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Isolation Of Bacteria With Potential As Mercury (Hg) Bioremediation From Kahayan River

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Abstract. Kayahan River, located in Central Kalimantan, is one of the rivers used for traditional gold mining with amalgamation techniques in its processing. The use of this technique has the potential to pollute and damage the environment. This study aims to analyze the ability of bacteria as mercury (Hg) bioremediation agents at various concentrations. The methods applied include inoculation of sediment samples into Zobell 2216 E media, purification of bacteria growing on NA media, and identification of bacteria and testing of Hg degradation capabilities using Atomic Absorption Spectrophotometer (AAS). The results showed that isolate 34 was able to accumulate 132.73 ppm of Hg from the initial 250 ppm Hg in the media (53.09% reduction), while isolate 108 was able to accumulate 139.68 ppm (55.87% reduction).

Keywords : amalgamation, AAS, Kahayan River, gold mine.

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Introduction

Indonesia has abundant natural resources, both biological and non-biological. One of the abundant non-biological natural resources in Indonesia is mineral resources. Minerals as part of non-biological resources can be gold, coal, silver, tin, and others [1]. Indonesia has an important role in the industrial sector as part of the national economy that contributes to increasing state revenues, developing the industrial sector, and creating new business opportunities that support equal distribution of community welfare. However, on the other hand, the mining industry often has negative impacts, such as environmental pollution and violations of the economic, social, and cultural rights of communities living around 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. Other impacts include sediment deposition and pollution by metal waste and hazardous materials [3]. Soil and water contamination can occur due to various factors, including industrial waste, mining waste, fertilizer and pesticide residues, and chemical weapons installation residues [4][5].

One of the significant impacts of industrial activities on the environment is pollution caused by heavy metal compound. Areas around mining can be the main source of heavy metal distribution [6]. Several industrial sectors that have the potential to produce large amounts of heavy metals include the machinery, metallurgy, metal plating, paint, leather processing industries and gold mine. Several types of heavy metals and toxic compounds that are often found in industrial wastewater include chromium (Cr), nickel (Ni), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cadmium (Cd), silver (Ag), lead (Pb), and 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 [7][8].

Mercury (Hg) can be found in nature in the form of cinnabar mineral (HgS) and is often used in gold mining and several chemical industry processes. In water, mercury can turn into toxic methylmercury and accumulate in the food chain. The impact of heavy metal mercury (Hg) pollutes water and soil, disrupts aquatic ecosystems, and causes accumulation in plants and animals [9]. Mercury (Hg) also has a negative impact on living things, including damaging the nervous system, disrupting fetal development, causing damage to internal organs, and causing respiratory and skin disorders. Mercury exposure poses a major risk to human health and ecosystem balance, so waste management must be carried out properly [10][11].

Kahayan River in Central Kalimantan is one of the main locations for traditional gold mining that uses a lot of mercury (Hg) in its processing. The technique commonly used is amalgamation, a method that uses mercury to bind gold particles from mining materials. The use of mercury has serious impacts on the environment and health [12]. Mercury waste that is not bound to gold is often dumped directly into rivers, causing water and sediment pollution, and can seep into the soil around the river flow, disrupting terrestrial ecosystems. In addition, mercury in water can turn into methylmercury, a more toxic compound that is easily absorbed by aquatic organisms. The accumulation of mercury in the food chain causes fish and river biota to be contaminated, which then poses a risk to humans who consume it [13][14]. Mercury exposure also has negative impacts on public health, especially for miners and local residents who are exposed through water, air, and food. The impacts can include nerve disorders, damage to internal organs, and health risks for pregnant women and children. In addition, mercury pollution in the Kahayan River causes a decrease in water quality which has an impact on the lives of fish and other organisms, and results in the loss of biodiversity due to heavy metal pollution [15].

Heavy metal pollution in waters can cause serious impacts on the environment and living things. Therefore, various efforts need to be made to reduce and overcome this pollution, both through natural methods and waste processing technology. One method that can be done is the bioremediation method [16]. Bioremediation is the process of restoring a polluted environment by using microorganisms, such as bacteria, fungi, or plants, to break down or remove pollutants, including heavy metals, oils, and hazardous chemicals[17]. This method is more environmentally friendly than conventional techniques because it does not cause additional negative impacts. In relation to this, research was conducted on the isolation of bacteria

that can be used to reduce the impact of mercury (Hg) pollution and have the potential to be used in others similar research [18][19].

Experimental

The tools used in this study were microscopes, beakers, Erlenmeyer flasks, measuring cylinders, volume pipettes, analytical scales, vernier calipers, dropper pipettes, incubators, autoclaves, shakers, stirring rods, hockey sticks, tweezers, slides, sample bottles, straight loops, spirit lamps, laminary air flow (LAF), test tubes, petri dish tube racks, and AAS (Atomic Absorption Spectrophotometer).

The materials used in this study were soil samples contaminated with heavy metal waste from the Kahayan River gold mining area, aluminium foil, cling warp, sterile distilled water, 70% alcohol, H_2SO_4, $K_2Cr_2O_7$, HNO_3, $HClO_4$, NaCl, HCl 25%, Agar (NA), Nutrient Broth (NB), (HgCl2), Crystal violet, Gram's iodine mordant, safranin, ethyl alcohol, water peptone.

Sterilization of Tools and Mediums

All equipment used is sterilized first. Glass and metal equipment is sterilized in an oven at 180°C for 2 hours. Meanwhile, plastic equipment and those that cannot withstand high temperatures are sterilized using an autoclave at 121°C with a pressure of 2 atm for 15 minutes. Ose is sterilized by heating it over a flame from a spirit lamp. Sterilization of the medium is carried out using the moist heat method using an autoclave at 121°C with a pressure of 2 atm for 15 minutes.

Sampling

The samples used came from soil in the gold mining area in the Kahayan River, Central Kalimantan, on the grounds that the soil has a high content of heavy metals, especially mercury (Hg). Sampling was carried out using a simple method, namely by taking the soil directly using a spoon, then putting it in a sterile glass jar. After that, the sample was taken to the laboratory for the isolation process and stored at 37°C or room temperature.

Isolation and Selection of Lead-Reducing Bacteria

The bacterial isolation process begins by weighing 5 grams of sample and mixing it with 45 ml of distilled water until homogeneous, resulting in a dilution of 10^{-1}. The culture is then diluted gradually from 10^{-1} to 10^{-6}. Next, the plating process is carried out, where 0.1 ml of culture from each dilution level (10^{-1} to 10^{-6}) is pipetted and inoculated onto the surface of Nutrient Agar (NA) media that has been added with 0.2 mg Hg 2 ppm using the pour plate method. After that, the sample is leveled with a hockey stick that has been sterilized using 70% alcohol and a Bunsen flame. The inoculated media is then incubated in an inverted position at 37°C for ±72 hours until the bacterial colonies grow.

The single colony that appears is then purified using the streak plate method. One colony of bacterial isolate is taken aseptically using an ose needle and inoculated onto the surface of the NA medium, then incubated at 37°C for 48 hours. The process of transferring the colony to a new NA medium is repeated until a pure culture is obtained. Selection of bacteria capable of reducing mercury is carried out by observing the growth of the colony. Bacteria that can survive and grow on NA media containing mercury are considered to have the ability to reduce the metal. This is due to the fact that only certain bacteria are able to survive in an environment containing mercury.

Bacterial Characterization

a) Macroscopic Examination of Colony Shape

Macroscopic examination of colonies is carried out by assessing several characteristics, such as colony shape (punctiform, irregular, filamentous, or rhizoid), elevation (flat, protruding, or convex), optical characteristics (colored, opaque, translucent, or transparent), and surface texture (smooth or rough).

b) Microscopic Examination of Gram Staining

The prepared bacterial isolates were smeared on a glass slide, then dripped with crystal violet and left for 1 minute before being rinsed with running water. After that, Gram's iodine mordant solution (Emerck) was added and left for 1 minute, then washed again with running water. Furthermore, the bacterial isolates were given 95% ethyl alcohol for 30 seconds, then rinsed again with running water. The final stage, safranin was dripped and left for 1 minute before being washed again with running water, dried, and observed under a microscope. The results of the Gram test showed a purple color for Gram-positive bacteria and red for Gram-negative bacteria.

Bacterial Resistance Test to Mercury (Hg)

After the pure isolate was obtained, the bacteria were inoculated into a liquid Nutrient Broth (NB) medium enriched with Mercury ($HgCl_2$) at concentrations of 0, 50, 100, 150, 200, and 250 ppm. Each treatment was carried out in three replications. Furthermore, the culture was incubated in a shaker incubator at a speed of 220 rpm for 72 hours to observe the resistance of the bacteria to heavy metals. After incubation, the Optical Density (OD) value was measured using a spectrophotometer at a wavelength of 600 nm to determine the level of bacterial density. The higher the OD value at various concentrations, the denser the bacterial population and the greater its resistance to Hg [20].

Each bacterial treatment was then measured for its Optical Density (OD) value at a wavelength of 600 nm using a spectrophotometer to determine its density level. After the isolates that were able to withstand heavy metals were identified based on the density level, the study continued to the Hg level reduction test stage.

Result and Discussion

Isolation and Purification of Mercury (Hg) Reducing Bacteria

This study began with the stage of bacterial isolation using the pour agar method, which was then continued with the scratch plate method. The isolation process began with the dilution of the sediment sample, which aims to reduce the number of microbes in the solution so that the colonies formed can be observed more clearly. A sample of 1 mL was inoculated into sterile media, then poured into a petri dish. Media sterilization was carried out using an autoclave at a temperature of 121°C to kill any microbes that might be present, thus preventing contamination. After the inoculation process, the media was incubated for two days to create optimal conditions for bacterial growth as in **Figure 1** below.

Colonies that grow separately or are the largest in size are then transferred to NA media that has been added with 10 ppm mercury [21]. This media functions as a general medium for bacterial growth until a pure colony is obtained [22]. The results of isolation from the Gold Mining Area in the Kahayan River produced 108 isolates with different morphological characteristics of the colonies. Isolates that successfully grew were found in petri dishes coded A1 to A 108, each with one colony. The results of bacterial colony purification in this study are presented in **Figure 2.**

From the 108 isolates, the resistance of the bacteria was then tested with variations in mercury concentration from 10 ppm to 250 ppm in liquid media using nutrient broth. From this test, 2 isolates were produced that survived growth at a mercury concentration of 250 ppm. The two isolates are codes 34 and 108. Bacterial growth in liquid media is characterized by a change in color from clear yellow to cloudy yellow (**Figure 3**).

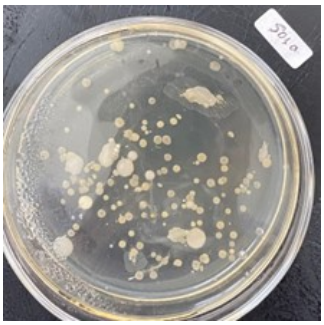

Figure 1. Bacterial growth after the inoculation process

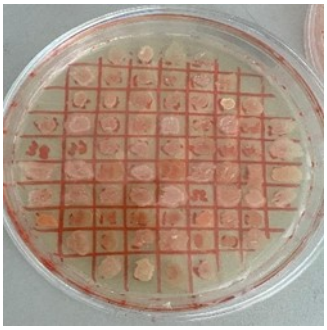

Figure 2. Isolates grown in a single colony on a petri dish

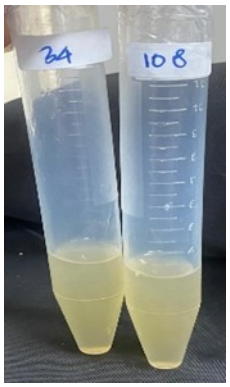

Figure 3. Bacterial Growth In Nutrient Broth Media

Bacterial Characterization

The two isolates were then observed macroscopically for several parameters [23].

Macroscopic Observation of Bacterial Colony Morphology

The morphology of bacterial colonies can be known by observing several parameters macroscopically. The parameters observed include the shape, surface, edge, and color of the colony. The results of observations regarding the morphology of bacterial colonies in this study are presented 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

According to previous research [24] showed that colonies that are round (circular), have a convex surface, flat edges, and are yellowish in color are categorized as Gram-negative bacteria. Meanwhile, research conducted revealed that colonies that are white with a filamentous and spindle-shaped shape, a slightly convex surface, appear thin, and have smooth edges are included in the Gram-positive bacteria group. Based on this information, it can be concluded that isolate 34 is most likely classified as Gram-negative, while isolate 108 is included in the Gram-positive group [25].

Large colonies are likely to come from the Gram-positive bacteria group, which can be associated with the structure of their cell walls. Gram-positive bacteria have thick cell walls, single cell membranes, and do not have an outer membrane. In contrast, Gram-negative bacteria have thinner cell walls, which are located between two layers of cell membranes [25].

Bacterial Resistance Test to Mercury (Hg)

Bacterial resistance test was conducted to determine its survival in media containing Hg. The survival of the bacteria can be seen based on the absorbance value (OD) on a spectrophotometer with a wavelength of 580 nm. The results of the bacterial resistance test added with mercury ($HgCl_2$) can be seen in **Figure 4.**

The results of the resistance test showed that both isolates had varying OD values. The difference in OD values at each concentration reflects the difference in bacterial cell density levels. In this study, at 0 ppm Hg used as a control, isolate 108 had the highest OD value, while the OD value of isolate 34 was lower than that of isolate 108.

According to [26], isolates that are able to survive and grow on synthetic media with heavy metal content ≥ 5 ppm are categorized as isolates with high levels of resistance to heavy metals. The resistance of bacteria to heavy metals in growth media is due to their ability to accumulate metals

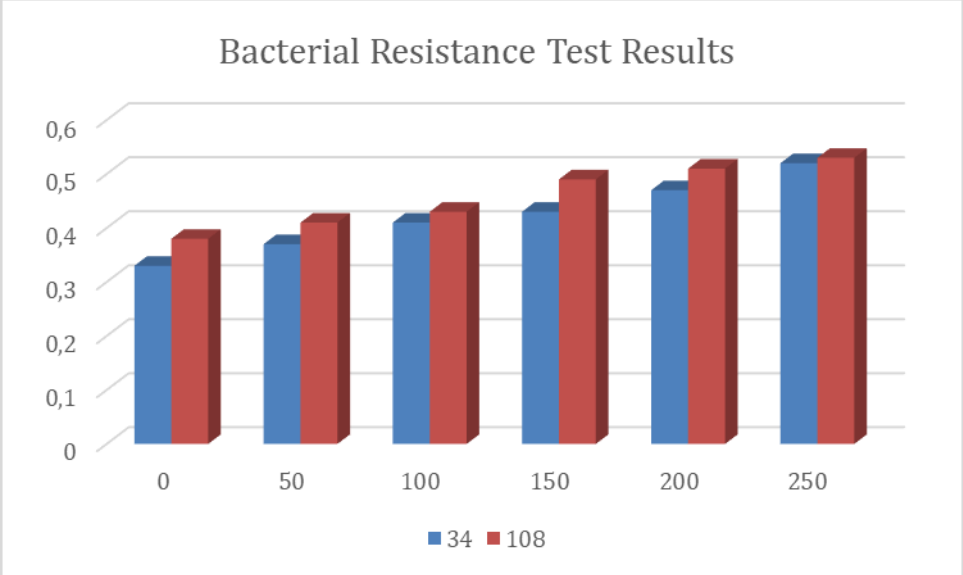

Figure 4. Results of bacterial resistance tests from the Sungai Kahayan Gold Mining Area to Hg Based on OD Wavelength 580 nm

through two main mechanisms, namely active uptake and passive uptake. Bioaccumulation is an example of active uptake, where this process involves the metabolism of living cells for growth or accumulation of metals intracellularly. Meanwhile, biosorption is an example of passive uptake, namely the process of metal absorption that occurs due to the interaction between metal ions and the surface of dead bacterial cells. The surface of bacterial cells is negatively charged because it is composed of various anion structures, while heavy metals have positively charged ions, allowing bonds to form between the two.

Both bacterial isolates given Hg at concentrations of 50, 100, 150, 200, and 250 ppm showed varying changes in OD values. Previous research by Zumaidar et al. (2022) revealed that the higher the concentration of Hg given, the lower the OD value obtained. This is thought to occur because heavy metals in the media inhibit bacterial growth, thus affecting the cell metabolism process and causing a decrease in cell density. However, in this study, the results obtained for isolates 108 and 34 actually showed a pattern that was opposite to previous studies. This is because these unidentified bacteria are able to survive in high Hg concentrations and can still adapt, where the biosorption ability of bacteria is also influenced by their type. In addition, these results also show that these bacteria have a longer growth transfer phase, so that during this observation period they have not entered the death phase or decreased cell density, which causes the graph to continue to show bacterial resistance to Hg metal.

Lead Level Reduction and Measurement Test by Bacteria

The mechanism of resistance to Hg plays a role in reducing the levels of heavy metal pollutants, especially mercury in the environment. This proves that bacteria that are able to survive (resistant) in media containing heavy metals also have the ability to reduce the levels of these metals. In this study, the analysis of the ability of bacteria to reduce Hg levels was carried out using the highest concentration, namely 250 ppm, as shown in **Table 2**.

Table 2 shows the results of the Hg reduction test by bacteria, where each isolate was given the same Hg level, which was 250 ppm in its growth medium. After going through an incubation process for 4x24 hours, there was a difference in the amount of Hg that was successfully accumulated by each isolate. This Hg accumulation was analyzed using an AAS tool to determine the level of absorbed metal.

Based on the results of the study, isolate 108 showed 132.73 the highest ability to reduce Hg levels. This can be seen from the amount of Hg accumulated in its cells of 139.68 ppm, so that the Hg level in the media was reduced to 110.32 ppm with a reduction efficiency reaching 55.87%. The next bacterial isolate that showed a decrease in Hg levels was isolate 34, which was able to accumulate Hg by 132.73 ppm. This caused the Hg level in the media to decrease to 117.27 ppm, with a reduction efficiency reaching 53.09%. According to [28] the difference in the ability of bacterial isolates to reduce Hg levels can be caused by differences in the metabolism of each isolate. Bacterial metabolism plays an important role in the mechanism of tolerance or resistance to the heavy metal Hg. Most bacteria that are tolerant to heavy metals use the efflux mechanism to remove metals from the cell as a form of defense against their toxicity.

Conclusion

Based on the research results obtained, it can be concluded that bacterial isolation from samples taken from the Kahayan River gold mining area, Central Kalimantan produced 2 bacterial isolates that could live or had resistance in Hg content up to 250 ppm, which were coded isolates 34 and 108. From the identification results, isolate 34 was included in the Gram-negative group, while isolate 108 was classified as Gram-positive. The bacteria found have the potential as bioremediation agents

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

Bacteria Initial	Hg Level (ppm)	Hg Level Accumulated by Bacterial Cells (ppm)	Final Hg Level (ppm)	Percentage Decrease
34	250	132,73	117,27	53,09 %
108	250	139,68	110,32	55,87 %

because of their ability to reduce Hg heavy metal levels. Isolate 34 was able to reduce mercury levels by 53.09%, while isolate 108 reduced mercury levels by 53.09%.

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