

PUTATIVE HEAVY METAL-TOLERANT *Azotobacter* AND *Pseudomonas* FROM PALM OIL MILL EFFLUENT (POME): PRELIMINARY EVALUATION OF BIOREMEDIATION AND BIOFERTILISER TRAITS

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ABSTRACT

Palm oil mill effluent (POME) is a nutrient-rich agro-industrial byproduct harbouring diverse microbial communities. This study aimed to isolate and characterise putative heavy metal-tolerant and plant growth-promoting (PGPR) bacteria from POME. Bacterial isolates were obtained using nitrogen-free Ashby agar and tryptic soy agar (TSA). Genus-level identification was based on colony morphology and selective growth characteristics. Hemolytic activity, heavy metal tolerance to Pb²⁺ and Cd²⁺, Cd²⁺ removal efficiency, and indole-3-acetic acid (IAA) production were evaluated under laboratory conditions. Five morphologically distinct isolates were obtained, tentatively assigned to the genera *Azotobacter* (three isolates) and *Pseudomonas* (two isolates). Most isolates exhibited γ -hemolysis, while one isolate showed β -hemolysis. All isolates demonstrated growth at 115 mg/L Pb²⁺ and 11 mg/L Cd²⁺ in agar-based assays. In liquid culture containing 15 mg/L Cd²⁺, removal efficiencies exceeded 98%. IAA production ranged from 44.15 to 74.91 μ g/mL in tryptophan-supplemented medium. These findings indicate that POME may serve as a source of putative heavy metal-tolerant and IAA-producing bacteria. However, molecular identification, expanded biosafety evaluation and soil-based validation studies are required to confirm their applicability in bioremediation and sustainable agriculture.

Keywords: *Azotobacter*, cadmium (Cd²⁺), lead (Pb²⁺), POME, *Pseudomonas*.

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INTRODUCTION

Palm oil mill effluent (POME) is one of the largest agro-industrial waste streams in Southeast Asia, particularly in Indonesia and Malaysia, where oil palm (*Elaeis guineensis* Jacq.) plantations dominate the agricultural sector. Approximately 2.8–3.3 t of POME are produced for every tonnes of crude palm oil (CPO) processed, resulting in large volumes of effluent each year (Abdurahman et al., 2013; Mohammad et al., 2021; Vijaya et al., 2008). It is classified as highly polluted wastewater due to its elevated biological oxygen demand (BOD, 25,000 mg/L) and chemical oxygen

demand (COD, 51,000 mg/L) levels (Ahmad et al., 2003; Bello & Raman, 2017). On the other hand, POME also contains abundant organic matter and diverse microbial communities, indicating its potential to be utilised as a biological resource rather than merely treated as waste (Nisha et al., 2026; Wu et al., 2010).

In addition to the large volume of POME generated during palm oil processing, heavy metal contamination has become a significant environmental issue in agricultural systems (Odigie, 2024; Okeke et al., 2024; Rashid et al., 2023). Metals such as cadmium (Cd^{2+}) and lead (Pb^{2+}) persist in soils, bioaccumulate in crops, and threaten food safety and human health. In oil palm agroecosystems, metal inputs may originate from phosphate fertilisers, agrochemicals, industrial discharge and processing effluents. Conventional physicochemical remediation methods are often costly and may generate secondary pollutants (Iskandar et al., 2018). Consequently, microbial-based bioremediation strategies have gained increasing attention as sustainable and eco-friendly alternatives (Ayangbenro & Babalola, 2017; Manikandan et al., 2026).

Microorganisms from wastewater environments are generally able to survive under heavy metal stress through mechanisms such as biosorption, intracellular accumulation, enzymatic detoxification, and metal efflux systems (Gadd, 2010; Igiri et al., 2018). Some of these bacteria can also promote plant growth through nitrogen fixation and indole-3-acetic acid (IAA) production, making them potential candidates for both bioremediation and biofertiliser applications (Jacob et al., 2018; Verma & Jaiswal, 2016). Among these, *Azotobacter* and *Pseudomonas* are well known for their environmental adaptability and plant growth-promoting (PGPR) traits (Ajmal et al., 2022; Lugtenberg & Kamilova, 2009). Previous studies have demonstrated that Cd^{2+} -resistant *Pseudomonas* strains, isolated from contaminated environments, exhibit a strong biosorption capacity and can support plant growth under heavy metal stress conditions (Ma et al., 2016; Ojuederie & Babalola, 2017).

Although many studies have explored microbial bioremediation in contaminated environments, research focusing on indigenous bacteria from POME with both heavy metal tolerance and PGPR traits is still limited. Most POME-related studies continue to emphasise biogas and biohydrogen production (Arisht et al., 2020; Lam & Lee et al., 2011; Nasrin et al., 2022, 2024), composting, or physicochemical treatment, while the potential microbial diversity within POME remains underexplored. Therefore, this study aimed to isolate and preliminarily characterise putative *Azotobacter* and *Pseudomonas* isolates from POME and evaluate their tolerance to Cd^{2+} and Pb^{2+} , Cd^{2+} removal ability, hemolytic activity and

IAA production under laboratory conditions. By addressing this gap, the study seeks to provide preliminary evidence that POME may serve as a source of multifunctional bacteria relevant to sustainable agriculture and environmental biotechnology.

MATERIALS AND METHODS

Sample Collection

POME samples were collected aseptically from a local palm oil processing facility in North Sumatra, Indonesia. Samples were taken from Anaerobic Pond 4, which was selected due to its high nutrient content. Approximately, 1 L of effluent was collected in sterile bottles and transported to the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, University of North Sumatra, under cooled conditions (4°C). Samples were processed within 24 hr of collection. Before isolation, samples were homogenised and serially diluted (10^{-1} to 10^{-6}) using sterile 0.85% sodium chloride (NaCl) solution.

Bacterial Isolation and Purification

Bacterial strains were isolated using selective and general-purpose media. For the isolation of putative *Azotobacter* spp., nitrogen-free Ashby agar was used and incubated at $30 \pm 2^{\circ}\text{C}$ for 72 hr. Colonies exhibiting mucoid morphology typical of diazotrophic bacteria were selected.

For the isolation of putative *Pseudomonas* spp., diluted samples were plated on tryptic soy agar (TSA) and incubated at $30 \pm 2^{\circ}\text{C}$ for 48 hr. TSA is a general-purpose medium and not selective for *Pseudomonas*. Therefore, genus-level identification was based solely on colony morphology and growth characteristics (Wiegand et al., 2008). No biochemical profiling or molecular identification (such as 16S rRNA gene sequencing) was conducted. Accordingly, isolates are referred to as putative *Azotobacter* and *Pseudomonas* spp.

Purification and Preservation

Distinct colonies were subcultured repeatedly to obtain pure isolates. Pure cultures were preserved on agar slants at 4°C for routine use and stored in 20% (v/v) glycerol at -20°C for long-term preservation.

Morphological Characterisation

Morphological characteristics, including colony shape, margin, elevation, pigmentation and texture, were recorded following standard

microbiological protocols (Cappuccino & Sherman, 2014). Observations were conducted after 48–72 hr incubation.

Hemolytic Activity

Preliminary biosafety screening was performed using 5% (v/v) sheep blood agar. Fresh cultures were streaked and incubated at 30°C for 24–48 hr. Hemolysis was categorised as β -hemolysis (clear zone), α -hemolysis (greenish zone), or γ -hemolysis (no visible change). This assay served only as an initial biosafety screening. Further evaluations, such as antibiotic susceptibility testing and molecular screening of virulence genes, were not performed and are recommended for future studies.

Heavy Metal Tolerance Assay

Heavy metal tolerance was assessed on nutrient agar supplemented with graded concentrations of lead (II) nitrate ($\text{Pb}(\text{NO}_3)_2$, 25, 50, 75, 100 and 115 mg/L) and cadmium chloride (CdCl_2 , 2, 5, 8 and 11 mg/L). Standardised bacterial suspensions were prepared by adjusting overnight cultures to $\text{OD}_{600} \approx 0.1$ (approximately 10^7 CFU/mL). The suspension (10 mL) were spot-inoculated onto agar plates. Plates were incubated at $30 \pm 2^\circ\text{C}$ for 48–72 hr. Growth was recorded as the presence or absence of visible colony formation at each. The maximum tolerance concentration (MTC) was defined as the highest tested concentration at which visible bacterial growth was observed. It is acknowledged that agar-based visible growth assessment provides qualitative tolerance data and does not represent minimum inhibitory concentration (MIC).

Cadmium (Cd^{2+}) Removal Assay

Cd^{2+} removal was quantified in liquid culture using batch experiments. Each isolate was inoculated (2% v/v) into nutrient broth supplemented with 15 mg/L Cd^{2+} (CdCl_2) and incubated at 30°C under shaking conditions (150 rpm) for 72 hr. After incubation, cultures were centrifuged at 10,000 rpm for 15 min. Supernatants were filtered through 0.22 μm syringe filters. Residual Cd^{2+} concentrations were measured using atomic absorption spectrophotometry (PerkinElmer Analyst 400, PerkinElmer Inc., USA). The Cd^{2+} removal efficiency (%) was calculated using Equation (1).

$$\text{Removal efficiency (\%)} = \frac{C_0 - C_t}{C_0} \times 100\% \quad (1)$$

where, C_0 represents the initial Cd^{2+} concentration and C_t the residual concentration after incubation.

Indole-3-Acetic Acid (IAA) Production

IAA production was determined using the Salkowski colourimetric method. Bacterial cultures were inoculated into Luria-Bertani (LB) broth supplemented with 0.1% L-tryptophan and incubated at 30°C for 72 hr under shaking (150 rpm). A control treatment without L-tryptophan supplementation was included to evaluate background indolic compound production. Cultures were centrifuged at 8,000 rpm for 10 min. The supernatant (1 mL) was mixed with 2 mL Salkowski's reagent and incubated in the dark for 30 min. Absorbance was measured at 530 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Japan). IAA concentration was quantified using a standard curve (10–100 $\mu\text{g/mL}$).

Data Analysis

All experiments were conducted in quadruplicate ($n = 4$). Mean values were analysed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

RESULTS AND DISCUSSION

Isolation of Bacteria

Isolation of bacterial strains from POME using nitrogen-free Ashby agar and TSA resulted in five morphologically distinct colonies. Three isolates obtained on Ashby medium were tentatively assigned to the genus *Azotobacter* based on their mucoid colony morphology and growth on nitrogen-free medium, suggesting diazotrophic characteristics. Two additional isolates obtained on TSA were tentatively categorised as *Pseudomonas* spp. based on colony morphology and growth features (Table 1).

Since identification was based solely on morphological characteristics and selective media, genus assignment should be considered tentative. Molecular confirmation, particularly through 16S rRNA gene sequencing, is required to validate the taxonomic identity of the isolates (Clarridge, 2004; Janda & Abbott, 2007). The putative *Azotobacter* isolates exhibited mucoid colonies, a characteristic commonly associated with exopolysaccharide (EPS) production in several *Azotobacter* species (Dhevagi et al., 2021; Rasulov et al., 2013). EPS has been reported to contribute to heavy metal binding and biosorption because of the presence of negatively charged functional groups such as carboxyl and phosphate moieties (Bala et al., 2014, 2015; Gadd, 2010).

The putative *Pseudomonas* isolates exhibited smooth, moist colonies with entire margins (Table 1). Although the pigmentation was faint, no biochemical assay was conducted to confirm the nature of the pigment; therefore, the pigment could not be definitively identified. Members of the genus *Pseudomonas* are widely recognised for their metabolic versatility and environmental adaptability (Leedj  rv et al., 2008; Lugtenberg & Kamilova, 2009; Macin & Akyon, 2017). Several studies have shown that *Pseudomonas* spp. possess multiple heavy metal resistance mechanisms, including biosorption, intracellular sequestration, metal efflux systems, and detoxification pathways (Gadd, 2010; Ma et al., 2016; Ojuederie & Babalola, 2017).

Hemolytic Activity

Hemolysis assays indicated that four isolates exhibited γ -hemolysis, suggesting the absence of detectable red blood cell lysis under the experimental conditions (Table 2). In contrast, one isolate (putative *Pseudomonas* sp2) exhibited β -hemolysis. Although hemolysis testing can provide an initial indication of potential virulence traits, it does not constitute a comprehensive assessment of biosafety (Urganci et al., 2022). Additional analyses, including antibiotic susceptibility profiling and molecular screening of virulence-associated genes, are necessary before environmental application can be considered (Chadha et al., 2022). Therefore, these findings should be interpreted as preliminary screening results rather than definitive evidence of biosafety.

The high prevalence of γ -hemolytic isolates indicates that most of the putative *Azotobacter* and

Pseudomonas isolates in this study did not show detectable hemolytic activity. Several studies have reported that non-pathogenic strains of *Pseudomonas* and *Azotobacter* are commonly utilised as PGPR because of their roles in nutrient mobilisation, phytohormone production and adaptation to environmental stress (Lugtenberg & Kamilova, 2009; Ojuederie & Babalola, 2017). Nevertheless, the β -hemolytic activity observed in *Pseudomonas* sp2 indicates that additional biosafety evaluation is still required before practical application.

Heavy Metal Resistance

All isolates were still able to grow on nutrient agar containing Pb^{2+} and Cd^{2+} , even at the highest concentrations tested, namely 115 mg/L Pb^{2+} and 11 mg/L Cd^{2+} (Table 3). These results suggest that the putative *Azotobacter* and *Pseudomonas* isolates possess tolerance to both heavy metals under laboratory conditions. Previous studies have also demonstrated that indigenous bacteria resistant to Cd^{2+} and Pb^{2+} can play a crucial role in the bioremediation of heavy metals, particularly in wastewater treatment systems (Bala et al., 2014, 2015). The presence of naturally resistant bacteria is considered beneficial because their resistance mechanisms are closely associated with environmental conditions and metal concentrations (Dixit et al., 2015), which may contribute to more effective removal of Cd^{2+} and Pb^{2+} . Nevertheless, the growth test used in this study was qualitative and based only on agar observations, so the results cannot be used to determine MIC values (Bruins et al., 2000; Jebril et al., 2022).

TABLE 1. MORPHOLOGICAL CHARACTERISTICS OF BACTERIAL ISOLATES

Isolate	Shape	Margin	Elevation	Color	Texture
<i>Azotobacter</i> sp1	Circular	Entire	Convex	Milky white	Mucoid
<i>Azotobacter</i> sp2	Circular	Undulate	Umbonate	Cream	Mucoid
<i>Azotobacter</i> sp3	Wrinkled	Undulate	Umbonate	Transparent	Mucoid
<i>Pseudomonas</i> sp1	Circular	Entire	Flat	Cream	Moist
<i>Pseudomonas</i> sp2	Circular	Entire	Convex	Cream	Moist

TABLE 2. HEMOLYTIC ACTIVITY OF BACTERIAL ISOLATES

Isolate	Hemolysis type	Description
<i>Azotobacter</i> sp1	γ -hemolysis	No change around colony (non-hemolytic)
<i>Azotobacter</i> sp2	γ -hemolysis	No change around colony (non-hemolytic)
<i>Azotobacter</i> sp3	γ -hemolysis	No change around colony (non-hemolytic)
<i>Pseudomonas</i> sp1	γ -hemolysis	No change around colony (non-hemolytic)
<i>Pseudomonas</i> sp2	β -hemolysis	Clear zone with faint green pigment (<i>pyocyanin</i>) indicating RBC lysis

Note: RBC - red blood cell.

TABLE 3. MAXIMUM TOLERANCE CONCENTRATIONS OF Pb²⁺ AND Cd²⁺

Isolate	Growth at 115 mg/L Pb ²⁺	Growth at 11 mg/L Cd ²⁺
<i>Azotobacter</i> sp1	Yes	Yes
<i>Azotobacter</i> sp2	Yes	Yes
<i>Azotobacter</i> sp3	Yes	Yes
<i>Pseudomonas</i> sp1	Yes	Yes
<i>Pseudomonas</i> sp2	Yes	Yes

Note: Pb²⁺ - lead; Cd²⁺ - cadmium.

Bacterial tolerance to heavy metals is often linked to mechanisms such as extracellular sequestration, biosorption, precipitation, and bioaccumulation within cells, EPS production and detoxification processes (Gadd, 2010; Gupta & Diwan, 2017). In *Azotobacter*, EPS may contribute to metal binding and reduce metal interaction with cellular components (Rasulov et al., 2013). Meanwhile, species of *Pseudomonas* are known to possess efficient metal resistance systems and are frequently reported as bacteria with potential roles in both bioremediation and plant growth promotion (Ojuederie & Babalola, 2017). The tolerance observed in this study may reflect adaptation to the physicochemical characteristics of POME, which contains high organic matter and complex waste components. Nevertheless, further molecular characterisation and soil-based studies are still needed to gain a clearer understanding of the resistance mechanisms and environmental performance of these isolates.

Cadmium (Cd²⁺) Removal Efficiency

Cd²⁺ removal was evaluated in liquid culture with an initial concentration of 15 mg/L Cd²⁺ for 72 hr. As presented in Table 4, all isolates reduced measurable Cd²⁺ concentrations under the tested conditions, with residual levels ranging from 0.140 to 0.183 mg/L, corresponding to removal efficiencies above 98.00%. *Azotobacter* sp2 exhibited the lowest residual Cd²⁺ concentration (0.140 mg/L) and the highest removal efficiency (99.07%). Statistical analysis using DMRT indicated significant differences among the isolates ($p < 0.05$), although the numerical variation was relatively small under the applied conditions. Only isolates confirmed as non-pathogenic based on hemolysis and phytopathogenicity assays were included in this analysis, whereas *Pseudomonas* sp2 was excluded due to pathogenic responses observed during the assay. Therefore, it was not presented in Table 4.

It is important to distinguish between the agar-based tolerance assay and the liquid removal

experiment. The agar method provides qualitative evidence of growth at defined concentrations, whereas the liquid system reflects dynamic interactions between bacterial cells and dissolved metal ions (Bruins et al., 2000; Gadd, 2010). Differences in metal bioavailability and medium characteristics may explain why removal was observed at 15 mg/L despite the highest agar-tested concentration being 11 mg/L.

This experiment was conducted at a single initial Cd²⁺ concentration and did not include abiotic or killed-biomass controls. Therefore, the data demonstrate removal capacity under specific laboratory conditions but do not distinguish between biosorption, bioaccumulation, or physicochemical precipitation. Several studies have reported that microbial Cd²⁺ removal may involve multiple mechanisms, including adsorption to cell surfaces, intracellular accumulation and extracellular binding processes (Gadd, 2010; Ojuederie & Babalola, 2017). Further studies incorporating appropriate controls and multi-concentration assays are required to clarify the underlying mechanisms.

Overall, these findings indicate that the putative *Azotobacter* and *Pseudomonas* isolates are capable of interacting with dissolved Cd²⁺ under controlled laboratory conditions. The results should be regarded as preliminary screening data that warrant further evaluation in more complex systems.

TABLE 4. RESIDUAL CADMIUM (Cd²⁺) CONCENTRATION AND REMOVAL EFFICIENCY AFTER 72 HR INCUBATION

Isolate	Residual Cd ²⁺ (mg/L)	Removal efficiency (%)
<i>Azotobacter</i> sp1	0.163 ^b	98.91 ^{ab}
<i>Azotobacter</i> sp2	0.140 ^a	99.07 ^a
<i>Azotobacter</i> sp3	0.169 ^{bc}	98.87 ^b
<i>Pseudomonas</i> sp1	0.183 ^c	98.78 ^b

Note: Initial Cd²⁺ = 15 mg/L. Values represent the mean of four replicates (n = 4). Means followed by different letters within the same column are significantly different according to DMRT at $p < 0.05$.

Indole-3-Acetic Acid (IAA) Production

All isolates were able to produce detectable amounts of IAA when grown in medium supplemented with L-tryptophan (Table 5). The highest concentration was observed in *Azotobacter* sp1 (74.91 µg/mL), followed by *Pseudomonas* sp1 (67.64 µg/mL). These results indicate that several isolates possess potential PGPR traits under laboratory conditions.

TABLE 5. INDOLE-3-ACETIC ACID (IAA) PRODUCTION BY BACTERIAL ISOLATES

Isolate	IAA concentration (µg/mL)
<i>Azotobacter</i> sp1	74.91 ^a
<i>Azotobacter</i> sp2	44.15 ^b
<i>Azotobacter</i> sp3	48.09 ^c
<i>Pseudomonas</i> sp1	67.64 ^d

Note: Values represent the mean of four replicates (n = 4). Means followed by different letters within the same column are significantly different according to DMRT at $p < 0.05$.

IAA is one of the major phytohormones involved in root development, cell elongation, and nutrient uptake. Many soil and rhizosphere bacteria are known to synthesise IAA, which may contribute to improved plant growth and stress tolerance (Khan et al., 2021). The ability of *Azotobacter* and *Pseudomonas* isolates to produce IAA in this study is consistent with previous reports describing these genera as important rhizobacteria PGPR (Lugtenberg & Kamilova, 2009; Ojuederie & Babalola, 2017).

Differences in IAA concentration among isolates may reflect variations in metabolic activity and tryptophan utilisation at the strain level. However, it should be noted that IAA production in this study was measured using the Salkowski colourimetric method, which may also react with other indolic compounds. It should also be noted that the assay was performed in L-tryptophan-supplemented medium, indicating that the results reflect the potential capacity for IAA synthesis under induced conditions rather than natural production under soil conditions. Therefore, further studies using more specific analytical methods and plant-based assays are needed to confirm the ecological significance of these isolates.

CONCLUSION

This study demonstrated that POME contains bacterial isolates capable of tolerating Pb²⁺ and Cd²⁺ under laboratory conditions and producing IAA in supplemented media. Three putative *Azotobacter* and two putative *Pseudomonas* isolates were characterised based on morphological traits. Most isolates exhibited no detectable hemolytic activity, although a comprehensive biosafety assessment is still required. High Cd²⁺ removal efficiencies (>98%) were observed at a single tested concentration, reflecting the potential capacity for metal removal under controlled conditions. IAA production ranged from 44.15 to 74.91 µg/mL, indicating the presence of PGPR traits.

Overall, these findings suggest that POME may serve as a potential source of beneficial bacteria with applications in bioremediation and PGPR.

Nevertheless, molecular identification, expanded biosafety evaluation, and validation under soil conditions are still needed. Further research is also needed to assess the applicability of these isolates in agricultural soils and to support the sustainable utilisation of POME in agroecosystems.

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