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MOLECULAR TECHNIQUE IDENTIFICATION OF THE MICROBIAL POPULATION IN PALM OIL MILL EFFLUENT (POME)

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ABSTRACT

Bioconversion of palm oil mill effluent (POME) to generate methane gas via anaerobic digestion involves a consortium of microbes which are responsible in several steps of the biodegradation process. In this study, the microbial community from a selected POME was characterised via molecular techniques and through culture-based plating in order to determine their composition, and subsequently understand their function in the anaerobic community. Genomic DNA of the microbial community was extracted using direct extraction technique, followed by PCR targeting the 16S rDNA region. Distinct fragments of approximately 1100 bp in sizes were successfully amplified using PCR and cloned onto Escherichia coli *XL-1 Blue. Upon sequencing of the fragments, BLAST queries identified the bacteria as* Thermoanaerobacterium *sp. In addition, two other bacterial species were successfully isolated from the POME by culturing on DVS agar. The sequencing result of these bacterial isolates showed both isolates belonged to the* Bacillus genus*. By understanding the bacterial community present in the POME, this will lead to the improvement of the anaerobic digestion process to enhance the production of biogas such as methane.*

Keywords: palm oil mill effluent, anaerobic digestion, microbial community.

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INTRODUCTION

Elaeis guineensis or more commonly known as oil palm is one of the major crops in Malaysia and Indonesia. Malaysia produced approximately 89 million tonnes of fresh fruit bunch per year (Singh *et al*., 2010). However, oil extraction process requires huge amount of water and it has been estimated that more than 50% of the water ends up as palm oil mill effluent (POME) (Ahmad *et al*., 2003). Approximately 53 million cubic metres POME is produced every year based on oil palm production in 2005 of 14.8 million tonnes (Lorestari, 2006). Although POME is non-toxic, it is identified as major source of aquatic pollutions when discharged untreated into nearby water system due to high concentration of organic matter, total solid, oil and grease, chemical oxygen demand (COD) as well as biological oxygen demand (BOD) (Rupani *et al*., 2010).

Many attempts to treat POME are currently being employed worldwide. One of the treatments introduced is the ponding system. Ponding system which is also known as waste stabilisation pond has been used in Malaysia since 1982 (Rupani *et al*., 2010). However, ponding systems have some disadvantages such as the need for large areas, long hydraulic retention time (HRT), bad odour and difficulties in maintaining liquid distribution

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(Rupani *et al*., 2010). Due to these limitations, anaerobic treatment of POME using newer technologies such as anaerobic digesters offer more attractive solutions for methane gas production and clean development mechanism (CDM).

One of the key factors in determining the efficiency of anaerobic digesters is the optimal composition of the bacterial community involved in the anaerobic degradation process, as the roles of the microbial consortia in this process are still not completely understood. Thus, it is critical to have an accurate understanding of the microbial population of the POME in order to provide an optimum condition for microbial propagation and to monitor the microbial activities which could contribute to greater methane production. In this study, the aim of the present work was to determine the microbial community in POME by using 16S rDNA clone library and traditional culture-based techniques.

MATERIALS AND METHODS

Samples Collection

POME was collected from the anaerobic pond from Bau Palm Oil Mill (BAPOM), Kuching, Sarawak, Malaysia. The samples were stored in sealed container immediately after collection and preserved at 4°C in order to avoid biodegradation due to microbial activities.

Isolation of Bacteria from POME

POME was cultured on DVS agar (Savant *et al*., 2002). The DVS medium has the following composition (per litre of distilled water): 6.0 g NaCl, 0.8 g $CaCl_2.2\ H_2O$, 1.0 g $MgCl_2.6\ H_20$, 10.0 g peptone, 10.0 g tryptone, 25.0 g CH_3CO_2K, 3.0 g KH_2PO_4, 3.0 g K_2HPO_4, 15.0 g $(NH_4)_2SO_4$, 20.0 g yeast and 30.0 g agar. Grown colonies were selected and purified by streaking on agar plate. All cultures were incubated at 50°C in anaerobic jar.

DNA Extraction and PCR Amplification

Colonies from the agar plates were selected and used for colony PCR whereas direct extraction of DNA from POME was carried out using Power Soil™ DNA Isolation Kit (Mo Bio Laboratories, USA), according to the manufacture's instruction. The DNA obtained was confirmed through electrophoresis in 1% agarose gel and amplified using the primer set 10f and 1100r (Miqueletto *et al*., 2011). PCR reactions were prepared in 25 µl final volume containing 50-100 ng of DNA, 10X *Taq* DNA polymerase buffer, 1.0 µl of 10 mM dNTP mix (Fermentas, Canada), 2.0 µl of 25 mM $MgCl_2$, 1.0 µl of each primer and 0.5 µl of 5 U AmpliTaq DNA polymerase (Fermentas, Canada). The PCR amplifications were then performed using an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 30 s at 96°C, 1 min at 54°C, and 1 min at 72°C; and a final extension of 7 min at 72°C.

Cloning 16S rDNA

PCR products were purified according to the manufacturer's instruction (Mo Bio Laboratories, USA). Purified colony PCR products were sequenced while the purified PCR fragments from direct extraction technique were cloned into pGEM-T Easy vector and transformed into *E.coli* XL-1 blue using the heat shock method (Sambrook and Russell, 2006). 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 compared to known 16S rDNA sequences in GeneBank database by using basic logical alignment tool (BLAST). Closely related sequences were aligned with PCR sequences using the program CLUSTAL W and further edited manually. Phylogenetic tree were constructed by neighbour-joining method (Saitou and Nei, 1987) using MEGA ver 5.0 (Tamura *et al*., 2011).

RESULTS AND DISCUSSION

The 16S rDNA region was successfully amplified and cloned into pGEM-T Easy vector. From nine clones screened, only three (Isolate C, E and H) were successfully cloned with the PCR fragment size of 1100 bp. Upon cloning, the extracted plasmids from the successful clones were re-amplified, purified and sequenced (*Figure 1*). As for the Isolates A and D from solid agar, PCR product of 1100 bp was successfully amplified (*Figure 1*). *Table 1* shows the result of the DNA sequencing.

In order to further understand the microbial community in POME, the isolates were included in the phylogenetic tree and *Kluyveromyces lactis* was used as the outgroup (*Figure 2*). The sequences obtained in this study have been deposited in the GenBank database under accession numbers KF539415-KF539419.

From the phylogenetic tree, all five isolates were classified in the phylum of *Firmicutes*. Three of the *Firmicutes* (C, E and H) were clustered in the class of *Clostridia* while Isolates A and D were assigned to the class of *Bacilli*. Isolate C, E and H were closely associated with *Thermoanaerobacterium* sp. from the class *Clostridia* with 99% similarity to the uncultured *Thermoanaerobacteriaceae* bacterium clone THPB-7 that

TABLE 1. SEQUENCES HOMOLOGY OF THE ISOLATES

Isolates	Bacteria	Gen Bank database	Sequences homology (%)
A	*Bacillus thermoamylovorans*	FN397520	98
C	Uncultured *Thermoanaerobacteriaceae*	AM408569	99
D	*Bacillus coagulans*	AB830332	99
E	Bacterium *Uncultured Thermoanaerobacteriaceae*	AM408569	99
H	Uncultured *Thermoanaerobacteriaceae bacterium*	AM408569	99

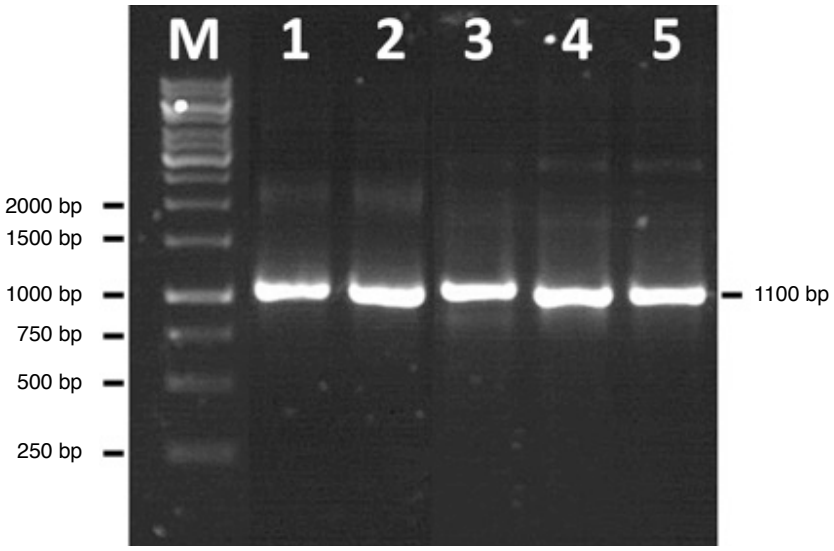

Figure 1. Agarose gel electrophoresis of amplified 16S rDNA region of the isolates. The extracted plasmid from 16S rDNA clone library and PCR products of isolated bacteria from solid agar which were successfully amplified (1100 bp). Lane M, 1 kb ladder (Fermentas); lane 1, 2 and 3, PCR products amplified by using extracted plasmid from Isolates C, E and H respectively. Lane 4 and 5, DNA template of bacteria A and D isolated from DVS agar.

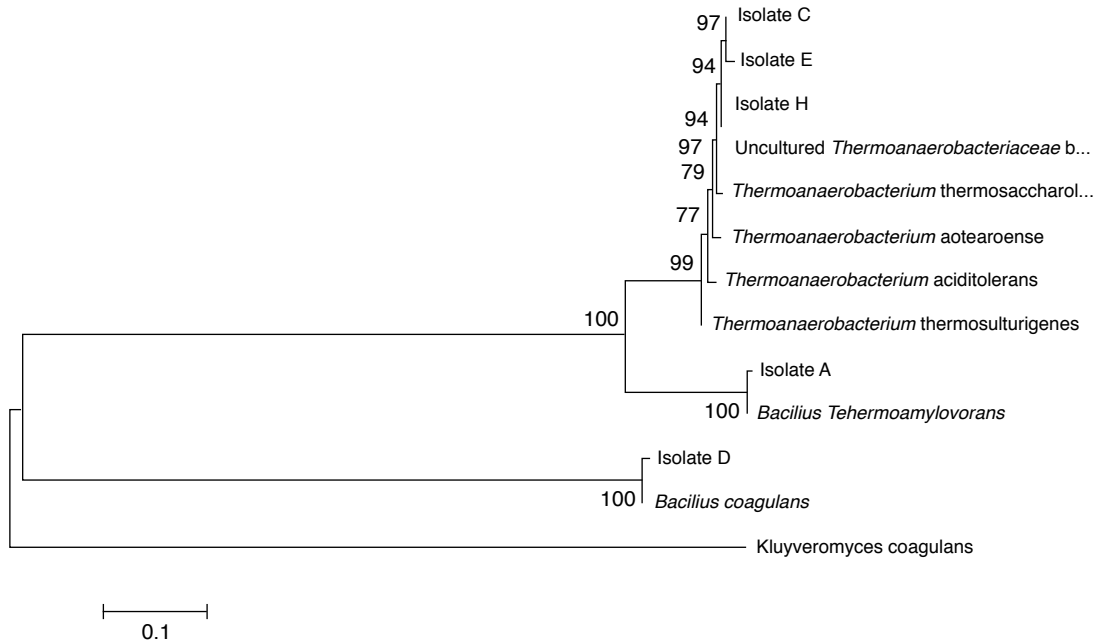

Figure 2. Dendrogram of partial sequence of 16S rDNA from palm oil mill effluent (POME), grouped by class. The dendrogram was constructed by the neighbour-joining method. The number at the nodes of the tree indicates bootstrap value of each node out of 100 bootstrap resampling. The scale bar represents 0.1 substitutions per base position.

had been previously found in environment sample taken from anaerobic sequencing batch reactor (AM408569, NCBI database). The *Thermoanaerobacter* sp. had also been reported in POME sludge (Khemkhao *et al*., 2011; O-Thong *et al*., 2011), paper mill and waste water from breweries (Suihko *et al*., 2005; Sommer *et al*., 2004). The *Thermoanaerobacter* sp. is known to be associated with the fermentation of glucose into ethanol, acetic acid, butyric acid, hydrogen and carbon dioxide (Koskinen *et al*., 2008; Shin and Youn, 2005; Lynd *et al*., 2002).

The sequences of isolates from solid agar (Isolate D and A) were identified as member of the bacterial genera *Bacillus*, *Bacillus coagulan* and *Bacillus thermoamylovorans* with 99% and 98% similarities respectively. The presence of *Bacillus* genus bacteria in sludge and agriculture wastes was also shown in previous findings (Ivanov *et al*., 2004; Vossoughi *et al*., 2001). It is known that *Bacillus thermoamylovorans* and *Bacillus coagulans* are capable of producing ethanol, acetate and lactate from glucose utilisation (Tay *et al*., 2002). Similar results were also reported by other researchers (Kotay *et al*., 2007; Pantamas *et al*., 2003).

Bacillus coagulans, Bacillus thermoamylovorans, *Thermoanaerobacter* sp. are thermophilic bacteria. Metabolically, they are facultative and/or strict anaerobes and moderately acidophile (Kublanov *et al*., 2007; Vecchi and Dargo, 2006; CombetBlanc *et al*., 1995). These characteristics enable the bacteria to survive in POME which is acidic with pH in between 4 to 5 (Madaki and Lau, 2013). This is supported by previous findings regarding isolation of these bacteria from acidic and/or extreme environments (Longo *et al*., 2010; Koskinen *et al*., 2008; Kublanov *et al*., 2007).

The capability of *Bacillus coagulans*, *Bacillus thermoamylovorans*, and *Thermoanaerobacter* sp. in utilising different substrates (Su and Xu, 2014; Chang *et al*., 2008; Kublanov *et al*., 2007) holds a promising future in the studies of anaerobic digestion treatment of POME. The ability to convert variety of substrates can help to improve hydrolysis process in anaerobic digestion of POME by employing co-digestion. Digestion of waste rich in nitrogen concentration such as POME (Baharuddin *et al*., 2010) together with high carbon content waste will balances the carbon to nitrogen (C/N) ratio (Lehtomaki *et al*., 2007). Additionally, co-digestion increases the digestion, stabilisation as well as biogas yield (Lo *et al*., 2010; Jingura and Matengaifa, 2009).

CONCLUSION

The microbial community of the POME was reflected from 16S rRNA clone library and traditional culture-based technique. The *Thermoanaerobacter* sp. from Clostridia class was isolated through 16S rRNA clone library while *Bacillus coagulans* and *Bacillus thermoamylovorans* were isolated using traditional culture-based technique. High sequence similarities (>90%) were found in both isolation methods.

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