Potential bacteria capable of remediating mercury contaminated soils

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Abstract. Mercury content in ex-artisanal and small-scale gold mining areas in Cianjur District, Province of West Java, Indonesia was 7 to 36 mg L⁻¹. It has exceeded the threshold value for industrial land. Bioremediation of mercury using mercury-resistant bacteria is attractive to remove mercury from the environment because it is more effective and less expensive. The objective of this study was to obtain potential bacteria capable of accumulating mercury to be used to remediate mercury contaminated soils in ex-gold mining areas. Potential bacteria isolates were characterized for their phenotypic and biochemical properties using the Biolog system. Thirty-two mercury-resistant bacteria were successfully isolated from the rhizosphere of Pityrogramma tartarea growing predominantly around tailings of ex-artisanal gold mining. After screening the presence of mercury, the three best isolates showing high resistances are Pseudomonas putida R2.13 and P. maculicola R4.27 that are capable to tolerate 180 mg L⁻¹ mercury, and Enterobacter aerogenes R3.24 that is capable to survive at 170 mg L⁻¹. Furthermore, the three bacteria also can fix atmospheric nitrogen and solubilize phosphate, but they cannot solubilize potassium. These indicate that P. maculicola R4.27, P. putida R2.13, and E. aerogenes R3.24 are potential as bioaccumulation agents on mercury-contaminated soils.

1. Introduction

Illegal gold mining activities generally use mercury to extract gold from ore to form an amalgam with gold. In the amalgamation process, around 25 to 30% of the mercury-added is lost to the environment [1]. Mercurial compounds are also used in agricultural pesticides which cause mercury pollution in the aquatic and soil systems [2]. Hence, mercury is being applied in many techniques such as informatics, production of batteries, and light bulbs. The addition of mercury as part of fertilizers, lime, sludges, and manures usually causes soil contamination [3], so that mercury contaminant needs to be removed from the soil.

Recently, an increasing interest in mercury pollution remediation technologies in ex-gold mining areas is the bioremediation using mercury resistant bacteria [4]. Mercury-resistant bacteria exist in the environment and can be isolated and then used to remove mercury. They have several different mechanisms to survive in the presence of high concentrations of mercuric salts. The mechanisms are enzyme reduction to elemental mercury (Hg0) and volatilization; formation of insoluble HgS; biomineralization of Hg²⁺ as an insoluble mercury-sulfur other than HgS due to the production of volatile...
thiol [2]. They are capable of degrading mercury pollutants and change them into less-and/or non-toxic substances in the soil environment. Bioremediation technologies are preferable due to their lower costs and environmentally friendly method for environmental clean-up, and they can either be carried out ex situ or in situ [5].

Many bacteria have been reported to have novel genetic mechanisms to transform the toxic form of mercury into less toxic forms for cleanup of contaminated environments [6,7]. One of the best-studied bacterial detoxification mechanisms is the mer operon-mediated mechanism. Mercury resistant bacteria harbored mer operons contain many functional genes that encode for mercury reduction (merA and/or merB), regulation (merR), mercury uptake (merT, merP, and/or merC). The main gene in mer operon encodes for mercuric ion reductase enzyme is merA, a flavoprotein catalyzes the reduction of Hg\(^{2+}\) into volatile Hg\(^{0}\) by using NADPH located in the cytoplasm as the source of electrons for the reduction of Flavin Adenine Dinucleotide [8,9]. The gene merB degrades organomercurials compounds such as phenylmercury, methylmercury, and ethylmercury by protonolysis. The genes merA and merB are related to the spectrum, broad-spectrum mercury-resistant bacteria have both, while narrow-spectrum bacteria have the only merA [10]. The objective of this study was to obtain potential bacteria capable of accumulating mercury to be used to remediate mercury-contaminated soil.

2. Materials and methods

2.1. Soil sampling
Mercury contaminated soil samples were collected from ex-artisanal gold mining sites in Cikondang Village, Campaka Sub-district, Cianjur District, West Java Province, Indonesia (geographic coordinate: 7°0'0.92” S and 107°0'3'56.71” E). The composite soil samples were taken from the rhizosphere of Pityrogramma tartarea plants which grow predominantly in the ex-gold mining area at four points with regular space intervals about 1 m which started from tailings of ex-gold mining. The samples were retained in clean and strongly closed polyethylene bags and stored in an iced box. Soil acidity and heavy metals concentration (Hg\(^{2+}\), Cd\(^{2+}\), Cr\(^{3+}\), Pb\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\)) of soil samples were characterized. Heavy metals contents were determined with atomic absorption spectrometry at 254 nm.

2.2. Isolation of mercury resistant bacteria
Isolation of mercury resistant bacteria was performed using the primary enrichment culture method and directly plating on Soil Extract Agar (SEA, Difco) in the presence of 10 mg L\(^{-1}\) Hg\(^{2+}\) [11]. Mercury stock solution was made in high concentration solution of Hg\(^{2+}\), sterilized using a sterile syringe filter 0.22 \(\mu\)m pore size (Millipore), and was added to cool 40 to 45 °C medium. Each composite soil sample of 10 g was suspended in 90 ml of sterile physiological saline to make soil suspensions and incubated for 3 hours at 37°C on a rotary shaker at 180 rpm. The suspensions were then allowed to settle down. As much as 1 ml of supernatant was homogenized using vortex and then diluted with 9 ml NaCl 0.85% solution up to 10\(^{-3}\). In triplicate, 0.1 ml of each diluted samples were spread on SEA medium supplemented with 10 mg L\(^{-1}\) of HgCl\(_2\). The plates were incubated for 2 to 7 days at 28°C and observed every day. From the same sample, colony types differing in shape, color, and margin were isolated and purified in SEA medium. Pure cultures were maintained on the slant nutrient agar (NA, difco) medium and stored at 4°C. For longer storage, the cultures were kept in Viabank bacterial storage (Himedia) at -20°C.

2.3. Screening the potential mercury resistant bacteria
All bacterial isolates were further tested for their resistance to mercury and were performed following the methodology described by Amin and Latif [12] with some modification. The fresh culture of each bacterium isolate was cultivated in a 300 ml Erlenmeyer flask containing 100 ml Nutrient Broth (NB, Difco) and incubated for less than 24 hours at 37°C on a rotary shaker at 180 rpm. Some 20 \(\mu\)l of 10\(^8\)


cells ml⁻¹ of the isolates were subcultured immediately by spot inoculating method to the SEA plates containing increased concentrations of mercury from 10 to 200 mg L⁻¹. Bacterial colonies resistant and sensitive to mercury were monitored along with SEA medium without HgCl₂ as a negative control after 2 to 7 days of incubation at 28°C to find out minimum inhibitory concentration (MIC). All experiments were performed in triplicate. The minimal inhibitory concentration was determined by the concentration of mercury chloride at which bacterial isolates failed to grow on plates even after 48 h of incubation. While bacteria’s resistance concentration of mercury chloride is in the plate with the concentration before of MIC. The isolates resistance ability at the highest mercury concentrations were considered to be potential bioremediation agents of mercury-contaminated soils. The responses of bacteria on increased mercury concentrations in media were observed morphologically i.e. the edges and the colors of the colony. They were identified and used for further tests.

2.4. Identification of potential mercury resistant bacteria
The shape and motility of all potential mercury resistant bacteria isolates were observed using the hanging drop method under a microscope Olympus DP2-BSW magnification 1000x. The phenotypic and biochemical properties of three selected pure strains were identified by the Biolog system model ELX808BLG serial 1306184 using buffer IF-A. The pure cultures were grown on NA medium and then suspended in inoculating fluid IF-A at the recommended cell density using a turbidimeter. Then the cell suspension was inoculated into the GEN III MicroPlate containing 100 μl per well and incubated for 16 to 24 hours at room temperature to allow the phenotypic properties. This plate analyzes a bacterium in 94 Phenotypic tests, they are 71 wells for carbon source utilization assays and 23 wells for chemical sensitivity assays. The phenotypic fingerprint of purple wells was then compared to Biolog’s extensive species library (http://www.biolog.com).

2.5. Functional characterization
The three potential bacteria were determined for the following function traits: nitrogen fixation, phosphate, and potassium solubilization ability using the selected James Nitrogen Free Bromothymol Blue, Pikovskaya, and Aleksandrov agar medium, respectively. The cultures of bacteria were spot inoculated on the center of agar plates containing each of the selective media. After 7 days of incubation at 28°C, the bacteria that could grow in the N-free medium indicated its ability to fix the free nitrogen. Phosphate and potassium solubilization capacities were observed for the formation of a clear zone around the colonies and then the ratio between the halo diameter and the diameter of the colony was recorded. Each test was performed in triplicate and each was repeated twice.

3. Results and discussion
3.1. Heavy metals analysis of soil
The physicochemical characterization of the soil samples used in this study showed pH values of 4.2 to 5.2 and four heavy metals: 7.4 to 36.1 mg kg⁻¹ Hg²⁺, 135 to 837 mg kg⁻¹ Pb²⁺, 13.7 to 25.5 mg kg⁻¹ Cr³⁺, 1.4 to 19.7 mg kg⁻¹ Cd²⁺, and 1.1 mg kg⁻¹ Co²⁺ (table 1). Heavy metals contamination in the artisanal gold mining area in Wonogiri were Hg 1 to 874 ppm, Pb 1 to 598 ppm, Fe 1.65 to 7.04%, Mn 0.04 to 0.60%, As 2 to 238 ppm, and Co did not detected [13], while in the soil from some gold mining in South Africa were Hg 0.06 to 0.13 ppm, Pb 1.58 to 10.22 ppm, Cd 0.04 to 0.05 ppm, Cr 77.50 to 861.67 ppm, and Co 11.82 to 33.68 ppm [14]. Xiao et al. [15] reported heavy metals contamination in the artisanal gold mining in Tongguan, Shaanxi, China i.e. Hg, Pb, and Cd were 0.16 to 14.5 ppm, 599 to 2.105 ppm, 2.39 to 16 ppm, respectively in the tailing gold mining and 0.69 to 23.7 ppm, 0.004 to 5.73 ppm, and 252 to 1.295 ppm, respectively in the soil.

The concentration of mercury, lead, and cadmium was observed greater than the threshold value for soil i.e. 0.5 mg kg⁻¹, 60 mg kg⁻¹, and 1 mg kg⁻¹ respectively, while the concentration of chromium, cobalt, and nickel was observed lower than the threshold value for soil [16]. Artisanal gold miners in soil sampling location still use traditional methods by adding liquid mercury to ground-up ore to form...
an amalgam. Waste runoff from these artisanal mines often ends up in the waterways and the environment. Furthermore, mercury contaminants can be absorbed by plants, get into the food chain, and affect human health. Consequently, these areas need to be reclaimed to remove toxic mercury and other heavy metals.

Table 1. Heavy metals content in the rhizosphere of *Pityrogramma tartarea* in the tailing areas of ex-gold mining and the number of bacteria isolates could be obtained from the sampling point.

| Sampling point from tailing (m) | pH | Heavy metals contents (mg kg⁻¹) | No. of isolates |
|--------------------------------|----|---------------------------------|----------------|
| 0                              | 4.2| Hg 36.1 Pb 837 Cd 19.7 Cr 16.2 Nd* Nd*  | 8              |
| 1                              | 5.2| Hg 11.5 Pb 135 Cd 1.4 Cr 13.7 Nd*  | 7              |
| 2                              | 5.2| Hg 27.6 Pb 703 Cd 9.0 Cr 16.0 Nd*  | 10             |
| 3                              | 5.2| Hg 7.4 Pb 286 Cd 3.1 Cr 25.5 Nd*  | 7              |
| Threshold value (MEF, 2007)    |    | Hg 0.5 Pb 60 Cd 1 Cr 100 Nd 20 Co 50 Ni  |                |

*a* Nd, not detected

3.2. Isolation and screening of mercury resistant bacteria

Thirty-two isolates of mercury-resistant bacteria having different colony characteristics were successfully obtained from the rhizosphere of *Pityrogramma tartarea* plant which grows predominantly around ex-gold mine tailings. This fern is adaptive to environments contaminated with mercury and other heavy metals. There were 8, 7, 10, and 7 isolates found from the rhizosphere of *P. tartarea* in the range of 1, 2, 3, and 4 m from tailing, respectively (table 1). Chasanah *et al.* [17] isolated mercury resistant bacteria from the tailing of small-scale gold mining in Sekotong District, West Lombok Regency, using cyanidation processing facilities and found four isolates that grew on nutrient broth containing 5 ppm Hg. They were *Brevundimonas vesicularis, Nitrococcus mobilis, Fusobacterium aquatile,* and *Fusobacterium necrogenes.*

Of the 32 bacterial isolates that were successfully obtained from solid media supplemented with 5 ppm mercury, 15 isolates (46.9%) were gram-positive bacteria, while 17 other isolates (53.1%) were gram-negative bacteria (table 2). Canstein *et al.* [18] stated that the highly mercury-resistant bacteria were those that can grow on synthetic media with a minimum of 5 ppm HgCl₂. The advantages of isolating bacteria from contaminated soil were adaptation in natural population and growing well in the environment. Furthermore, plant root exudates contained amino acids, organic acids, and simple sugars, as well as polysaccharides, and proteins [19] that stimulate microbial activities.

All bacterial isolates had different minimum inhibitory concentrations to mercury. There was only slight toxicity in the presence of mercury up to 140 mg L⁻¹ in the media, and there were 27 isolates (84.4%) survive. In the medium containing more than 140 mg L⁻¹ of Hg²⁺, the number of isolates decreased drastically to become 13 isolates which survive at 160 mg L⁻¹ of Hg²⁺, and more decreased by adding mercury concentration. All of the bacteria isolates were completely inhibited at the presence of 185 mg L⁻¹ of mercury. Three isolates of potential mercury-resistant bacteria were obtained with isolates code R2.13 and R4.27 (tolerate and aerobically grow in medium containing up to 180 mg L⁻¹ Hg²⁺) and R3.24 tolerate and aerobically grow in medium containing up to 170 mg L⁻¹ Hg²⁺ (figure 1). Ball *et al.* [6] isolated mercury resistant bacteria from gold mine tailings pond water and found that out of a total of 53 mercury resistant bacterial strains obtained, 73.58% could live in media containing 27.2 ppm Hg²⁺ and only 1 bacterial strain resistant to 81.6 Hg²⁺.
### Table 2. The macroscopic and microscopic morphology of mercury resistant bacteria.

| No | Isolate code | Colony morphology (form, edge, elevation, color, pigmentation) | Cell morphology (optical, Gram’s staining, shape, motility) |
|----|--------------|---------------------------------------------------------------|------------------------------------------------------------|
| 1  | R1.1         | Filamentous, undulate, convex, grey-white, negative           | Translucent, positive, rod, motile                         |
| 2  | R1.2         | Rhizoid, lobate, convex, creamy white, negative               | Translucent, negative, rod, motile                         |
| 3  | R1.3         | Irregular, undulate, raised, yellowish-white, positive        | Translucent, negative, rod, motile                         |
| 4  | R1.4         | Circular, entire, convex, red, negative                      | Translucent, positive, rod, motile                         |
| 5  | R1.5         | Circular, filiform, raised, creamy white, negative           | Translucent, negative, rod, motile                         |
| 6  | R1.6         | Circular, entire, raised, white, negative                     | Opaque, positive, rod, motile                              |
| 7  | R1.7         | Circular, lobate, convex, white, negative                    | Opaque, positive, rod, motile                              |
| 8  | R1.8         | Circular, undulate, flat, yellowish-white, negative           | Translucent, negative, rod, motile                         |
| 9  | R2.9         | Circular, entire, raised, dull-white, negative                | Translucent, negative, rod, motile                         |
| 10 | R2.10        | Circular, entire, raised, dull-white, negative                | Translucent, negative, rod, non-motile                     |
| 11 | R2.11        | Circular, undulate, convex, creamy white, negative            | Translucent, positive, rod, motile                         |
| 12 | R2.12        | Circular, undulate, convex, yellowish-white, negative         | Translucent, positive, cocci, non-motile                   |
| 13 | R2.13        | Circular, undulate, convex, white, negative                  | Translucent, negative, rod, motile                         |
| 14 | R2.14        | Circular, entire, convex, creamy white, negative              | Translucent, positive, rod, motile                         |
| 15 | R2.15        | Circular, undulate, convex, yellowish-white, negative         | Translucent, negative, rod, motile                         |
| 16 | R3.16        | Circular, undulate, convex, white, negative                   | Translucent, positive, rod, motile                         |
| 17 | R3.17        | Circular, undulate, raised, creamy white, negative            | Translucent, positive, cocci, non-motile                   |
| 18 | R3.18        | Circular, undulate, raised, milky white, negative             | Translucent, positive, cocci, non-motile                   |
| 19 | R3.19        | Circular, undulate, raised, translucent white, negative       | Translucent, negative, rod, motile                         |
| 20 | R3.20        | Circular, lobate, convex, creamy white, negative              | Translucent, negative, rod, motile                         |
| 21 | R3.21        | Circular, lobate, convex, yellowish-white, negative           | Translucent, negative, rod, motile                         |
| 22 | R3.22        | Rhizoid, filiform, raised, yellow, positive                   | Translucent, negative, rod, motile                         |
| 23 | R3.23        | Irregular, filiform, raised, light yellow, positive            | Translucent, negative, rod, motile                         |
| 24 | R3.24        | Circular, lobate, convex, yellow, positive                   | Translucent, positive, rod, non-motile                     |
| 25 | R4.25        | Circular, entire, raised, milky white, negative               | Translucent, positive, rod, non-motile                     |
| 26 | R4.26        | Circular, undulate, convex, white, negative                  | Translucent, negative, rod, motile                         |
| 27 | R4.27        | Circular, undulate, convex, translucent white                 | Translucent, positive, rod, motile                         |
| 28 | R4.28        | Circular, undulate, convex, creamy white, negative            | Translucent, positive, rod, motile                         |
| 29 | R4.29        | Circular, entire, umbonate, yellowish-white, negative         | Translucent, positive, rod, motile                         |
| 30 | R4.30        | Circular, lobate, convex, yellow, positive                   | Translucent, negative, rod, motile                         |
| 31 | R4.31        | Circular, entire, umbonate, milky white, negative             | Translucent, negative, rod, motile                         |
| 32 | R4.32        | Circular, entire, convex, milky white, negative               | Translucent, positive, cocci, non-motile                   |
Figure 1. The number of mercury resistant bacteria isolates from the rhizosphere of *Pityrogramma tartarea* plants predominantly grow in the tailing areas of ex-gold mining.

The growth responses of potential bacteria on high mercury concentrations in media were morphological changes as the colony surfaces were rougher and slightly wrinkled edges, and the colors changed to dull and dark. Morphologically, bacterial colonies become coarser and wrinkled to survive in stressful or toxic environments. Microorganisms can affect the migration and transformation of heavy metals by changing their physical and chemical characteristics, which are undegradable and un-destroyable. The bioremediation mechanisms include extracellular complexion, precipitation, oxidation-reduction reactions, and intracellular accumulation. The *mer* operon genes that occur on chromosomes, plasmids, and transposons cause bacterial resistance to inorganic and organic mercury compounds (HgR).

3.3. Identification of bacteria

On SEA solid medium after 7 days of incubation at room temperature, R2.13 mercury resistant isolate was found to be rounded in shape and brownish-white in color, whereas the colony margin and elevation were convex and undulating. Bacteria cells were rod-shaped motile. The colony of R4.27 isolate was found to be rounded shape, white color, convex colony margin, and lobate margins, and the bacteria cells were rod motile in shape. The R3.24 isolate colony was rounded in shape and cream in color, and its colony margin and elevation were convex and undulating. Meanwhile, the R3.24 isolate bacteria were rod motile in shape (table 3).

Three potential bacteria isolates were successfully identified for their biochemical properties using the Biolog microplates system up to species level as Gram-negative bacteria. The R2.13 and R4.27 isolate codes each belonged to non-enteric bacteria of *Pseudomonas putida* (similarity 0.518) and *P. maculicola* (similarity 0.575), while R3.24 isolate code belonged to enteric bacteria of *Enterobacter aerogenes* (similarity 0.569). Based on biochemical properties of the Biolog Gen III Micro Plate that contain 71 carbon sources utilization and 23 chemical sensitivity assays, the bacteria *E. aerogenes* R3.24 exhibit more of sugars and sugar alcohols than *P. maculicola* R4.27 and *P. putida* R2.13. On the other side, the *P. maculicola* R4.27 and *P. putida* R2.13 could use more amino, hexose, carboxylic, and fatty acids and esters than *E. aerogenes* R3.24 do (table 4).
Based on phenotypic and biochemical properties using the Biolog system, all the bacteria were able to grow on pH 5 to 6 and the R3.24 strain was more adaptive to salinity than the two other bacteria. The three bacteria were resistant to some antibiotics, bacteriostatic, or surfactant like troleandomycin, rifamycin SV, lincomycin, vancomycin, aztreonam, nalidixic acid, potassium tellurite, fusidic acid, D-serine, niaprofin, guanidine HCl, and also have the power to reduce tetrazolium violet and blue. Terán et al. [20] reported that P. putida exhibited resistance to a wide range of antibiotics such as ampicillin, carbenicillin, tetracycline, nalidixic acid, and chloramphenicol. Furthermore, Li and Ramakrishna [21] reported that under control of the Gac regulatory system, P. putida RW1051 produced promysalin (an antibiotic composed of salicylic acid and 2,8-dihydroxymyristamide) to protect many other pseudomonads, including the opportunistic pathogen P. aeruginosa. Phosphate-solubilizing bacteria such as Gram-negative P. putida also secrete antibiotics [22] and protect plants against soil-borne pathogens [23].

The strain of R2.13 and R3.24 were also resistant to minocycline while the E. aerogenes R3.24 was tolerant to 1% sodium lactate i.e. sodium butyrate and sodium bromate (table 5). Xu et al. [24] isolated P. putida from industrial wastes in Hefei, Anhui Province, China and reported that the bacteria could tolerate and aerobically grow in the medium containing up to 50 mg L\(^{-1}\) Hg\(^{2+}\) and remove 85.2% of Hg\(^{2+}\) at an initial concentration of 15 mg L\(^{-1}\). Furthermore, Zeng et al. [25] reported that the merR and merT gene of P. aeruginosa makes resistant to mercuric ion.

De et al. [26] isolated mercury-resistant bacteria from seawater and sediment samples and found that they belong to Pseudomonas, Proteus, Xanthomonas, Alteromonas, Aeromonas, and Enterobacteriaceae which are capable of growing in a high mercury concentration (50 mg L\(^{-1}\)). Furthermore, Fortunato et al. [27] reported that P. putida could reduce thiomersal or toxic organomercury with the strong bactericidal effect which was widely used as a preservative in producing the vaccine, especially in Europe. In addition to mercury bioremediation, Pseudomonas sp. has been used to Cr biosorption from the industrial waste tannery [28], accumulated metal ions Cu\(^{2+}\), Cd\(^{2+}\), and Ni\(^{2+}\) [29]. These bacteria have transport molecular to the uptake of essential metals such as Zn, Mo, Mn, and Ni [30].

The E. aerogenes bacteria have been used to bioremediate mercury and zink [31], chromium [32], cadmium, and copper [33]. This bacterium was found to have a symbiotic relationship with legume plants of Vicia faba, Phaseolus vulgaris, Pisum sativum, and non-legume plants of Cucumis sativus and Lycopersicon esculentum and help the plants to remediate the mercury in the soil [34]. The use of bacteria to remove toxic synthetic organic compounds could offer an efficient, economic, and sustainable remediation technology.

### Table 3. Characteristics of R2.13, R4.27, and R3.24 bacteria isolate on nutrient agar media after 7 days incubation at room temperature.

| Characteristics | Isolate code |
|-----------------|--------------|
|                 | R3.24        | R2.13        | R4.27        |
| Colony: Shape   | Rounded      | Rounded      | Rounded      |
| Elevation       | Convex       | Convex       | Convex       |
| Margin          | Lobate       | Undulating   | Entire       |
| Color           | Cream        | Brownish white | White       |
| Opacity         | Translucent  | Translucent  | Translucent  |
| Pigmentation    | Yellow       | Nd\(^a\)    | Nd           |
| Cell: Shape     | Rod-shaped   | Rod-shaped   | Rod-shaped   |
| Motility        | Motile       | Motile       | Motile       |

\(^a\) Nd, not detected
3.4. Functional ability of potential bacteria

Nitrogen, phosphorus, and potassium are the primary macronutrient required by plants for their growth and development. Bacteria *P. putida* R2.13, *E. aerogenes* R3.24, and *P. maculicola* R4.27 had no activity to fix nitrogen and also to dissolve potassium. Qualitatively, the three bacteria had an activity to soluble phosphate through the formation of clear zones around their colonies grown on solid medium containing tribasic calcium phosphate as sole phosphorus with solubilization index of 1.5, 0.2, and 0.4 respectively. Phosphate solubilizing bacteria can convert the insoluble to soluble forms of phosphate and make it available for plants. Collavino *et al.* [35] reported that phosphate solubilizing activity of *E. aerogenes* was 590 μg mL⁻¹. Mercury resistant bacteria produced organic acid binding the heavy metals. Therefore, phosphorus bound with heavy metals becomes available for the plant.

| Carbon source | *Pseudomonas putida* R2.13 | *Pseudomonas maculicola* R4.27 | *Enterobacter aerogenes* R3.24 |
|---------------|-----------------------------|--------------------------------|-------------------------------|
| Polymers      |                             |                                |                               |
| Dextrin       | +                           | +                              | +                             |
| N-Acetyl-D-neuraminic acid | + | + | + |
| Tween 40      | -                           | -                              | -                             |
| Sugars and sugar derivates |              |                                |                               |
| N-Acetyl-D-galactosamine | - | - | + |
| N-Acetyl-D-glucosamine | - | - | + |
| N-Acetyl-β-D-mannosamine | - | - | + |
| D-Arabitol    | -                           | -                              | +                             |
| D-Cellobiose  | -                           | -                              | +                             |
| D-Fructose    | +                           | +                              | +                             |
| L-Fucose      | +                           | +                              | +                             |
| D-Fucose      | -                           | -                              | -                             |
| D-Galactose   | -                           | -                              | +                             |
| Gentiobiose   | -                           | -                              | +                             |
| α-D-Glucose   | +                           | +                              | +                             |
| 3-Methyl glucose | - | - | + |
| myo-Inositol  | -                           | -                              | +                             |
| α-D-Lactose   | -                           | -                              | -                             |
| D-Salcin      | -                           | -                              | +                             |
| D-maltose     | -                           | -                              | -                             |
| D-Mannitol    | -                           | -                              | +                             |
| D-Mannose     | -                           | -                              | +                             |
| D-Melibiose   | -                           | -                              | +                             |
| β-Methyl-D-glucoside | - | - | + |
| Stachyose     | -                           | -                              | +                             |
| D-Raffinose   | -                           | -                              | +                             |
| L-Rhamnose    | -                           | -                              | +                             |
| D-Sorbitol    | -                           | -                              | +                             |
| Sugars and sugar derivates |              |                                |                               |
| Sucrose       | -                           | -                              | +                             |
| Carbon source                  | *Pseudomonas putida* R2.13 | *Pseudomonas maculicola* R4.27 | *Enterobacter aerogenes* R3.24 |
|-------------------------------|-----------------------------|-------------------------------|-------------------------------|
| D-Trehalose                   | -                           | -                             | +                             |
| D-Turanose                    | -                           | -                             | ±                             |
| Alcohols                      |                             |                               |                               |
| Glycerol                      | ±                           | ±                             | +                             |
| D-Glucose-6-PO₄               | ±                           | -                             | +                             |
| D-Fructose-6-PO₄              | ±                           | ±                             | +                             |
| Methyl esters                 |                             |                               |                               |
| D-Lactic acid methyl ester    | -                           | -                             | ±                             |
| Methyl pyruvate               | ±                           | -                             | +                             |
| Carboxylic acids              |                             |                               |                               |
| Acetic acid                   | +                           | ±                             | +                             |
| Acetoacetic acid              | ±                           | ±                             | ±                             |
| Citric acid                   | +                           | +                             | +                             |
| Formic acid                   | ±                           | -                             | +                             |
| L-Galactonic acid-g-lactone   | -                           | -                             | ±                             |
| D-Galacturonic acid           | +                           | +                             | ±                             |
| D-Glucic acid                 | +                           | ±                             | +                             |
| D-Malic acid                  | +                           | +                             | +                             |
| L-Malic acid                  | +                           | +                             | +                             |
| D-Glucuronic acid             | +                           | +                             | +                             |
| α-Hydroxy-butyric Acid        | -                           | -                             | -                             |
| β-Hydroxy-D, L-butyric acid   | +                           | ±                             | +                             |
| p-Hydroxy phenylacetic acid   | -                           | -                             | +                             |
| D-Saccharic acid              | +                           | ±                             | +                             |
| Mucic acid                    | +                           | +                             | ±                             |
| α-keto-butyric Acid           | -                           | -                             | -                             |
| α-Keto glutaric acid          | +                           | ±                             | +                             |
| L-Lactic acid                 | +                           | ±                             | +                             |
| Carboxylic acids              |                             |                               |                               |
| Propionic acid                | ±                           | ±                             | -                             |
| Quinic acid                   | +                           | +                             | +                             |
| Bromo succinic acid           | ±                           | ±                             | +                             |
| Pectin                        | ±                           | ±                             | +                             |
| Amides                        |                             |                               |                               |
| Glucuronamide                 | +                           | +                             | ±                             |
| Amino acids, peptides, related chemicals |                     |                               |                               |
| L-Alanine                     | +                           | ±                             | +                             |
| L-Aspartic acid               | +                           | ±                             | +                             |
| L-Glutamic acid               | +                           | ±                             | +                             |
| L-Histidine                   | +                           | ±                             | +                             |
| Glycl-L-proline               | -                           | -                             | +                             |
| L-Pyroglutamic acid           | -                           | ±                             | +                             |
| L-Serine                      | ±                           | ±                             | +                             |
| D-Serine                      | +                           | +                             | +                             |
| D-Aspartic acid               | -                           | -                             | +                             |
| L-Arginine                    | +                           | ±                             | +                             |
| γ-Aminobutyric acid           | +                           | ±                             | +                             |
| Gelatin                       | -                           | -                             | ±                             |
| Carbon source | Pseudomonas putida R2.13 | Pseudomonas maculicola R4.27 | Enterobacter aerogenes R3.24 |
|---------------|--------------------------|-----------------------------|-----------------------------|
| Nucleosides   |                          |                             |                             |
| Inosine       | ±                        | ±                           | +                           |

*a* Utilized.

*b* Half utilized.

*c* Not utilized.

**Table 5.** Chemical sensitivity characteristics of bacteria strain R2.13, R4.27, and R3.24 in a Biolog Gen III microplate simple table.
Chemical sensitivity | *Pseudomonas putida* R2.13 | *Pseudomonas maculicola* R4.27 | *Enterobacter aerogenes* R3.24 |
--- | --- | --- | --- |
Sodium bromate | - | - | ± |
^a Utilized.  
^b Half utilized.  
^c Not utilized.

4. Conclusions
Thirty-two isolates of mercury-resistant bacteria have been isolated from rhizosphere of *Pityrogramma tartarea* around ex-gold mine tailings. Three bacteria isolates survived at 170 to 180 mg L\(^{-1}\) mercury and had the ability to fix atmospheric nitrogen and solubilize fixed phosphate. Morphological observation of the colonies of these isolates showed rounded shape, convex, translucent, with a variety of colors from white to cream. Most of bacterial cells were rod-shaped and motile. Biochemical properties using the Biolog microplates system, these three bacteria were identified as Gram-negative bacteria of *Pseudomonas putida* R2.13 (similarity 0.518), *P. maculicola* R4.27 (similarity 0.575), and *Enterobacter aerogenes* R3.24 (similarity 0.569). *E. aerogenes* R3.24 exhibited use more sugars and sugar alcohols, while the *P. maculicola* R4.27 and *P. putida* R2.13 used more amino, hexose, carboxylic, and fatty acids and esters. These bacteria are potential to be used to remove mercury contaminants for environmental clean-up.

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References
[1] Velásquez-López P C, Veiga M M and Hall K 2010 Mercury balance in amalgamation in artisanal and small-scale gold mining: Identifying strategies for reducing environmental pollution in Portovelo-Zaruma, Ecuador J. Clean. Prod. 18(3) 226–232.
[2] Essa A M M, Macaskie L E and Brown N L 2002 Mechanisms of mercury bioremediation Bioch. Soc. Trans. 30(4) 672–674.
[3] Crespo-López M E, Macêdo G L, Pereira S I D, Arrifano G P F, Picanço-Diniz D L W, do Nascimento J L M and Herculano A M 2009 Mercury and human genotoxicity: Critical considerations and possible molecular mechanisms Pharmacol. Res. 60(4) 212–220.
[4] Giovanella P, Cabral L, Costa A P, de Oliveira-Camargo F A, Gianello C and Bento F M 2017 Metal resistance mechanisms in gram-negative bacteria and their potential to remove Hg in the presence of other metals Ecotoxicol. Environ. Saf. 140 162–169.
[5] Azubuike C C, Chikere C B and Okpokwasili G C 2016 Bioremediation techniques-classification based on site of application: Principles, advantages, limitations, and prospects World J. Microbiol. Biotechnol. 32 180.
[6] Ball M M, Carrero P, Castro D and Yarzábal L A 2007 Mercury resistance in bacterial strains isolated from tailing ponds in a gold mining area near El Callao (Bolívar State, Venezuela) Curr. Microbiol. 54 149–154.
[7] Pepi M, Focardi S, Tarabelli A, Volterrani M and Focard S E 2013 Bacterial strains resistant to inorganic and organic forms of mercury isolated from polluted sediments of the Orbetello Lagoon, Italy, and their possible use in bioremediation processes E3S Web of Conferences 1 DOI: 10.1051/20130131002 1 e3sconf 31002 (2013).
[8] Schottel J L 1978 The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of *Escherichia coli* J. Biol. Chem. 253(12) 4341–4349.
[9] Furukawa K and Tonomura K 1972 Metallic mercury-releasing enzyme in mercury-resistant *Pseudomonas Agr. Biol. Chem. 36(2) 217-226.
[10] Naguib M M, El-Gendy A O and Khairalla A S 2018 Microbial diversity of mer operon genes and potential roles in mercury bioremediation and resistance Open Biotechnol. J. 12 56-77.

[11] Narita M, Chiba K, Nishizawa H, Ishii H, Huang CC, Kawabata Z, Silver S and Endo G 2003 Diversity of mercury resistance determinants among Bacillus strains isolated from sediment of Minamata Bay FEMS Microbiol. Lett. 223 73-82.

[12] Amin A and Latif Z 2016 Screening of mercury-resistant and indole-3-acetic acid producing bacterial-consortium for growth promotion of Cicer arietinum L: Olant growth promotion by Hg-resistant bacteria J. Basic Microbiol. 57(3) 204-217.

[13] Nurcholis M, Yudiantoro D F, Haryanto D and Mirzam A 2017 Heavy metal accumulation and health risks associated with artisanal gold mining in Tongguan, Shaanxi, China Ecotoxicol. Environ. Saf. 141 17-24.

[14] Xiao R, Wang S, Li R, Wang J J and Zhang Z 2017 Soil heavy metal contamination and health risks associated with artisanal gold mining in Tongguan, Shaanxi, China Ecotoxicol. Environ. Saf. 141 17-24.

[15] MEF, Ministry of the Environment Finlandia 2007 Government decree on the assessment of soil contamination and remediation needs. March 1 2007. p.6 https://www.finlex.fi/en/laki/kaannokset/2007/en20070214.pdf.

[16] Chasanah U, Nuraini Y and Handayanto E 2018 The potential of mercury-resistant bacteria isolated from small-scale gold mine tailings for accumulation of mercury J. Ecol. Eng. 19(2) 236-245

[17] von Canstein H, Li Y, Timmis K N, Deckwer W D and Wagner-Döbler I 1999 Removal of mercury from chloralkali electrolysis wastewater by a mercury-resistant Pseudomonas putida strain Appl. Environ. Microbiol. 65(12) 5279-5284.

[18] Li K and Ramakrishna W 2011 Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth J. Hazard. Mater. 189(1-2) 531–539

[19] Taurian T, Anzuay M S, Angelini J G, Tonelli M L, Ludueña L, Pena D, Ibáñez F and Fabra A 2010 Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities Plant Soil 329(1) 421–431

[20] Singh N, Kumar S, Bajpai V K, Dubey R C, Maheshwari D K and Kang S C 2010 Biological control of Macrophomina phaseolina by chemotactic fluorescent Pseudomonas aeruginosa PN1 and its plant growth promotory activity in chir-pine Crop Prot. 29 1142–1147

[21] Zeng X, Tagi J, Jiang P, Liu H, Dai Z and Liu X 2010 Isolation, characterization, and extraction of mer gene of Hg2+ resisting strain D2 Trans. Nonferrous Met. Soc. China. 20(3) 507–512

[22] De J, Ramaiah N, Mesquita A and Verlekar X N 2003 Tolerance to various toxicants by marine bacteria highly resistant to mercury Mar. Biotechnol. 5(2) 185–193

[23] Fortunato R, Crespo J G and Reis M A 2005 Biodegradation of thiomersal containing effluents by a mercury resistant Pseudomonas putida strain Water Res. 39(15) 3511–3522

[24] Hussein H, Ibrahim S F, Kandeel K and Moawad H 2004 Biosorption of heavy metals from waste water using Pseudomonas sp Electron. J. Biotechnol. 7(1) 38–46

[25] Vieira R H S F and Volesky B 2000 Biosorption: A solution to pollution Int. Microbiol. 3 17–24
[30] Canovas D, Cases I and De Lorenzo V 2003 Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis *Environ. Microbiol.* 5(12) 1242–1256

[31] Ravikumar S, Williams G P, Shanthy S, Gracelin N A A, Babu S and Parimala P S 2007 Effect of heavy metals (Hg and Zn) on the growth and phosphate solubilizing activity in halophilic phosphobacteria isolated from Manakudi mangrove *J. Environ. Biol.* 28(1) 109–114

[32] Panda J and Sarkar P 2012 Bioremediation of chromium by novel strains *Enterobacter aerogenes* T2 and *Acinetobacter* sp. PD 12 S2 *Environ. Sci. Pollut. Res.* 19(5) 1809–1817

[33] Huang Q, Chen W and Xu L 2005 Adsorption of copper and cadmium by Cu- and Cd-resistant bacteria and their composites with soil colloids and kaolinite *Geomicrobiol. J.* 22(5) 227–236

[34] Sorkhoh N A, Ali N, Al-Awadhi H, Dashti N, Al-Mail M, Eliyas M and Radwan S S 2010 Phytoremediation of mercury in pristine and crude oil contaminated soils: Contributions of rhizobacteria and their host plants to mercury removal *Ecotoxicol. Environ. Saf.* 73(8) 1998–2003

[35] Collavino M M, Sansberro P A, Mroginski L A and Aguilar O M 2010 Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth *Biol. Fert. Soils* 46 727–738