



**BIOMARKER SCREENING, IDENTIFICATION, AND EVALUATION FOR
BIOMONITORING PALM OIL MILL EFFLUENT POLLUTION IN RIVER
WATER**

By

SITI SUHAILAH BINTI SHARUDDIN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Chairman : Assoc. Prof. Norhayati Ramli, PhD

Faculty : Biotechnology and Biomolecular Sciences

The palm oil industry plays a vital role in Malaysia's socioeconomic development but generates a significant amount of wastewater, known as palm oil mill effluent (POME). The treated POME, known as POME final discharge, is commonly released into nearby rivers or plantations, leading to river contamination. With the expansion of palm oil production, the volume of POME also increases, posing a growing threat to water ecosystems. Current monitoring of POME pollution is based on physicochemical assessments like biochemical oxygen demand (BOD₅) and chemical oxygen demand (COD), but these methods cannot distinguish between pollution sources, are time-consuming, involve harmful chemicals, and do not provide insight into the biological impact of pollutants. Given these limitations and the sensitivity of bacterial communities to pollution, this study aimed to investigate the effects

of POME final discharge on the structure and function of bacterial communities in rivers, with the goal of identifying potential biomarkers that could serve as more precise indicators of POME pollution. This study employed metatranscriptomics to elucidate the structural and transcriptional profiles of the active bacterial community in POME final discharges and receiving rivers from two study sites, correlating these profiles with physicochemical assessments. Potential biomarkers were identified through differential expression analysis, and their correlation with community metabolomics detected using gas chromatography-mass spectrometry (GC-MS) was explored. The candidate biomarker was validated by quantifying corresponding metabolites using high-performance liquid chromatography with a fluorescence detector (HPLC-FLD) and further correlated with its expression using reverse transcription quantitative PCR (RT-qPCR) across multiple sites.

The findings revealed that despite different POME treatment systems at the two study sites, the bacterial functional profiles in POME final discharge samples were similar. Notably, metabolic pathways related to carbohydrates, lipids, and amino acids were significantly upregulated in the downstream parts of rivers receiving POME compared to upstream parts (FDR <0.05), highlighting the greater impact of POME on bacterial functional profiles than suggested by BOD₅ and COD alone. Amino acids emerged as the dominant metabolites, with cysteine synthase, an enzyme involved in L-cysteine biosynthesis, identified as a candidate biomarker due to its high expression levels. HPLC-FLD analysis confirmed higher L-cysteine concentrations in POME final discharge (1.273 – 1.545 µM) and downstream rivers (0.476 – 0.583 µM) compared to upstream parts. RT-qPCR further validated the

significant prevalence of cysteine synthase, showing a strong positive correlation with RNA-seq data and a 2.0 to 4.5-fold increase in its copy number/L in downstream rivers impacted by POME. Additionally, the study found a statistically significant difference in cysteine synthase levels between downstream POME-receiving rivers and control urban rivers not affected by POME. These expression patterns support cysteine synthase as a promising biomarker for POME pollution, particularly given its much higher expression in POME-impacted rivers compared to unaffected areas. Overall, this study provides the first comprehensive molecular-level analysis of POME's impact on river ecosystems, identifying cysteine synthase as a potential biomarker for POME pollution. This biomarker-based monitoring approach could allow for more sensitive and rapid detection of POME-related pollution, promoting sustainable practices within the palm oil industry.

Keywords: Biomonitoring, Metabolomics, Metatranscriptomics, Pollution Biomarker, Palm Oil Mill Effluent

SDG: GOAL 6: Clean Water and Sanitation, GOAL 11: Sustainable Cities and Communities

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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**SARINGAN, PENGENALPASTIAN, DAN PENILAIAN BIOPENANDA BAGI
BIOPEMANTAUAN PENCEMARAN EFLUEN KILANG MINYAK SAWIT
DALAM AIR SUNGAI**

Oleh

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Industri minyak sawit memainkan peranan penting dalam pembangunan sosioekonomi Malaysia tetapi menghasilkan jumlah besar air sisa yang dikenali sebagai efluen kilang minyak sawit (POME). POME yang telah dirawat, dikenali sebagai pelepasan akhir POME, sering dilepaskan ke dalam sungai atau ladang berdekatan, menyebabkan pencemaran sungai. Dengan pertumbuhan pengeluaran minyak sawit, penghasilan POME juga meningkat, seterusnya mengancam kestabilan ekosistem air. Pemantauan pencemaran POME pada masa kini berdasarkan penilaian fizikokimia seperti keperluan oksigen biokimia (BOD_5) dan keperluan oksigen kimia (COD), namun kaedah ini tidak dapat membezakan antara sumber pencemaran, memakan masa, melibatkan bahan kimia berbahaya, dan tidak memberikan gambaran tentang kesan biologi pencemaran. Memandangkan kekangan ini dan sensitiviti komuniti bakteria terhadap pencemaran, kajian ini bertujuan menyiasat kesan

pelepasan akhir POME terhadap struktur dan fungsi komuniti bakteria di sungai, dengan tujuan mengenal pasti biopenanda yang berpotensi sebagai petunjuk yang lebih tepat bagi pencemaran POME. Kajian ini menggunakan metatranskriptomik untuk menerangkan profil struktur dan transkripsi komuniti bakteria aktif dalam pelepasan akhir POME dan sungai yang menerima POME di dua tapak kajian, serta mengaitkan profil ini dengan penilaian fizikokimia. Calon biopenanda dikenal pasti melalui analisis ekspresi pembezaan, dan hubungannya dengan metabolomik komuniti dikesan menggunakan kromatografi gas-spektrometri jisim (GC-MS). Calon biopenanda tersebut disahkan dengan pengkuantitian metabolit menggunakan kromatografi cecair berprestasi tinggi dengan pengesan pendarfluor (HPLC-FLD) dan dikaitkan dengan ekspresinya menggunakan PCR kuantitatif transkripsi terbalik (RT-qPCR) di pelbagai lokasi.

Dapatan kajian menunjukkan bahawa walaupun sistem rawatan POME berbeza di kedua-dua tapak kajian, profil fungsi bakteria dalam sampel pelepasan akhir POME adalah serupa. Metabolisme yang berkaitan dengan karbohidrat, lipid, dan asid amino didapati meningkat dengan ketara di bahagian hilir sungai yang menerima POME berbanding dengan bahagian hulu ($FDR < 0.05$), menonjolkan kesan POME yang lebih besar terhadap profil fungsi bakteria daripada apa yang dicadangkan oleh BOD5 dan COD. Asid amino dikenal pasti sebagai metabolit utama dan sisteina sintase, enzim yang terlibat dalam biosintesis L-sisteina, dikenal pasti sebagai calon biopenanda berdasarkan tahap ekspresi yang tinggi. Analisis HPLC-FLD mengesahkan kepekatan L-sisteina yang lebih tinggi dalam pelepasan akhir POME ($1.273 - 1.545 \mu\text{M}$) dan bahagian hiliran sungai penerima POME ($0.476 - 0.583 \mu\text{M}$)

berbanding dengan bahagian hulu. Analisis RT-qPCR selanjutnya mengesahkan kelaziman sisteina sintase yang ketara, menunjukkan korelasi positif dengan data RNA-seq dan peningkatan 2.0 hingga 4.5 kali ganda di sungai hilir yang terjejas oleh POME. Kajian ini juga mendapati perbezaan signifikan dalam tahap sisteina sintase antara hilir sungai yang menerima POME dan sungai bandar yang tidak terjejas. Corak ekspresi sisteina sintase ini menyokong potensinya sebagai biopenanda bagi pencemaran POME. Secara keseluruhan, kajian ini menyediakan analisis menyeluruh pertama di peringkat molekul mengenai kesan POME terhadap ekosistem sungai, dan mengenal pasti sisteina sintase sebagai biopenanda yang berpotensi untuk pemantauan pencemaran POME. Pendekatan ini membolehkan pengesanan pencemaran POME yang lebih sensitif dan pantas, serta mempromosikan amalan mampan dalam industri minyak sawit.

Kata Kunci: Biopemantauan, Biopenanda Pencemaran, Metabolomik, Metatranskriptomik, Efluen Kilang Minyak Sawit

SDG: MATLAMAT 6: Air Bersih dan Sanitasi, MATLAMAT 11: Bandar dan Komuniti Lestari

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CHAPTER 1

INTRODUCTION

The palm oil industry is a key contributor to Malaysia's economy, with 5.74 million hectares of oil palm planted area and a staggering 18 million tonnes of crude palm oil (CPO) in 2021. This accounts for approximately 8.5% of global oils and fats production (Parveez et al., 2022). However, the industry faces scrutiny over the substantial volumes of wastewater it generates, known as palm oil mill effluent (POME) (Leela et al., 2018). The wet extraction method used in palm oil mills necessitates 5-7 tonnes of water to produce a single tonne of CPO, leading to about half of this water becoming POME (Mohammad et al., 2021).

Further exacerbating the issue, the treated or partially treated POME, known as POME final discharge, is commonly released into the river water or plantation, ultimately seeping into the soil and contaminating water bodies (Soo et al., 2022). As palm oil production continues to soar, with an average annual increment of 300,000 tonnes over the past 50 years (FAO, 2022), it is expected that the amount of POME generated will also escalate in parallel. Since raw POME contains high concentrations of organic compounds, continuous discharging of large volumes of POME final discharge into river water systems could alter the natural physicochemical properties of the rivers, ultimately threatening the ecological stability of the water system (Isaiah and Blessing, 2020).

Extensive research has highlighted the significance of microbes in effluent treatment and ecosystem functionality (Yang et al., 2021; Sharma et al., 2023). However, studies on the impact of POME final discharge on receiving rivers have primarily focused on physicochemical properties, with fewer examining the effects on bacterial communities (Sharuddin et al., 2017). This knowledge gap is concerning, especially considering reports of significant alterations in bacterial communities due to changes in the physicochemical properties of effluent-receiving rivers (Reddington et al., 2020; Staley et al., 2014; Xiaofei et al., 2016). While the current impact of POME final discharge on these communities may not be significant at present, the continuous increase in effluent volume could disrupt the ecosystem balance. Therefore, it's imperative to comprehensively explore the influence of POME final discharge on the functional profiles of bacterial communities integral to the biogeochemical cycle.

Considering the annual increase in POME generation and the strict discharge of biochemical oxygen demand (BOD) limit of 20 mg/L in sensitive areas (Chin et al., 2020), there is an urgent need to increase the scale and frequency of river water monitoring. Currently, pollution monitoring predominantly involves conventional physicochemical parameter measurements, such as BOD and chemical oxygen demand (COD) (Mohamad-Zainal et al., 2021). These methods, however, do not pinpoint the pollution source, complicating effective pollution management in rivers exposed to diverse anthropogenic pollutants. Additionally, the time-intensive nature of certain physicochemical analyses can delay essential corrective measures, posing risks to ecosystem health.

Furthermore, the reliance on hazardous chemicals in some physicochemical analyses also poses additional risks to both ecosystem and human health.

Addressing these challenges calls for an advanced, more efficient monitoring approach like biomonitoring, which assesses environmental conditions or changes through biological systems, organisms, or their responses (Baird and Hajibabaei, 2012; Yates et al., 2021). This approach includes the use of bioindicators, reflecting environmental changes in habitats, communities, or ecosystems (Sagova-Mareckova et al., 2020) and biomarkers, known for their cutting-edge potential in biomonitoring technology. While previous research has identified potential bioindicator bacteria associated with POME final discharge (Mohamad-Zainal et al., 2021; Mohd-Nor et al., 2018; Sharuddin et al., 2017; Zolkefli et al., 2020), this study pivots towards biomarkers, due to their superior sensitivity and technological advancements (Bourhane et al., 2022).

Biomarkers, identifiable through cellular or biochemical shifts triggered by environmental stimuli, including xenobiotics, offer rapid and precise reflections of environmental changes, due to the fast mRNA turnover in microbial ecosystems (Yates et al., 2021). This sensitivity renders biomarkers a more effective alternative to bacterial indicators (taxonomic profiling) for detecting shifts in environmental conditions (Cordier et al., 2019). Their use in environmental risk assessment is increasingly recognised due to their specificity and accuracy, as demonstrated in various studies (Sangkaew et al., 2021; Zlender and Rupnik 2023; Reid et al., 2020). Additionally, the advent of biosensor technology further amplifies the practicality of biomarkers in

environmental monitoring, establishing them as indispensable tools for early warning systems in environmental quality assessment (Palchetti et al., 2018).

Metatranscriptomics is the comprehensive analysis of all RNA transcripts in a microbial community, providing insights into the functional and active components of these communities. Hence, this study employed metatranscriptomics to analyse the transcriptional profiles in POME final discharge and receiving river water, gaining insights into how POME affects bacterial community functional profiles. Genes showing increased patterns in the downstream part of the river were shortlisted as candidate POME biomarkers for further examination. Community metabolomics was conducted to correlate these genes with the biochemical profile of the bacterial community. Subsequently, the potential biomarker was verified using reverse transcription quantitative polymerase chain reaction (RT-qPCR), with specific primer designed for the biomarker. Its correlation with targeted metabolites was also explored using reverse-phase high-performance liquid chromatography coupled with a fluorescence detector (RP-HPLC-FLD). Given the importance of understanding POME's impact on bacterial communities and the need for effective POME pollution biomonitoring, this research was driven by the following objectives:

- i) To assess the effect of POME final discharge exposure towards active bacterial community in receiving river water at the whole-transcriptome level using metatranscriptomics.
- ii) To determine the significantly upregulated isoforms in correlation with untargeted community metabolomics through gas chromatography-

mass spectrometry (GC-MS) in POME-receiving rivers for biomarker selection.

- iii) To determine the expression of potential biomarker isoform using reverse transcription quantitative PCR (RT-qPCR) in correlation with targeted metabolites through high-performance liquid chromatography with a fluorescence detector (HPLC-FLD) for biomarker validation.

Through this approach, the study aims to offer crucial insights into potential biomarkers, fostering more effective and precise POME pollution monitoring methods and contributing to a more sustainable palm oil industry.

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