



UNIVERSITI PUTRA MALAYSIA

**BACTERIAL AND METHANOGENIC ARCHAEAL COMMUNITY
CHANGES DURING TREATMENT OF PALM OIL MILL EFFLUENT AND
BIOLOGICAL INDICATORS FOR FINAL DISCHARGE**

DIANA MOHD NOR

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By

DIANA BINTI MOHD NOR

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulmiment of the Requirements for the Degree of
Doctor of Philosophy**

April 2019

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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April 2019

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The sustainable practice in the palm oil industry which supplies the most demanded edible oil in the world, has a long way to be well developed. One of the most challenging problems is the management of wastewater generated from the oil palm processing, known as palm oil mill effluent (POME). The most popular treatment method applied to treat POME in the palm oil mills is anaerobic ponding system. Bioconversion of POME to generate methane gas via anaerobic digestion involves a consortium of microbes which are responsible in several steps of the biodegradation process. However, the biodegradation potential of the microorganisms in the full-scale treatment system in the palm oil mill is yet to be explored. POME is also known to have the adverse environmental effects if it is not properly treated, including contamination of land and aquatic ecosystem and the loss of biodiversity. A proper treatment is needed to ensure POME can be discharged into the nearby river water or land according to the requirement set by the authority. However, the current monitoring system using physicochemical characterisation is not sensitive enough to indicate the actual source of contamination in the water bodies.

In general, a detailed evaluation of the compositions of the bacterial communities in the POME final discharges obtained from four different palm oil mills and composition of bacterial community during the anaerobic treatment of POME were elucidated in this study using PCR-denaturing gradient gel electrophoresis (DGGE) and high-throughput MiSeq approaches, aided by advanced bioinformatics analysis in analysing the bacterial community structures. The correlation relationships were also carried out which allow deeper understanding of the interactions between the shift of bacterial community compositions and the changes of physicochemical properties of POME, including pH, temperature,

biochemical oxygen demand (BOD₅) and chemical oxygen demand (COD). The compounds analyses were also done to correlate the biodegradation potential of bacteria during the treatment of POME.

The findings demonstrated a significant difference of bacterial species richness and evenness among the four POME final discharges. However, the bacterial community compositions in the different final discharges exhibited almost similar patterns in that the phylum *Proteobacteria* was dominant in all the samples. Interestingly, the proposed bioindicators to indicate the river water contamination due to POME final discharge, the *Alcaligenaceae* and *Chromatiaceae* families, were found to be present in all the four final discharges despite the different characteristics of the mills and the different biotreatment processes used by them. In addition, both bioindicators were also shown to be strongly and positively correlated with the concentration of BOD₅, hence make them reliable bioindicators to indicate the river water contamination due to POME final discharge.

Furthermore, in order to elucidate the biodegradation potential of microorganisms in the POME treatment, a thorough analysis of bacterial and archaeal communities in different stages of POME treatment was carried out which comprised of anaerobic, facultative anaerobic and aerobic processes, including the mixed raw effluent (MRE), mixing pond, holding tank and final discharge phases. The bacterial and archaeal communities were shown to be shifted according to their biodegradation potential and the changes of physicochemical properties of POME. Based on the data obtained, the following biodegradation processes were suggested to take place in the different treatment stages: (1) *Lactobacillaceae* (35.9%) dominated the first stage that contributed to high lactic acid production; (2) higher population of *Clostridiaceae* in the mixing pond (47.7%) and *Prevotellaceae* in the holding tank (49.7%) contributed to the higher acetic acid production; (3) the aceticlastic methanogen *Methanosaetaceae* (0.6–0.8%) played a role in acetic acid degradation in the open digester and closed reactor for methane generation; (4) *Syntrophomonas* (21.5–29.2%) might be involved in fatty acids and acetic acid degradations by syntrophic cooperation with hydrogenotrophic methanogen, *Methanobacteriaceae* (0.6–1.3%); (5) phenols and alcohols detected in the early phases but not in the final discharge indicated the successful degradation of lignocellulosic materials. A sustainable palm oil industry could be developed with better POME pollution management by adopting a reliable and accurate monitoring system. To our knowledge, this is the first study reported on the biodegradation mechanisms involved in the different stages of the full-scale treatment of POME.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PERUBAHAN KOMUNITI BAKTERIA DAN ARKEA METANOGENIK
SEMASA RAWATAN EFLUEN KILANG MINYAK KELAPA SAWIT DAN
INDIKATOR BIOLOGI UNTUK PELEPASAN AKHIR**

Oleh

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Amalan lestari dalam industri minyak sawit menyediakan minyak makan yang paling banyak di dunia masih mempunyai perjalanan yang jauh untuk dibangunkan dengan baik. Salah satu cabaran pengurusan yang paling mencabar adalah pengurusan air sisa yang dihasilkan daripada pemprosesan minyak sawit, dikenali sebagai efluen kilang minyak sawit (POME). Kaedah rawatan POME yang paling popular digunakan dalam kilang minyak kelapa sawit adalah sistem kolam anaerobik. Biopertukaran POME untuk menjana gas metana melalui proses anaerobik melibatkan konsortium mikrob yang bertanggungjawab dalam beberapa langkah proses biopenguraian POME. Walaubagaimanapun, potensi biodegradasi mikroorganisma dalam sistem rawatan skala penuh di kilang kelapa sawit masih belum diterokai. POME juga telah diketahui memberi kesan buruk terhadap alam sekitar jika ia tidak dirawat dengan baik, termasuk pencemaran ekosistem darat dan akuatik serta kehilangan biodiversiti. Pelaksanaan kaedah rawatan yang betul diperlukan untuk memastikan POME boleh dilepaskan ke dalam air sungai atau tanah yang berdekatan mengikut ketetapan pihak berkuasa. Walaubagaimanapun, sistem pemantauan semasa menggunakan pencirian fizikokimia tidak cukup sensitif untuk menunjukkan sumber sebenar pencemaran di dalam badan air.

Secara umumnya, penilaian terperinci mengenai komposisi komuniti bakteria dalam pelepasan akhir POME yang diperolehi daripada empat kilang minyak sawit dan komposisi komuniti bakteria semasa rawatan anaerobik POME telah dijelaskan di dalam kajian ini menggunakan PCR-gel elektroforesis penyahasian kecerunan (DGGE) dan Illumina MiSeq, dibantu oleh analisis bioinformatik dalam menganalisis struktur komuniti bakteria. Hubungan korelasi juga dijalankan bagi memahami lebih mendalam tentang interaksi antara

peralihan komposisi komuniti bakteria dan perubahan sifat fizikokimia POME, termasuk pH, suhu, permintaan oksigen biokimia (BOD₅) dan permintaan oksigen kimia (COD). Analisis sebatian juga dilakukan untuk mengaitkan potensi biopenguraian bakteria semasa rawatan POME.

Hasil kajian menunjukkan terdapat perbezaan yang ketara dalam kekayaan dan kesamarataan spesies bakteria antara empat pelepasan akhir POME. Walau bagaimanapun, komposisi komuniti bakteria dalam pelepasan akhir yang berbeza mempamerkan corak yang hampir serupa yang mana filum *Proteobacteria* adalah dominan dalam semua sampel. Menariknya, bioindikator yang dicadangkan untuk menunjukkan pencemaran air sungai disebabkan oleh pelepasan akhir POME, iaitu keluarga *Alcaligenaceae* dan *Chromatiaceae*, didapati hadir dalam kesemua empat pelepasan akhir walaupun keempat-empat pelepasan akhir ini mempunyai ciri-ciri fizikokimia serta melalui proses bio-rawatan yang berbeza. Di samping itu, korelasi positif yang kuat terhadap kepekatan BOD₅ ditunjukkan bagi kedua-dua bioindikator ini, maka bioindikator ini diyakini menjadi bakteria penunjuk yang boleh digunakan untuk menunjukkan pencemaran air sungai disebabkan oleh pelepasan akhir POME.

Tambahan pula, untuk menjelaskan potensi biodegradasi mikroorganisma dalam rawatan POME, analisis menyeluruh komuniti bakteria dan arkea dalam pelbagai peringkat rawatan POME juga dijalankan, yang terdiri daripada proses anaerobik, fakultatif anaerobik dan aerobik, termasuk campuran efluen mentah (MRE), kolam pencampuran, tangki takungan dan fasa pelepasan akhir. Peralihan komuniti bakteria dan arkea mengikut potensi biopenguraian dan perubahan sifat fizikokimia POME telah ditunjukkan. Berdasarkan data yang diperolehi, proses biopenguraian berikut dicadangkan telah berlaku dalam peringkat rawatan yang berbeza: (1) *Lactobacillaceae* (35.9%) mendominasi peringkat pertama yang menyumbang kepada penghasilan asid laktik yang tinggi; (2) populasi *Clostridiaceae* yang lebih tinggi di kolam pencampuran (47.7%) dan *Prevotellaceae* dalam tangki takungan (49.7%) menyumbang kepada penghasilan asid asetik yang lebih tinggi; (3) metanogen asetiklastik *Methanosaetaceae* (0.6-0.8%) memainkan peranan dalam penguraian asid asetik di dalam tangki pencernaan terbuka dan tangki reaktor tertutup bagi penjaan metana; (4) *Syntrophomonas* (21.5-29.2%) mungkin terlibat dalam penguraian asid lemak dan asid asetik yang berhubung secara sintrofik dengan metanogen hidrogenotropik, *Methanobacteriaceae* (0.6-1.3%); (5) fenol dan alkohol yang dikesan pada fasa awal tetapi tidak dikesan dalam pelepasan terakhir, menunjukkan proses penguraian bahan lignoselulosa yang berjaya. Industri minyak kelapa sawit yang lestari boleh dibangunkan dengan pengurusan pencemaran POME yang lebih baik melalui sistem pemantauan yang diyakini dan tepat. Setakat yang diketahui, kajian ini merupakan kajian pertama yang melaporkan berkenaan mekanisme biopenguraian yang terlibat dalam pelbagai peringkat rawatan POME berskala penuh.

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5.11 Schematic flow process diagram of biodegradation in the different stages during the treatment of POME catalysed by dominant bacteria and archaea populations for the generation of methane as end-product

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LIST OF ABBREVIATIONS

ACE	Abundance-based Coverage Estimator
APHA	American Public Health Association
ARDRA	Amplified ribosomal DNA restriction analysis
BOD	Biochemical oxygen demand
bp	Base pair
BPA	Bisphenol A
CaCl ₂	Calcium chloride
CO ₂	Carbon dioxide
CCA	Canonical correspondence analysis
COD	Chemical oxygen demand
CPO	Crude palm oil
ddNTPs	Dideoxy nucleoside triphosphates
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOE	Department of Environment
FeCl ₃	Ferric chloride
FELDA	Federal Land Development Authority
FFA	Free fatty acid
FFB	Fresh fruit bunch
g	Gram
H ₂	Hydrogen
HNA	High nucleic acid
HRT	Hydraulic retention time

IC	Inorganic carbon
kg	Kilogram
LNA	Low nucleic acid
LH-PCR	Length heterogeneity polymerase chain reaction
m ³	Cubic metre
mg/L	Milligram per litre
MgSO ₄	Magnesium sulphate
min	Minutes
mL	Millilitre
mm	Millimetre
MPOB	Malaysian Palm Oil Board
MPOC	Malaysian Palm Oil Council
ng	Nanogram
nm	Nanometre
NGS	Next generation sequencing
O ₂	Oxygen
OTU	Operational taxonomic unit
NP	4-Nonylphenol
PCR	Polymerase chain reaction
POME	Palm oil mill effluent
RDP	Ribosomal database project
rRNA	Ribosomal RNA
RISA	Ribosomal intergenic spacer analysis
SSCP	Single-strand conformation polymorphism
SCFA	Short chain fatty acids
TAE	Tris-acetate-EDTA

TEMED	Tetraethylmethylenediamine
TOC	Total organic carbon
TSS	Total suspended solids
T-RFLP	Terminal restriction fragment length polymorphism
VSS	Volatile suspended solids
wt/vol	Weight per volume
μL	Microlitre



CHAPTER 1

INTRODUCTION

The oil palm industry is an active contributor to the nation's economic growth with high annual export profit of palm products which was estimated at RM 78 billion just in the year 2017 (Kushairi et al., 2017). The production of palm oil will continue to increase to meet the global demand for oil and fats products. Although the oil palm industry appears to be one of Malaysia's most structured agro-industry, the milling process produces massive colloidal wastewater during the oil extraction process at the palm oil mill, known as palm oil mill effluent (POME). The traditional milling process normally employs steam for the sterilisation of fruit, which finally lead to the formation of POME (Law et al., 2016). Typically, 1 tonne of crude palm oil production requires approximately 5 to 7 tonnes of water which over 50% of it ends up as POME (Wang et al., 2015). Due to the higher discharge of POME into the receiving water bodies, the oil palm industry has been claimed as one of the largest water polluters in Malaysia.

The huge generation of POME from the palm oil industry has become a serious issue that not only affects the industry, but also people and the environment. POME is brownish as it contains appreciable amounts of organic matter which eventually increases both the biochemical oxygen demand (BOD) and the chemical oxygen demand (COD) that originate from steam extraction process (Bala et al., 2015; Saeed et al., 2015). Without a proper treatment, POME will inflict serious environmental pollution. The anaerobic digestion applied for the treatment of POME could increase the rate of biodegradation (Poh and Chong, 2009), in addition to the conventional POME treatment using the ponding system. However, if it is not being managed efficiently, the discharge of treated or partially treated POME into a nearby river could lead to severe environmental pollution (Rupani et al., 2010).

Therefore, it is important to have a reliable assessment method to indicate the source of contamination due to POME final discharge. Current assessment via determination of the physicochemical properties of the affected river water is considered inaccurate as they may contain pollutants originated from other anthropogenic sources such as from the residential areas and agricultural practices. The use of an indicator could be regarded as a potential approach for assessing the specific cause of pollution in the effluent receiving river water (Zhang et al., 2014). The *Alcaligenaceae* and *Chromatiaceae* have been proposed in the previous study to be used as potential bioindicators to indicate the river water contamination due to POME final discharge. Both of them which were reported to be present in the effluent receiving river but not in the unpolluted river water were also determined to be originated from the POME final discharge (Sharuddin et al., 2017).

Nevertheless, the aim of POME treatment is alike which is to reduce the polluting power of this wastewater below the effluent discharge standard before it is being released into the environment, but different biotreatment processes have been employed in the different palm oil mills, generating different properties of POME final discharge. The generation of waste from palm oil mills is also dependent on the amount of fresh fruit bunch (FFB) processed (Liew et al., 2017). Hence, it remains unclear how these different biotreatment processes and capacities could affect the result of the previous study which determined that *Alcaligenaceae* and *Chromatiaceae* were the bioindicators. Since the bacteria are highly sensitive to disturbances and could react differently depending on the properties of pollutants, environmental factors and treatment processes, it is important to assess the practical relevance of the bioindicator for monitoring the river water quality.

In addition, as emphasis is placed on the concept of biodegradability in biological treatment of POME, utilisation of vast microbial consortia in the pond and anaerobic treatment systems in the mills (Choong et al., 2018; McHugh et al., 2003) needs to be looked into more thoroughly. Up to date, insights into the bacterial and archaeal communities and key players catalysing a complex series of biochemical reactions to reduce the polluting power of POME in the treatment system are still unclear. Therefore, the substrates causing inefficient treatment of POME are not ascertained. Digestions problems may also arise from insufficient understanding of the biodegradation mechanism which in turn resulting in failure of the treatment system.

Current studies have focused largely on the microbial community structure of POME in various bioreactor configurations (Rana et al., 2017) with little attention given to the bacterial and archaeal consortia involved in the biodegradation in the different stages of full-scale treatment of POME. The management of POME remains unclear and challenging without understanding the biodegradative pathways involved throughout the treatment. Therefore, to ensure a sustainable industrial practice, it is essential to have more knowledge on the bacterial population, diversity and how they are related to the biodegradation process and severity of pollution.

The current conventional culturing methods used in analysing the bacterial community structure is inadequate as most of the important environmental bacterial species are unculturable (Lu et al., 2015; Rani et al., 2008). However, with the application of molecular phylogenetic methods and the emergence of next generation sequencing (NGS), it is now possible to obtain a view on the bacterial community that was previously inaccessible (Tan et al., 2015). The integration of PCR-denaturing gradient gel electrophoresis (DGGE) approach and high-throughput MiSeq serves as valuable tools to study the bacterial community composition and structure (Yu et al., 2015). The databases containing information regarding the relative abundance and activity of bacterial communities could provide thorough insights on the bacterial community

composition in the POME final discharges taken from different biotreatment processes of POME, as well as the bacterial and archaeal diversity and composition in the different stages of POME treatment. The biodegradation potential of bacterial community throughout the treatment process could also be obtained by correlating the shift of bacterial community with the changes of physicochemical properties of POME.

This study aimed to assess bacterial community changes in the POME final discharges generated from different biotreatment processes, as well as during the full-scale treatment of POME. The specific objectives of this research were:

- I. To assess the presence of bioindicators in the POME final discharge generated by different biotreatment processes and correlate to the changes of pollutant properties;
- II. To assess the changes of bacterial and methanogenic archaeal communities in the different stages during the treatment of POME.

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BIODATA OF STUDENT



Diana Mohd Nor was born on March 21, 1981. She attended her preliminary school at Sekolah Kebangsaan Seberang Takir, Kuala Terengganu, Terengganu from 1988 to 1993. She continued her secondary education at Sekolah Menengah Kebangsaan Agama Tok Jiring, Kuala Terengganu, Terengganu (1994 – 1996) and MARA Junior Science College, Kuala Berang, Terengganu (1997-1998). Later, in 1999, she went to MARA Seremban Matriculation College, Seremban, Negeri Sembilan. After one year completion of her matriculation program, she was promoted to continue his First Degree in Bachelor Science of Plant Biotechnology, a three year program at Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor and graduated in July, 2003. She was offered to join a master's degree in Molecular Biology at UKM under the supervision of Dr. Roohaida Othman and graduated in 2012. In February 2014, she started her PhD study in the field of Environmental Biotechnology under supervision of Dr. Norhayati Ramli. She registered for PhD study with the School of Graduate Studies, Universiti Putra Malaysia (UPM) under Dual Degree Program (DDP) with Kyushu Institute of Technology (Kyutech), Japan. Throughout her study, she has been given the opportunities to carry out her research in Kyushu Institute of Technology, Japan under the joined research work. She also has experiences in guiding undergraduate students on the final year project, assisting in handling the laboratory class and paper publication. Up to date, she managed to publish two manuscripts in ISI journals. She also has participated in several conferences and workshops.

LIST OF PUBLICATIONS

Paper Publications

- Mohd-Nor, D., Ramli, N., Sharuddin, S.S., Hassan, M.A., Mustapha, N.A., Amran, A., Sakai, K., Shirai, Y. and Maeda, T. 2018. *Alcaligenaceae* And *Chromatiaceae* as Reliable Bioindicators Present in Palm Oil Mill Effluent Final Discharge Treated by Different Biotreatment Processes. *Ecological Indicators* 95: 468-473.
- Sharuddin, S.S., Ramli, N., Hassan, M.A., Mustapha, N.A., Amran, A., Mohd-Nor, D., Sakai, K., Tashiro, Y., Shirai, Y. and Maeda, T., 2017. Bacterial community shift revealed *Chromatiaceae* and *Alcaligenaceae* as potential bioindicators in the receiving river due to palm oil mill effluent final discharge. *Ecological Indicators*, 82, 526–529.
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- Mohd-Nor, D., Ramli, N., Hassan, M.A., Sakai, K., Tashiro, Y., Maeda, T. and Shirai, Y. 2014. Microbial community changes in different stages of palm oil mill effluent treatment. Symposium of Applied Engineering and Sciences 2014 (SAES 2014). 10-11th November 2014, Kyushu Institute of Technology, Japan. (Poster Presentation).
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