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Comparative Granular Characteristics of Mesophilic and Thermophilic UASB Producing Biogas from Palm Oil Mill Effluent

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This study investigated granular characteristics and microbial profiles in granules from the upflow anaerobic sludge blanket (UASB) reactor treating palm oil mill effluent at a thermophilic temperature of 60° C in comparison with a mesophilic UASB. Phylogenetic analysis of microbial communities in sludge granules from both reactors based on bacterial 16S rRNA and archaeal *mcr*A genes revealed different bacterial and archaeal communities under the different operating conditions. The bacterial community under the mesophilic condition was composed mainly of *Proteobacteria*, *Firmicutes* and *Chloroflexi* while a less complex community comprising mainly *Proteobacteria* and *Actinobacteria* was present under the thermophilic condition. The mesophilic methanogen assemblage was found to be comprised mainly of those related to uncultured methanogens in class *Methanomicrobiales* and *Methanobacteriales*. The thermophilic methanogen community was dominated by those related to class *Methanosarcinales* in addition to some *Methanobacteriales*. Most were closely related to *Methanosaeta thermophilia*. The microbial community structures suggested that different methanogenesis pathways dominated under the mesophilic and thermophilic conditions and implied different capabilities of extracellular polymer production which affected reactor performance.

1. Introduction

Palm oil mill effluent (POME) is regarded as toxic waste because it has high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) and contains a wide-range of biological substances from complex biopolymers such as proteins, starches and hemicelluloses to simple sugars and amino-acids. POME may also contain dissolved oil and fatty acids, glycerin, crude oil solids and short fibers as well as soluble materials that are harmful to the environment. POME was selected for this study because it is usually discharged at temperatures around 80–90°C. The high discharge temperatures mean that both mesophilic and thermophilic treatments are possible without external heating. Thermophilic operation (55-60 °C) of anaerobic reactors has been shown to be a feasible alternative to mesophilic operation (30-35 °C). It has been demonstrated that the thermophilic operation yielded higher efficiency in the reduction of pathogens, higher rates of COD removal, and biogas production than mesophilic operation (Ahn and Forster, 2003). However, some disadvantages of thermophilic operation, such as lower stability than mesophilic conditions, have also been reported (Fernandez and Forster, 1993). A number of studies have been performed to understand the characteristics of microbial granules grown under mesophilic conditions (Quarmby and Forster, 1995; Liu et al., 2003). Relatively little research has been performed to understand the microbial characteristics of granular sludge

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produced during thermophilic operation of POME treatment. In this work, the characteristics granules produced during biogas production and treatment of POME in mesophilic (37^{o}C) and thermophilic (60^{o}C) UASB reactors were investigated. The phylogeny of the microbial communities in sludge granules produced under both of the operating conditions was studied by a method based on analysis of bacterial 16S rRNA and methanogenic archaeal mcrA genes using denaturing gradient gel electrophoresis (DGGE) followed by a clone library analysis.

2. Materials and Methods

2.1 Wastewater and inoculum

The raw POME was collected from the wastewater collection tank of the factory (Suksomboon Palm Oil Co., Ltd., Chonburi province, Thailand). The POME preparation and characteristics were previously described (Tanikkul and Pisutpaisal, 2014). The supernatant portion of the POME was used to feed the UASB reactors in this study. Anaerobic granular sludge was obtained from the UASB reactors treating fruit canning wastewater of Malee Sampran factory (Nakonpathom province, Thailand). The sludge was visible as granules and had an average volatile suspended solid (VSS) concentration of 72.5 kg VSS m^{-3}.

2.2 UASB start-up and operation

Two identical UASB reactors, with a working volume of 3 L, were employed. The granular sludge was inoculated into the reactors to a final VSS concentration of 12 kg m^{-3}. The reactor temperatures were maintained by circulating hot water through the reactor jackets. One reactor was operated at a mesophilic condition of 37^{o}C (POME_T37), while another reactor was operated at a thermophilic condition of 60^{o}C (POME_T60). The POME was diluted with tap water to achieve a COD of either 5,000 mg L^{-1} or 6,000 mg L^{-1}. The hydraulic retention times (HRT) in both reactors were 7 days. The effluent from the reactors was recirculated to the reactors with the ratio of influent:effluent to 1:50 in order to maintain an upflow velocity of 0.3 m·hr^{-1}. Samples from the reactor influent and effluent were collected periodically for analyses of COD removal, biogas production and biomass washout. On day 115, 145 and 175, granule samples were taken from the bottom of both reactors for the analysis of granular size distribution and microbial community structures.

2.3 Microbial diversity and 16S rRNA gene clone library analyses

Total genomic DNA was extracted from the microbial samples using the benzyl chloride method (Zhu et al., 1993). The purified DNA was used as a template for amplification of the partial bacterial 16S rRNA gene fragment using *Taq* DNA polymerase (Fermentas, Vilnius, Lithunia) according to the manufacturer's protocol with 338GC-F forward primer and 518R reverse primer. Amplification of 16S rRNA gene, DGGE, transformation of 16S rRNA gene were performed following Wongwilaiwalin et al. (2010). The 16S rRNA gene sequences have been deposited in GenBank under the accession number HM440280-HM440317.

2.4 *mcr*A clone library analysis for archaeal diversity

The purified environmental DNA was used as the template for amplification of methyl-coenzyme-M reductase (mcrA) gene (470 bp) targeting for methanogenesis using primers MLf and MLr (Luton et al. 2002). The amplification reaction was performed using Taq DNA polymerase according to the manufacturer's protocol. Reaction conditions were pre-denaturation at 94^{o}C, 3 min; 30 cycles of denaturation at 94^{o}C, 1 min, annealing at 50^{o}C, 1 min, and extension at 72^{o}C, 1 min, followed by prolonged extension at 72^{o}C, 10 min. The amplicons were cloned into pTZ57R/T for DNA sequencing as previously described. The mcrA gene sequences in this study have been deposited in GenBank under the accession number HM440318-HM440337. Sequences were initially compared to the GenBank using the BLASTn server to determine their phylogeny. The construction of a phylogenetic tree was described in the previous work (Wongwilaiwalin et al., 2010).

2.5 Analytical methods

The analyses of COD, total alkalinity, suspended solids (SS), VSS, dissolved solids (DS), total solids (TS), total nitrogen (TN), potassium (K), sulfur (S) and total phosphate (TP) were performed according to the Standard Methods (Tanikkul and Pisutpaisal, 2014). Oil content was determined according to ASTM PS74-98 with a Soxtec^{TM} 2043 (Foss, Denmark). Volatile fatty acid (VFA) concentration and biogas composition were determined by gas chromatography (Tanikkul and Pisutpaisal, 2014). Granular size distributions in both reactors were performed by an image analysis using a microscope (Moticam 2000, Meiji, Japan). One-thousand granules were representatives of each reactor. Extracellular polymers (ECP) extraction and analysis was performed according to Quarmby and Forster (1995). Thermal extraction-solvent precipitation techniques were used to extract polymeric substances from the samples as described by Morgan et al. (1991). Dried polymer was dissolved in distilled water to give a 1 g L^{-1} solution. Carbohydrates and protein were determined using the phenol-sulphuric acid method (Dubois et al., 1956), and the Folin-Ciocalteau method.

3. Results and Discussion

3.1 Reactor performances and granular characteristics

The UASB reactors were operated under two phases of feeding conditions for 175 days. The first phase was operated under an organic loading rate (OLR) of 2.54 kg COD m^{-3} d^{-1} for 124 days, while the second phase was operated under OLR of 3.05 kg COD m^{-3} d^{-1} for 51 days. In the first phase, the mesophilic reactor gave satisfactory and superior performances to the thermophilic reactor. The POME_T37 yielded 87-94% COD removal, 1.2-1.5 L of biogas at STP d^{-1}, 0.176-0.260 m^3CH_4 kg^{-1}COD and 77-319 mg effluent VSS L^{-1}. In the second phase, the performance of POME_T37 dropped with increasing OLR. When the OLR was increased from 2.54 kg COD m^{-3} d^{-1} at the end of 124 days to the higher rate of 3.05 kg COD m^{-3} d^{-1}, the COD removal initially dropped to 60% but then subsequently climbed up to a steady state of 91% for the higher OLR. The biogas production in the second phase was higher than that in the first phase because of the higher organic loading rate in the second phase. The effluent VSS slightly increased to be between 189 and 334 mg L^{-1}, which indicated that the OLR slightly affected the degree of biomass washout. The increase in biogas production due to the increase in OLR can cause more hydrodynamic turbulence which in turn can lead to more biomass washout (Liu et al., 2003). In contrast, biomass washout was found to be a serious problem in the thermophilic operation. The loss of the microbial biomass with the effluent was clearly observed during the first phase. The POME_T60 yielded 60-91% COD removal, 0.51-0.79 L STP d^{-1}, 0.086-0.126 m^3CH_4 kg^{-1}COD and 159-654 mg effluent VSS L^{-1}. The biogas production of the POME_T60 was only slightly more than half of that of the POME_T37, but the methane compositions in both reactors were similar. When the OLR was increased approximately 25% in the second phase, the biomass washout became more severe. The COD removal dropped significantly to 36-70% and the VSS in the effluent increased to 644-941 mg L^{-1}. The biogas production of the POME_T60 became less than half of that of the POME_T37.

Granular size distribution

It was found that the granular size distribution was affected by the operating temperature (Figure 1). The granules had size between 1 and 2 mm. After operating for 175 days, the highest proportions of granules from both POME_T37 and POME_T60 were retained as 1 mm particles. The proportion of 2 mm granules in the POME_T37 was approximately double that of the POME_T60. Overall, the POME_T37 had higher average particle sizes, which is in agreement with its lower biomass washout.

VFA and pH profiles

The VFA profiles in both reactors were relatively similar. The VFA concentrations were in the order of 1 mM indicating that there was no VFA accumulation. It has been suggested that the propionic acid to acetic acid ratio can be used as an indicator of digester imbalance (Buyukkamaci, 2004). Hill et al. (1987) proposed that an anaerobic system failure occurs when the propionic acid to acetic acid ratio is greater than 1.4. The ratios in both POME_T37 and POME_T60 were less than 1 and indicated that the system was fairly balanced and the severe biomass washout in POME_T60 was not related to VFA accumulation or VFA imbalance. Besides the VFA data, the pH data which oscillated in a range between 6.5 and 8 throughout the experimental course (data not shown) could not indicate the severe biomass washout in the POME_T60.

ECP

Microbial ecology and ECP have been thought to influence the stability of anaerobic granules (Forster, 1991). ECP typically contains a high amount of protein, carbohydrate and some inorganic molecules (Morgan et al., 1991) and approximately 1-4% of the total solids consist of extractable ECP (Forster, 1991; Morgan et al., 1991). The amounts of extractable ECP from the granules in this study are shown in Figure 2. The ECP amounts from the three samples and their protein to carbohydrate ratios were in the same order of magnitude of the values reported in the literature. The ECP amounts in the initial granules were significantly lower than those in the mesophilic reactor. However, it is difficult to make a comparison between the ECP in the initial granules and those in the granules from POME_T37 and POME_T60 because they had been grown in different substrates and conditions. The amount of ECP is known to depend on the nature of the substrate. Quarmby and Forster (1995) reported that the ECP content from a UASB treating coffee wastewater was 2-3 times lower than that from a UASB treating POME. The results showed that the ECP content in the mesophilic granules was 36% higher than that in the thermophilic granules. The carbohydrate content in the ECP of the thermophilic granule was significantly lower than that of the mesophilic granules and the protein to carbohydrate increased from 1.39 in the mesophilic ECP to 2.52 in the thermophilic ECP. It is known that the amount of ECP produced is dependent on the types of bacteria present in the granule and that the number of bacteria present in the granule can affect the amount of ECP produced (Quarmby and Forster, 1995). Therefore, the microbial community structures of both granular samples were further investigated by DGGE and clone library analysis.

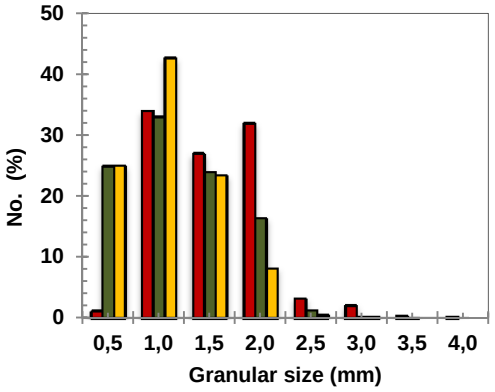

Figure 1 Granular size distribution as a function of operating temperatures. One-thousand granules were representatives of each reactor. Granular size at the start up (red), 37^{o} C, day 175 (green), 60^{o} C, day 175 (yellow).

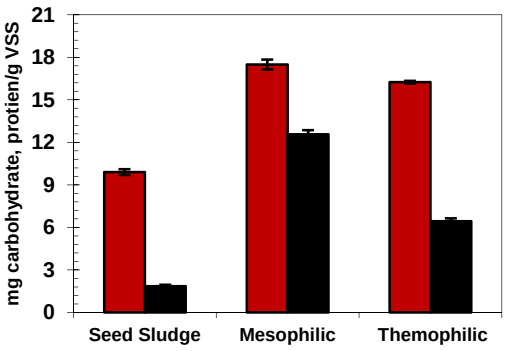

Figure 2 ECP protein (red) and ECP carbohydrate (black) of the seed sludge, mesophilic and thermophilic granules.

3.2 Microbial communities under mesophilic and thermophilic conditions

Figure 3 shows the DGGE patterns of the bacterial communities from POME_T37 and POME_T60. Even though they originated from the same seed culture, the bacterial communities under the two conditions were remarkably different in terms of their composite microbial members. The DGGE analysis based on partial prokaryotic 16S rRNA gene sequences revealed that the bacterial communities were relatively unchanged during day 115-175 of the mesophilic and thermophilic operations, although some significant changes in composite microbes were detected on day 175 under the thermophilic condition. The DGGE patterns also suggested higher diversity of bacterial strains under the mesophilic condition in comparison to the thermophilic one, potentially due to higher selective pressure under the thermophilic operation. The lower degree of bacterial diversity corresponded with the lower ECP content in the granules from the thermophilic reactor.

The granule samples collected on day 175 were analyzed for the phylogenetics of the microbial communities. The prokaryotic diversity was studied based on full-length 16S rRNA gene sequences of 40 randomly selected clones from each operational condition, which resulted in 25 and 13 RFLP patterns for the mesophilic and thermophilic methane producing communities, respectively. Higher bacterial diversity was observed for the mesophilic microbial community (Table 1). The mesophilic community consisted of 6 major bacterial phyla, predominated by *Proteobacteria* (mainly *Beta-* and *Gammaproteobacteria*), *Firmicutes* (e.g. *Clostridiaceae*), and *Chloroflexi*, in addition to minor phyla including *Bacteroidetes*, *Planctomycetes* and *Verrucomicrobia*. Some were related to those in facultative anaerobic and aerobic genera including *Stenotrophomonas*, *Butyribacterium*, *Burkholderia*, *Variovorax*, *Serratia*, and *Sphingomonas*. The thermophilic bacterial community was significantly less complex possibly due to higher selective pressure at the high operating temperature. They comprised only 2 major bacterial phyla including *Proteobacteria* (*Beta-* and *Gammaproteobacteria*) and *Actinobacteria*. Many were related to those in genera *Shigella*, *Propionibacterium*, *Burkholderia*, *Achromobacter*, *Pseudomonas*, and *Serratia*. Some of these bacterial phyla have been previously reported in methane production systems operated under different conditions with various substrates (Dedysh et al., 2006) by involving in the hydrolysis of polymeric substrates and formation of acid intermediates. These bacteria would adapt along with the archaeal community and metabolically linked to the methanogenic archaea coexisted in the systems. *Clostridium* and *Bacteroidetes* are producers of hydrolytic enzymes e.g. lipases, proteases and non-starch polysaccharide degrading enzymes which would play a key role on hydrolysis of the lipids, protein and lignocellulosic substrates. Some genera e.g, *Clostridium*, *Bacteroidetes* and *Propinibacterium* have also been reported to provide intermediates and metabolites including volatile fatty acids (e.g. acetate, propionate and buytyrate) and hydrogen for the subsequent methanogenesis step (Quesada-Chanto et al. 1998). The methanogenic archaeal community structures were assessed based on *mcr*A sequences of 10 randomly selected clones from *mcr*A clone libraries of the mesophilic and thermophilic communities. Remarkable differences were observed in the methanogen community structures at the two temperatures. The methanogen members of both temperatures were clearly separated in the phylogenetic tree, indicating overall differences in methanogenic archaea present at different temperatures. The mesophilic methanogen assemblage was found to comprise mainly of those related to uncultured methanogens in class

Methanomicrobiales and *Methanobacteriales*. The thermophilic methanogen community was predominated by those related to class *Methanosarcinales* in addition to some *Methanobacteriales*. Most were closely related to *Methanosaeta* (previously *Methanothrix) thermophila*. *Methanosaeta* has been known to have slow growth (up to 12 days doubling time) and lower growth yield than other microbes (Alber and Ferry, 1994). *M. thermophila* has also been known to obtain their energy as a thermophilic obligately-aceticlastic methane-producing archaeon. That is, it produces methane from acetate only. The overall difference in methanogen taxa suggested that different methanogenesis pathways proceeded under the mesophilic and thermophilic conditions (Garsia et al. 2000).

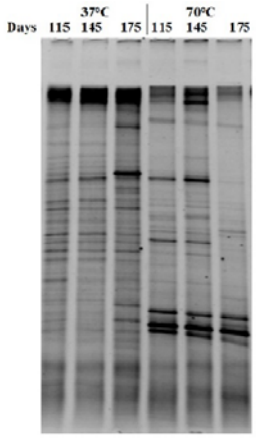

Figure 3 DGGE analysis of bacterial community structures from the methane fermentation systems under mesophilic (37°C) and thermophilic (60°C) conditions at different operating times.

According to the methanogen taxa, hydrogenotrophic pathway would represent the predominant methanogenesis process under the mesophilic conditions while acetotrophic/methylotrophic pathways contributed significantly under the thermophilic conditions. The result also implied the differences in intermediates for methanogenesis and thus the difference in metabolic linkages to the bacterial communities under the thermophilic conditions. The result also implied the differences in intermediates for methanogenesis and thus the difference in metabolic linkages to the bacterial communities. The microbial community data also suggested that the granulation under the mesophilic condition resembled the Cape Town Hypothesis, which is based on excessive ECP production of *Methanobacterium* (Sam-Soon et al., 1987). The hypothesis is the only theory that explains granulation by organisms other than *Methanosaeta* and *Methanosarcina* (Hulshoff Pol et al., 2004).

Table 1 Classification of bacterial community taxa in methane producing communities at mesophilic and extreme thermophilic conditions. Relative abundances are calculated based on 40 clones from 16S rRNA gene libraries from each operational conditions

Phylum	**Class**	**Abundance (%)**	
		37°C	**60°C**
Proteobacteria	Alphaproteobacteria	4	-
	Betaaproteobacteria	16	46
	Deltaproteobacteria	4	-
	Gammaproteobacteria	8	38
Bacteriodetes	Sphingobacteria	8	-
Planctomycetes	Planctomycetia	8	-
Verrucomicrobia	Verrucomicrobiae	4	-
Firmicutes	Cloastridia	28	-
Actinobacteria	Actinomycetales	-	16
Chloroflexi	Chloroflexi	20	-

In contrast, the dominant methanogen under the thermophilic condition was *M. thermophila*. It was likely that the source of the thermophilic species was the POME rather than the mesophilic granular seed, suggesting that the thermophilic condition was too harsh for the methanogens in the granular seed to survive. In addition, the optimal growth for *Methanosaeta* has been known to be between 55 and 60°C (Ahring, 1995) and

therefore the thermophilic condition may not be suitable for the growth of the dominant methanogen in the reactor.

4. Conclusions

UASB operation under the thermophilic condition resulted in an adverse effect on biomass granules, while the effluent VFA and pH profiles were similar in both mesophilic and thermophilic reactors. The mesophilic UASB had higher COD removal, biogas production and lower biomass washout. The low performances of the thermophilic reactor were associated with relatively low ECP content and significantly lower bacterial diversity in the sludge granules. The mesophilic methanogen assemblage was found to be comprised mainly of those related to uncultured methanogens in class *Methanomicrobiales* and *Methanobacteriales*, while the thermophilic methanogen community was dominated by *M. thermophila*.

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