Isolation and Characterization of Hg and Pb Reducing Bacteria in Several Contaminated Habitats

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Abstract

Wastes contaminated with heavy metals mercury (Hg) and lead (Pb) are hazardous and usually found in various habitats. Impacts of heavy metal Hg are more dangerous than Pb, not only in contamination of the environment but also destruction of organisms. One of the techniques to reduce the harmful effects of heavy metals is bioremediation. In this study, the bioremediation process was carried out using indigenous bacteria isolated from several contaminated habitats. Indigenous bacteria were obtained by taking soil sediments samples from brick factory, Bogor Botanical Gardens river sediment, and former gold mining side, Pongko and liquid samples from the river at Bogor Botanical Gardens. The best potential isolates of Hg (HGKRB5) and Pb (PBBTK1) reducing bacteria were obtained. HGKRB5 was isolated from the river at Bogor Botanical Gardens and PBBTK1 from the soil of the brick factory. The result of the bioassay test showed HGKRB5 was capable to remediate 10 ppm Hg contaminated water after 2 hours of inoculation. On the other hand, PBBTK1 was also able to remediate 10 ppm Pb contaminated water. In this study, the effectiveness of bioremediation HGKRB5 was 99% (mortality 67%) and PBBTK1 reached 100% (no mortality).

Keywords: *Bioremediation, Environment, Heavy Metals, Waste*

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I. INTRODUCTİON

Pollution by heavy metals is a major environmental problem worldwide due to their uncontrolled pollution levels caused by industrial growth and increased use of fossil fuel. Heavy metal contamination can also be caused by mining, metal smelting, industry, agricultural activities and domestic activities [1]. Heavy metals are classified as environmental pollutants because of their toxic effects on plants, animals and humans. Heavy metals are also persistent so they can accumulate in soil and plants. Metal contamination has harmful effects on biological systems because it cannot be degraded or destroyed. Toxic heavy metals such as Pb, Co, Cd and Hg can accumulate in living organisms, causing various diseases and disorders and even death. Heavy metals are one of the major threats to aquatic fauna, especially fish, which are one of the main sources of protein-rich food for humans [2].

Various methods have been developed to reduce heavy metal contamination in the environment, including bioremediation, which is a process of alleviating environmental pollution using plants and microbes, both intracellularly and extracellularly. Some of the principles used to remove heavy metals included biosorption, bioaccumulation, bioprecipitation, bioreduction, and bioleaching [3]. This research aims to isolate, characterize, and assess the bioremediation effectiveness of heavy metal reducing bacteria Hg (Mercury) and Pb (Lead) taken from different contaminated habitats.

II. MATERIALS AND METHODS

Isolation of Hg and Pb Reducing Bacteria

Hg and Pb reducing bacteria were isolated from three soil samples from brick factory soil (PBTK), Bogor Botanical Gardens river sediment (PKRB), former Pongkor mining soil (PPKR) and one liquid sample from BIOTROP liquid waste (CBIO). About 10g from each soil sample was dissolved in 90 ml of physiological solution in a 250 ml flask to make a 1: 10 dilution. The samples

were stirred using a shaker for 30 minutes to break up the soil. Then a series of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} dilutions were made. From each dilution, 0.1 ml was transferred to a petri dish filled with LB media (Luria-Bertani) plus 10 ppm $HgCl_2$ and PbCl2, pH 7.2-7.4. The solution was then spread evenly in a petri dish using a glass rod spreader using the spread plate method and incubated at 30 ± 1°C for 7 days, then a clear zone formed was observed. Bacterial colonies that can grow and form clear zones on the media were considered to have the ability to reduce Hg and Pb. The bacterial density at each location was calculated using the plate count method and characterized morphologically, physiologically and biochemically.

Characterization of Hg and Pb Reducing Bacteria Morphologically, Physiologically and Biochemically

Macroscopıc Observation

Morphological characterizations were carried out through macroscopic observations by describing their shapes, elevation, margin and colour of bacterial colonies growing on LB media mix with 10 ppm of $HgCl_2$ or $PbCl_2$.

Hypersensitivity Test

The pathogenicity reaction test was carried out by injecting inoculum bacterial into the tobacco leaves (at least 3 leaves). A 0.5 ml inoculum bacterial was injected into the leaves using a sterilized siren [4] and a hemolysis test was carried out by injecting bacterial inoculum into the blood agar. The bacterial isolates were incubated for 48 hours and then observed.

Gram Staining

Microscopic examination was carried out through a Gram staining test. The bacterial culture that had been grown in Nutrient Agar (NA) for 24 hours was collected using a loop and placed on a glass slide. Afterwards, it was dripped with violet dye for 20 seconds and then rinsed with water and then finally dripped

with iodine for 1 minute. After that, the samples were rinsed using 95% alcohol for 10-20 seconds until the medium became clear, and then rinsed with water to stop the reaction. The culture was dripped with safranin dye for 20 seconds and then rinsed with water. The bacterial morphology was observed under a microscope to describe the shape and colour of the cells [5].

Catalase Test

The catalase test was carried out to determine the nature of the bacteria that produce the catalase enzyme, which is used by bacteria for the decomposition of hydrogen peroxide (H_2O_2) into the air (H_2O) and oxygen (O_2). Hydrogen peroxide is formed during aerobic metabolism so that bacteria growing in an aerobic environment can decompose these toxic substances. One loop of bacterial colonies was taken aseptically, then inoculated onto a glass object. The inoculant slide was dripped with a sufficient amount of 3% H_2O_2 solution. The presence of catalase can be seen from the formation of air bubbles around the colony after the addition of 3% H_2O_2 solution [6].

Oxidase Test

The oxidase test was carried out to determine the nature of the bacteria that produce the oxidase enzyme, which was determined by the colour change of the oxidase paper. Colonies, which had been cultured for 24 hours, were collected using a loop under aseptic conditions. The samples were placed and rubbed with oxidase paper, and then the changes that occurred were observed. A positive reaction is indicated by a change within 1-20 seconds of the colour of the paper to blue [7].

Motility Test

A motility test was conducted to determine the ability of bacteria to move. The isolates of Hg and Pb reducing bacteria were inserted into semisolid Sulfide-İndole-Motility (SIM) media and then incubated for 24 hours and then observe the colony formation. A positive test indicates the presence of colony

distribution, suggesting that the bacteria is motile [6].

Bacteria Growth Test at Various pH

The Nutrient Broth (NB) media was conditioned to have a pH of 3, 5, 7, and 9. The NB media was sterilized. A total of 1 ml of liquid from the stock was placed into 9 ml NB and incubated for 24 hours. The observation was made on the turbidity that occurs in the medium after incubation [8].

Effectiveness Test of Hg and Pb Reducing Bacteria

A total of 5 ml inoculum of the isolate was mixed into 20 ml of LB media with concentrations of 10 ppm Hg or 10 ppm Pb. The standard solubility curve was made in the range of 0.1, 2, 3, 4, 5, 6, 7, 9, and 10 ppm for Pb. The inoculum was incubated for 1 week. Before measuring the Pb solubility, the inoculum was centrifuged for 10 minutes at 3500 rpm. After being centrifuged the samples were filtered using Whatman filter paper. The filtered pellet was discarded and the supernatant was collected for measurement of metal contents. The supernatant was filtered and prepared for testing using an AAS (Atomic Absorption Spectrophotometry). Meanwhile, Hg-reducing bacteria were measured at the Botanical Laboratory, LIPI Cibinong, Indonesia. The bioremediation's effectiveness of Hg and Pb reducing bacteria was calculated using the formula proposed by Imron (2019) [9].

Bioassay Test

The purpose of the bioassay test was to determine the effectiveness of Hg and Pb reducing bacteria to decrease the level of toxicity of Hg and Pb. The bioassay test was carried out by testing the isolates of Hg and Pb reducing bacteria on goldfish (*Cyprinus carpio*) in an aquarium measuring 10 cm x 10 cm x 15 cm, with an air volume of 700 ml. Each aquarium was given 3 fish with 3 replications. The ppm concentrations tested were 0, 1 and 10 ppm Hg or Pb. The treatments included: (1) Water + fish + 10 ppm Pb, (2) Water + fish + 10 ppm Hg, (3) Water + fish + 1 ppm Pb, (4)

Water + fish + 1 ppm Hg, (5) Water + fish + 10 ppm Hg + Isolate Hg, (6) Water + fish + 10 ppm Pb + Isolate Pb, (7) Water + fish + 1 ppm Hg + Isolate Hg, (8) Water + fish + 1 ppm Pb + Pb isolate, (9) Water + fish + Hg isolate, (10) Water + fish + Pb isolate, (11) Water + fish. In each treatment, the mortality data of the fish were recorded on 1, 2, 3, 4, 5, up to 24 hours after treatment. The data were processed using the SAS 9.0 program and analyzed for variance and further testing with the DMRT (Duncan Multiple Range Test) test at an alpha (α) 5% level. The test fish were kept as well as possible with adequate nutrition. Before the experiment, the test fish were placed in test containers for 1 day without being fed. Toxicity testing of Hg and Pb was carried out during an exposure time of 24 hours with observations in the form of recording fish mortality. The dead test fish were removed immediately to prevent contamination of the water.

III. RESULTS AND DISCUSSION

Results

Isolation, Characterization, and Effectiveness of Bioremediation of Isolated Hg and Pb Bacteria from Several Contaminated Habitats

The initial screening isolated 28 bacterial isolates, consisting of 11 isolates suspected as Hg reducing bacteria and 17 isolates as Pb reducing bacteria. These isolates were successfully grown at serial dilutions of 10^{-5}-10^{-10} from CBIO samples, suggesting that they were products of PBTK, PKRB, and PPKR samples at serial dilutions 10^{-9}-10^{-10}. Further screening through pathogenicity and hemolysis tests using tobacco leaves and blood agar resulted in the isolation of 17 pathogenic bacteria and 11 non-pathogenic bacteria. The non-pathogenic bacteria consisted of 2 bacteria that were thought to reduce Hg (HGKRB1 and HGKRB5) and 9 bacteria to reduce Pb (PBBTK1, PBBTK2, PBBTK3, PBBTK4, PBBTK5, PBBIO5, PBBIO7, PBKRB1, and PBPKR4). Results of morphological, physiological and biochemical characterization of bacteria are

shown in Tables 1 and 2.

Table 1. Results of Observation Morphological and Physiological Characterization of Non-Pathogenic Hg and Pb Reducing Bacteria

No.	Code of Isolate	Heavy Metal	Shape	Elevation	Margin	Surface	Color of Colony	Shape Cell	Type of Gram	Catalase (H_2O_2 3%)	Oxidase	Motility
1.	HGKRB1	Hg	Circular	Raised	Entire	Dull	White	Coccus	+	++	+	+
2.	HGKRB5	Hg	Irregular	Flat	Undulate	Smooth	White	Coccus	+	++	-	+
3.	PBPKR4	Pb	Irregular	Raised	Entire	Glistening	Light Orange	Coccus	+	++	-	+
4.	PBBTK1	Pb	Irregular	Flat	Entire	Dull	Yellow	Coccus	-	+++	-	+
5.	PBBTK2	Pb	Irregular	Flat	Undulate	Wrinkled	Red	Coccus	+	++	-	+
6.	PBBTK3	Pb	Irregular	Flat	Entire	Dull	Yellow	Coccus	+	++	-	+
7.	PBBTK4	Pb	Irregular	Flat	Entire	Dull	Yellow	Coccus	+	++	-	+
8.	PBBTK5	Pb	Irregular	Flat	Entire	Dull	Yellow	Coccus	+	+++	-	+
9.	PBKRB1	Pb	Irregular	Flat	Undulate	Smooth	White	Coccus	-	+++	+	+
10.	PBBIO5	Pb	Irregular	Flat	Entire	Dull	Yellow	Coccus	+	+	-	+
11.	PBBIO7	Pb	Irregular	Flat	Undulate	Dull	Dull White	Coccus	+	+	+	+

Biological Response Observation

Results of the Bioassay test (Table 3) revealed significant differences of mortality rates of golden fish (*Cyprinus carpio)* as affected by the different heavy metals (Hg and Pb) concentrations ($p < 0.05$). Bacterial isolate HGKRB5 was able to remediate 10 ppm Hg contaminated water after 2 hours of inoculation (G). On the other hand, PBBTK1 bacterial isolate was also able to remediate 10 ppm Pb contaminated water (H). In this study, the effectiveness of HGKRB5 bioremediation was 99% (67% mortality) and PBBTK1

reached 100% (no mortality). Treatment of Hg and Pb heavy metal reducing bacteria isolates at various concentrations (E, F, G, H) was able to reduce the number of deaths of *Cyprinus carpio* compared to treatment without bacterial isolates (A, B, C, D) (Table 3). The percentage value of the bioremediation effectiveness of the eleven isolates of Hg and Pb heavy metal reducing bacteria are shown in Table 2.

Table 2. Results of Observation Biochemical Characterization and Effectiveness Bioremediation Non Pathogenic Hg and Pb Reducing Bacteria

No.	Code of Isolate	Heavy Metal	Bacterial Growth Test at Various pH				Bacterial Growth Test At Various Concentrations of Heavy Metals Hg and Pb (ppm)			Bioremediation Effectiveness (%)
			3	5	7	9	50	250	1250	
1.	HGKRB1	Hg	-	-	++	+	-	-	-	98%
2.	HGKRB5	Hg	-	-	++	+	-	-	-	99%
3.	PBPKR4	Pb	+	-	-	-	+	-	-	99%
4.	PBBTK1	Pb	+	+	++	++	+	+	++	100%
5.	PBBTK2	Pb	-	-	+	+	+	+	-	98%
6.	PBBTK3	Pb	-	-	++	+++	+	+	+	97%
7.	PBBTK4	Pb	-	-	+	+	-	-	-	97%
8.	PBBTK5	Pb	++	++	++	++	-	-	-	99%
9.	PBKRB1	Pb	-	++	++	++	++	+	+	97%
10.	PBBIO5	Pb	-	+	+	+++	-	-	-	99%
11.	PBBIO7	Pb	++	++	+++	+++	-	-	-	99%

Table 3. Mortality of Golden Fish (*Cyprinus carpio*) for 24 Hours of Treatment

Code	Treatment	Concentration of Contaminants (ppm)	Number of Fish Deaths	Time of Fish Death (hours)	Mortality (%)
A	W+F+1 ppm Pb	1 ppm Pb	1^{c}	24	33
B	W+F+1 ppm Hg	1 ppm Hg	2^{b}	24	67
C	W+F+10 ppm Pb	10 ppm Pb	3^{a}	1	100
D	W+F+10 ppm Hg	10 ppm Hg	3^{a}	1	100
E	W+F+1 ppm	1 ppm Hg	0^{d}	0	0
F	W+F+1 ppm Pb+PBBTKI	1 ppm Pb	0^{d}	0	0
G	W+F+10 ppm	10 ppm Hg	2^{b}	2	67
H	W+F+10 ppm	10 ppm Pb	0^{d}	0	0
I	W+F+HGKRB5	0 ppm	0^{d}	0	0
J	W+F+PBBTKI	0 ppm	0^{d}	0	0
K	Control (W+F)	0 ppm	0^{d}	0	0

W=water, F=golden fish (*Cyprinus carpio*), HGKRB5= Hg reducing bacteria isolate, PBBTKI= Pb reducing bacterial isolate. *The number in one of the same columns followed by the same letter showed no significant difference in each factor, according to the Duncan Multiple Range Test with α =5%

Discussion

Isolation, Characterization, and Effectiveness of Hg and Pb Reducing Bacterial

Isolation of Hg and Pb heavy metal reducing bacteria from several contaminated habitats generated 28 isolates of bacteria, which are suspected to reduce Hg and Pb metals. However, based on the selection results of the pathogenicity test, 11 isolates of bacteria were not pathogenic and motile. Motility test observations showed a positive reaction indicated by the distribution of colonies when grown on Sulfide-İndole-Motility (SIM) semisolid media.

Colony distribution was observed after being incubated for 24 hours on SIM media. On the other hand, the results of Gram staining and observation of cell shape revealed that all 11 isolates had coccus cell shape, generally Gram (+). Most of the colony edges are flat with smooth surface. Mercury-resistant bacteria includes those from the Gram-positive group of *Staphylococcus* spp., *Streptococcus pyogenes*, and *Streptococcus pneumoniae*, which have a coccus cell shape [10]. The bioremediation agent for lead-resistant bacteria is known to have coccus form in both isolates B and C isolated from Lake Tempe, South Sulawesi, Indonesia [11].

Similarly, a catalase is an antioxidant enzyme that is capable to degrade hydrogen peroxide into water and oxygen [12]. In this study, isolates PBBTK1, PBBTK5, and PBKRB1 produced more catalase enzymes, which were detected by the formation of more bubbles produced from three isolates than others. Some pathogenic bacteria produce catalase to defend themselves against hydrogen peroxide attack, a weapon commonly used by the host immune system, in addition to oxidative stress.

In addition to the catalase test, an oxidase test was also carried out. Three bacterial isolates PBKRB1, PBBIO7, and HGKRB1 showed positive reactions on the oxidase test. The positive reaction was determined through the appearance of a purple color on the oxidase paper, indicating that there was a cytochrome oxidase

enzyme in the isolates when the test was carried out. Oxidase enzymes play an important role in the implementation of the electron transport system in an aerobic respiration [7].

Based on the results of biochemical characterization (Table 2), seven bacterial isolates PBBTK1, PBBTK2, PBBTK3, PBBTK5, PBKRB1, PBBIO5, and PBBIO7 had shown optimal growth at pH 7 and 9. Bacterial growth is strongly influenced by pH as it affects the function of the enzymatic system and the formation of energy in bacterial cells [13]. However, at low pH or in an acidic environment, Hg and Pb reducing bacteria have poor growth and low population. Data also suggest that the isolates of microbes degrade Hg and Pb belong to the group of alkaliphiles.

It was also observed that three isolates, PBBTK1, PBBTK3 and PBKRB1, were able to grow at various concentrations of Pb (i.e. 50 ppm, 250 ppm, and 1250 ppm). The high tolerance of these isolates to high level of Pb concentrations could be explained by their adaptation because the three isolates were suspected to come from soils containing high Pb and therefore able to develop genetic and physiological adaptability and able to degrade Pb. Differences in bacterial growth responses occur due to differences in the metabolism of microorganisms in response to the pollutants that contaminated the environment, which is carried out through bio-absorption and bioaccumulation mechanisms [14]. The PBBTKI isolates had the highest bioremediation effectiveness of 100%. This is the most resistant bacteria against Pb (Table 2), hence, the most effective Pb reduction ability compared with other Pb reducing isolates.

The HGKRB5 isolate, which samples were collected from Bogor Botanical Gardens River sediment, also showed 99 percent bioremediation effectiveness (Table 2), suggesting that this isolate has the most effective Hg reduction ability. Areas that are relatively close to rivers are often potentially contaminated with heavy metals such as Hg in which it is possible to find new bacteria such as Staphylococcus epidermidis that are adapted to a Hg polluted environment, and able to lower the Hg concentration through the presence of Mer Operon [15].

The Effect of Applying Hg and Pb Reducing Bacteria To The Number of Cyprinus carpio Mortality

Based on the measurement of the bioremediation effectiveness of the eleven bacterial isolates, two superior bacteria were found to reduce Hg (HGKRB5) and Pb (PBBTK1) which had the highest percentage of bioremediation effectiveness compared to other bacterial isolates. The percentage effectiveness of the bioremediation of HGKRB5 isolates was 99%, while PBBTK1 isolates were 100% (Table 2). The presence of bacterial isolates HGKRB5 and PBBTK1 had a significant effect in reducing the number of deaths of *Cyprinus carpio* compared to treatment without applying the two isolates. Both isolates were able to reduce heavy metals Hg and Pb at concentrations of 1 ppm and 10 ppm.

The PBBTK1 isolate, which was designated as treatment code F (1 ppm Pb contaminated water) and H (10 ppm Pb contaminated water) gave a significant effect on the life of *Cyprinus carpio*. When it is applied at doses of 1 ppm and 10 ppm Pb, all fish were still alive (no mortality) up to 24 hours after inoculation of bacterial, suggesting that isolate PBBTK1 able to neutralize the toxicity of Pb at a dose of 1 ppm and 10 ppm. Similarly, the HGKRB5 isolate also showed positive indication in neutralizing the toxicity of Hg as indicated by high survival rates of the fishes compared with the control treatments.. All fish survived when the HGKRB5 isolate was added at a dose of 1 ppm Hg into the contaminated water (E). Increasing the dose of Hg to 10 ppm resulted in the 67 percent survival of fish after 2 hours exposure in the contaminated water. These observations suggest that the HGKRB5 was 100 percent effective at low levels (1 ppm) and reduced by 33 percent at 10 ppm concentration level. The reduced survival rates of fish at 10 ppm Hg levels could be attributed to the carcinogenic and easily oxidizable charateristics of Hg, which is present in this study because of aerator installed in the container.In addition, Hg is also easily used in the lipid layer and damages cell membranes. Fish that were treated with *E. Coli* which can reduce Hg, after treatment was able to reduce levels of accumulation of Hg_2+ in fish muscles, Hg_2+, accumulated Hg_2+

were excreted in the form of faeces [16].

IV. CONCLUSION AND RECOMMENDATIONS

Based on the results of morphological, physiological, biochemical characterization, a potential isolate of Hg reducing bacteria (HGKRB1) was obtained, which was isolated from sediment samples from the river of Bogor Botanical Gardens. The HGKRB1 isolate was able to synthesize both catalase and oxidase enzymes, motile and alkaliphilic. Other potential isolates were Pb reducing bacteria (PBBTK1) isolated from soil samples from the brick factory. The PBBTK1 isolate can synthesize catalase enzyme, motile, and grow at various pH (3, 5, 7, and 9) and also can survive at various concentrations of Pb metal contamination (50 ppm, 250 ppm, up to 1250 ppm). The result of the bioassay test showed HGKRB5 was capable to remediate 10 ppm Hg contaminated water after 2 hours of inoculation. Similarly, PBBTK1 was also able to remediate 10 ppm Pb contaminated water. In this study, the effectiveness of bioremediation HGKRB5 was 99% (mortality 67%) and PBBTK1 reached 100% (no mortality).

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