UNIVERSITI PUTRA MALAYSIA

BIOMONITORING PALM OIL MILL EFFLUENT FINAL DISCHARGE POLLUTED RIVERS USING MOLECULAR-BASED APPROACHES

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By

NURHASLIZA BINTI ZOLKEFLI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Palm oil industry has been associated with water quality issues from the practice of palm oil mill effluent (POME) final discharge disposal into nearby river. Nevertheless, it is hardly proven since the river is exposed to many other anthropogenic pollutants. The current method for the pollution assessment using physicochemical profiling is less specific. This necessitates the establishment of a complementary molecular-based biomonitoring system where the bacterial indicator and genetic biomarker detections specific for POME pollutants can be utilized. Present study was done to determine the dominant presence of *Alcaligenaceae* and *Chromatiaceae* in the POME final discharge polluted rivers in comparison to the other rivers polluted by mining drainage, automotive oil and chemical-related industrial effluents. Based on the capability of *Alcaligenaceae* to biodegrade lignin derived phenolic compounds, this research was also done to identify the functional genes involved in POME pollutants biodegradation. To achieve the objectives, physicochemical characterization was done to correlate with the detection of the potential bioindicators. 16S rRNA next generation amplicon sequencing was done by Illumina MiSeq, while the bacterial cells viability and nucleic acid activities in all studied rivers were evaluated by utilizing double nucleic acid-based assay of the flow cytometry. In addition, the significant functional genes carried by the bacterial communities involved in the POME biodegradation were screened in the final discharge and polluted rivers by conventional polymerase chain reaction (PCR). Overall, it was confirmed that *Alcaligenaceae* (0.53% - 0.96%) and *Chromatiaceae* (0.81% - 3.44%) are dominant in the POME final discharge polluted rivers and not in other polluted rivers. The principle coordinate (PCO) analysis had proved the strong positive correlation between these two bacterial families with 5-day biological oxygen demand (BOD_5). Plus, the environmental changes lead to a higher bacterial viability (58.3% - 69.5%) and high nucleic acid (HNA) composition (75.0% - 82.2%) in the POME final discharge polluted rivers as compared to the other polluted rivers with cell viability ranging from 4.4% - 57.6% and HNA from 2.2% - 13.4%. The functional gene screening had resulted with the characterization of phenol

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hydroxylase and protocatechuate 3,4-dioxygenase genes which are consequentially involved in the degradation of lignin derived phenolic compounds via the β-ketoadipate metabolic pathway. It is impressive that the high intensity of the protocatechuate 3,4-dioxygenase gene at 800 bp was shared only by the bacterial communities in POME final discharges and the receiving downstream rivers but not in the other comparative rivers. These findings prove on the specificity of *Alcaligenaceae* and *Chromatiaceae* as the POME final discharge pollution bioindicators in the river and the potential of protocatechuate 3,4-dioxygenase to be used as a molecular biomarker for the detection of this pollutant in the river.

KEYWORDS: *amplicon sequencing; bioindicator; palm oil mill effluent; phenol hydroxylase; protocatechuate 3,4-dioxygenase*

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Sarjana Sains

BIOPEMANTAUAN SUNGAI YANG DICEMARI PELEPASAN AKHIR EFLUEN KILANG MINYAK SAWIT MENGGUNAKAN PENDEKATAN BERASASKAN MOLEKUL

Oleh

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Industri minyak kelapa sawit telah dikaitkan dengan isu-isu kualiti air daripada amalan pelupusan pelepasan akhir efluen kilang minyak sawit (POME) ke dalam sungai berhampiran. Walaubagaimanapun, ianya sukar untuk dibuktikan kerana sungai juga terdedah kepada banyak bahan cemar antropogenik lain. Kaedah semasa bagi penilaian pencemaran tersebut menggunakan pemprofilan fizikokimia adalah kurang spesifik. Ini memerlukan satu pembentukan sistem biopemantau pelengkap berasaskan molekul dimana pengesanan bakteria penunjuk dan biopenanda genetik yang spesifik bagi bahan pencemar POME boleh digunakan. Kajian ini telah dijalankan untuk menentukan kehadiran dominan *Alcaligenaceae* dan *Chromatiaceae* di dalam sungai-sungai yang dicemari oleh pelepasan akhir POME berbanding sungai-sungai lain yang dicemari oleh saliran pelombongan, minyak automotif dan efluen industri berkaitan kimia. Berdasarkaan keupayaan *Alcaligenaceae* untuk biodegradasi sebatian fenolik yang berasal dari lignin, kajian ini juga telah dilakukan untuk mengenalpasti gen-gen berfungsi yang terlibat dalam biodegradasi bahan pencemar POME. Bagi mencapai objektif ini, pencirian fizikokimia dilakukan untuk menghubungkaitkannya dengan pengesanan bioindikator yang berpotensi. Penjujukan amplikon generasi akan datang 16S rRNA dilakukan dengan penjujukan “Illumina MiSeq”, sementara kebolehhidupan sel bakteria dan aktiviti asid nukleik di dalam semua sungai yang dikaji dinilai dengan menggunakan sitometri aliran berasaskan asai asid nukleik berganda. Di samping itu, gen berfungsi penting yang dibawa oleh komuniti bakteria yang terlibat dalam biodegradasi POME telah disaring di dalam efluen akhir serta sugai-sungai tercemar menggunakan reaksi rantai polimerase (PCR) konvensional. Secara keseluruhannya, disahkan bahawa *Alcaligenaceae* (0.53% - 0.96%) dan *Chromatiaceae* (0.81% - 3.44%) adalah dominan di dalam sungai-sungai dicemari pelepasan akhir POME dan tidak di dalam sungai tercemar lain. Analisis penyelarasan koordinat utama (PCO) telah membuktikan hubungan positif antara kedua-dua keluarga bakteria ini dengan permintaan

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oksigen biologi 5 hari (BOD_5). Tambahan lagi, perubahan persekitaran ini membawa kepada kebolehhidupan bakteria (58.3% - 69.5%) dan komposisi asid nukleik tinggi (HNA) (75.0% - 82.2%) yang lebih tinggi di dalam sungai yang dicemari pelepasan akhir POME berbanding dengan sungai tercemar yang lain dengan kebolehhidupan sel antara 4.4% - 57.6% dan HNA antara 2.2% - 13.4%. Penyaringan gen berfungsi menghasilkan pencirian gen fenol hidroksilase dan protokatekuat 3,4-dioksigenase yang terlibat secara berturutan dalam biodegradasi sebatian fenolik dari lignin melalui laluan metabolik β-ketoadipate. Ianya mengagumkan apabila intensiti protokatekuat 3,4-dioksigenase pada 800 bp hanya dikongsi oleh komuniti bakteria di dalam pelepasan akhir POME dan hilir sungai yang dicemarinya sahaja tetapi tidak di sungai-sungai lain yang dibandingkan. Penemuan ini membuktikan kekhususan *Alcaligenaceae* dan *Chromatiaceae* sebagai bakteria penunjuk bagi pencemaran pelepasan akhir POME di dalam sungai dan potensi gen protokatekuat 3,4-dioksigenase untuk digunakan sebagai biopenanda molekul untuk mengesan pencemaran ini di dalam sungai.

KATA KUNCI: *penjujukan amplikon; biopenanda; pelepasan akhir efluen kilang minyak sawit; fenol hidroksilase; protokatekuat 3,4-dioksigenase*

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LIST OF ABBREVIATIONS

°C	Degree Celsius
× g	Gravitational force
α-	Alpha-
β-	Beta-
1,2-CTD	Catechol 1,2-dioxygenase
2,3-CTD	Catechol 2,3-dioxygenase
3-D	Three-dimensional
3,4-Pcd/PCD	Protocatechuate 3,4-dioxygenase
4,5-PCA	Protocatechuate 4,5-dioxygenase
AOP	Automotive oil polluted river
APHA	American Public Health Association
BLASTn	Basic Local Alignment Search Tool for nucleotide
BOD_3	Biological oxygen demand after 3 days
BOD_5	Biological oxygen demand after 5 days
bp	Base pair
CCMEWQI	Canadian Council of Ministers of the Environment Water Quality Index
CIP	Chemical industry polluted river
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CPO	Crude palm oil
DAPI	4’,6-diamino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxynucleic acid
DO	Dissolved oxygen
DOE	Department of Environment
DOM	Dissolved organic compounds
E’	Evenness index
EDTA	Ethylenediaminetetraacetic acid
EFB	Empty fruit bunch
EQA	Environmental Quality Act
FFB	Fresh fruit bunch
FSC	Forward-scattered light
g	Gram
GAE	Gallic acid equivalent

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H’	Shannon-Weaver index
HNA	High nucleic acid
kbp	Kilo base pair
kg	Kilogram
L	Liter
LNA	Low nucleic acid
m-	*Meta-*
MDP	Mining drainage polluted river
MEGA	Molecular Evolutionary Genetics Analysis
mg/L	Milligram per liter
mL	Milliliter
mM	Milli molar
MMWQI	Malaysian Marine Water Quality Index
MPOB	Malaysian Palm Oil Board
NA	Not available
Na_2CO_3	Sodium carbonate
ND	Not detected
ng	Nanogram
nm	Nanometer
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NH_3-N	Ammoniacal nitrogen
NO_3^-	Nitrate
NSFWQI	National Sanitation Foundation Water Quality Index
o-	*Ortho-*
OPF	Oil palm frond
OTU	Operational taxonomic unit
OWQI	Oregon Water Quality Index
p-	*Para-*
PAH	Polycyclic aromatic hydrocarbon
PAST	Paleontological Statistics
PCO	Principal coordinate
PCR	Polymerase chain reaction
PFD	POME final discharge polluted river
Phe/PHE	Phenol hydroxylase
PI	Propidium iodide
PKC	Palm kernel cake

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PKS	Palm kernel shells
POME	Palm oil mill effluent
PPF	Palm pressed fiber
QIIME	Quantitative Insights into Microbial Ecology
qRT-PCR	Quantitative real-time polymerase chain reaction
rDNA	Ribosomal deoxynucleic acid
rRNA	Ribosomal ribonucleic acid
SSC	Side-scattered light
SRA	Short reads archive
T-RFLP	Terminal-restriction fragment length polymorphism
TAE	Tris-Acetate-EDTA
TCC	Total cells concentration
TN	Total nitrogen
TS	Total solid
TSS	Total suspended solid
VSS	Volatile suspended solid
WF	Waterfall
WQI	Water quality index
WWTP	Wastewater treatment plant
µL	Microliter
µM	Micro molar

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CHAPTER 1

INTRODUCTION

Palm oil is an important agro-based industry which contributes to economic growth not only for South East Asian countries but also some Sub Saharan African countries (Robins, 2018) and Papua New Guinea (Bonneau, 2019). With the continuous increase in demand for palm oil worldwide (Silalertruksa et al., 2017), there is an urgency in terms of environmental monitoring of the released generated wastewater known as palm oil mill effluent (POME). POME is of great concern as it causes mass depletion of oxygen in the aquatic ecosystem which negatively affects the aquatic communities and water resources including bacterial community changes in the receiving waterways (Sharuddin et al., 2017). In conforming with the requirement for POME to be treated prior to being released into nearby rivers, it is common for palm oil mills to use anaerobic and aerobic processes for the treatment of POME (Mohd-Nor et al*.,* 2019). In the palm oil industry, physicochemical parameters outlined for the released POME are being defined such as biological oxygen demand (BOD), chemical oxygen demand (COD) and pH which act as means to monitor the environmental impact of the POME discharges into the environment (Iskandar et al., 2018). However, other than POME final discharge, the receiving rivers could also be polluted by many other anthropogenic pollutants (Geissen et al., 2015) which make the management and monitoring of the released POME final discharge into the river water challenging. Thus, a more specific complementary monitoring method is required to aid palm oil mill operators in ensuring the POME being discharged into the rivers fulfills the environmental requirements.

Bacterial bioindication is receiving an increasing attention in the aquatic system monitoring due to the direct involvement of microorganisms in the biogeochemical cycle of the river ecosystem (Sims et al., 2013). This makes it an inevitable factor to complement the current use of the physicochemical monitoring system. Therefore, a more specific bacterial indicator as well as molecular-based monitoring tool can be the reliable alternatives or complementary for POME final discharge detection in the river water due to their direct response in reflecting the variability of ecological consequences of the environment (Shade et al., 2012). Moving closer to this vision, Illumina sequencers have been applied to generate metagenomic data from various samples including environmental metagenomes (Mason et al., 2014; Ju et al., 2014) such as river (Lu et al., 2016) and wastewater treatment systems (Bai et al., 2017). From these, identifiable bacterial communities can be analyzed in terms of their patterns, dominances, abundances and diversities. Moreover, by utilizing the Illumina MiSeq technology, the use of bacteria namely *Alcaligenaceae* and *Chromatiaceae* as specific bioindicators to indicate POME final discharge contamination in receiving rivers has been proposed since they were found to be dominant in POME final discharges (Sharuddin et al., 2017; Mohd-Nor et al., 2018).

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In concurrence with the discovery of the pollution bacterial indicators, the biodegradation mechanisms taking place in the POME could be predicted. Conceptually, resistant or adaptable species is able to emerge in a polluted environment due to their capabilities to metabolize either the organic or inorganic compounds whereby this is determined by their genetic constitutions and physiological tolerances (Potvin & Tousignant, 1996). The detection of catabolic genes expressed or carried by dominant bacterial community can be used to develop an advanced molecular technique to identify and monitor targeted microbial subpopulation biodegrading the concerned pollutants (Táncsics et al., 2008).

The detection of *Alcaligenaceae* among other dominant *Proteobacteria* in POME is relatable to the presence of lignin-derived phenolic compounds in the wastewater. This knowledge opens the path to develop a molecular-based probe which is specific for the detection of functional genes involved in the biodegradation of POME final discharge carried by the bacterial community. Besides, *Proteobacteria* with the ability to biodegrade lignin derived aromatic compounds had been thoroughly reported in previous studies (Pérez-Pantoja et al., 2012; Pérez-Pantoja et al., 2017). This fact harbors the possibility of *ortho*- and *meta*-cleavage pathways of the intermediary catechol or protocatechuate to occur in the partially treated POME, final discharge and the receiving downstream rivers which were dominated by *Alcaligenaceae* (Sharuddin et al., 2017).

With the proposal of *Alcaligenaceae* and *Chromatiaceae* as the potential bioindicators for POME final discharge pollution in the river, it is important to prove that these two bacteria are only dominant in POME final discharge polluted rivers, so that their specificity as bioindicators can be securely established. Morever, with the understanding of POME biodegradation mechanisms by *Alcaligenaceae*, a molecular-based monitoring system specific for POME pollution in the environment could be developed by profiling the functional genes involved in the biodegradation of aromatic compounds in the POME. For this research, Illumina MiSeq sequencing platform was utilized to obtain the bacterial communities in POME final discharge polluted rivers compared with unpolluted rivers and rivers polluted by other pollutants not related to POME. The sequencing data was statistically correlated further with the quantitative data of physicochemical characteristics and flow cytometry analyses. Upon confirming the distinct presence of *Alcaligenaceae* in the POME final discharge polluted rivers, the potential genes carried by this bacterial family were screened by conventional PCR in order to pioneer a basis for genomic biomarker development specific for the detection of such pollution.

In this study, it was hypothesized that the *Alcaligenaceae* and *Chromatiaceae* are dominant in POME final discharge polluted rivers, thus can be used as specific bioindicators to indicate the river water pollution due to this pollutant. On another note, with the knowledge of *Alcaligenaceae* as the lignin derived phenolic compounds degrader, it was hypothesized that the functional gene involved in the degradation of aromatic compound within the targeted bacterial

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community in POME final discharge and its receiving river water could be amplified. In the future, a molecular based-indicator to indicate the river water contamination due to POME final discharge could be developed.

Therefore, the objectives of this study are:

1. To determine the dominant presence of pollution bacterial indicators, *Alcaligenaceae* and *Chromatiaceae* associated with POME final discharge polluted rivers.

2. To identify the functional genes involved in the biodegradation of aromatic compounds in POME final discharge and the receiving river.

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