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Microbial Diversity in Thermophilic Adaptation on Pome Treatment

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Abstract

Microbial diversity in a thermopilic UASB reactor producing biogas from palm oil mill effluent (POME) was investigated. The PCR-based DGGE analysis showed that the microbial population profiles changed with the temperature transition from mesophilic to thermophilic conditions. Acetotrophic and hydrogenotrophic methanogens were found in all three phases: the seed sludge, transition from mesophilic to thermophilic condition and operation under the thermophilic condition. The hydrolytic and acidogenic bacteria were found in the seed sludge, while only hydrolytic bacteria were found during the transition and hydrolytic, acidogenic and acetogenic bacteria were presented under the thermophilic condition. The microbial profiles were highly diversified under the thermophilic condition. The results suggested that the temperature transition strategy was suitable for proliferation of the microorganisms both in the seed sludge and in POME.

Keywords: Anaerobic, methanogens, POME, UASB, thermophilic

1. Introduction

Palm oil mill effluent (POME) is a highly polluting wastewater generated from the palm oil extraction process, and is high in chemical oxygen demand (COD; 80,000 - 120,000 mg l^{-1}) and biochemical oxygen demand (BOD; 50,000 - 80,000 mg l^{-1}) [1-3]. Various anaerobic digestion systems have been applied for POME treatment. It has been known that temperature

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is a major factor that significantly affects digester performances [2, 4, 5]. A thermophilic (55-65^{o}C) operation yields high substrate degradation and biogas production rates [2, 5-7]. In addition, temperature may affect the microbial diversity in an anaerobic digestion. The microorganisms involve in anaerobic digestion consist of four steps. Hydrolytic bacteria are the first to hydrolyze complex organic compound in POME to simple organic compounds. In the second step which is known as fermentation, acidogenic bacteria utilize the simple organic compounds and produce organic acids. The metabolites from the fermentation is converted by acetogenic bacteria to acetic acid, hydrogen and carbon dioxide. Finally, hydrogen and carbon dioxide are utilized by hydrogenotropic methanogens while acetic acid and carbon dioxide are utilized by acetoclastic methanogens to give methane as a final product [4, 8, 9]. The profiles of dominant microorganisms in anaerobic granules developed in a UASB under a transition from a mesophilic to thermophilic condition have scarecely been documented.

The objective of this study was to determine domain bacteria and methanogen in the anaerobic granules developed in a UASB treating POME under a transition from a mesophilic to thermophilic condition.

2. Materials and Methods

2.1 Reactor system, acclimation, start-up, and operation

A UASB reactor with a working volume of 5.3 l, was of a cylindrical shape. The sludge was a mesophilic anaerobic sludge obtained from Nong-Khaem Water Quality Control Plant (Bangkok, Thailand). The POME wastewater was obtained from Suksomboon Palm Oil Co., Ltd. (Chonburi province, Thailand). The POME was pretreated with aluminum sulfate to remove oil and grease. $NaHCO_3$ was added to the samples to obtain an alkalinity concentration of 500 mg l^{-1} and 6 N NaOH was added to obtain a pH of 7.

Sludge with an initial VSS concentration of 12 kg VSS m^{-3} was inoculated into the reactors. The POME substrate prepared by diluting POME with tap water at a ratio of 1:10 (pretreated POME: tap water) was fed to the reactor with hydraulic retention time (HRT) of 2.4 days. The reactor starting temperature was 37^{o}C. After the COD removal, the HRT, the organic loading rate (OLR) of reactor was increased by feeding the 2:10, 3:10, 4:10 and 5:10 diluted substrate, respectively. Simultaneously, the temperature was increased by a step of 5^{o}C to 42^{o}C, 47^{o}C, 52^{o}C and 57^{o}C, respectively. The effluent from the reactors was recirculated to the reactors with the ratio of influent: effluent to 1:50 in order to maintain the upflow velocity of 0.3 m hr^{-1}.

The influent and effluent samples from the UASB reactors were collected for analyses of pH, COD, VSS according to the Standard Methods [10]. Gas composition was determined by a gas chromatograph (GC) equipped with a thermal conductivity detector (GC-2014; SHIMADZU, Japan).

2.2 Microbial community analysis by denaturant gradient gel electrophoresis (DGGE)

Total genomic DNA was extracted from the seed sludge and sampled on day 21 and 123 using a modified protocol of Zhou *et al.* [11]. For microbial community analysis *Eubacteria* and *Archaea*, a 1,500 bp fragment was amplified with the primers EUB8F/U1492R and A20F/U1492R for *Eubacteria* and *Archaea* 16S rRNA genes, respectively [12-14]. The specific primer sets of 338GC-F/518R and 344GC-F/522R were used for *Eubacteria* and *Archaea* 16S rRNA fragments, respectively [12, 15-17].

The 200 bp PCR fragments were analyzed by DGGE. The DGGE analysis was performed as described by Muyzer [16] and Øvreås *et al.* [18] using a DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, CA, USA). The nested PCR products were loaded to 8% polyacrylamide gels in 1xTAE (Tris-acetate-EDTA) buffer, with a gradient of between 40% to 65% for *Eubacteria* and 35 to 75% for *Archaea*. Gradients were created by

polyacrylamide containing 0–80% denaturant (5.6M urea and 40% (v/v) formamide). Electrophoresis was performed at 200V for 5 h and at a constant temperature of 60°C. After the electrophoresis, the gels were stained with SYBR Gold nucleic acid stain (Molecular Probes, Eugene, OR, USA) for 20 min. The image was visualized on a UV transilluminator and captured using Biovision CN 1000/26M (Vilber Lourmat, France) [18-20]. Most bands were excised from the gel and re-amplified with the primers 338GC-F/518R and 344GC-F/522R. The PCR products were purified using Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions. The purified PCR products were sequenced using the 1st BASE Laboratories Sdn Bhd (Malaysia). All sequences for partial 16S rRNA gene were identified with similar sequences of the reference organisms using BLAST search [21].

3. Results and Discussion

3.1 Reactor performances

Throughout the 123 operating days, the OLR ranged between 2.2-9.5 kg COD $m^{-3}d^{-1}$. The effluent pH was steadily higher than 7, ranging between 7.22-7.91. The biogas production and methane production ranged between 0.42-13.18 l d^{-1} and 0.03-10.57 l d^{-1}, respectively. At the OLR of 9.5 kg COD $m^{-3}d^{-1}$, the COD removal efficiency was higher than 80%. Table 1 summarizes the reactor performance.

Table 1 Summary of the reactor performance

Phase of Operation	Temperature	Steady-state OLR	Steady-state COD removal	Steady-state Biogas production	Steady-state Methane production
Day 21	37 °C	2.25 kg COD $m^{-3}d^{-1}$	83.5%	4.0 l d^{-1}	3.4 l d^{-1}
Day 123	57 °C	9.49 kg COD $m^{-3}d^{-1}$	81.2%	13.2 l d^{-1}	10.6 l d^{-1}

3.2 Bacterial diversity analysis

In the process of degrading complex molecules in POME into methane, there is a sequence of reactions involving both bacteria and archaea. It is known in the literature that anaerobic bacteria consist of hydrolytic, acidogenic and acetogenic bacteria [4]. The community of *Eubacteria* in the anaerobic sludge and that during the start up and under the thermophilic condition are shown in Figure 1. Table 2 summarizes the observed bands, their related microorganisms along with the degree of similarlity. Thirteen DGGE bands were observed for the microbial community of the seed sludge. Five of them, A3, A5, A8, A9 and A13, were identified as hydrolytic bacteria. Bands A1 and A12 were identified as the members of acidogenic bacteria. None of acetogenic bacterium was detected in the seed sludge, but four bands, A2, A4, A6 and A10, were identified as some other bacteria not involving in methane production.

The bacterial community structure during the start up on day 21 was similar to that presented in the seed sludge. Thirteen DGGE bands were observed. Most band patterns of the hydrolytic bacteria were similar to those of the seed sludge. As in the seed sludge, an acetogenic bacterium was not detected. In addition, three bands, B1, B3 and B7, were found not to be the main microorganisms involving in methane production. A difference in the DGGE bands of the seed sludge and the sludge granule during the start up was that band A3, which was related to *Halomonas* sp. 3026 (90% similarity), disappeared during the start up. Another difference was that acidogenic bacteria were not detected during the start up.

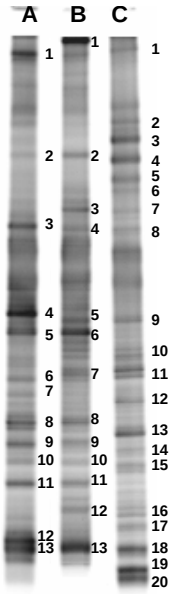

Figure 1 DGGE profile of bacterial community. Lane A: seed sludge; lane B: start up period at 37^{o}C; lane C: thermophilic operation at 57^{o}C.

However, three additional bands, B4, B5 and B12, were found during the start up. They were the member of hydrolytic bacteria.

Twenty DGGE bands of the granular samples during the thermophilic operation on day 123 were found. All hydrolytic, acidogenic and acetogenic bacteria were detected. Nine bands, C3, C5, C8, C9, C10, C14, C16, C17 and C18, belong to hydrolytic bacteria. Band C18 presented in both seed sludge (A13) and start up peroid (B13). Two bands, C8 and C9, also presented in the start up. Six new bands, C3, C5, C10, C14, C16 and C17, were detected. For acidogenic bacteria, only band C1 was previously found in the seed sludge (A1), while four new bands, C4, C12, C19 and C20, were observed. There were three bands belong to acetogenic bacteria; C6, C13 and C15. Under the thermophilic condition, three bands, C2, C7 and C11, were found to associate with other bacteria which are not the main microorganisms in the methane production.

3.3 Archaeal diversity analysis

Figure 2 illustrates the DGGE profile of the archaeal community. Table 3 summarizes the observed bands, their related microorganisms along with the degree of similarlity. At least seven bands were associated with those of the seed sludge. Four of them, D4, D6, D7 and D9, belong to acetotrophic methanogen. One of them, D5, was identified as a member of hydrogenotrophic methanogen. Band D1 was identified as methanogenic archaeon, while two bands, D2 and D3, were analyzed as non-methanogen archaeon.

Table 2 Summary of the partial 16S rRNA gene sequences of DGGE bands determined by BLAST searches

Band	Closet match	Sequence similarly	Accession No.	Anaerobic bacteria group
A3	*Halomonas* sp. 3026	90%	AM110981.1	Hydrolytic bacteria
A5, B6	*Sphingobacterium* sp. P-7	91%	AM411964.1	
A8, B8	*Bacillus* sp. N18	94%	FJ577666.1	
A9, B9	*Brevundimonas* sp. AP-5	99%	AY145543.1	
A13,B13,C18	*Tepidiphilus* sp. enrichment culture clone D_8m3_OTU2	91%	F895352.1	
B4, C8	*Pseudomonas* sp. W399	86%	GU826599.1	
B5, C9	*Bacillus pumilus* strain LCR103	99%	FJ976612.1	
B12	Uncultured *Clostridium* sp. clone 026	89%	GU556243.1	
C3	*Sediminibacterium* sp. I-28	99%	AM990456.1	
C5	Uncultured *Bacteroides* sp. clone IPL 21	98%	EU037943.1	
C10	Uncultured Caldilineaceae bacterium clone REV_R1PII_4C	91%	FJ933454.1	
C14	*Caulobacter* sp. KIN53	96%	AY136076.1	
C16	*Denitratisoma oestradiolicum* strain AcBE2-1	92%	AY879297.1	
C17	Uncultured *Massilia* sp. Clone G13-S-5	80%	EJ192248.1	
A1, C1	Uncultured *Acidobacterium* sp. Clone MBT1	96%	FJ538114.1	Acidogenic bacteria
A12	*Pasteurella multocida* subsp. Multocida str.	95%	AE004439.1	
C4	*Robinsoniella peoriensis* strain SWP02	100%	GU811874.1	
C12	Uncultured Firmicutes bacterium	93%	CU922223.1	
C19	Uncultured *Thermoanaerobacteriaceae* bacterium clone MRE50b18	96%	AY684101.1	
C20	Uncultured *Thermoanaerobacteriaceae* bacterium clone MRE50b23	92%	AY684101.1	
C6	Uncultured *Sulfurospirillum* sp.	93%	EU628134.1	Acetogenic bacteria
C13	Uncultured *Smithella* sp. clone 3.29	96%	GQ183319.1	
C15	Uncultured *Syntrophus* sp. clone 153	98%	GU112190.1	
A2, B2	*Parasporobacterium paucivorans* strain SYR1	100%	NR025390.1	Other
A4	Uncultured *Geobacter* sp.	94%	AM941471.1	
A6	*Desulfovibrio* sp. enrichment culture clone Ecwsrb030	99%	GQ503867.1	
A10, B10	*Phormidium autumnale* CYN53	94%	GQ451413.1	
B1	Iron-reducing bacterium enrichment culture clone HN19	94%	FJ269054.1	
B3, C7	Uncultured *Sulfurovum* sp. clone ZZ-S12H6	93%	EF613484.1	
B7, C11	*Desulfovibrio desulfuricans* strain SRB-22	93%	FJ873799.1	

Five acetotrophic methanogen, E2, E5, E6, E7 and E9, of the start up period, resembled those of the seed sludge. One hydrogenotrophic methanogen, E3, and one non-methanogen archaeon, E1, were previously detected in the seed sludge (D5 and D3, respectively). Two bands, E4 and E8, first appeared during the start up. E4 was identified to be a member of acetotrophic methanogen, while E8 was identified as hydrogenotrophic methanogen.

Under the thermophilic condition, there were two bands, F5 and F7, identified as acetotrophic methanogen. It should be noted that microorganisms presented throughout the experiment. One band, F4, was identified as hydrogenotrophic methanogen. F1 was identified as a non-methanogen archaeon which existed throughout the experiment. Two new bands, F2 and F3, identified as the acetotrophic and hydrogenotrophic methanogens, respectively, and one band, F4, identified as non-methanogen archaeon first appeared under the thermophilic condition.

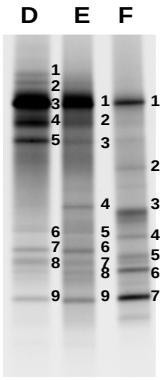

Figure 2 DGGE profile of archaeal community. Lane A: seed sludge; lane B: start up period at 37^{o}C; lane C: thermophilic operation at 57^{o}C.

Table 3 Summary of the partial 16S rRNA gene sequences of DGGE bands determined by BLAST searches

Band	Closet match	Sequence similarly	Accession No.	Anaerobic methanogen
D4, E2	Uncultured *Methanosarcinales* archaeon gene	96%	AB353207.1	Acetotrophic methanogen
D6, E5	Uncultured *Methanosaeta* sp. clone A01	99%	EU888804.1	
D7, E6	Uncultured *Methanosaeta* sp. clone A06	99%	EU888805.1	
D9, E9,F7	*Methanothrix soehngenii*	96%	X51423.1	
E4	Uncultured *Methanosaeta* sp. clone A12	98%	EU888812.1	
F2	Uncultured *Methanosarcinaceae* archaeon clone ARK2_66	90%	GQ995122.1	
D5, E3	*Methanofollis liminatans*	88%	AY196677.1	Hydrogenotrophic methanogen
E8, F6	Uncultured *Methanolinea* sp.	96%	AB479399.1	
F3	Uncultured *Methanomicrobiales* archaeon gene	85%	AB077222.1	
D1	Uncultured methanogenic archaeon clone SMPFLSS56m 8	98%	FJ982756.1	Other methanogen
D2	Uncultured archaeon clone SB_WN41	98%	EF6395791	Non-methanogen
D3, E1, F1	*Candidates Nitrososphaera gargensis*	93%	GU797786.1	
F4	Uncultured Thermoplasmata archaeon clone NRA9	92%	HM041910.1	

4. Conclusions

Most hydrolytic bacteria appeared in both the seed sludge and the anaerobic granular sludge during the start up. Three hydrolytic bacteria could adapt from the start up to the thermophilic condition. Six thermophilic hydrolytic bacteria first appeared under the thermophilic condition. Most acidogenic and acetogenic bacteria were observed only under the thermophilic condition. It was evident that the growth of these groups was favored at the high temperature. The domain archaea, acetotrophic and hydrogenotrophic methanogen of the seed sludge had similar microbial community patterns to the sludge during the start up. All methanogen in the seed sludge prolonged to the start up period. In contrast, only few methanogen in the seed sludge could prolong to the thermophilic condition. Two acetotrophic and one hydrogenotrophic methanogen under the thermophilic condition were found to adapt from the start up. One acetotrophic and one hydrogenotrophic methanogen first appeared under the thermophilic condition and they were presumed to originate from the substrate POME. The results indicated that the thermophilic condition was suitable for methane production as all bacterial and archaeal consortium involved in anaerobic digestion were observed under this condition.

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References

[1] Yacob, S., *et al.* **2006**. Baseline study of methane emission from anaerobic ponds of palm oil mill effluent treatment. *Science of the Total Environment*, 366(1), 187-196.

[2] Choorit, W. and Wisarnwan, P. **2007**. Effect of temperature on the anaerobic digestion of palm oil mill effluent. *Electronic Journal of Biotechnology*, 10(3), 376-385.

[3] Prasertsan, S. and Prasertsan, P. **1996**. Biomass residues from palm oil mills in Thailand: An overview on quantity and potential usage, *Biomass and Bioenergy*, 11(5), 387-395.

[4] Poh, P. E. and Chong, M. F. **2009**. Development of anaerobic digestion methods for palm oil mill effluent (POME) treatment. *Bioresource Technology*, 100(1), 1-9.

[5] Yu, H. -Q., Fang, H. H. P. and. Gu, G. -W **2002**. Comparative performance of mesophilic and thermophilic acidogenic upflow reactors. *Process Biochemistry*, 38(3), 447-454.

[6] Quarmby, J. and Forster, C. F. **1995**. A comparative study of the structure of thermophilic and mesophilic anaerobic granules. *Enzyme and Microbial Technology*, 17(6), 493-498.

[7] Ahn, J. H. and Forster, C. F. **2000**. A comparison of mesophilic and thermophilic anaerobic upflow filters. *Bioresource Technology*, 73(3), 201-205.

[8] Lee, C., *et al*. **2009**. Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters. *Water Research*, 43, 157-165.

[9] Yang, Y., Tsukahara, K. and Sawayama, S. **2008**. Biodegradation and methane production from glycerol-containing synthetic wastes with fixed-bed bioreactor under mesophilic and thermophilic anaerobic conditions. *Process Biochemistry*, 43(4), 362-367.

[10] APHA, AWWA, WEF. **2005**. Standard methods for the examination of water and wastewater. Eaton A. D., L. S. Clesceri, and A. E. Greenberg, Editors. Washington, D.C.
[11] Zhou, J.,. Bruns, M. A and Tiedje, J. M. **1996**. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology*, 62(2), 316-322.
[12] Amann, R., Ludwig, W. and Schleifer, K. H. **1995**. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Reviews*, 59, 143-169.
[13] Moyer, C. L., Dobbs, F. C. and Karl, D. M. **1994**. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Applied and Environmental Microbiology*, 60(3), 871-879.
[14] Orphan, V. J., *et al.* **2000**. Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum resevoirs. *Applied and Environmental Microbiology*, 66, 700-711.
[15] Amann, R.I., *et al.* **1990**. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, 56(6), 1919-1925.
[16] Muyzer, G., De Waal, E. C. and Uitterlinden, A. G. **1993**. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, 59(3), 695-700.
[17] Raskin, L., *et al.* **1994**. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Applied and Environmental Microbiology*, 60, 1241-1248.
[18] Øvreås, L., *et al.* **1997**. Distribution of bacterioplankton in meromictic lake Sælenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Applied and Environmental Microbiology*, 63(9), 3367-3373.
[19] Shigematsu, T., *et al.* **2006**. Microbial diversity of mesophilic methanogenic consortium that can degrade long-chain fatty acids in chemostat cultivation. *Journal of Bioscience and Bioengineering*, 102(6), 535-544.
[20] Keyser M, *et al.* **2006**. PCR-based DGGE fingerprinting and identification of methanogens detected in three different types of UASB granules. *Systematic and Applied Microbiology*, 29, 77-84.
[21] Keyser, M., *et al.* **2006**. PCR-based DGGE fingerprinting and identification of methanogens detected in three different types of UASB granules. *Systematic and Applied Microbiology*, 29, 77-84.