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Identification of Methane-producing Bacteria from Palm Oil Mill Sludge (POMS) with Solid Cud from Ruminant Stomach

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Abstract

Biological generation in anaerobic environments such as enteric fermentation and anaerobic waste treatment from agriculture sector are the major contributor of methane gas which has the potential as biogas. The aimed of this study was to identify methane-producing bacteria in anaerobic vessel which contained a mixture of Palm Oil Mill Sludge (POMS) and solid cud taken from the first compartment of cow's stomach (1:2 and 2:1 ratio) as co-mixture. The co-mixture was incubated at 50 °C in a 2 L vessel with initial starter of 400 ml and sampling was conducted every 4 weeks interval during 12 weeks of incubation. For specific detection of methanogens, 16S rRNA-cloning analysis was carried out. *Methanobrevibacter* sp. and *Methanosaeta* sp. were confirmed to be presence within the 2:1 ratio of co-mixture while only *Methanobrevibacter* sp. was found in 1:2 ratio of co-mixture on both Week 0 and Week 4. No methanogens were detected for both co-mixtures on Week 8 and Week 12.

Introduction

Crude palm oil (CPO) production in Malaysia has been increasing continuously over the years, from 4.1 million tonnes in 1985 to 6.1 million tonnes in 1990. The production is further increased by 11.29% to 18.9 million tonnes in 2011 [1]. However, increase of production leads to generation of huge quantities of wastes. During oil extraction process, about 50% of water used results in palm oil mill effluent (POME) while others are lost as steam, mainly through sterilizer exhaust, piping leakages as well as wash water [2]. POME contains suspended solids and total dissolved solids in the range between 18,000 mg L^{-1} and 40,000 mg L^{-1} respectively [3]. Both solids are known as palm oil mill sludge (POMS). POMS consists of 3.6, 0.9 and 2.1 mg L^{-1} of total nitrogen, phosphorus and potassium, respectively, which results in bad odors and is consider as a source of ground pollution [4].

POMS can be applied as fertilizer as it has high nutrient value [5]. However, during rainy season, the drying process of POMS becomes difficult as the rate of drying become slower. Due to this limitation, anaerobic treatment of POMS such as anaerobic digester offer more attractive solutions for biogas production and clean development mechanism (CDM).

Anaerobic digestion process involves a wide variety of microbial community. In order to produce higher biogas yield, inoculum source is crucial for optimization of inoculum ratio. In this study, the aim of the present work was to determine the methane-producing bacteria community in POMS with solid cud from ruminant stomach using 16S rRNA clone library techniques.

Material and Methods

Samples Collection

Palm Oil Mill Sludge (POMS) was collected from the anaerobic pond from Bau Palm Oil Mill (BAPOM), Kuching, Sarawak. The solid cud from the first compartment of cow's stomach was collected from a slaughter house located at Ladang Lapan, Kuching. Both samples were stored in sealed container immediately after collection and preserved at 4 °C in order to avoid biodegradation due to microbial activities.

Anaerobic vessel set up

Co-mixture with different ratio (Table 1.0) were incubated at 50 °C in a 2 L vessel with initial starter of 400 ml. Sampling for both ratio were conducted every 4 weeks interval during 12 weeks of incubation.

DNA Extraction and PCR Amplification

Bacterial DNA of both ratio of co-mixture were extracted using Power Soil ™ DNA Isolation Kit (Mo Bio Laboratories, USA) and amplification of 16S rRNA region was amplified using

Table 1: Different ratio of Poms and solid cud in co-digestion

Ratio	Co-digestion mixture
1:2	POMS: Solid cud
2:1	POMS: Solid cud

Met86F and Met1340R primers [6]. PCR of the 16S rRNA was run in 25 µl reactions comprising 50-100 ng of DNA, 10X Taq DNA polymerase buffer, 0.5 µl of 10 mM dNTP mix (Fermentas, Canada), 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of each primer and 0.2 µl of 5 U AmpliTaq DNA polymerase (Fermentas, Canada). The amplification condition included 95 °C for 10 minutes followed by 34 cycles of 94 °C for 40 seconds, 54 °C for 50 seconds and 72 °C for 90 seconds. On the 35th cycles, the final elongation step was increased to 10 minutes at 72 °C.

Cloning 16S rDNA

PCR products of both co-mixture (1:2 and 2:1) were purified according to the manufacturer's instruction (Mo Bio Laboratories, USA). 16S rRNA clone libraries were constructed by cloning purified PCR fragments into pGEM-T Easy vector using the heat shock method. White colonies were randomly selected from the agar plates and plasmids were extracted using a plasmid extraction kit (Promega, USA). The extracted plasmids were re-amplified through PCR reaction and sent for sequencing.

Phylogenetic analysis

The sequences obtained were trimmed and further analysed using Bellerophon program [7] to remove chimera rRNA clones. Sequences similarities with 16S rRNA sequences in GeneBank ™ database were conducted using basic logical alignment tool (BLAST). PCR sequences were combined with closely related sequences. MEGA 5 [8] was used to construct neighbour-joining tree, which was bootstrap resampled 1000 times.

Results and Discussion

16S rRNA region was successfully amplified from the DNA extracted from the different ratio (1:2 and 2:1) of co-mixture. From 24 clones screened (6 from both Week 0 and Week 4 of 1:2 ratio and 6 from both Week 0 and Week 4 of 2:1 ratio) only 12 clones contained the correct size of DNA insert (1300bp) (Figure 1). No amplification of PCR products for Week 8 and 12 of both co-mixtures.

The diversity and phylogeny of the isolates were investigated by constructing phylogenetic tree with *Kluyveromyces lactis* as the outgroup (Figure 2). The sequences obtained in this study have been deposited in the GenBank database under accession numbers KJ522696-KJ522706.

From the phylogenetic tree, 66.6% of clones isolated, displayed 95% or greater genus-level sequence homology to species belonging to *Methanobrevibacter*. Within this genus, 41.6% (5/12) of all clones had 97% or greater species-level sequence similarity to *Methanobrevibacter millerae*. In contrast, only 0.083% of library clones were identified as *Methanobrevibacter olleyae*, *Methanobrevibacter arboripilus* and *Methanobrevibacter thaueri* respectively.

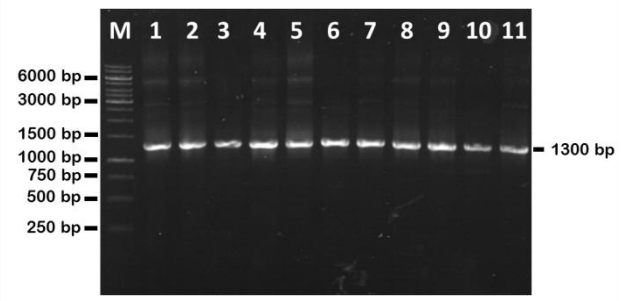

Figure 1: Agarose gel electrophoresis of amplified 16S rDNA region. Lane M, 1kb ladder (Fermentas); Lane 1, 2 and 3, PCR products of mixed sample with 1:2 ratio on Week 0 amplified using extracted plasmid from transformed bacteria; Lane 4,5, and 6, PCR products of mixed sample with 2:1 ratio on Week 0 amplified using extracted plasmid from transformed bacteria; Lane 7,8, and 9, PCR products of mixed sample with 1:2 ratio (Week 4) amplified using extracted plasmid from transformed bacteria; Lane 10 and 11, PCR products of mixed sample with 2:1 ratio (Week 4) were amplified by using extracted plasmid from transformed bacteria.

The other four clone libraries were divided into three different phylogenetic groups. Two of the clones showed 90% or greater sequences homology belonging to *Methanosaeta concilii* while the remaining clones were identified as *Methanolinea tarda* and *Aciduliprofundum boonei* respectively with 97% sequence similarities.

Majority of the clones from co-mixture of 1:2 (anaerobic sludge: solid cud) ratio belonged to the genus *Methanobrevibacter*. Presence of *Methanobrevibacter* sp. had also been reported in ovine and bovine content [9] and dairy cow [10]. In addition, Singh *et al.*[11] also reported presence of *Methanobrevibacter* sp. in ruminal fluid of buffalo. Clones from environment samples which show genus-level sequence similarity of more than 95% to *Methanobrevibacter* sp. are most abundance in gastrointestinal samples from herbivores [12, 13, 14]. In contrast, different methanogens such as *Methanosaeta concilii*, *Methanolinea tarda* and *Aciduliprofundum boonei* were found in co-mixture (2:1 ratio) which contains larger volume of anaerobic sludge. The presence of *Methanosaeta* sp., *Methanolinea* sp. and *Aciduliprofundum* sp. in sludge and agriculture wastes are also demonstrated in previous findings [15, 16, 17].

Conclusion

Methanogens population in co-mixture was reflected from 16S rRNA clone library in this study. *Methanobrevibacter* sp., *Methanosaeta concilii*, *Methanolinea tarda* and *Aciduliprofundum boonei* were presented in the co-mixture of anaerobic sludge with solid cud. With the knowledge of methanogens community in co-mixture, a better understanding in enhancing biogas production using anaerobic digester can be achieved in reduction of greenhouse gases emission.

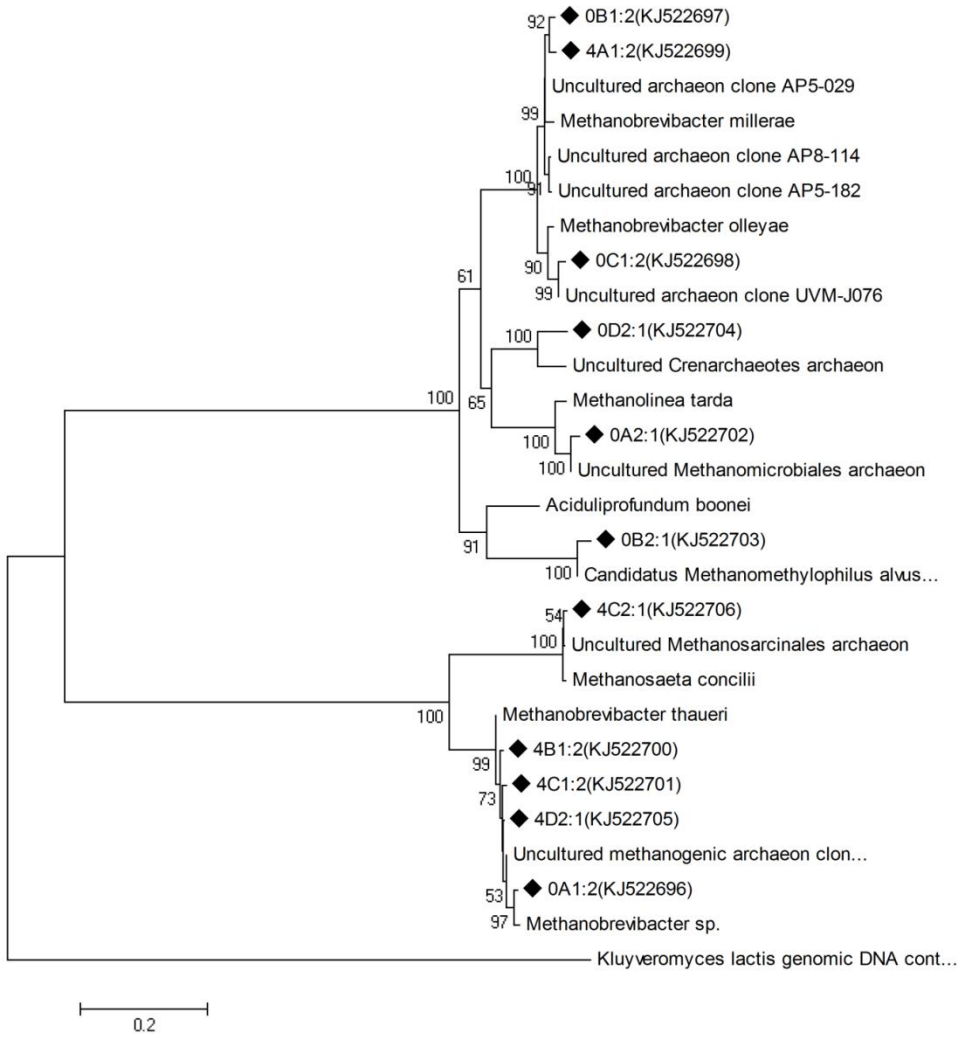

Figure. 2: Dendrogram of partial sequence of 16S rRNA of clone libraries from different ratio (1:2 and 2:1) co-mixture. The number at the nodes of the tree indicates bootstrap value of each node out of 1000 bootstrap resampling. The scale bar represents 0.2 substitutions per base position.

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References

[1] Alam F. Good Vibes for Palm Oil: An Industry Overview. Global Oils Fats Bus. Mag.. 2012; 9(1). ISSN No. 2180-4486.

[2] Ahmad A, Ismail S and Bhatia S. Water Recycling from Palm Oil Mill Effluent (POME) Using Membrane Technology. Desalination. 2003; 157:87-95.

[3] Ma AN. Environmental Management for the Oil Palm Industry. Palm Oil Dev. 2000; 30:1-10

[4] Yaser AZ, Rahman RA and Kahlil MS, Co-composting of palm oil mill sludge-sawdust. Pak. J. Biol. Sci. 2007; 10(24):4473-8.

[5] Rupani PF, Singh RP, Ibrahim MH and Esa N. Review of Current Palm Oil Mill Effluent (POME) Treatment Methods: Vermicomposting as a Sustainable Practice. WASJ. 2010; 11(1):70-81.

[6] Wright AG. and Pimm, C. Improved strategy for presumptive identification of methanogens using 16 riboprinting. J. Microbiol. Methods. 2003; 55(2):337-49.

[7] Huber T, Faulkner G and Hugenholtz P. Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics. 2004; 20:2317-19.

[8] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis

using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 2011; 28:2731-39.

[9] Wright ADG, Kennedy P, O'Neill C, Toovey AF, Popovski S, Rea SM, Pimm CL and Klein L. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine. 2004a; 22:3976–85.

[10] King EE, Smith RP, St-Pierre B and Wright AG. Differences in the Rumen Methanogen Populations of Lactating Jersey and Holstein Dairy Cows under the Same Diet Regimen. Appl. Environ. Microbiol. 2011; 77(16). doi: 10.1128/AEM.05130-11

[11] Singh KM, Tripathi AK, Pandya PR, Parnerkar S, Kothari RK, and Joshi CG. Molecular Genetic Diversity and Quantitation of Methanogen in Ruminal Fluid of Buffalo (*Bubalus bubalis*) Fed Ration (Wheat Straw and Concentrate Mixture Diet). Genet. Res. Int. 2013. doi:10.1155/2013/980191

[12] Hook SE, Wright ADG and McBride B. Methanogens: methane producers of the rumen and mitigation strategies. Archaea. 2010; 1–11.

[13] Wright ADG, Auckland CH, and Lynn DH. Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. Appl. Environ. Microbiol. 2007; 73(13):4206–10.

[14] Whitford MF, Teather RM, and Forster RJ. Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol. 2001; 1(5). doi:10.1186/1471-2180-1-5

[15] Wright ADG and St-Pierre B. Molecular analysis of methanogenic archaea in the forestomach of the alpaca (*Vicugna pacos*). BMC Microbiol. 2012; 12:1. doi:10.1186/1471-2180-12-1

[16] Tabatabaei M, Zakaria MR, Rahim RA, Wright AG, Shirai Y, Abdullah N, Sakai K, Ikeno S, Mori M. PCR-based DGGE and FISH analysis of methanogens in an anaerobic closed digester tank for treating palm oil mill effluent. J. Biotechnol. 2009; 12(3). DOI: 10.2225

[17] Imachi H, Sakai S, Sekiguchi Y, Hanada S, Kamagata Y, Ohashi A and Harada H. *Methanolinea tarda* gen. nov., sp. nov., a methane producing archaeon isolated from a methanogenic digester sludge. Int. J. Syst. Evol. Microbiol. 2008; 58, 294–301.