

Isolation and Identification of Bacteria with Potential for Mercury (Hg) Bioremediation from the Kahayan River

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ABSTRACT

The Kahayan River in Central Kalimantan is one of the waterways utilized for traditional gold mining that employs amalgamation techniques. The application of this method poses a significant risk of environmental pollution and degradation. This study was conducted to evaluate the capacity of bacteria to act as bioremediation agents for mercury (Hg) at different concentrations. The methodology involved inoculating sediment samples into Zobell 2216E medium, isolating and purifying bacterial colonies on Nutrient Agar (NA), followed by bacterial identification and assessment of mercury degradation using an Atomic Absorption Spectrophotometer (AAS). The findings revealed that isolate 34 was able to remove 132.73 ppm of Hg from an initial concentration of 250 ppm (equivalent to a 53.09% reduction), whereas isolate 108 removed 139.68 ppm, corresponding to a 55.87% reduction.

Introduction

Indonesia is rich in natural resources, encompassing both biological and non-biological types. Among its abundant non-biological resources are minerals, including gold, coal, silver, tin, and others [1]. These mineral resources play a significant role in supporting Indonesia's industrial sector and national economy by contributing to state revenue, fostering industrial development, and generating new business opportunities that promote equitable social welfare. Nevertheless, mining activities also pose considerable challenges, as they can lead to environmental degradation and infringe upon the economic, social, and cultural rights of communities residing near mining areas [2]. Mining activities are also one of the main causes of environmental damage due to the disposal of operational waste into rivers, forests, estuaries, and even reaching marine areas. Additional impacts include sediment accumulation and contamination

from metal residues as well as hazardous substances [3]. Pollution of soil and water may arise from multiple sources, such as industrial effluents, mining waste, residues of fertilizers and pesticides, and remnants from chemical weapons facilities. [4,5].

One of the major environmental consequences of industrial activities is contamination by heavy metal compounds. Mining areas, in particular, often serve as primary sources of heavy metal dispersion [6]. Various industrial sectors are known to generate substantial quantities of heavy metals, including machinery manufacturing, metallurgy, electroplating, paint production, leather processing, and gold mining [7,8]. Common heavy metals and toxic substances frequently detected in industrial wastewater include chromium (Cr), nickel (Ni), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cadmium (Cd), silver (Ag), lead (Pb), as well as cyanide compounds. Wastewater pollution

containing heavy metals has become an environmental issue that has received widespread attention because of its impacts that have the potential to endanger the lives of living things, including humans [9,10].

Mercury (Hg) naturally occurs in the form of cinnabar (HgS) and is widely used in gold mining as well as in various chemical industrial processes. In aquatic environments, mercury can be transformed into the highly toxic compound methylmercury, which readily accumulates along the food chain. Contamination by mercury leads to water and soil pollution, disrupts aquatic ecosystems, and results in bioaccumulation in both plants and animal [11]. Furthermore, mercury poses serious health risks to living organisms, including damage to the nervous system, interference with fetal development, impairment of internal organs, and the onset of respiratory and skin disorders. Due to these severe impacts on human health and ecological balance, proper waste management is essential [12,13].

The Kahayan River in Central Kalimantan is one of the primary sites for traditional gold mining, where mercury (Hg) is extensively used in the extraction process. The commonly applied method is amalgamation, which utilizes mercury to bind gold particles from ore materials. However, the use of mercury poses serious environmental and health risks [14]. Mercury that does not bind with gold is often discharged directly into the river, leading to contamination of water and sediments, and may also infiltrate surrounding soils, thereby disturbing terrestrial ecosystems. In aquatic systems, mercury can be converted into methylmercury, a more toxic form that is readily absorbed by aquatic organisms. The bioaccumulation of mercury in the food chain results in contamination of fish and other aquatic life, posing health risks to humans who consume them [15,16]. Moreover, mercury exposure adversely affects public health, particularly among miners and nearby communities who are exposed through water, air, and food. These effects include neurological disorders, damage to internal organs, and increased health risks for pregnant women and children. Additionally, mercury pollution in the

Kahayan River leads to a decline in water quality, negatively impacting aquatic organisms and contributing to biodiversity loss due to heavy metal contamination [17].

Heavy metal contamination in aquatic environments can have severe consequences for ecosystems and living organisms. Therefore, various strategies are required to mitigate and address this pollution, including both natural approaches and technological waste treatment methods. One such approach is bioremediation [18]. Which involves the use of microorganisms—such as bacteria, fungi, or plants—to degrade, transform, or remove pollutants, including heavy metals, hydrocarbons, and hazardous chemicals [19]. Compared to conventional methods, bioremediation is considered more environmentally friendly because it minimizes additional negative impacts. In this context, the present study focuses on isolating bacteria capable of reducing mercury (Hg) contamination, with the potential for application in similar future studies [20,21].

Material and Method

The equipment utilized in this study included microscopes, beakers, Erlenmeyer flasks, graduated cylinders, volumetric pipettes, analytical balances, vernier calipers, dropper pipettes, incubators, autoclaves, shakers, glass stirring rods, spreaders (hockey sticks), forceps, microscope slides, sample bottles, inoculating loops, spirit lamps, laminar air flow (LAF) cabinets, test tubes, test tube racks, Petri dishes, and an Atomic Absorption Spectrophotometer (AAS).

The materials used consisted of soil samples contaminated with heavy metals collected from the Kahayan River gold mining area, aluminum foil, cling wrap, sterile distilled water, 70% alcohol, H_2SO_4 , $\text{K}_2\text{Cr}_2\text{O}_7$, HNO_3 , HClO_4 , NaCl, 25% HCl, Nutrient Agar (NA), Nutrient Broth (NB), HgCl_2 , crystal violet, Gram's iodine (mordant), safranin, ethanol, and peptone water.

Sterilization of Tools and Mediums

All equipment was sterilized prior to use. Glassware and metal instruments were sterilized in an oven at 180°C for 2 hours. In contrast, plastic materials and heat-sensitive items were sterilized using an autoclave at 121°C under a pressure of 2 atm for 15 minutes. The inoculating loop (ose) was sterilized by flaming it over a spirit lamp. Media sterilization was performed using the moist heat method in an autoclave at 121°C and 2 atm pressure for 15 minutes.

Sampling

The samples were obtained from soil collected in the gold mining area along the Kahayan River, Central Kalimantan, based on the assumption that the soil contains high levels of heavy metals, particularly mercury (Hg). Sampling was conducted using a simple technique by directly collecting soil with a spoon and placing it into a sterile glass container. The samples were then transported to the laboratory for isolation procedures and stored at 37°C or at room temperature.

Isolation and Selection of Lead-Reducing Bacteria

The bacterial isolation procedure began by weighing 5 g of the sample and mixing it with 45 mL of distilled water until a homogeneous suspension was obtained, corresponding to a 10^{-1} dilution. Serial dilutions were then performed stepwise from 10^{-1} to 10^{-6} . Subsequently, 0.1 mL from each dilution level (10^{-1} to 10^{-6}) was pipetted and inoculated onto the surface of Nutrient Agar (NA) medium supplemented with 0.2 mg Hg (2 ppm) using the pour plate technique. The inoculum was evenly spread using a sterilized spreader (hockey stick), previously disinfected with 70% alcohol and flamed. The inoculated plates were then incubated in an inverted position at 37°C for approximately 72 hours until bacterial colonies developed.

Individual colonies that appeared were subsequently purified using the streak plate technique. A single bacterial colony was aseptically picked with an inoculating loop and streaked onto the surface of Nutrient Agar (NA)

medium, followed by incubation at 37°C for 48 hours. This subculturing process was repeated on fresh NA medium until a pure culture was obtained. The selection of mercury-resistant bacteria was based on colony growth observations. Bacteria that were able to survive and proliferate on NA medium containing mercury were considered to possess the capability to reduce the metal. This is because only specific bacteria can tolerate and grow in mercury-contaminated environments.

Procedure of AAS

Sample preparation begins with careful digestion to prevent Hg volatilization. Solid samples (e.g., sediment or biological tissue) are weighed (approximately 0.2–0.5 g) and then digested using concentrated HNO_3 or a mixture of HNO_3 – H_2SO_4 under closed heating (microwave digestion is recommended to minimize Hg loss). Oxidizing agents such as KMnO_4 or $\text{K}_2\text{Cr}_2\text{O}_7$ are often added to stabilize the Hg as Hg^{2+} during the process. After digestion is complete and the solution is clear, the excess oxidizer is neutralized (e.g., with hydroxylamine hydrochloride), and the solution is then diluted to a specified volume using metal-free distilled water. For liquid samples, acidification (HNO_3 to pH <2) and mild oxidation, if necessary, are sufficient. All containers must be made of inert materials (e.g., borosilicate glass or Teflon) and acid-washed to avoid contamination.

Calibration curves are prepared using a certified Hg standard solution (e.g., 1000 mg/L), which is diluted into a series of low-concentration standards, such as 0; 1; 2; 5; 10; and up to 20 $\mu\text{g/L}$ (ppb), as Hg is typically analyzed at trace levels. The standards should be prepared in the same matrix (containing the same acid and oxidizing agent as the sample). In CV-AAS, each standard is reacted with a reducing agent such as SnCl_2 or NaBH_4 to produce Hg^0 vapor, whose absorbance is then measured. The calibration curve is constructed by plotting absorbance against concentration and should exhibit high linearity with an $R^2 \geq 0.995$. Verification is performed by periodically measuring the middle standard to ensure there is no instrument drift.

Determining the limit of detection (LOD) and limit of quantitation (LOQ) is crucial in Hg analysis due to its low concentrations. The LOD is calculated as 3 times the standard deviation of the blank measurement (at least 10 times) divided by the slope of the curve ($LOD = 3\sigma/m$), while the $LOQ = 10\sigma/m$. In the CV-AAS method, the LOD for Hg is typically in the sub-ppb ($\mu\text{g/L}$) range, depending on instrument sensitivity and operational conditions. It is important to ensure that all sample results are above the LOQ for quantitative reporting.

During the measurement, instrument parameters must be set specifically for Hg, such as a wavelength of approximately 253.7 nm and the use of a cold vapor system with a carrier gas flow (usually air or argon). The reduction reaction of Hg^{2+} to Hg^0 must proceed consistently, so reagent flow rates and reaction times need to be controlled. Each sample is measured in at least duplicate, accompanied by a blank, a spike (standard added to the sample), and, if possible, a certified reference material (CRM) to evaluate accuracy and recovery (generally accepted at 80–120%).

Bacterial Characterization

a) Macroscopic Examination of Colony Shape

Macroscopic observation of colonies is performed by evaluating several characteristics, including colony shape (such as punctiform, irregular, filamentous, or rhizoid), elevation (flat, raised, or convex), optical properties (colored, opaque, translucent, or transparent), and surface texture (smooth or rough).

b) Microscopic Examination of Gram Staining

The prepared bacterial isolates were spread onto a glass slide, followed by the application of crystal violet, which was left for 1 minute before rinsing with running water. Next, Gram's iodine mordant solution (Merck) was added and allowed to stand for 1 minute, then rinsed again. The slides were then treated with 95% ethanol for 30 seconds and washed once more with running water. In the final step, safranin was applied and left for 1 minute, after which the slide was rinsed, dried, and examined under a microscope. Gram staining results indicated that Gram-positive bacteria appeared

purple, while Gram-negative bacteria appeared red.

Bacterial Resistance Test to Mercury (Hg)

After obtaining pure isolates, the bacteria were inoculated into liquid Nutrient Broth (NB) medium supplemented with mercury (HgCl_2) at concentrations of 0, 50, 100, 150, 200, and 250 ppm, with each treatment performed in triplicate. The cultures were then incubated in a shaker incubator at 220 rpm for 72 hours to evaluate bacterial resistance to heavy metals. Following incubation, the Optical Density (OD) was measured using a spectrophotometer at a wavelength of 580 nm to determine cell density. Higher OD values at increasing mercury concentrations indicate greater bacterial growth and higher resistance to Hg [22]

Subsequently, OD measurements for each treatment were used to assess bacterial density. Isolates that demonstrated the ability to tolerate high mercury concentrations, as indicated by their density, were selected for further analysis in the mercury (Hg) reduction assay.

Result and discussion

Isolation and Purification of Mercury (Hg) Reducing Bacteria

This study commenced with bacterial isolation using the pour plate method, followed by the streak plate technique. The isolation process began with serial dilution of the sediment sample to reduce microbial density, allowing individual colonies to be more clearly observed. A 1 mL sample was then inoculated into sterile medium and poured into a Petri dish. The medium was sterilized in an autoclave at 121°C to eliminate any existing microorganisms and prevent contamination. After inoculation, the plates were incubated for two days to provide optimal conditions for bacterial growth, as shown in Figure 1 below.

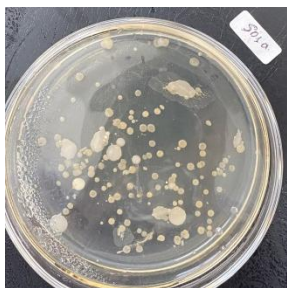


Figure 1. Bacterial growth after the inoculation process

Colonies that grow separately or are the largest in size are then transferred to NA media that has been added with 10 ppm mercury [23]. This medium served as a general growth medium to obtain pure bacterial colonies [24]. The isolation process from the gold mining area of the Kahayan River yielded 108 isolates with diverse colony morphologies. Successfully grown isolates were identified in Petri dishes labeled A1 to A108, each containing a single colony. The results of bacterial colony purification in this study are shown in Figure 2.

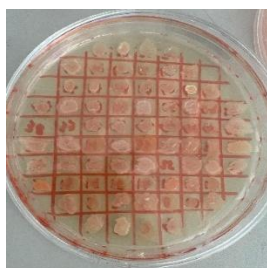


Figure 2. Isolates grown in a single colony on a petri dish

From the 108 isolates obtained, bacterial resistance was further evaluated using varying mercury concentrations ranging from 10 ppm to 250 ppm in liquid Nutrient Broth media. The results showed that only two isolates were able to survive and grow at the highest concentration of 250 ppm, namely isolates coded 34 and 108. Bacterial growth in the liquid medium was indicated by a change in color from clear yellow to turbid yellow (Figure 3).



Figure 3. Bacterial Growth In Nutrient Broth Media

Bacterial Characterization

The two isolates were then observed macroscopically for several parameters [25].

Macroscopic Observation of Bacterial Colony Morphology

The morphology of bacterial colonies can be determined through macroscopic observation of several characteristics, including colony shape, surface texture, margin, and color. The results of these observations in this study are summarized in Table 1.

Table 1. Morphological Characteristics of Bacterial Colonies Isolated from the Kahayan River Gold Mining Area

Isolate Code	Colony Morphology			
	Shape	Surface	Edge	Color
34	Circular	Convex	Flat	Yellowish
108	Irregular	Slightly convex	Choppy	White

Previous studies [26] have shown that colonies with a circular shape, convex elevation, smooth margins, and a yellowish coloration are typically classified as Gram-negative bacteria. In contrast, other research indicates that colonies that are white, filamentous or spindle-shaped, slightly convex, thin in appearance, and have smooth edges are characteristic of Gram-positive bacteria. Based on these criteria, isolate 34 is likely to belong to the Gram-negative group, while isolate 108 is classified as Gram-positive [27] as illustrated in Figure 4.

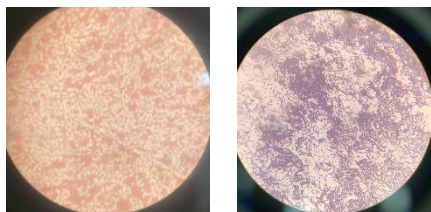


Figure 4. The results of the staining of the bacteria are code 34 (gram negative) and code 108 (gram positive).

Larger colonies are often associated with Gram-positive bacteria, which may be related to the structure of their cell walls. Gram-positive bacteria possess thick cell walls, a single cell membrane, and lack an outer membrane. In contrast, Gram-negative bacteria have thinner cell walls that are situated between two membrane layers [27].

Bacterial Resistance Test to Mercury (Hg)

The bacterial resistance test was performed to evaluate the ability of the isolates to survive in media containing mercury (Hg). Bacterial survival was assessed based on the absorbance (OD) values measured using a spectrophotometer at a wavelength of 580 nm. The results of the resistance test with the addition of mercury (HgCl_2) are presented in Figure 5.

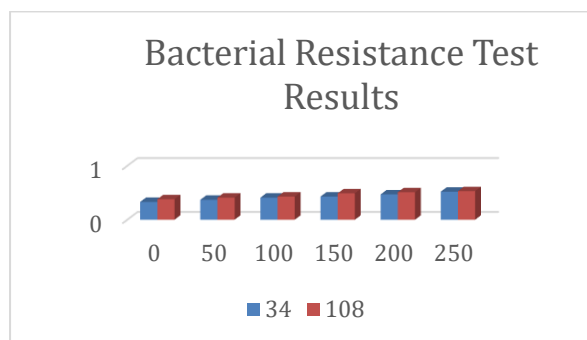


Figure 5. Results of bacterial resistance tests from the Sungai Kahayan Gold Mining Area to Hg Based on OD Wavelength 580 nm.

The results of the resistance test indicated that both isolates exhibited different

OD values. Variations in OD at each mercury concentration reflect differences in bacterial cell density. In this study, at 0 ppm Hg (control), isolate 108 showed the highest OD value, whereas isolate 34 had a lower OD compared to isolate 108.

According to [28], isolates that are capable of surviving and growing in synthetic media containing ≥ 5 ppm of heavy metals are classified as highly resistant. Bacterial resistance to heavy metals in growth media is associated with their ability to accumulate metals through two primary mechanisms: active uptake and passive uptake. One form of passive uptake is biosorption, which involves the binding of metal ions to the surface of non-living bacterial cells. The bacterial cell surface carries a negative charge due to the presence of various anionic groups, while heavy metals are positively charged, enabling electrostatic interactions and binding between them.

Isolates that can survive and proliferate in synthetic media containing ≥ 5 ppm of heavy metals are considered highly resistant. This resistance is linked to the ability of bacteria to accumulate metals through two main pathways: active uptake and passive uptake. Biosorption represents a passive mechanism, in which metal ions adhere to the surface of non-living bacterial cells. The cell surface is negatively charged due to the presence of various anionic groups, while heavy metals carry positive charges, allowing electrostatic attraction and binding between them.

Lead Level Reduction and Measurement Test by Bacteria

The mechanism of mercury (Hg) resistance contributes to the reduction of heavy metal pollutants, particularly mercury, in the environment. This indicates that bacteria capable of surviving in media containing heavy metals also possess the ability to decrease metal concentrations. In this study, the evaluation of bacterial capacity to reduce Hg levels was conducted at the highest tested concentration, 250 ppm, as presented in Table 2.

Table 2. Ability of Bacteria to Reduce Hg Levels Using the AAS Method

Bacteria Initial code	Hg Level (ppm)	Hg Level Accumulated by Bacterial Cells (ppm)	Final Hg Level (ppm)	Percentage Decrease (%)
Negative Control (No Bacteria)	250	-	250	0%
34	250	132,73	117,27	53,09 %
108	250	139,68	110,32	55,87 %

The negative control in this study was a mercury (Hg) solution without the addition of bacterial biological agents. The purpose of using a negative control in this study was to ensure that the changes in Hg concentrations occurring in the main treatment were truly caused by bacterial biological activity, and not by other factors. In a closed system and without reducing agents or microorganisms, mercury in solution generally exists in a relatively stable ionic form [29]. Therefore, the Hg concentration in the negative control tended to remain constant or did not experience significant changes (stable) throughout the observation period. This stability occurred because there were no processes that could alter the chemical form or distribution of Hg, such as the reduction of Hg^{2+} to Hg^0 , the enzymatic activity of microorganisms, or other reactive chemical reactions [24].

Table 2 presents the results of the mercury (Hg) reduction test, in which each isolate was exposed to the same initial Hg concentration of 250 ppm in the growth medium. After an incubation period of 4×24 hours, differences were observed in the amount of Hg reduced by each isolate. The incubation duration was maintained for approximately 4×24 hours to allow the bacterial cells to reach their optimal growth phase, thereby maximizing the activity of enzymes involved in mercury reduction. During the exponential to early stationary phase, the production of enzymes such as mercury reductase increases, which plays a role in the redox reaction of converting Hg^{2+} to Hg^0 . This time also allows for effective

expression of mercury resistance genes. Therefore, this incubation period was chosen to achieve stable and reproducible reduction efficiency, although the optimal value may vary depending on culture conditions and bacterial species [13]. This Hg reduction was analyzed using an AAS tool to determine the level of absorbed metal.

Based on the study results, isolate 108 demonstrated the highest capacity for mercury (Hg) reduction. This is indicated by its ability to accumulate 139.68 ppm of Hg within its cells, reducing the Hg concentration in the medium to 110.32 ppm, with a removal efficiency of 55.87%. The next isolate showing significant Hg reduction was isolate 34, which accumulated 132.73 ppm of Hg, lowering the Hg concentration in the medium to 117.27 ppm, with a reduction efficiency of 53.09%.

According to [30] variations in the Hg reduction capability among bacterial isolates may be attributed to differences in their metabolic processes. Bacterial metabolism plays a crucial role in determining tolerance or resistance to heavy metals such as Hg. Many metal-tolerant bacteria employ efflux mechanisms to expel toxic metals from the cell as a protective strategy against their harmful effects.

Bacteria are able to reduce mercury (Hg) through a biotransformation process, namely the change in the chemical form of mercury due to enzymatic activity within the cell. This mechanism is generally controlled by a genetic system known as the mer operon, which allows bacteria to survive in environments contaminated with heavy metals. In this process, highly toxic inorganic mercury ions (Hg^{2+}) will enter the bacterial cell, then be reduced to elemental mercury (Hg^0) by the mercury reductase enzyme encoded by the *merA* gene. This reduction reaction involves electron donors such as NADPH, so that Hg^{2+} changes to Hg^0 which is volatile and less soluble in air. The formed elemental mercury will then diffuse out of the cell and move into the gas phase, thereby causing a decrease in mercury concentration in the environment. In addition,

in organomercury compounds such as methylmercury (CH_3Hg^+), bacteria first decompose it into Hg^{2+} through the action of the organomercurial lyase enzyme encoded by the merB gene, before finally being further reduced to Hg^0 . Overall, this biotransformation process is an important detoxification mechanism for bacteria, because it converts the highly toxic form of mercury into a relatively less toxic form that is easier to remove from the cellular system [28].

Conclusion

Based on the findings of this study, it can be concluded that bacterial isolation from samples collected in the gold mining area of the Kahayan River, Central Kalimantan, yielded two isolates capable of surviving in mercury (Hg) concentrations up to 250 ppm, namely isolates 34 and 108. Identification results indicated that isolate 34 belongs to the Gram-negative group, whereas isolate 108 is classified as Gram-positive. These bacteria show potential as bioremediation agents due to their ability to reduce Hg levels. Isolate 34 achieved a mercury reduction of 53.09%, while isolate 108 demonstrated a slightly higher reduction efficiency of 55.87%.

Author Contribution

Material preparation, data collection, and analysis were performed by Chucita and acted as the corresponding author. Zahrotun Nafisah was responsible for designing the methodology. Zimon Pereiz and Efriyana Oksal contributed to data analysis. Miranti Maya Sylvani and Ahmad Irawan assisted in developing the conceptual framework of the manuscript. Febrianto Alfic contributed by reviewing and improving the English translation. All authors read and approved the final version of the manuscript. All authors read and approved the final manuscript.

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