UNIVERSITI PUTRA MALAYSIA

Alcaligenaceae AND Chromatiaceae AS POTENTIAL POLLUTION BIOINDICATORS IN PALM OIL MILL EFFLUENT AND ITS RELATIONSHIP WITH ENVIRONMENTAL PARAMETERS AND ZERO-EMISSION SYSTEM

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FBSB 2021 18

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By

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

February 2021

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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October 2020

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Alcaligenaceae and *Chromatiaceae* have previously identified as unique pollution bioindicators in rivers polluted by palm oil mill effluent (POME) final discharge. Since bacteria are sensitive towards environmental stresses, it is crucial to evaluate survivability of both bacteria in different environmental parameters, proving their credibility as POME pollution bioindicators. Therefore, the effects of temperature (25 – 40°C), pH (7 – 9), durations of UV irradiation (30 – 120 minutes) and during low/high crop seasons of oil palm towards *Alcaligenaceae* and *Chromatiaceae* were assessed in the first objective of this study. The POME samples were collected from the facultative pond, algae (aerobic) pond and final discharge point. Following the recent breakthrough in POME management where the zero-emission technology was being introduced, the second objective of this study was conducted to evaluate the implementation effect of the zero-emission of POME final discharge towards bacterial community dynamics in a constructed artificial river water system. For both objectives, the viability status and composition of bacterial community were assessed using flow cytometry-based assay and 16S rRNA amplicon sequencing using Illumina MiSeq, respectively, in correlation with the changes of physicochemical properties. The shifts of physicochemical properties, nutrient contents and bacterial community dynamics were correlated using principal coordinate (PCO) analysis. Even though the changes of environmental parameters changed the physicochemical properties, as well as the viability and nucleic acid contents of bacterial cells in POME, the *Alcaligenaceae* and *Chromatiaceae* remained detected in all POME samples, each comprising more than 0.5% from the total bacterial population, and thus could be regarded as the reliable bacterial indicators. Both were shown to be positively correlated with biological oxygen demand (BOD_5); hence they could be used in complementary with the current adopted physicochemical assessment to specifically indicate the contaminated river due to POME final discharge. Following the implementation of zero-emission of POME final discharge, the polluting properties of the affected river

water were reduced, as shown by the physicochemical properties, nutrient contents and bacterial cell's viability (from 43.0% to 24.5%). Being the bacterial indicators to detect the presence of POME final discharge in the river water, *Alcaligenaceae* and *Chromatiaceae* were shown to be carried over by the effluent into the artificial river water system with total bacterial compositions of 1.0 – 1.3% and 2.2 – 5.1%, respectively. As a result of the implementation of zero-emission system, the *Chromatiaceae* was shown to be reduced from 2.2% on day 8 until it was undetected on day 15. Even though still being detected after the zero-emission system implementation, *Alcaligenaceae* was constantly reduced from 1.2% on day 8 to 0.9% on day 15. The positive correlation between both *Alcaligenaceae* and *Chromatiaceae* and BOD_5 had strengthen the possibility of further reduction of *Alcaligenaceae* with the reduction of contamination level, indicated by BOD_5. Overall, it can be concluded that the *Alcaligenaceae* and *Chromatiaceae* showed to be the reliable POME bacterial indicators despite the changes of environmental conditions. Their reduction following the implementation of zero-emission also proved the efficiency of zero-emission system in reducing the polluting properties of the affected river water.

Keywords: bacterial indicator; environmental parameters; Illumina MiSeq; palm oil mill effluent; zero-emission

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

Alcaligenaceae DAN _Chromatiaceae_ SEBAGAI BIOPENUNJUK PENCEMARAN YANG BERPOTENSI DALAM EFLUEN KILANG MINYAK SAWIT DAN HUBUNGANNYA DENGAN PARAMETER ALAM SEKITAR DAN SISTEM PEMBEBASAN SIFAR

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Alcaligenaceae dan *Chromatiaceae* telah dikenalpasti sebagai biopenunjuk pencemaran unik di sungai yang tercemar oleh pembuangan akhir efluen kilang minyak sawit (POME). Oleh kerana bakteria peka terhadap tekanan persekitaran, adalah sangat penting untuk menilai ketahanan kedua bakteria tersebut dalam parameter persekitaran yang berbeza, dan akhirnya membuktikan kredibiliti mereka sebagai biopenunjuk pencemaran POME. Sehubungan dengan itu, kesan suhu (25 – 40°C), pH (7 – 9), jangka masa penyinaran UV (30 – 120 minit) dan semasa musim tanaman kelapa sawit yang rendah/tinggi terhadap *Alcaligenaceae* dan *Chromatiaceae* telah dinilai dalam objektif pertama kajian ini. Sampel POME dikumpulkan dari kolam fakultatif, kolam alga (aerobik) dan titik pelepasan akhir. Berikutan kejayaan terbaharu dalam pengurusan POME di mana teknologi pelepasan sifar diperkenalkan, objektif kedua kajian ini dijalankan untuk menilai kesan pelaksanaan pelepasan sifar bagi pelepasan akhir POME terhadap dinamik komuniti bakteria dalam sistem air sungai buatan yang dibina. Untuk kedua-dua objektif tersebut, status kebolehhidupan dan komposisi komuniti bakteria masing-masing dinilai menggunakan pengujian berasaskan sitometri aliran dan penjujukan 16S rRNA amplikon menggunakan Illumina MiSeq, berkorelasi dengan perubahan sifat fizikokimia. Perubahan pada sifat fizikokimia, kandungan nutrien dan dinamik komuniti bakteria dihubungkaitkan dengan menggunakan analisis penyelarasan koordinat utama (PCO). Walaupun perubahan parameter persekitaran mengubah sifat fizikokimia serta kebolehhidupan dan kandungan asid nukleik sel bakteria dalam POME, *Alcaligenaceae* dan *Chromatiaceae* tetap dikesan dalam semua sampel POME dengan masing-masing terdiri lebih daripada 0.5% daripada jumlah populasi bakteria, oleh itu mereka boleh dianggap sebagai bakteria penunjuk yang boleh dipercayai. Keduanya juga terbukti berkorelasi positif dengan permintaan oksigen biologi (BOD_5), oleh itu itu mereka dapat digunakan sebagai pelengkap penilaian fisikokimia yang digunakan untuk

menunjukkan secara khusus sungai yang tercemar akibat pelepasan akhir POME. Berikutan pelaksanaan pelepasan sifar bagi pelepasan akhir POME, sifat pencemaran air sungai yang terjejas turut berkurang, seperti yang ditunjukkan oleh sifat fizikokimia, kandungan nutrien dan kebolehhidupan sel bakteria (daripada 43.0% hingga 24.5%). Sebagai bakteria penunjuk untuk mengesan kehadiran pelepasan akhir POME di dalam sistem air sungai, *Alcaligenaceae* dan *Chromatiaceae* terbukti dibawa oleh efluen tersebut ke dalam sistem air sungai buatan dengan komposisi masing-masing 1.0 – 1.3% dan 2.2 – 5.1%. Hasil daripada pelaksanaan sistem pelepasan sifar, *Chromatiaceae* terbukti berkurang daripada 2.2% pada hari ke-8 hingga tidak dapat dikesan pada hari ke-15. Walaupun masih dikesan setelah pelaksanaan sistem pelepasan sifar, *Alcaligenaceae* terus berkurangan daripada 1.2% pada hari ke-8 hingga 0.9% pada hari ke-15. Korelasi positif antara kedua-dua *Alcaligenaceae* dan *Chromatiaceae* dan BOD_5 telah menguatkan kemungkinan penurunan *Alcaligenaceae* dengan penurunan tahap pencemaran, yang ditunjukkan oleh BOD_5. Secara keseluruhannya, dapat disimpulkan bahawa *Alcaligenaceae* dan *Chromatiaceae* terbukti menjadi bakteria penunjuk POME yang boleh dipercayai walaupun terdapat perubahan keadaan persekitaran. Pengurangan mereka setelah pelaksanaan pelepasan sifar juga membuktikan keberkesanan sistem pelepasan sifar dalam mengurangi sifat pencemaran air sungai yang terjejas.

Kata Kunci: penunjuk bakteria; parameter persekitaran; Illumina MiSeq; efluen kilang minyak sawit; pelepasan sifar

ACKNOWLEDGEMENT

First and foremost, I would like to express my tremendous gratitude and all praises to Allah s.w.t. for the opportunity to pursue Master study and exposing me to a glimpse of His great knowledge. Blessings upon our prophet Muhammad SAW. for from him I learnt perseverance and non-stop effort in pursuing knowledge. I explored new great things throughout my Master journey with hope these could help me to improve my interpersonal and research skills.

I am forever indebted to my incredible supervisor, Assoc. Prof. Dr. Norhayati binti Ramli, for her never-ending guidance and support both physically and mentally and keep me on path. You deserve a medal for the best supervisor! An utmost respect to Prof Dato' Dr. Mohd Ali bin Hassan for sharing his insight and wisdom for my research and enlighten me on matters that was uncertain. I would like to express my thankfulness to Assoc. Prof. Dr. Toshinari Maeda for his generosity and kindness for giving me opportunity to explore new technology, Illumina MiSeq and provided me with his great insight.

To Nurhasliza binti Zolkefli, my wonderful co-researcher and English teacher, thank you for complementing my weaknesses and your willingness to help me while juggling with your own difficulties, which without you I might lost. Other T.E.A.M. members, Siti Suhailah binti Sharuddin, Diana binti Mohd-Nor, Nik Ida Mardiana binti Nik Pa and Muhammad Farhan bin Sobri, as well as Fatini binti Mat Arisah for being amazing seniors and friend that gave me helping hand and being my inspiration. I would like also to express my appreciation to my peculiar soulmate, Salma Malihah binti Mohamad for being my all ears and my source of information, Aimi Atiqah binti D. Azman for keep emphasizing me that the journey is what matter, Fatin Ayuni binti Rahman for always cheering me up on my gloomy days, Nur Inani binti Azizan and Husmaruzaini Syaza binti Hussin for keeping me company whenever I needed. Space is not enough to express my gratefulness to all friends and personnel that aided me directly or indirectly throughout my Master journey.

Finally, to my beloved Mama, Asmah binti Idris (*Allahyarhamah*) for being my first supporter and real reason I embarked to my Master journey. There were no words to express my gratefulness and love towards you. May we meet again in Jannah. My Papa, Mohamad Zainal bin Hussin for entrusting me to further my study and always being there to aid me during my hardship. My Ibu, Nafisah binti Ismail for bringing back smile to our small family and helped me realized there is always rainbow after storm. My seven beautiful stars that shine bright with purple light, thank you for always be my greatest euphoria whenever I am blue and letting me know that making mistakes is fine as long as I am willing to learn from them and keep moving forward for the better me. Last but not least to myself, Lyana, congratulations you did it!

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

°C	Degree Celsius
× g	Gravitational force
ANOVA	Analysis of variance
APHA	American Public Health Association
BOD	Biological oxygen demand
cell/µL	Cell per microliter
CFU	Colony forming unit
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CPO	Crude palm oil
DO	Dissolved oxygen
DNA	Deoxynucleic acid
DOE	Department of Environment
DGGE	Denaturing gradient gel electrophoresis
E’	Evenness index
EFB	Empty fruit bunch
EQA	Environmental Quality Act
EDTA	Ethylenediaminetetraacetic acid
FD	Final discharge
FFB	Fresh fruit bunch
FISH	Fluorescence *in-situ* hybridization
GJ/m^2	Gigajoule per meter square
H’	Shannon-Weiner index
HNA	High nucleic acid
ICP-MS	Inductively Coupled Plasma-Mass Spectroscopy

J/m^2	Joule per meter square
K	Potassium
L	Liter
L/min	Liter per minute
L/ton	Liter per ton
LNA	Low nucleic acid
LSD	Fisher's Least Significant Difference
m	meter
M	Molar
m^3	Cubic meter
mg	Milligram
mL	Milliliter
mM	Micromolar
mg/L	Milligram per liter
mm	Millimeter
MPOB	Malaysian Palm Oil Board
NH_3	Ammonia
nm	Nanometer
NO_3-N	Nitrate
NH_3-N	Ammonia nitrogen
NGS	Next-generation sequencing
NCBI	National Centre for Biotechnology Information
NWQS	National Water Quality Standard
OTU	Operational taxonomic unit
ppb	Part per billion
ppm	Part per million
psi	Pound per square inch

PAST	Paleontological Statistics
POME	Palm oil mill effluent
PCO	Principle coordinate
PCR	Polymerase chain reaction
QIIME	Quantitative Insights into Microbial Ecology
rpm	Revolutions per minute
SO_2	Sulfur dioxide
SS	Suspended solids
SSCP	Single-strand conformation polymorphism
TAE	Tris-acetate EDTA
TCC	Total cell concentration
TiO_2	Titanium oxide
T-RFLP	Terminal-restriction fragment length polymorphism
TSS	Total suspended solids
UV	Ultraviolet
VSS	Volatile suspended solids
WQI	Water Quality Index
µm	Micrometer
µL	Microliter

CHAPTER 1

INTRODUCTION

The rise in demand towards palm oil worldwide which is worth USD 50 billion annually (Paterson and Lima, 2018) has contributed to the expansion of the economy of major palm oil producers, especially Malaysia and Indonesia. Both countries contributed around 87% of world's total oil palm production (Stichnothe and Schuchardt, 2011). However, the positive growth in the production of crude palm oil (CPO) leads to the huge generation of wastewater known as palm oil mill effluent (POME), which is a difficult effluent to be treated that requires higher cost (Madaki and Seng, 2013). In order to address this issue, the bio-treatment process was incorporated into palm oil mill operation to reduce the polluting properties of POME before it can be safely discharged into nearby rivers (Zahrim *et al*., 2014). Currently, the law enacted regarding the standard discharge limit of wastewater as listed in the Environmental Quality Act 1974 only emphasizes on the physicochemical properties of the wastewater. Therefore, it could not provide specific indication to differentiate the source of pollution in the river water, specifically caused by POME final discharge. Hence, a more specific monitoring system or tool is needed to complement the current monitoring system.

The current technology in environmental monitoring has been upgraded with the implementation of bacterial indicators to complement the current assessment using physicochemical properties (Dhawde *et al*., 2018). This is supported with the ability of bacteria that could provide information on the current pollution condition due to their high abundancies and high adaptation to the changing environmental stressor (Guo *et al.*, 2016). In order to achieve this aim, 16S rRNA amplicon sequencing using Illumina MiSeq, among other platforms such as DNA fingerprinting and fluorescence-based technology, has been widely adopted to assess the bacterial community shift in the contaminated receiving waterways. For instance, the ability of *Escherichia coli* as an indicator for fecal contamination in the urban wastewater (Hu *et al.,* 2018) and the use of *Pseudomonas*, *Enterobacteriaceae* and *Chryseobacterium* as potential bacterial indicators for water contaminated with abattoirs wastewater (Handley *et al.,* 2018) have been confirmed.

A previous study had proposed either the *Chromatiaceae* or *Alcaligenaceae* or both as the potential bacterial indicators to indicate the river water contamination due to POME final discharge, based on the fact that they only emerged in the downstream but not present in the upstream part of the receiving river (Sharuddin *et al*., 2017). Following that, Mohd-Nor *et al.* (2018) had further confirmed the presence of *Chromatiaceae* and *Alcaligenaceae* in the POME final discharge collected from different palm oil mills despite the different biotreatment systems applied. A recent study also proved the presence of *Chromatiaceae* and

Alcaligenaceae only in POME final discharge and the polluted rivers, but they have not been detected in the other non-POME contaminated rivers (Zolkefli *et al.,* 2020).

However, the major issue in using bacteria as pollution bioindicators is their reliability at inconsistent environmental conditions. As a tropical country, Malaysia experiences a yearlong exposure from sunlight with heavy rainfall (Azmuda *et al.*, 2019) which affects the ambient temperature, hence changing the bacterial community composition (Abu Bakar *et al*., 2015; Sabajo *et al*., 2017). The diversity and composition of bacterial communities in terrestrial and aquatic environments were also affected by pH (Kuang *et al.,* 2013; Wang *et al.,* 2012). UV irradiation could also induce the changes in the heterotrophic bacterial community structure, growth and production (Hernandez *et al*., 2016). Besides, the amount and polluting properties of POME discharge depended on the fresh fruit bunch (FFB) production, which contributed to the changes in physicochemical properties of POME (Poh *et al*., 2010), thus eventually affecting the function and composition of bacterial community in the sediment (Saarenheimo *et al.*, 2014). The increased of sediment was reported to correspond to the increased of total suspended solids (TSS), moisture content and nutrients (Rupani *et al.,* 2010), hence increasing the bacterial population in the sample (Xia *et al.,* 2006).

In addition, the bacterial community was also claimed to be shifted following the implementation of zero-emission system. This ideal technology is proposed to be applied in the palm oil mills as a continuous effort to remove the possibility of pollution in receiving waterways due to POME final discharge (Tabassum *et al*., 2015). The treated effluent is proposed to be used as recycled water for oil palm processing (Othman *et al.*, 2013), instead of discharging it into the river. Even though there is no previous report concerning the effect of zero-emission of POME final discharge towards the bacterial community shift in the river, the previous study had strengthened the hypothesis where microbial community was reported to be changed following the implementation of zero-discharge of wastewater from the mariculture system (Cytryn *et al.,* 2003). In addition, the zero-discharge in the aquaculture system also exhibited the shift in microbial community (Kandel *et al.,* 2014).

Due to the ongoing concern about evolving environmental conditions, it is critical to demonstrate the reliability of both proposed bacterial indicators, *Chromatiaceae* and *Alcaligenaceae*, as pollution bioindicators to specifically indicate pollution in the rivers caused by POME final discharge. Other than that, the implementation of zero-emission system for POME final discharge in palm oil mills has shown to minimize waste generation. However, no information was available about how the bacterial population in river water polluted with POME final discharge was affected after the zero-emission system was being implemented.

In this study, the pollution bacterial indicators, *Chromatiaceae* and *Alcaligenaceae* that were originated from the later stages of POME treatment system (Sharuddin *et al.,* 2017), were hypothesized able to survive in the POME and final discharge despite the changes occurred in the environment. In order to prove this hypothesis, the POME and final discharge were treated at different temperatures, pH, duration of UV irradiation and TSS concentrations based on low/high crop seasons of oil palm. Their reliability to be used as bacterial indicators in detecting the river water contamination caused by POME final discharge was confirmed. Meanwhile, the bacterial community in the receiving river that is carried over by this effluent, represented by *Chromatiaceae* and *Alcaligenaceae*, was hypothesized to be rebound following the implementation of zero-emission of POME final discharge, hence the original ecosystem in the river water can be restored. In order to test this hypothesis, an artificial river water system was constructed to mimic the usual practice of discharging POME and the following implementation of zero-emission system. For both hypotheses, 16S rRNA amplicon sequencing using Illumina MiSeq was utilized to assess the bacterial community composition, which was then correlated statistically with the physicochemical properties and the functional status of bacterial cell analyzed using flow cytometry.

Therefore, the objectives of this study are:

1. To determine the effects of temperature, pH, durations of UV irradiation and low/high crop seasons of oil palm on the survivability of bacterial community, particularly *Chromatiaceae* and *Alcaligenaceae* in different stages of POME treatment.
2. To assess the bacterial community shift in a constructed artificial river water system receiving POME final discharge before and after the implementation of zero-emission of POME final discharge.

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