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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, Ismiani S, Hazra F, Santosa 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 *Mycolicibacterium 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, Krisnayanti 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 µg/L to 6.09 µg/L has been reported causing mercury accumulation in the fish up to 0.77 µg/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

around 6-18.5 ppm (Cikaniki River and Cipanganten River) and third class interpreted as not polluted area (1-6 ppm). Meanwhile, the mercury concentration level in the soil was divided into three classes: first class (60-400 ppm), second class (10-60 ppm) and third class as not polluted area (1-6 ppm). The first category was located at PETI Cikoret, Pasir Jawa, Ciguha, around the Cipanganten River, around the Cikaradak River and around the Cimarinten River, and the second class has reported including around the Cikaniki River in the Cihiris area and around the Citeureup River (Juliawan 2006).

Several types of microbes and the mechanism of bioremediation of metal pollutants in the environment have been successfully carried out (Kiran et al. 2017; Retnaningrum and Wilopo 2017; Du et al. 2021). Metal-resistant microbes have a critical role in optimizing bioremediation as an effective and low-cost alternative to removing metal pollutants, one of which is mercury. Many exploration results have been reported massively to support mercury remediation development. Hindersah et al. (2017) have isolated *Azotobacter* as mercury-resistant bacteria from mercury-contaminated agriculture soil on Buru Island, Maluku. Four bacteria that can accumulate mercury have been discovered in Mandor District, West Kalimantan. They consisted of *Bacillus subtilis* HgTA1 and HgTL2, *Burkholderia cepacia* HgRL, and *Burkholderia cenosepacia* HgRA (Ekyastuti and Setyawati 2015). In West Lombok, *Brevundimonas vesicularis* and *Fusobacterium aquatile* have been reported to have mercury accumulation ability by 75 % in small-scale gold mine tailing with initial HG concentration 41.37 ppm (Chasanah et al. 2018). Imron et al. (2019) isolated *Pseudomonas aeruginosa* from the Keputih non-active sanitary landfill leachate, which can be mercury-reducing agents in bioremediation. Apart from bacteria, several species of fungi have also been reported to have mercury resistance. Hindersah et al. (2018a) carried out the isolation 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, two 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°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} $HgCl_2$, 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 $HgCl_2$ were prepared, filter-sterilized, and added to NB (Nutrient Broth) medium to obtain the final concentration levels provided (10-3000 ppm $HgCl_2$ 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 ^oC 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, H_2S 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-AGA GTT 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 TCC GCT 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 16SrRNA 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 ($1x10^8$ 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^oC 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 ^oC 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 Pongkor area	Hg I	-6^{o}38’35”, 106^{o}33’54”	460
	Hg II	-6^{o}38’11”, 106^{o}33’16”	406
	Hg III	-6^{o}37’28”, 106^{o}33’2”	385
	Hg IV	-6^{o}37’23”, 106^{o}33’2”	349
	Hg V	-6^{o}37’23”, 106^{o}33’2”	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 crap (*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 treatmented 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 $HgCl_2$ 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 treatmented water with an artificial aerator fitted to maintain oxygen levels for an e 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 50 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 (^oC), 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 out it 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 $HgCl_2$. We used Lurient Bertani Agar medium with supplemented 10 ppm $HgCl_2$ for screening 128 prospective isolates in this research. All 128 potential bacterial isolates were inoculated in Lurient Bertani Broth with the multilevel concentration of $HgCl_2$ (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	-6º37’28”;106º33’2”	385	3000
Hg37	-6o37’23”;106o33’2”	349	3000
Hg43	-6o37’23”;106o33’2”	349	800
Hg44	-6o37’23”;106o33’2”	349	1000

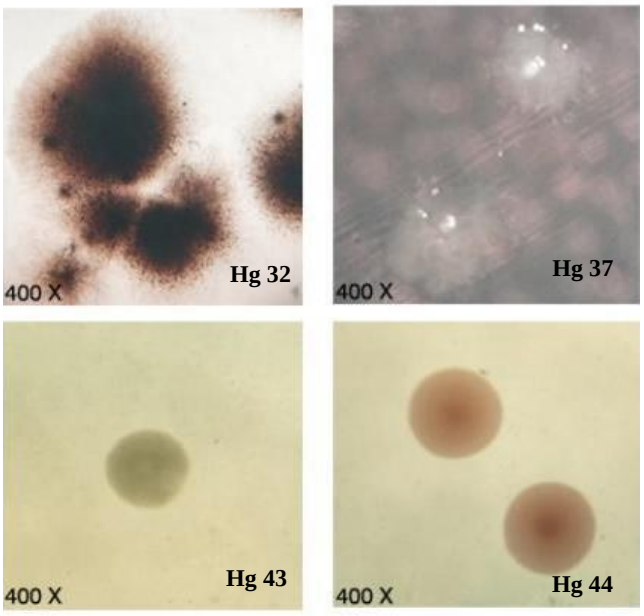

Figure 2. Colony shape under 400 times stereo microscope magnification

Table 3. Morphological and physiological characteristic of Mercury-resistant microbes isolated from gold mine area in Mount Pongkor

Characteristic	Isolate code			
	Hg32	**Hg37**	**Hg43**	**Hg44**
Cell Morphology	*n/d*	Coccus	Coccus	Bacil
Colony Shape	Filamentous	Circular	Irregular	Circular
Colony Elevation	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 Facultative	Microaerofil	Anaerobe Facultative	Anaerobe Facultative
Motility	*n/d*	+	+	-
Catalase	+	+	+	+
Oxidase	+	-	-	-
Citric acid	-	-	-	+
Glucose	+	+	+	+
Lactose	-	+	+	-
Sucrose	-	-	-	-
TSIA medium	Red-Yellow	Red-Yellow	Red-Yellow	Red-Yellow

Note: *n/d*: undefined

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 is 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 (Schaefer 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.

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).

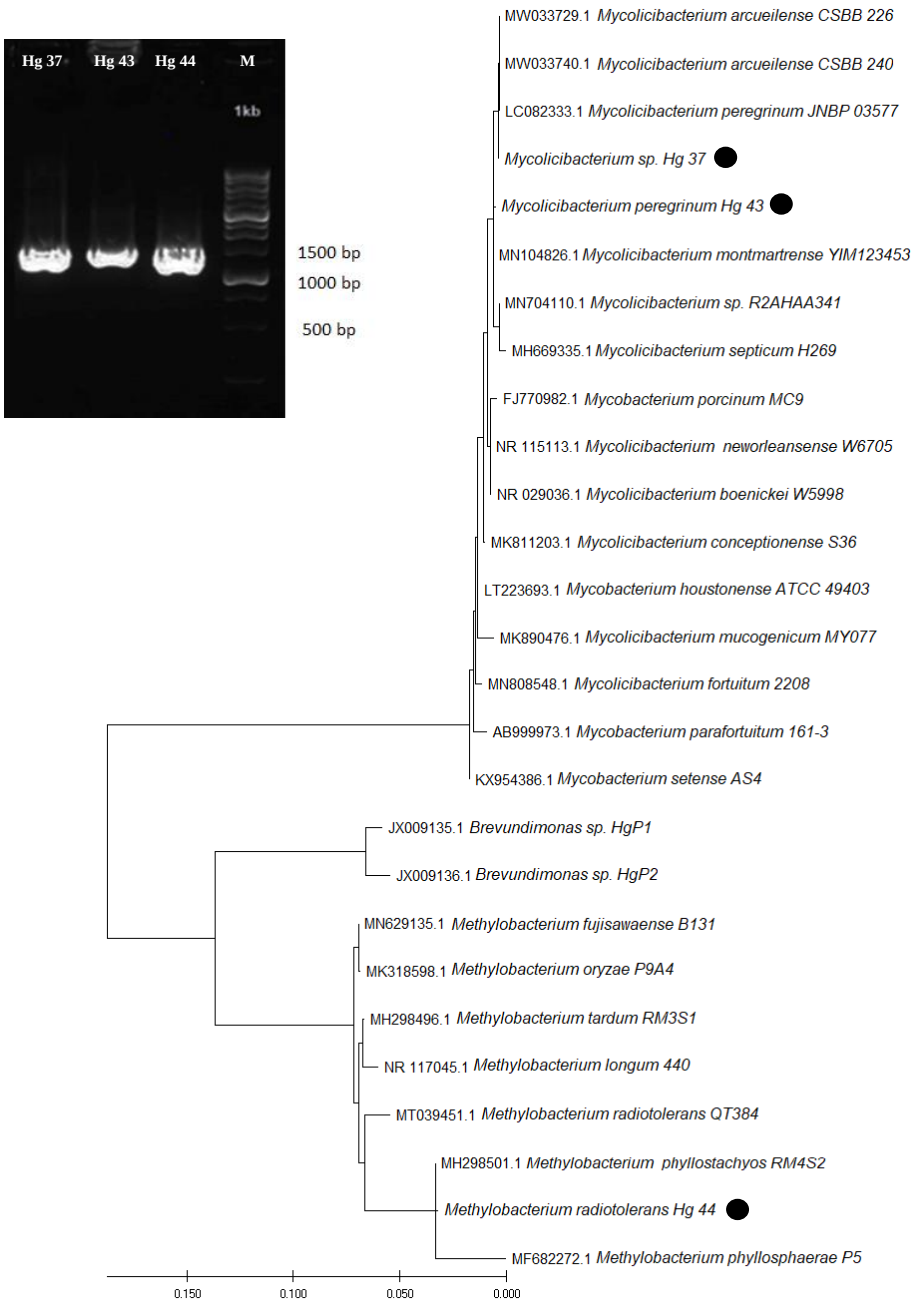

Figure 3. Phylogenetic tree of *Mycobacterium* sp. Hg 43, *Mycolicibacterium peregrinum* Hg 37, and *Methylobacterium radiotolerans* Hg 44, based on 16s rRNA. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions per site.

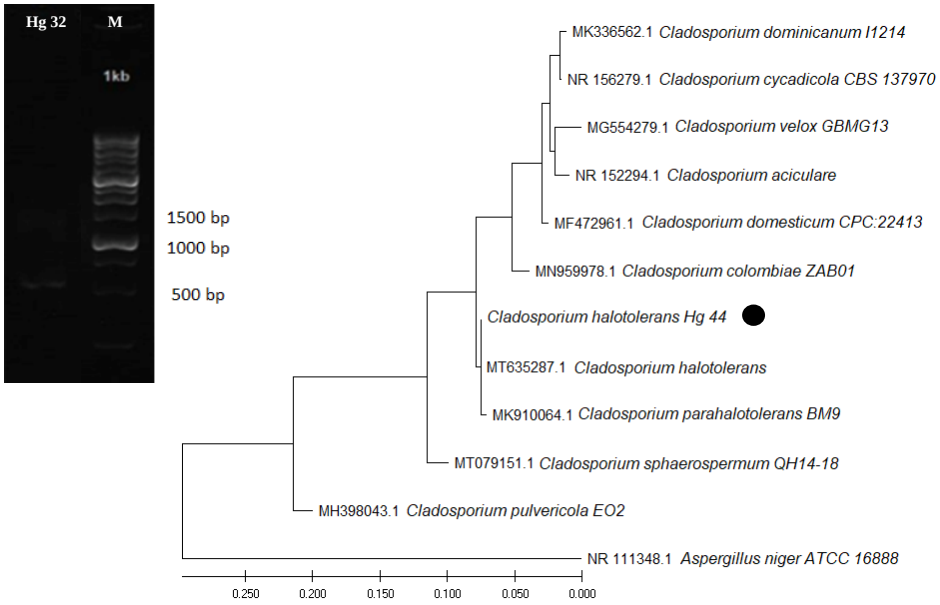

Figure 4. Phylogenetic tree of *Cladosporium sphaerospermum* Hg 32 based on ITS 3. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of base substitutions 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.

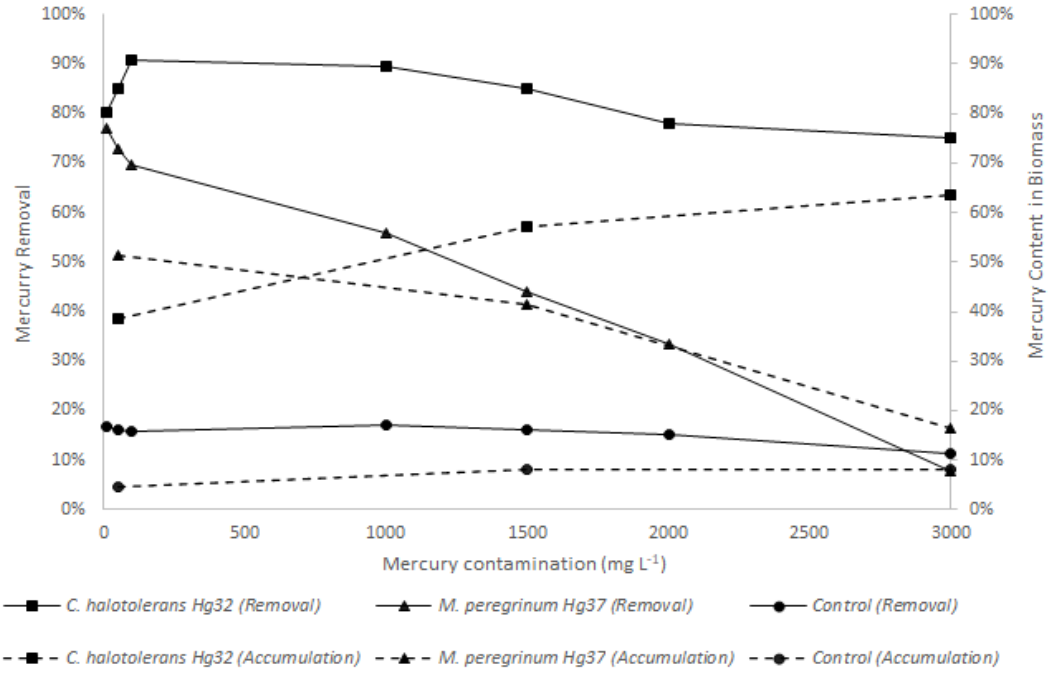

Figure 5. Percentage of removal of Hg^{2+} from solution following treatment with *Cladosporium halotolerans* Hg32 and *Mycolicibacterium peregrinum* Hg37 at the various concentrations

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 (*merA* and *merB*). Furthermore, Barrio-Duque et al. (2020) also reported that *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 *Mycolicibacterium* to be obtained as a bioaugmentation agent for heavy metals, including mercury in contaminated soil.

The motility of *C. carpio* and *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. *Cyprinus carpio* and *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, *C. Carpio* was incubated in water containing 0.1 mg L^{-1} mercury which was decontaminated by using *C. halotolerans* Hg32 and *M. peregrinum* Hg37. Meanwhile, *G. affinis* specimens were incubated in water containing 0.08 mg L^{-1}. The *G. affinis* was incubated at lower concentrations due to their lower lethal concentration level than *C. Carpio* (Ahmad 2011). Gupta and Jawale (2013) have reported that *G. affinis* have 96-h LC50 by 0,097 mg L^{-1} and 96-h LC100 by 0.148 mg L^{-1}. The bioassay results showed that the mortality rate of both *C. Carpio* and *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 $HgCl_2$ 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, *C. halotolerans* Hg42 and *M. peregrinum* Hg37.

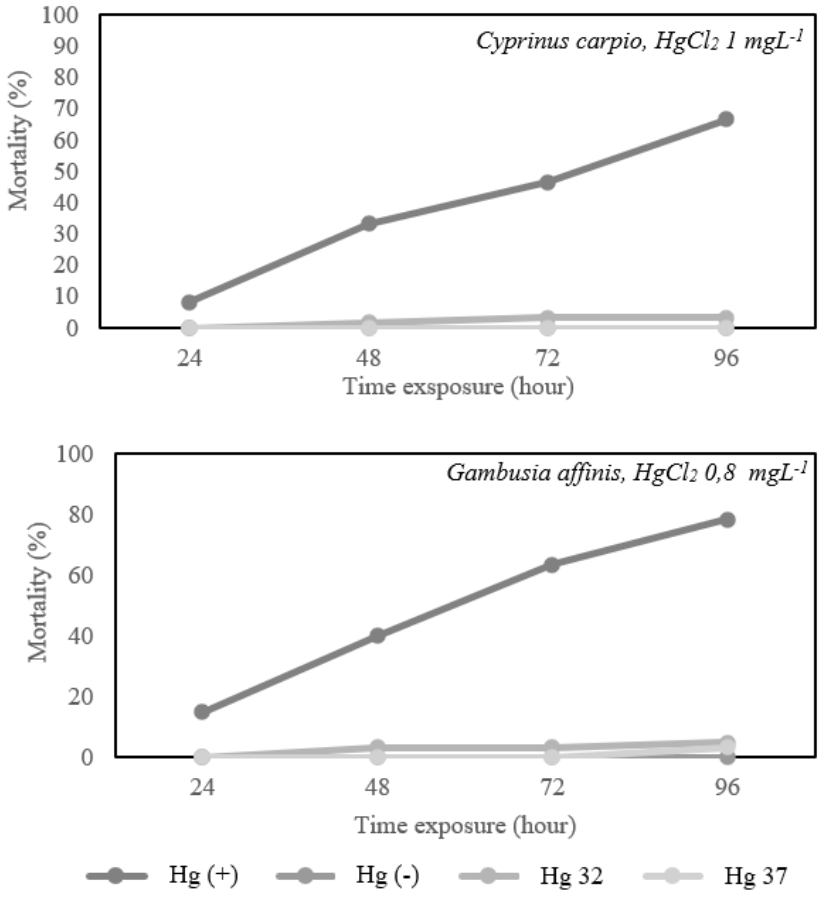

Figure 6. Percentage mortality of *Cyprinus carpio* and *Gambusia affinis* and after 96 h exposure to $HgCl_2$. Hg (+): contaminated water (negative control), Hg (-): noncontaminated water (positive control), Hg 32: decontaminated water using *Cladosporium halotolerans* Hg32, Hg 37: decontaminated water using *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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