 98% similarity to uncultured bacterium clone BUT1_OTUB16 which similar (86%) to cultured species of *Dehalogenimonas lykanthroporepellens,* within phylum *Chloroflexi*. The details of these two sequences were described above in the resulted of bacterial DGGE profile of CD-UASB reactor.

This result revealed that most 16S rDNA sequences of dominant bacteria obtained from DGGE method belonged to uncultured bacteria in the phyla *Proteobacteria* class *Betaproteobacteria* (B3, B9, B10 and B14), *Deltaproteobacteria* (S1 and S3) and *Gammaproteobacteria* (B12, B13 and B15), *Firmicutes* class *Clostridia* (B2, B8 and S4), *Bacteroidetes* class *Bacteroidia* (B4, B6, B7, B16 and B17, S2), *Chloroflexi* (S5), *Spirochaetes* (B11) and *Cloacamonas* (B5 and B18) indicated the coexistence and ability of these bacterial groups to thrive on a variety of substrates. Bacterial members in phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes* are known as hydrolytic and fermentation degrader. They have ability to degrade various complex organic matters, including protein, carbohydrate and lipid in various anaerobic habitats. Besides, some bacterial member in these phyla were potential cellulose utilize. These results are in agreement with many previous studies which found members of these phyla in abundant and play important role in the degradation of complex organic matters in anaerobic reactors, while other bacterial phyla such as *Chloroflexi* and *Spirochaetes* are sporadically found (Wang *et al*., 2009; Insam *et al*., 2010; Merlino *et al*., 2012; Panichnumsin *et al*., 2012).

These results also showed that bacteria in original seed, especially band S4 were the most dominant at all reactor, can adapt to new condition and play crucial role in the digestion. Band S4 was a member in class *Clostridia* phyla *Firmicutes*. Many previous studies also found that member in this class were dominant bacteria in anaerobic reactor. Krause *et al*. (2008) observed that the *Clostridiales* from the phylum *Firmicutes* were the most prevalent phylogenetic order in a full-scale biogas plant fed with a mixture of maize, green rye, and chicken manure. Wang *et al*. (2009) showed that microorganism from the class of *Firmicutes* and *Bacteroidetes* were the major groups in the co-digestion of grass silage with cow manure. Kröber *et al*. (2009) also found the abundance of the order *Clostridiales* and *Bacteroiales* in full-scale biogas digester fed with maize silage, green rye, and liquid manure.

Moreover, many sequences were related to their closet sequences with less than 97% similarity indicating that they are potentially new bacterial species and have never been reported. Knowledge regarding bacterial species in anaerobic co-digestion is still lacking underlines the need for further research on classical isolation and characterization studies.

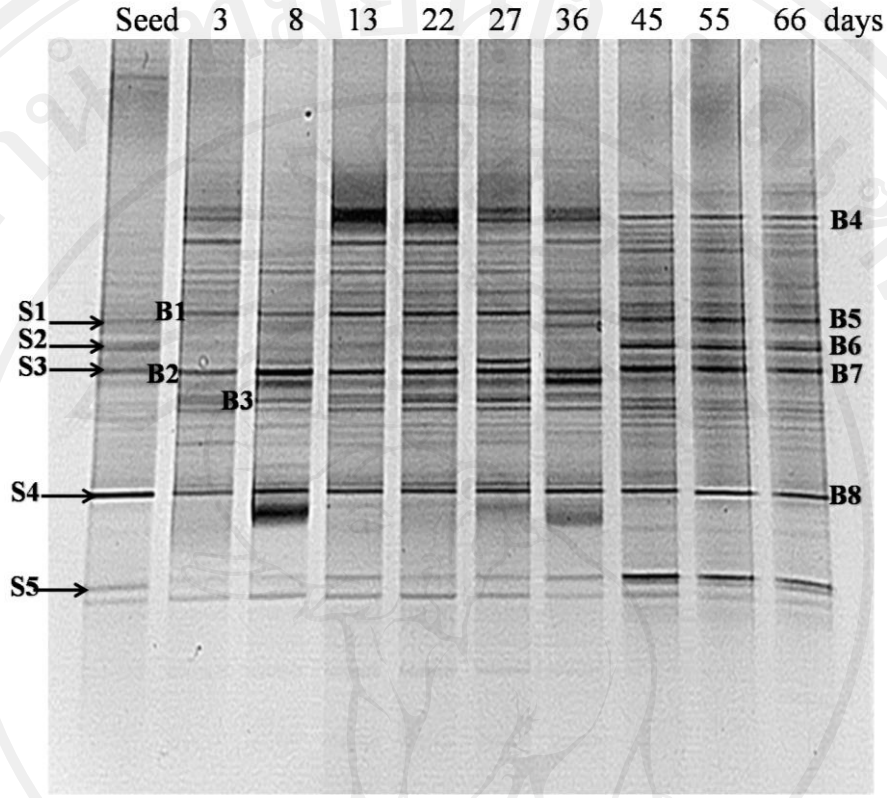

Figure 4.4 DGGE profile of bacterial 16S rDNA fragments amplified from sludge in CD-UASB reactor co-digested with Napier grass at HRT 30 days.

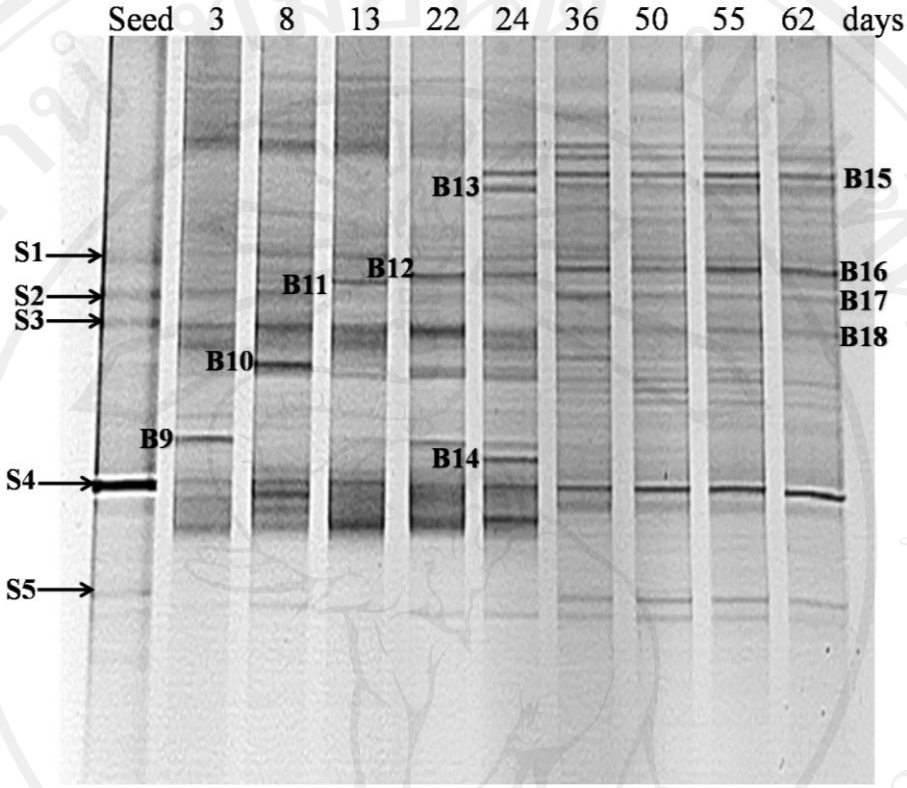

Figure 4.5 DGGE profile of bacterial 16S rDNA fragments amplified from sludge in CSTR co-digested with Napier grass at HRT 30 days.

Table 4.1 Phylogenetic affiliation of the bacterial 16S rDNA sequences from DGGE bands using BLAST search in GenBank and EzTaxon database from sludge in CD-UASB and CSTR co-digested with Napier grass at HRT 30 days.

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
S1	KJ630802	Uncultured bacterium clone QEDV3AD12, Mesophilic anaerobic digester which treats municipal wastewater sludge (CU919758)	94	*Smithella propionica* R4b16, Granular sludge (AF482441)	94	*Proteobacteria*/ *Deltaproteobacteria*
S2	KJ630803	Uncultured bacterium clone QEDR1AH11, Mesophilic anaerobic digester which treats municipal wastewater sludge (CU922702)	95	*Prolixibacter bellariivorans* F2, Sediment (AY918928)	85	*Bacteroidetes*/ *Bacteroidia*
S3	KJ630804	Uncultured bacterium clone KID28_P3_G08, DHS reactor treating sweage after UASB reactor (AB902603)	99	*Smithella propionica* R4b16, Granular sludge (AF482441)	97	*Proteobacteria*/ *Deltaproteobacteria*

Table 4.1 (Continued)

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
S4	KJ630805	Uncultured bacterium clone PB 94, Sediment (HQ330563)	99	*Thermovenabulum ferriorganovorum* Z-9801, Terrestrial hydrothermal source (AY033493)	84	*Firmicutes/ Clostridia*
S5	KJ630789	Uncultured bacterium clone BUT1_OTUB16, Anaerobic UASB bioreactor fed with butyrate (JN995355)	98	*Dehalogenimonas lykanthroporepellens* BL-DC-9, Chlorinated solvent contaminated groundwater (CP002084)	86	*Chloroflexi/ Dehalococcoidetes*
B1	KJ630772	Uncultured bacterium clone 4C1_cons, Anaerobic wastewater treatment (EF688191)	99	*Desulfovibrio simplex* DSM 4141, (FR733678)	83	*Proteobacteria/ Deltaproteobacteria*
B2	KJ630773	*Clostridium* sp. D3RC-2, Ruman (DQ852338)	99	*Clostridium* sp. D3RC-2, Ruman (DQ852338)	99	*Firmicutes/ Clostridia*

Table 4.1 (Continued)

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
B3	KJ630774	Uncultured unclassified bacterium clone QEDQ1BD11, Mesophilic anaerobic digester treats municipal wastewater sludge (CU923077)	95	*Simplicispira metamorphum* DSM 1837, Activated sludge (Y18618)	94	*Proteobacteria*/ *Betaproteobacteria*
B4	KJ630775	Uncultured Bacteroidetes bacterium clone QEDR1CA07, Mesophilic anaerobic digester treats municipal wastewater sludge (CU922534)	92	*Natroflexus pectinivorans* AP1, Sediment (GQ922844)	86	*Bacteroidetes*/ *Bacteroidia*
B5	KJ630776	Uncultured bacterium clone R2B11L, Anaerobic EGSB bioreactors treating a synthetic sewage-based wastewater (GQ423886)	99	*Cloacamonas acidaminovorans* Evry, Anaerobic digester (CU466930)	92	*Cloacamonas*/ *Cloacamonas*

Table 4.1 (Continued)

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
B6	KJ630777	Uncultured Bacteroidetes bacterium clone QEDN11AC08, Mesophilic anaerobic digester treats municipal wastewater sludge (CU926896)	99	*Prolixibacter bellariivorans* F2, Sediment (AY918928)	87	*Bacteroidetes*/ *Bacteroidia*
B7	KJ630778	Uncultured unclassified bacterium clone QEDR1BG07 , Mesophilic anaerobic digester treats municipal wastewater sludge (CU922180)	94	*Prolixibacter bellariivorans* F2, Sediment (AY918928)	84	*Bacteroidetes*/ *Bacteroidia*
B8	KJ630779	Uncultured bacterium clone PB 94, Sediment (HQ330563)	99	*Thermovenabulum ferriorganovorum* Z-9801, Terrestrial hydrothermal source (AY033493)	85	*Firmicutes*/ *Clostridia*

Table 4.1 (Continued)

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
B9	KJ630782	*Azonexus* sp. D7 (KC693725)	99	*Azonexus hydrophila* d8-1, Freshwater (EF158390)	97	*Proteobacteria*/ *Betaproteobacteria*
B10	KJ630780	*Janthinobacterium lividum* HC-3, Soli (KF993615)	99	*Janthinobacterium lividum* DSM 1522 (Y08846)	97	*Proteobacteria*/ *Betaproteobacteria*
B11	KJ630781	Uncultured bacterium clone SAMBR300bact5, Anaerobic digester treating municipal solid waste (HQ111178)	99	*Spirocheata caldaria* DSM 7334 (CP002868)	87	*Spirochaetes*/ *Spirochaetes*
B12	KJ630783	*Acinetobacter* sp. Wuba16, Aquifer (AF336348)	99	*Acinetobacter lwoffii* DSM 2403 (X81665)	98	*Proteobacteria*/ *Gammaproteobacteria*
B13	KJ630785	*Pseudomonas* sp. Iso-13, Effluent water treatment process (KC768739)	99	*Pseudomonas gessardii* CIP 105469, Natural mineral waters (AF074384)	99	*Proteobacteria*/ *Gammaproteobacteria*

Table 4.1 (Continued)

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
B14	KJ630786	Uncultured bacterium clone M02-1-Bac-H11_27F_1_H11_081, 250 m depth borehole (AB704063)	99	*Azonexus hydrophila* d8-1, Freshwater (EF158390)	97	*Proteobacteria*/ *Betaproteobacteria*
B15	KJ630784	*Pseudomonas* sp. a101-18-2, Wastewater treatment plant treats tannery wastewater (HM468081)	100	*Pseudomonas fragi* ATCC 4973 (AF094733)	99	*Proteobacteria*/ *Gammaproteobacteria*
B16	KJ630790	Uncultured bacterium clone B1-15, Sediment (HQ636120)	99	*Cytophaga* sp. MEBiC 08093, Sedimemt (KF620113)	89	*Bacteroidetes*/ *Bacteroidia*
B17	KJ630787	Uncultured Bacteroidetes bacterium clone 207, anaerobic fermentation of pig manure (GQ468584)	99	*Prolixibacter bellariivorans* F2, Sediment (AY918928)	85	*Bacteroidetes*/ *Bacteroidia*

Table 4.1 (Continued)

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
B18	KJ630788	Uncultured bacterium clone 13, Lab scale anaerobic sequencing batch reactor (FR836447)	99	*Cloacamonas acidaminovorans* Evry, Anaerobic digester (CU466930)	90	*Cloacamonas/ Cloacamonas*

* Data taken from GenBank database

** Data taken from EzTaxon database

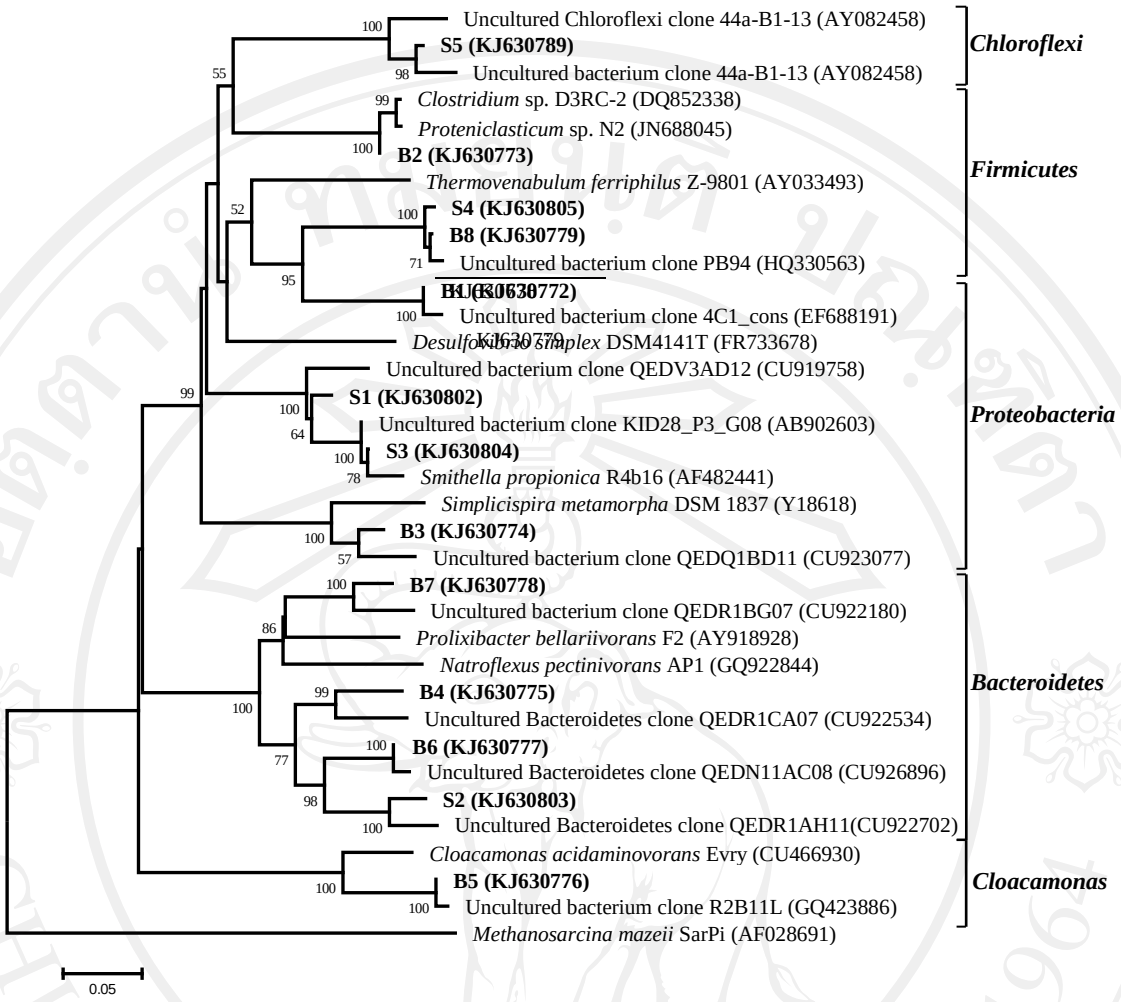

Figure 4.6 Phylogenetic tree of 16S rDNA bacterial sequences from sludge DNA in CD-UASB reactor co-digested with Napier grass at HRT 30 days as determined by the neighbor-joining method. *Methanosarcina mazeii* was used as an out group. The percentage of 1000 bootstrap samplings that supported a cluster is indicated. The scale bar indicates 0.05 nucleotide substitution per site. Number in round brackets indicates contributing GenBank accession number of reference nucleotide sequence.

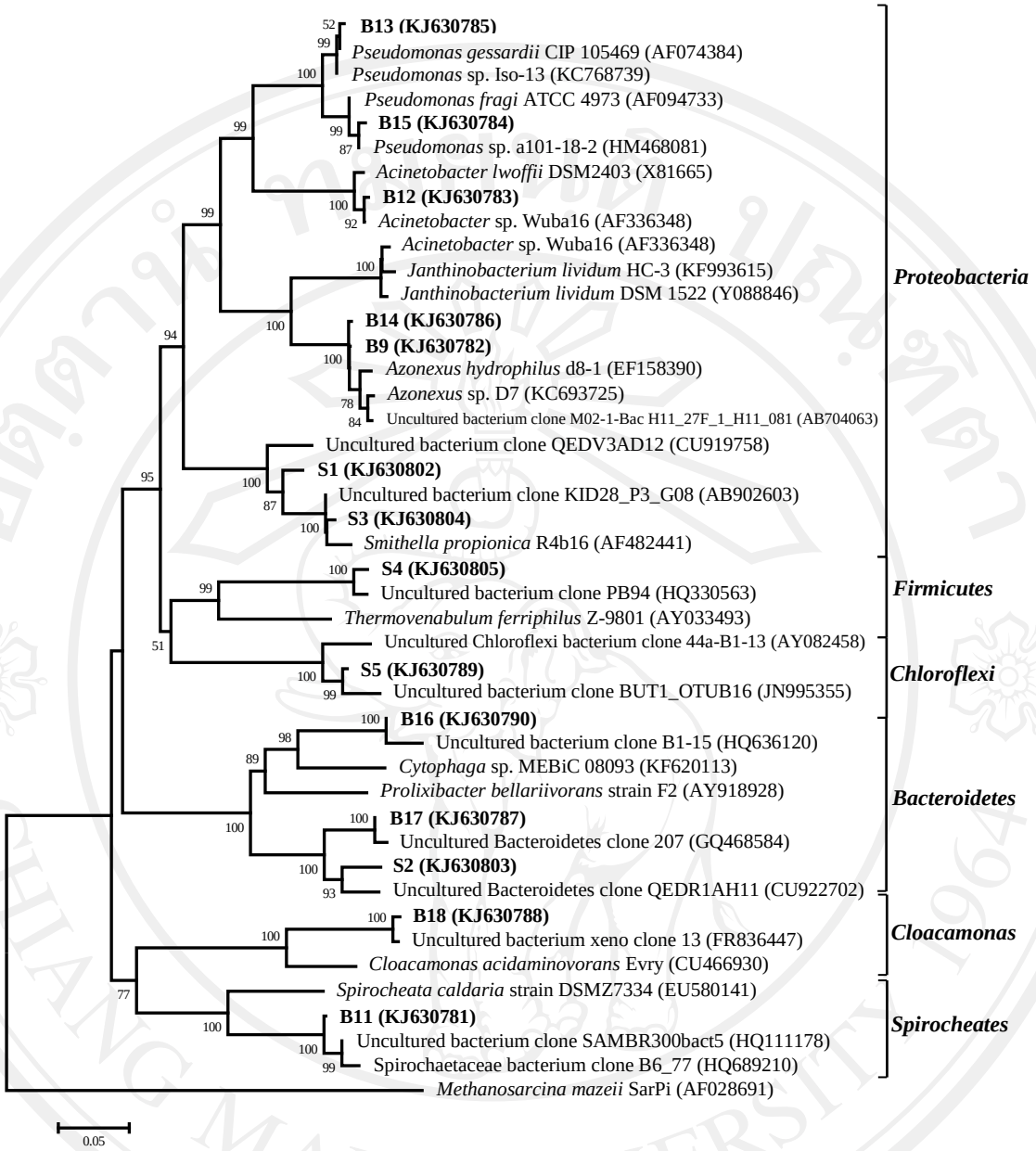

Figure 4.7 Phylogenetic tree of 16S rDNA bacterial sequences from sludge DNA in CSTR co-digested with Napier grass at HRT 30 days as determined by the neighbor-joining method. *Methanosarcina mazeii* was used as an out group. The percentage of 1000 bootstrap samplings that supported a cluster is indicated. The scale bar indicates 0.05 nucleotide substitution per site. Number in round brackets indicates contributing GenBank accession number of reference nucleotide sequence.

2) Archaeal population

2.1) Archaeal population profile

The result of monitoring change in archaeal population in CD-UASB reactors under HRT 10, 20 and 30 days showed that archaeal pattern had similar trend as shown in Figure 4.8. Each DGGE profile showed five DNA bands, bands 1, 2, 3, 4 and 5. Archaeal pattern of HRT 10 and 20 days were relatively stable throughout the operation. However, band 5 tended to increase band intensity from day 45 onward, which is steady state period of operation. Those of HRT 30 day seem to be more shift than HRT 10 and 20 days, especially bands 3 and 5 which faded in certain day of operation. However, band 5 tended to increase band intensity from day 45 onward.

The archaeal population in CSTRs under HRT 10, 20 and 30 days had similar pattern as shown in Figure 4.9. Each DGGE profiles showed five DNA bands, bands 1, 2, 3, 4 and 5. Band 3 of HRT 10 and 20 days changed during beginning and middle of operation. Moreover, band 5 from both HRT increased intensity during the end of operation from day 24-36 onward. The archaeal population of HRT 30 days was rather stable throughout the operation.

The DGGE profile of archaeal in all reactors displayed a similar band pattern indicated that the archaeal population remained stable during operation related with Gómez *et al*. (2011) who found the archaeal community was stable during operation of anaerobic digester treating municipal sewage sludge with increasing the organic loading rate. This observation suggested that HRT and type of reactor had less effect on archaeal population. Each profile displayed five bands throughout the operation compared to bacterial population that presented about 21-30 bands in DGGE profile, implied that archaeal population in the reactors was less diverse than bacteria. Because archaea can only utilize substrates like acetate, H_2 and CO_2 or methyl compound for energy (Gerardi, 2003). The low diversity of archaeal population is in accordance with Wang *et al*. (2009) which found that archaeal community in reactor co-digesting with grass silage and cow manure was lower than the bacterial community.

Each profile displayed five bands throughout the process, therefore, the PCR product of 16S rDNA of archaea from the sludge of each reactor and seed were re-run on DGGE to confirm that they migrated to the same position. As shown in Figure 4.10,

the five dominant DNA bands (A1, A2, A3, A4 and A5) from the sludge in each reactor and seed migrated to the same position on the DGGE gel. This demonstrated that the dominant archaea found in each reactor were the same taxa and might have originated from seed.

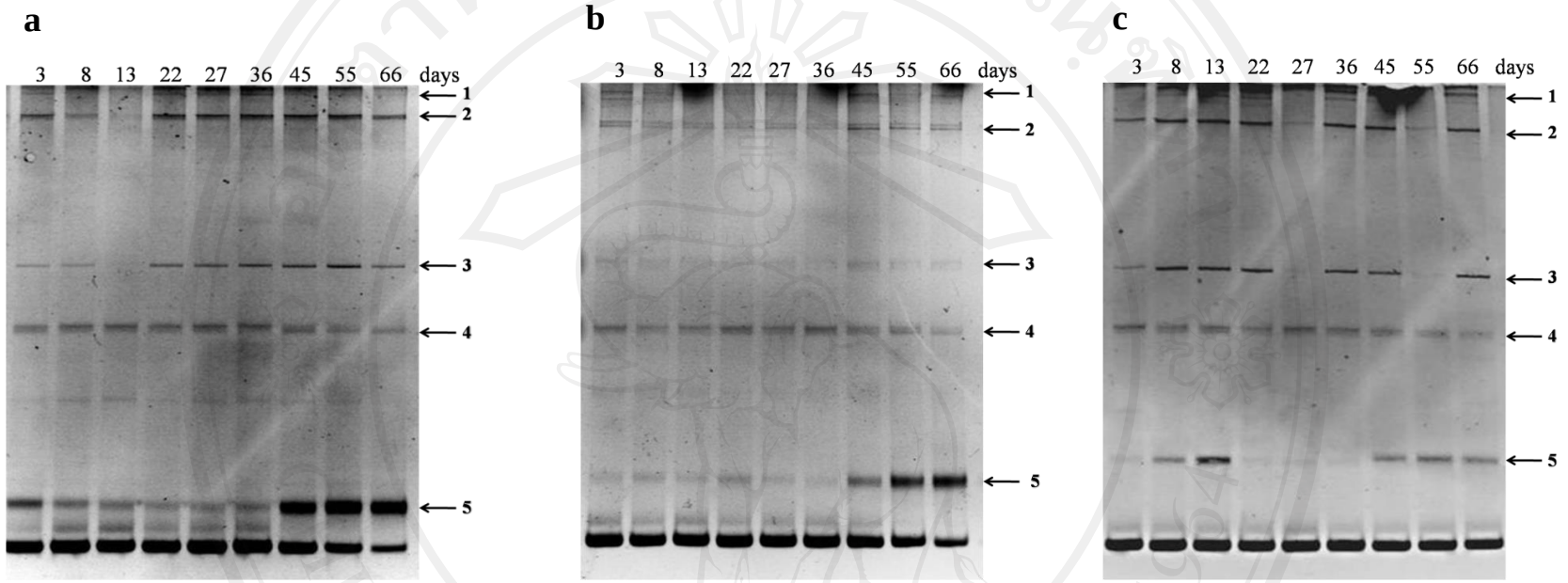

Figure 4.8 DGGE profiles of archaeal 16S rDNA fragments amplified from sludge in CD-UASB reactor co-digested with Napier grass under different HRT; HRT 10 days (a), HRT 20 days (b) and HRT 30 days (c).

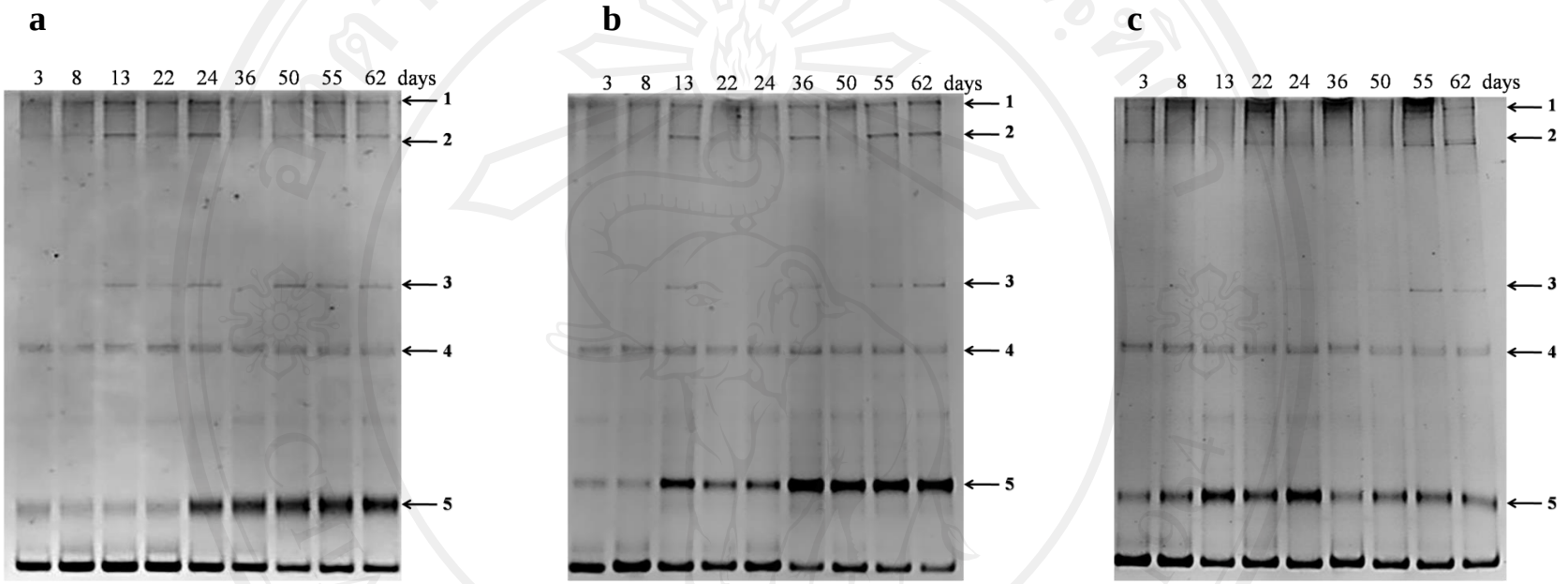

Figure 4.9 DGGE profiles of archaeal 16S rDNA fragments amplified from sludge in CSTR co-digested with Napier grass under different HRT; HRT 10 days (a), HRT 20 days (b) and HRT 30 days (c).

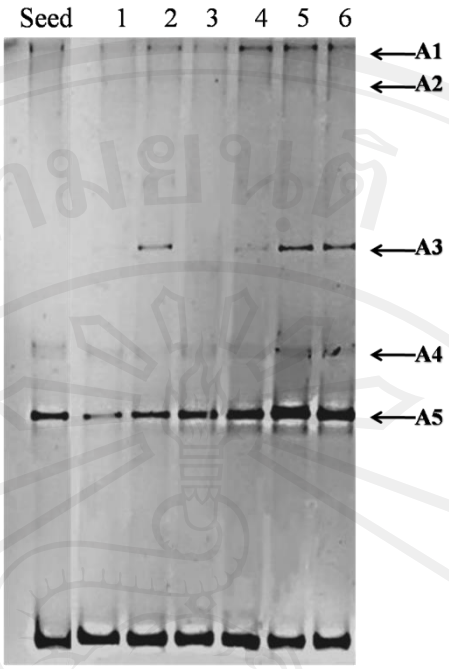

Figure 4.10 DGGE profile of archaeal 16S rDNA fragments amplified from seed and sludge samples in CD-UASB and CSTR co-digested with Napier grass.

Lane 1, 2 and 3: 16S rDNA fragments amplified from sludge in CD-UASB reactor under HRT 10, 20 and 30 days, respectively.

Lane 4, 5 and 6: 16S rDNA fragments amplified from sludge in CSTR under HRT 10, 20 and 30 days, respectively.

2.2) Identification of dominant bands from DGGE profiles

Like the identification of dominant bands from bacterial DGGE profile, the identification of dominant archaeal DGGE bands was performed with HRT 30 days due to this HRT gave high methane yields with better consistency in removal of organic compounds than other HRT. The dominant band from each profile was visually detected and excised from the gel for subsequent sequencing analyses in order to determine the composition of dominant archaeal population. The dominant 16S rDNA sequences were compared with available sequences in the NCBI database and compared with cultured species in the EzTaxon database.

The DGGE analysis of archaea in CD-UASB and CSTR displayed similar band pattern during operation of the reactor as shown in Figure 4.11 and 4.12, respectively. The DGGE profile of 16S rDNA from the sludge of each reactor migrated to the same

position (Figure 4.10) as describe above. The five dominant bands, A1, A2, A3, A4 and A5, were excised from the gel, sequence and identified. The phylogenetic affiliations of archaeal sequences were presented in Table 4.2.

Bands A1, A2, A4 and A5 were related to uncultured clone with 83-96% similarity and related to cultured specie of *Methanocorpusculum sinense* with 78-96% similarity. Band A3 showed 87% similarity to *Methanoseata concilii*. However, the sequences obtained from the DGGE band were relatively short (~200 bp). Therefore, the archaeal clone library was constructed from seed. The proportions of phyla and genera in the archaeal clone library are shown in Figure 4.13.

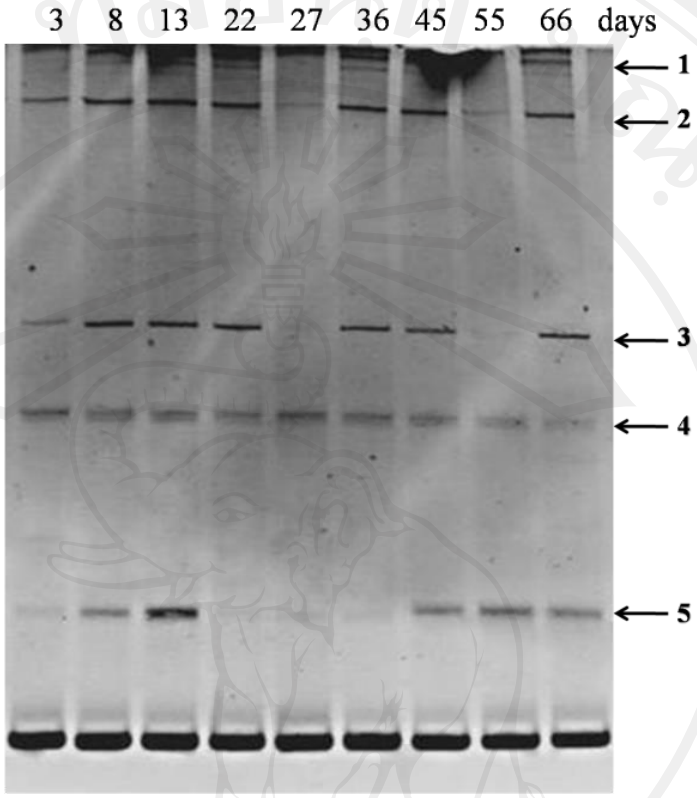

Figure 4.11 DGGE profile of archaeal 16S rDNA fragments amplified from sludge in CD-UASB reactor co-digested with Napier grass under HRT 30 days.

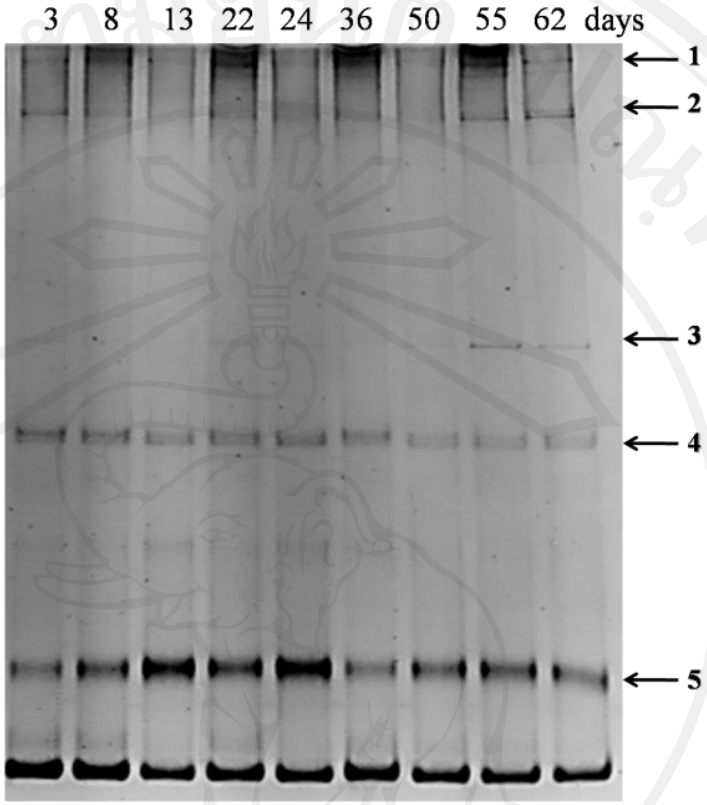

Figure 4.12 DGGE profile of archaeal 16S rDNA fragments amplified from sludge in CSTR co-digested with Napier grass under HRT 30 days.

Table 4.2 Phylogenetic affiliation of the archaeal 16S rDNA sequences from DGGE bands using BLAST search in GenBank and EzTaxon database from sludge in CD-UASB and CSTR co-digested with Napier grass at HRT 30 days

DGGE band	Accession number	Closest sequence match* with BLASTN (accession number)	Similarity (%)	Closest type strain** (accession number)	Similarity (%)	Taxonomic assignment
A1	-	Uncultured archaeon clone Arch8, Seed sludge inoculum for methane production (JQ822093)	83	*Methanocorpusculum sinense* DSM 4274 (FR749947)	78	*Methanomicrobiales*
A2	-	Uncultured archaeon clone SC3, high-rate bioreactor (DQ160191)	92	*Methanocorpusculum sinense* DSM 4274 (FR749947)	92	*Methanomicrobiales*
A3	-	-	-	*Methanoseata concilii* GP-6 (CP002565)	87	*Methanosarcinales*
A4	-	Uncultured archaeon clone SC3, high-rate bioreactor (DQ160191)	96	*Methanocorpusculum sinense* DSM 4274 (FR749947)	95	*Methanomicrobiales*
A5	-	Uncultured archaeon clone SC3, high-rate bioreactor (DQ160191)	96	*Methanocorpusculum sinense* DSM 4274 (FR749947)	96	*Methanomicrobiales*

*Data taken from GenBank database ** Data taken from EzTaxon database

The clone library of archaeal population showed three orders (Fig.4.13), namely *Methanomicrobiales*, *Methanosarcinales*, and *Methanobacteriales*, accounting for 88, 8 and 4%, respectively. The majority of archaea in the reactors were member of the order *Methanomicrobiales*, especially the genus *Methanocorpusculum* (71%). *Methanocorpusculum* sp. is a hydrogenotrophic methanogen that uses H_2/CO_2 and formate as a substrate for methanogenesis (Garcia *et al*., 2006). Additional genera (*Methanolinea* and *Methanoregular*) in the order *Methanomicrobiales* were also found in lower proportion. Other hydrogenotrophic *Methanobrevibacter* in the order *Methanobacteriales* was found in very small fraction, indicated that they may represent a nondominant population. The genus *Methanoseata* belonging to the order *Methanosarcinales* was detected in small number (8%). *Methanoseata* sp. is an acetotrophic methanogen that uses acetate as the sole substrate and occur only at low acetate concentration (Kendall and Boone, 2006). These findings are in agreement with the common hypothesis that biogas is normally produced by both hydrogenotrophic and acetoclastic methanogens (Gerardi, 2003). Members of the genus *Methanocorpusculum* and *Methanoseata* have frequently been detected in agricultural biogas plants (Nettmann *et al*., 2008; Wirth *et al*., 2012).

These results were similar to those obtained from the DGGE profile, which found the same two genera of *Methanocorpusculum* and *Methanoseata*. The DGGE profile in this study clearly reflected the dominant composition of archaea in the reactors. Our results demonstrated that the hydrogenotrophic methanogens were dominated with relatively low representation of acetotrophic methanogens in the biogas community, which is consistent with previous studies. Souidi *et al*. (2007) observed a similar result, with 71% of all archaeal OTUs in a lab-scale CSTR supplied with maize silage and cattle liquid manure were affiliated with hydrogenotrophic methanogen. Nettmann *et al*. (2008) found that the clone libraries of archaea in a reactor supplied with cattle liquid manure and maize silage were assigned to three orders: *Methanomicrobiales*, *Methanosacinales* and *Methanobacteriales*. Over 70% of all archaea OTUs belonged to the order *Methanomicrobiales*. Mostly are hydrogenotrophic methanogens. Liu *et al*. (2009) found that hydrogenotrophic methanogens were the dominant methanogens in a biogas digester treating pig manure. In addition, Ziganshin *et al*. (2013) found that the archaeal community was dominated by methanogenic

archaea of the orders *Methanomicrobiales* and *Methanosarcinales* in a laboratory-scale biogas reactor fed with different agricultural waste materials. Nettmann *et al*. (2008) also suggested that hydrogenotrophic methanogens are the dominant group of archaea in their reactors, indicating that hydrogenotrophic methanogenesis may be the major pathway of methane formation. Most methane formation was resulted from the conversion of H_2 and CO_2.

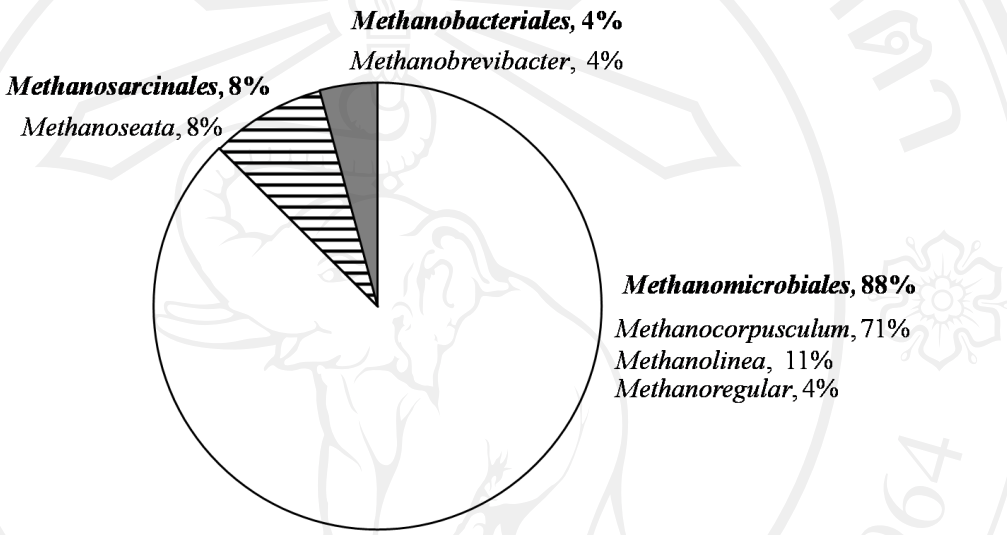

Figure 4.13 Relative abundance of methanogenic archaea in the clone library.