

The diversity of indigenous and potential bacteria as bioremediator of palm oil mill secondary effluent (POMSE) by metagenomics assessment

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Abstract. Karno R, Arisoesilaningih E, Mustafa I, Siswanto D. 2024. The diversity of indigenous and potential bacteria as bioremediator of palm oil mill secondary effluent (POMSE) by metagenomics assessment. *Biodiversitas* 25: 1194-1200. This study aimed to identify the indigenous bacteria for potential bioremediation agents of palm oil mill secondary effluent (POMSE). Samples were obtained from the secondary pond of palm oil mill treatment. The bacterial diversity on the POMSE water samples was revealed by Next Generation Sequencing (NGS) of 16S rRNA gene amplicons. The results showed that the high abundance of bacteria phylum was Pseudomonadota or Proteobacteria (50%), Bacillota (31%), Bacteroidota (5%), and Thermotogae (3%). A photosynthetic sulfur bacterium, *Allochromatium humboldtianum*, was the most abundant species detected in the POMSE, with more than 8% of the total Operational Taxonomic Unit (OTU) detected. It was followed by *Allochromatium phaeobacterium* (6%), *Allochromatium* sp. (6%), and *Macromonas bipunctata* (6%). Meanwhile, the level of physicochemical parameters in POMSE were relatively high for several main parameters of the quality standard of palm oil industry waste, such as Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD). In contrast, oil and fat are low and meet the quality standards. The value of POMSE physicochemical parameters, including COD, BOD, and oil and fat, were 2,492.46, 852.96, and 6.20 mg/L, respectively. Metagenomic analysis showed that the bacterial community at POMSE was highly diverse. Nine dominant and codominant species found are *Allochromatium humboldtianum*, *Allochromatium phaeobacterium*, *Allochromatium* sp., *Allochromatium vinosum*, *Macromonas bipunctata*, *Tepidibaculum saccharolyticum*, *Rhodocyclus purpureus*, *Comamonas nitratorans*, and *Fervidobacterium riparium*. Several indigenous bacterial species found in this research were bacteria that have potential as bioremediation agents for palm oil wastewater.

Keywords: Bioremediation, indigenous bacteria, metagenomics, NGS, POMSE

Abbreviations: POME: Palm Oil Mill Effluent, POMSE: Palm Oil Mill Secondary Effluent, NGS: Next Generation Sequencing, OTU: Operational Taxonomic Unit, COD: Chemical Oxygen Demand, BOD: Biological Oxygen Demand, TSS: Total Suspended Solid

INTRODUCTION

Palm oil industry wastewater contains high levels of organic matter, causing environmental pollution around palm oil mills. The palm oil industry has tried to treat wastewater by implementing a lagoon system. However, the operation of the lagoon system has not been optimized, so the resulting outlet still does not meet the quality standards of palm oil wastewater. Some microorganisms have the potential to hydrolyze fats and oils, which are some of the wastewater pollutants generated from palm oil processing, apart from other organic pollutants such as BOD, TSS, and N Total. Based on this, it is necessary to research bacteria that decompose organic matter in palm oil industry wastewater for environmental wastewater treatment technology or bioremediation.

Bioremediation is a promising approach for cleaning contaminated environments by intervening in microbial communities (Medina-Bellver et al. 2005; Qaderi et al. 2018). Bioremediation effectively degrades the BOD and COD in palm oil mill effluent (POME) (Bala et al. 2014;

Karim et al. 2021). Bioremediation using *Meyerozyma guilliermondii* effectively reduces BOD, COD, total nitrogen, ammonia, and organic carbon levels in POME (Ganapathy et al. 2019). The process of waste bioremediation generally utilizes populations of microorganisms or other products, such as microorganism enzymes, to break down organic compounds in naturally polluted environments (Megharaj et al. 2014). These microorganisms produce lipase extracellular enzymes to reduce the oil and fat levels in the waste. Several bacteria, especially indigenous bacteria with extracellular enzymes, can degrade and reduce pollutants from POME/primary effluent.

Several authors have reported the isolation of bacteria from palm oil industrial waste. However, little literature still reports the isolation of indigenous bacteria that have the potential for bioremediation and degradation of palm oil mill secondary effluent (POMSE). The POMSE is a product of the secondary treatment of POME. The POME, commonly treated in ponds, starts with an acid pond followed by subsequent steps performed anaerobically,

aerobically, and facultatively (Darajeh et al. 2016). One of the methods for knowing the characteristics and composition of microorganisms, especially bacteria, that have the potential to degrade wastewater is the metagenomics pathway. Metagenomics, as a next-generation sequencing (NGS) field, offers a modern way to determine the diversity and structure of microorganisms (Rawat et al. 2013). The metagenomic analysis is a technique using next-generation sequencing based on culture-independent microorganisms. Metagenomic is a precise way to determine the uncultured microbial diversity in an environment (Yarlina et al. 2022). Metagenomic based on DNA analysis from a community using the 16S rRNA gene. The 16S rRNA marker is used to amplify DNA and identify various diverse and structured microbes. The 16S rRNA gene amplicon is a microbiome analysis technique where different samples are analyzed simultaneously using multiplexing. The results can be used to perform microbial annotation diversity by phylum, class, order, family, genera, family, and species levels. There have been many recent studies on structure, diversity, and environment quality evaluation using metagenomics, including the microbial community's metagenomic assessment and biosolids' methanogenic pathways (Bedoya et al. 2019).

The diverse microbiota community is known to participate effectively in the bioremediation of POME or POMSE. Therefore, the study of the microbiological characteristics of POMSE is necessary to enhance a better understanding of the types and nature of microorganisms that reside in POMSE. Standard microbiological methods, especially through NGS, are needed for a more comprehensive analysis. This study was designed to explore the microorganisms associated with the POMSE and identify the potential indigenous bacteria for bioremediation agents POMSE from palm oil mills in Riau Province.

MATERIALS AND METHODS

The study was exploration research without repetition to evaluate information on the bacterial community and the dominant and codominant bacteria in POMSE.

Sample collection and preservation

Raw POMSE water samples were collected aseptically from the Sei Rokan palm oil mill industry, which had utilized the ponding system. The ponds for effluent were started with a cooling pond, anaerobic pond, aerobic pond, and facultative or stabilization pond. The samples used in this study were taken from the secondary aeration pond (aerobic and facultative pond). Samples were taken by purposive sampling with three sampling points in a composite manner. The sample was taken using a sterile bottle (5 L) and kept in an ice box container, while transported to PT Genetica Science Indonesia and preserved at 4°C until further treatment to stop the wastewater from undergoing biodegradation due to microbial action (APHA 2005). Before use, the sample was

moved from the refrigerator and left at room temperature until it returned to its nature so the sample ready to be processed further.

Physicochemical evaluation of samples

Physicochemical parameter observation for POMSE was required for environmental quality evaluation, especially for wastewater. In Indonesia, the minimum concentration limit of palm oil industry effluent was controlled by the Regulation of the Minister of Environment of the Republic of Indonesia No. 5 of 2014 (Table 1). Temperature (°C) and pH were measured in situ using a portable meter (Eutech Expert pH, Thermo Scientific, Sweden). When arriving at the laboratory, chemical oxygen demand (COD) measurements were made after decomposing in the COD reactor (HACH 45600) according to the HACH 8000 method. The 5-day biological oxygen demand (BOD₅) measurement was conducted after the COD test following APHA 5210-B (APHA 2005). Total Suspended Solid (TSS) Titrimetric method (SNI 6989.3-2019); Oil/lipid (Indonesian Standard: 6989.10-2011) by Gravimetric method; total nitrogen test was conducted by Kjeldahl method as described by Bremner and Mulvaney (1982); water acidity was assessed by pH meter.

Bioinformatics and research workflow

The research began by taking raw samples of POMSE waste according to predetermined sampling points. Furthermore, DNA extraction was carried out, which was then analyzed using Oxford Nanopore Technology (Figure 1). DNA concentration was determined using both NanoDrop spectrophotometers and Qubit fluorometers. Library preparations were conducted using Kits from Oxford Nanopore Technology to obtain full length 16S rRNA-gene sequences. Nanopore sequencing was operated by MinKNOW software version 23.04.5. Basecalling was performed using Guppy version 6.5.7 with the high-accuracy model (Wick et al. 2019). The quality of FASTQ files was visualized using NanoPlot, and quality filtering was performed using NanoFilt (Nygaard et al. 2020).

Reads were classified using a centrifuge classifier (Kim et al. 2016). The bacteria and archaea index was built using the NCBI 16S RefSeq database (<https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/>). Downstream analysis and visualizations were performed using Pavian (<https://github.com/fbreitwieser/pavian>), Krona Tools (<https://github.com/marbl/Krona>), and RStudio using R version 4.2.3 (<https://www.R-project.org/>).

Table 1. Indonesian standard of wastewater in the palm oil industry

| Parameters | Indonesian standard limit |
|-------------------------|---------------------------|
| pH | 6-9 |
| BOD ₅ (mg/L) | 100 |
| COD (mg/L) | 350 |
| TSS (mg/L) | 250 |
| Oil and fat (mg/L) | 25 |
| Total nitrogen (mg/L) | 50 |

Source: Indonesian Minister of Environment Regulation (2014)

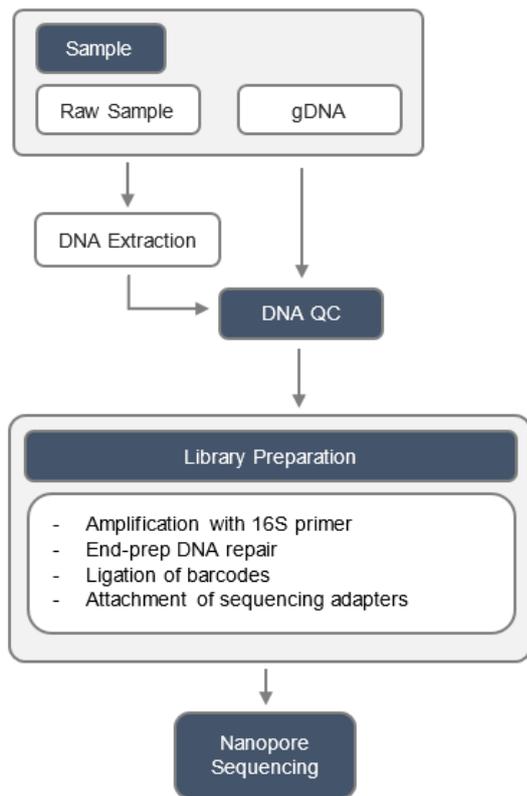


Figure 1. Research workflow

RESULTS AND DISCUSSION

Physicochemical properties of POMSE

A list of regulatory guidelines for the physicochemical properties of wastewater for POME and POMSE was compiled by the Indonesian Ministry of Environment number 5 of 2014. The highlighted quality of POMSE wastewater showed that almost all physicochemical parameters did not meet the set guidelines for most parameters (Table 1) except pH, oil, and fat (Table 2).

Several of these physicochemical properties of POMSE, such as BOD₅, COD, TSS, and total nitrogen, were far above the recommended limits set by the Indonesian standard for the palm oil industry (Tables 1 and 2). Further treatments must be done to improve POMSE quality to meet the standard.

Taxonomic and abundance of bacteria in POMSE

The electrophoresis results for the amplified DNA (labeled as 1), which was accompanied by a marker (M) and no template control (NTC), were shown in Figure 2. The fragments of bacterial 16S rRNA-gene amplified from POMSE DNA samples were solved using agarose gel electrophoresis and showed a thickened band at approximately 1,500 bp, and in NTC as a control, there is

no band detected. Further, according to the metagenomic analysis, as many as 82,910 sequences were obtained after quality filtering, showing a high number of OTUs. The effluent also developed a bacterial community with high diversity and a relatively even distribution of species, as indicated by the Shannon and Simpson index of 4.955 and 0.973, respectively. Taxonomically, the result shows that two of the dominant phyla in POMSE were Pseudomonadota (50%), and Bacillota (31%) (Figure 3). The Pseudomonadota (*Proteobacteria*) phylum was often present in wastewater.

Figure 4 shows the sequence collection, representing ten established bacteria families. *Chromatiaceae* was the most dominant family on the POMSE, with more than 26% of the total OTUs of bacterial families found. The analysis showed that ten taxa were the dominant species. Overall, 2,480 species were detected; *Allochrotaium humboldtianum* was the one with the highest abundance (8%), followed by *A. phaeobacterium* (6%), *Allochrotaium* sp. (6%), *Macromonas bipunctata* (6%) and, *Tepidibaculum saccharolyticum* (4%) (Figure 5).

Table 2. Physicochemical measurements of the POMSE

| Parameters | Values |
|-------------------------|----------|
| pH | 7.6 |
| BOD ₅ (mg/L) | 852.96 |
| COD (mg/L) | 2,492.46 |
| TSS (mg/L) | 423.00 |
| Total Nitrogen (mg/L) | 203.63 |
| Oil and fat (mg/L) | 6.20 |

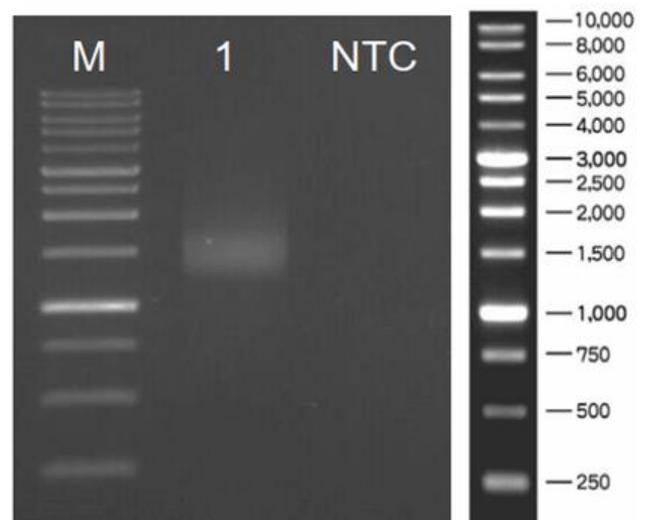


Figure 2. An image of a gel post electrophoresis of marker (M), sample (1) and no template control (NTC)

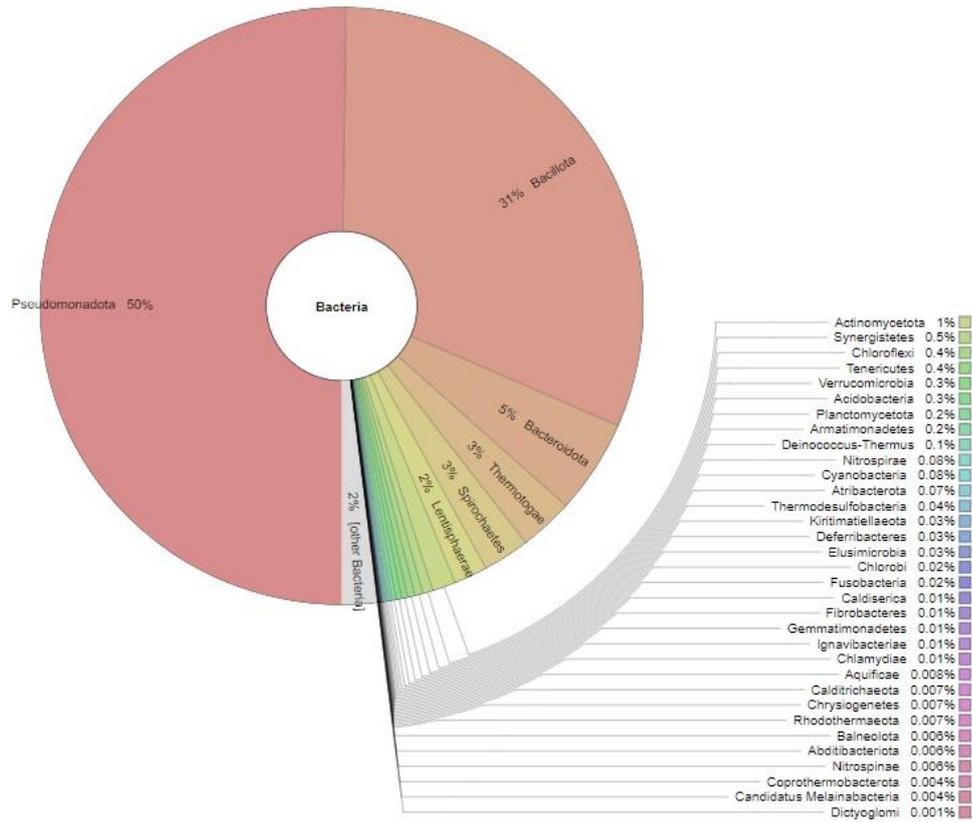


Figure 3. Taxonomic classification of bacteria at the phylum level. The top ten phyla are represented, the remaining are grouped within the "others" category

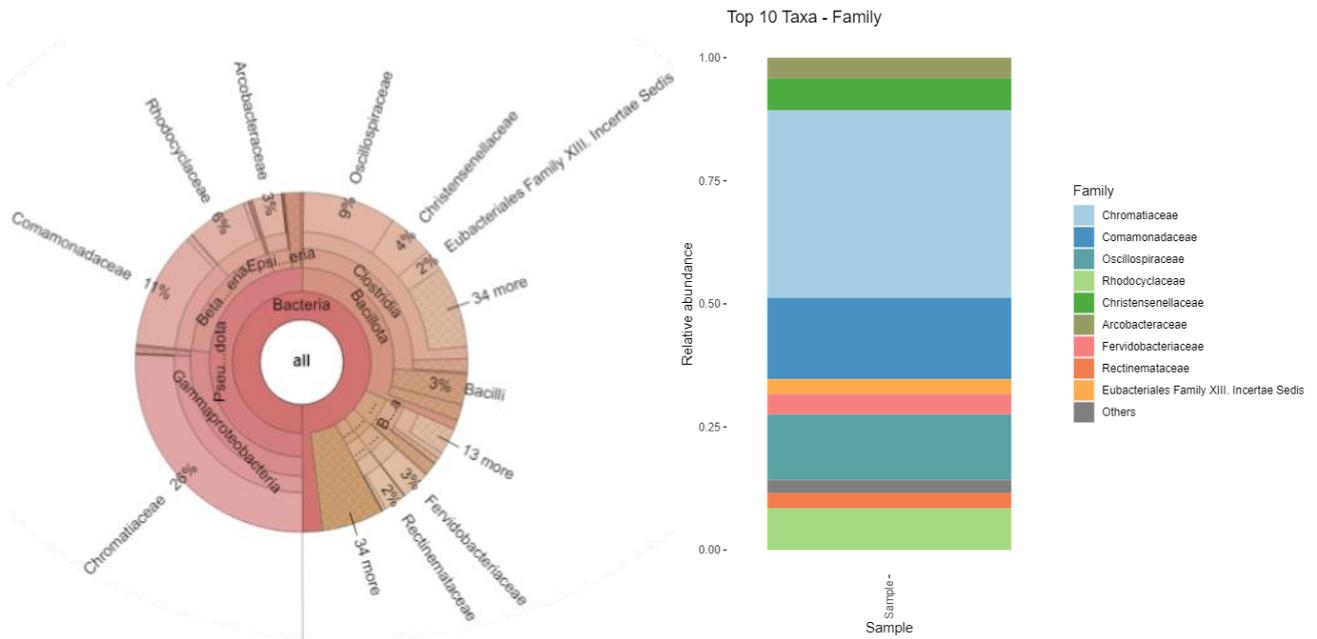


Figure 4. Taxonomic classification of bacteria at the family level. The top ten families are represented, and the remaining are grouped within the "others" category

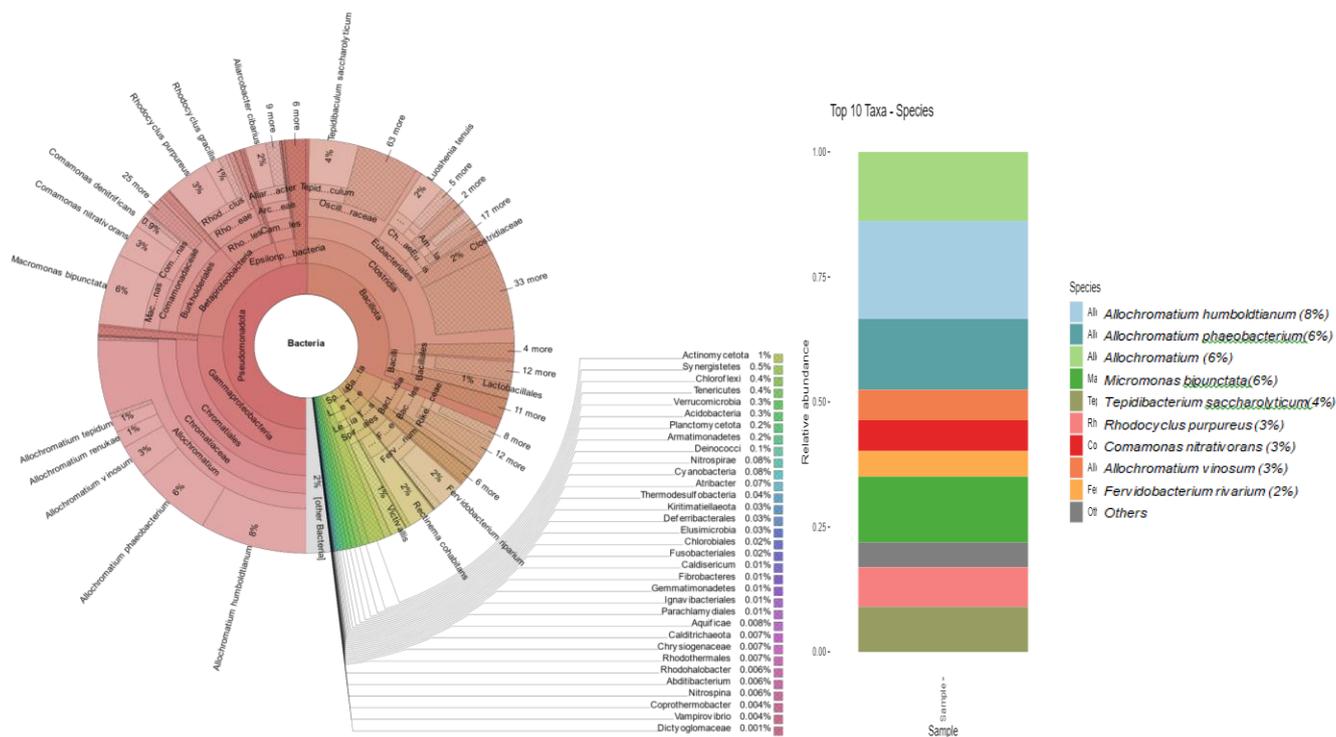


Figure 5. Taxonomic classification of bacteria at the species level. The top ten species are represented, and the remaining are grouped within the "others" category

Discussion

Physicochemical of POMSE

Four ponds were used in the waste processing carried out by the research partner, a palm oil factory. The first pond was a cooling or acidification pond whose temperature and pH could not be measured because the waste sample was very hot. The second pond was a primary anaerobic pond, which had an average temperature of 39.6°C and an average pH of 8.1; the next pond was an anaerobic pond and facultative with an average temperature of 37.7°C and an average pH of 7.62, while the last pool was an aerobic and facultative pool with an average temperature of 43.2°C and an average pH of 7.61. The waste from the third pond called POMSE.

The physicochemical values of POMSE in this analysis did not differ from those of other studies. However, the TSS value was lower than in other studies. Physicochemical parameters in the effluent affect the growth of microorganisms. In the palm oil industry effluent, the main pollutants used as limits and standards are the levels of organic pollutants, including COD, BOD, and TSS. Furthermore, pH and temperature are also determinant factors. In POMSE, the physicochemical parameters in this study and several other studies show lower levels of pollutants when compared to those in POME waste; however, they have not met the Indonesian palm oil waste industrial standard. Oil and fat results in this study were found to be similar to Karno et al. (2023), which were lower than the Indonesian standard, reflecting the factory's efficient oil extraction process; some of the physicochemical parameters of POMSE that were lower

than those of POME may affect the type and number of microorganisms, including bacteria contained therein.

Taxonomic and abundance of bacteria in POMSE

Agarose gel electrophoresis has proven to be an effective and efficient way to separate nucleic acids. The high strength of agarose gels allows for low percentage gel handling for the separation of large DNA fragments. On average, the bacterial phyla found in POMSE effluents are most often found in wastewater. Proteobacteria is the most dominant phylum, as suggested by other studies, which reported as the most frequently encountered taxa in the river at many locations. Taxonomically, the major phyla observed in all samples belong to Proteobacteria and Bacteroidetes (Sharuddin et al. 2017), and the bacterial community was dominated by Proteobacteria, Firmicutes, Actinobacteria, and Cyanobacteria (Cai et al. 2016). Pseudomonadota (Proteobacteria) is the most abundant phylum in the sediments of the collapsed lake area due to coal mining in Huaibei, China (Shen et al. 2022). Microbial communities dominated by the Proteobacteria, Bacteroidota, and Actinobacteriota at nine large-scale WWTPs treat municipal sewage from Moscow, Russia (Begmatov et al. 2022). The metagenomic analysis identified the predominant phyla of Proteobacteria present in wastewater samples from the Covicorti wastewater treatment plant (PTARC) are Bacillota, Pseudomonadota, Bacteroidota, Actinomyketon, and Campylobacterota (De La Cruz-Noriega et al. 2023); the phyla Firmicutes (46.24%), Bacteroidetes (34.19%), and Proteobacteria (15.11%), with the particular presence of Spirochaetes,

Verrucomicrobia, and Synergistetes dominant on POME from industrial plants harbored. In comparison, those from artisanal production were colonized by Firmicutes (92.06%), Proteobacteria (4.21%), and Actinobacteria (2.09%). Proteobacteria, Bacteroides, and Firmicutes are commonly found in wastewater due to their ability to decompose organic pollutants. In the primary and secondary stages of wastewater treatment, bacteria such as Proteobacteria, Bacteroidetes, and Firmicutes are predominant and help break down organic pollutants (Chen et al. 2022). Other than organic wastes, Proteobacteria and Bacteroidetes were also found in wastewaters with high heavy metals. The most abundant phyla in wastewater treatment plants containing heavy metals located in chemical industrial zones for all the samples are Proteobacteria, Chloroflexi, Actinobacteriota, Bacteroidetes, and Planctomycetes, in the order of their relative proportions (Zeng et al. 2022). Proteobacteria, Bacteroidota, Patescibacteria, and Chloroflexi were the dominant phyla from active sludge in China's tropical municipal wastewater treatment plant (Gu et al. 2022). However, different results were seen by Khalid et al. (2019), which showed that the dominant phylum in POME was Chloroflexi (23%), although the phyla Firmicutes (19%), Bacteroidetes (16%), Proteobacteria (16%) were also found in smaller abundance. Hence, the bacterial phylum found in POMSE is not much different from that found in POME in several previous research studies. This leads to the assumption that the high organic material contained in POMSE increases the growth and development of bacteria. These bacteria grow until the waste treatment process ends or the organic material contained in the POMSE runs out.

Meanwhile, the bacterial classes in the POMSE that have the highest abundance include Gammaproteobacteria (27%), Clostridia (26%), and Betaproteobacteria (19%). Gammaproteobacteria, such as *Pseudomonas*, *Stenotrophomonas*, and *Acinetobacter* spp., were found in wastewater-irrigated fields. Isolate bacteria from soils irrigated with wastewater for 100 years revealed that 46% were affiliated with the Gammaproteobacteria and 50% with the Bacilli (Broszat et al. 2014). Gammaproteobacteria, Alphaproteobacteria, Deltaproteobacteria, and Thermotogae are the dominant classes in the activated sludge, digestion sludge, influent, and effluent samples of a full-scale wastewater treatment plant treating saline sewage (Ye and Zhang 2013). Based on 16S rDNA analyses, Gammaproteobacteria, Epsilonproteobacteria, and Betaproteobacteria codominated the microbial consortium of the MFC fed with POME (Jong et al. 2011). Alcaligenaceae and Chromatiaceae from the class Betaproteobacteria and GammaProteobacteria were detected in all four final discharges in Malaysia (Mohd-Nor et al. 2018). Family Chromatiaceae and Alcaligenaceae on the river are proposed as indicator bacteria to indicate the river water contamination caused by POME final discharge (Sharuddin et al. 2017; Zolkefli et al. 2020). Chromatiaceae were found in several POME landfills, indicating that the bacteria originated from the treatment system, regardless of

the different biotreatment processes used in the palm oil mill (Mohd-Nor et al. 2018).

Result revealed the identified bacteria from POME containing the ten dominant bacteria at the species level in our study. This comparison revealed that some of the bacterial species identified in this study are different from previous studies, and even some studies also did not reveal any *Allochrochromatium* species dominance due to the differences in the waste treatment technology used and the wastewater sampled types.

In the present research, the samples were obtained from the POME secondary treatment ponds (POMSE) that use processing technology with a land application processing system; the POMSE has slightly different characteristics from POME. POMSE is characterized by a thick, brownish color with a higher pH (7-9 pH) but has lower BOD and COD effluent than POME (Darajeh et al. 2016). The difference in the effluent's physical and chemical characteristics is likely to cause the difference in bacterial species that have been found. Moreover, the pH fluctuations and temperature changes affect microorganisms in POME treatment (Ahmad et al. 2003). Most microorganisms like bacteria and fungi prefer to degrade PAHs at neutral pH (Sawadogo et al. 2014; Al-Hawash et al. 2019). The dominance of *Allochrochromatium* bacterial is quite a new discussion for palm oil mills because these genera are often found in effluents with high metal and sulfur contents. However, some genera also exist in effluents with high organic content. *Allochrochromatium* is a tolerant bacteria found in WWTPs with high levels of metals and organics (Akinwole et al. 2022).

In conclusion, the indigenous bacterial species found in this research have potential as bioremediation agents for palm oil wastewater. There are *Allochrochromatium humboldtianum*, *Allochrochromatium phaeobacterium*, *Allochrochromatium* sp., *Allochrochromatium vinosum*, *Macromonas bipunctata*, *Tepidibaculum saccharolyticum*, *Rhodocyclus purpureus*, *Comamonas nitratorans*, and *Fervidobacterium riparium*. BOD₅, COD, TSS, and total nitrogen values of water samples have not met the minimum quality limits of Indonesian palm oil industry waste, respectively, with values of 852.96, 2492.46, 423.00, and 203.63 mg/L. In contrast, the pH value and oil contents have met the quality standards set. The POMSE metagenomic test analysis showed the three dominant phyla, such as Pseudomonadota/Proteobacteria (50%), Bacillota (31%), and Bacteroidota (5%). Some of the dominant species found are species from the *Allochrochromatium* group of genera.

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