CHAPTER 2

LITERATURE REVIEW

2.1 Palm oil mill effluent

Palm oil is one of the main agricultural products of many countries in the world. Oil World expected global palm oil output to reach 47.2 million tons during the 2009, October season which was 6.5% higher than the year before. Palm oil production in the world within year 2006-2009 was shown in Table 2.1. Thailand is the third of top five countries of palm oil production and total palm oil production in 2009/10 was 1,420 metric tons.

Table 2.1 Top five countries of palm oil production and export (Oil world, 2009)

Year/Country	2006/07	2007/08	2008/09	2009/10	Year/Country	2006/07	2007/08	2008/09	2009/10
Production (metric tons)					**Export (metric tons)**				
Indonesia	16,730	18,880	20,450	22,090	Indonesia	12,465	14,100	16,110	16,840
Malaysia	15,294	17,567	17,259	18,200	Malaysia	13,768	15,041	15,990	16,180
Thailand	989	1,273	1,310	1,420	PNG	406	385	451	422
Nigeria	752	812	853	880	Ecuador	180	178	173	189
Colombia	830	779	758	773	Thailand	327	399	123	160
World	**37,591**	**42,666**	**44,262**	**47,154**	**World**	**29,638**	**32,850**	**35,480**	**36,350**

Besides the main product, crude palm oil (CPO), the mills also generate many by-products and liquid wastes as shown in a chart of mass balance of various products generated from a palm oil mill (Figure 2.1), which may have a significant impact on the environment if they are not dealt with properly. The mill has the capacity to handle 50 tons h^{-1} of fresh fruit bunch (FFB). This FFB is processed into crude palm oil and a number of other useful by-products. During the processing of the FFB, a significant amount of wastewater is produced, with high content of organic waste materials, known as palm oil mill effluent (POME). The ratio of POME produced is approximately 0.6 tons per tons of FFB processed, with a typical COD value of around 78,000 mg l^{-1} (CDM feasibility study for palm oil mill effluent treatment co-benefits project, 2009).

POME cannot be discharged without first being treated because POME is acidic and has a very high l organic content. Raw POME is a colloidal suspension containing 95–96% water, 0.6–0.7% oil and 4–5% total solids. Included in the total solids are 2–4% SS, which are mainly constituted of debris from palm fruit mesocarp generated from three main sources, i.e. sterilizer condensate, separator sludge and hydrocyclone wastewater. If the untreated effluent is discharged into watercourses, it is certain to cause considerable environmental problems due to its high biochemical oxygen demand (25,000 mg l^{-1}), chemical oxygen demand (53,630 mg l^{-1}), O&G (8,370 mg l^{-1}), total solids (43,635 mg l^{-1}) and SS (19,020 mg l^{-1}) (Ahmad et al., 2003). Wastewater and solid waste rich in lipids as POME are potentially attractive for biogas production owing to their high theoretical methane yield (Angelidaki and Ahring, 1992).

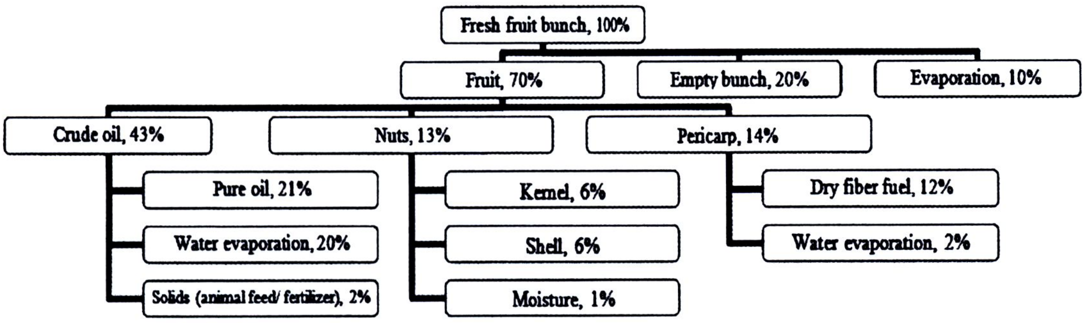

Figure 2.1 Typical fruit and production composition chart of a palm oil (Muttamara et al., 1987)

2.2 POME treatment technology

There are several POME treatment technologies. Relatively simple wastewater treatment technologies can be designed to provide low cost sanitation and environmental protection. General consideration for the choice of anaerobic or anaerobic wastewater treatment systems. If a producer of wastewater has to decide whether to install an aerobic or an anaerobic waste or wastewater treatment system, several points should be considered (Gallert and Winter, 2005):

- Highly concentrated wastewater should in general be treated anaerobically, because of the possibility of energy recovery in biogas and the much lower amounts of surplus sludge to be disposed of. For aerobic treatment, a high aeration rate is necessary and much surplus sludge is generated. Aeration causes aerosol formation and eventually requires off-gas purification.
- The efficiency of COD degradation for the bulk mass in concentrated wastewater or sludge (degradability of organic pollutants) generally seems to be about similar in aerobic or anaerobic bacteria. However, the degradation rates may be faster in aerobic treatment procedures than in anaerobic treatment procedures.
- Anaerobic treatment in general does not lead to the low pollution standards of

COD, BOD_5, or TOC that can be met with aerobic systems and which are required by environmental laws. Anaerobic treatment of wastes and wastewater is often considered a pretreatment process to minimize the oxygen demand and surplus sludge formation in a subsequent aerobic post-treatment stage. Only after a final aerobic treatment can the COD, BOD_5, or TOC concentration limits stated in the environmental laws is met. If limiting concentrations for nitrogen and phosphate also have to be achieved, further treatment steps such as nitrification, denitrification, and biological or chemical phosphate removal, must be considered.

- Wastewater with a low concentration of organic pollutants should be treated aerobically due to its higher process stability at low pollutant concentrations, although aerobic treatment is more expensive and more sludge remains for disposal. If mineralized sludge is required, aerobic treatment at a low loading or at prolonged hydraulic retention times is necessary to reinforce respiration of all endogenous reserve material.
- Anaerobic treatment systems are more expensive to construct but less expensive to operate than aerobic treatment systems.

The current treatment technology of POME typically consists of biological aerobic and anaerobic digestion. Biologically treated effluent is disposed of via land application system, thus providing essential nutrients for growing plants. This method may be a good choice for disposal of treated effluent (Wong et al., 2002). In general, the advantages of using natural biological processes relate to their low technology, which means that these systems are relatively easy to construct and operate, and to their low cost, which makes them attractive to communities with limited budgets.

Various aerobic treatments for POME were readily available. The common system with only few palm oil mills using advanced activated sludge system. Different aerobic pond systems, which vary in the type of the oxygen supply system (aeration system) and the design loading rate, are facultative ponds (maturation ponds), oxidation pond, aerated lagoons and polishing pond (Environment Advisory Assistances Industry, 1997). The efficiency of the activated sludge process was evaluated by treating anaerobically digested and diluted raw POME with HRT 36 h, the COD removal efficiencies were found to be 83%, 72%, 64%, 54% and 42% of initial COD concentrations at 1000, 2000, 3000, 4000 and 5000 mg l^{-1}, respectively (Vijayaraghavan et al., 2007).

Membrane technology shows high potential to treat POME for eliminating the environmental problem, and in addition, this alternative treatment system offers water recycling. A pilot plant was designed and constructed by Ahmad (2003) for POME treatment; two stages of treatment have been conducted whereby coagulation, sedimentation and adsorption play their roles at first stage as a membranes pretreatment process, and ultrafiltration and reverse osmosis membranes are combined for the membrane separation treatment. It resulted in 100% of turbidity reduction, COD and BOD removal moved up to 98.8 and 99.4%, respectively, with final pH of 7.

Anaerobic digestion has been widely used for POME treatment with large emphasis placed on capturing the methane gas released as a product of this biodegradation treatment method (Poh and Chong, 2009). Today 85% of POME treatment is based on an anaerobic and facultative ponding system, which is followed by another system consisting of an open tank digester coupled with extended aeration in a pond (Ma, 1999b). Compared to conventional aerobic methods of wastewater treatment, the anaerobic wastewater treatment concept indeed offers fundamental benefits such as low costs, energy production, relatively small space requirement of modern anaerobic wastewater treatment systems, very low sludge production (10-20% of COD removed) with very high dewaterability, stabilized sludge and high tolerance to unfed conditions (Metcalf and Eddy, 2003).

2.3 Anaerobic POME digestion

Anaerobic digestion is the degradation of complex organic matters under the absence of oxygen. This process is time consuming as microbial consortia responsible for the degradation process requires time to adapt to the new environment before they start to consume on organic matters to grow. Organic matters are degraded to methane and carbon dioxide in discrete steps by the concerted action of several different metabolite groups of microorganism (Pavlostathis and Giraldo-Gomez, 1990).. The main pathways of anaerobic digestion are shown in Figure 2.2.

Under strictly anaerobic conditions, soluble carbon compounds of wastes and wastewater are degraded stepwise to methane, CO_2, NH_3, and H_2S via a syntrophic interaction of fermentative and acetogenic bacteria with methanogens or sulfate reducers. The complete methanogenic degradation of biopolymers via hydrolysis/fermentation, acetogenesis, and methanogenesis can proceed only at a low H_2 partial pressure, which is maintained mainly by interspecies hydrogen transfer. Interspecies hydrogen transfer is facilitated when acetogens and hydrogenolytic methanogenic bacteria are arranged in proximity in flocs or in a biofilm within short diffusion distances. The reducing equivalents for carbon dioxide reduction to methane or sulfate reduction to sulfide are derived from the fermentative metabolism, e.g., of clostridia or *Eubacterium* sp., from β-oxidation of fatty acids, or the oxidation of alcohols. Methane and CO_2 are the main products in anaerobic environments where sulfate is absent, but sulfide and CO_2 are the main products if sulfate is present (Gallert and Winter., 2005). In the process of biodegrading POME into methane, carbon dioxide and water, there is a sequence of reactions involved: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Gerardi, 2003), which are the same as general anaerobic digestion path way. The salient features of those bacteria involved in the stabilization process are as follows:

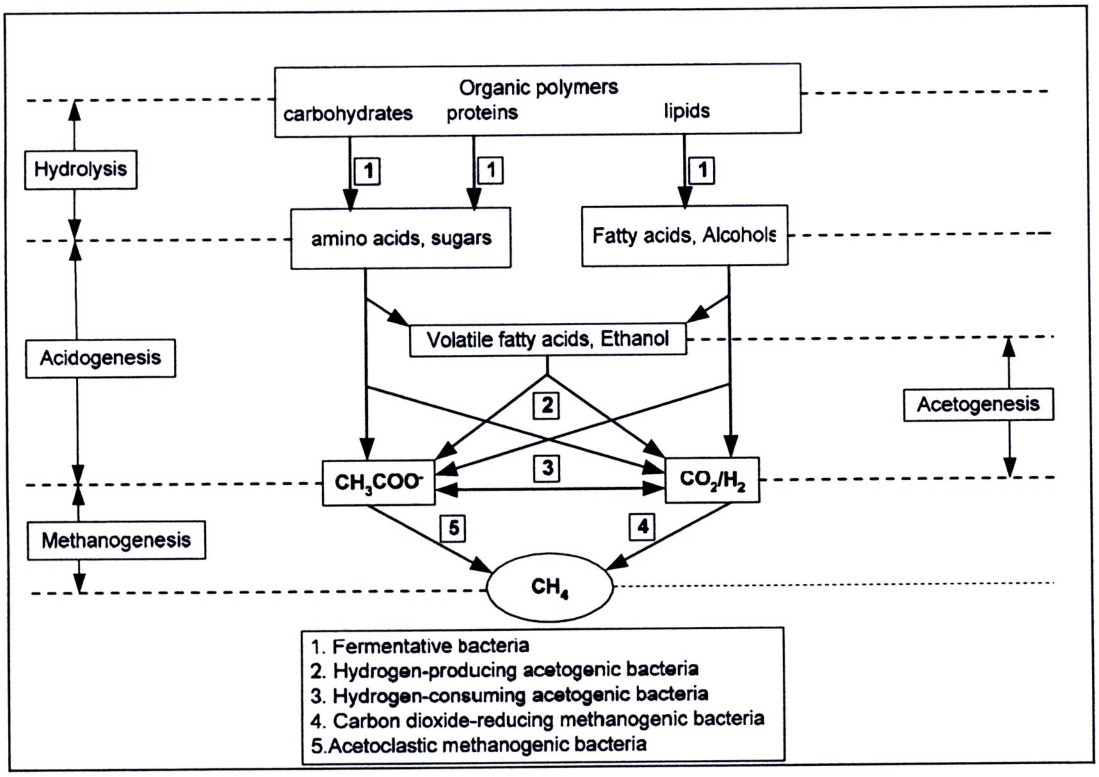

Figure 2.2 Anaerobic conversion of organic matter to methane (Pavlostathis and Giraldo-Gomez, 1990)

2.3.1 Hydrolysis

The first step for most digestion processes is hydrolysis during which particulate matters are converted to soluble compounds that can be hydrolyzed further to simple monomers to be subsequently utilized by fermentative bacteria. The group of non-methanogenic microorganisms responsible for the fermentation process consists of facultative and obligate anaerobic bacteria (Metcalf and Eddy, 2003). Hydrolysis is where complex molecules (i.e., carbohydrates, lipids, proteins) are converted into simpler molecule as sugar, amino acid and etc. Extracellular enzymes excreted by the fermentative bacteria catalyze the hydrolysis reactions. As no mineralization of organics is involved, this conversion results in no reduction in COD (Eckenfelder, 2000). Although most biopolymers are readily degradable, the cellulose of highly lignified plant material (straw, wood, etc.) has been shown to be resistant to hydrolysis (Lynd et al., 2002). Anaerobic degradation of lignocellulose and cellulose to methane and CO_2 illustrated in Figure 2.3. The rate of hydrolysis is a function of factors such as pH, temperature, composition and particle size of the substrate (Veeken et al., 2000; Paramsothy et al., 2004).

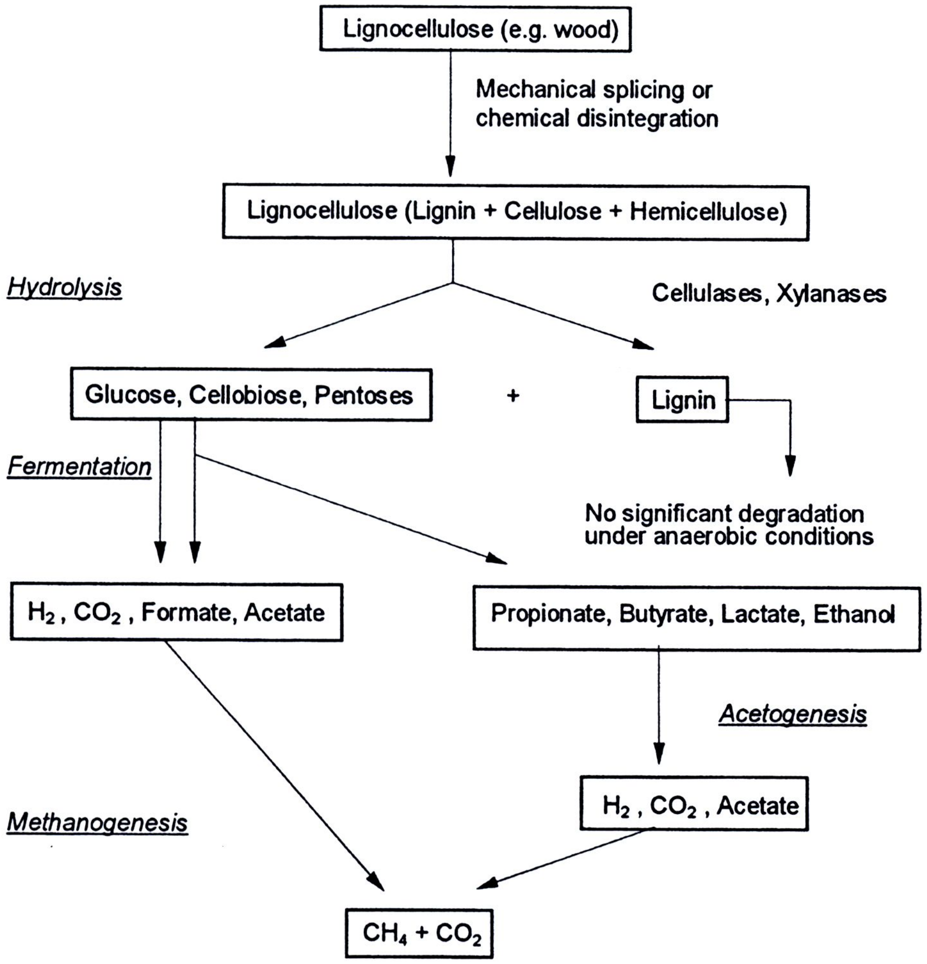

Figure 2.3 Anaerobic degradation of lignocellulose and cellulose to CH_4 and CO_2 (AVT, 1994)

2.3.2 Acidogenesis

In the acidogenesis step, the hydrolysis products are absorbed by the cells of fermentative bacteria to be fermented or anaerobically converted into compounds such as alcohols, short-chain fatty acids, formic acid, carbon dioxide, hydrogen, ammonia and sulfide. The organic substrates serve as both the electron donors and acceptors. The final products of the metabolic activities of these bacteria depend upon the initial substrate (Figure 2.2). As an example, consider the following reactions of glucose metabolism (Mosey, 1983).

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad (2.1)$$

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O \qquad (2.2)$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2\,CH_2COOH + 2CO_2 + 2H_2 \qquad (2.3)$$

The first reaction is the most preferred. It produces acetic acid which is the major precursor of CH_4. The other two reactions occur when there is an accumulation of H_2 in the system. In Equation 2.2, there is a clear utilization of H_2 while in Equation 2.3, there is also hydrogen production but of lesser quantity (two molecules against four in the first reaction). The increase in the acid load of the system is also lower (one mole butyric acid against two moles acetic acid in the first reaction).

2.3.3. Acetogenesis

2.3.3.1 Acetogens (Hydrogen-producing bacteria)

In the previous step of acidogenesis, acidogenic bacteria will break down these simpler molecules into organic acids and acetogenic bacteria, then convert these resulting organic acids into acetic acid, along with additional ammonia, hydrogen, and carbon dioxide which mainly consist of acetic acid (from acetogenesis) together with hydrogen and carbon dioxide (Poh and Chong, 2009). Propionate and butyrate are thought to be converted to acetate only by syntrophic acetogens in concert with hydrogen-utilizing methanogens (Lowe et al., 1993). Propionate-oxidizing Syntrophobacter-like bacteria have been identified in microcolonies in intimate association with methanogens (De Bok et al., 2004). These bacteria are responsible for converting organic products of fermentative bacterial activity, such as alcohols, propionic acid and butyric acid into acetic acid, CO_2 and H_2O (Rittmann and McCarty, 2001) as follows:

$$CH_3CH_2OH + H_2O \longrightarrow CH_3COO^- + H^+ + 2H_2$$
$$\Delta G^0 = +9.6\ kJ\ mol^{-1} \qquad (2.4)$$
$$CH_3CH_2COO^- + H_2O \longrightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2$$
$$\Delta G^0 = +76.1 kJ\ mol^{-1} \qquad (2.5)$$
$$CH_3CH_2CH_2COO^- + 2H_2O \longrightarrow 2CH_3COO^- + H^+ + 2H_2$$
$$\Delta G^0 = +48.1 kJ\ mol^{-1} \qquad (2.6)$$

In the bioconversion of organic matter to methane and carbon dioxide, acetate is the major intermediate which it about 70 % of the total methane produced in anaerobic digestion. Thus, the production of methane from acetate is an important step in the anaerobic digestion process (Rittmann and McCarty, 2001). A peculiar characteristic of these reactions is that they remain thermodynamically unfavorable (ΔG^0 =+ve) unless the H_2 produced is constantly removed from the system. The utilization of the hydrogen produced by the acidogenenic bacteria and other anaerobes by the methanogens is termed interspecies hydrogen transfer (Metcalf and Eddy, 2003).

2.3.3.2 Acetogens (Hydrogen-utilizing bacteria)

The H_2-utilizing or homoacetogenic bacteria are a group of obligatory anaerobic bacteria which utilize the acetyl coenzyme A (CoA) pathway to synthesize acetate from C_1 precursors. These bacteria grow autotrophically on H_2 and CO_2 and/or heterotrophically on a variety of organic compounds, with mixotrophic growth on H_2 and a suitable organic substrate being observed in some species (Breznak and Kane, 1990; Wood and Ljungdahl, 1991). These bacteria also contribute towards the acetic acid pool in anaerobic digestion for subsequent conversion to methane. They are thermodynamically highly efficient because they do not generate H_2 and CO_2 during growth on multi-carbon compounds including glucose, fructose, lactose, pyruvate, etc. The reaction is presented as follows:

$$4H_2 + 2CO_2 \longrightarrow CH_3COOH + 3H_2O \qquad (2.7)$$

However, the release of molecular hydrogen during acetogenesis of fatty acids or of other reduced metabolites may occur only when hydrogen does not accumulate, for thermodynamic reasons. Molecular hydrogen is consumed by methanogens (Table 2.2, reaction 1) or, alternatively, by sulfate reducers (Table 2.2, reaction 2) via interspecies hydrogen transfer. In the rumen and in sewage sludge digesters, the hydrogen concentration can be decreased by acetate formation from CO_2 and H_2 (Table 2.2, reaction 3) by bacteria such as *Acetobacterium woodii* and *Clostridium thermoaceticum.* Some additional reactions consuming hydrogen to decrease its concentration are also listed in Table 2.2 (reactions 4–6). To maintain a low H_2 partial pressure, a syntrophism of acetogenic, hydrogenproducing and methanogenic, hydrogen-utilizing bacteria is essential (Ianotti et al., 1973).

Table 2.2 Hydrogen-consuming reactions in anaerobic ecosystems (Schink, 1997)

Substrates (mol)	Products (mol)	ÄG$^{\circ\prime}$ (kJ mol^{-1})
(1) $4H_2 + CO_2$	$\rightarrow CH_4 + 2H_2O$	–131.0
(2) $4H_2 + SO_4^{2-}$	$\rightarrow S^{2-} + 4H_2O$	–151.0
(3) $4H_2 + 2CO_2$	$\rightarrow CH_3COO– + H^+ + 2H_2O$	–0.9
(4) $H_2 + S^0$	$\rightarrow H_2S$	–0.9
(5) $H_2C(NH_3{}^+)COO^- + H_2$	$\rightarrow CH_3COO– + NH_4{}^+$	0.0
(6) $COOH–CH–CH–COOH + H_2$	$\rightarrow COOH–CH_2–CH_2–COOH$	0.0

2.3.4 Methanogenesis

Hydrogen and carbon dioxide will be utilized by hydrogenotropic methanogens while acetic acid and carbon dioxide will be utilized by acetoclastic methanogens in methanogenesis step to give methane as a final product and this process is also indicated to the rate limiting step in anaerobic digestion of POME (Ibrahim et al., 1984).

2.4 Anaerobic treatment technology of POME

2.4.1 Conventional treatment systems

Ponding system is the most common treatment system which is employed to treat POME with more than 85% of the mills having adopted this method. Ponding system consists of de-oiling tank, acidification ponds, anaerobic ponds and facultative or aerobic ponds (Lettinga, 1995). Number of ponds varies according to the capacity of the POME. Facultative or aerobic ponds are necessary to further reduce BOD concentration in order to produce effluent before release it to nearby environment. Open digesting tank is the one choice using for POME treatment when limited land area is available for ponding system. It was investigated on the methane emission from open digesting tanks where each tanks was half the capacity of anaerobic ponds with retention time of 20 d (Yacob et al., 2005).

2.4.2 Anaerobic filtration

A few improved high rate bioreactors have been tested in the treatment of POME such as the anaerobic filter and anaerobic fluidized bed reactor (Borja and Banks, 1995), thermophilic upflow anaerobic filter (Mustapha et al., 2003). Experimental results indicated better treatment of POME compared to conventional practices. For anaerobic filtration, the packing allows biomass to attach on the surface when raw POME feed enters from the bottom of the bioreactor while treated effluent together with generated biogas will leave from the top of the bioreactor. These technology is selected for wastewater treatment because it requires a smaller reactor volume which operates on a shorter hydraulic retention times (HRTs) and high substrate removal efficiency (Ugoji, 1997). Moreover, it has ability to maintain high concentration of biomass in contact with the wastewater without affecting treatment efficiency (Metcalf, 2003), and tolerance to shock loadings (Gerardi, 2003). However, filter clogging by suspended solid is a major problem in the continuous operation of anaerobic filters (Chan and Chooi, 1984) and the large scale implementation of any of the improved system is still lacking.

In general, anaerobic filter is capable of treating wastewaters to give good effluent quality with at least 70% of COD removal efficiency with methane composition of more than 50% such as brewery wastewater treatment (Alvarado et al., 2008). In terms of POME treatment, the highest COD removal efficiency recorded was 94% with 63% of methane at an OLR of 4.5 kg COD $m^{-3}d^{-1}$, while overall

COD removal efficiency was up to 90% with an average methane gas composition of 60% (Borja and Banks, 1994b).

2.4.3 Up-flow anaerobic sludge blanket (UASB) reactor

UASB has been applied for the treatment of agro-industry wastewater and other such as domestic and pharmaceutical wastewater. Anaerobic treatment of POME in a single UASB and two stages UASB reactors was investigated (Borja and Banks, 1994; Borja et al, 1996). Achieving COD removal efficiency at 96% with the highest is operating OLR of 10.6 g COD l^{-1} d^{-1} in the single UASB reactor, whereas a 90% reduction in COD at an OLR of 15 g COD l^{-1} d^{-1} was achieved in the two-stage UASB reactor.

Characteristic of POME generally composts of suspended and colloidal, which in the form of fat, protein and cellulose have adverse impact on UASB reactor performance and can cause deterioration of microbial activities and wash out of active biomass. This method is required since SS in POME have high potential for gas production while extra costs from sludge disposal can be avoided. Finally, two-stages (acidogensis and methanogenesis) using a pair of UASB reactors was developed. The methanogenic reactor was found to adapt quickly with the feed from the acidogenic reactor and also tolerate higher OLRs (Poh and Chong, 2009). It seems UASB reactor can overcome the problem of high SS containing wastewater treatment that may clog reactors with packing material and also provide higher methane production (Yacob et al., 2005). However, this reactor face long start-up periods when seeded sludge is not granulated (Goodwin et al., 1992).

2.4.4 Up-flow anaerobic sludge fixed-film (UASFF)

Anaerobic treatment technology has growth along with the recovery of usable energy with good process efficiency and stability. Anaerobic hybrid technology, which combination of different anaerobic systems into a single bioreactor, is the one of developed anaerobic treatment technologies applying to POME treatment system. The up-flow anaerobic sludge fixed film (UASFF) bioreactor as an anaerobic hybrid reactor is a combination of an up-flow anaerobic sludge blanket (UASB) reactor and an immobilized cell or fixed film (FF) reactor (Metcalft and Eddy, 2003). The FF portion positioned above the UASB section prevents sludge washout and helps in retaining a high biomass concentration in the reactor. Several researchers have successfully used the UASFF reactor to treat various kinds of wastewaters such as starch, swine and slaughterhouse (Tong, 2006). UASFF implementing in POME treatment was invested that the granular sludge was rapidly developed within 20 d by its size increased from an initial pinpoint size to reach 2 mm and high process performance in

COD removals of 89% and 97% at HRT of 1.5 and 3 d were achieved, respectively (Najafpour et al., 2006).

Some UASFF are made up of glass columns which consist of three sections; bottom, middle and top. The bottom part of the column is operated as a UASB reactor, the middle part of the column is operated as a fixed film reactor and the top part of the bioreactor served as a gas–solid separator. The middle section of the column was packed with packing material such as plastic or nylon fiber (Borja and Banks, 1995). The treatment of POME found that internal packing and high ratio of effluent recycle are both vital to control the stability of the UASFF reactor (Najafpour et al., 2006).

2.5 Microbial community in anaerobic digestion

Anaerobic digestion consists of a series of microbiological processes that convert organic compounds to methane. Consortia of microorganisms are involved in the transformation of complex high-molecular-weight organic compounds to methane. Furthermore, there are synergistic interactions between the various groups of bacteria implicated in anaerobic digestion of wastes (Drost, 1997). Anaerobic microbial communities can be classified into two domains, *Bacteria* and *Archaea.* Stable anaerobic digestion is accomplished by representatives of four major metabolic groups: hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotrophic methanogens, and aceticlastic methanogens (Zinder et al., 1984).

2.5.1 Anaerobic fermentative bacteria

Hydrolysis of biopolymers and fermentation or respiration of monomers can be catalyzed by strictly anaerobic, facultative anaerobic and aerobic microorganisms. The bacterial group directly involved in the decomposition of wastewater to generate substrates directly utilized by methanogenic. *Clostridium stercorarium* produces a variety of plant cell wall hydrolyzing enzymes, especially for the hydrolysis of hemicellulose. However, one of the most efficient cellulose-degrading microorganisms known so far is *Clostridium thermocellum* (Lynd et al., 2002; Zhang et al., 2005). Lignocellulose and cellulose were insoluble organic matters composting in POME which widely accepted to be the rate limiting substrate in the anaerobic digestion of organic solid wastes to methane and carbon dioxide and it has been shown that cellulose solubilisation in bioreactor systems is facilitated by surface attached bacteria (Chynoweth and Pullammanappallil, 1996; Song et al., 2005).

However, the cellulose solubilisation rate in rumen was not limited by the activity of the cellulolytic population but rather by the amount of cellulose surface area available for enzymatic attack and it was constant on a per unit surface area basis once the surface was fully colonized (Sanders et al., 2000; Song, 2003). Other study reported that the rate of cellulose solubilisation is depressed during the early stages of the digestion when the cellulose particles are not fully colonized by solubilizing bacteria and the rate also falls towards the end of the digestion due to the formation of complex, multilayer biofilms. Moreover, change in microbial community was observed that the increase in the *B. cellulosolvens*-like bacterial population which occurred after the *C. thermocellum*-like bacteria began to fall suggests that there may be a competitive interaction between these two groups of bacteria (O'Sullivan et al., 2005)

In terms of POME, fats, oil and greases which major problem in treatment system. Brooksbank et al. (2007) investigated the ability of commercial microbial supplements to degrade fat, oil and grease under laboratory. The results show that bacteria associated with wastewater preferentially degrade unsaturated fatty acids producing semi-solid, sticky material likely to block sewers. Multi-species microbial inocular can degrade significant amounts of a variety of fats and oils without significantly modifying the fatty acid composition and may thus help keep sewer lines free of grease deposits. Olive mill solid residual anaerobic treatment which high consisted of SS and O&G as same POME was studied the microbial community in anaerobic digestion by Rinc´on et al., 2008. At low Firmicutes, mostly represented by the genus *Clostridium* and other bacterial communities such as *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Deferribacteres* were the most abundant at high OLR.

2.5.2 Methanogens

The carbonic products from organic fermentation are either acetate and hydrogen or intermediate compounds, such as propionate and butyrate, which may later be converted to acetate and hydrogen. Methane is mostly produced from acetate or hydrogen (H_2) and carbon dioxide (CO_2) or formate. The acetoclastic methanogens can compete with the acetate oxidizing bacteria. The acetate oxidizing bacteria can convert acetate to H_2 and CO_2 or they can also use the reverse reaction to produce acetate from them. Therefore, the term ''reversible acetogenesis'' was created, as it was assumed that it was even the same ''Reversibacter'' enabling both reactions (Zinder 1994). At high concentrations of H_2 ($\leq$ 500 Pa), acetogenesis is favoured (or the methanogenesis from H_2 and CO_2), and at low concentrations ($\leq$ 40 Pa), the acetate oxidation occurs. Hydrogen is used as an electron acceptor to form methane, by hydrogenotrophic methanogens, while many H_2-using methanogens can also use formate as an electron donor for the reduction of CO_2 to CH_4. On the other hand, acetate is cleaved to form methane from the methyl group by methyltrophic methanogens and carbon dioxide from the carboxyl group by acetotrophic methanogens. Classification of methanogenic bacteria and general characteristics of some methanogenic bacteriain is outlined in Table 2.3 and 2.4, respectively.

Table 2.3 Classification of methanogens (Whitman et al., 2001; Garrity et al., 2004)

- Class I. Methanobacteria (known to grow on H_2/CO_2 and formate as C source)
  - Order I. Methanobacteriales
    - Family I. Methanobacteriaceae
      - Genus I. Methanobacterium
      - Genus II. Methanobrevibacter
      - Genus III. Methanosphaera
      - Genus IV. Methanothermobacter
    - Family II. Methanothermaceae
      - Genus I. Methanothermus

- Class II. Methanococci (known to grow on H_2/CO_2 and formate as C source)
  - Order I. Methanococcales
    - Family I. Methanococcaceae
      - Genus I. Methanococcus
      - Genus II. Methanothermococcus
    - Family II. Methanocaldococcaceae
      - Genus I. Methanocaldococcus
      - Genus II. Methanotorris

- Class III. Methanomicrobia (known to grow on H_2/CO_2 and formate as C source)
  - Order I. Methanomicrobiales
    - Family I. Methanomicrobiaceae
      - Genus I. Methanomicrobium
      - Genus II. Methanoculleus
      - Genus III. Methanofollis
      - Genus IV. Methanogenium
      - Genus V. Methanolacinia
      - Genus VI. Methanoplanus
    - Family II. Methanocorpusculaceae
      - Genus I. Methanocorpusculum
    - Family III. Methanospirillaceae (known to be hydrogenotrophic)
      - Genus I. Methanospirillum
  - Order II. Methanosarcinales (known to be acetato- and methylotrophic)
    - Family I. Methansarcinaceae
      - Genus I. Methanosarcina
      - Genus II. Methanococcoides
      - Genus III. Methanohalobium
      - Genus IV. Methanohalophilus
      - Genus V. Methanolobus
      - Genus VI. Methanomethylovorans
      - Genus VII. Methanimicrococcus
      - Genus VIII. Methanosalsum
    - Family II. Methanosaetaceae
      - Genus I. Methanosaeta

Table 2.4 General characteristics of some methanogens (Vogels et al., 1988; Boone et al., 1993a)

Species	Morphology	Cell width/length	Substrate	Optimal temperature (C)	Optimum pH range
Methanobacterium bryantii	Long rods to filaments	0.5–1.0/1.5	H_2/CO_2	37	6.9–7.2
Methanobacterium formicicum	Long rods to filaments	0.4–0.8/2–15	H_2/CO_2, formate	37–45	6.6–7.8
Methanobacterium thermoalcaliphilum	Rods	0.3–0.4/3–4	H_2/CO_2	58–62	8.0–8.5
Methanothermobacter thermoautotrophicum	Long rods to filaments	0.3–0.6/2–7	H_2/CO_2	65–70	7.0–8.0
Methanothermobacter wolfeii	Rods	0.4/2.4–2.7	H_2/CO_2	55–65	7.0–7.5
Methanobrevibacter smithii	Short rods, short chains	0.6–0.7/1.0–1.5	H_2/CO_2, formate	37–39	–
Methanobrevibacter ruminantium	Short rods, short chains	0.7/ 0.8–1.7	H₂/CO2, formate	37–39	–
Methanothermus fervidus	Short rods	0.3–0.4/1–3	H_2/CO_2, formate	83	\7.0
Methanothermococcus thermolithotrophicus	Regular to irregular cocci	–	H_2/CO_2, formate	65	–
Methanococcus voltaei	Regular to irregular cocci	1.5 (diameter)	H_2/CO_2, formate	35–40	6.0–7.0
Methanococcus vannielii	Regular to irregular cocci	1.3 (diameter)	H_2/CO_2, formate	65	7–9 (pH range)
Methanomicrobium mobile	Short rods	0.7/1.5–2.0	H_2/CO_2, formate	40	6.1–6.9
Methanolacinia paynteri	Short irregular rods	0.6/1.5–2.5	H_2/CO_2	40	7.0
Methanospirillum hungatei	Regular curved rods to long spiral filaments	0.5/7.4	H_2/CO_2, formate	30–40	–
Methanosarcina acetivorans	Irregular cocci	–	Methanol, acetate	35–40	6.5
Methanosarcina barkeri	Irregular cocci, forming irregular packets	–	H_2/CO_2, methanol, methyamines, acetate	35–40	5–7
Methanosarcina mazeii	Irregular cocci, forming cysts and packets	–	Methanol, methyamines, acetate	30–40	6–7
Methanosarcina thermophila	Irregular cocci, forming aggregates	–	H_2/CO_2, methanol, methyamines, acetate	50	6–7
Methanococcoides methylutens	Irregular cocci	0.8–1.2 (diameter)	Methanol	42	7.0–7.5
Methanosaeta concilii (soehngenii)	Rod	0.8 9 2.5–6.0 (dimensions)	Acetate	35–40	7.0–7.5
Methanosaeta thermophila	Rod	0.8–1.3 9 6.0 (dimensions)	Acetate	55–60	7

Oleic and palmitic acid are the major long chain fatty acid (LCFA) components of POME in ratios of approximately 40.5 and 42.8%, respectively (Kines 1985). Synthetic LCFA wastewater with oleic acid (1.75 mM) and palmitic acid (1.75 mM) was treated in anaerobic chemostat using completely stirred tank reactor (CSTR) under temperature at 37°C. The major groups of methanogens within the archaeal community: the aceticlastic genera *Methanosaeta* and *Methanosarcina* and the hydrogenotrophic genus *Methanospirillum* were detected by 16S rRNA gene sequencing. Fatty-acid oxidizing bacteria affiliated with the family Syntrophomonadaceae and bacteria belonging to the phyla *Bacteroidetes* and *Spirochaetes* was detected that they play important roles in LCFA degradation within the chemostat (Shigematsu et al., 2006).

Three major methanogens, *Methanobacteriales, Methanococcales and Methanomicrobiales* within the domain archaea were monitored with rRNA probes specifically targeting these groups during start-up of acidogenic anaerobic reactors by Liu et al., (2002). The results showed that *Methanomicrobiales* was the most abundant among these methanogens monitored in the seed sludge, followed by *Methanobacteriales* and *Methanococcales* and the dominant methanogens identified by DGGE profiles were *Methanosaeta* and *Methanobacterium.* The former belongs to family *Methanosarcinaceae* and the latter order *Methanobacteriales.* These methanogenic populations significantly decreased during the start-up of these two acidogenic reactors.

Yoshiyuki (2008) has studied microbial population in a thermophilic packed-bed reactor for methanogenesis from volatile fatty acids for investigate reactor proformance. The results show that the methanogenesis in packed-bed reactor could be accomplished by the interrelated and complementary actions of the microorganisms in the biofilm and fermentation broth. High performance operations at high OLR could be accomplished by mutual and cooperative interactions that are established by the microorganisms in both biofilm and fermentation (Goodwin et al., 1992).

2.6 Molecular techniques

Modern molecular techniques provide an exciting opportunity to overcome the requirement for culturing and have therefore greatly increased our understanding of microbial diversity and functionality in the environment. These methods rely on the characterization of cellular constituents such as nucleic acids, proteins, fatty acids and other taxa-specific compounds (Rossello-Mora and Amann, 2001). Such molecules can be extracted directly from environmental samples without the need for culturing and analysis of the molecular composition can be used to elucidate the composition of the microbial community (Amann et al., 1995; Greene and Voordouw, 2003) and bacterial identification, especially those including the sequencing of genes coding for ribosomal 16S rDNA, has become a very important tool in studying bacterial communities in environmental samples (Suraruk et al., 1998).

Population dynamics of resident microbes in anaerobic treatment processes have also been analyzed by using rRNA and rRNA-gene based methods. For example, Diaz et al. (2006) and Zheng et al. (2006) studied the community changes along with the maturation of sludge granules in UASB reactors using rRNA gene-based denaturing gradient gel electrophoresis (DGGE) analysis and FISH. Similarly, Hori et al. (2006) analyzed changes in the microbial community succession in a thermophilic methanogenic reactor under deteriorative and stable conditions, which were induced by acidification and neutralization, using rRNA gene-based single-strand conformation polymorphism (SSCP), quantitative PCR, and FISH. The results indicated that the methanogenic community in the process was significantly affected by volatile fatty acid concentrations.

2.6.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) has the ability to produce millions of copies of a portion of a desired gene, entire gene or gene clusters with high fidelity within 3 to 4 h. It is the most widely used method for the amplification of 16S rRNA, or its gene, prior to fingerprinting studies (Malik et al., 2008). All living cells contain ribosomes, which are part of the cells apparatus for translating deoxyribonucleic acid (DNA) into protein and rRNA is a dominant cellular macromolecule. Most bacterial cells have somewhere between 10^3 and10^5 ribosomes. This natural amplification results in excellent sensitivities of hybridization assays (Suraruk et al., 1998). The cellular RNA content varies depending on the general metabolic activity or growth rate of a given species. Moreover, rRNA are excellent molecules for discerning evolutionary relationships among bacteria because RNA molecules contain conserved and variable regions which make it possible to find general as well as specific target sites for probes. These regions are used for identification purposes. A practical reason for using rRNA is the public availability of large databases. They have enough sequence information to be used as a phylogenetic marker (Borja et al., 1998).

For the detection of organisms or genes from contaminated environments, two variants of the PCR technique are often used: simple PCR and multiplex PCR. Simple PCR uses a pair of primers in a single amplification reaction, while multiplex PCR uses multiple primer pairs simultaneously to amplify several genes in a single reaction (Markoulatos et al., 2002). Multiplex PCR technique was employed for the detection of naphthalene dioxygenase, biphenyl dioxygenase, toluene dioxygenase, xylene monooxygenase, phenol monooxygenase and ring-hydroxylating toluene monooxygenase genes in a single PCR reaction (Baldwin et al., 2003). Multiplex PCR seems can serve time and resources in the detection of microorganisms or genes involved in biodegradation, however, the successful application depends on the combination of several primer pairs being able to perform reliably in a single reaction. Primer dimmer formation between the various primers can occur and this may lead to poor sensitivity and preferential amplification of certain targets (Gilbride et al., 2006).

PCR-based methods have also used in the detection of microbial communities of one-stage anaerobic digestion of two-phase olive mill solid residue. The results showed that at low OLR, Firmicutes, mostly represented by the genus *Clostridium*, were the predominant bacteria and other bacterial communities such as *Gammaproteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Deferribacteres* were the most abundant at high OLR. This study remarks the relating wastewater decomposing bioreactor performance with the microbial communities carrying out the process in order to better understand and monitor this anaerobic digestion (Rincon et al., 2008). PCR amplification is dependent on the extraction and purification of nucleic acids of sufficient yields and quality from environmental samples. In addition, its efficiency can severely be obstructed by the presence of inhibitory substances (Kirk et al., 2004). Therefore, PCR is a very sensitive technique and in some cases may produce false positive or false negative signals due to contamination (Spiegelman et al., 2005).

2.6.2 Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE)

The trend is towards culture independent methods because they are believed to overcome problems associated with selective cultivation and isolation of bacteria from natural samples. Genetic fingerprinting techniques are able to make available a profile representing the genetic diversity of a microbial community from a specific environment. Denaturing gradient gel electrophoresis (DGGE) is possibly the most commonly used among the culture-independent fingerprinting techniques. It is based on the separation of polymerase chain reaction (PCR) amplicons of the same size but of different sequences (Ercolini, 2004).

The most recent applications of this technique are aimed to study the structure and evolution of microbial communities from soil (Anaerobic digestion, 2009). PCR-DGGE has been also used as a tool for identification and typing of microbial. Many other applications of PCR-DGGE in microbial ecology have been previously described and masterfully reviewed by other authors (Schink, 1997). Moreover, the application of PCR and/or sequencing to restriction fragment length polymorphism (RFLP) analysis has also been successfully tested for the identification of red seaweeds (Booksblank et al., 2007).

The microbial community structures of anaerobic sludge, alternately exposed to mesophilic and thermophilic condition, were investigated by Young Chae Song et al, (2006) (Kincs, 1985). PCR-DGGE profiles for the eubacterial and archaeal communities from thermophilic sludge alternately exposed to mesophilic condition (TSEM) and mesophilic sludge alternately exposed to thermophilic condition (MSET) had a few populations in common, these are probably thermophilic microorganisms. The population profiles for archaeal communities of TSEM and MSET were quite different from each other.

2.6.3 Quantitative Real-Time PCR (qPCR)

Reverse transcription (RT) followed by a polymerase chain reaction (PCR) represents the most powerful technology to amplify and detect trace amounts of mRNA (Heid *et al.*, 1996; Lockey, 1998). To quantify these low abundant expressed genes in any biological matrix, the real-time quantitative RT-PCR or qPCR is the method of choice. The advantage of using qPCR is that traditional PCR is measured at End-Point (plateau), while qPCR collects data in the exponential growth phase. An increase in Reporter fluorescent signal is directly proportional to the number of amplicons generated, because of its high sensitivity, high specificity, good reproducibility, and wide dynamic quantification range (Schmittgen et al., 2000; Bustin, 2000). qPCR is the most sensitive and most reliable method, in particular for low abundant transcripts in tissues with low RNA concentrations, partly degraded RNA, and from limited tissue sample (Freeman et al., 1999; Steuerwald et al., 1999; Mackay et al., 2002). The levels of expressed genes may be measured by an ‘absolute’ quantification or by a ‘relative or comparative’ real-time qPCR (Pfaffl, 2004).

The ‘absolute’ quantification approach relates the PCR signal to input copy number using a calibration curve (Fronhoffs et al., 2002). Calibration curves can be derived from diluted PCR products, recombinant DNA or RNA, linearized plasmids, or spiked tissue samples. The so-called ‘absolute’ quantification is misrepresentative, because the quantification is shown relative to the used calibration curve. The mRNA copy numbers must be correlated to some biological parameters, amount of total RNA or DNA, a defined amount of cells, or compared with a reference gene copy number. The ‘absolute’ quantification strategy using various calibration curves and applications are summarized elsewhere in detail (Donald et al., 2005; Lai et al., 2005; Pfaffl et al., 2002).

Whereas relative quantification or comparative quantification measures the relative change in mRNA expression levels, it determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of another RNA. Relative quantification does not require a calibration curve or standards with known concentrations and the reference can be any transcript, as long as its sequence is known (Bustin, 2002).

Quantitative real-time PCR has been use in various environmental studies such as the identification and quantification of the arsenate reductase gene (arsC) in soil and aromatic oxygenase genes (Baldwin et al., 2003; Sun et al., 2004). It also has been used in quantifying the proportion of microorganisms containing alkane monooxygenase and the subsequent assessment of microbial community changes in hydrocarbon-contaminated Antarctic soil (Powell et al., 2006). Methanogenic population dynamics in sludge granule in UASB treating swine wastewater was determined by qPCR. 16S rRNA gene concentrations of the domain archaea were quantified as 1.9×10^7–5.7×10^7 copies ml^{-1}. The orders *Methanobacteriales* ranged from copies ml^{-1} and *Methanosarcinales* from 4.3×10^5 to 1.1×10^6 copies ml^{-1} (Song et al., 2009)