Isolation and characterization of mercury-resistant microbes from gold mine area in Mount Pongkor, Bogor District, Indonesia

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Abstract. Sanjaya WTA, Khoirunnisa NS, Ismiati S, Hazra F, Santos DA. 2021. Isolation and characterization of mercury-resistant microbes from gold mine area in Mount Pongkor, Bogor District, Indonesia. Biodiversitas 22: 2656-2666. Exploring novel wild-type microbes is very important to give more flexibility for bioremediation implementation. It is related to discovering strain with higher detoxification ability and more reliable degradation mechanisms. Moreover, novel strain can be used as genetic material for strain development by molecular genetic engineering and production design formulation. The aims of this experiment were to characterize and identify new mercury-resistant microbes, investigate their capacity to accumulate mercury, and analyze the reducing mercury toxicity in bioassay. Four strains of bacteria selected through the screening stage were characterized for their morphological, biochemical, physiological, and molecular genetic characteristics. Considering their characteristics and mercury resistance levels, there are two selected microbial strains: fungus strain Cladosporium halotolerans Hg32 and the bacterial strain Mycobacterium peregrinum Hg37 with a mercury resistance level up to 3000 mg L\(^{-1}\). The C. halotolerans Hg32 could remove mercury with the highest potency up to 90.72\% at a mercury concentration of 100 mg L\(^{-1}\), while \(M\). peregrinum Hg37 removes up to 77.10\% at mercury concentrations of 10 mg L\(^{-1}\). Toxicity bioassay tests using fish confirmed that \(C\). halotolerans Hg32 and \(M\). peregrinum Hg37 had the ability to detoxify mercury in contaminated water. Both have successfully proven to reduce the mortality rate to below 5\%.

Keywords: ASGM, bioremediation, contamination, fish bioassay, mercury removal

Abbreviations: ASGM: Artisanal and Small-scale Gold Mining; MIC: Minimum Inhibitory Concentration; PCR: Polymerase Chain Reactions; AAS: Atomic Absorption Spectrophotometry

INTRODUCTION

Nowadays, mercury (Hg) contamination extends to environments such as soils, sediments, seawater, etc. In soil environment, Krishnayanti et al. (2012) reported that soil at Sekotong District of West Lombok was contaminated by mercury ranging from 25 to 40 mg kg\(^{-1}\). It also caused plant poisoning symptoms (chlorosis, brown plant root, root hood damage) in the region, and the accumulation of mercury in plant seeds around 0.20 mg kg\(^{-1}\). Meanwhile, in aquatic environment of southeastern coast of the Mediterranean Sea, total dissolved mercury ranged from 0.04 \(\mu\)g L\(^{-1}\) to 6.09 \(\mu\)g L\(^{-1}\) has been reported causing mercury accumulation in the fish up to 0.77 \(\mu\)g/g on the Siganus luridus (Abdallah 2020). In general, the concentration level of mercury in fishes was influenced by feeding habitat for each species. While for the human, organic mercury compounds (methyl mercury and phenyl mercury) which are highly reactive and can attack the human nervous system through the bloodstream (Rasmussen et al. 2008).

The Mount Pongkor area is an area with the biggest Artisanal and Small-scale Gold Mining (ASGM) in Indonesia using mercury for gold leaching. There are 850 ASGM hotspots mined by more than 150,000 miners (Ismawati et al. 2013). The location of ASGM in Mount Pongkor is similar to the majority of ASGM in Indonesia generally which take place at the upland area around rice fields or residence. It makes mercury easily transported through water flow from the upland to the lowland area.

Activity of ASGM at Mount Pongkor area is handled traditionally similar to other ASGM in Indonesia which excavate vertically and horizontally the soil. The gold is extracted by amalgamation technology using Hg. Residual mud from the extraction process is usually reprocessed through cyanidation. Then, the residue is discharged into land around the site, even agricultural land (Suhartini and Abubakar 2017). Yoga et al. (2014) have reported that mercury contamination caused by ASGM in Cikaniki River was higher than the maximum limit. In Cisarua Village, 60\% of villagers have been reported poisoned by mercury (Hg) which was proven by mercury accumulation in their hair counted between 2.03 to 9.04 ppm (Sumantri et al. 2014). Until 2015, the Ministry of Environment reported that 90\% of land, including residential housing in Mount Pongkor area, has been contaminated by heavy metal (Ismawati et al. 2015).

Generally, there were three classes of the value range of the element mercury in active river sediments in the Mount Pongkor area, consisted of first-class around 18.5-220 ppm (ASGM Cikoret, Pasir Jawa and Ciguha), second class...
of several species of fungi that are resistant to mercury from mercury-contaminated agricultural land. Meanwhile, Pietro-Souza et al. (2020) reported the role of endophytic fungi as bioremediation agents.

In the previous study in Mount Pongkor area, four mercury-resistant microbes (Brevundimonas sp. HgP1 and Brevundimonas sp. HgP2) have been discovered that can survive with a Minimum Inhibitory Concentration (MIC) of 575 ppm. They can accumulate Hg\(^{2+}\) at the stationary phase in the medium supplemented with 50 ppm and 100 ppm HgCl\(_2\) (Irawati et al. 2012). However, based on preliminary exploration in 2017, some microbes have higher mercury resistance. There four microbes isolated from Mount Pongkor area can survive in the medium supplemented by 600 ppm HgCl\(_2\). In this study, four new microbes are isolated with higher MIC of 600-3000 ppm. The research aims to characterize new mercury-resistant microbes, to investigate their capability to accumulate mercury, and to analyze the reducing mercury toxicity in bioassay.

MATERIALS AND METHODS

Study area

The study area and sampling locations located in Mount Pongkor area, Bantar Karet Village, Nanggung Sub-district, Bogor District, West Java Province, Indonesia are shown in Figure 1. Besides the gold mining company, villagers also mined Mount Pongkor area illegally and used mercury for gold purification. In the present study, soil samples were collected from five sampling sites (Table 1). All samples were collected using 0.5 L pre-sterilized glass containers with screw-cap lids, which were immediately stored at \(-2^\circ\)C upon arrival at the laboratory until they would be analyzed.

Figure 1. Location of the five sample point in Mount Pongkor area, Bantar Karet Village, Nanggung Sub-district, Bogor District, West Java Province, Indonesia
Isolation and selection of microbes

Ten grams of soil sample was mixed with 90 mL of sterile 0.85% NaCl solution for 30 minutes. Serial dilution was used for each sample up to seven concentrations (10^1, 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7). Then 0.5 mL suspension was poured into a nutrient agar medium that had been supplemented with 10 mg L^-1 HgCl₂, and then an incubation period was conducted for 48 hours. A single colony was inoculated using streak plate methods and incubated for 24 hours to obtain a pure isolate.

Generally, to define mercury resistance, the isolates that grew in the presence of 10 mg L^-1 Hg were considered to be mercury-resistant microbes. Stock solutions containing analytical grades of HgCl₂ were prepared, filter-sterilized, and added to NB (Nutrient Broth) medium to obtain the final concentration levels provided (10-3000 ppm HgCl₂). NB composition used in the experimentation was 1/5 normal recipe, consisting of 2 g peptone, 2 g beef extract, and 1 g sodium chloride in one liter water. The NB tubes were inoculated with the tested isolate and then incubated at 37°C for 72 h. The highest concentration of the tested mercury in which the isolate was able to grow was considered the maximum tolerable concentration (MTC).

Morphological and biochemical characterization of mercury resistant microbes

The collected bacteria were then subjected to biochemical tests that included carbohydrate fermentation, H₂S production, motility, oxygen consumption, citrate use, catalase, and oxidase test. A Carbohydrate fermentation test was conducted using 15% glucose, 0.5% lactose, and 0.5% sucrose media dissolved in 100 mL of peptone water, 10% NaCl and 1 mL of 0.1% bromothymol blue indicator. Each biochemical test was performed following standard procedures for microbial biochemical testing according to Cappuccino and Sherman (2002), Harley and Prescott (2005). Morphological characters were observed through microscopic observations with stereo microscopy to illustrate colony characteristics and compound microscopy for cell histological images.

Molecular identification based 16S rRNA gene and ITS region analysis

Identification of selected isolates was done by 16S rRNA approach for bacteria and ITS 3 region for fungi. Genomic DNA was extracted using Presto™ Mini gDNA Bacteria Kit (Genaid). Amplification for 16S rRNA gene was achieved using two primers consisted of E8F: 5'-GGT TGA TCC TGG CTC 3' for forward and 1541R: 5'-AAG GAG GTG ATC CAG CCG CA-3' for reverse, while ITS region amplification used ITS-1: 5'-TCC GTA GGT GAA CCT GCG G-3 as a forward primer and ITS-4: 5'-TCC GCT GTC TAT TGA TAT GC-3 as reverse primer in a 50 ul reaction volume using 1U Taq DNA polymerase (My Taq Red Mix, Bioline) in a My-Cycler thermal cycler (Bio-Rad) with the following program: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C denaturations for 30 sec, 55°C of annealing for 30 sec, and extended to 72°C for 1.5 min. A final extension step was set at 72°C for 10 min. Amplified products were verified by agarose gel electrophoresis and Gel doc system (BioRad, USA). Verified DNA samples were sent to First Base (Malaysia) for DNA sequencing.

The initial sequence analysis was undertaken using the Basic Local Alignment Search Tool (BlastN). Phylogenetic analyses were carried out with MEGA X using Maximum Likelihood method. The tree was used maximum composite likelihood method for determining evolutionary distances (Tamura et al. 2013). After removing gaps and missing data, the multiple sequence alignment of 16S rRNA gene sequence was done employing ClustalW (Larkin et al. 2007). The evolutionary distances of harboring organisms were computed using the Poisson correction method.

Mercury removal potential analyses

Two microbes with the highest resistance ability (3000 ppm) were selected for the mercury removal test. The mercury removal experiment was performed in 50 mL Nutrient Broth (NB) and Potato Dextrose Broth (PDB). Log phase microbes culture (1x10⁸ cell/mL) was inoculated into medium NB (for bacteria) and PDB (for fungi). The mercury's initial concentration was set to 500 ppm, 1000 ppm, 1500 ppm, 2000 ppm, 2500 ppm, 3000 ppm in the separate flask. All isolates were incubated at 37°C on a shaker condition of 120 rpm. Nutrient broth containing mercury was maintained as a negative control flask. Mercury removal from the nutrient broth was studied four days after inoculation. Mercury removal was determined by withdrawing the samples' aliquots from the flask (culture with mercury) at various concentration intervals. Sample centrifugation at 8,000 rpm for 10 minutes was carried out to separate bacterial biomass and culture medium. Then, supernatant and pellets were moved separately, and stored at 0°C for Hg quantification using Atomic Absorption Spectrophotometry (AAS) at Laboratory of Productivity and Aquatic Environment, IPB University, Indonesia. Mercury concentration measured in the supernatant represents soluble mercury in media, while mercury content in the pellet illustrates accumulated mercury in the cells.

Table 1. Altitude and coordinates of the five sampling point

| Location       | Sample | Coordinate          | Height (m) |
|----------------|--------|---------------------|------------|
| Mount          | Hg I   | -6°38'35", 106°33'54" | 460        |
| Pongkor area   | Hg II  | -6°38'11", 106°33'16" | 406        |
|                | Hg III | -6°37'28", 106°33'12" | 385        |
|                | Hg IV  | -6°37'23", 106°33'12" | 349        |
|                | Hg V   | -6°37'23", 106°33'12" | 371        |
Toxicity bioassay of decontaminated water
Toxicity Bioassay was tested in decontaminated water which has been detoxified by selected resistant isolates. It was carried out on cultured common carp (Cyprinus carpio) and western mosquitofish (Gambusia affinis). Fish were taken from an aquaculture pond and transferred to the laboratory aquarium using polythene bags. After transferring, the fish were acclimatized for one week before moving to the aquarium with treated water. There were three treatments including synthetic contaminated water (SCW) as positive control, no contaminated water as negative control, and decontaminated water by selected isolates. The SCW was water that has been added with HgCl₂ to a concentration of 1 mg L⁻¹ for C. carpio and 0.8 mg L⁻¹ for G. affinis. The decontaminated water was obtained from SCW which has already been inoculated by selected isolates and incubated four days before use. The fish were raised in treated water with an artificial aerator fitted to maintain oxygen levels for an exposure time of 96 h. Feeding was not carried out during exposure period. There were three treatments for each fish species with three replication units (aquarium set) for each treatment. Ten fishes with 30 L treatment water were put in each aquarium set. The fish used in the experiment had 8.42 (±0.51) g for the average wet weight (SD). Environmental data measured consisted of dissolved oxygen (mg L⁻¹), temperature (°C), and pH. Those data collections were observed individually in each aquarium at 24 h, 48 h, 72 h, and 96 h incubation time to determine experimental tank water quality. Fish mortalities were recorded at 24, 48, 72, and 96 h of exposure, and the dead fish were removed regularly from the test solution.

RESULTS AND DISCUSSION
Isolates of mercury resistant microbes
Even though research related to mercury-resistant microbes from Mount Pongkor area have been carried out earlier by Irawati et al. (2012), we carried it out again to explore more pronouncedly. Sampling was done at the sediment area point, a microbial hotspot with a high exchangeable mercury ratio (Abdallah 2020). We discovered several isolates with higher resistance levels to mercury exposure in screening tests. In Earlier Brevundimonas sp. HgP1 (Accession Number JX009135) and Brevundimonas sp. HgP2 (Accession Number JX009136) had been reported as strains with mercury accumulation ability that could survive in medium supplemented by 575 ppm HgCl₂. We used Lurient Bertani Agar medium with supplemented 10 ppm HgCl₂ for screening 128 prospective isolates in this research. All 128 potential bacterial isolates were inoculated in Lurient Bertani Broth with the multilevel concentration of HgCl₂ (50 ppm, 100 ppm, 200 ppm, 400 ppm, 500, and 600 ppm). Four selected bacteria survive in NB medium supplemented 600 ppm, including Hg32, Hg37, Hg43, and Hg44. The addition of mercury doses to the media in stages was continuously carried out until the microbial isolates could not grow.

Two isolates showed the highest resistance to mercury at concentrations up to 3000 ppm, while Hg43 has a resistance level of up to 800 ppm and Hg44 by 1000 ppm (Table 2). Based on resistant-mercury bacteria collected in Indonesia, none of the isolates could survive at the same mercury concentration level. It is also higher than two isolates isolated from Mount Pongkor area in the previous study (Irawati et al. 2012). In the other region of Indonesia, Febria et al. (2016) have isolated bacteria with the highest resistance mercury level from Sijunjung District; West Sumatra was only 250 ppm. Meanwhile, Ginting et al. (2021) have reported the highest resistance mercury level up to 170 ppm around ex-gold mine tailings. The mercury-resistant fungus from contaminated agricultural soil in Buru Island had been reported to grow well at 25 ppm (Hindersah et al. 2018a). In Egypt, Naguib et al. (2019) have reported that 14 isolates have the highest resistance to mercury at concentrations up to 160 ppm.

Table 2. Four selected isolates with the highest resistance to mercury

| Isolated code | Coordinated | Height (m) | Resistant level (ppm) |
|---------------|-------------|-----------|----------------------|
| Hg32          | -6°37'28";106°33'2" | 385       | 3000                 |
| Hg37          | -6°37'23";106°33'2" | 349       | 3000                 |
| Hg43          | -6°37'23";106°33'2" | 349       | 800                  |
| Hg44          | -6°37'23";106°33'2" | 349       | 1000                 |

Figure 2. Colony shape under 400 times stereo microscope magnification
Morphological and biochemical characterization of mercury resistant microbes

The four isolates had different colony morphology, which indicated diversity at the species level (Table 3). Meanwhile, based on the cell morphology observation, the isolates code Hg37 and Hg43 had the same coccus shape, and the Hg44 isolate was bacil shape. Hg37 and Hg43 were Gram-positive bacteria, while Hg44 was Gram-negative bacteria. In general, both Gram-positive and Gram-negative were reported to have mer genes that regulate the mercury resistance mechanism. The family of mer genes could be discovered in both bacterial groups’ genome or plasmids (Barkay et al. 2003). However, Kannan and Krishnamoorthy (2006) stated that the isolated Gram-negative bacteria with bacilliform exhibits lower resistance to heavy metals than Gram-positive bacteria. In contrast, Chasanah et al. (2018) reported that Gram-negative bacteria were more resistant and dominant to pollutants than Gram-positive bacteria.

Based on the growth environment, the four isolates could grow at a pH of 4-8. Dash et al. (2013) reported the isolate that can volatilize mercury efficiently under environmental parameters, pH of 7 to 8. The pH is an essential factor affecting microbial growth. It strongly influences abiotic factors, such as carbon availability, nutrient availability, and metals’ solubility (Rousk et al. 2009). Kannan and Krishnamoorthy (2006) reported that the increased pH (above 9) would inhibit the organomercurial lyase enzyme activity and usually stimulate deprotonation of microbial surfaces.

The three isolates (Hg32, Hg43, and Hg44) grew in the facultative anaerobic state, and only the Hg37 isolate grew under microaerophilic conditions. It is influenced by isolation techniques carried out in aerobic conditions. Mercury cycle can be found in various ecosystems, including soil, water, and sediment (Obrist et al. 2018; Hindersah et al 2018b). Its process was significantly influenced by the biological activities of the microbial community played in various oxic and anoxic reactions (Barkay and Wagner-Döbler 2005). Exploring mercury-reducing bacteria is very important because it shows facilitated Hg (II) transport activity under aerobic and anaerobic conditions (Schaef et al. 2011).

Biochemical tests showed that all isolates were positive for catalase and glucose fermentation tests but negative for sucrose fermentation tests. Only Hg37 and Hg43 isolates were able to ferment lactose. In the oxidase and citric acid test, only Hg32 and Hg44 showed positive results. All oxidase-positive bacteria were aerobic and can use oxygen as a terminal electron acceptor in respiration. Bacteria which were oxidase-negative can be anaerobic, aerobic, or anaerobe facultative bacteria. The negative oxidase result shows that these organisms do not have cytochrome c oxidase activity. Imron et al. (2019) reported mercury-resistant bacteria characteristics were positive for glucose fermentation and citric utilization and negative for lactose and sucrose fermentation. Chasanah et al. (2015) said that four isolates of mercury-resistant microbes were negative to citric and lactose utilization, only one positive to oxidase test, and only one harmful to sucrose fermentation.

| Characteristic | Hg32 | Hg37 | Hg43 | Hg44 |
|---------------|------|------|------|------|
| Cell Morphology | n/d | Coccus | Coccus | Bacil |
| Colony Shape | Filamentous | Circular | Irregular | Circular |
| Colony Elevating | Flat | Raised | Raised | Raised |
| Colony Margin | Entire | Undulate | Entire | Entire |
| Colony Color | Black | White | White | Red |
| Colony Size | 1-1.5 mm | 0.5-0.7 mm | 0.9-1 mm | 0.5-0.9 mm |
| Gram stain | n/d | positive | positive | negative |
| PH | 4-9 | 4-8 | 4-8 | 4-8 |
| Oxygen using | Anaerobe Faculative | Microaerofil | Anaerobe Faculative | Anaerobe Faculative |
| Motility | n/d | + | + | - |
| Catalase | + | + | + | + |
| Oxidase | + | - | - | - |
| Citric acid | - | + | + | + |
| Glucose | + | + | + | + |
| Lactose | - | + | + | - |
| Sucrose | - | - | - | - |
| TSIA medium | Red-Yellow | Red-Yellow | Red-Yellow | Red-Yellow |

Note: n/d: undefined

Molecular characteristic of gene 16S rRNA and ITS region

The identification of selected isolates was performed by several morphological and biochemical tests followed by molecular characterization. Because there was one strain indicated as mold, and three strains were bacteria based on their morphological and biochemical characteristics. So molecular identification was based on two genes, consisting of 16S rRNA for bacteria and ITS region for mold. Based on 16S rRNA sequences, three microbial strains had been identified as Mycobacterium sp. (strain Hg43), Mycolicibacterium peregrinum (strain Hg37), and Methylobacterium radiotolerans (strain Hg44) (Figure 3).
Meanwhile, strain Hg32 was identified as a mold, *Cladosporium sphaerospermum*, based on ITS 3 sequences (Figure 4). As shown in Figure 3, *M. peregrinum* Hg37 and *Mycobacterium* sp. Hg43 were still one genera with a very close relationship. Meanwhile, *M. radiotolerans* Hg44 had the most distant relatives from the three and has a relatively higher closeness to *Brevundimonas* sp. HgP1 and HgP2 (JX009135 and JX009136) isolated by Irawati et al. (2012). It indicates that the level of similarity of morphological and biochemical characters is completely unrelated to their ability to defend against mercury exposure.

The *Mycobacterium* sp. Hg43 and *M. peregrinum* Hg37 are members of the phylum Actinobacteria. Meanwhile, *Methylobacterium radiotolerans* Hg44 is a member of Proteobacteria. Long-term mercury contamination could drive microbiota composition change in microcosm. Both phyla can survive microbiota composition changes and have a high population in mercury-contaminated soil (Frossard et al. 2018; Najar et al. 2020; Zhu et al. 2021). Even these phyla proportions in the contaminated soil relatively increased compared to other microbial groups in several cases (Mahbub et al. 2020).

**Figure 3.** Phylogenetic tree of *Mycobacterium* sp. Hg 43, *Mycolicibacterium peregrinum* Hg 37, and *Methylobacterium radiotolerans* Hg 44, based on 16s rRNA. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site.
Several factors cause composition change, including limitation of horizontal gene transfer, specific mercury resistant mechanism, and soil characteristic related mercury mobilization (Thomas and Nielsen 2005; Naguib et al. 2018; Wang et al. 2020). Meanwhile, *C. sphaerospermum* is a mold species often found in soil ecosystems. *C. sphaerospermum* has been reported in various agricultural fields and has an essential role in increasing plant growth by producing volatile organic compounds (MVOCs). These compounds may bind to metal ions in the soil in certain circumstances (Hamayun et al. 2009; Li et al. 2020). Even though mercury resistance mechanisms in fungi have not yet been fully elucidated, resistance fungi with the ability to degrade mercury have been reported, such as *Cladosporium cladosporioides*, which resistant towards Hg$^{2+}$ because of bio-volatilization as the primary mechanism (Pietro-Souza et al. 2017).

**Mercury removal by resistant isolates**

The high mercury resistance levels indicated that the strain would be more promising for detoxifying mercury, thus the test was narrowed by selecting the two strains with the highest performance, i.e. *Cladosporium halotolerans* Hg32 and *Mycolicibacterium peregrinum* Hg37 for further experiment. Nevertheless, the isolates were not selected because the resistance ability was not always correlated with mercury's detoxification efficiency in some studies (Mangesa et al. 2019). The selection was made after the identification and construction process of phylogenetic trees to observe the correlation between kinship and the ability of microbial resistance to mercury. The phylogenetic tree indicates that the close genetic relationship level does not correlate with microbes' resistance (Figure 5). It strengthens the possibility of a disjunction between genetic data and microbes' ability to degrade mercury, including the relationship with the level of resistance of bacteria to mercury exposure.

The two strains have similar abilities at low concentrations below 10 mg L$^{-1}$. At a mercury concentration of 10 mg L$^{-1}$, 80.30% of the mercury was removed by *C. halotolerans* Hg32 and 77.1% of mercury removed by *M. peregrinum* Hg37 (Figure 5). The *C. halotolerans* Hg32 and *M. peregrinum* Hg37 showed different mercury removal patterns. The ability of *C. halotolerans* Hg32 to remove mercury increased when the concentration raised up to 100 mg L$^{-1}$ which reached 90.72% efficiency then stable as the mercury level increased. While, the *M. peregrinum* Hg37 removal ability decreased periodically when mercury concentration increased. Both strains have high mercury removal ability compared to previous reports ranging from 70 to 90% removal efficiency (Saranya et al. 2017). Typically, mercury removal ability will increase following mercury initial concentration up to a particular limit but will steady or decrease if the mercury concentration beyond limit circumstances (Upadhyay et al. 2017). However, according to this study, there was a strain, *C. halotolerans* Hg32, which preserved its ability to detoxify mercury at 3000 mg L$^{-1}$ exposure.
According to mercury accumulation in biomass, *M. peregrinum* Hg37 can accumulate higher mercury amounts in low concentration (<50 mg L\(^{-1}\)) than *C. halotolerans* Hg32. Then, the accumulation ability of *M. peregrinum* Hg37 would fall gradually following a decrease in mercury removal ability due to most bacterial cells unable to survive at high mercury exposure. Meanwhile, *C. halotolerans* had an opposite accumulation trend and was positively correlated with a decrease in removal ability, increase in mercury concentration. The *M. peregrinum* Hg37 was assumed to have the ability to uptake soluble mercury and transform it into cell biomass as the main detoxifying mechanism. On the other hand, mercury content in biomass also proved that there is a possibility that *C. halotolerans* Hg32 had another mechanism besides mercury accumulation. It was indicated by the mercury accumulation in low concentration that there was a gap between mercury loss in solution and accumulated mercury in biomass.

Generally, fungi have a higher resistant-mercury ability than bacteria. Some fungi had been reported to survive with a MIC of more than 1000 mg L\(^{-1}\). Besides enzymatic reactions, fungi can retain Hg and decrease metal uptake through their filaments. They can also secrete organic acid to increase heavy metal mobilization (Hindersah et al. 2018a). Urík et al. (2014) reported that *Cladosporium* volatilized almost 80 % of initial mercury content during 7-day static cultivation in the dark. Mercury bio-volatilization is the major filamentous fungal detoxification mechanism rather than its deposition or efflux in non-volatile forms. In the last updated study of fungal mercury resistance, the highest mercury concentration tested is 600 mg mL\(^{-1}\) towards two genera of endophytic fungi (Pietro-Souza et al. 2020).

The detoxification stability of *C. halotolerans* Hg42 indicates that it has a specific mercury bioremediation mechanism activated by the presence and amount of mercury. It’s regulation was also reported in *Westerdykella* sp. P71 has a particular resistance mechanism activated by mercury's existence and amount (Sun et al. 2017). However, whether *C. halotolerans* Hg42 has the same relationship pattern remains uncertain. There is a possibility that *C. halotolerans* Hg32 also has another mechanism apart from its mercury detoxification mechanism which allows stable detoxification processes amid increasing mercury concentrations.

The *Mycolicibacterium peregrinum* Hg37 has almost similar mercury degradation efficiency to *C. halotolerans* Hg32 strains at concentrations below 10 mg L\(^{-1}\). However, as mercury exposure increases, its degradation ability decreases further down to 10%. The *M. peregrinum* Hg37 is gram-negative bacteria, which generally have merD as a gene repressor. It can relate to *M. peregrinum*’s ability to decrease along with mercury concentration increase. In exploratory studies of mercury degradation, genus *Mycolicibacterium* is relatively rarely reported to have the ability to detoxify mercury compared to various other genera, such as *Brevundimonas, Klebsiella, Pseudomonas, Serratia, Streptococcus,* and *Enterococcus* (Pushkar et al. 2019; Kepel et al. 2020). However, Augelletti et al. (2020) have reported the results of gene sequences of various genes related to resistance to heavy metals in *Mycolicibacterium frederiksbergense*, including copper (copA, copC, and copD), arsenic (arsRBCDA), and...
mercury (\textit{merA} and \textit{merB}). Furthermore, Barrio-Duque et al. (2020) also reported that \textit{Mycolicibacterium} has several gene copies that have implications for plant resistance, including arsenic, mercury, chromium, copper, cadmium, cobalt, and zinc. It shows the potential for genus \textit{Mycolicibacterium} to be obtained as a bioaugmentation agent for heavy metals, including mercury in contaminated soil.

**The motility of \textit{C. carpio} and \textit{G. affinis} in mercury decontaminated water**

In the high mercury concentration level, there are significant relations between the mercury chloride concentration and the mortality rate of organisms (Gupta and Jawale 2013). It is inspired to design bioassay tests to confirm the effectiveness of the mercury decontamination process. We confirmed that the two selected isolates did not have pathogenicity to fish in previous experiments. \textit{Cyprinus carpio} and \textit{Gambusia affinis} were used in this study because they are sensitive to environmental conditions and are often used in standard bioassay procedures. Two fish species were placed on mercury-contaminated water that had been incubated using mercury-reducing microbial strains (Figure 6).

In this study, \textit{C. Carpio} was incubated in water containing 0.1 mg L\textsuperscript{-1} mercury which was decontaminated by using \textit{C. halotolerans} Hg32 and \textit{M. peregrinum} Hg37. Meanwhile, \textit{G. affinis} specimens were incubated in water containing 0.08 mg L\textsuperscript{-1}. The \textit{G. affinis} was incubated at lower concentrations due to their lower lethal concentration level than \textit{C. Carpio} (Ahmad 2011). Gupta and Jawale (2013) have reported that \textit{G. affinis} have 96-h LC50 by 0.097 mg L\textsuperscript{-1} and 96-h LC100 by 0.148 mg L\textsuperscript{-1}. The bioassay results showed that the mortality rate of both \textit{C. Carpio} and \textit{G. affinis} were significantly lower compared to the control that was not incubated with microbial strains which no mortality was observed during the testing period in the negative controls (without HgCl\textsubscript{2} contamination). Mercury existence exceeding lethal concentration can cause the death of fish. It is related to toxic effects produced by protein precipitation, enzyme inhibition, and generalized corrosive action (Bjørklund et al. 2017). Water that has been treated with microbes can maintain a mortality rate of less than 5%. It indicates a mercury degradation effectiveness of the two bacterial isolates, \textit{C. halotolerans} Hg42 and \textit{M. peregrinum} Hg37.

![Figure 6](image_url)

**Figure 6.** Percentage mortality of \textit{Cyprinus carpio} and \textit{Gambusia affinis} and after 96 h exposure to HgCl\textsubscript{2}. Hg (+): contaminated water (negative control), Hg (-): noncontaminated water (positive control). Hg 32: decontaminated water using \textit{Cladosporium halotolerans} Hg32. Hg 37: decontaminated water using \textit{Mycolicibacterium peregrinum} Hg37.
In conclusion, microbes with higher resistant mercury levels have been discovered during the present study from the Mount Pongkor area. Data showed that four isolates could survive in the conditions with more than 600 mg L\(^{-1}\) with two strains that have survivability at 3000 mg L\(^{-1}\) of HgCl\(_2\). Based on morphological, biochemical and molecular characteristics, the strains with highest tolerance level were identified as Cladosporium halotolerans Hg32 (mold) and Mycolicibacterium peregrinum Hg37 (bacterial strain). Quantitative analysis of mercury removal confirmed that both strains have the ability to detoxify mercury. The C. halotolerans Hg32 and M. peregrinum Hg37 have different mercury degradation patterns influenced by increased mercury exposure. Even though C. halotolerans Hg32 have the best removal ability at 100 mg L\(^{-1}\) of HgCl\(_2\), it can still remove mercury at high concentrations up to 3000 mg L\(^{-1}\) with 75% removal efficiency. Meanwhile, M. peregrinum Hg37 has best performance at 10 mg L\(^{-1}\) of HgCl\(_2\) (77% removal efficiency), and dropped with increasing mercury exposure level. The removal mechanism was also confirmed by a fish bioassay study in mercury-contained water detoxified by both strains. Both strains were confirmed to reduce mercury levels, proven by low fish mortality. We estimate that the two strains have interesting mercury detoxification mechanisms to advanced studies. The explanation regarding it needs to be elaborated further up to the molecular level. Overall, we can conclude that both strains have great potential to be developed as bioremediation agents in environments exposed to mercury. The difference in characteristics of both strains in mercury detoxification can be considered for designing bioremediation applications in the field.

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REFERENCES

Abdallah MAM. 2020. Mercury speciation in aquatic environment southeastern coast of the Mediterranean Sea, Egypt. Emerg Contam 6: 194-203. DOI:10.1016/j.emc.2020.04.003.

Ahmad Z. 2011. Acute toxicity and haematological changes in common carp (Cyprinus carpio) caused by diazinon exposure. Afr J Biotechnol 10 (63): 13852-13859. DOI: 10.5897/AJB11.1247.

Aguelletti F, Tremblay J, Agathos SN, Jousset A, Stenuit B. 2020. Draft whole-genome sequence of the anthropogenic-degrading strain Mycolicibacterium frederiksbergense LB501T, isolated from a polycyclic aromatic hydrocarbon-contaminated soil. Microb Resour Announc 9 (43): e00671-20. DOI: 10.1128/MRA.00671-20.

Barkey T, Miller SM, Summers AO. 2003. Bacterial mercury resistance from atoms to ecosystems. PEMS Microbiol Rev 27 (2-3): 355-84. DOI: 10.1016/S0168-6445(03)00046-9.

Barkey T, Wagner-Dohler I. 2005. Microbial transformations of mercury: potentials, challenges, and achievements in controlling mercury toxicity in the environment. In: Allen I, Laskin JWB, Geoffrey MG (eds) Adv Appl Microbio, vol 57. Academic Press, New York. DOI: 10.1016/S0065-2165(05)/57001-1.

Barrio-Duque A, Samad A, Nymbro O, Antonelli L, Sesitsch A, and Compan S. 2020. Interaction between endophytic Proteobacteria strains and Serendipita indica enhances biocontrol activity against fungal pathogens. Plant Soil 451: 277-305. DOI: 10.1007/s11104-020-04512-5.

Bjerkhvid G, Dedar M, Mutter J, Aaseth J. 2017. The toxicology of mercury: Current research and emerging trends. Environ Res 159: 545-554. DOI: 10.1016/j.envres.2017.08.051.

Cappuccino JG, Sherman N. 2002. Techniques for Isolation of Pure Culture. Microbiology: A Laboratory Manual, 6th edn. Pearson Education Inc., Singapore.

Chasanah U, Nurani Y, Handayanto E. 2018. The potential of mercury-resistant bacteria isolated from small-scale gold mine tailings for accumulation of mercury. J Ecol Eng 19 (2). DOI: 10.12911/22908993/83565.

Dash HR, Mangwani N, Das S. 2013. Characterization and potential application in mercury bioremediation of highly mercury-resistant marine bacterium Bacillus thuringiensis PW-05. Environ Sci Pollut Res 21 (4): 2642-2653. DOI: 10.1007/s11356-011-2206-8.

Du H, Sun T, Liu Y, An S, Xie H, Wang D, Igarashi Y, Imanaka T, Luo F, Ma M. 2021. Bacteria and archaea involved in anaerobic mercury methylation and methane oxidation in anaerobic sulfate-rich reactors. Chemosphere 274: 129773. DOI: 10.1016/j.chemosphere.2021.129773.

Ekyastuti W, Setyawati TR. 2015. Identification and in vitro effectiveness test of four isolates of mercury-resistant bacteria as bioaccumulation agents of mercury. Procedia Environ Sci 28: 258-264. DOI: 10.1016/j.proenv.2015.07.033.

Febria FA, Zakaria IJ, Syukriani L, Rahayu SF, Fajar MA. 2016. The highest mercury resistant bacteria as a mercury remediator from gold mining soil in West Sumatera, Indonesia. J Chem Pharm Res 8 (1): 394-397.

Frossard A, Donhauser J, Mestrot A, Gygax S, Bååth E, Frey B. 2018. Microbial transformations of mercury: potentials, challenges, and achievements in controlling mercury toxicity in the environment. In: Allen I, Laskin JWB, Geoffrey MG (eds) Adv Appl Microbio, vol 57. Academic Press, New York. DOI: 10.1016/S0065-2165(05)/57001-1.

Harley JP, Prescott LM. 2005. Laboratory Exercises in Microbiology, 6th edn. Pearson Education Inc., Singapore.

Hendriks R, Mulyani O, Osok R. 2017. Proliferation and exopolysaccharide production of Azotobacter in the presence of mercury. Biodivers J 8 (1): 21-26.

Hendriks R, Asda KR, Herdiyantoro D, NN Kamaludinn. 2018a. Isolation of mercury-resistant fungi from mercury-contaminated agricultural soil. Agriculture 8 (3). DOI: 10.3390/agriculture08030033.

Hendriks R, Risamasu R, Kalay AM, Dewi T, Makatita L. 2018b. Mercury contamination in soil, tailing and plants on agricultural fields near closed gold mine in Buru Island, Maluku. J Degraded Mining Lands Manag 5 (2): 1027-1034. DOI: 10.15243/jdmlm.2018.052.1027.

Imron MF, Kurniawan SB, Soegianto A. 2019. Characterization of mercury-reducing potential bacteria isolated from Keputih non-active sanitary landfill leachate, Surabaya, Indonesia under different saline conditions. J Environ Manag 241: 113-122. DOI: 10.1016/j.jenvman.2019.04.017.

Irawati W, Soraya Y, Baskoro AH. 2012. A study on mercury-resistant bacteria isolated from a gold mine in Mount Pongkor Village, Bogor, Indonesia. Hayati J Biosci 19 (4): 197-200. DOI: 10.4306/hjb.19.4.197.

Ismawati Y, Jindrich P, Dangaq J. 2013. Mercury Hotspots in Indonesia. IPEN Mercury-Free Campaign Report. BaliFokus (Indonesia) - Arniika Association (Czech Republic) - IPEN Heavy Metals Working Group, Denpasar. DOI: 10.13140/RG.2.2.62150.73282.

Ismawati Y, Lelitasari, Buffelth S. 2015. Gold production in rural areas of Bogor Regency and its hidden hazards implication. The 5th Environmental Technology and Management Conference - Green
Technology towards Sustainable Environment*, Bandung, Indonesia. [Indonesian]

Juliawan N. 2006. Distribution of Mercury in mining areas in Mount Pongkor, Bogor District, West Java. Proceeding of Disclosure of The Results of Field and Non-field Activities in 2006, Center of Geological Resources, Ministry of Energy and Mineral Resources of the Republic of Indonesia. Jakarta. [Indonesian]

Kannan SK, Krishnamoorthy R. 2006. Isolation of mercury resistant bacteria and influence of abiotic factors on bioavailability of mercury—a case study in Pulicat Lake north of Chennai, South East India. Sci Total Environ 367 (1): 341-353. DOI: 10.1016/j.scitotenv.2005.12.003.

Kepel BJ, Gani MA, Taille TE. 2020. Comparison of bacterial community structure and diversity in traditional gold mining waste disposal site and rice field by using a metabarcoding approach. Intl J Microbiol. DOI: 10.1155/2020/1858732.

Kiran MG, Pakshirajan K, Das G. 2017. Heavy metal removal from multicomponent system by sulfate-reducing bacteria: Mechanism and cell surface characterization. J Hazard Mater 324: 62-70. DOI: 10.1016/j.jhazmat.2015.12.042.

Krisnayanti BD, Anderson CW, Utomo WH, Fung X, Handayanto E, Mudarisna N, Ikrarn H. 2012. Assessment of environmental mercury discharge at a four-year-old artisanal gold mining area on Lombok Island, Indonesia. J Environ Monit 14 (10): 2598-2607.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGreggor PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD. (2007). Clustal W and Clustal X version 2.0. Bioinformatics 23 (21): 2947-2948. DOI: 10.1093/bioinformatics/btm404.

Li J, Zhang M, Sun J, Mao X, Wang J, Liu H, Zheng H, Li X, Zhao H, Zou D. 2020. Heavy metal stress-associated proteins in rice and arabidopsis: Genome-wide identification, phylogenetics, duplication, and expression profiles analysis. Front Genet 11: 477. DOI: 10.3389/fgene.2020.00477.

Mahbub KR, King WL, Siboni N, Nguyen VK, Rahman MM, Megharaj M, Seymour JR, Franks AE, Lobbate M. 2020. Long-lasting effect of mercury contamination in the soil microbiome and its co-selection of antibiotic resistance. Environ Pollut. DOI: 10.1016/j.envpol.2020.115057.

Mangesa R, Kasimawiti K, Durma D, Lisaholit S, Setiaji AB, Umanaltio MCB. 2019. Identification and testing resistance against bacteria isolated mercury from gold mining in Gogorea Buru. Int J Biotechnol Res 8 (11): 5-8.

Nagub MM, El-Gendy AO, Khairalla AS. 2018. Microbial diversity of mer operon genes and their potential rules in mercury bioremediation and resistance. Open Biotech J 12 (1). DOI: 10.2174/1874070701812010056.

Nagub MM, Khairalla AS, El-Gendy AO, Elkhatib WF. 2019. Isolation and characterization of mercury-resistant bacteria from wastewater sources in Egypt. Can J Microbiol 65 (4): 308-321. DOI: 10.1139/cjm-2018-0379.

Najar IN, Sherpa MT, Das S, Das S, Thakur N. 2020. Diversity analysis and metagenomic insights into the antibiotic resistance and metal resistances among Himalayan hot spring bacteriome- insinuating inherent environmental baseline levels of antibiotic and metal tolerance. J Glob Antimicrob Resist. DOI: 10.1016/j.jgar.2020.03.026.

Obrist D, Kirk JL, Zhang L, Sunderland EM, Jiskra M, Selin NE. 2018. A review of global environmental mercury processes in response to human and natural perturbations: Changes of emissions, climate, and land use. Ambio 47, 116-140. DOI: 10.1007/s13280-017-0104-9.

Pietro-Souza W, Melio IS, Vendrusculo SJ, Silva GFD, Cunha CND, White JF, Soares MA. 2017. Endophytic fungal communities of Polygonum acuminatum and Aeschynomene flaminensis are influenced by soil mercury contamination. PLoS One 12 (7): e0182017. DOI: 10.1371/journal.pone.0182017.

Pietro-Souza W, de Campos Pereira F, Melio IS, Stachack FFF, Terezo AJ, Cunha CND, White JF, Li H, Soares MA. Mercury resistance and bioremediation mediated by endophytic fungi. Chemosphere 240: 124874. DOI: 10.1016/j.chemosphere.2019.124874.

Pushkar B, Sevak P, Singh A. 2019. Bioremediation treatment process through mercury-resistant bacteria isolated from Mithi River. Appl Water Sci 9: 117. DOI: 10.1007/s13201-019-0998-5.

Rasmussen LD, Zawadksy C, Binnerup SJ, Oregaard G, Sorensen SJ, Kroer N. 2008. Cultivation of hard to culture subsurface mercury-resistant bacteria and discovery of new merA gene sequences. Appl Environ Microbiol 74: 3795-3803. DOI: 10.1128/AEM.00499-08.

Ratnamurghi E, Wilopo W. 2017. Removal of sulphate and manganese on synthetic wastewater in sulphate reducing bioreactor using Indonesian natural zeolite. Indon J Chem 17 (2): 203-210. DOI: 10.22146/ijc.22710.

Rousk J, Brookes PC, Baath E. 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl Environ Microbiol 75 (6): 1589-1596. DOI: 10.1128/AEM.02775-08.

Saranya K, Sundaramaniakam A, Shekar S, Swanaminathan S, Balasubramanian T. 2017. Bioremediation of mercury by Vibrio flavidus screened from industrial effluents. BioMed Res Int 2017: 6509648. DOI: 10.1155/2017/6509648.

Scharfner JK, Rocks SS, Zheng W, Liang L, Gu B, Morel FM. 2011. Active transport, substrate specificity, and methylation of Hg(II) in anaerobic bacteria. Proc Natl Acad Sci USA 108 (21): 8714-8719. DOI: 10.1073/pnas.1105781108.

Suhartini, Abubakar. 2017. Socio-economic impacts and policy of artisanal small-scale gold mining in relation to sustainable agriculture: a case study at Sekotong of West Lombok. J Degraded Mining Lands Manag 4 (3): 789-796. DOI: 10.15243/jdmlm.2017.043.789.

Sumantri A, Lelasari E, Junita NR, Nasrudson N. 2014. Logam merkuri pada pekerja penambangan emas tanpa izin. Kesmas: Natl Publ Health J 8 (8): 398-403. DOI: 10.21109/kesmas.v8i8.411.

Sun Y, Aguila B, Perman J, Earl LD, Abney CW, Cheng Y, Wei H, Nguyen N, Wojtas L, Ma S. 2017. Postsynthetically modified covalent organic frameworks for efficient and effective mercury removal. J Am Chem Soc 139: 2786-2793. DOI: 10.1021/jacs.6b11285.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30 (12): 2725-2729. DOI: 10.1093/molbev/mst197.

Thomas CM, Nielsen KM. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Micro 3: 711-721. DOI: 10.1038/nrmicro1234.

Upadhyay KH, Vaishnav AM, Tipre DR, Patel BC, Dave SR. 2017. Kinetics and mechanisms of mercury biosorption by an etoposucaracide producing marine isolate Bacillus licheniformis. 3 Biotech 7 (5): 313. DOI: 10.1007/s13205-017-0958-4.

Urik M, Hlodák M, Mikušová P, Matúš P. 2014. Potential of microscopic fungi isolated from mercury-contaminated soils to accumulate and volatilize mercury (II). Water Air Soil Pollut 225 (12): 1-11. DOI: 10.1007/s11270-014-2219-z.

Wang L, Hou D, Cao Y, Ok YS, Tack FMG, Rinklebie J, O’Connor D. 2020. Remediation of mercury-contaminated soil, water, and air: A review of emerging materials and innovative technologies. Environ Int 134: 105281. DOI: 10.1016/j.envint.2019.105281.

Yoga GP, Lumbanbatu D, Riani E, Wardiatno Y. 2014. Pengaruh pencemaran merkuri di sungai Cakanimi terhadap biota Trichoptera (Insecta), Limnol-Perairan Darat Tropis di Indonesia 21 (1). [Indonesian]

Zhu H, Teng Y, Wang X, Zhao L, Ren W, Luo Y, Christie P. 2021. Changes in clover rhizosphere microbial community and diazotrophs in mercury-contaminated soils. Sci Total Environ 767: 145473. DOI: 10.1016/j.scitotenv.2021.145473.