Biofilm formation by mercury resistant bacteria from polluted soil small-scale gold mining waste

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Abstract. *Nurfitriani S, Arisoesilaningsih E, Nuraini Y, Handayanto E. 2022. Biofilm formation by mercury resistant bacteria from polluted soil small-scale gold mining waste. Biodiversitas 23: 992-999.* Small-scale gold mining has a major impact on living things and the environment due to mercury contamination. Bacteria are known to have a defense mechanism in mercury-contaminated soil. The biofilm produced by bacteria helps them survive and protect them from environmental stresses. This study aimed to find mercury-resistant bacteria capable of forming biofilms from mercury-contaminated soil in small-scale gold mining. Samples for bacterial isolation were mercury-contaminated soil from three gold processing sites in Lombok, Indonesia. The obtained mercury-resistant bacteria were tested to form biofilms on the media according to the test dose. The bacterial biofilms formed were observed, and each biofilm's adsorption capacity was measured. The three bacteria with highest adsorption values were identified molecularly using 16S rDNA. The results showed that most mercury-resistant bacteria were able to form biofilms at a dose of 25 ppm. However, only four bacteria were able to produce biofilms at a dose of 50 ppm. Biofilms of three bacteria had the highest adsorption values, ranging between 2.78-3.5. The three bacteria were identified as *Bacillus toyonensis* (JGT-F1), *Burkholderia cepacia* (PJT-K), and *Microbacterium chocolatum* (PJT-D). This study indicates that biofilm-producing bacteria are one of the remediation agents and can be used in mercury bioremediation.

Keywords: Bacterial biofilm, mercury-resistant bacteria, soil pollution, small-scale gold mining

INTRODUCTION

Soil contamination due to heavy metal contamination is a serious problem that requires rapid treatment. Heavy metal contamination is caused by various industrial activities, agriculture, mining, and adequate waste and wastewater recycling (Fabietti et al. 2010; Hindersah et al. 2018). The effects of high heavy metal toxicity, persistent properties, reactive at low concentrations and can accumulate in the food chain cause metal pollution above the threshold, which poses a great threat to all forms of living things, including microbes (Widyawati et al. 2007; Ayangbenro and Babalola 2017; Prabhakaran et al. 2016).

Mercury is a heavy metal that is commonly found in soil and has a great impact on living things. Mercury pollution is mostly contributed by small-scale gold mining with a total annual emission of 37% of global mercury emissions (Hagan et al. 2015; Esdaile and Chalker 2018). Gold mine tailings have a pH that tends to be acidic (Ekyastuti et al. 2016). This causes the level of available mercury in the soil to increase and increases the chances of mercury pollution. Mercury pollution in soils above threshold agriculture causes most food crops to accumulate mercury. Its accumulation in food crops above the threshold can lead to mercury contamination through the food chain (Hindersah et al. 2018). Mercury contamination can also reduce the population of bacteria in the soil. The results showed that mercury-contaminated soil in Lombok, Indonesia, only had a microbial population of 1.46-1.93×10^6 CFU and 8.5×10^7 CFU (Nurfitriani et al. 2020).

Microbes that can live in harsh environments are known to have defense mechanisms. Defense mechanisms allow microbes such as bacteria to convert metals into less harmful forms. This mechanism can also be reduced to form microbes that are resistant to heavy metals (Prabhakaran et al. 2016). Several interactions and defense mechanisms are formed due to the stress response to heavy metals, one of which is the formation of aggregates and biofilms (Prabhakaran et al. 2016). Bacteria in nature can be present in the form of a consortium attached (aggregate) and/or the surface of a substrate (biofilm) forming a community that can adapt to a less favorable environment. Cells in aggregates and biofilms are used by extracellular polymeric substances and adhere to surfaces (Davey and O'toole 2000). The bond formed by the extracellular polymeric substance allows the bacterial cell envelope to occur and protects the bacteria from physical contact with the environment. Extracellular polymers can bind heavy metal ions (Ianeva 2009). Bacteria that adhere to each other and/or adhere to surfaces (biofilms) are 2-600 times more resistant to heavy metals than single cells (Teitzel and Parsek 2003; Koechler et al. 2015). Formation of biofilm by *Pseudomonas* sp. survive on Cd-contaminated media with a concentration of 2 nM (Chien et al. 2013). *Sinorhizobium meliloti* is also known to survive various high concentrations of Hg and As elements by forming

biofilms (Nocelli et al. 2016). Bacteria that have defense mechanisms can be used as biological cleaning agents (Adhami et al. 2017). This study aimed to find mercury-resistant bacteria capable of forming biofilms from soil contaminated with small-scale gold mercury.

MATERIALS AND METHODS

Location of samples

Samples for bacterial isolation were the mercury-contaminated soil from three gold processing sites in Lombok, West Nusa Tenggara Province Indonesia. The sampling location was in the Sekotong sub-district (Lombok Barat District) with coordinates 8°44'49.43" S 115°57'43.69"E, Pujut sub-district (Lombok Tengah District) (8°53'27.00" S 116°14'54.00" E), and Jonggat sub-district (Lombok Tengah District) (8°38'26.39" S 116°13'23.20" E) (Figure 1).

Procedures

Bacterial isolate and growth medium

Previous research by Nurfitriani et al. (2020) has found several bacterial strains from gold mining tailings that were resistant to mercury. These strains were PJT-B, PJT-D, PJT-F, PJT-I, PJT-K, PJT-M (from Pujut), JGT-D3, JGT-D3.1, JGT-F1, JGT-F2 (from Jonggat), SKT- B, and SKT-C1 (from Sekotong). Bacterial isolates showed high resistance to HgCl2 at a test dose of 125 ppm. Bacterial isolates were grown in Luria Bertani (LB) broth containing the following (per liter): tryptone 10 g, yeast extract 5 g, and NaCl 10 g (Irawati et al. 2012).
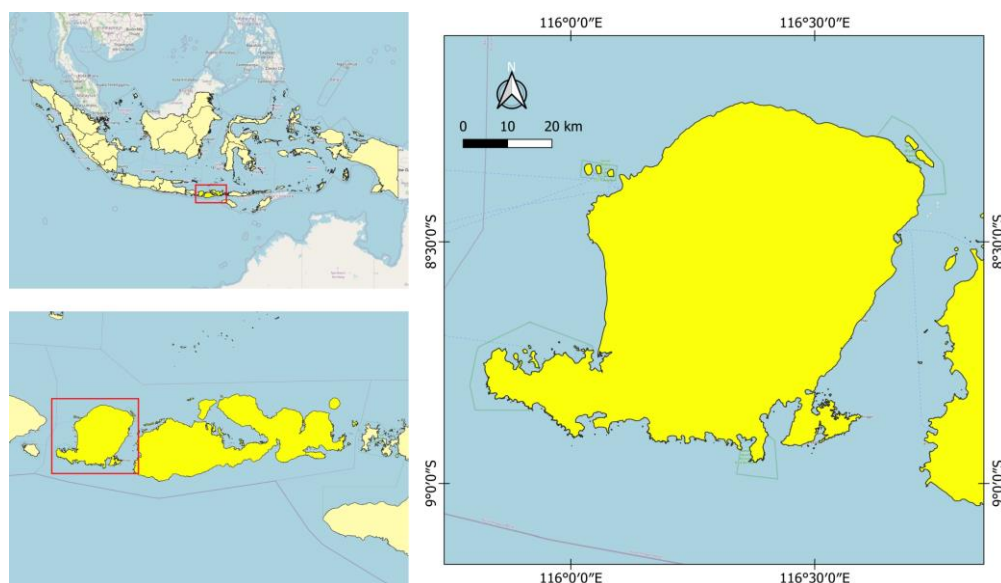
Qualitative test of the ability of bacteria to produce biofilm

Isolates of resistant bacteria that could accumulate mercury were grown on LB-broth medium and incubated until the concentration reached 2×10^8 CFU/ml. Isolate with a concentration of 2×10^8 CFU/ml was taken 5 l to add to 125 l of Hg-enriched LB-broth medium (at doses of 0, 25, 50, and 100 ppm) on a 96-well microplate (Corning, New York). Bacteria were incubated in microplate wells for 48 hours at room temperature. Bacterial incubation was carried out under static and dynamic conditions on a shaker at a speed of 120 rpm (Prihanto 2011). The suspension on the microplate well was carefully removed. Sterile LB-broth medium was added to the microplate wells with a volume of 100 l. Each well in the microplate well was added with 1% crystal violet dye to ensure the formation of a biofilm ring and waited for 1 hour. Crystal violet dye was added until each well was filled. The crystal violet dye was carefully removed after 1 hour and then wells were washed three times with sterile distilled water. Each well in the microplate well was visually examined for the presence of a crystal-violet stained ring. The ring in the well indicates the formation of a biofilm on the well's wall. This test was repeated three times for each isolated strain tested (Koczan et al. 2009). Three biofilms from the best bacterial isolates were analyzed for their structure using SEM (Scanning Electron Microscopy) and FTIR (Fourier Transform Infrared Spectroscopy) (Koczan et al. 2011; Consumi et al. 2020).

Quantitative test of the ability of bacteria to produce biofilm

The biofilm formed on the microplate well was measured using a caliper. The difference in the biofilm formation of each bacterium was measured by the concentration of the dye solution produced in the microplate well. Each well was added 200 l of a solution consisting of 40% methanol and 10% glacial acetic acid to remove the crystal violet dye. The dissolved crystal violet was measured using spectrophotometry at an adsorbance of 600 nm using a microplate reader (Tecan, Research Triangle Park, NC). Each isolate was tested in a 96-well microplate and the experiment was repeated three times (Koczan et al. 2009). The three best isolates were then identified at molecular level.

Figure 1. Location of mercury-contaminated soil samples

Identification of biofilm-forming bacteria

The three best bacterial isolates capable of producing biofilms were identified using 16S-rRNA gene sequence PCR (Chien et al. 2013). Physiological tests were also carried out to determine the biochemical characteristics of bacteria. DNA isolation of the selected isolates was carried out using the Quick-DNA Fungal or Bacterial Microprep Kit extraction kit (Zymo research corp., Tustin, USA). DNA isolation was performed from bacteria aged 24 hours (Dliyauddin et al. 2020). The pure DNA was then put into a PCR (Polymerase chain reaction) machine to detect RNA and the PCR results were visualized to determine the bands formed. The pure DNA was sequenced at 1st BASE DNA Sequencing Service, Malaysia. The pure DNA was compared with the 16S rDNA gene sequences available on the National Center for Biotechnology Information (NCBI) website and used the "Basic Local Alignment Search Tool" (BLAST) to identify matches between the obtained gene sequences and existing references. Finally, the BLAST results were matched with GenBank species (Sanjay et al. 2018).

Statistical analysis

Data of bacterial biofilm were analyzed by Analysis of Variance (ANOVA) using SPSS 16.0 software for windows. The significant differences was determined based on Duncan multiple range Test (DMRT) analysis with p < 0.05.

RESULTS AND DISCUSSION

Formation of biofilm by mercury-resistant bacteria (Qualitative test)

The biofilm formation test was carried out on 12 mercury-resistant bacteria capable of accumulating mercury, namely PJT-B, PJT-D, PJT-F, PJT-I, PJT-K, PJT-M, JGT-D3, JGT-D3.1, JGT -F1, JGT-F2, SKT-B, and SKT-C1. The test results showed that mercury-resistant bacteria had different abilities to produce biofilms (Table 1). The difference in the ability of bacteria to form biofilms was closely related to the defense mechanism of each bacterium in an unfavorable environment, such as the presence of heavy metal stress. It was also observed that all isolates could form biofilms at a concentration of 0 ppm mercury. However, the biofilm produced by bacteria was not confirmed as a form of tolerance to mercury. At a dose of 25 ppm mercury, most bacteria produced biofilm, while at a dose of 50 ppm only 4 bacteria were able to produce biofilm.

The formation of bacterial biofilm in a medium may also be influenced by the conditions of the medium (static and dynamic). The results showed that some bacteria required different conditions to produce biofilms (Table 2). However, some bacteria can produce biofilms under two different conditions. Isolates with codes JGT-F1 and PJT-K were able to produce biofilms under static and dynamic conditions, while other isolates could only form biofilms on static media.

Quantitative test of mercury-resistant bacterial biofilm

The thickness of the biofilm produced in each bacteria was different. Measurements were made on isolates capable of forming biofilms at a test dose of 50 ppm (Table 1). The measurement results showed that in static conditions, biofilm produced by JGT-F1 isolates was greater than the others (Figure 2). The adsorbance value on the microplate well also showed that the color adsorption bound to the biofilm was very high (Figures 3 and 4).

Based on the results of statistical tests, it was found that there was no interaction between the dose of HgCl in the media and the thickness of biofilm. However, it was noted that there was an interaction between the amount of color bound in the biofilm and the dose of HgCl. This indicates that the volume of bacterial biofilm may increase in media with higher HgCl doses. The adsorbance value of PJT-K and JGT-F1 isolates solution at a test dose of 50 ppm (Figure 3) was exceeded, so they had the same value. This shows that the adsorption value of the solutions of the two isolates is very high. However, based on observations of the bacterial biomass attached to the wall of the microplate well, it was revealed that the biofilm of JGT-F1 isolate was thicker (Figure 2) and the color produced after staining was more concentrated (Figure 4).

Table 1. Results of the ability of mercury-resistant bacteria in forming biofilms
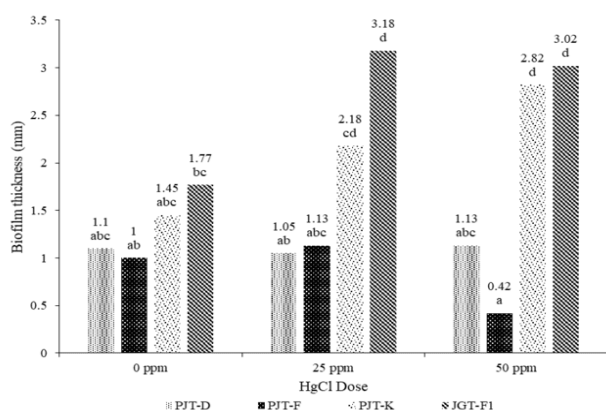
Isolate codes	Doses			
	0 ppm	25 ppm	50 ppm	100 ppm
PJT-B	√	-	-	-
PJT-D	√	√	√	-
PJT-F	√	√	√	-
PJT-I	√	√	-	-
PJT-K	√	√	√	-
PJT-M	√	-	-	-
JGT-D3	√	√	-	-
JGT-D3.1	√	√	-	-
JGT-F1	√	√	√	-
JGT-F2	√	√	-	-
SKT-B	√	-	-	-
SKT-C1	√	√	-	-

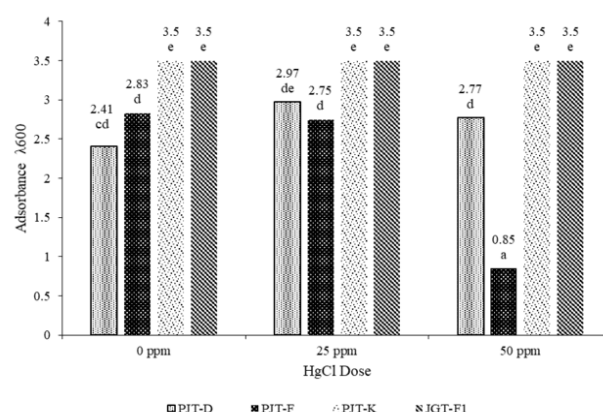Remarks: √ (capable of producing biofilms/biofilm positive), - (unable to produce biofilm/biofilm negative).

Table 2. Formation of bacterial biofilms on different conditions

Isolate codes	Medium conditions	
	Static	Dynamic
PJT-B	√	-
PJT-D	√	-
PJT-F	√	-
PJT-I	√	-
PJT-K	√	√
PJT-M	√	-
JGT-D3	√	-
JGT-D3.1	√	-
JGT-F1	√	√
JGT-F2	√	-
SKT-B	√	-
SKT-C1	√	-

Note: √: capable of producing biofilms/biofilm positive, -: unable to produce biofilm/biofilm negative.

Figure 2. Biofilm thickness in static condition. Numbers followed by different letters showed significant differences based on post hoc DMRT test at 5% level ($p < 0.05$)
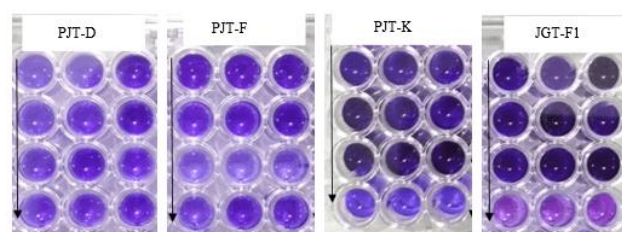

Figure 3. Adsorption on biofilms produced by bacteria. Numbers followed by different letters showed significant differences based on the post hoc DMRT test at 5% level ($p < 0.05$)
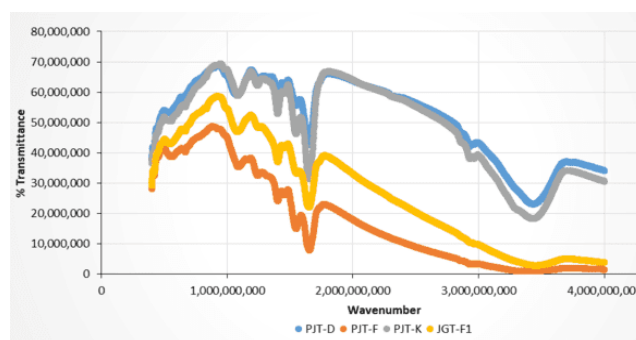
SEM and FTIR of bacterial biofilm

Scanning electron micrographs on three bacteria with the highest biofilm adsorption values are shown in Figure 5. Figure 5 shows the biofilm that has grown on the microplate well for 48 hours. The results of scanning electron micrographs showed that the structure and thickness of biofilm of each bacterium were different. The loose structure and lack of adhesion between biofilms indicate that the biofilm was in a more advanced stage of development. Longer incubation can make the bacterial biofilm structure more robust.
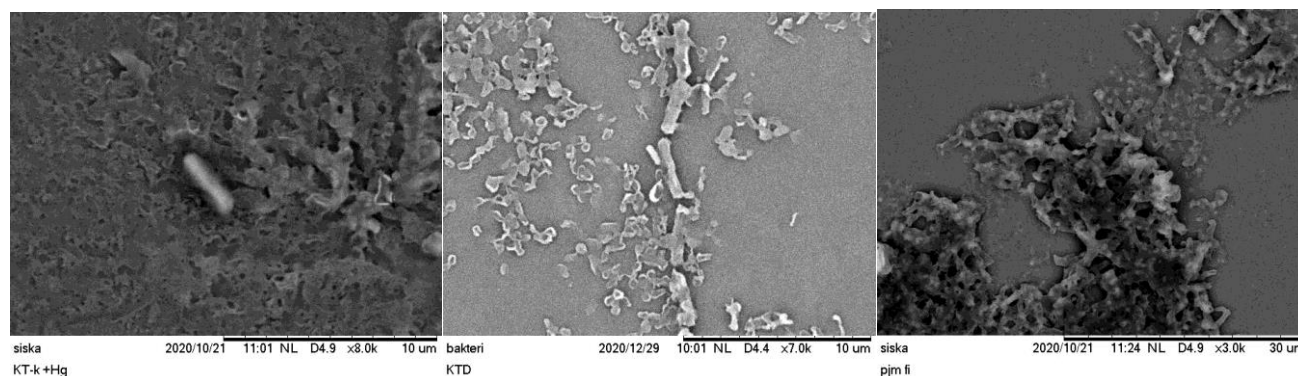
The FTIR result showed that there were NH stretching vibrations at 3433 cm-1 (in PJT-D), 3462 cm-1 (JGT-F1), and 3433 cm-1 (PJT-K1) which can be interpreted as the presence of amines (NH) in bacterial biofilms. There were aromatic ring bending vibrations in the three samples including 1650; 1544; 1401 cm-1 in PJT-D; 1651; 1556; 1401 cm-1 in JGT-F1; 1654; 1653; and 1546; 1401 cm-1 in PJT-K (Figure 6). The discovery of N-H stretching vibrations (amine group) and aromatic ring bending vibrations in the biofilms of the three bacteria can be predicted that the biofilms may contain aromatic amine species. The percent transmittance ratio of each resulting spectrum shows the quality of the aromatic amine species in each sample was different.

Figure 4. The thickness of color produced by the biofilm of each bacterium

Figure 6. Measurement of adsorption spectra using FTIR on bacterial biofilm

Figure 5. Scanning electron micrograph (SEM) biofilm formed from mercury-resistant bacteria

Identification of biofilm-producing mercury-resistant bacteria

The results of the identification of three biofilm-producing bacteria with the highest biofilm adsorption values showed that PJT-D was *Microbacterium chocolatum* (Figure 7), PJT-K was *Burkholderia cepacia* (Figure 8), and JGT-F1 was *Bacillus toyonensis* (Figure 9). All the three best bacteria were identified as gram-negative bacteria with rods or bacilli (Table 3).

Discussion

A group of bacteria with the same or different types that can be found attached to the surface or bound to each other in a certain environment which is usually called a biofilm. Biofilm is a form of a bacterial resistance mechanism and an unfavorable environment causes bacteria to have this kind of mechanism. The results of this study showed that most of the tested bacter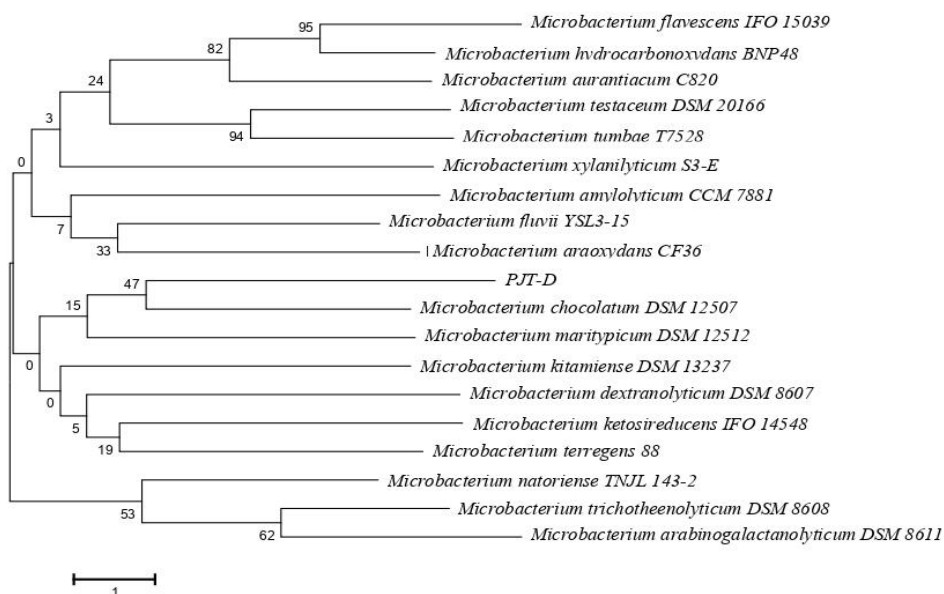ia were able to form biofilms at a test dose of 25 ppm. But when the test dose was increased to 50 ppm HgCl only 4 bacteria were able to form biofilms. This indicates the level of ability of biofilm formation by bacteria is different.

Bacterial biofilms can be formed when bacteria are grown on HgCl-enriched test media because they are able to produce extracellular polymeric substances that function as barriers. The barrier of extracellular polymeric substances serves to prevent metals from entering the cell. In the extracellular barrier mechanism, the cell wall, plasma membrane or capsule can prevent metal ions from entering the cell through the adsorption of metal ions (adsorption). The extracellular barrier consisting of carboxyl, amino, phosphate and hydroxyl groups is also a passive process so that dead bacterial cells are also able to bind metal ions (Ianieva 2018).
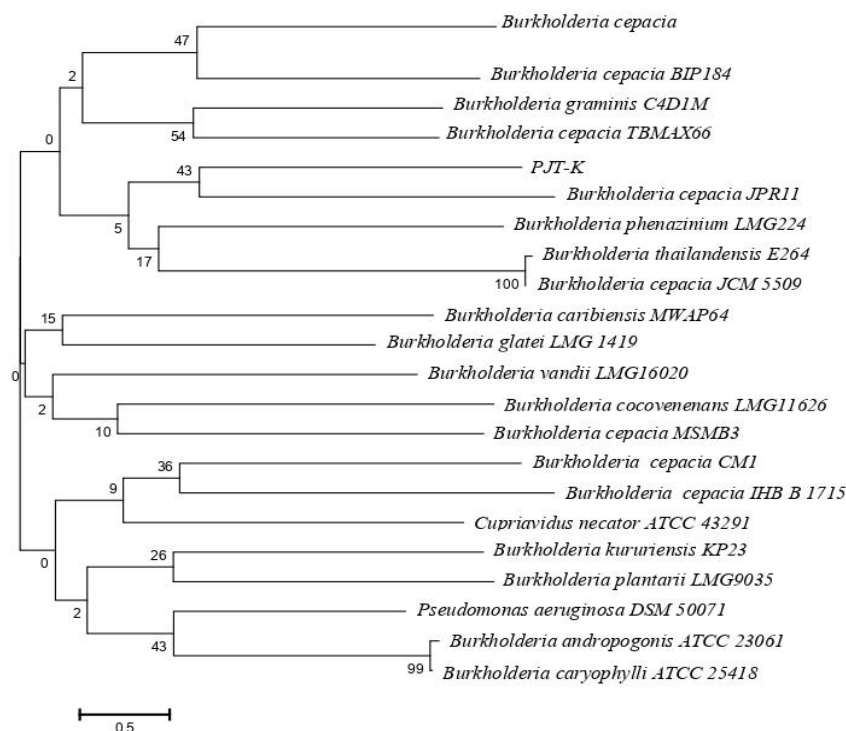
Table 3. Morphological characters of biofilm-forming mercury-resistant bacteria

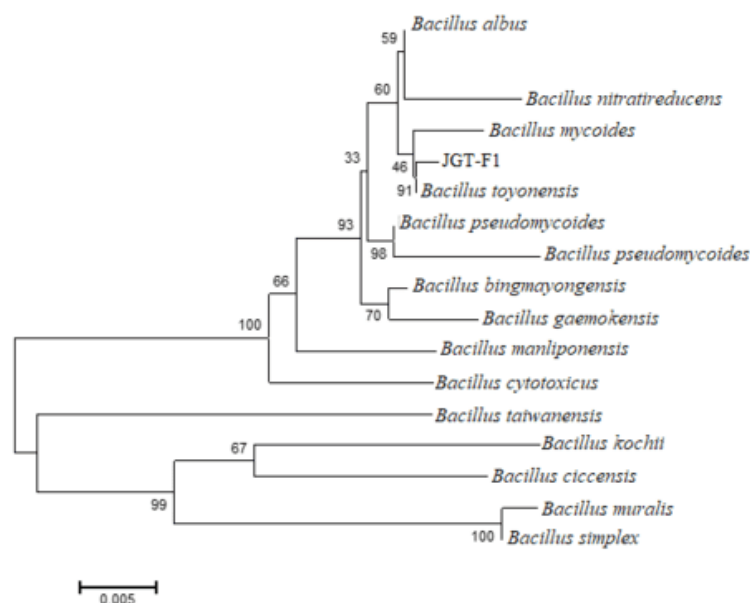| Tests	Isolate codes		
	PJT-D	PJT-K	PJM-F1
Colour of the colony at TSA	Beige	Beige	Beige
Colony diameter (mm)	2.29	2.03	2.29
Colony form	Circular	Oval	Circular
Colony margin	Undulate	Undulate	Undulate
Colony elevation	Convex	Convex	Convex
Colony consistency	Wet	Dry	Wet
Gram reaction	Negative	Negative	Negative
Cell form	Bacilli	Bacilli	Bacilli
Motility	Motile	Motile	Motile
Oxidase	-	+	-
Catalase	-	-	-
Indol production	-	-	-
Use of carbon from citrate	+	+	+
TSIA test	Alk/As	Alk/Alk	Alk/As

Note: Alk/As (fermented lactose or sucrose), Alk/Alk (unfermented sugar)

Figure 7. Phylogenetic tree of PJT-D

Figure 8. Phylogenetic tree of PJT-K

Figure 9. Phylogenetic tree of JGT-F1

Four bacteria capable of producing biofilms on medium enriched with high concentrations of Hg, namely PJT-D, PJT-F, PJT-K, and JGT-F1 can be used in bioremediation. This is because the formation of microbial biofilms and production of extracellular polymeric substances are generally associated with metal resistance, tolerance, and bioremediation (Chien et al. 2013). Biofilm formation is strongly influenced by the presence of heavy metals. Biofilm formation begins with exopolysaccharide polymer substances that act as a barrier to isolate bacterial cells from environmental stress. Furthermore, cell metabolism changes and cells that develop slowly or do not grow (persistently) begin to appear. In multispecies biofilms the capacity to survive is greater than that in single-species biofilms due to evolving metabolic interactions (Koechler et al. 2015). Other studies have shown that biosorption or bioaccumulation of heavy metals by extracellular polymeric substances or exopolysaccharide (EPS)

produced by *Pseudomonas* sp., is one of the important mechanisms that contribute to its resistance to heavy metals. The accumulation of metal ions by living cells (in *Pseudomonas putida*, *Brevibacterium* sp., and *Bacillus* sp.) occurs in two steps, such as, initial rapid nonspecific adsorption by the cell wall and then slowing the active transport of metal ions into the cytoplasm (Ianieva 2018). Biofilm formation is not only influenced by the presence of heavy metals but also the condition of medium. Static and dynamic medium conditions determine the formation of biofilms. Most of the biofilms formed under static conditions, but isolates JGT-F1 and PJT-K formed more biofilms on dynamic media. The difference in biofilm thickness under dynamic conditions indicates that the movement of the media makes bacterial cells multiply or divide under unfavorable conditions. It is also possible for bacteria to produce more extracellular polymeric substances to protect themselves. Most bacteria only form biofilms under static conditions because shaking causes bacteria to not stick to the surface of the substrate. Fimbria in bacteria plays an important role in the formation of biofilms. In static conditions, bacterial fimbria can modify the substrate surface by adsorbing components that are in contact with the substrate surface, such as inorganic salts, proteins, and glycoproteins. Shaking may cause the adsorption of the substrate surface by the fimbria to be smaller so that the biofilm is not formed (Prihanto 2011). Besides fimbriae, other bacterial surface structures such as flagella, outer membrane proteins, colanic acid, and poly-b-1,6-N-acetyl-d-glucosamine are known to contribute to biofilm formation under static growth conditions (Naves et al. 2008).

The biofilm formation produced by each bacterium can be compared by measuring manually using a caliper and crystal violet staining. In crystal violet staining, the color density is a measure of the amount of biofilm produced by bacteria. The darker the color produced when the biofilm was washed after staining, the more dye bound to the biofilm wall and the more biofilm produced by bacteria. The color density produced after staining the biofilm was shown through the measurement results of the adsorbance on each sample. The ability of bacteria to produce biofilms can be easily observed on the walls of the test site using crystal violet dye. This is because the biofilm is a biologically active cell-matrix and the extra-cellular substances are associated with solid surfaces (Garrett et al. 2008).

The result showed that the three bacteria with the highest biofilm adsorption values were PJT-D, PJT-K, and PJT-F1. Results exhibited that the relationship between microbial cell layer of the three bacteria (which consisted of a biologically active cell-matrix and extra-cellular substances) and the solid surface (microplate well wall) was not very strong. This can be seen from the results of scanning electron micrograph (SEM) biofilms which show loose and less attached structures between biofilms. This result is different from the research of Wagner-Dobler et al. (2000), where the biofilm grown in a sterile wastewater model reactor with an operating time of 14 days showed a single layer of microbial cells was attached to the surface

of the carrier material. In this system, the bacterial biofilm adheres strongly and acts as a catalyst to remove mercury (Wagner-Dobler et al. 2000). One of the three bacteria with the highest biofilm formation ability in this study was JGT-F1, had a denser biofilm structure than the others. The addition of an incubation time that is longer than the initial incubation time (48 hours) allows the formation of a dense biofilm structure and adheres firmly to the surface. The best condition for biofilm formation of *Rhodococcus rhodochrous* and *Rhodococcus rhodnii* isolate was determined after incubation at 96 hours (Adhami et al. 2017).

The three bacteria with the highest biofilm adsorption values in this study, namely PJT-D, PJT-K, and PJT-F1 were identified as *Microbacterium chocolatum*, *Burkholderia cepacia*, and *Bacillus toyonensis*. *Burkholderia cepacia* (PJT-K) has been known as a biofilm-forming bacterium. *B. cepacia* is a member of the *Burkholderia cepacia* complex. The *B. cepacia* complex consists of 17 closely related and phenotypically similar bacterial species. *B. cepacia* is a bacterial pathogen that causes severe lung infections in people with weakened immune systems. *B. cepacia* biofilm makes it more persistent and resistant to antibiotics (Murphy and Caraher 2015; Van Acker et al. 2013). *Microbacterium chocolatum* is known to be effective in supporting soybeans in the phytoremediation of deltamethrin contaminated soil (Erguven and Koçak 2019). *Bacillus toyonensis* (PJT-F1) is a bacterium that is commonly found in soil and lives as a saprophyte (Agamennone et al. 2019). These bacteria are also used to produce microbial flocculants. Microbial flocculants from *B. toyonensis* are good substitutes for hazardous chemical flocculants currently used in drinking water or sewage treatment (Okaiyeto et al. 2015). Additional knowledge about biofilms in this study makes it possible to utilize *B. toyonensis* for bioremediation of heavy metals, especially mercury.

Based on this research, it can be concluded that bacteria isolated from soil contaminated with small-scale gold mines in Lombok were able to form biofilms on mercury-contaminated media up to a test dose of 50 ppm. The adsorption value on the biofilm produced by bacteria ranged from 0.8-3.5. Of the three, two bacteria, namely *Burkholderia cepacia* and *Bacillus toyonensis*, had a higher adsorbance values than others. The bacteria found can be used as bioremediation agents for mercury-containing waste. Further research needs to be done to determine the maximum biofilm production by bacteria and the appropriate incubation time to produce mature biofilms.

REFERENCES

Adhami E, Aghaei S, Zolfaghari MR. 2017. Evaluation of heavy metals resistance in biofilm cells of native *Rhodococcus* spp. isolated from soil. Arch Hyg Sci 6 (3): 235-243. DOI: 10.29252/archhygsci.6.3.235.

Agamennone V. Agamennone V, van Straalen J, Brouwer A, de Boer TE, Hensbergen PJ, Zaagman N, Braster M, van Straalen NM, Roelofs D, Janssens TKS. 2019. Genome annotation and antimicrobial properties of *Bacillus toyonensis* VU-DES13, isolated from the *Folsomia candida* gut. Entomol Exp Appl 167 (3): 269-285. DOI: 10.1111/eea.12763.

Ayangbenro AS, Babalola OO. 2017. A new strategy for heavy metal polluted environments: A review of microbial biosorbents. Intl J Environ Res Public Health 14 (1): 1-16. DOI: 10.3390/ijerph14010094.

Chien CC, Lin BC, Wu CH. 2013. Biofilm formation and heavy metal resistance by an environmental *Pseudomonas* sp. Biochem Eng J 78: 132-137. DOI: 10.1016/j.bej.2013.01.014.

Consumi M, Jankowska K, Leone G, Rossi C, Pardini A, Robles E, Wright K, Brooker A, Magnani A. 2020. Non-destructive monitoring of *P. fluorescens* and *S. epidermidis* biofilm under different media by fourier transform infrared spectroscopy and other corroborative techniques. Coatings 10 (10): 930. DOI: 10.3390/coatings10100930.

Davey ME, O'toole GA. 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64 (4): 847-867. DOI: 10.1128/mmbr.64.4.847-867.2000.

Dliyauddin M, Ardyati T, Suharjono. 2020. Evaluation of enzymatic activity and identification of potent proteolytic and chitinolytic bacteria isolated from crab shell waste. Biodiversitas 21 (1): 211-218. DOI: 10.13057/biodiv/d210127.

Ekyastuti W, Astiani D, Roslinda E. 2016. Prospect of indigenous plant species for revegetation in the tailings area of ex community gold mine. Biodiversitas 17 (2): 764-768. DOI: 10.13057/biodiv/d170252.

Erguven GO, Koçak E. 2019. Determining the detoxification potential of some soil bacteria and plants on bioremediation of deltamethrin. Eurasian J Agric Res 3 (1): 36-47.

Esdaile LJ, Chalker JM. 2018. The mercury problem in artisanal and small-scale gold mining. Chem-Eur J 24 (27): 6905-6916. DOI: 10.1002/chem.201704840.

Fabietti G, Biasioli M, Barberis R, Ajmone-Marsan F. 2010. Soil contamination by organic and inorganic pollutants at the regional scale: The case of piedmont, Italy. J Soil Sediment 10 (2): 290-300. DOI: 10.1007/s11368-009-0114-9.

Garrett TR, Bhakoo M, Zhang Z. 2008. Bacterial adhesion and biofilms on surfaces. Prog Nat Sci 18 (9): 1049-1056. DOI: 10.1016/j.pnsc.2008.04.001.

Hagan N, Robins N, Hsu-Kim H, Halabi S, Gonzales RDE, Ecos E, Richter D, Vandenberg J. 2015. Mercury hair levels and factors that influence exposure for residents of Huancavelica, Peru. Environ Geochem Health 37 (3): 507-514. DOI: 10.1007/s10653-014-9665-9.

Hindersah R, Risamasu R, Kalay AM, Dewi T, Makatita I. 2018. 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.

Ianieva OD. 20018. Mechanisms of bacteria resistance to heavy metals. Mikrobiolohichnyĭ zhurnal 71 (6): 54-65.

Irawati W, Patricia, Soraya Y, Baskoro AH. 2012. A Study on mercury-resistant bacteria isolated from a gold mine in Pongkor Village, Bogor, IndonesiA. HAYATI J Biosci 19 (4): 197-200. DOI: 10.4308/hjb.19.4.197.

Koczan JM, Lenneman BR, McGrath MJ, Sundin GW. 2011. Cell surface attachment structures contribute to biofilm formation and xylem colonization by *Erwinia amylovora*. Appl Environ Microbiol 77 (19) 7031-7039. DOI: 10.1128/AEM.05138-11.

Koczan JM, McGrath MJ, Zhao Y, Sundin GW. 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: Implications in pathogenicity. Phytopathology 99 (11): 1237-1244. DOI: 10.1094/PHYTO-99-11-1237.

Koechler S, Farasin J, Cleiss-Arnold J, Arsene-Ploetze F. 2015. Toxic metal resistance in biofilms: Diversity of microbial responses and their evolution. Res Microbiol 166 (10): 764-773. DOI: 10.1016/j.resmic.2015.03.008.

Murphy MP, Caraher E. 2015. Residence in biofilms allows *Burkholderia cepacia* complex (Bcc) bacteria to evade the antimicrobial activities of neutrophil-like dHL60 cells. Pathog Dis 73 (8): 1-13. DOI: 10.1093/femspd/ftv069.

Naves P, del Prado G, Huelves L, Gracia M, Ruiz V, Blanco J, guez-Cerrato VR, Ponte MC, Soriano F. 2008. Measurement of biofilm formation by clinical isolates of *Escherichia coli* is method-dependent. J Appl Microbiol 105 (2): 585-590. DOI: 10.1111/j.1365-2672.2008.03791.x.

Nocelli N, Bogino PC, Banchio E, Giordano W. 2016. Roles of extracellular polysaccharides and biofilm formation in heavy metal resistance of rhizobia. Materials 9 (6): 1-19. DOI: 10.3390/ma9060418.

Nurfitriani S, Arisoesilaningsih E, Nuraini Y, Handayanto E. 2020. Bioaccumulation of mercury by bacteria isolated from small scale gold mining tailings in Lombok, Indonesia. J Ecol Eng 21 (6): 127-136. DOI: 10.12911/22998993/123247.

Okaiyeto K, Nwodo UU, Mabinya LV, Okoh AI. 2015. *Bacillus toyonensis* strain AEMREG6, a bacterium isolated from South African marine environment sediment samples produces a glycoprotein bioflocculant. Molecules 20 (3): 5239-5259. DOI: 10.3390/molecules20035239.

Prabhakaran P, Ashraf MA, Aqma WS. 2016. Microbial stress response to heavy metals in the environment. RSC Adv 6 (111): 109862-109877. DOI: 10.1039/c6ra10966g.

Prihanto AA. 2011. Penggunaan software Image-J untuk penghitungan dan visualisasi 3D tutupan biofilm *Vibrio Cholerae* El Tor pada Kondisi Tumbuh Berbeda. Rekayasa 4 (2): 113-118. [Indonesian]

Sanjay MS, Sudarsanam D, Raj GA, Baskar K. 2020. Isolation and identification of chromium reducing bacteria from tannery effluent. J King Saud Univ Sci 32 (1): 265-271. DOI: 10.1016/j.jksus.2018.05.001.

Teitzel GM, Parsek MR. 2003. Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Appl Environ Microbiol 69 (4): 2313-2320. DOI: 10.1128/AEM.69.4.2313-2320.2003.

Van Acker H, Andrea S, Bazzini S, De Roy K, Udine C, Messiaen T, Riccardi G, Boon N, Nelis HJ, Mahenthiralingam E, Coenye T. 2013. Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. Plos One 8 (3): 1-12. DOI: 10.1371/journal.pone.0058943.

Wagner-Dobler I, Lunsdorf H, Lubbehusen T, Von Canstein HF, Li Y. 2000. Structure and species composition of mercury-reducing biofilms. Appl Environ Microbiol 66 (10): 4559-4563. DOI: 10.1128/AEM.66.10.4559-4563.2000.

Widyawati W, Wiryanto, Listyawati S. 2007. The effect of sambung nyawa (*Gynura procumbens*) leaves extract to methyl mercury concentration in blood and characteristics of erythrocyte of rat after exposed by methyl mercury chloride. Biofarmasi 5: 26-37. DOI: 10.13057/biofar/f050104.