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Research Article

Assessment and bioremediation of mercury pollutants by highly mercury-resistant bacteria immobilized in biochar from small-scale artisanal gold mining areas

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Abstract

Small-scale gold mining activities in Indonesia still use amalgamation techniques, which have the potential to cause mercury (Hg) pollution and affect the quality and number of microorganisms. Mercury-resistant bacteria can survive and adapt to mercury-exposed environments and can be developed as bioremediation agents. The bioremediation activity of these bacteria can be increased through immobilization using biochar. The results of observations of physicochemical qualities in three samples in the mining area, showed significant differences. The TOC in the rhizosphere soil sample of *Calliandra calothyrsus* L. showed the significantly highest value at 14.5%, and the pH of the three samples indicated acidity and exhibited no difference ($p<0.05$). The highest concentration measured in the tailing sample was 9.9 ng/g ($p<0.05$). The number of heterotrophic bacteria in the rhizosphere soil was the highest at 7.2×10^8 CFU/g. On the other hand, the number of mercury-resistant bacteria in the tailing sample showed the highest value of 6.3×10^3 CFU/g. In the selection based on the toxicity profile of 30 mercury-resistant bacteria obtained, the highest results were observed in the LMP1B5 bacterial isolate from the river sediment, with 50% effective concentration (EC50) and minimum inhibitory concentration (MIC) values of 225 and 250 mg/L, respectively. Polyphasic identification based on phenotypic and genotypic characteristics using the 16S rRNA gene showed that the bacterial isolate was identified as *Escherichia fergusonii*. The growth and mercury removal activity of *E. fergusonii* LMP1B5 increased by 21% and 52%, respectively, after the immobilization with biochar. Thus, immobilized *E. fergusonii* LMP1B5 was effective in removing mercury pollutants.

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Introduction

Indonesia has gold reserves that are evenly distributed in almost all provinces. Gold production in Indonesia occupies the seventh position in the world. As much as 20% of the national scale gold production comes from small-scale gold mining activities, which are spread across 32 provinces in Indonesia (Hutamadi, 2007). However, in small-scale gold mining activities, gold ore is extracted by an amalgamation technique using inorganic mercury. During the extraction process, large amounts of mercury move and pollute aquatic and terrestrial environments (Zolnikov and Ramirez-Ortiz, 2018; Mantey et al., 2020). Furthermore,

mercury pollutants can react with various organic compounds in the environment, bind to sulfhydryl groups in proteins, and accumulate in the food chain. Both inorganic and organic mercury are highly toxic to organisms. Exposure to these pollutants can cause brain damage, damage to the nervous system, memory loss, corrosive bronchitis, and pneumonitis in humans (Narváez et al., 2017; Pinto et al., 2019; Jiang et al., 2021).

One of the small-scale gold mining activities is in Cineam, Tasikmalaya, West Java Province. Gold mining activities at this location have been operating since 1968 using amalgamation techniques, resulting in symptoms of environmental degradation, such as the cloudy gray river water and sedimentation phenomenon. In addition, mercury (Hg) pollutants in the environment have an impact on reducing the abundance of microbes, especially bacteria. Meanwhile, some bacteria can survive and adapt to the environment through the mechanism of mercury resistance.

Mercury-resistant bacteria can convert toxic ionic compounds and inorganic forms of mercury into less harmful components in the form of vaporized Hg^0 and Hg sulfide, which can be absorbed and immobilized by bacterial cells. The mechanism of mercury resistance is caused by bacteria with a *mer operon* gene, which plays a role in detoxifying mercury pollutants in the environment. The *mer operon* is a functional gene consisting of metal regulatory genes (*merR*), mercury transport genes (*merT, merP,* and *merC*), mercury reductase (*merA*), and *organomercury lyase* (*merB*). The mercury reductase produced by bacteria catalyzes toxic inorganic mercury ions (Hg^{2+}) into volatile and non-toxic forms of mercury (Hg^0) (Priyadarshanee et al., 2022). Given their ability to detoxify mercury pollutants in the environment, mercury-resistant bacteria can be applied in the bioremediation of these pollutants.

Several physicochemical techniques for the removal of metal pollutants from the environment have been developed, and they include adsorption, chemical precipitation, nanomaterial-based removal, reverse osmosis ion exchange removal, and membrane filtration (Rajasulochana and Preethy, 2016; Karna et al., 2017; Xu et al., 2018). However, these physicochemical techniques require high operational and energy costs and emit large amounts of sewage sludge and toxic secondary pollutants. By contrast, bioremediation techniques that remove heavy metal pollutants using microbial cell factories offer more advantages. The bioremediation technique is natural, environmentally friendly, and sustainable. Thus, it is highly recommended for the removal of toxic metal pollutants from the environment without producing secondary pollutants nor harmful effects (Retnaningrum and Wilopo, 2016; Kumari et al., 2020; Rani et al., 2021). Several metal-resistant bacteria with the ability to detoxify several metal pollutants, namely Cd, Cu, Zn, Mn, and Hg, have been investigated (Mahbub et al., 2016; Zhang and Wang, 2016; Retnaningrum and Wilopo, 2017; Andriyanto et al., 2020; Huang et al., 2021).

The bioremediation ability of bacteria for pollutants can be further enhanced by immobilization techniques (Chen et al., 2019; Farsi et al., 2021; Fu et al., 2021). In these techniques, bacterial cells are immobilized using material carriers via physical or chemical methods (Bera and Mohanty, 2020; Lo et al., 2020). The nature of the immobilized bacteria is superior to that of free cell bacteria; an increase can be observed in the physiological abilities of cells, including metabolic activity, growth, and resistance to environmental exposure, then free cell bacteria. In addition, these immobilized bacteria can be regenerated and thus can be used sustainably (Żur et al., 2016; Bouabidi et al., 2018). Biochar is one of the abundant carrier materials in Indonesia and has the potential to be used in this bacterial immobilization technique.

Metal-resistant bacteria are very useful for the bioremediation of metal-contaminated environments and can be obtained from polluted environments. Tailings, river sediments, and rhizosphere soil of *Calliandra calothyrsus* L., which is the dominant plant at the gold mining site in Cineam, Tasikmalaya, West Java Province, Indonesia, have high potential for mercury-resistant bacteria. To date, no research has investigated the potential of highly resistant mercury bacteria immobilized by biochar from these three sample sources for bioremediation of mercury pollutants. Therefore, this study aimed to analyze the physicochemical qualities, mercury content, and the number of heterotrophic and mercury-resistant bacteria in three samples of tailings, river sediment, and rhizosphere soil of *C. calothyrsus* from small-scale gold mining. Furthermore, bacterial isolates were selected based on their mercury toxicity profile, which was determined based on the highest minimum inhibitory concentration (MIC) and 50% effective concentration (EC50), and polyphasic identification was based on the phenotypic character and 16S rRNA gene. The bioremediation capability of mercury was calculated by immobilization using biochar.

Materials and Methods

Study sites and sampling

Sampling was carried out in March 2019 at three selected sampling sites of gold mining at Dusun Cikurawet, Pasirmukti Village, Cineam District, Tasikmalaya, Indonesia. Two sites were sampled along the river at Dusun Cikurawet, and one site was sampled at the tailing well. The Cineam gold mine is located about 180 km southeast of Bandung City and 30 km from Tasikmalaya City at an altitude of 400 m

above the mean sea level. Gold minerals in Cineam are associated with epithermal veins and other minerals, such as Cu, Pb, and Zn (Rohmah and Retnaningrum, 2020).

Gold mining activities in Cineam are carried out around the Cikurawet River, with the dominant vegetation in the form of *C. calothyrsus*. Therefore, samples for this study were obtained in the form of tailings (7°26'44.67" S/108°20'40.09" E), Cikurawet River sediment (7°26'44.78" S/108°20'43.46" E), and *C. calothyrsus* rhizosphere soil (7°26'44.11" S/108°20'43.39" E). Tailings were collected from gold mining activity reservoirs, and river sediments and *C. calothyrsus* rhizosphere soil were collected along the banks of the Cikurawet River. Cikurawet River sediment samples at a position below the river water depth of about 50 cm were collected manually using an acrylic tube with a diameter of 8-10 cm. The sediment samples were collected at a depth of about 20 cm. The samples of *C. calothyrsus* rhizosphere soil were obtained from the area around the roots of the plant at a depth of 0-20 cm. Four samples were collected from each sampling site. Then, 200 g of each sample was extracted and held at 4 °C before testing.

Physicochemical qualities and mercury concentration

The total organic carbon (TOC) concentration, pH, and mercury in the sample were measured at each location. Prior to analysis, as much as 5 g samples of the tailings and rhizosphere soil were prepared and air dried. The samples were then pulverized with a mortar and sieved through a 0.15 mm sieve. Meanwhile, the sediment samples, which were previously washed with a HNO_3 solution (10%), were then rinsed with Milli-Q water to remove the contaminants. The samples were dried in an oven at a temperature of 50 °C, homogenized with a mortar and pestle, and sieved through a 2 mm-mesh sieve.

The TOC concentration of a sample was analyzed using the loss of weight on ignition method based on the reference of Heiri et al. (2001). To remove the total inorganic carbon concentration of the sample, we previously soaked 10 g of the sample in 6 M HCl and rinsed it with distilled water. The sample was then dried in an oven. Subsequently, the dry sample was placed in a muffle furnace and heated at 550 °C for 5-6 h until all samples turned into ash. The sample was cooled in a desiccator, and its final weight was weighed. The TOC content of the sample was calculated using the following formula:

$$\text{TOC}\ (\%) = \frac{\text{W0} - \text{Wt}}{\text{W0}} \times 100\ \%$$

where:

TOC = total organic carbon (%)
W0 = initial sample weight (g)
Wt = final sample weight (g)

The sample was digested with a mixture of 10 mL HF, 15 mL $HClO_4$, and 5 mL HNO_3. The acid mixture was added slowly to the sample and left overnight before being heated. The sample was heated for 2 h on a hot plate at a temperature of approximately 200 °C, allowed to cool, and filtered to remove contaminants. The solution was diluted to a final volume of 100 mL. The mercury concentration of the sample was then detected with a Mercury Instruments Lab Analyzer LA 254. In addition, the sample pH was analyzed using a pH meter.

Number of heterotrophic and mercury-resistant bacteria

An analysis of the number of heterotrophic and mercury-resistant bacteria was determined using Luria–Bertani (LB) agar medium and LB agar with the addition of 10 mg/L $HgCl_2$, respectively. The composition of the LB agar medium consisted of the following (g/L): tryptone, 10; yeast extract, 5; NaCl, 10; nystatin, 0.050. Each 5 g sample was diluted in series with sterile water saline (0.85% w/v) to attain a sample concentration in the range of 10^{-2} to 10^{-8}. The diluted sample was then inoculated by pour plating in the LB agar medium with the addition of 10 mg/L $HgCl_2$.

After incubation for 48 h at 30 °C, bacterial colonies growing in each medium were counted and expressed in CFU/g sample. Furthermore, each bacterial colony with different morphological characteristics grown in LB agar medium with the addition of 10 mg/L $HgCl_2$ was inoculated into a new medium until purified culture bacteria were obtained.

Toxicity profile of mercury-resistant bacteria

The collected bacterial isolates were each evaluated for toxicity against varying mercury concentrations to determine the MIC and EC50. Bacterial cultures aged 24 h were inoculated into a 96-well microplate (Iwaki AGC Techno Co., LTD., Japan) containing an LB broth medium. The turbidity of the bacterial culture was adjusted to enable the measurement of the final optical density (OD) at 0.6 on a 595 nm spectrophotometer. Next, the bacterial culture in the microplate was exposed to 3 mL LB broth medium containing various concentrations of mercury (0, 25, 25, 75, 100, 150, 200, and 250 mg/L). The bacterial culture was then incubated at 30 °C for 72 h. Bacterial growth was observed on the basis of the OD value of the culture using an enzyme-linked immunosorbent assay reader (BioTek) (λ = 595 nm).

Based on these growth results, the MIC and EC50 values of each mercury-resistant bacterial isolate were determined. The most mercury-resistant bacterial isolates were screened and determined based on the highest EC50 and MIC values. The selected bacterial isolates were then immobilized by biochar and analyzed for their growth and removal ability for mercury pollutants.

Identification of mercury-resistant bacteria

The polyphasic identification of selected mercury-resistant bacteria with the highest mercury removal ability was carried out based on phenotypic characteristics, including morphology, biochemical activity tests, and molecular characteristics based on the 16S rRNA gene. Bacterial morphology observations included colony morphological (shape, color, surface texture, elevation, and margins) and cell morphological (cell shape, gram staining and endospores) characteristics. The biochemical activity of the selected mercury-resistant bacteria consisted of motility tests, fermentation of maltose, cellobiose, lactose, and D-mannose. In addition, the biochemical properties of bacterial isolates were observed based on their ability to produce indole, lysine decarboxylase, ornithine decarboxylase, alkaline phosphatase, b-D-glucosidase, and b-xylosidase.

The molecular characterization of mercury-resistant bacteria from amalgamation waste was analyzed based on the 16S rRNA gene. The selected bacterial isolates were purified using the streak-plate method. The colonies that grew separately were inoculated in 50 mL nutrient broth (Himedia) and then incubated at 37°C for 48 h. Bacterial cultures were extracted to obtain pure DNA isolates using the Quick DNA Fungal Bacterial Minirep Kit (Zymo Research). The isolated pure DNA was amplified by polymerase chain reaction (PCR) using MyTaq Red Mix (Bioline). The 16S rRNA gene amplification was carried out using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Hossain et al. 2020).

The final volume of the PCR reaction used in the amplification process was 25 µL, which included 1 µL template DNA, 12.5 µL 2x MyTaq Red Mix, 1 µL 0.5 µM primer 27F and 1429R, and 9.5 µL double-distilled water. The PCR conditions consisted of pre-denaturation at 95 °C for 30 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were further confirmed by 0.8% (w/v) agarose gel electrophoresis and stained with cyber safe. The PCR amplification products were then purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and bi-directional sequencing. The sequencing results were edited using GeneStudio software.

A second bacterial isolate was analyzed using Basic Local Alignment Search Tools (BLAST) from the database available on the website www.ncbi.nlm.nih.gov. The bacterial isolate sequences were aligned using CLUSTAL W. The phylogenetic tree was reconstructed using the maximum likelihood method with the Molecular Evolutionary Genetics Analysis X software.

Removal of mercury by the highest mercury-resistant bacteria immobilized in biochar

Before the analysis of mercury removal ability by selected immobilized bacteria, biochar was determined and prepared beforehand. In this study, the coconut shell charcoal biochar was selected as a carrier material for immobilized bacteria in heavy metal bioremediation based on the results of prior experiments because of its high yield and outstanding performance (Liu et al., 2018; Rahim et al., 2020). The coconut shell charcoal biochar was pyrolyzed at 700 °C for 4 h after being chemically activated with $ZnCl_2$ and Na_2CO_3. The biochar product with a diameter range of 0.8-1 mm was selected after being passed through a sieve (Retnaningrum et al., 2021).

Pellets of 25 mg mercury-resistant bacterial culture were prepared and suspended in a 4% mixture of xanthan gum and olive oil (1:1) to obtain a mercury-resistant bacterial culture biopolymer. The next culture biopolymer was distributed and gently rolled evenly for 10 min onto the surface of the activated biochar with a ratio of 1:25. The prepared immobilized bacterial culture was inoculated into 100 mL liquid LB medium containing $HgCl_2$ 250 mg/L in a 500 mL Erlenmeyer flask. The cultures were then incubated at 30 °C with shaking at 200 rpm for 72 h. The control as a comparison was prepared and treated exactly the same as the treatment sample, using selected mercury-resistant bacterial isolates without biochar immobilization. The growth and bioremediation ability for mercury of the immobilized bacteria was observed by a batch process during observation intervals of 0, 24, 48, and 72 h of the growth of immobilized bacteria was calculated by pour plate. Meanwhile, testing of mercury bioremediation activity by immobilized bacteria was carried out by preparing 3 mL bacterial culture at predetermined intervals. Each culture sample was collected and separated between the cell pellet and medium using centrifugation at 1300 rpm for 2 min at 4 °C. The sample pellets were then dried at 50 °C. Furthermore, the dry sample pellets and the sample medium were digested using 1 mL 70% nitric acid by heating at 60 °C for 16 h. The digested sample was then analyzed for mercury concentration using the Mercury Instruments Lab Analyzer LA 254.

Data analysis

The mean value and standard deviations of the triplicate determination are presented in this research. One-way analysis of variance was followed by Duncan's new multiple range test. The significant difference test was performed using SPSS 25.0 to detect significant differences in the physicochemical parameters, mercury content, the number of bacteria, and mercury removal efficiency. Correlations between the number of bacteria (heterotrophic and mercury-resistant bacteria), physicochemical qualities (TOC and pH), and mercury concentration data were also

analyzed using Pearson's correlation coefficients. Significant difference was defined as $p<0.05$.

Results and Discussion

Physicochemical qualities and mercury concentration

Samples of tailings, river sediment, and rhizosphere soil of *C. calothyrsus* were collected from small-scale gold mining products in Cikurawet Subvillage, Pasirmukti Village, Cineam District, Tasikmalaya, Indonesia. Table 1 displays the results of the tests used to determine the TOC concentration, pH, and mercury values in the samples. The TOC in the rhizosphere soil sample of *C. calothyrsus* showed the highest significant value at 14.5%, followed by those of river sediment and tailing samples at 10.35% and 0.17%, respectively ($p<0.05$). The higher TOC in the rhizosphere soil samples of *C. calothyrsus* and river sediments was most likely due to the number and diversity of organisms in the form of plants or microbes around the river, where the sample was collected considerably more than the tailing sample. The banks of the river are overgrown with plants dominated by *C. calothyrsus*. Some researchers reported that the primary source of soil organic matter and river sediments can come from the decomposition of plant organic tissues in the form of leaves, twigs and branches, stems, fruits, and roots and the microbes associated with these plants. In addition, the excretion of plant and microbial metabolites is a source of organic material that will increase the TOC value of the samples (Carneiro et al., 2021)

Table 1. TOC, pH, and Hg concentrations in the tailing, river sediment, and rhizosphere soil *C. calothyrsus* samples from a small-scale artisanal gold mining in Cikurawet Subvillage.

Sampling site	Sampling site characteristics		
	TOC (%)	pH	Hg (ng/g)
River sediment	10.35 ± 0.01^{b}	5.1 ± 0.4^{a}	16.33 ± 0.02^{b}
Tailing	0.17 ± 0.0009^{c}	4.9 ± 0.5^{a}	79.90 ± 0.01^{a}
Rhizosphere soil of *C. calothyrsus*	14.50 ± 0.03^{a}	5.3 ± 0.4^{a}	0.27 ± 0.03^{c}

Note: numbers followed by different letters in the same column show significant differences ($p<0.05$).

From the results of pH measurements, all samples had an acidic pH of 5, and the concentration of Hg showed significant differences between samples (Rohmah and Retnaningrum, 2020). The sample pH describes the solubility of hydrogen ions and sample acidity (Wang et al., 2015). The lower the pH of the sample, the higher the solubility of Hg in the sample, which affects the pollution of the environment (Bowman et al., 2020). In addition, acidic pH conditions will affect the growth and activity of organisms in the environment. The highest Hg concentration was measured in the tailing sample, followed by river sediment and rhizosphere soil samples at 9.9, 16.33, and 0.27 ng/g, respectively ($p<0.05$). The detection of Hg pollutant in river sediment samples and rhizosphere soil proved that the tailings released from gold mining activities can contaminate the area around the location. Concentration Hg of the tailing was lower by 1/3 compared with that observed by Mantey et al. (2020), who investigated the tailing concentration in gold mining activities in the western region of Ghana, West Africa. The difference of Hg concentration from the tailing in the different gold mining activities is strongly influenced by the scale of operation, intensity of use, and the amount of mercury used (Mantey et al., 2017).

Number of heterotrophic and mercury-resistant bacteria

The number of heterotrophic and mercury-resistant bacteria from the sample showed a variation in values with a range between 3.4×10^2 and 7.2×10^8 CFU/g, as shown in Table 2. The number of heterotrophic bacteria in the rhizosphere soil showed the highest yield of 7.2×10^8 CFU/g, followed by river sediment and tailing samples of 2.4×10^8 and 1.3×10^3 CFU/g ($p<0.05$). On the other hand, the calculated number of mercury-resistant bacteria showed the highest value of 6.3×10^3 CFU/g in the tailing sample, followed by those in the river sediment and rhizosphere soil samples, with values of 8.2×10^2 and 3.4×10^2 CFU/g, respectively.

Based on Pearson's correlation coefficient analysis, the number of heterotrophic bacteria showed a positive correlation with the TOC concentration ($R^2 = 0.8274$), and the number of mercury-resistant bacteria exhibited a positive correlation with the concentration of Hg ($R^2 = 0.9858$) ($p<0.05$). This finding proves that a high concentration of TOC in the rhizosphere soil will provide the organic C compounds needed by heterotrophic bacteria. Furthermore, the available organic C compounds will be hydrolyzed by heterotrophic bacteria and produce energy for cell metabolism, resulting in the increased number of heterotrophic bacteria, allowing them to reach the highest value. On the other hand, the high concentration of Hg in the tailings caused bacteria to adapt to exposure to these pollutants through resistance mechanisms. The ability of bacteria to adapt to mercury in tailings was indicated by the highest number of mercury-resistant bacteria in the agar plate medium containing 10 mg/L $HgCl_2$ compared with the river sediment and rhizosphere soil samples. Mercury-

resistant bacteria from river sediment and rhizosphere soil samples showed a higher diversity with 12 bacterial isolates, whereas the tailings sample revealed six bacterial isolates (Table 3). The high diversity of mercury-resistant bacteria in the samples from the two sites may be related to the high TOC.

Table 2. Number of heterotrophic and mercury-resistant bacteria in the tailing, river sediment, and rhizosphere soil *C. calothyrsus* samples from a small-scale artisanal gold mining in Cikurawet Subvillage.

Sample site	Number of bacteria (CFU/g)	
	Heterotrophic bacteria	Mercury-resistant bacteria
River sediment	2.4×10^{8b}	8.2×10^{2b}
Tailing	1.3×10^{3c}	6.3×10^{3a}
Rhizosphere soil of *C. calothyrsus*	7.2×10^{8a}	3.4×10^{2c}

Note: numbers followed by different letters in the same column show significant differences ($p < 0.05$)

Toxicity profile of mercury-resistant bacteria

Although all mercury-resistant bacteria strains can grow on media containing 10 mg/L $HgCl_2$, the toxicity profile of strains due to exposure to variations in $HgCl_2$ concentrations (25, 25, 75, 100, 150, 200, and 250 mg/L) showed different results (Table 3). The highest toxicity profile was observed in LMP1B5 bacterial isolates with EC50 and MIC values of 225 and 250 mg/L, respectively. Meanwhile, the lowest toxicity profile was observed in isolate RHZC7 with EC50 and MIC values of 38 and 50 mg/L, respectively. The toxicity profile of a bacterial strain indicates its strength of resistance to mercury exposure. These bacterial strains gradually adapt to the presence of Hg and continually grow and reproduce under long-term metal stress. Bacteria may develop various resistance mechanisms to mercury exposure. Therefore, bacterial strains play an important role in the bioremediation of these pollutants. The mechanisms of mercury resistance in bacterial strains include be efflux pumps, chelation by biopolymers, precipitation, biomethylation, and *mer operon* (Sharma and Malaviya, 2016).

Table 3. EC 50 and MIC of mercury-resistant bacteria in tailing, river sediment, and rhizosphere soil of *C. calothyrsus* samples from small-scale artisanal gold mining in Cikurawet Subvillage.

No	River sediment			Tailing			Rhizosphere soil of *C. calothyrsus*		
	Strain	EC 50 (mg/L)	MIC (mg/L)	Strain	EC 50 (mg/L)	MIC (mg/L)	Strain	EC 50 (mg/L)	MIC (mg/L)
1	LMP1A1	70	100	LMP2A1	138	150	RHZB1	40	100
2	LMP1A2	68.8	100	LMP2A2	62	100	RHZB2	40	74
3	LMP1A3	112	150	LMP2B1	89	100	RHZB3	40	74
4	LMP1B2	70	150	LMP2C1	125	150	RHZB4	58	150
5	LMP1B3	123	150	LMP2C3	65	72	RHZB5	62	73
6	LMP1B4	160	200	LMP2C5	60	72	RHZB6	165	200
7	LMP1B5	225	250				RHZC1	92	250
8	LMP1C1	175	200				RHZC3	60	72.5
9	LMP1C2	145	150				RHZC4	130	250
10	LMP1C4	152	100				RHZC5	58	70
11	LMP1C5	140	150				RHZC6	40	72.5
12	LMP1C6	135	150				RHZC7	38	50

The MIC results of the LMP1B5 bacterial isolate against $HgCl_2$ showed higher results compared with that in the study by Mahbub et al. (2017), who measured the MIC of *Sphingopyxis* sp. SE2 (33.57 mg/L). Abu-Dieyeh et al. (2019) also reported five bacterial strains with lower MIC values for $HgCl_2$ compared with the results of this study. The four bacterial strains consisted of *Bacillus marisflavi* HHA6, *Bacillus pumilus* ZA3-S, *Bacillus infantis* HA4, and *Bacillus pumilus* HA3, which showed an MIC value of 100 mg/L on $HgCl_2$, whereas one strain of the bacterium *Acinetobacter schindleri* HA9 showed an MIC of 200 mg/L. Joshi et al. (2021) also reported several genera of bacteria, including *Bacillus* sp. NIOT-EQR_J86, *Pseudoalteromonas* sp. NIOT-EQR_J178, and *Pseudomonas stutzeri* NIOT-EQR_J179, which each showed an MIC value of 100 mg/L for $HgCl_2$.

Identification of mercury-resistant bacteria

Table 4 shows the observation results of the phenotypic characteristics of LMP1B5 bacterial isolates, which included colony, cell morphology, and biochemical activities. The colony morphology of LMP1B5 bacterial isolates had a circular colony shape, beige color, smooth surface structure, convex

elevation, and entire margin. As for morphology, the LMP1B5 bacterial cells were rod-shaped, Gram-negative, and non-spore. Biochemical character analysis revealed that the LMP strain was positive motility. In addition, it can ferment maltose, cellobiose, lactose, and D-mannose. The strain was positive for indole production, lysine decarboxylase, and ornithine decarboxylase. However, it was negative for alkaline phosphatase, ß-D-glucosidase, and ß-xylosidase. Based on Bergey's Manual of Determinative Bacteriology, the bacterial isolate LMP1B5 has phenotypic characteristics referring to the *Escherichia* genus (Brenner et al., 2015).

Table 4. Phenotypic character of LMP1B5 isolated from river sediment samples of a small-scale artisanal gold mining in Cikurawet Subvillage.

No	Key tests	Results
1	**Morphological properties**	
	Colony shape	Circular
	Colony color	Beige
	Surface structure	Smooth
	Colony elevation	Convex
	Colony margin	Entire
	Cell shape	Rod
	Gram staining	Negative
	Endospore	-
2	**Biochemical properties**	
	Motility	+
	Fermentation of maltose	+
	Fermentation of cellobiose	+
	Fermentation of lactose	+
	Fermentation of D-mannose	+
	Indol production	+
	Lysine decarboxylase	+
	Ornithine decarboxylase	+
	Alkaline phosphatase	-
	ß-D-glucosidase	-
	ß-xylosidase	-

The results of the genotypic analysis using the 16S rRNA gene on the bacterial isolate showed a visualization of the PCR amplification product with a size of 1,500 bp (Figure 1). Table 5 presents the results of the 16S rRNA gene sequencing of LMP1B5 isolates, which have been edited using GeneStudio software and matched with the consensus 16S rRNA gene sequence contained in the GeneBank database through the BLASTn program at National Center for Biotechnology Information. The results of BLASTn showed that the LMP1B5 isolate had the highest identity value of 99.16% with *Escherichia fergusonii* ATCC 35460 (Rohmah and Retnaningrum, 2020). Referring to the research of Schlaberg et al. (2012), these bacterial isolates were categorized in the same species because the percentage of 16S rRNA gene sequence similarity was 99%.

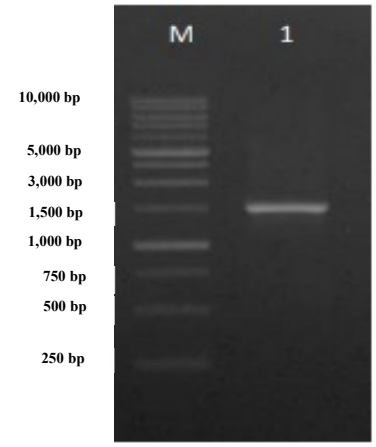

Figure 1. Result of 16S rRNA gene amplification using 27F and 1429R primers (M: 1 Kb DNA ladder marker (Geneaid), 1: LMP1B5 isolate).

In addition, based on the results of phylogenetic tree reconstruction (Figure 2), the LMP1B5 bacterial isolate was closely related to *Escherichia fergusonii* ATCC 25469 (NR_027549.1). It also formed a combined cluster with *Escherichia fergusonii* ATCC 25469 (NR_027549.1), *Escherichia fergusonii* NBRC 102419 (NR_114079.1), *Escherichia fergusonii* ATCC 35469 (NR_074902.1), and *Shigella flexneri* ATCC 29903 (NR_026331.1) with a bootstrap value of 89%. Referring to the work of Hillis and Bull (1993), the results of phylogenetic tree reconstruction with bootstrap values ≥70% can be trusted. In this phylogenetic tree reconstruction, *Staphylococcus aureus* ATCC 12600 was used as an outgroup to generate the polarization of apomorphic and plesiomorphic characters. The two analysis results proved that the BLAST findings support the reconstruction of the phylogenetic tree, and thus, the LMP1B5 bacterial isolate was identified as *Escherichia fergusonii* (Rohmah and Retnaningrum, 2021).

Removal of mercury by the highest mercury-resistant bacteria immobilized in biochar

Escherichia fergusonii LMP1B5, which showed the highest mercury toxicity profile, was then tested for growth and its bioremediation ability toward 250 mg/L mercury using free and immobilized cells. Biochar was used as an inorganic carrier for bacterial cell attachment to immobilize *E. fergusonii* LMP1B5. In this study, biochar was produced by pyrolysis (pyrochar) from coconut shell charcoal biomass under limited oxygen conditions. Figure 3 shows differences in growth and bioremediation ability of *E. fergusonii* LMP1B5 on free and immobilized cells. During the observation time of 72 h, free and immobilized cells showed an increased growth followed by an increase in mercury removal with the increase in incubation time.

Table 5. BLAST results of LMP1B5 bacterial isolates from the river sediment of a small-scale gold mining area in Cikurawet Subvillage.

Homolog species	Query cover	Identity	Accession number	Source of isolate	Reference
Escherichia fergusonii ATCC 35469	99%	99.16%	NR_027549.1	Clinical samples	Paradis et al. (2005)
Shigella flexneri ATCC 29903	99%	98.89%	NR_026331.1	Culture Collection	Wang et al. (1997)
Shigella sonnei CECT 4887	99%	98.82%	NR_104826.1	Culture Collection	Yarza et al. (2013)
Escherichia fergusonii ATCC 35469	99%	98.68%	NR_074902.1	American Type Culture Collection	Genoscope (2008) (Unpublished)
Escherichia fergusonii NBRC 102419	99%	98.61%	NR_114079.1	Culture Collection NITE Biological Resource Center	Nakagawa et al. (2011) (Unpublished)
Shigella dysenteriae ATCC 13313	99%	98.61%	NR_026332.1	Culture Collection	Wang et al. (1997)
Escherichia coli NBRC 102203	99%	98.54%	NR_114042.1	Culture Collection NITE Biological Resource Center	Nakagawa et al. (2011) (Unpublished)
Shigella boydii P288	99%	98.54%	NR_104901.1	Culture Collection	Yarza et al. (2013)
Escherichia marmotae HT073016	99%	98.47%	NR_136472.1	Fresh fecal samples of *Marmota himalayana*	Liu et al. (2015)
Escherichia albertii Albert 19982	99%	98.20%	NR_025569.1	Diarrheal stools	Huys et al. (2003)
Escherichia coli U 5/41	98%	98.18%	NR_024570.1	Culture Collection	Cillia et al. (1996)

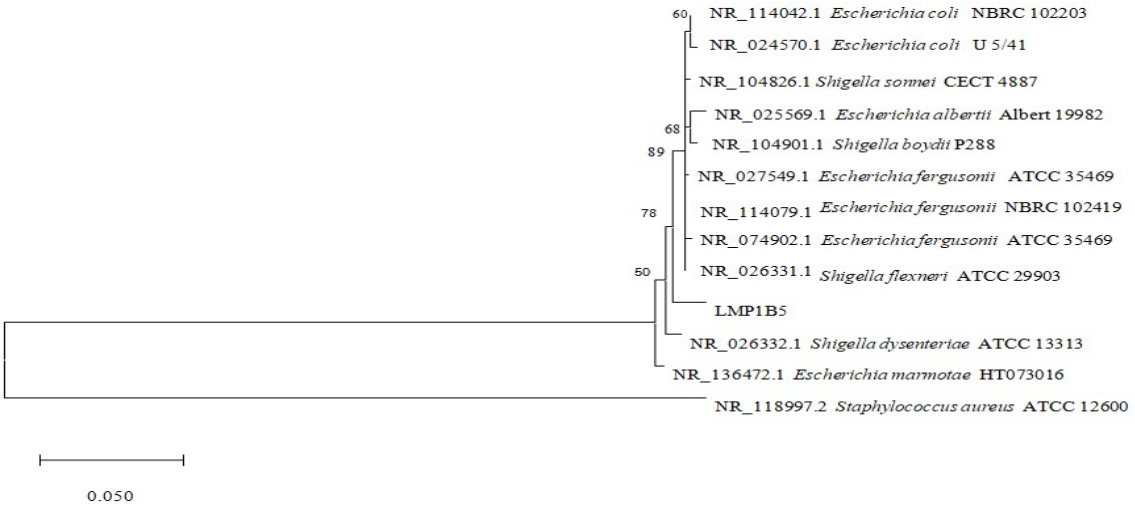

Figure 2. Phylogenetic tree of LMP1B5 isolate obtained using the maximum likelihood method (bootstrap = 1000).

Free cell *E. fergusonii* LMP1B5 showed increased growth after 72 h of incubation. Thus, the number of cells increased to 1.7×10^9 CFU/mL. The increase in free cell growth of *E. fergusonii* LMP1B5 during the incubation was followed by an increase in mercury removal by 63%. The results of mercury removal by *E. fergusonii* LMP1B5 were considerably more effective because the levels of mercury added to the medium were substantially higher (250 mg/L), and the incubation period was shorter compared with previous

studies. Dash et al. (2017) reported that *Bacillus thuringiensis*, *Pseudomonas aeruginosa*, *Exiguobacterium* sp., *Vibrio* sp., and *Escherichia coli* grown in a medium containing 10 ppm $HgCl_2$ showed the ability to remove mercury after 48 h of incubation (99%, 98%, 80%, 78%, and 50%, respectively).

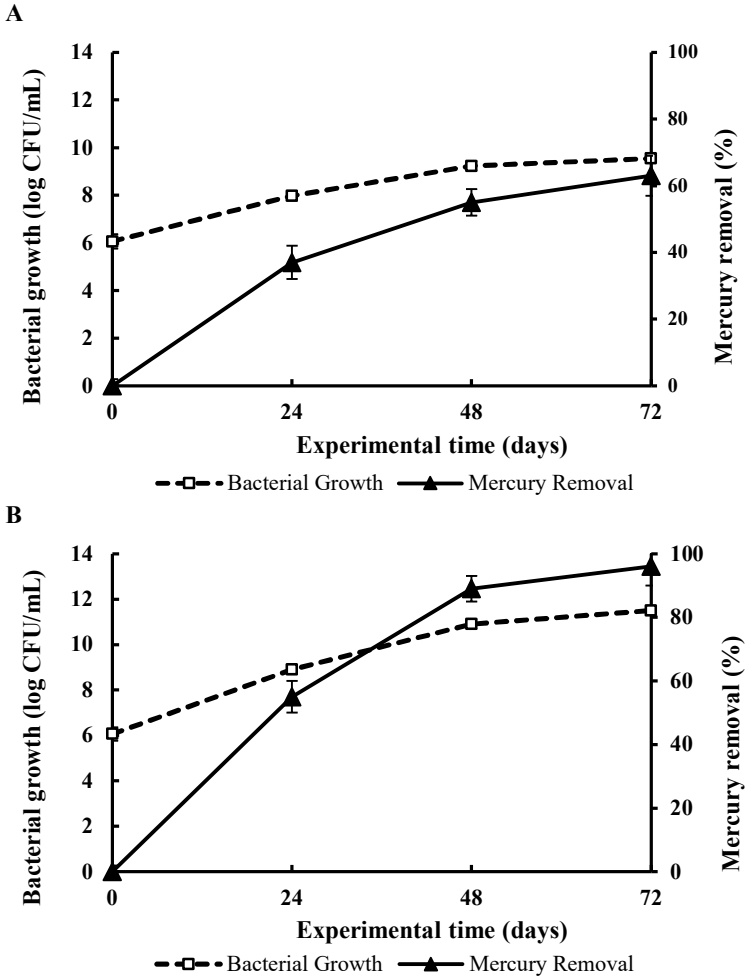

Figure 3. Growth and bioremediation ability of free (A) and immobilized cells (B) of *E. fergusonii* LMP1B5.

Sphingopyxis sp. SE2 on a medium containing 3.2 mg/L $HgCl_2$ removed 44% mercury after 6 days of incubation (Mahbub et al., 2017). *Bacillus* sp. HA6 and *Acinetobacter* sp. HA9 showed mercury removal in medium containing 7.9 mg/L $HgCl_2$ after 6 days of incubation, with values of 96.7% and 98.9%, respectively (Abu-Dieyeh et al., 2019). Although *Bacillus* sp. NIOT-EQR_J86, *Pseudoalteromonas* sp. NIOT-EQR_J178, and *Pseudomonas stutzeri* NIOT-EQR_J179, which were reported by Joshi et al. (2021), can remove 85%, 75%, and 80% mercury, respectively, within a short incubation period of 48 h, the concentration of $HgCl_2$ in the medium was notably lower at 100 mg/L $HgCl_2$. *Marinomonas* sp. RS3 can also remove mercury under the same incubation time for the results of this study (72 h). However, 50% of mercury removal from the strain occurred in a medium containing 50 mg/L $HgCl_2$ (Al-Ansari, 2022). The variation in the bioremediation strength of each bacterial strain against mercury pollutants was strongly related to the magnitude of the *merA* gene expression, as reported by Dash et al. (2017) on five bacterial strains observed after 48 h incubation. The highest *merA* gene expression was observed in *Bacillus thuringiensis* bacterial strains, followed by the expression values of the *merA* gene in *Pseudomonas aeruginosa*, *Exiguobacterium* sp., *Vibrio* sp., and *Escherichia coli*.

Compared with free cells, cell immobilization treatment using biochar can induce an increase in cell growth, which in turn led to a significant increase in mercury removal ($p<0.05$). Growth and mercury removal of *E. fergusonii* LMP1B5 increased by 21% and 52%, respectively, due to cell immobilization with biochar. Similar studies have shown that immobilization of bacteria using a variety of biochar,

including rice husk, sludge, and *Eucalyptus* leaf biochar, also led to increased removal of some metals. Chen et al. (2019) reported the results of a study on the immobilization of *Enterobacter* sp. using rice husk biochar, which removed 24.1% more Pb(II) than the control after 3 days of incubation. Meanwhile, the immobilization of *Enterobacter* sp. with sludge biochar removed more than 60.9% Pb(II) withing the same incubation period. Other researchers observed a decrease in Mn(II) removal by *Streptomyces violarus,* which can remove mercury pollutants by 74.8%, immobilized by biochar from *Eucalyptus* leaves after 5 days of incubation (Youngwilai et al., 2020).

In this study, the success of immobilization of *E. fergusonii* LMP1B5 using a biochar for bioremediation was strongly influenced by the nature of the material. Several researchers reported that biochar is a porous carbonaceous material that has a large specific surface area, abundant oxygen-containing functional groups (OFGs), and low cytotoxicity. These superior properties facilitate the attachment and proliferation of bacterial cells during the immobilization process. In addition, biochar is economically very promising (Xiong et al., 2017; Youngwilai et al., 2020). Given the nature of biochar, the immobilization of bacteria uses the adsorption principle, which is based on physical interactions. The surface functional groups of microorganisms (carboxyl, amine, hydroxyl, phosphate, and sulfhydryl) and OFGs on the surface of material carriers (hydroxyl, carboxyl, and carbonyl) interact with each other through several bonds, including van der Waals forces, ionic interactions, and hydrogen bonding (Jesionowski et al., 2014). This interaction results in a weak bond affinity between the microorganism and the carrier material, which causes no change in the natural structure of the immobilized microbe. Therefore, this method is very suitable for applications to live bacterial cells in bioremediation (Jesionowski et al., 2014; Lu et al., 2020).

Conclusion

The amalgamation technique, which is still used in small-scale gold mining activities in Cineam, Tasikmalaya, West Java Province, Indonesia, has been proven to cause low TOC and pH concentrations and high mercury contaminants in the area. The decrease in environmental quality has an impact on the decreased number of heterotrophic bacteria and an increased number of mercury-resistant bacteria. The number of heterotrophic bacteria was positively correlated with the concentration of TOC. Meanwhile, the number of mercury-resistant bacteria showed a positive correlation with the concentration of Hg. *Escherichia fergusonii* LMP1B5, which was isolated from the river sediment, was selected as the isolate with the highest mercury toxicity profile. Based on growth observations in a medium containing 250 mg/L mercury and its ability to remove mercury, the strain offers great prospects for application in the bioremediation of environments contaminated with high mercury concentrations. The activity of *E. fergusonii* LMP1B5 was increased by immobilization technique using biochar. Growth and ability to remove mercury increased by 21% and 52%, respectively. Understanding of the knowledge of bacterial cell immobilization techniques using biochar is useful in increasing our capacity to develop technologies for the remediation of mercury-contaminated waste. Therefore, our findings may provide scientific guidance for the assessments and bioremediation of small-scale gold mining.

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