CHAPTER 3
METHODOLOGY

3.1 Reactor configuration

The schematic diagram of the anaerobic hybrid sludge bed-fixed film reactor (AHR) used in this study is shown in Figure 3.1. AHR was made up of acrylic column with an internal diameter of 9.6 cm and an overall height 90 cm. Total volume of the reactor was 5,800 ml with working volume of 5,000 ml. Reactor working volume occupied by 50% of sludge zone in the bottom part of reactor and 50% of packed zone at the upper part of reactor. This packed zone contained nylon fibers with the specific surface area of 2 m^2m^{-3} as supporting media for microbial attachment as biofilm formation. Seven sampling ports were distributed at different height level of sludge and packed zones in AHR. The biogas system components contained of anaerobic reactor with sampling ports, influent and effluent tanks, peristaltic pump, and gas counter.

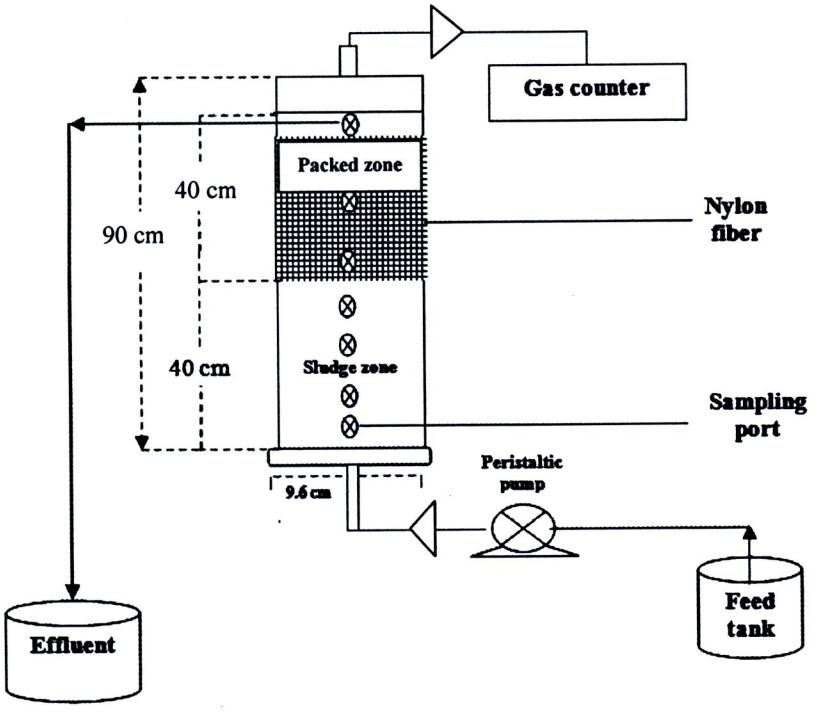

Figure 3.1 Schematic diagram of the laboratory scale AHR

3.2 Palm oil mill effluent (POME)

3.2.1 Raw POME characteristics

Raw POME was collected directly from palm oil production plant at Surat Thani province. The characteristics of raw POME were determined chemical oxygen demand (COD), biochemical oxygen demand (BOD), total solid (TS), suspended solid (SS), oil and grease (O&G), pH, total volatile fatty acid (TVA) and alkalinity (Alk) and volatile suspended solid (VSS) by APHA standard methods (2005) as shown in Table 3.1 The characteristics of discharged POME from production plants are normally fluctuated by nature.

Table 3.1 Characteristics of raw POME

Parameters	Value
pH	4.6 ± 0.2
Alk (mg l^{-1} $CaCO_3$)	1200 ± 0.2
TVA (mg l^{-1} CH_3COOH)	2700 ± 0.5
TCOD (mg l^{-1})	60300 ± 1.3
SCOD (mg l^{-1})	41800 ± 1.0
BOD (mg l^{-1})	30200 ± 1.2
TS (mg l^{-1})	51300 ± 1.5
SS (mg l^{-1})	11600 ± 0.3
VSS (mg l^{-1})	9850 ± 0.1
O&G (mg l^{-1})	5800 ± 0.1

Values are the mean of *n* values ± standard deviation (%); whereby *n* in the range of three values.

3.2.2 Preparation and characteristics of POME used in this study

To control the concentration of SS residues for feedstock to feed in the reactor, POME was prepared by settling in water bath at 65°C for 6-8 h. It is separated into three layers: top layer was O&G, middle layer was clearify wastewater and bottom part was SS. Top layer was removed while clearify wastewater and SS were separately collected. Feed stocks at various concentrations of SS were prepared by mixing of the SS and clarify wastewater to obtain the influent characteristics as summarized in Table 3.2. It can be seen that although O&G layer was removed, but there were partial O&G remained in the influent by combining with SS and settle in bottom part. Therefore, O&G concentration increased according to SS concentration. Both of SS and O&G were hardly biodegradable and they cause the operational problems in anaerobic digester, however, they were potential organic matters of methane production. It is therefore important to determine the concentrations of SS and O&G in POME that able to handle feeding into reactor with having high

efficiency performance in term of biodegradation, organic acid formation and finally methane production.

Table 3.2 Influent characteristics and organic loading rate of AHR operation

Operation time (d)	Influent (g l^{-1})				OLR (g TCOD l^{-1} d^{-1})
	SS	O&G	TCOD	SCOD	
1 - 30	5.0 ± 0.2	0.9 ± 0.5	15.0 ± 0.3	9.0 ± 0.2	3.0 ± 0.1
32 - 115	7.0 ± 0.2	1.4 ± 0.6	19.0 ± 0.2	12.0 ± 0.2	3.8 ± 0.1
116 - 144	10.0 ± 0.1	1.9 ± 0.1	20.0 ± 0.3	8.0 ± 0.3	4.0 ± 0.1
145 - 196	11.0 ± 0.3	2.3 ± 0.2	24.0 ± 0.6	10.0 ± 0.2	4.8 ± 0.2
197 - 222	12.5 ± 0.3	2.7 ± 0.2	30.0 ± 0.5	13.0 ± 0.3	6.0 ± 0.1
224 - 290	10.0 ± 0.2	1.8 ± 0.3	20.0 ± 0.2	8.2 ± 0.3	4.0 ± 0.2

Values are the mean of *n* values ± standard deviation (%); whereby *n* in the range of three values.

3.3 Reactor startup and operation

Reactor was operated under ambient temperatures (~30-35°C). POME was continuously up-flow feeding from the bottom of sludge bed zone up to the top of packed zone. The inoculum seed for startup in AHR was taken from the anaerobic wastewater treatment system of POME. The starter with the specific methanogenic activity (SMA) 0.12 gCOD-CH_4 g^{-1} VSS d^{-1} was cultivated in the reactor. The inoculum seed was inoculated with seed sludge concentration 5 g VSS l^{-1}. In order to allow the adaptation of the mesophilic microbial seed sludge to the new environment, the reactor was semi-continuous fed once a day with the diluted POME (5,000 – 6,000 mg COD l^{-1}) and ran for 7 d at hydraulic retention time (HRT) of 5 d. Reactor was then fed with stepwise increasing of SS concentration at 5, 7, 10, 11 and 12.5 g l^{-1} (Table 3.2) and flow rate and HRT kept constant at 5 d and 130 ml min^{-1}, respectively. During reactor operation, process performance (TCOD, SCOD, SS and O&G) and stability (pH, TVA and Alk) were monitored in sludge and packed zones. After reactor ran to steady state, microbial quality (non-methanogen and methanogen activity), microbial quantity (non-methanogen and methanogen population) and microbial community in sludge and packed zones of AHR were monitored. Microbial activities were studied by spicing glucose and acetic acid as substrate for non-methanogen and methanogen, respectively. Microbial population and community were studied by molecular techniques using 16S rDNA quantitative PCR and PCR-DGGE and DNA sequencing technique, respectively. The flow diagram of this study is shown in Figure 3.2.

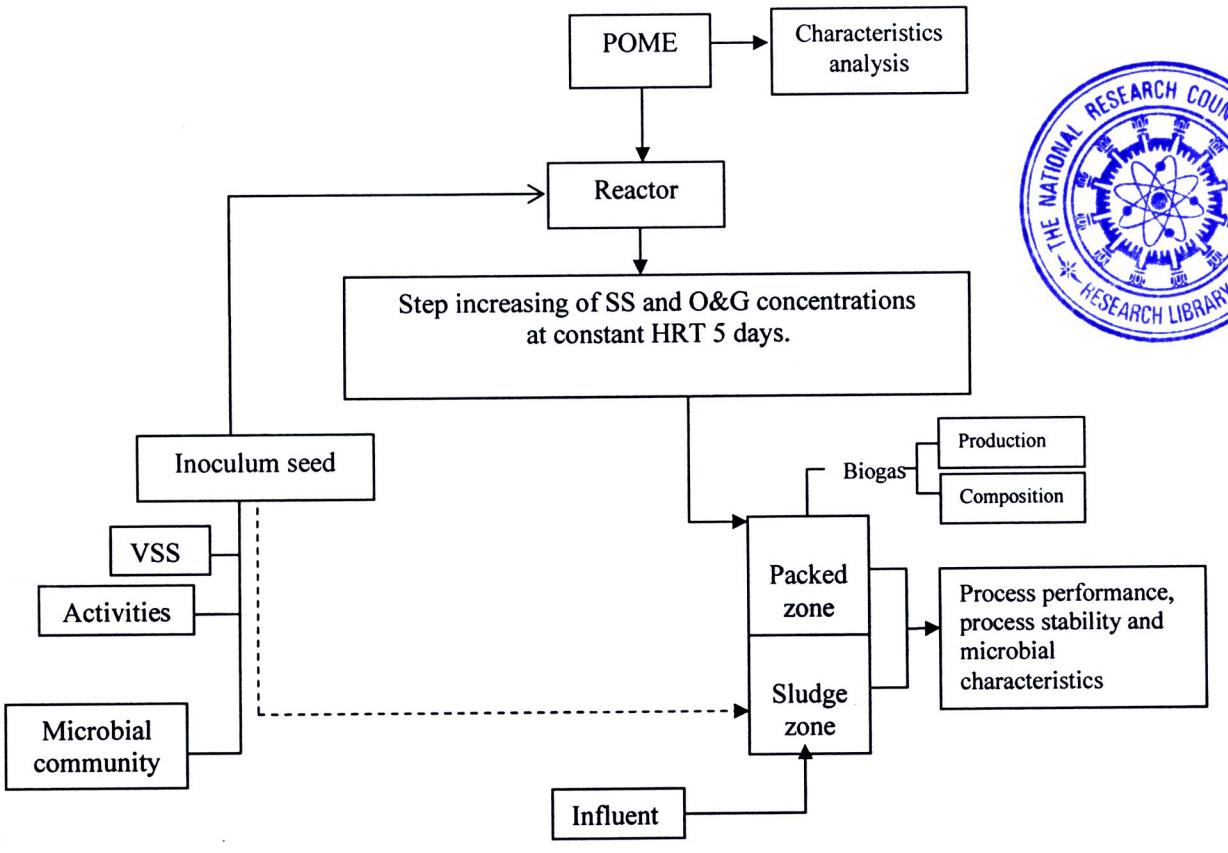

Figure 3.2 Flow diagram of the study

3.4 Reactor monitoring

The performance and stability of AHR was routinely monitored through the measurements of TCOD, SCOD, SS, O&G, TVA, Alk and pH (Table 3.3) according to APHA Standard Method (2005) as well as biogas production and composition. Before the system ran to steady state, all parameters were monitored daily, and during steady state, they were monitored every three days.

Table 3.3 Methods for characterization of palm oil mill effluent

Parameter	Analysis Method
pH	pH meter
Alkalinity	Titration Method
TVA	Titration Method
TCOD	Close Reflux (APHA, 1995)
SCOD	Close Reflux (APHA, 1995)
TS	Standard Method (APHA, 2005)
SS	Standard Method (APHA, 2005)
VSS	Standard Method (APHA, 2005)
O&G	Hexane extraction

3.5 Microbial characteristics

Anaerobic digestion is the process of bacterial consortia responsible for the organic matter degradation in the absence of oxygen. During reactor operation under different conditions, microorganisms have been adapted with new environmental and consumed organic matters to growth (Markoulatos et al., 2002). Therefore, microbial community, activity and quantity will be change according to environmental change. This study monitored microbial diversity and community by PCR-DGGE and DNA sequencing technique, while using 16S rDNA quantitative PCR to study of microbial quantity. Microbial quality was determined in term of non-methanogenic (EUB) and methanogenic activities (ARC).

3.5.1 Microbial activities determination

Sludge samples of sludge and packed zones were collected at each steady state. In the sludge zone, suspended sludge was collected from wastewater in AHR. In the packed zone, attached sludge on supporting media was separated by ultra-sonication for 10 min. They were analyzed on the activities of methanogen and non-methanogen by spicing acetic acid and glucose as substrate (Nopharatana et al., 1998).

3.5.2 Analysis of microbial community structure

DNA was extracted from the samples and PCR was conducted using the DNA as template. The PCR samples were subjected to the DGGE analysis. Each step of the DGGE analysis is as follows:

3.5.2.1 Nucleic acid extraction

Total genomic DNA was extracted from the microbial samples. DNA was extracted using a modified version of Zhou et al. (1996). All samples were collected in duplicate. Genomic DNA was used as the template for the Polymerase Chain Reaction (PCR) (Hori et al., 2006). Before extraction, the granular samples were homogenized with a homogenizer. The nucleic lysis buffer or extraction buffer was added into pellet samples. The extraction buffer consisted of 100 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0 and 1.5 M NaCl. The pellet samples were mixed with 100 µl of proteinase K (10 mg ml^{-1}). Then, the samples were mixed by horizontal shaking at 225 rpm for 30 min at 37°C. For Gram positive bacteria, 100 µl of 10 mg ml^{-1} lysozyme is required for efficient lysis. After shaking, 100 µl of 20% SDS was added to the pellet samples. The samples were incubated in a 65°C water bath for 20 min with gentle mixing by inversion every 2 min. The supernatants were mixed with an equal volume of chloroform-isoamyl alcohol (24:1; vol·vol^{-1}). The aqueous layer was recovered by centrifugation, taking care to avoid the white protenacious material. The DNA in the aqueous layer was precipitated with 0.6 volume of isopropanol. Then, pelleted nucleic acids were washed with ice-cold 70% ethanol and absolute ethanol. The pellet samples were air-dried. The pellet of crude nucleic acid was re-suspended in 50 µl of TE buffer (pH 7.2) or sterile deionized water (Zhou et al., 1996; Pender et al., 2004; Hori et al., 2006).

Extracted DNA was visualized by the UV excitation after electrophoresis in a 0.8% (w/v) 1X TAE buffer agarose gel containing ethidium bromide (1 mg·ml^{-1}). This buffer consisted of 40 mM Tris-base, 1 mM EDTA and 1.14 mM glacial acetic acid at pH 8.0. The aliquots of the DNA samples were used for PCR amplification immediately. The remainder was stored at -20°C (Pender et al., 2004; Enright et al., 2009).

3.5.2.2 Polymerase chain reaction (PCR) amplification

PCR is the generation of the DNA copies by a repeated sequence of denaturation of double-stranded DNA followed by the next round of denaturation and synthesis (Howe, 1997). The nested PCR is an elimination of nonspecific products from the first primer set. Nested PCR uses two sets of amplification primers. The second (nested) set of primers anneal to a sequence internal to the region flanked by the first set (Singh et al., 1996; Loeffelholz and Deng, 2006). On the first round PCR, a full length 1,500 bp fragment of 16S rRNA gene was used as the DNA template for the second round GC clamps of 16S PCR. The PCR products of the second round are around 200 bp. Table 3.4 summarizes the primer sets was used in this study. The bacterial 16S rRNA gene was amplified by PCR with the primer EUB8F/U1492R in the first round and the specific primer set 338GC-F/518R in the second round (Moyer et al., 1994; Amann et al., 1995; Ophan et al., 2000). The archaeal 16S rRNA gene was

amplified by PCR with the primer A20F/U1492R in the first round and the specific primer set 344GC-F/522R in the second round (Muyzer et al., 1993; Raskin et al., 1994; Amann et al, 1995).

The reaction components used for PCR amplification are 10 mM of deoxynucleoside triphosphates (dNTPs), 10X PCR buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer, 1 U of Taq DNA polymerase (Invitrogen, USA) and 50–100 ng of DNA template. These components were brought to 25 µl total volume with deionized water. The cyclical parameter conditions used for each microbial group by the thermocycler are described in Table 3.5. The amplified products were examined by a 0.8% agarose gel with visualization of DNA by ethidium bromide fluorescence (Hori et al., 2006).

3.5.2.3 Denaturing gradient gel electrophoresis (DGGE)

The 16S rRNA gene is partially amplified by PCR with bacterial and archaeal primers. These PCR samples give a mixture of DNA fragments of the same length. The DNA mixture is separated by denaturant gradient electrophoresis on an acrylamide gel with an increasing urea/formamide gradient (Keyser et al., 2006).

The DGGE was performed as described by Muyzer (1993). The 200 bp PCR fragments were analyzed by DGGE on the DGGE-2000 system apparatus (CBS Scientific Co. Inc., CA, USA). The nested PCR products were loaded onto 8% polyacrylamide gels in 1xTAE (Tris-acetate-EDTA) buffer. The gradients were created by the addition of 0–80% denaturant (5.6M urea and 40% $v \cdot v^{-1}$ formamide) into polyacrylamide. (Kayser et al., 2006; Shigematsu et al., 2006; A 40% to 65% denaturing gradient was used for the domain Bacteria, while a 35 to 75% denaturing gradient was used for the domain of Archaea. Electrophoresis was performed at 200V for 5 h and at a constant temperature of 60°C. After the electrophoresis, the gels were stained with SYBR Gold nucleic acid stain (Molecular Probes, USA) for 20 min. The image was visualized on a UV transilluminator and was captured using Biovision CN 1000/26M (Vilber Lourmat, France).

3.5.2.4 16S rRNA gene sequencing

Most of the bands were excised by Gel Cutting Tips (Cleaver Scientific, England) from the gel and re-amplified with the primer 338GC-F/518R and 344GC-F/522R for the bacterial and archaeal 16S rRNA genes, respectively. The PCR products were purified using Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions. The purified PCR products were sequenced using the 1st BASE Laboratories Sdn Bhd (Malaysia). Sequences were compared to known 16S rRNA sequences in the GenBank™ database using the BLASTn to locate nearly exact matches in the GenBank database (Altschul et al., 1990).

Table 3.4 Primers using in this study

Primer	Position	Sequence (5′-3′)	Target	References
EUB8F		AGAGTTTGATCCTGGCTCAG	*Eubacteria*	Amann et al., 1995
A1F		ATTCCGGTTGATCCTGC	*Archaea*	Tajima et al, 2001
U1492R	1510-1492	GGTTACCTTGTTACGACTT	Universal	Moyer et al., 1994
338GC-f	338-355	GCTGCCTCCCGTAGGAGT	*Bacteria*, V3 region	Amann et al., 1990
518r	518-534	ATTACCGCGGCTGCTGCTGG	Universal, V3 region	Muyzer et al., 1993
344GC-f	340-357	CCCTACGGGG(C/T)GCA(G/C)CAG	*Archaea*, V3 region	Raskin et al., 1994
522r	534-522	GWATTACCGCGGCKGCTG	*Archaea*, V3 region	Amann et al., 1995
GC-camp		CGCCCGCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGG		Muyzer et al., 1993

Table 3.5 The cyclical parameters of the thermocycler program for the 16S rRNA gene amplification

PCR primer set	No. of cycle	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cooling (°C)
EUB8f/U1492r	30	95°C/5 min	95°C/50 s	55°C/30 s	72°C/2 min	72°C/7 min	4
338GC-f/518r	30	95°C/5 min	95°C/50 s	60°C/30 s	72°C/50 s	72°C/7 min	4
A1f/U1492r	30	95°C/5 min	95°C/50 s	55°C/2 min	72°C/2 min	72°C/7 min	4
344GC-f/522r	30	95°C/5 min	95°C/50 s	60°C/30 s	72°C/50 s	72°C/7 min	4

3.5.3 16S rDNA quantitative PCR

Copy numbers of 16S rDNA gene of EUB and ARC were quantified by relative quantification real-time PCR (qPCR). KAPA SYBR® Fast qPCR Kit was used for real-time reactions (KAPA, Brazil). The qPCR assay was performed using a fluorescence-detecting thermocycler (Stratagene Mx3005P). The two-step amplification protocol was as follows: initial denaturation for 10 min at 95°C followed by 40 cycles of 30 sec at 95°C and combined annealing and elongation for 30 sec at 60°C. Standard curves were generated using 16s rDNA of standard EUB and ARC strains. The primer 8F/U1492R was used to amplify 16S rDNA of standard EUB. The primer A1F /U1492R was used to amplify 16S rDNA of standard ARC. Amplicons of EUB and ARC were cloned to plasmid vector (pGEM®-T Easy vector, Promega) and inserted in chemically competent cells (*E.coli* DH5α). The inserted plasmids were extracted according to the manufacturer's instructions of High-Speed mini kit (Geneaid). Plasmids DNA were serially diluted in the range of 10^2 - 10^7 copies rDNA/µl and directly used as template for qPCR with primers and amplification protocol as defined. The copy concentrations were calculated using the following equation (Whelan et al., 2003).

$$\text{DNA copy} = \frac{(6.02 \times 10^{23}\ \text{copy mole}^{-1}) \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ (\text{g mol}^{-1}\text{bp}^{-1})}$$

Threshold cycle (C_t) values were plotted against the logarithm of their initial template copy numbers. DNA fragments were analyzed by qPCR as described above and their C_t were compared with standard curves.