

## Bioaccumulation of Mercury by Bacteria Isolated from Small Scale Gold Mining Tailings in Lombok, Indonesia

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### ABSTRACT

The activity of small scale gold mining (ASGM) in Indonesia has expanded to Lombok Island, West Nusa Tenggara. The use of mercury (Hg) and the traditional mining process cause that the ASGM tailings waste still contains Hg. The tailings waste is piled up near paddy fields and residential areas so that it has the potential to pollute the environment. This study aimed at isolating the bacteria that can accumulate Hg from the ASGM tailings in Lombok. The tailing samples were taken from Sekotong, Pujut, and Jonggat Districts, Lombok. The bacteria that have been isolated from the tailing samples were tested for their ability of Hg hemolysis resistance, and Hg bioaccumulation. Three bacteria with the highest Hg bioaccumulation ability were identified using the 16S rDNA gene squad. The results showed that out of 32 bacterial isolates, 27 isolates were found to be tolerant to Hg. Two bacterial isolates that were able to accumulate the highest amount of Hg and were identified as *Fictibacillus nanhainensis* (SKT-B) 82.25% and *Bacillus toyonensis* (PJM-F1) 81.21%. This study proved that the bacteria isolated from ASGM tailings have a great potential to be used as Hg bioaccumulation agents.

**Keywords:** Hg resistant bacteria, Hg bioaccumulation, small scale gold mine tailings

### INTRODUCTION

Small Scale Gold Mining (ASGM) activities are increasing in many low and middle-income countries, one of which is Indonesia (Basu et al., 2015). One of the islands in Indonesia that is the location of ASGM is Lombok, West Nusa Tenggara (Krisnayanti et al., 2012). The gold mining in Lombok is carried out using the amalgamation and cyanidation methods (Muddarisna et al., 2013). However, in some areas in Lombok, such as Sekotong, miners use the amalgamation method to separate gold from tailings (Utomo et al., 2014). In the amalgamation method, gold is extracted from gold ore using ‘gelondong’ (small iron cylindrical tubes) and mercury (Hg) (Krisnayanti, 2018). The traditional mining process causes an increase of the Hg contamination from

tailings to the environment. Every gram of gold produced through the amalgamation process releases 1–3 g of Hg into the environment through the tailings that are piled around the mining sites (Krisnayanti et al., 2012). Hg is one of the toxic elements that negatively affect the quality of the soil environment. The presence of Hg in the soil environment is associated with the presence of this metal in the lithosphere. Hg is present in very small amounts, but it can pose a threat to the animal and human organisms (Małuszyński et al., 2019). The human activities are increasing the heavy metals concentration in the ecosystem (Tjahjono and Suwarno, 2018).

Bioremediation is one of the remediation techniques that are considered to be more environmentally friendly than others (Ojuederie and Babalola, 2017). Besides, microbes are known

to be able to live under extreme conditions. The community of bacteria reflects the relationship between diversity and environmental factors (Fagorzzi et al., 2019). The bacteria that live in tailings have various mechanisms to protect themselves from the Hg toxicity, one of which is accumulation. Through the accumulation mechanism, the concentration of Hg in the bacterial cell tissue is higher than that in the environment in which it grows. This is because the metabolism and excretion of Hg from the bacterial body are slower than the absorption of Hg. As a result, Hg accumulates in the body of bacteria (Puspitasari, 2007).

Several studies have been conducted to explore the types of bacteria that can live in the soils with high Hg concentrations. Irawati et al., (2017) reported that the *Brevundimonas* sp. bacteria isolated from the ASGM soil in Pongkor Village, Bogor was able to accumulate 2.7 mg Hg g<sup>-1</sup> of the bacterial dry weight. Other bacteria found in the Sekotong District, Lombok by Chasanah et al., (2018) were able to accumulate 74.2–76.1% Hg in the Nutrient Broth (NB) medium enriched with HgCl<sub>2</sub> in the laboratory. *Bacillus subtilis* and *Burkholderia cenosepacia* isolated from the West Kalimantan ASGM tailings can accumulate Hg in their cells (Ekyastuti and Rima, 2015). This study aimed at elucidating the accumulation of Hg by the bacteria isolated from the ASGM tailings in Lombok.

## MATERIALS AND METHODS

### Tailing Samples

The tailings samples for bacterial isolation were collected from three gold processing sites in Lombok (Table 1) at a depth of 0–5 cm (Kuterbekov, 2019). The tailing samples were analyzed for pH (Moore, 1968), organic carbon content (Walkley and Black, 1934), organic matter content (Waksman and Stevens, 1930), available P content (Olsen et al., 1954), total N content (Kjeldahl, 1883), and total K content (Affinih et al., 2014). The results of the sample analysis are presented in Table 3.

### Bacterial Isolation

Isolation was carried out on Nutrient Agar (NA) medium using serial dilution and Pour Plate methods up to 10<sup>-7</sup> dilution series. The culture was incubated for 48 hours. The bacterial colonies that grew on each Petri were counted. The total bacterial population was calculated using the following formula (Husen et al., 2007):

$$\text{Total bacteria (CFU g}^{-1}\text{)} = \frac{\text{total colony} \times \text{fp}}{\text{soil weight}} \quad (1)$$

where: *fp* = dilution factor in Petri dishes whose colonies are counted

**Table 1.** Description of the study location

No	Location	District Code	Coordinate	Status of tailings collection	Vegetation / Land Use
1.	Sekotong Tengah village, Sekotong District, West Lombok	SKT	8°44'49.43" S 115°57'43.69" E	Not active	Meadows and tailings ponds
2.	Mawun Street, Mount Prabu Village, Pujut District, Central Lombok	PJT	8°53'27.00" S 116°14'54.00" E	Active	Without vegetation
3.	Batu Guling Street, Bonjeruk village, Jonggat District, Central Lombok	JGT	8°38'26.39" S 116°13'23.20" E	Not active	Tegalan, irrigated rice fields, and freshwater fish farming ponds

**Table 2.** Chemical properties of tailings

No	District	pH		C-Organic (%)	N Total (%)	C/N	Organic Matter (%)	P-Olsen (mg/Kg)	K (me/100g)	Hg (ppm)
		H <sub>2</sub> O	KCl							
1	JGT	7.2	7	0.24	0.03	7	0.42	11.69	0.56	23.19
2	PJT	8.1	7.6	0.16	0.03	6	0.28	29.13	0.28	9.87
3	SKT	7.3	7.2	0.08	0.03	3	0.14	43.46	0.3	15.69

Remarks: JGT: Jonggat; SKT: Sekotong; PJT: Pujut

**Table 3.** Identification of isolated bacteria with the highest Hg bioaccumulation ability

No.	Parameter	Isolate code	
		PJM-F1	SKT-B
1.	Colour of the colony at TSA	Beige	Beige
2.	Colony diameter (mm)	2,29	2, 39
3.	Colony form	Circular	Circular
4.	Colony margin	Undulate	Entire
5.	Colony Elevation	Convex	Convex
6.	Colony consistency	wet	wet
7.	Gram reaction	negative	negative
8.	Sel form	Rod	Rod
9.	Motility	Motil	Motil
10.	Oxidase	-	-
11.	Catalase	-	-
12.	Indol production	-	+
13.	Use of carbon from citrate	+	-
14.	TSIA test	Alk/As	Alk/As
15.	Oxidase	-	-
16.	Motility	+	+

Remarks: Alk / As (lactose or sucrose fermentation)

Morphology of the bacterial colonies was identified based on the Bergey's Manual Determinative of Bacteria system.

### Bacterial Tolerance Test

The bacterial tolerance test was carried out in Nutrient Agar (NA) enriched with  $\text{HgCl}_2$  (Irawati et al., 2013) according to treatment, namely 0 ppm (control), 25, 50, 75, 100, and 125 ppm using the streak method plates. The bacterial isolates were incubated for 14 days. The colonies that are resistant to Hg will grow and form new colonies on the streak line (Ekyastuti and Setyawati, 2015).

### Bacterial Pathogenicity Test

The bacterial pathogenicity test was used to determine the potential of bacterial pathogenicity through its ability to dissolve blood. The pathogenicity test was carried out on the Blood Agar medium (Tryptic Soy Agar (TSA) medium enriched with 5% sheep blood) using the streak plates method and incubated for 24 hours. The observation of the ability of bacterial hemolysis was carried out over 24 hours. The bacteria are capable of hemolyzing blood if a clear zone ( $\beta$  hemolysis) or media discolouration ( $\alpha$  hemolysis) around the colony turns to green or brown (Buxton, 2005). The bacteria that are unable to hemolyze blood ( $\gamma$  hemolysis) were used in further tests.

### Bacterial growth curve

The bacterial isolates that were unable to hemolyze blood were tested for their growing ability on the Luria Bertani (LB) medium enriched with  $\text{HgCl}_2$ . The bacteria that have been made inoculum stock were transferred to 25 mL of LB +  $\text{HgCl}_2$  medium with a new dose of 125 ppm with a concentration of 10% (v / v). The culture was incubated for 24 x 5 hours in the shaker (Irawati et al., 2013; Ekyastuti and Setyawati, 2015). The bacterial growth was observed every 24 hours by measuring the cell density using a spectrophotometer at a wavelength of 600 nm. The bacterial growth test in enriched media was repeated three times. The changes in the colour of bacterial colonies grown on  $\text{HgCl}_2$  enriched medium and without  $\text{HgCl}_2$  were also observed (Irawati et al., 2013).

### Hg accumulation test

The Hg accumulation test was carried out on 25 mL of LB medium enriched with 125 ppm  $\text{HgCl}_2$ . 2.5 mL of bacterial inoculum stock was added to 25 ml LB. The accumulation test of each bacterial isolate was repeated three times (Irawati et al., 2013). The culture was incubated for 24 x 5 hours at room temperature. The culture was observed on the fifth day or at the 120<sup>th</sup> hour. The culture inoculum was shaken using a centrifuge

at 10,000 rpm with a temperature of 4°C. The precipitate and supernatant from the culture were separated. The supernatant was transferred to a sterile test tube to measure the Hg concentration using an Atomic Absorption Spectrometer. The level of Hg accumulation by bacteria was determined through the formula (Ekyastuti and Setyawati, 2015). The ability of bacterial accumulation of each isolate was distinguished based on the percentage of bioaccumulation. Two isolates with the highest accumulation ability were selected for use in the next research stage.

$$\text{Bioaccumulation (\%)} = \frac{\text{total Hg of bacterial isolate}}{\text{total Hg in growing medium}} \times 100 \% \quad (2)$$

### Identification of potential bacterial species from tailings

Three selected bacterial isolates were identified through the biochemical and molecular tests using the 16S rDNA gene squad (Irawati et al., 2013; Cappuccino and Welsh, 2017). The identification was carried out on pure bacteria aged 24 hours using the Quick-DNA Fungal or Bacterial Microprep Kit (Zymo research corp., Tustin, USA). The extracted pure DNA was sent to Genetics Science Jakarta and 1<sup>st</sup> Base, Selangor Malaysia for identification. After the 16S rDNA gene from the bacteria was identified, the gene squad was entered into the Basic Local Alignment Search Tool (BLAST) which can be accessed through the National Center for Biotechnology Information (NCBI) website to identify the matches between the gene squares obtained. The final results from BLAST were matched with names or species in GenBank (Sanjay et al., 2018).

## RESULTS AND DISCUSSION

### Total bacterial population

The three gold processing districts have different bacterial populations. The highest bacterial population was obtained from Jonggat District (JGT) tailings with a total of  $8.50 \times 10^7$  CFU, followed by Sekotong District (SKT) tailings of  $1.93 \times 10^6$  CFU, and Pujut District (PJT) tailings of  $1.46 \times 10^6$  (Figure 1). The difference in bacterial populations is related to the different condition of the tailings in the three districts (Table 1). Bacteria are sensitive indicators for determining the changes in organic matter and other chemical properties in soil. Adoption of a different management system can have a negative or positive effect on the chemical properties of the soil (Araujo et al., 2012).

The chemical properties of soil affect the total bacterial population (Rao, 1977). The results of chemical analysis of tailings from the three districts showed different results. The tailings from Jonggat (JGT) has organic carbon (C-organic), total nitrogen (N), phosphorus (P), and exchangeable potassium (K) higher than others (Table 3). Carbon, nitrogen, and phosphorus are important elements that support the bacterial growth and metabolism, so the presence of these elements greatly affects the bacterial population (Lay and Hastowo, 1992; Subandi, 2014). Organic carbon is a source of carbon and energy for bacteria. The decomposition of complex organic materials into simpler organic materials or inorganic materials will produce energy in the form of ATP. The energy produced by the bacteria from the decomposition process will be used in the synthesis of macromolecules, such as nucleic acids, lipids, and polysaccharides (Lay and Hastowo, 1992).



**Figure 1.** The total bacterial population in tailings. JGT: Jonggat; SKT: Sekotong; PJT: Pujut

The formation of nucleic acids (RNA and DNA) in cells requires nitrogen and phosphorus. Nitrogen also plays a role in the formation of proteins responsible for the activity of cell metabolism and phosphorus needed in the synthesis of high-energy organic compounds such as ATP (adenosine triphosphate) (Subandi, 2014).

Cellular metabolism in bacteria also requires other elements in the form of minerals, such as potassium (Subandi, 2014). Potassium is the main intracellular cation in bacteria as well as in eukaryotic cells. The potassium absorption in cells is regulated by the ion and turgor concentrations. Potassium acts as a signal giver in the cytoplasm, activating and/or inducing the enzymes and transportation systems that allow cells to adapt to increased osmolarity (Epstein, 2003). Potassium is obtained by bacteria from basic compounds in the soil, so its availability greatly affects the bacterial population (Subandi, 2014).

The Hg concentration (Table 2) is directly proportional to the bacterial population (Figure 1). The Jonggat District tailing with the highest concentration of Hg has the highest bacterial population and vice versa. The results of this study differed from the previous studies that have been conducted. Most of the results showed that the bacterial population was negatively affected by the Hg concentration. The bacterial population and protozoa will decrease with the increase of the Hg concentration (Muller et al., 2001). Hg has been shown to negatively affect the conjugate transfer to plasmid (Rasmussen et al., 2008). However, considering the long history of the Hg contamination and the low concentration of the bioavailable Hg, a plausible explanation for the high diversity of the Hg-resistant population is a horizontal transfer of mobile genetic elements carrying the *mer*-operon (Rasmussen et

al., 2008). This results in the formation of bacteria that are tolerant of Hg, contributing to high bacterial populations in contaminated soil.

### The tolerance ability of isolates to Hg

There were 27 isolated bacterial isolates that could tolerate Hg at a test dose of 125 ppm (Figure 2). The ability of bacteria to tolerate Hg is because bacteria have the *mer*-operons that contain the encoding genes that can make the bacteria tolerant and have different mechanisms for adapting to the Hg stress (Ianieva, 2009).

The genes encoded by the *mer*-operons can be found in plasmids, chromosomes, transposomes, and integrins. Cellular elements (plasmids, transposons, and integrons) play an important role in the spread of mercury resistance throughout the microbial community through horizontal transfer. In addition, the resistant bacteria can also effectively transfer phenotypes (Hg resistant genes) to potentially pathogenic species (Morel et al., 1998; Kahl, 2015). This causes the mechanism of resistance that can also be found in the pathogenic bacteria.

The structure of *mer*-operons is different for each type of bacteria (Barkay et al., 2003). The key genes of the *mer*-operon are *merA* (gene reductase), *merT* (transport protein gene), *merP* (extracellular mercury-binding protein gene) and *merR* (regulatory protein gene). However, operons can contain additional genes, such as *merB* (organomercurial lyase gene), *merC* (transport protein gene), *merD* (regulatory protein gene), *merE* (transport protein gene), *merF* (transport protein gene) and *merG* (a gene that provides resistance to phenyl mercury) (Ianieva, 2009). The bacteria that possess only the mercury reductase (*merA*) gene are called narrow spectrum mercury

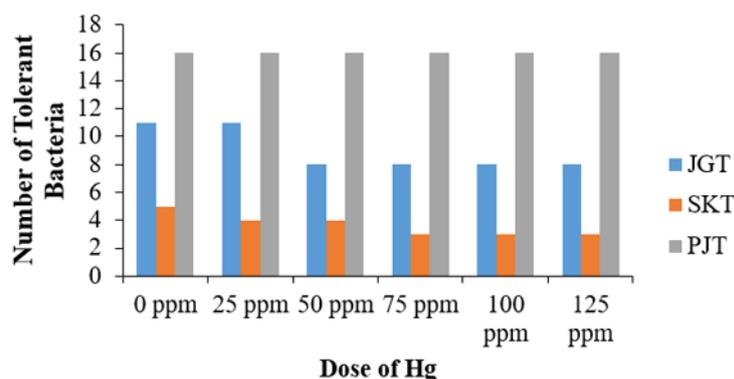


Figure 2. The ability of isolated bacteria to tolerate Hg. JGT: Jonggat; SKT: Sekotong; PJT: Pujut

resistant bacteria. Some bacteria that have genes other than *merA* or *merB* are called broad-spectrum mercury resistant bacteria. The *MerA* protein has the function of reducing the toxic mercury ions to mercury (Hg (0)) which is less toxic and volatile at room temperature. The *MerB* protein has the function of catalyzing the breaking of mercury-carbon bonds to produce organic compounds and Hg (II) ions (Barkay et al., 2003).

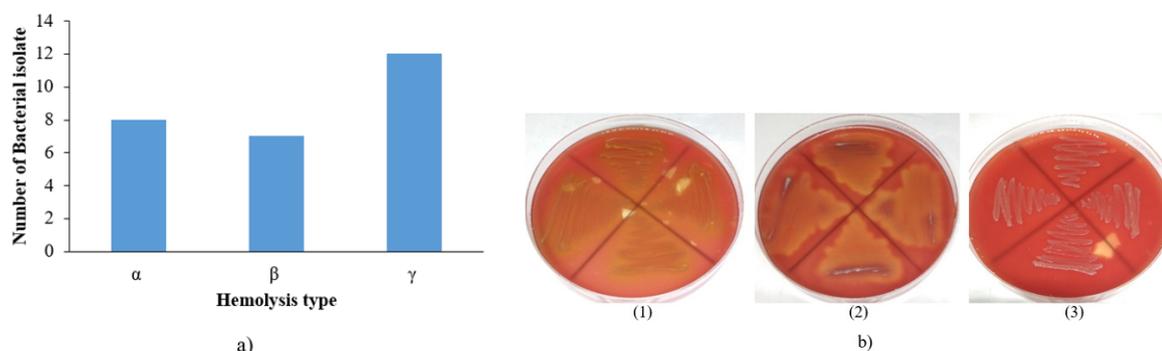
The ability of bacteria to transmit the resistance-coding genes or tolerance to the pathogenic bacteria causes a high potential for obtaining these bacteria during isolation. Reducing the potential use of pathogenic bacteria for the next stage of this study was carried out by testing the ability of blood hemolysis or pathogenicity tests. The results of the pathogenicity tests showed that 15 of the 27 isolates obtained were capable of the blood hemolysis. The ability of hemolysis is demonstrated by the formation of clear zones ( $\beta$  hemolysis) or changes in the color of the media ( $\alpha$  hemolysis) to green or brown around the bacterial colony (Buxton, 2016). There were 12 bacterial isolates that were unable to hemolyze blood ( $\gamma$  hemolysis) (Figure 3). *Escherichia coli* is a pathogenic bacterium that is known to be resistant to Hg. *E. coli* is known to be able to store the Hg resistant genetic markers that make this bacterial species a promising alternative to the bioremediation process (Costa et al., 2013). In addition, *E. coli* also passed the test using it as a Hg-resistant transgenic bacteria (Irawati et al., 2013).

### Bioaccumulation of Hg by isolated bacteria

The bacterial isolate with the highest Hg bioaccumulation ability was JGT-F2 (89.56%), followed by SKT-B (82.25%) and JGT-F1 (81.21%)

(Figure 4). Two bacteria with the highest Hg bioaccumulation ability were obtained from the Jonggat District tailings having the highest Hg concentration. One bacterial isolate with the highest ability to accumulate Hg was obtained from the Sekotong district tailings having the second-highest Hg concentration. In addition, the three highest accumulator bacteria also had the best ability to adapt to Hg in the test media. Figure 5 shows that JGT-F2, SKT-B, and JGT-F1 isolates were able to adapt quickly, and their growth was quite stable on LB + Hg media compared to other isolates. This shows that the higher the Hg concentration in a soil, the greater the ability of bacteria to adapt to toxic metals.

The differences in the ability of bacteria to accumulate Hg are caused not only by the differences in the Hg concentrations in their habitats but also by the differences in the response mechanisms. The mechanism of bacterial response to high Hg in the environment is done in three ways, namely: (1) by inhibiting cell metabolism so that cells grow slowly, even dying, (2) inducing an operon system that plays a role in Hg resistance so that cells remain alive under stress, and (3) having a plasmid containing the Hg resistance genes (Ekyastuti and Setyawati, 2015). In the Hg resistant and Hg accumulator bacteria, Hg in the soil is transported across the lipid membrane that surrounds the cell. The absorption of Hg by bacteria is a crucial step in bioaccumulation. Most metals enter cells through special transmembrane cation transporters or through other metal transporters. Hg at high concentrations is transported by bacteria through protein transport or *MerT*, whereas at low concentrations, Hg is absorbed through the lipid membrane in the form of water-soluble Hg. The chemical bonds



**Figure 3.** The blood hemolysis ability of the isolated bacteria. a) the number of bacteria based on the ability of hemolysis. b) the appearance of bacterial hemolysis in the Blood agar media, (1)  $\beta$  hemolysis, (2)  $\alpha$  hemolysis, (3)  $\alpha$  hemolysis

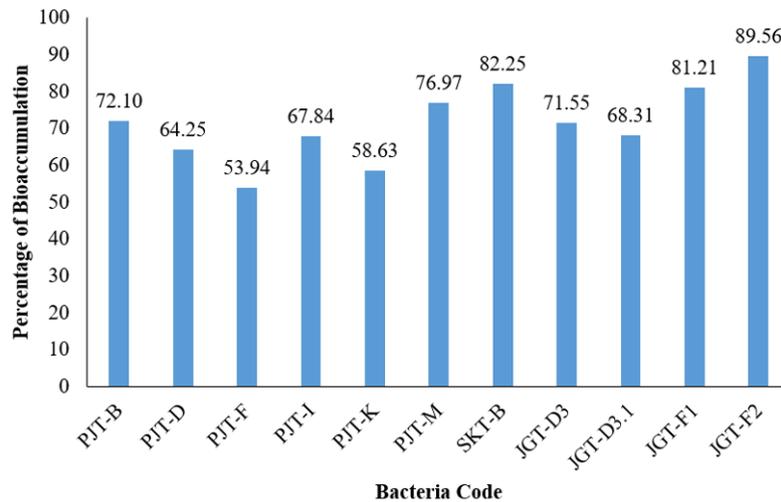


Figure 4. The ability of bioaccumulation of bacterial isolates on Luria Bertani Broth enriched Hg

in the  $HgCl_2$  complex are mostly covalent rather than ionic, so the complexes are relatively non-polar in charge and have sufficient fat solubility. Fat solubility is generally measured by the octanol-water partition coefficient, which measures the relative solubility of a compound in octanol and water and ranging from almost zero for very hydrophilic molecules to 108 for highly hydrophobic (Morel et al., 1998).

The pattern of bacterial cell growth rate is influenced by the characteristics of individual bacterial cells. The bacterial growth on the media containing heavy metals is influenced by the type of bacteria and the effect of heavy metals on bacterial cells. Different bacterial species have various tolerance abilities, which are caused by the toxic properties of Hg and the strength of

bacterial affinity for chelating agents in cells such as proteins, lipids and nucleic acids (Ekyastuti and Setyawati, 2015).

#### Identification of Hg bioaccumulator bacteria

The results of bacterial identification showed that the JGT-F1 is *Bacillus toyonensis* (Figure 6), and the SKT-B is *Fictibacillus nanhaiensis* (Figure 7). However, the identification results also showed that the JGT-F2 was not a pure isolate, so the isolate was eliminated from the list of bacteria with the best ability. The two best bacteria were identified as gram-negative bacteria in the form of rod (bacilli) (Table 3).

The operons in Gram-negative bacteria can encode some proteins. This is not found in the

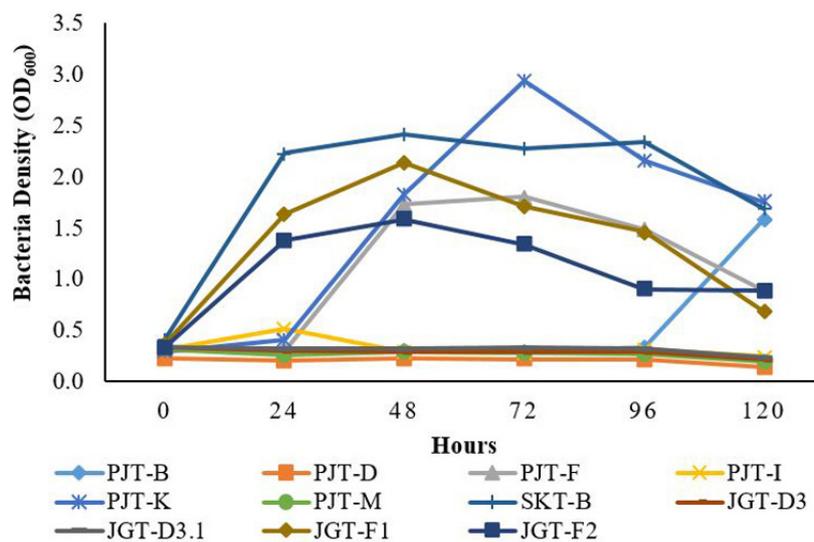


Figure 5. The growth curve of bacterial isolates on Luria Bertani Broth enriched with Hg

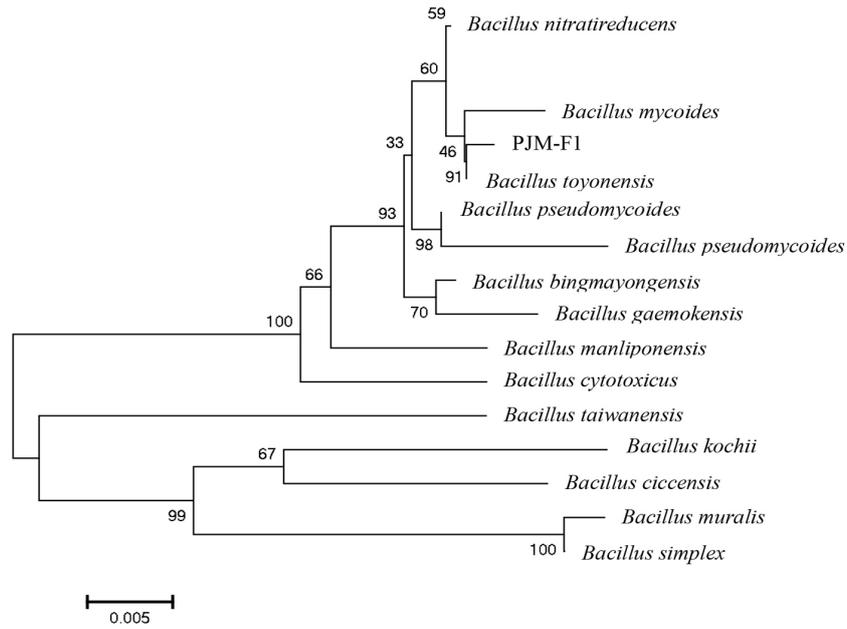


Figure 6. Phylogenetic tree of JGT-F1

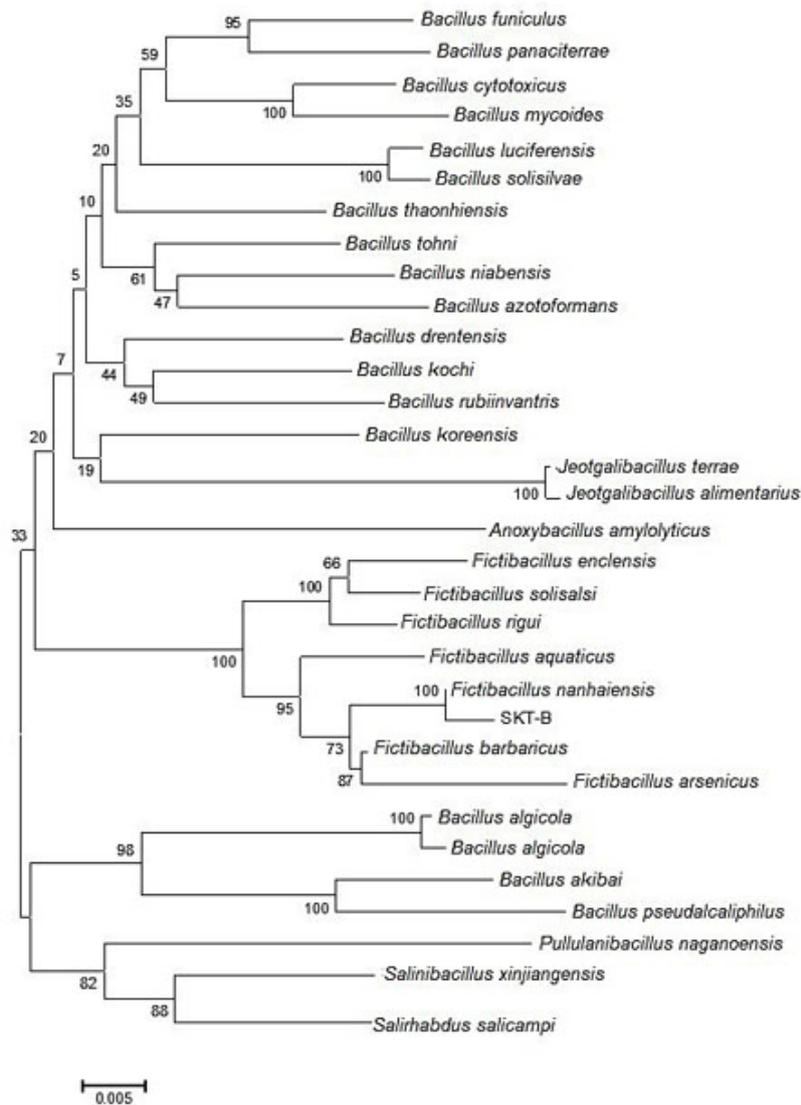


Figure 7. Phylogenetic tree of SKT-B

gram-positive bacterial operons. Some of them are membrane proteins or *MerC*, as well as *MerF* and *MerE*. *MerE* and *MerF*, which replace *MerC* in several operons, can work alone in the absorption of Hg (II), except *mer* on *Thiobacilli*, which is always known to be replaced by *MerT*. *Periplasmic* protein (*MerG*) is also found in some Gram-negative bacteria. This protein gives a slight increase in the organomercurial resistance, even in strains that lack *MerB*. This causes that in many Gram-negative bacteria there is an additional protein called *MerD*, which appears to be hostile to the *MerR* activation for the transcription of *mer*-operons (Barkay et al., 2003).

## CONCLUSION

The bacteria isolated from Pujut, Sekotong, and Jonggat Districts in Lombok Island, West Nusa Tenggara, were able to accumulate Hg with bioaccumulation capabilities of 53.94–89.56%. The bacteria found can be used as remediation agents for tailings so that the contamination of Hg from tailings can be reduced. Exploration of the ability of bacteria needs to be carried out further to find out the extent of the ability of bacteria to accumulate Hg and its mechanism.

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