# CHAPTER 3

# Materials and methods

## 3.1 Sludge sampling

Sludge samples were collected at different time from laboratory scale reactors which were channel digester-upflow anaerobic sludge blanket (CD-UASB) and completely stirred tank reactor (CSTR) with working volume 1000 L and 500 L, respectively. Each reactor types were fed with pig manure with Napier grass and pig manure with food waste in ratio 70:30 and 60:40 base on volatile solid (VS), respectively and operated under different hydraulic retention time (HRT) of 10, 20 and 30 days (Rerkkriangkrai *et al*., 2009 ; Muenjee, 2010). The sludge samples were stored at -20 °C until use. The sampling time is shown in Table 3.1. The sampling point of CD-UASB and CSTR were shown in Figure 3.1 and 3.2, respectively.

**Table 3.1** Sampling time

| Substrate | HRT (days) | Sampling time (days) |
|---|---|---|
| **Pig manure + Napier grass** | | |
| CD-UASB reactor | 10, 20, 30 | 3, 8, 13, 22, 27, 36, 45, 55, 66 |
| ASBR | 10, 20, 30 | 3, 8, 13, 22, 24, 36, 50, 52, 62 |
| **Pig manure + food waste** | | |
| CD-UASB reactor | 10, 20, 30 | 3, 6, 10, 15, 22, 36, 45, 55, 62 |
| ASBR | 10, 20, 30 | 3, 8, 12, 17, 22, 36, 43, 52, 64 |

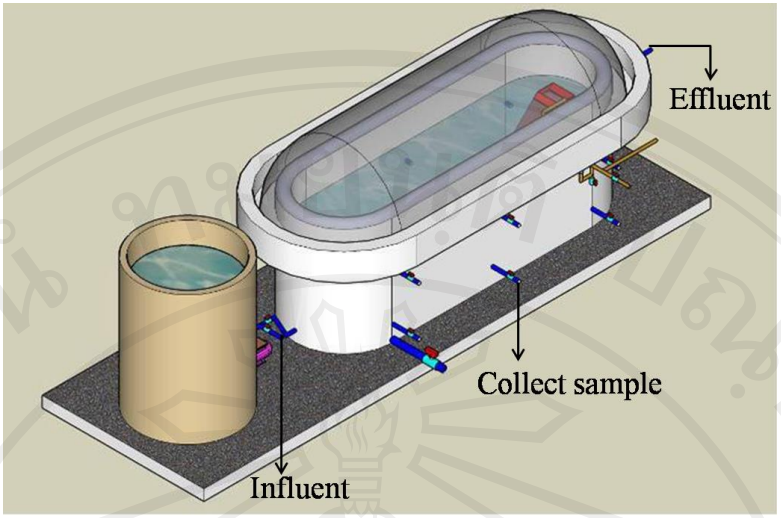


**Figure 3.1** Sampling point of the lab scale CD-UASB reactor (Rerkkriangkrai *et al*., 2009).

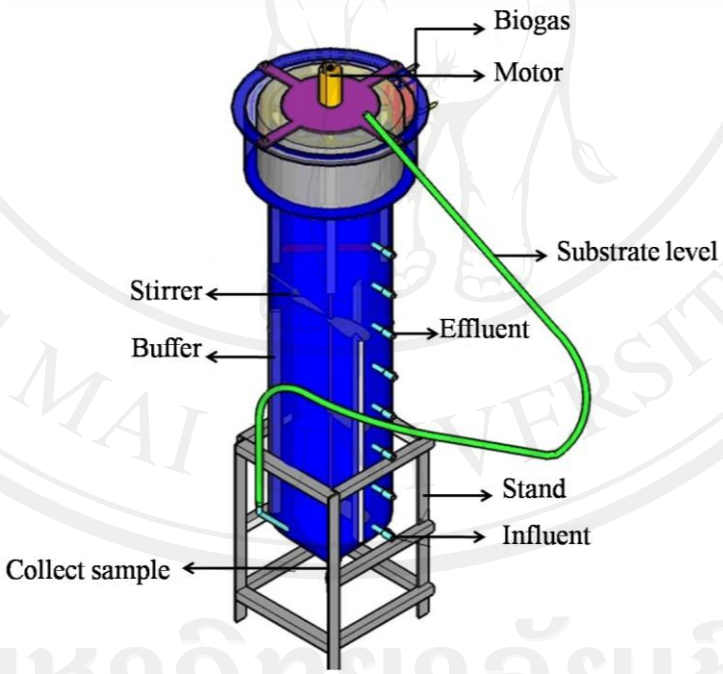


**Figure 3.2** Sampling point of the lab scale CSTR (Muenjee, 2010).

### 3.2 Microbial community analysis

#### 3.2.1 Genomic DNA extraction

A 500 µl of sludge sample (0.2 g) was added to a 1.5 ml microcentrifuge tube and centrifuged at 14,000×*g* for 10 min. Sludge pellet was lysed by adding an equal volume of sterile glass beads (≤ 106 µm diameter ; Sigma-Aldrich, USA) and 500 µl of hexadecyltrimethyl ammonium bromide (CTAB) (Fluka, USA) extraction buffer (Griffiths *et al*., 2000). The suspension was mixed by vortexing and 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture. The resultant suspension was vortexed for 1 min and frozen on ice for 1 min. This step was carried out 3 times. The mixture was centrifuged at 14,000×*g* for 10 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube. Phenol extraction was repeated with 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous phase was transferred to a new microcentrifuge tube and phenol removed by mixing with 500 µl of chloroform: isoamyl alcohol (24:1), followed by centrifugation at 14,000×*g* for 10 min at 4 °C. The DNA was precipitated with 0.1 volume of sodium acetate (NaOAc) pH 5.5 and 0.6 volume of isopropanol at -20 °C overnight. DNA pellet was washed with 70% (v/v) iced cold ethanol, air-dried and resuspended in 40 µl of TE buffer containing 0.002% RNase (w/v). The size and concentration of extracted DNA were estimated by agarose gel electrophoresis in a 1.0% (w/v) agarose gel.

#### 3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze genomic DNA and PCR products. 5 µl of products was mixed well with 1 µl of 6X gel loading dye before loaded into well of the agarose gel (in 1X TAE buffer). Agarose gel electrophoresis was carried out in 1X TAE buffer at 100 volt for 1 hour. The gels were stained with ethidium bromide (EtBr) and were visualized under a UV light. A 1 kilobase pair (kb) and 100 base pair (bp) plus DNA ladder from Fermentas, Canada were used to estimate the genomic DNA and PCR products, respectively.

### 3.2.3 Polymearse chain reaction (PCR) amplification of 16S rDNA

#### 1) Bacteria

The variable V3-V5 region of bacterial 16S rDNA was amplified by PCR using a set of universal bacterial primer 357f-GC and 907rM. The GC clamp was attached on the 5' end of the primer 357f to enable fragment separation by DGGE. The size of PCR product is 586 bp. Sequence of the primers used are listed in Table 3.2

The preparation of PCR reaction mixture is shown in Table 3.3. PCR condition was as follow: initial denaturation at 94 ºC for 4 min, 30 cycles of denaturation at 94 ºC for 30 sec, annealing at 51 ºC for 30 sec and elongation at 72 ºC for 30 sec. Final extension was at 72 ºC for 10 min before cooling down to 4 ºC. All PCR amplifications were performed in MyCycler$^{TM}$ thermal cycler (Bio-Rad, USA). The size and concentration of PCR products were estimated by agarose gel electrophoresis in a 1.4% (w/v) agarose gel.

#### 2) Archaea

The variable V3 region of archaeal 16S rDNA was amplified by PCR using two sets of universal archaeal primer. The primers PRE46f and PREA1100r were used to produce 1,072 bp fragment and these PCR products were then used as template for the amplification of 179 bp fragments using the set of primer PARCH340f-GC and PARCH519r which attached a GC-clamp at 5' end of forward primer to enable fragment separation by DGGE. Sequence of the primers used are listed in Table 3.2

The preparation of PCR reaction mixture with both 2 sets of primer is shown in Table 3.4. PCR condition was as follow: initial denaturation step at 92 °C for 2 min, followed by 30 cycles of denaturation at 92 °C for 1 min, annealing at 55 °C for 30 sec, elongation at 72 °C for 1 min. Final extension was performed at 72 °C for 6 min before cool down to 4 °C. All PCR amplifications were performed in a MyCycler$^{TM}$ thermal cycler (Bio-Rad, USA). The size and concentration of PCR products of first and second round were estimated by agarose gel electrophoresis in 1.0% and 1.4% (w/v) agarose gel, respectively.

**Table 3.2** Sequence of the primers used

| Target microorganism and Primer[a] | 16S ribosomal DNA target (position)[b] | Sequence (5'-3') | References |
|---|---|---|---|
| **Bacteria** | | | |
| 357f | 341–357 | CCTACGGGAGGCAGCAG | Muyzer *et al*. (2004) |
| 357f-GC | 341–357 | CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCAC GGGGGGCCTACGGGAGG CAGCAG | Muyzer *et al*. (2004) |
| 907rM | 907–926 | CCGTCAATTCMTTTGAG TTT | Muyzer *et al*. (2004) |
| **Archaea** | | | |
| PRE46f | 46–60 | (C/T)TAAGCCATGC(G/A) AGT | Øvreås *et al*. (1997) |
| PREA1100r | 1100–1117 | (T/C)GGGTCTCGCTCGTT (G/A)CC | Øvreås *et al*. (1997) |
| PARCH340f | 340–357 | CCCTACGGGG(C/T)GCA (G/C)CAG | Øvreås *et al*. (1997) |
| PARCH340f-GC | 340–357 | CGCCCGGGGCGCGCCCC GGGCGGGGCGGGGGCAC GGGGGCCCTACGGGG (C/T)GCA(G/C)CAG | Øvreås *et al*. (1997) |
| PARCH519r | 519–533 | TTACCGCGGC(G/T)GCTG | Øvreås *et al*. (1997) |

[a] *f* forward primer; *r* reverse primer

[b] Numbering referred to the position in *E. coli* 16S rDNA

**Table 3.3** Reaction mixtures for amplification of bacterial 16S rDNA

| Material for PCR reaction mixture | Volume (µl) | Final concentration |
|---|---|---|
| 10X PCR buffer | 2.5 | 1X |
| 10 mM dNTPs | 1 | 0.4 mM |
| 50 mM $MgCl_2$ | 0.7 | 1.4 mM |
| 99% (v/v) DMSO | 1 | 3.96% (v/v) |
| 10 µM 357f-GC (forward primer) | 1 | 0.4 µM |
| 10 µM 907rM (reverse primer) | 1 | 0.4 µM |
| 5U *Taq* DNA polymerase | 0.3 | 1.5 U |
| Sterile deionized water | 16.5 | - |
| DNA template | 1 | - |
| Total volume | 25 | - |

**Table 3.4** Reaction mixtures for amplification of archaeal 16S rDNA

| Material for PCR reaction mixture | Volume (µl) | Final concentration |
|---|---|---|
| 10X PCR buffer | 2.5 | 1X |
| 10 mM dNTPs | 1 | 0.4 mM |
| 25 mM $MgCl_2$ | 3 | 3 mM |
| 99% (v/v) DMSO | 1 | 3.96% (v/v) |
| 10 µM forward primer | 1 | 0.4 µM |
| 10 µM reverse primer | 1 | 0.4 µM |
| 5U *Taq* DNA polymerase | 0.3 | 1.5 U |
| Sterile deionized water | 14.2 | - |
| DNA template | 1 | - |
| Total volume | 25 | - |

### 3.2.4 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). 6% and 8% (w/v) polyacrylamide gel (40% acrylamide/bis solution, 37:1) with a denaturing gradient of 30-55% and 20-45% (100% denaturant contain 7 M urea and 40% (v/v) formamide) were used for bacteria and archaea, respectively. The DGGE gel solution mixture is shown in Table 3.5. 45 µl of PCR product of each sample was mixed well with 10 µl of 6X loading dye and loaded into the well of polyacrylamide gel. Electrophoresis was performed in 1X TAE buffer at 120 volts for 5 hours at 60 ºC. After electrophoresis, the gels were stained in EtBr for 20 min and visualized under UV light.

**Table 3.5** Preparation of DGGE gel solution for a polyacrylamide gel with a denaturant gradient between 20-45% and 30-55%.

| **Component of DGGE gel solution** | **20%** | **45%** | **30%** | **55%** |
|---|---|---|---|---|
| 40% Acrylamide/Bis (µl) | 2,600 | 2,600 | 1,950 | 1,950 |
| 50X TAE buffer (µl) | 260 | 260 | 260 | 260 |
| 40% Formamide (µl) | 1,040 | 2,340 | 1,560 | 2,860 |
| 7 M Urea (g) | 1.09 | 2.46 | 1.64 | 3.00 |
| Sterile deionized water | 8,010 | 5,340 | 7,590 | 4,930 |
| 10% Ammonium persulphate (µl) | 50 | 50 | 50 | 50 |
| TEMED (µl) | 4 | 4 | 4 | 4 |
| DCode dye solution | - | 25 | - | 25 |

### 3.2.5 Statistical analysis of DGGE profile

Jaccard similarity coefficients were calculated for the DGGE patterns, which were clustered by unweighted pair group method with arithmetic mean (UPGMA) algorithm and 1000 bootstrap replicates to obtain confidence estimates. These calculations were performed using FreeTree (Hampl *et al*., 2001) and the result displayed using TreeView (Page, 1996).

### 3.2.6 Clone library construction of single DGGE band

Prominent DGGE bands were excised from the DGGE gel with sterile blade and placed in a 1.5 ml microcentrifuge tube. The gel were crushed and incubated 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 but without GC-clamp under the same condition described above. The PCR products were then purified with GF-1 Ambiclean Kit (Vivantis, Malaysia) and cloned into the pGEM-T Easy vector (Promega, USA) according to the manufacturer's instructions. The ligated products were transformed into *Escherichia coli* JM109 competent cell with ampicillin and blue/white screening. White colonies were picked and incubated at 37 °C overnight in LB broth with ampicillin and then insert was checked by colony PCR amplification using M13 primer set (M13F 5'-GTA AAA CGA CGG CCA G-3' and M13R 5'-CAG GAA ACA GCT ATG AC-3').

The preparation of PCR reaction mixture is shown in Table 3.6. PCR condition was as follow: initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, elongation at 72 °C for 30 sec. Final extension was performed at 72°C for 7 min before cool down to 4°C. PCR amplifications were performed in MyCycler™ thermal cycler (Bio-Rad, USA). Then, plasmid was purified with PureYield™ Plasmid Miniprep System (Promega, USA) and sent for sequencing.

### 3.2.7 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 describe 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 vector and checked insert using the same method as describe above.

**Table 3.6** Reaction mixtures for colony PCR amplification.

| Material for PCR reaction mixture | Volume (µl) | Final concentration |
|---|---|---|
| 10X PCR buffer | 1.0 | 1X |
| 10 mM dNTPs | 0.4 | 0.4 mM |
| 50 mM $MgCl_2$ | 0.3 | 1.5 mM |
| 10 µM M13F (forward primer) | 0.4 | 0.4 µM |
| 10 µM M13R (reverse primer) | 0.4 | 0.4 µM |
| 5U *Taq* DNA polymerase | 0.1 | 0.5 U |
| Sterile deionized water | 5.4 | - |
| DNA template (broth) | 2 | - |
| Total volume | 10 | - |

### 3.2.8 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).