

Research Article

Microbial Communities and Their Performances in Anaerobic Hybrid Sludge Bed-Fixed Film Reactor for Treatment of Palm Oil Mill Effluent under Various Organic Pollutant Concentrations

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The anaerobic hybrid reactor consisting of sludge and packed zones was operated with organic pollutant loading rates from 6.2 to 8.2 g COD/L day, composed mainly of suspended solids (SS) and oil and grease (O&G) concentrations between 5.2 to 10.2 and 0.9 to 1.9 g/L, respectively. The overall process performance in terms of chemical oxygen demands (COD), SS, and O&G removals was 73, 63, and 56%, respectively. When the organic pollutant concentrations were increased, the resultant methane potentials were higher, and the methane yield increased to 0.30 L CH₄/g COD_{removed}. It was observed these effects on the microbial population and activity in the sludge and packed zones. The eubacterial population and activity in the sludge zone increased to 6.4×10^9 copies rDNA/g VSS and 1.65 g COD/g VSS day, respectively, whereas those in the packed zone were lower. The predominant hydrolytic and fermentative bacteria were *Pseudomonas*, *Clostridium*, and *Bacteroidetes*. In addition, the archaeal population and activity in the packed zone were increased from 9.1×10^7 copies rDNA/g VSS and 0.34 g COD-CH₄/g VSS day, respectively, whereas those in the sludge zone were not much changed. The most represented species of methanogens were the acetoclastic *Methanosaeta*, the hydrogenotrophic *Methanobacterium* sp., and the hydrogenotrophic *Methanomicrobiaceae*.

1. Introduction

Palm oil production is the second largest edible oil output and accounts for approximately 23% of the world's fat and oil supply, which is approximately 2.8×10^{10} tons [1]. Palm oil is now not only being used as edible oil, but also in the production of biodiesel as a renewable energy source. In 2009, Thailand consumed 24,872 ktoe of alternative energy sources, and biodiesel accounted for 3.2%, the demand for which is continually increasing [2]. Biodiesel is set as one of the renewable energy sources in Thailand's strategic plan,

which recently used 1.62×10^6 L/day. The target for biodiesel production by the year 2021 is planned to produce 5.97×10^6 L/day [3]. Therefore, the palm oil industry in Thailand is expanding rapidly for production of edible oil, biodiesel, and other applications. The expansion of crude palm oil production will generate additional wastewater at an increasing rate.

Environmental impacts from wastewater of palm oil production known as palm oil mill effluent (POME) are a matter of great concern. POME contains large quantities of high organic pollutants. 42.7 million tons of palm oil were produced globally in 2008 [4]. POME is one of the most

significant pollutants associated with its voluminous production [5]. For every ton of palm oil production, 2.5–3.0 tons of POME is generated [6, 7]. It has been classified as a high-strength wastewater due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), consisting of high organic pollutant concentrations in suspended solids (SS) and lipid or oil and grease (O&G). Fresh POME is an acidic (pH 3.5–4.5), brownish, viscous, and voluminous colloidal suspension being 95–96% water, 10–44 g/L of BOD, 16–100 g/L of COD, 5–45 g/L of SS, 1–15 g/L of O&G, and 0.2–0.5 g/L of total nitrogen [8, 9].

Biological treatment of POME is the most frequently used treatment method. Since it contains high concentrations of organic matter, adoption of anaerobic digestion (AD) in the first stage of the process is needed to convert the bulk of the wastewater to biomethane. AD is a multistep degradation of the organic compounds into biogas, methane, and carbon dioxide, by the action of a microbial consortium [10]. The metabolic reactions that occur during the anaerobic digestion of the substrates involve four important reactions: hydrolysis, acidogenesis, acetogenesis, and methanogenesis [11]. In general of AD, methanogenesis is the rate-limiting step. As such, conventional anaerobic digesters require long hydraulic retention time and large volume reactors to ensure the complete treatment of the influent. Nonetheless, high-rate anaerobic bioreactors have been proposed to reduce reactor volume, shorten retention time, and capture methane gas for utilization. The anaerobic hybrid reactor (AHR), like the upflow anaerobic sludge bed-fixed film reactor, was found to be high performing in COD removal efficiency and methane production [5]. This hybrid system can overcome the existing deficiencies of the original upflow anaerobic sludge blanket (UASB) reactors by shortening the biogranule formation time [12]. Upflow AHRs can work well for the high-suspended solid pollutants like cassava starch wastewater, slaughterhouse waste, and POME [12–15]. Therefore, this study applied AHR, a combination of two zones in the reactor, namely, the sludge zone (part of the microbial granules) and the packed zone (part of the biofilm on the packing material) for the anaerobic treatment of POME.

The major components in biological anaerobic digestion, microorganisms, play an important role as the main function in controlling reactor performance and stability. The performance and stability of an anaerobic digester is directly related to the quantity and quality of the microbial community present in the digester. Furthermore, the operational and environmental parameters of the process obviously affect the microbial behavior resulting in wastewater treatment and biogas production performances [11]. SSs containing palm fiber and O&G are the main organic pollutants in POME. In this study, we looked specifically at the concentrations of these organic pollutants in the organic digestion of AHR. This work focused on the effect of the organic pollutant concentrations on the process performance and stability, that is, the microbial communities and the microbial performance in the sludge and packed zones of the AHR. The work will lead to an understanding of the operational efficiency of the AHR system, depending on the structure of the microbial

TABLE 1: Average composition of POME influents under various operating conditions.

Condition	OLR (g COD/L day)	TCOD (g/L)	SCOD (g/L)	SS (g/L)	O&G (g/L)
C1	6.2	30.6	18.4	5.2	0.9
C2	7.6	38.4	24.6	7.1	1.4
C3	8.2	40.4	28.6	10.2	1.9

Values are averages of three determinations with standard deviations lower than 5% between analyses.

communities present in the system and the environmental conditions needed to control the system.

2. Materials and Methods

2.1. Wastewater Characteristics. Raw POME was collected from a palm oil production plant in Thailand. The raw POME characteristics were determined according to the procedures of the standard methods of wastewater analysis [16]. Its characteristics were in the range of total chemical oxygen demand (TCOD) 57–63 g/L, soluble chemical oxygen demand (SCOD) 40–44 g/L, SS 25–38 g/L, and O&G 9–13 g/L with pH 4.5–4.8. To study the influence of SS combining with O&G concentrations, influent POME was prepared by varying the SS and O&G concentrations into three conditions, C1, C2, and C3. These three operating conditions of the POME were fed into the reactor as shown in Table 1.

2.2. Reactor Configuration and Operation. A schematic diagram of the anaerobic hybrid sludge bed-fixed film reactor (AHR) used in this study is shown in Figure 1. The AHR was made up of an acrylic column with a working volume of 5.0 L. The bottom half of the reactor was occupied by the sludge zone and the upper half was occupied by the packed zone. This packed zone was fitted with nylon fibers having a specific surface area 150 m²/m³ for microbial attachment as biofilm formation. Seven sampling ports were distributed at several heights in the sludge and packed zones in the AHR. The influent was fed upflow from the bottom to the upper part of the AHR, and the treated wastewater was discharged through an effluent port. The AHR was initially fed with 5.0 L of diluted POME and inoculated with startup seed, which was collected from the AD of the POME treatment at a final concentration of 5.0 g VSS/L. For acclimatization of the startup seed to a new environment, 1 L of diluted POME influent was fed semicontinuously once a day with a hydraulic retention time (HRT) of 5 days for 20 days. Upon reactor startup operation, three experiments were set up by increasing the organic pollutant concentrations to C1, C2, and C3 (Table 1). The influent was upflow fed semi-continuously at constant HRT of 5 days. The reactor was operated in each condition until the process performance reached a steady state and was kept continually running for more than 3 cycles of HRT.

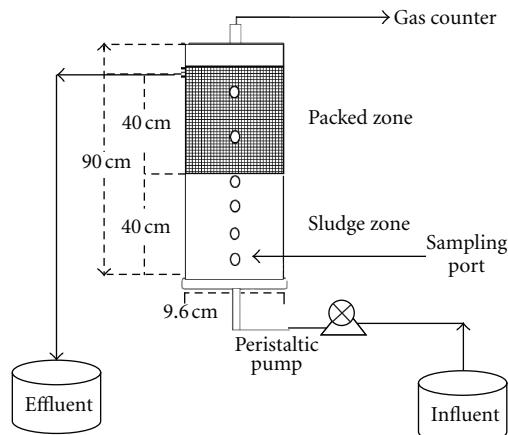


FIGURE 1: Schematic diagram of the laboratory-scale AHR.

The process performance of the sludge and packed zones, as well as the overall AHR, was routinely monitored through measurement of the TCOD, SCOD, alkalinity (Alk), total volatile acid (TVA), O&G, SS, and the pH of the effluent POME. The TCOD, SCOD, SS, Alk, and TVA analyses were carried out according to the procedures of the standard methods of wastewater analysis [16]. The O&G was analyzed by Soxhlet with hexane extraction according to AOAC methods [17]. The pH and biogas production were determined daily. The remaining parameters were measured three times a week throughout the steady-state period to ensure that representative data were obtained. Biogas composition was determined using gas chromatography [18].

2.3. Microbial Characteristics. At the end of each operating condition of C1, C2, and C3, suspended sludge samples from the sludge zone and the attached biofilm samples from the supporting media in the packed zone were collected. Microbial characteristics were determined by PCR-DGGE. DNA sequencing was carried out to determine the microbial community; 16S rDNA quantitative real-time PCR was used for the microbial quantity; microbial activity of non-methanogens (eubacteria: EUB) and methanogens (archaea: ARC) was used for the microbial qualities.

2.3.1. PCR-DGGE and DNA Sequencing. To study the microbial communities, samples were collected aseptically from the reactor and immediately stored in a freezer for community analysis and prepared with a centrifugation method prior to DNA extraction. DNA extraction and amplification [19] and denaturing gradient gel electrophoresis (DGGE) were performed as previously described [20]. Sequences were initially compared to known 16S rRNA gene sequences in the GenBank database using the BLASTn to locate nearly exact matches in the GenBank database [21].

2.3.2. 16s rDNA Quantitative Real-Time PCR. Copy numbers of 16S rDNA of EUB and ARC were quantified by relative quantification real-time PCR (qPCR). A KAPA SYBR Fast qPCR Kit was used for real-time reactions (KAPA,

Brazil). The qPCR 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 eubacteria and methanobacteria as standard EUB and ARC stains, respectively. 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 [20]. Amplicons of EUB and ARC were cloned to plasmid vector (pGEM-T Easy vector, Promega) and inserted in chemically competent cells (*E. coli* DH5 α). Plasmids DNA were serially diluted in the range of 10^2 – 10^7 copies rDNA/ μ L and used as templates for qPCR with primers and amplification protocol as defined. The copy concentrations were calculated using the method of Whelan et al. [22].

2.3.3. Microbial Activity. Determination of microbial activity was carried out in triplicate using 120 mL vials with 50 mL of working volume. The inoculum-substrate ratio in the final volume was 30:70 v/v. Glucose and acetate were used as the substrates for activity analysis of the EUB and ARC, respectively. Determination of microbial activity was performed using the method of Nopharatana et al. [23].

2.4. Statistical Analysis. In this study, the standard errors were all within 5% of the mean value. A test of significant difference based on the paired t-statistic was performed using the MINITAB software (Minitab Inc., USA). The difference was regarded as nonsignificant if the paired t-statistic showed Probability, $P > 0.05$ and significant if $P \leq 0.05$.

3. Results and Discussion

3.1. Reactor Performances and Stabilities. The AHR was operated for 144 days consecutively by increasing the organic pollutant (SS and O&G) concentrations under the three conditions, C1, C2, and C3 (Table 1). The operating dates for the C1, C2, and C3 conditions were 40, 51, and 53 days, respectively. Once each operating condition reached a steady state of process performance, the reactor was run for a further 20 to 25 days (>3 cycles of HRT) to ensure that representative data from the steady-state period were obtained and could yield shown values in mean and standard deviation from ten to fifteen determinations. Figure 2 shows the overall process performance during the steady state of the anaerobic bioreactor under C1, C2, and C3 conditions. Production of biogas was obtained at 2100 ± 60 , 3300 ± 35 , and 6400 ± 75 mL/day for C1, C2, and C3, respectively. The methane yield coefficient is defined as the ratio of methane produced in this experiment to the COD utilized. The methane content in the biogas was approximately $65 \pm 2\%$. The paired t-test analysis of the methane yielded ($P \leq 0.05$), indicating that C1, C2, and C3 produced methane yield at significantly different rates. The methane yield increased corresponding to the increase of the SS and O&G concentration. Under C1 and C2, methane yields of 0.13 ± 0.01 and

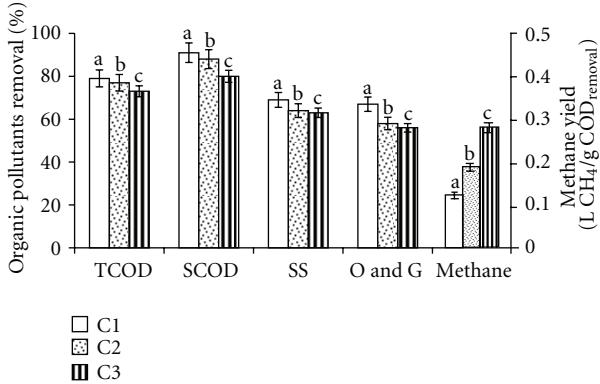


FIGURE 2: Overall process performances in organic pollutants removal and methane yields.

0.20 ± 0.01 L CH₄/g COD_{removed}, respectively, were obtained. Under C1 operation, a low methane yield was obtained, which was similar to the result found by Chaiprasert et al. [14] and Poh and Chong [5], likely due to the organic carbon (COD) being consumed by microorganisms to build more cells during the initial startup period. Under the C3 operation, the methane yield was up to 0.30 ± 0.02 L CH₄/g COD_{removed}. This value was close to the theoretical methane yield of 0.35 L CH₄/g COD_{removed}. Considering a theoretical methane yield, the whole of the organic matter is transformed into methane, accounting for virtually negligible biomass growth and cell maintenance [24]. The AHR in this study showed the high effectiveness of the this reactor in converting the POME into methane at mesophilic temperatures with an organic loading rate (OLR) of 8.2 g COD/L day consisting of 10.2 g SS/L and 1.9 g O&G/L with 5 days of HRT.

The process performance in terms of the efficiency of TCOD and SCOD removal was higher than 70%. The SCOD was easily biodegradable and almost completely removed while the TCOD removal efficiency was between 70–80%. Insoluble COD in the form of SS and O&G was slightly harder to biodegrade, and its removal was lower than 70% (Figure 2). The SS removal efficiency varied in the range of 60 to 70%. SS is one of major organic components of POME. This suspended organic matter is cell walls, short fibers, hemicelluloses, and nitrogenous compounds of proteins, which are less biodegradable and require a longer retention time for satisfactory digestion [25]. In addition, the O&G or lipid content was one of the least biodegradable organic materials. The increase of O&G concentration from the C1 to the C3 operation showed a decrease in O&G removal. An inhibitory AD process by lipids was found in the other studies and indicated as an impeding step of hydrolysis. The overall conversion rate was limited either by degradation of long-chain fatty acids (LCFAs) or by the physical processes of dissolution and mass transfer of these acids [26–28]. However, the removal efficiency of O&G in this study was in the range of 50 to 60%. O&G was generally hydrolyzed by bacterial enzymes under anaerobic conditions at an optimum pH value to a neutral value [29, 30]. The slightly high

O&G removal obtained in this study might arise from the suitable pH and stability of process with the ratio of TVA/Alk < 0.4. The values of pH and ratio of TVA/Alk were 6.9 ± 0.4 and 0.35 ± 0.02 , respectively. At this point, the environmental condition in the AD process can control the system to neutralize the pH with a high buffer capacity and less acidification risk leading to the high process stability [31].

Each zone of the AHR, the sludge and packed zones, was monitored and considered for its process performance and stability, as shown in Figures 3 and 4, respectively. Removal of the TCOD, SS, and O&G in the sludge zone (Figure 3(a)) varied with an increase of the organic pollutant concentration. With a low organic pollutant concentration under the C1 condition, organic removal of TCOD, SS, and O&G was more than 70%. When the reactor was operated under the C2 and C3 conditions, increasing the organic pollutants, the removal decreased to 50%. High concentrations of SS and O&G in the POME influent caused a reduction in their removal because of the complex structure and less biodegradable compounds. Remaining undigested matter, which settled and accumulated in the sludge zone, was observed. Under the C2 and C3 operating conditions, TVA concentration sharply increased and was detected at 1400 ± 15 mg CH₃COOH/L in the sludge zone (Figure 4(a)). Increasing the TVA concentration indicated that hydrolysis and acidogenesis had occurred in this sludge zone. The increase in the TVA concentration led to an increase in the ratio of TVA/Alk to 0.66 ± 0.05 , and the pH was decreased to 6.5 ± 0.1 (Figure 4(a)). A high TVA concentration in the sludge zone affected O&G removal because an inappropriate pH level inhibited the activity of the extracellular lipase enzyme.

Organic pollutant removal in the packed zone is shown in Figure 3(b). High organic pollutant removal was achieved (>60%), possibly due to the lower molecular weight or shorter chain compounds in the TCOD, SS, and O&G obtained from the sludge zone, where they were more easily digested by anaerobic microorganisms. In the packed zone, the TVA concentration was lower than 800 ± 10 mg CH₃COOH/L, leading to the pH and the ratio of TVA/Alk increasing to 6.9 ± 0.2 – 7.4 ± 0.2 and 0.20 ± 0.01 – 0.50 ± 0.02 , respectively (Figure 4(b)). This environmental condition in the packed zone was suitable for supporting methanogens. Microbial consortiums were attached and grew on supporting media as biofilm in the packed zone, and they biodegraded organic acids to methane. The result was similar to the study of Suraruksa et al. [32], and this phenomenon showed that methanogenesis took place in the packed zone. The microbial community and characteristics will be explained further below.

Moreover, this study further increased the organic loading rate (OLR) from 8.2 (C3) to 9.6 g COD/L day consisting of 11.3 g SS/L and 2.3 g O&G/L. It was found that the process performance, that is, the organic pollutants removal and biogas production, was decreased, and the process stability tended to be unstable (data not shown). A short operation was run, and the reactor started to fail. This AHR with 50% of suspended growth and 50% of attached growth by volume

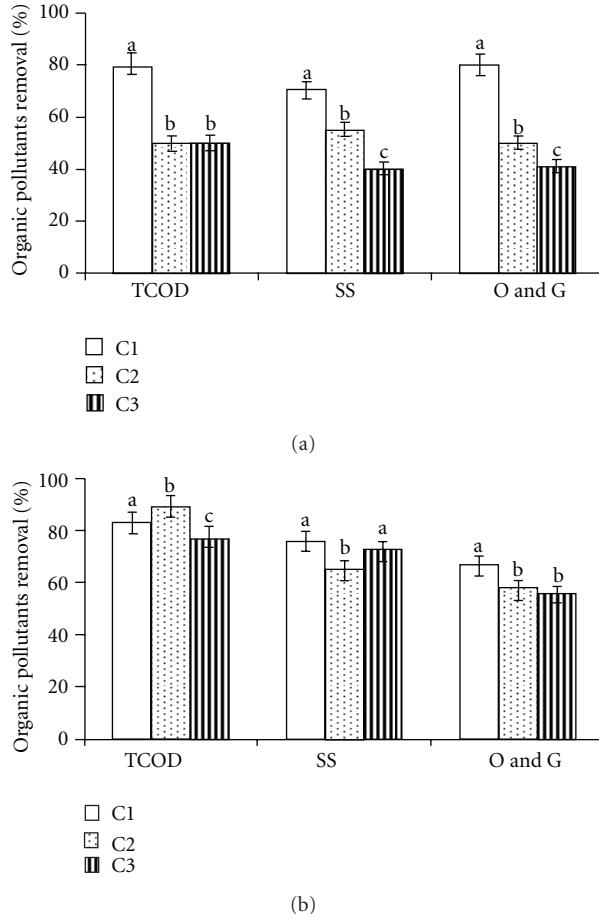


FIGURE 3: Process performances in (a) the sludge zone and (b) the packed zone of AHR.

can load high proportions of organic matter to 8.2 g COD/L day containing high SS (10.2 g/L) and O&G (1.9 g/L). High process performance and stability were found. The capacity of organic loading fed to a high-rate anaerobic hybrid reactor for POME in this study was close to that in the study of Najafpour et al. [12].

3.2. Eubacterial and Archaeal Community. PCR-DGGE targeting the 16S rRNA genes of EUB and ARC was performed to investigate the microbial communities in the sludge and packed zones of the AHR at the operating conditions C1, C2, and C3. The DGGE profiles of the EUB (lane S, A-F) and the ARC (lane G-M) communities from all the samples in the startup seed and both the sludge and packed zones are illustrated in Figures 5(a) and 5(b), respectively. From Figures 5(a), and 5(b), the EUB band patterns were more complicated than those of the ARC due to the relatively higher diversity of domain EUB in most microbial complexes of hydrolytic, acidogenic, and acetogenic bacteria [33]. The partial 16S rRNA gene fragments from the selected bands in the DGGE profiles, twenty-two EUB and fifteen ARC bands, were sequenced, and affiliations were determined by comparison with the Genbank (Tables 2 and 3).

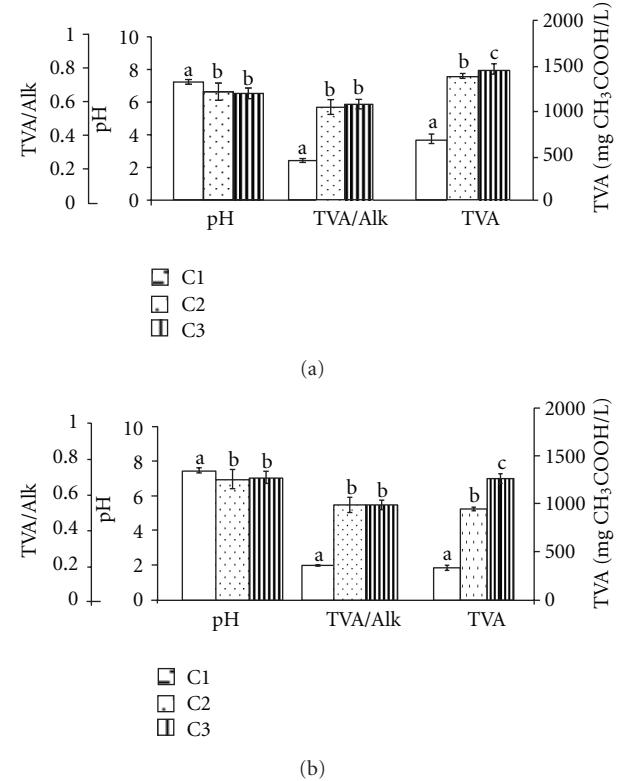


FIGURE 4: Process stabilities in (a) the sludge zone and (b) the packed zone of AHR.

In the startup seed, fourteen DGGE bands of the EUB communities (S1-14) and five bands of ARC communities (G1-5) as shown in Figures 5(a), and 5(b), respectively, were observed. These EUB communities were represented by hydrolytic bacteria—*Pseudomonas* sp. (S1, S3, S5-6, and S8-9) and Uncultured γ -proteobacterium (S10), acidogenic bacteria—uncultured *Bacteroidetes* bacterium (S7, S11-13), and acetogenic bacteria—*Clostridiales* bacterium (S2), *Acetobacter* sp. (S14), and uncultured *Actinobacterium* (S4), as shown in Table 2. Three of the five ARC bands were closely related to *Methanobacterium* sp. (G1-2) and *Methanomicrobiaceae* (G4), which were classified as hydrogenotrophic methanogens. The other two bands were closely related to the uncultured *Methanococcoides* sp. (G5) and uncultured *Methanosaeta* sp. (G3) belonging to the acetoclastic methanogens (Table 3).

The initial C1 condition was operated with the startup seed in the AHR for 40 days. The EUB communities in the packed zone (lane A) and in the sludge zone (lane B) were analyzed and are shown in Figure 5(a) and Table 2. Similar in the DGGE profiles of the EUB between the startup seed and C1, we found in S1-A1-B1 (*Pseudomonas* sp. M130), S5-A4-B4 and S8-A7-B7 (*Pseudomonas* sp.), S7-A6-B6, S11-A9-B8, S12-A10-B9, and S13-A11-B10 (uncultured *Bacteroidetes* bacterium), S2-A2-B2 (*Clostridiales* bacterium), and S14-A12-B11 (*Acetobacter* sp.). The band intensity of A3, B3, and A8 increased during the operation period under C1

TABLE 2: The partial 16S rRNA gene sequences of EUB domain and organism with the best-matching sequences determined by BLAST searches.

Affiliation	Seed	DGGE band						Similarity (%)	Accession no.	
		C1	C2		C3					
Hydrolytic bacteria										
<i>Pseudomonas</i> sp. M130	S1	A1	B1	C1	D1	E1	F1	92	AB088750.2	
<i>Pseudomonas entomophila</i> str. L48	S3		B3	C3	D3			92	CT573326.1	
<i>Pseudomonas</i> sp.	S5	A4	B4					97	AM886092.1	
<i>Pseudomonadaceae bacterium</i>	S6	A5	B5					94	AB545745.1	
<i>Pseudomonas</i> sp.	S8	A7	B7	C8	D8	E7	F6	97	AM886092.1	
<i>Pseudomonas pseudoalcaligenes</i>	S9	A8			D9			79	AF140011.1	
Uncultured γ -proteobacterium	S10							82	EU167353.1	
Uncultured <i>Firmicutes</i> bacterium				C5	D5			94	FM896934.1	
Uncultured γ -proteobacterium				C9		E9	F8	82	EU167353.1	
Uncultured γ -proteobacterium						E8	F7	82	EU167353.1	
Uncultured <i>delta</i> -proteobacterium						E10		73	FN429803.1	
Acidogenic bacteria										
Uncultured <i>Bacteroidetes</i> bacterium	S7	A6	B6			E6	F5	94	CU926845.1	
Uncultured <i>Bacteroidetes</i> bacterium	S11	A9	B8	C10	D10		F9	98	EU810898.1	
Uncultured <i>Bacteroidetes</i> bacterium	S12	A10	B9			E11	F10	76	GU955023.1	
Uncultured <i>Bacteroidetes</i> bacterium	S13	A11	B10	C11	D11	E12	F11	91	GU955023.1	
Uncultured <i>Bacteroidetes</i> bacterium				C6	D6	E5		86	AB433139.1	
Uncultured <i>Bacteroidetes</i> bacterium				C7	D7			94	CU926845.1	
<i>Bacteroides</i>						E3	F3	87	EU136682.1	
Acetogenic bacteria										
<i>Clostridiales</i> bacterium	S2	A2	B2	C2	D2	E2	F2	81	GU428556.1	
Uncultured <i>Actinobacterium</i>	S4	A3		C4	D4			91	GU194237.1	
<i>Acetobacter</i> sp.	S14	A12	B11	C12	D12	E13		98	GQ246703.1	
Uncultured <i>Clostridiaceae</i> bacterium						E4	F4	74	AB218300.1	

Remark: S: startup seed; A: packed zone and B: sludge zone at C1; C: packed zone and D: sludge zone at C2; E: packed zone and F: sludge zone at C3.

TABLE 3: The partial 16S rRNA gene sequences of ARC domain and organism with the best-matching sequences determined by BLAST searches.

Affiliation	Seed	DGGE band						Similarity (%)	Accession no.	
		C1	C2		C3					
Acetoclastic methanogens										
Uncultured <i>Methanococcoides</i> sp.	G5		I8	J9	K11	L12	M8	89	AY454739.1	
Uncultured <i>Methanosaeta</i> sp.	G3	H6	I6	J6	K8	L9	M6	85	AY454766.1	
<i>Methanocaldococcus vulcanius</i> M7			I2		K2	L2	M2	90	CP001787.1	
Hydrogenotrophic methanogens										
<i>Methanobacterium</i> sp.	G1	H4	I4	J4	K6	L7	M4	93	GU936489.1	
<i>Methanobacterium</i> sp.	G2	H5	I5	J5	K7	L8	M5	95	GU569395.1	
<i>Methanomicrobiaceae</i>	G4	H7	I7	J7	K9	L10	M7	87	GU129124.1	
Uncultured <i>Methanomicrobiales</i>		H1	I1	J1	K1	L1	M1	95	AY780566.1	
<i>Methanospirillum hungatei</i>		H2						99	AB517987.1	
<i>Methanobacterium</i> sp.		H3	I3	J3	K4	L4	M3	97	GU936489.1	
<i>Methanobacteriaceae</i>					K5			89	GU129060.1	
<i>Methanoculleus</i> sp.				J8	K10	L11		96	AB436897.1	
<i>Methanobacterium palustre</i>						L5		91	EU293795.1	
<i>Methanobacterium</i> sp.						L6		95	GU569395.1	

Remark: G: startup seed; H: packed zone and I: sludge zone at C1; J: packed zone and K: sludge zone at C2; L: packed zone and M: sludge zone at C3.

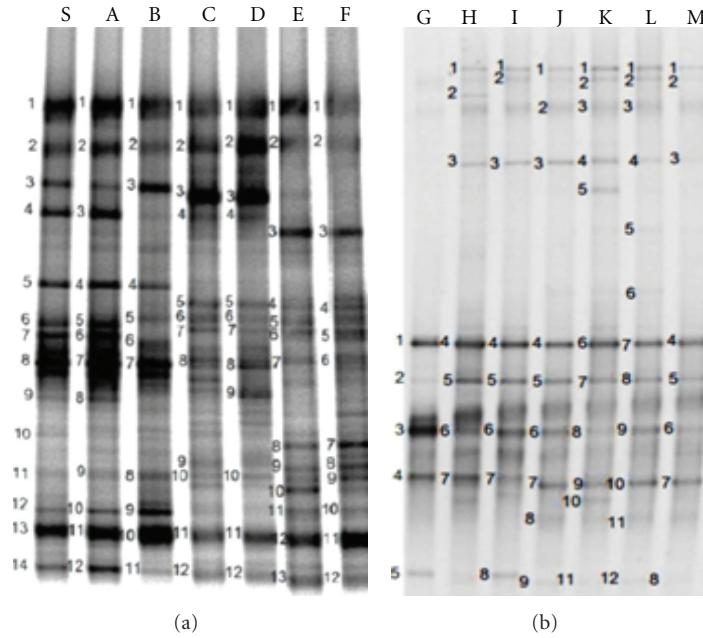


FIGURE 5: DGGE profiles of (a) EUB and (b) ARC of all reactor operations. Remark: (a) DGGE profile of EUB; S: startup seed; A: packed zone B: sludge zone at C1; C: packed zone and D: sludge zone at C2; E: packed zone and F: sludge zone at C3; (b) DGGE profile of ARC; G: startup seed; H: packed zone and I: sludge zone at C1; J: packed zone and K: sludge zone at C2; L: packed zone and M: sludge zone at C3.

(Figure 5(a)). A3, B3, and A8 were closely related to uncultured *Actinobacterium*, *Pseudomonas entomophila str. L48*, and *Pseudomonas pseudoalcaligenes*, respectively. The major EUB communities of hydrolytic, acidogenic, and acetogenic bacteria in the startup seed also existed during the initial reactor startup with slightly increased organic pollutant concentration (C1 condition) which confers community stability during the beginning stage of anaerobic waste treatment [34, 35].

The DGGE band profiles showed some changes in the EUB community structure during the operations of the C2 and C3 conditions with the increase of the organic pollutant concentration. The EUB communities in the packed and sludge zones under operating conditions C2 and C3 are shown in C-D and E-F, respectively, (Figure 5(a)). Three hydrolytic bacteria of unculture *Firmicutes bacterium* (C5, D5), Uncultured γ -*proteobacteria* (C9, E8-9, F7-8), and uncultured *delta proteobacterium* (E10) were first detected in the DGGE band intensity under these conditions. Some of the initially predominant EUB bands, B3-C3-D3, and A8-D9, under C1 and C2 conditions became practically undetectable in the DGGE profile after increasing the organic pollutants to the C3 condition. Those sequence were related to *Pseudomonas entomophila str. L48* and *Pseudomonas pseudoalcaligenes*, respectively. The sharp intensity bands of E3-F3 and E8-F7 were detected after increasing the organic pollutant to the C3 condition. These were related to *Bacteroides* and Uncultured *Clostridiaceae bacterium* in the groups of hydrolytic and acetogenic bacteria, respectively. The DGGE bands of E4-F4 (Uncultured *Clostridiaceae bacterium*) in the operating condition of C3 were also detected. *Pseudomonas*

sp. M130 (A1-F1), Uncultured *Bacteroidetes bacterium* (A11-B10-C11-D11-E12-F11), and *Clostridiales bacterium* (A2-F2) were observed throughout the operating conditions of C1 to C3 (Figure 5(a) and Table 2). The hydrolytic bacteria for lipid decomposition, *Pseudomonas* sp., is one of the predominant bacteria in the anaerobic digestion system. It has an ability to produce extracellular lipase enzymes that hydrolyze triglycerides to fatty acids and glycerol, and is generally found in lipid-contaminated wastewater [36]. With an increase of the organic pollutant of O&G concentration at 1.9 g/L under the C3 operating condition, *Pseudomonas entomophila str. L48* and *Pseudomonas pseudoalcaligenes* disappeared because of the inhibition of the O&G. However, *Pseudomonas* sp. M130 was found to exist in O&G at the concentration of 0.9–1.9 g/L during the operation of the C1 to C3 conditions. Moreover, with the increase of organic loading from C1 to C3, the SS concentration also increased, and γ -*proteobacteria* was found to be dominant at the high SS concentration of 10.2 g/L (C3 condition). This is a common representative of the microbial communities in anaerobic processes of solid substrates [37]. Uncultured *Bacteroidetes bacterium*, as an acidogenic bacterium, was only one species found under all operation conditions (C1–C3) in this study, and it is one of the major microbial components of acidogenesis in AD, as shown in other studies [38–40]. In acidogenesis, simple organic compounds are transformed into fermentation endoproducts such as lactate, propionate, acetate, and ethanol including H₂ and CO₂. This acidogenic bacterium can exist in low and high TVA concentrations, as found in this study at the range of 500–1500 mg CH₃COOH/L (Figure 4). The detected acetogenic bacteria were members

of *Firmicutes*, mostly represented by *Clostridiales bacterium* and *Acetobacter* sp., which were found in the sludge and packed zones throughout the reactor operation. The exception was Uncultured *Clostridiaceae bacterium*, which was only found under the C3 condition. The acetate product from this acetogenic activity was transformed into methane by methanogens [41]. However, Firmicutes and Actinobacteria are known to produce cellulases, lipases, proteases, and other extracellular enzymes [39], suggesting they are also involved in hydrolysis through acetogenesis.

The DGGE band profiles and sequenced bands of ARC during the C1–C3 operations are shown in Figure 5(b) and Table 3, respectively. At the beginning of the reactor operation (C1), Uncultured *Methanosaeta* sp. (G3-H6-I6), *Methanobacterium* sp. (G1-H4-I4 and G2-H5-I5), and *Methanomicrobiaceae* (G4-H7-I7) in the startup seed were also detected in both the sludge and packed zones, with the exception of Uncultured *Methanococcoides* sp. (G5-I8), which was found only in the sludge zone. An increase of ARC diversity was observed as the first detected bands of I2 and H1-3. These were represented by *Methanocaldococcus vulcanius* M7 for acetoclastic methanogens and Uncultured *Methanomicrobiales*, *Methanospirillum hungatei* and *Methanobacterium* sp. for hydrogenotrophic methanogens. During the increase of organic pollutants from the C1 to the C2 and C3 operations, Uncultured *Methanosaeta* sp. (H6-I6-J6-K8-L9-M6), *Methanobacterium* sp. (H4-I4-J4-K6-L7-M4 and H5-I5-J5-K7-L8-M5), *Methanomicrobiaceae* (H7-I7-J7-K9-L10-M7) and Uncultured *Methanomicrobiales* (H1-I1-J1-K1-L1-M1) were found in both the sludge and packed zones. The low intensity of the DGGE bands I8-J9-K11-L12-M8 belonged to Uncultured *Methanococcoides* sp.; *Methanosaeta* sp. Is one of the acetoclastic methanogens presented in the startup seed and all operating conditions. *Methanosaeta* sp. is commonly found in stable anaerobic digestion systems and often represents the major ARC in methanogenic communities [36, 42]. More diversity of hydrogenotrophic methanogens was detected with an increase in the organic pollutant concentration (Table 3). Not only *Methanobacterium* sp. and *Methanomicrobiaceae*, but also other hydrogenotrophic methanogens such as *Methanobacteriaceae* (K5), *Methanoculleus* sp. (J8-K10-L11), *Methanobacterium palustre* (L5), and *Methanobacterium* sp. (L6) were also found among the sludge and the packed zones of the AHR. Two types of methanogenic ARC, acetoclastic and hydrogenotrophic methanogens were found under all the operating conditions for this study. Methanogenic acetate degradation was converted to methane by acetoclastic methanogens (Uncultured *Methanosaeta* sp.), whereas hydrogen and CO₂ were carried out by hydrogenotrophic methanogens (*Methanobacterium* sp, *Methanomicrobiaceae*, and Uncultured *Methanomicrobiales*). During the methanogenic mineralization process, oxidation of reduced compounds (alcohols and short-chain fatty acids) by acidogenic bacteria and/or acetogenic bacteria is thermodynamically unfavorable. The oxidation of these reduced compounds can proceed only if hydrogen partial pressure is kept low by coupling with hydrogen consuming methanogenesis [43]. Thus, interspecies hydrogen transfer between syntrophic fatty

acid-oxidizing bacteria (Uncultured *Bacteroidetes bacterium*, *Clostridiales bacterium*, and *Acetobacter* sp.) and the hydrogenotrophic methanogens appeared for the oxidation of these substrates. Acetate is one of the most important intermediates for the methane production step in the anaerobic mineralization of organic substrates. Hattori [44] reported that methanogenic acetate degradation is carried out by either an acetoclastic reaction or an anaerobic acetate-oxidizing reaction (syntrophic acetate oxidation and hydrogenotrophic methanogenesis). *Methanosaeta* sp. has a high affinity for acetate, and the growth of this ARC is affected by the concentration of acetate. In addition, high concentrations of ammonia and volatile fatty acids are considered important factors for acetate metabolism. Acetoclastic methanogens are also known to be more sensitive to these compounds than hydrogenotrophic methanogens. Some studies [45, 46] found syntrophic acetate-oxidizing bacteria in the class of Clostridia within the phylum Firmicutes (acetate-oxidizing bacteria strain AOR and *Clostridium ultunense*) and hydrogenotrophic methanogens (*Methanobacterium* and *Methanoculleus* sp.) for methane production under these stresses. We seemed to observe a similar result in the operation of C3 for high concentration of SS and O&G influent. There was the first detection of Uncultured *Clostridiaceae bacterium* within the phylum of Firmicutes in both the sludge zone (F4) and the packed zone (E4) (Figure 5(a) and Table 2) and hydrogenotrophic methanogens (*Methanobacterium paluster*, L5, *Methanobacterium* sp., L6, and *Methanoculleus* sp., L11) in the packed zone (Figure 5(b) and Table 3).

3.3. Qualitative and Quantitative Microorganism. Quantitative changes in the 16S rRNA gene concentration were determined by real-time PCR, and microbial quality was studied by microbial activity determination in the sludge and packed zones under the three operating conditions. The microbial populations and activity of EUB and ARC are described in Table 4. Among the operating conditions of C1, C2, and C3, there was an evident variation in the EUB and ARC populations and activity in the sludge and packed zones of AHR. The level of concentration of organic pollutants had an effect on the microbial populations and activity as well as on the structure of the microbial communities in each zone. This result will impact the environmental condition (TVA/Alk and pH) and the process performance. Microbial populations in the startup seed, the 16S rRNA gene concentrations of the EUB and ARC were 1.2×10^7 and 8.2×10^4 copies rDNA/g VSS, while their activity were 1.00 g COD/g VSS a day and 0.12 g COD-CH₄/g VSS a day, respectively. At the beginning of the reactor operation (C1), the overall microbial populations increased, resulting mostly from organic carbon being utilized for microbial cell development. The paired *t*-test analysis of the EUB and ARC activity ($P \leq 0.05$) indicated that under each of the conditions C1, C2, and C3 there was significantly different microbial activity in the reactor operation. The activity of the EUB and ARC in the sludge zone were close to those in the startup seed, while those in the packed zone were different.

TABLE 4: Microbial populations and activities in startup seed, sludge, and packed zones of AHR under various operating conditions.

Condition/zone	EUB		ARC	
	Population (Copies rDNA/g VSS)	Activity (g COD/g VSS day)	Population (Copies rDNA/g VSS)	Activity (g COD-CH ₄ /g VSS day)
Startup seed	1.2×10^7	1.00	8.2×10^4	0.12
Sludge zone				
C1	$3.5 \times 10^{8^a}$	1.08 ^a	$1.1 \times 10^{5^a}$	0.10 ^a
C2	$4.4 \times 10^{8^b}$	1.58 ^b	$4.8 \times 10^{5^b}$	0.11 ^b
C3	$6.4 \times 10^{9^c}$	1.65 ^c	$7.5 \times 10^{5^c}$	0.14 ^c
Packed zone				
C1	$9.0 \times 10^{7^a}$	0.43 ^a	$3.3 \times 10^{5^a}$	0.18 ^a
C2	$4.3 \times 10^{8^b}$	0.74 ^b	$8.4 \times 10^{5^b}$	0.25 ^b
C3	$8.6 \times 10^{8^c}$	0.91 ^c	$9.1 \times 10^{7^c}$	0.34 ^c

Values are the averages of three determinations taken at the end of operating conditions.

Averages followed by the different letter in the same column among operating condition C1–C3 are statically different at 95% level by the paired *t*-test.

EUB activity decreased and ARC activity slightly increased compared to that in the startup seed. When the organic pollutant concentration was increased to the C2 and C3 conditions, the population and activity of the EUB and ARC in the sludge and packed zones were investigated for the development of these characteristics and compared to those under the C1 conditions. In the sludge zone, the EUB population and activity were significantly increased from 3.5×10^8 to 6.4×10^9 copies rDNA/g VSS, and 1.08 to 1.65 g COD/g VSS, respectively, while the ARC population and activity were slightly increased from 1.1×10^5 to 7.5×10^5 copies rDNA/g VSS and 0.10 to 0.14 g COD-CH₄/g VSS a day. In addition, when comparing the packed and the sludge zones, the EUB population had decreased slightly, and the EUB activity was lower, whereas the ARC population and activity were higher, from 10^5 to 10^7 copies rDNA/g VSS and 0.1 to 0.3 g COD-CH₄/g VSS a day. The sludge zone is the bottom part of the AHR, where there is the first contact with the influent POME. In this zone, most of the organic carbon was hydrolyzed to a simpler molecule and then converted to volatile fatty acids by hydrolytic, acidogenic and acetogenic bacteria. This resulted in the high TVA concentration and TVA/Alk ratio (Figure 4(a)). Contrary to the ARC characteristics, a higher population and activity were found in the microbial biofilm of the packed zone (Table 4). The packed zone was located in the top part of the AHR, where the remaining organic compounds were continuously converted to short chains of volatile fatty acids. This was reflected in the low activity of EUB, while the dominant ARC characteristics were evident in this packed zone. The ARC activity in the packed zone under operating conditions C1 to C3 was increased from 0.18 to 0.34 g COD-CH₄/g VSS a day. This might relate to the uncomfortable environment for these ARC in the sludge zone due to the high TVA concentration, lower pH, and high TVA/Alk; whereas, the dominant ARC biofilm can resist more than that in suspended cells and was able to utilize the TVA for its growth and methane production in the packed zone. This kept the process stable, with pH 6.9 ± 0.4 and TVA/Alk 0.35 ± 0.05 . An increased ARC population and activity reflected the low TVA concentration

($<800 \pm 10$ mg CH₃COOH) with a pH in the neutral range and a lower TVA/Alk ratio (<0.5) in the packed zone. More methanogenesis occurred in this zone. The action of the sludge and packed zones, which work as hydrolysis or fermentative and methanogenesis zones, respectively, were mostly responsible for a properly enhanced reactor performance and maintained the process stability of the AHR. For POME, anaerobic digestion could be achieved completely within one reactor of an anaerobic hybrid reactor.

4. Conclusions

The process performance and stability, as well as the microbial characteristics, varied according to the organic pollutant concentrations. The organic pollutants were studied at OLR of 6.2–8.2 g COD/L day consisting of 5.2–10.2 g SS/L and 0.9–1.9 g O&G/L. The AHR utilized in the study can handle the OLR to 8.2 g COD/L day containing 10.2 g SS/L and 1.9 g O&G/L with high performance and stability. Process stability in terms of the TVA/Alk ratio and pH was in the range of 0.2–0.5 and 6.5–7.0, respectively. The increase of organic pollutant concentration affected the EUB and ARC communities, populations, and activity in the sludge and packed zones, which was reflected in the organic removal of TCOD, SS, and O&G. Throughout the graduated C1 to C3 operating conditions, high organic hydrolysis/fermentation took place in the sludge zone of the AHR, and the dominant eubacteria were represented by *Pseudomonas* sp., Uncultured *Bacteroidetes* bacterium, and *Clostridiales* bacterium. Methane was produced from both of acetoclastic and hydrogenotrophic methanogens. The dominant archaeal bacteria found under all the operating conditions related to acetoclastic methanogens as Uncultured *Methanosaeta* sp. and hydrogenotrophic methanogens as *Methanobacterium* sp., *Methanomicrobiaceae*, and Uncultured *Methanomicrobiales*. Higher levels of archaeal population and activity were found in the packed zone within the microbial biofilm. From the results of the microbial characteristics, this implied that the sludge and packed zones in the AHR acted overall as acidification and methanation zones, respectively.

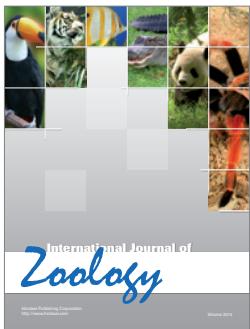
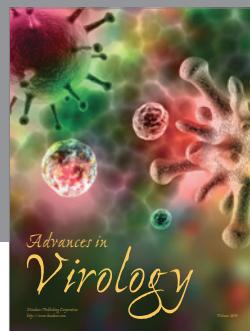
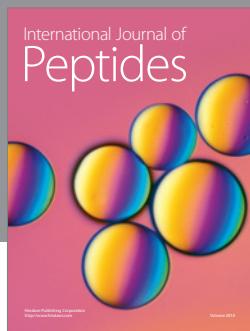
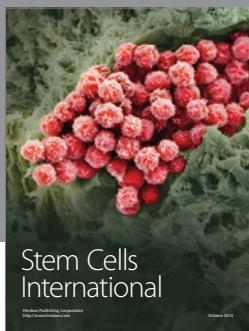
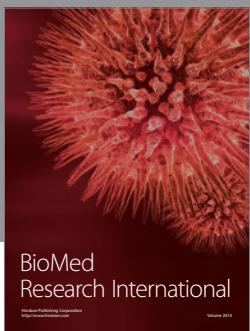
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