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# Resistance of bacteria isolated from leachate to heavy metals and the removal of Hg by *Pseudomonas aeruginosa* strain FZ-2 at different salinity levels in a batch biosorption system

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## Abstract

Leachate is produced from sanitary landfills containing various pollutants, including heavy metals. This study aimed to determine the resistance of bacteria isolated from non-active sanitary landfill leachate to various heavy metals and the effect of salinity levels on the removal of Hg by the isolated bacterium. Four dominant bacteria from approximately  $33 \times 10^{17}$  colony-forming units per mL identified as *Vibrio damsela*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, and *Pseudomonas fluorescens* were isolated from non-active sanitary landfill leachate. Heavy metal resistance test was conducted for Hg, Cd, Pb, Mg, Zn, Fe, Mn, and Cu ( $0\text{--}20 \text{ mg L}^{-1}$ ). The removal of the most toxic heavy metals by the most resistant bacteria was also determined at different salinity levels, i.e., fresh water (0‰), marginal water (10‰), brackish water (20‰), and saline water (30‰). Results showed that the growth of these bacteria is promoted by Fe, Mn, and Cu, but inhibited by Hg, Cd, Pb, Mg, and Zn. The minimum inhibitory concentration (MIC) of all the bacteria in Fe, Mn, and Cu was  $> 20 \text{ mg L}^{-1}$ . The MIC of *V. damsela* was  $5 \text{ mg L}^{-1}$  for Hg and  $> 20 \text{ mg L}^{-1}$  for Cd, Pb, Mg, and Zn. For *P. aeruginosa*, MIC was  $> 20 \text{ mg L}^{-1}$  for Cd, Pb, Mg, and Zn and  $10 \text{ mg L}^{-1}$  for Hg. Meanwhile, the MIC of *P. stutzeri* was  $> 20 \text{ mg L}^{-1}$  for Pb, Mg, and Zn and  $5 \text{ mg L}^{-1}$  for Hg and Cd. The MIC of *P. fluorescens* for Hg, Pb, Mg, and Zn was 5, 5, 15, and  $20 \text{ mg L}^{-1}$ , respectively, and that for Cd was  $> 20 \text{ mg L}^{-1}$ . From the MIC results, Hg is the most toxic heavy metal. In marginal water (10‰), *P. aeruginosa* FZ-2 removed up to 99.7% Hg compared with that in fresh water (0‰), where it removed only 54% for 72 h. Hence, *P. aeruginosa* FZ-2 is the most resistant to heavy metals, and saline condition exerts a positive effect on bacteria in removing Hg.

**Keywords:** Bioremediation, Leachate, Mercury, Minimum inhibitory concentration, Heavy metal resistance, *Pseudomonas aeruginosa*

## Introduction

Heavy metal contamination in surface water and groundwater is a global concern. Heavy metals may pose chronic and even acute health hazards to living organisms, including humans. Heavy metal exposure causes

several health problems, including skin irritation, headache, nausea, cancer, kidney disorders, and depression, among humans [1]. Heavy metal contamination in the environment has several sources. Industrial wastewater, mining activities, and leaching metals from solid wastes are some causes of heavy metal pollution in water bodies [2]. Among these sources, leachate from landfills has

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elicited increasing concern due to its potential to pollute groundwater severely [3].

Landfill leachate is a percolated liquid flowing out of a pile of garbage that contains suspended, dissolved, and extracted materials; it is produced when rainwater or runoff infiltrates wastes [4]. Landfill leachate contains various heavy metals, such as Hg, Pb, Zn, Cr, Mg, and Cd. Agamuthu and Fauziah [5] reported that Cd, Cr, Cu, Pb, Zn, and Mg were found in leachate with concentrations of 0.002, 0.006, 0.005, 0.15, 0.15, and 7.48 mg L<sup>-1</sup>, respectively. Similarly, Xue et al. [6] found that the concentrations of Pb, Zn, Cr, Cd, and Cu in landfill leachate were 0.36, 0.27, 0.11, 0.2, and 0.21 mg L<sup>-1</sup>, respectively. The World Health Organization set 3, 50, 1, 100, and 10 µg L<sup>-1</sup> as the standards for the maximum level of Cd, Cr, Hg, Mn, and Pb in wastewater. If not handled properly, leachate can pollute groundwater and pose risks to human health, such as bloody diarrhea, dehydration, renal failure, cancer, hypertension, pneumonitis, and anemia [7].

Hg is one of the most toxic heavy metals, and it has become a worldwide concern because it has been found in groundwater in landfill areas with concentration of approximately 0.36–3.01 µg L<sup>-1</sup> [8]. Maiti et al. [9] reported that Hg was found in groundwater near the landfill in Kolkata, India with a concentration of 0.1 ± 0.05 mg L<sup>-1</sup>. Grassi and Netti [10] also detected more than 1 µg L<sup>-1</sup> Hg in saline groundwater (34‰) along the coast of southern Tuscany, Italy. Apart from the Hg content of groundwater in landfill areas, high levels of salinity were also measured in leachate and groundwater due to seawater intrusion [11]. Mangimbulude et al. [12] indicated that the groundwater along Keputih landfill in Indonesia are contaminated by leachate and seawater intrusion with a salinity level of approximately 0.26–6.5‰. High salinity levels will decrease the quality of groundwater and disrupt the groundwater treatment process [13]. In addition, high salinity will disrupt the natural bioremediation process.

Bioremediation is one of the effective biotechnologies used in heavy metal removal; it requires simple maintenance and operation and can be used in large areas, particularly sanitary landfills, under several conditions. The utilization of microbial metabolism plays a major role in biotechnology. Some microorganisms can produce enzymes to remove heavy metals from contaminated areas [14]. Indigenous microorganisms isolated from contaminated environments exhibit resilient and efficient capability to remove pollutants. Several previous studies have reported that many species of bacteria can be isolated from active landfill soil and leachate, including *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp., *Stenotrophomonas* sp., *Rhodococcus* sp., and *Burkholderia* sp. [15, 16]. Little information is available about the

isolation of bacteria from non-active sanitary landfill leachates. The metal leaching process in non-active sanitary landfills does not only occur through physico-chemical leaching, but also through a biological leaching process, providing bacteria with high resistance to heavy metals [17]. In Imron et al. [18], *Pseudomonas fluorescens* was isolated from non-active sanitary landfill leachate with a minimum inhibitory concentration (MIC) of 5 mg L<sup>-1</sup> for Hg. *Pseudomonas aeruginosa* was isolated from the leachate of an active solid waste disposal dumpsite with MIC values of 15, 10, 12, and 9 mg L<sup>-1</sup> for Pb, Cr, Ni, and Cd, respectively [19]. Apart from exhibiting high resistance, bacteria isolated from contaminated areas can also remove heavy metals. *P. aeruginosa* isolated from solid waste disposal dumpsite leachate can remove approximately 29, 30, 35, and 28% of Pb, Cr, Ni, and Cd, respectively [19]. Neneng and Gunawan [20] reported that *P. aeruginosa* KHY2 and *Klebsiella pneumonia* KHY3 isolated from gold mine wastewater exhibited the capability to remove 1000 mg L<sup>-1</sup> of Hg up to 60%. De et al. [21] claimed that *P. aeruginosa* strain CH07 isolated from marine sediments could remove up to 95% of Hg at 8 mg L<sup>-1</sup> of Hg and 8 g L<sup>-1</sup> of NaCl. Similarly, Dash et al. [22] determined that *Bacillus thuringiensis* PW-05 isolated from seawater removed up to 91% of Hg from a concentration of 50 mg L<sup>-1</sup> at 10‰ salinity level.

Previous studies on the isolation of bacteria have mostly focused on wastewater and active landfill soil and leachate. By contrast, research on the resistance of bacteria isolated from non-active sanitary landfill leachate to heavy metals remains limited, and the subject has not been thoroughly explored. Moreover, studies on the effect of different salinity levels on bacterial performance in Hg removal are scarce. To fulfill these gaps, the current research aims to determine (i) heavy metal content in non-active sanitary landfill leachate, (ii) the resistance of bacteria isolated from non-active sanitary landfill leachate to various heavy metals, and (iii) the effect of salinity level on the Hg removal of the most resilient isolated bacterium as a developmental strategy for the future remediation of heavy metals in contaminated areas.

## Materials and methods

### Preparation of media and stock solutions

All chemicals and media used were analytical grade reagents. Liquid and agar media were respectively made using nutrient broth (NB) and nutrient agar (NA) (Merck, Germany). The liquid medium was prepared from 13 g of NB powder in 1 L distilled water. Meanwhile, the agar medium was made with 20 g of NA powder in 1 L distilled water and then heated until dissolved. NaCl (0.85%) was provided by 8.5 g of NaCl powder

(Merck, Germany) in 1 L distilled water. The eight heavy metal salt solutions, namely,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{HgCl}_2$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{PbCl}_2$ ,  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ ,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{CdCl}_2$ , and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (Merck, Germany), used in the study were also analytical grade reagents with > 99% purity. Stock solutions, each with a concentration of  $100 \text{ mg L}^{-1}$ , were prepared with 0.84, 0.014, 0.45, 0.134, 0.3, 0.39, 0.16, and 0.49 g of Mg, Hg, Zn, Pb, Mn, Cu, Cd, and Fe powders, respectively, in 1 L distilled water. The desired concentration was adjusted using dilution with a certain ratio. All the prepared chemicals and media were then autoclaved at  $121^\circ\text{C}$  for 15 min at 101 kPa.

#### Collection of leachate

Leachate samples were collected from two monitoring wells (MWs) of a non-active sanitary landfill in Keputih, Surabaya, Indonesia with coordinate points of  $7^\circ17'67''$  S and  $112^\circ48'17''$  E for MW-1 and  $7^\circ17'67.5''$  S and  $112^\circ48'18.5''$  E for MW-2. Leachate (100 mL) from each MW was transferred into and mixed in a sterilized bottle. The leachate was filtered using Whatman 42 with  $2.5 \mu\text{m}$  of pore size (Merck, Germany) to remove interfering substances before analyzing its characteristics. Physicochemical characterizations, such as biological oxygen demand (BOD), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), total phosphorus (TP), pH, temperature, and heavy metals, were analyzed.

#### Isolation of bacteria

A serial dilution method based on the procedures of Imron et al. [15] was used to isolate bacteria from the leachate. Mixed samples (1 mL) from the two locations were transferred into tubes filled with 9 mL of 0.85% NaCl solution and diluted until single bacterium colonies could be obtained. Samples (0.1 mL) from the last 3 dilutions (up to 17 times dilution) were dispensed onto nutrient agar medium and then incubated in an incubator (Ogawa Seiki, Japan) for 24 h at  $37^\circ\text{C}$ . Thereafter, the growing colonies were transferred into the nutrient agar medium to purify the bacteria before characterization and identification [23].

#### Identification of bacteria

Bacteria were identified via biochemical characterization. Microbial identification kits (Microbact™ GNB 12A and 12B) (Oxoid, UK) were used to observe physiological characteristics, and then the characteristics were evaluated using Bergey's Manual of Determinative Bacteriology.

The most resistant bacteria were identified through 16S rDNA sequencing. A mini spin column was used to extract the total genomic DNA from bacteria. The  $1 \mu\text{L}$  bacterial DNA extract was amplified with  $0.5 \mu\text{M}$  primers: 27 F forward primer ( $5' - \text{AGAGTTT GATCCTG}$

$\text{GCTCAG-3}'$ ) and 1492R reverse primer ( $5' - \text{GGTTAC CTTGTTACGACTT-3}'$ ). The protocol for GoTaq® Hot Start Green Master Mix, 2X (Promega) was used to conduct polymerase chain reaction (PCR) analysis, and the PCR Clean-Up System (Promega) was utilized to purify the PCR product. BLAST was used to compare the nucleotide sequences obtained from the sequencing to the sequences in the GenBank database. The result from the GenBank database was adopted to construct the phylogenetic tree.

#### Heavy metal resistance test

The eight heavy metal salt solutions mentioned in Section Preparation of media and stock solutions were used in this research, with concentrations of 5, 10, 15, and  $20 \text{ mg L}^{-1}$  for each heavy metal. Heavy metal resistance test was conducted on the basis of the disk diffusion method adopted from Imron et al. [15]. Each bacterium was cultured in a conical flask filled with 100 mL of NB and shaken at 150 rpm for 24 h. Bacterial cultures (0.1 mL) were transferred to plates filled with NA. The sterilized 5 mm disk was saturated in heavy metal for 1 h. The heavy metal-saturated disks were placed on the surface of NA that contained bacteria by using sterile forceps, and then the agar was incubated at  $37^\circ\text{C}$  for 24 h. MIC, which is the lowest heavy metal concentration that completely inhibits microbial activity, was determined based on the diameter of clear zone (DCZ) on the surface of the agar. DCZ indicates the inhibition of bacterial growth.

#### Removal of Hg by bacteria

The Hg removal experiment was performed in 250 mL conical flasks containing 100 mL of  $5 \text{ mg L}^{-1}$  Hg solution and NB with different salinity levels, i.e., fresh water (0‰), marginal water (10‰), brackish water (20‰), and saline water (30‰) under sterile and aerobic conditions. NaCl was used to attain the salinity level. First, 2 mL (2% v/v) of bacterial suspension in exponential phase was inoculated into the solution. Then, the conical flasks were agitated in a shaking incubator at 120 rpm for 5 d. After incubation time, the solution sample was measured for Hg concentration via inductively coupled plasma optical emission spectrometry (ICP-OES).

#### Quality control, quality assurance, and parameter analysis method

Before the experiment, all pieces of glassware were washed with 30%  $\text{HNO}_3$  for 24 h and then flushed with distilled water before used. The purpose of this process was to remove all the contaminants attached onto glassware. BOD, COD, TKN, and TP were respectively analyzed using the Winkler method [24], open reflux method [24], Kjeldahl method [24], and

spectrophotometry (Thermo Spectronic Genesys 20, USA) [25]. The pH and temperature of the leachate were measured using a pH meter (Hanna, USA) and a digital thermometer (Thermo, Indonesia), respectively. Heavy metal concentration analysis was conducted in accordance with Giovanella et al. [26] and Imron et al. [15]. For the concentrations of Cd, Mg, Zn, Cu, Fe, Mn, and Pb, 100 mL of the samples were centrifuged at 5000 rpm for 10 min. Thereafter, the supernatant was digested using 20% HNO<sub>3</sub> and 10% HClO<sub>4</sub> to produce pH < 1.5. Then, the heavy metals were analyzed via atomic absorption spectroscopy. In contrast with Hg analysis, 100 mL of the samples were taken from the bacterial culture and centrifuged for 10 min at 8000 rpm. Thereafter, KMnO<sub>4</sub> (15 mL), H<sub>2</sub>SO<sub>4</sub> (5 mL), and HNO<sub>3</sub> (2 mL) were added to the samples for the subsequent digestion process. Then, K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (8 mL) was added to the samples, mixed, and the resulting solution was heated in a water bath (Memmert, Germany) for 2 h at 95 °C. In the last step, NaCl-(NH<sub>2</sub>OH)<sub>2</sub>-H<sub>2</sub>SO<sub>4</sub> (6 mL) was added to the samples after the sample temperature reached room temperature. ICP-OES was performed to determine Hg concentration. The sample analysis was conducted in triplicate and the data were presented in average ± standard deviation.

### Statistical analysis

Minitab 16.0 was used for statistical analysis. In this research, statistical analysis was conducted to determine (i) the significant difference in bacterial resistance to various heavy metals, and (ii) the significant difference in the Hg removal of the selected bacterium under different salinity levels and contact time. Before conducting statistical analysis, a normality test was performed using the Shapiro–Wilk method. The results were normally distributed on the basis of the normality test. Thereafter, analysis of variance (ANOVA) was also conducted [27]. General linear ANOVA was selected to determine the correlation between variables, and Tukey's honestly significant difference test was used to determine the significance of the result [28], with  $\alpha = 0.05$ .

## Result and discussion

### Characteristics of leachate

Leachate sample from the non-active sanitary landfill was analyzed for its physical and chemical properties to determine its organic (BOD, COD, TKN, and TP) and heavy metal concentrations. The characteristics of the leachate sample are listed in Table 1.

As indicated in Table 1, the pH and temperature of the leachate were ranged 7.5–8.2 and 31 ± 1.4 °C, respectively. BOD, COD, TKN, and TP concentrations were 134 ± 5, 258 ± 14, 25.8 ± 1.4, and 1.82 ± 0.14 mg L<sup>-1</sup>, respectively. Heavy metals, except for Mn (0.004 ±

**Table 1** Characteristics of the leachate sample

Parameter	Unit	Concentration	Limit of detection (LOD)
COD	mg L <sup>-1</sup>	258 ± 14	–
BOD	mg L <sup>-1</sup>	134 ± 5	–
TKN	mg L <sup>-1</sup>	25.8 ± 1.4	–
TP	mg L <sup>-1</sup> PO <sub>4</sub> – P	1.85 ± 0.14	–
pH	–	7.5–8.2	–
Temperature	°C	31 ± 1.4	–
Mg	mg L <sup>-1</sup>	< LOD	0.0003
Hg	mg L <sup>-1</sup>	< LOD	0.0085
Zn	mg L <sup>-1</sup>	< LOD	0.001
Pb	mg L <sup>-1</sup>	< LOD	0.01
Mn	mg L <sup>-1</sup>	0.004 ± 0.0	0.002
Cu	mg L <sup>-1</sup>	< LOD	0.003
Cd	mg L <sup>-1</sup>	< LOD	0.002
Fe	mg L <sup>-1</sup>	< LOD	0.006

0.0 mg L<sup>-1</sup>), were not detected. The low concentrations of organic compounds and heavy metals were due to the sanitary landfill being under reclamation/recovery and had not been used more than 20 yr.

### Isolation and initial identification of bacteria by using a biochemical test

Four dominant bacteria (FZ-1, FZ-2, FZ-3, and FZ-4) were isolated from the leachate and characterized on the basis of morphological differences. The four bacteria were then identified through biochemical tests, and the results are provided in Table 2. FZ-1, FZ-2, FZ-3, and FZ-4 were identified as *Vibrio damsela*, *P. aeruginosa*, *Pseudomonas stutzeri*, and *P. fluorescens*, respectively, with a similarity percentage of more than 90%. All the isolated bacteria were identified as Gram-negative bacteria. In accordance with Murínová and Dercová [29], Gram-negative bacteria have two layers of membranes and can be more resistant to pollutants than Gram-positive bacteria. Imron et al. [15] and Latorre et al. [16] also reported that Gram-negative bacteria are dominant among bacterial populations isolated from leachate.

### Heavy metal resistance and identification of resistant bacteria through PCR

The DCZ values are presented in Fig. 1. Based on the DCZ values, the most toxic among the heavy metals is Hg. All the bacterial isolates formed clear zones at 5, 10, 15 and 20 mg L<sup>-1</sup> Hg, except for *P. aeruginosa*, which formed a clear zone at 10 mg L<sup>-1</sup> Hg. Therefore, *P. aeruginosa* is the most resistant to heavy metals among the four bacteria. The highest DCZ value was 1.2 cm for *P. stutzeri* at 15 mg L<sup>-1</sup> of Hg. The second most toxic

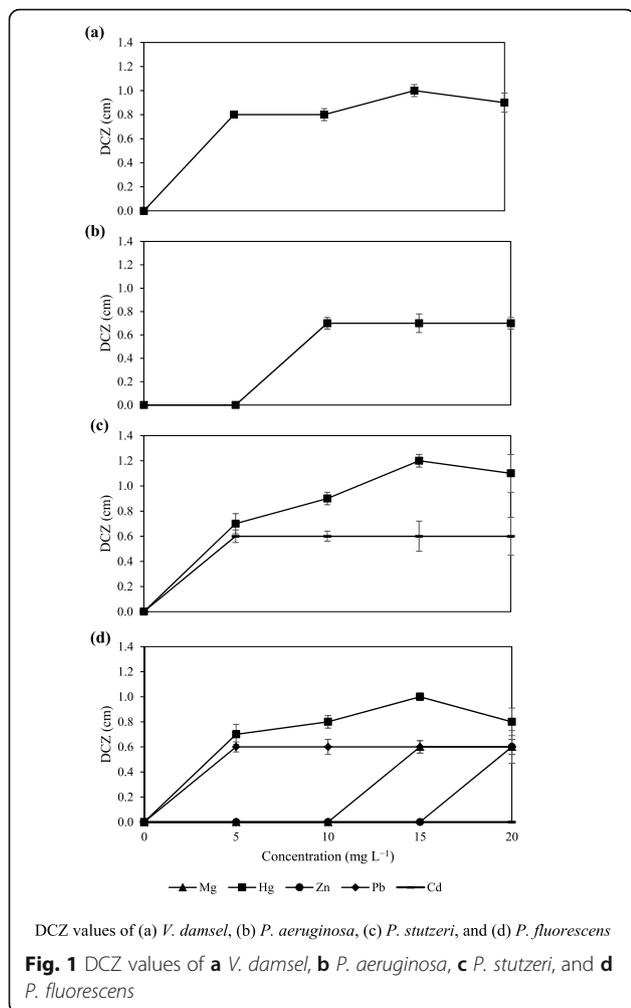
**Table 2** Biochemical test of isolated bacteria

Biochemical Properties	Responses			
	FZ-1	FZ-2	FZ-3	FZ-4
Oxidase	+	+	+	+
Ornithine	-	-	-	-
Lysine	+	-	+	+
H <sub>2</sub> S	-	-	-	-
Motility	+	+	+	-
Xylose	-	+	-	+
Nitrate	+	+	+	+
Glucose	+	+	-	+
Indole	+/-	+	-	-
Mannitol	-	-	-	-
Sucrose	-	-	-	-
ONPG	-	-	-	-
Urease	-	+	+	+
VP	-	-	-	-
Citrate	-	+	+	+
Adonitol	-	-	-	-
Sorbitol	-	-	-	-
TDA	+	+	-	-
Gelatin	+	+	-	-
Inositol	-	-	-	-
Rhamnose	-	-	-	-
Lactose	-	-	-	-
Arabinose	-	-	-	-
Arginine	+	+	+	-
Raffinose	-	-	-	-
Malonate	-	+	+	+
Salicin	-	-	-	-
Gram Staining	-ve	-ve	-ve	-ve
Shape	Rod	Rod	Rod	Rod
Diameter (mm)	2	1	0.5	1
Identified Species	<i>Vibrio damsela</i>	<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas stutzeri</i>	<i>Pseudomonas fluorescens</i>
Probability (%)	95.65	99.16	98.82	92.74

heavy metals are Cd and Pb. *P. stutzeri* and *P. fluorescens* formed clear zones at 5 mg L<sup>-1</sup> of Cd and Pb with a DCZ value of 0.6 cm. Jaishankar et al. [1] reported that Hg, Cd, and Pb are the most toxic heavy metals and they have carcinogenic effects on humans. The other heavy metals that presented clear zones are Mg and Zn for *P. stutzeri*. At 20 mg L<sup>-1</sup> of Mg and Zn, *P. stutzeri* inhibited its growth with a DCZ value of 0.6 cm. Fe, Mn, and Cu did not produce clear zones in the four bacteria; hence, *V. damsela*, *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens* exhibited high resistance to Fe, Mn, and Cu. *P. fluorescens* was the most inhibited because it formed clear

zones at low concentrations of Hg, Cd, Pb, Mg, and Zn. The results indicated that a higher heavy metal concentration up to 15 mg L<sup>-1</sup> produces a higher DCZ value indicating more severe toxicity, but at 20 mg L<sup>-1</sup> of heavy metal concentration, the DCZ value slightly decreases. Imron et al. [18] reported that the size of the inhibited zone will increase with increasing heavy metal concentration. Inhibited bacterial growth was due to the effect of heavy metals on the DNA structure and cell membranes that decreased microbial activities [30].

Microorganisms can enhance their metal resistance systems to protect their sensitive intracellular and



extracellular components. Limiting metal entrance or changing cellular components reduces their sensitivity to metals. Ianeva [31] indicated that the major mechanisms for the heavy metal resistance of bacteria had been characterized and identified as extracellular barrier, active transport of metal ions, extracellular sequestration, and intracellular sequestration, as illustrated in Fig. S1 of Supplemental Information. These mechanisms are commonly observed in microorganisms that are resistant to heavy metals. They may possibly occur in *V. damsela*, *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens*.

An extracellular barrier is a characteristic of microorganisms that prevents heavy metals from entering cells. The cell wall, plasma membrane, and capsule play important roles during this stage in inhibiting heavy metals from entering cells. Microorganisms can naturally produce extracellular polysaccharide (EPS). EPS is found on the outermost surface of a wide range of bacteria. EPS structures support the sequestration of metal ions and prevent them from penetrating into cell surface. Some bacteria, such as *P. aeruginosa*, *P. stutzeri*, *Arthrobacter*

sp., and *Rhizobium metallidurans*, exhibit the capability to bind metals extracellularly. Banerjee et al. [32] reported that EPS was produced by *Pseudomonas* sp. PFAB4 and induced by Ag ions. The production of EPS as an extracellular barrier can inhibit heavy metals from entering cells.

The active transport of metal ion systems indicates the powerful mechanism of metal resistance systems because it can be chromosomal or plasmid-encoded. This mechanism is used by microorganisms to release toxic metals from the cytoplasm. Essential metals, such as Fe<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, and Na<sup>+</sup>, can enter the active transport system, in contrast with nonessential metals, which are rapidly exported from the cytoplasm. The active transport system can be non-ATPase or ATPase-linked, and it is highly specific for the exported cation or anion [33]. Some examples of bacteria cause plasmid encoding in heavy metals, such as Hg<sup>2+</sup> resistance mediated by the *mer* operon in *P. stutzeri* strain 273 [33], Cd<sup>2+</sup> resistance mediated by the *czc* and *czr* operons in *P. aeruginosa* [34], and Pb<sup>2+</sup> resistance mediated by the *pbrA* gene in *Vibrio harveyi* strain M-11 and *P. stutzeri* strain M-9 [35]. Zheng et al. [33] reported that the operons of *P. stutzeri* strain 273 plasmids contain Hg<sup>2+</sup>-transport proteins, namely, *merT*, *merP*, *merA*, *merD*, and *merF*. These operons are essential for bacterial Hg resistance when exposed to Hg<sup>2+</sup>. The *merF* operon is a physiological response of *P. stutzeri* strain 273 to Hg stress. The *mer* operon encodes an ATPase efflux pump and the detoxifying enzyme Hg reductase.

After passing through the extracellular barrier, heavy metal ions will accumulate in the outer membrane and periplasm of microorganisms or form a complexation of metal ions as precipitated compounds. This mechanism is known as extracellular sequestration. Microorganisms can release heavy metals from the cytoplasm to sequester metals within the periplasm [30]. Miller et al. [36] have reported that *Pseudomonas putida* strain KT2440 is a Cd<sup>2+</sup>- and Cu<sup>2+</sup>-resistant bacterium that synthesizes the Cd-induced protein CzcR2 and the Cu-induced proteins CopR1 and CopB1 on the outer membrane, binding Cd<sup>2+</sup> and Cu<sup>2+</sup> as metal accumulations on the outer membrane.

Intracellular sequestration is a mechanism of heavy metal resistance among bacteria that occurs in the cytoplasm. During this stage, heavy metal ions will form complexations with various components produced by the cytoplasm and will precipitate and accumulate inside cells. A previous study showed that *V. harveyi* exhibits the capability to transform Pb<sup>2+</sup> into precipitated Pb phosphate compound [Pb<sub>9</sub>(PO<sub>4</sub>)<sub>6</sub>] and cause it to accumulate inside cells [37]. Pb precipitation was also observed in *P. fluorescens*; however, the chemical compositions were not determined [38].

All the isolated bacteria exhibited varied resistance to the heavy metals, and resistance significantly differed among all the bacterial species. The order of bacteria based on the  $F$ -value is as follows: *P. aeruginosa* [ $F(p < 0.05) = 6.0$ ] < *P. fluorescens* [ $F(p < 0.05) = 8.3$ ] < *P. stutzeri* [ $F(p < 0.05) = 13.8$ ] < *V. damsela* [ $F(p < 0.05) = 15.3$ ]. Low  $F$ -values indicate that the correlation between different heavy metals and DCZ values tends to be the same, and *P. aeruginosa* has a low  $F$ -value. Based on the results, the DCZ values of *P. aeruginosa* were similar in different heavy metals and concentrations; hence, *P. aeruginosa* was the most resistant bacteria and a clear zone only appeared in Hg. The results indicated a correlation between the metal resistance of bacterial species and the measured DCZ. The most evident correlation was observed in *P. stutzeri*, with a plot of  $y = 0.054x + 0.24$  with  $R^2 = 0.803$ , even exhibiting a weakness to Hg exposure.

In addition, ANOVA was used to determine the dominant factor in the bacterial resistance test, and it was found to be the DCZ value. The ANOVA results of the DCZ value are provided in Table S1. Based on Table S1, the types of bacteria, heavy metals, and different concentrations provide significant differences in DCZ values ( $p < 0.05$ ). The type of heavy metal exhibited the highest  $F$ -value, indicating that it is an influential factor in the bacterial resistance test due to different heavy metals having varying toxicity levels. In heavy metals with high toxicity, bacteria will be stressed and create a new metabolic mechanism to be able to survive in these heavy metals. In metals with low toxicity, bacteria will easily survive and will be utilized as macronutrients and micronutrients for metabolism via electrostatic interactions, regulating osmotic pressure [39].

The growth level of bacteria on contaminated agar indicates the level of bacterial resistance to each heavy metal [40]. The four bacteria responded differently to heavy metal exposure, and the results are provided in Table 3. All the bacteria exhibited a negative response at  $5 \text{ mg L}^{-1}$  Hg except for *P. aeruginosa*. *P. aeruginosa* demonstrated high resistance to Hg. *P. stutzeri* and *P. fluorescens* were more inhibited than the others at  $5\text{--}20 \text{ mg L}^{-1}$  of Cd and Pb, and thus, had low resistance to heavy metal exposure. All the bacteria grew well and presented high resistance to  $> 20 \text{ mg L}^{-1}$  Fe, Mn, and Cu. The order of bacterial resistance is as follows: *P. aeruginosa* > *V. damsela* > *P. stutzeri* > *P. fluorescens*. The level of heavy metal toxicity can be ranked as follows: Hg > Cd > Pb > Mg > Zn > Fe, Mn, and Cu. From the statistical analysis, a significant difference existed between *P. aeruginosa* and *P. fluorescens* in terms of resistance to heavy metal exposure, but the two bacteria did not exhibit a significant difference from *P. stutzeri* and *V. damsela* ( $p < 0.05$ ). In addition, all the heavy metals presented no significant difference, except for Hg ( $p < 0.05$ ).

Variations in heavy metal concentration also showed a significant difference in Hg. By contrast, variations in concentration did not present a significant difference in bacterial resistance level ( $p < 0.05$ ) in the other heavy metals. Type of microorganism, produced enzyme, and metabolism are factors that influence differences in bacterial response to heavy metal exposure. *P. aeruginosa* was highly resistant to all the heavy metals except for Hg.

The heavy metal resistance test can determine the MIC values of the heavy metals to bacteria. MIC is the lowest concentration of a heavy metal that inhibits bacterial growth. A higher MIC indicates less toxicity, and conversely, a lower MIC indicates that a heavy metal is more toxic. The MIC values of the eight heavy metals are provided in Table 4. The MIC values of Fe, Mn, and Cu were  $> 20 \text{ mg L}^{-1}$  in all the bacteria. The MIC values of all the heavy metals, except for Hg, were  $> 20 \text{ mg L}^{-1}$  for *V. damsela* and *P. aeruginosa*, and they were  $5 \text{ mg L}^{-1}$  and  $10 \text{ mg L}^{-1}$ , respectively, for Hg. The MIC values of Hg and Cd were both  $5 \text{ mg L}^{-1}$ , and those of the remaining heavy metals were  $> 20 \text{ mg L}^{-1}$  for *P. stutzeri*. *P. fluorescens* exhibited the lowest resistance among the bacteria, with MIC values of 5, 5, 15, and  $20 \text{ mg L}^{-1}$  for Hg, Pb, Mg, and Zn, respectively. The difference in MIC values indicates that various types of microorganisms exhibit varying responses when exposed to heavy metals [41]. Exposure to bacteria will affect the electron transport mechanisms, enzymatic reactions, and transformation mechanism of heavy metals into less toxic compounds [30]. Therefore, bacteria with high resistance to heavy metals exhibit potential as bioremediation agents.

Based on the heavy metal resistance test, *P. aeruginosa* is the most resistant to all the heavy metals among the four bacteria. The genus and species bacterium levels for FZ-2 were further identified through 16S rDNA sequencing. The neighbor-joining phylogenetic tree is shown in Fig. 2. From this figure, high bootstrap values were found for bacterium FZ-2 (93%), which was closely matched to *P. aeruginosa* strain DSM 50071 with an identity value of 99%. *P. aeruginosa* is one of the highly tolerant heavy metal-resistant bacteria isolated from different environments [15]. Purwanti et al. [42] reported that *P. aeruginosa* can remove and recover heavy metals from wastewater.

#### Removal of Hg by *P. aeruginosa* FZ-2

The effect of various salinity levels on the removal of Hg by *P. aeruginosa* FZ-2 was further investigated and provided in Fig. 3. As shown in the figure, the removal of Hg in fresh water is lower than those in other salinity levels, with the removal efficiency reaching 54%. In marginal water, brackish water, and saline water, the

**Table 3** Heavy metal resistance test

Heavy metal	Concentration (mg L <sup>-1</sup> )	<i>V. damsela</i>	<i>P. aeruginosa</i>	<i>P. stutzeri</i>	<i>P. fluorescens</i>
Mg	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	10	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	15	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
	20	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
Hg	0	*** f, D, H	*** F, D, H	*** f, D, H	*** f, D, H
	5	** f, D, h	*** F, D, H	** f, D, h	** f, D, h
	10	** f, D, h	** F, D, h	** f, D, γ	** f, D, h
	15	** f, D, h	** F, D, h	* f, D, γ	** f, D, h
	20	** f, D, h	** F, D, h	* f, D, γ	** f, D, h
Zn	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	10	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	15	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	20	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
Pb	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
	10	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
	15	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
	20	*** f, d, H	*** F, d, H	*** f, d, H	** f, d, h
Mn	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	10	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	15	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	20	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
Cu	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	10	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	15	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	20	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
Cd	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	** f, d, h	*** f, d, H
	10	*** f, d, H	*** F, d, H	** f, d, h	*** f, d, H
	15	*** f, d, H	*** F, d, H	** f, d, h	*** f, d, H
	20	*** f, d, H	*** F, d, H	** f, d, h	*** f, d, H
Fe	0	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	5	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	10	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	15	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H
	20	*** f, d, H	*** F, d, H	*** f, d, H	*** f, d, H

**Note:** F-f-α represent significant statistical differences at  $p < 0.05$  for different bacteria in the same heavy metal and concentration. D-d-β represent significant statistical differences at  $p < 0.05$  for different heavy metals in the same bacteria and concentration. H-h-γ represent significant statistical differences at  $p < 0.05$  for different concentrations in the same bacteria and heavy metal

\*\*\*: high growth (DCZ = 0 cm),

\*\* : medium growth (0 < DCZ < 1 cm),

\* : low growth (1 < DCZ < 2 cm),

–: no growth (DCZ > 2 cm)

**Table 4** MIC values of heavy metals in the bacteria

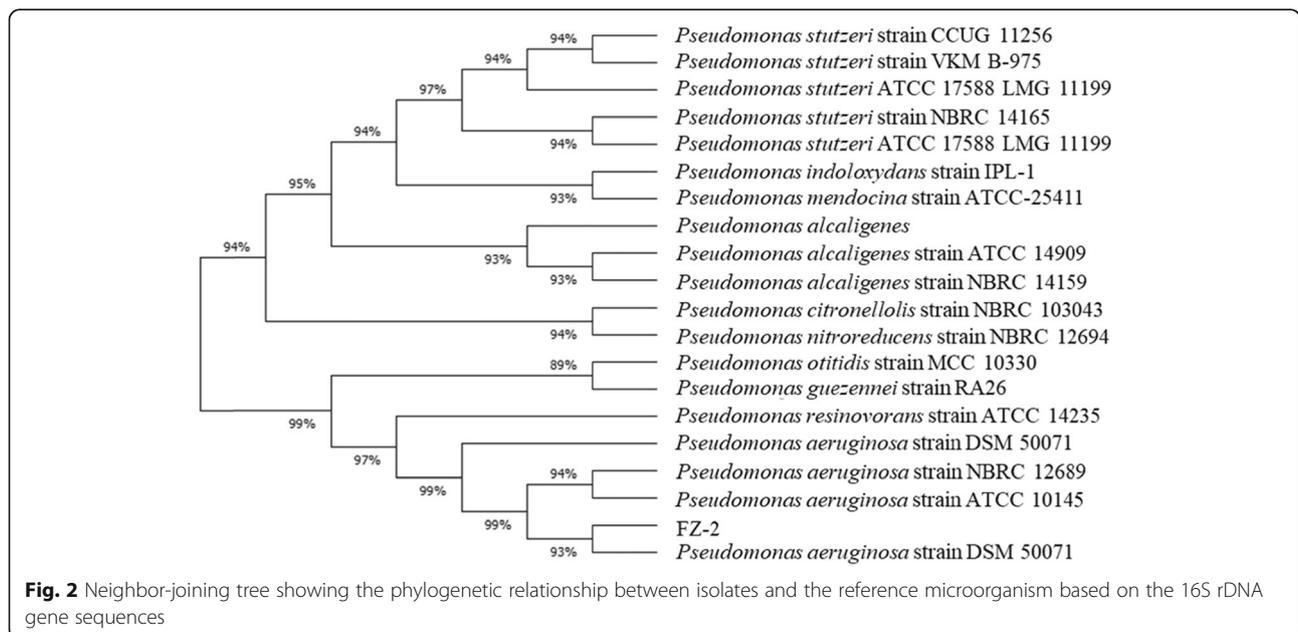
Isolated bacteria	Concentration (mg L <sup>-1</sup> )							
	Mg	Hg	Zn	Pb	Mn	Cu	Cd	Fe
<i>V. damsela</i>	> 20	5	> 20	> 20	> 20	> 20	> 20	> 20
<i>P. aeruginosa</i>	> 20	10	> 20	> 20	> 20	> 20	> 20	> 20
<i>P. stutzeri</i>	> 20	5	> 20	> 20	> 20	> 20	5	> 20
<i>P. fluorescens</i>	15	5	20	5	> 20	> 20	> 20	> 20

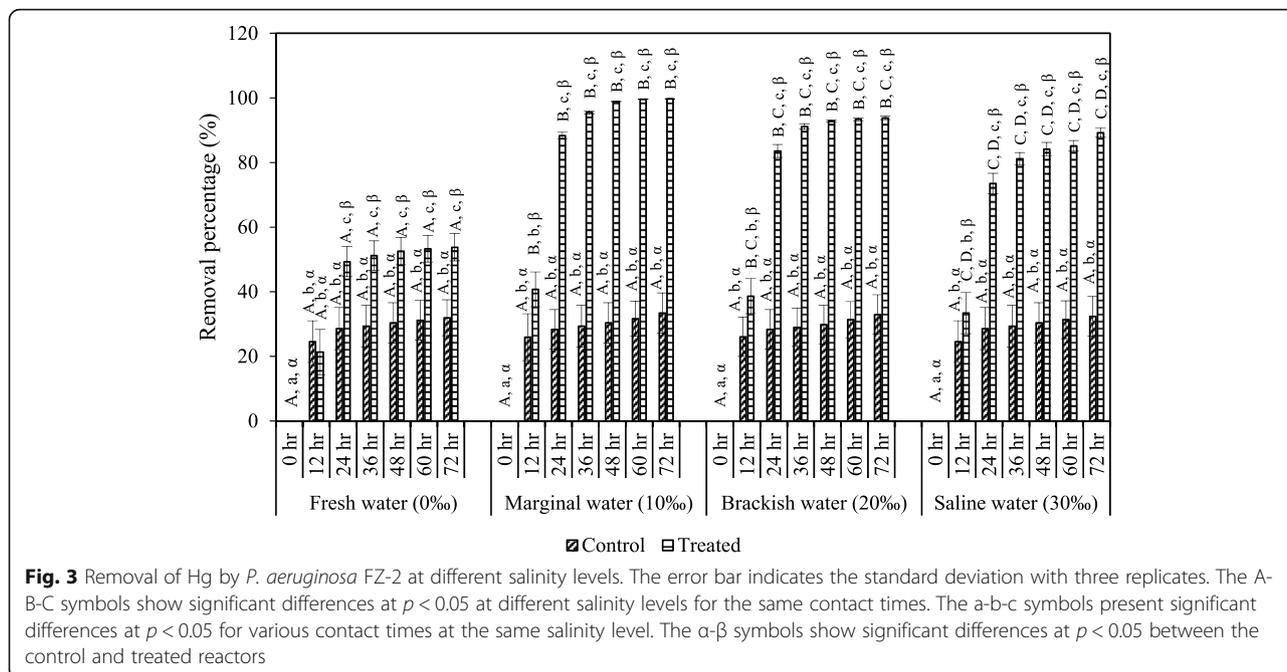
removal efficiencies are >99, 94, and 89%, respectively. Salinity exerts a significant positive effect on *P. aeruginosa* FZ-2 in removing Hg. Therefore, the finding that the concentration of Hg in the control was also slowly declining up to 33% should not be disregarded; it suggests that the medium component exerts some effects on Hg removal. Furthermore, the oxygen present in the reactor during agitation may oxidize Hg [15]. Thus, in addition to the role of the medium component, the removal efficiencies of Hg by *P. aeruginosa* FZ-2 were 22, 66, 61, and 57% at 72 h in fresh water (0‰), marginal water (10‰), brackish water (20‰), and saline water (30‰), respectively. Several previous studies have been conducted on the reduction of Hg by isolated bacteria. De et al. [21] reported that *P. aeruginosa* CH07 and Bro12 can remove up to 66% of Hg. Saranya et al. [43] found that *Vibrio fluvialis* reduced 250 µg mL<sup>-1</sup> of Hg with 60% removal. Table 5 summarizes several previous studies related to the removal of Hg by bacteria.

The optimum salinity for *P. aeruginosa* FZ-2 to reduce Hg is in marginal water (10‰). Saline condition influences the transport of proteins and periplasmic proteins

present on the cell surface of bacteria, and such influence can increase the role of these proteins at high salinity levels. Consequently, Hg will bind more on the surface of bacteria and increase the production of the *merA* gene. The presence of NaCl in the solution with Hg will increase the dissolution (decrease the solubility) of Hg because Cl<sup>-</sup> will form a stable complex compound with Hg, decreasing the toxicity level [10]. Otherwise, conditions with extremely high saline concentrations can reduce bacterial biomass due to osmotic stress and cell lysis [47]. Dash et al. [22] reported that the expression of Hg reductase (*merA*) from *B. thuringiensis* PW-05 under various saline conditions exert a significant effect on *B. thuringiensis* PW-05 in removing Hg. With an initial Hg concentration of 50 mg L<sup>-1</sup>, *B. thuringiensis* PW-05 can remove Hg up to 91% in 10‰ salinity. The *merA* gene is an enzyme that plays an important role in Hg reduction; this gene transforms Hg<sup>2+</sup> into Hg<sup>0</sup> [21].

Deng and Wang [48] reported that Hg is bound strongly to sulfur functional groups. Hg will bind to a functional sulfur group before being transformed by the *merA* gene. Al-Qadiri et al. [49] analyzed the functional group of *P. aeruginosa* via Fourier transform infrared spectroscopy, and the result showed the presence of amide I groups at 1650 cm<sup>-1</sup>, amide II groups at 1550 cm<sup>-1</sup>, CH<sub>3</sub> groups at 1455 cm<sup>-1</sup>, CH<sub>2</sub> groups at 1398 cm<sup>-1</sup>, and polysaccharide groups at 1200–900 cm<sup>-1</sup>. The presence of these groups indicates the interaction of heavy metals with functional groups on the surface of *P. aeruginosa*. Polysaccharides on *P. aeruginosa* bind heavy metals inside cells. *P. aeruginosa* also produces extracellular polymeric substances against heavy metals that





contribute to heavy metal resistance. *P. aeruginosa* exhibits high resistance to Hg, Cd, Cu, and Zn [15, 34].

**Application of bioremediation to heavy metal removal**

Understanding the response of bacteria related to heavy metal exposure is required before applying bioremediation techniques. Bioremediation can exhibit good performance when the bacteria used as bioremediation agent can grow well in heavy metal-polluted areas. Bacteria exhibit the capability to adsorb and accumulate heavy metal ions with high adsorption capacity [50]. The bioremediation of leachate by bacteria is considered one of the biotechnology approaches that is environment-friendly, cost-effective, and safe for decontaminating leachate. This process is strongly influenced by the resistance level of bacteria to heavy metals. This research approach will considerably help in identifying the type of bacteria that is highly resistant to heavy metals. MIC and bacterial resistance are useful in bioremediation, in which bacteria can remove heavy metals through bioaccumulation and biosorption [51].

Heavy metal bioremediation applications can be performed using bioreactors [52]. A bioreactor is a system that utilizes biological processes to reduce pollutants through biochemical processes. Types of bioreactors include biofilm reactors [53] and fixed bed bioreactors [52]. The bacteria applied in a bioreactor can be used as a biosorbent for binding heavy metals. Similar to Sinha et al. [54], *Bacillus cereus* cells were immobilized with alginate for Hg bioremediation in wastewater by using a bioreactor with an efficient removal of more than 80%.

The utilization of bacteria as a biosorbent can be achieved with living or dead cells. Moreover, the bioaugmentation process can be implemented in bioremediation applications by using a bioreactor [55]. Bacteria isolated from polluted areas, particularly from leachate, can be used in the bioremediation of leachate-contaminated soil through bioaugmentation. Exogenous bacteria isolated from polluted areas with similar conditions are added to the bioaugmentation process and used as degrading agent to improve their performance and exert a positive effect on the bioremediation process. Indigenous bacteria isolated from polluted areas will be effective in reducing pollutants due to their high resistance to pollutants [56]. Bacteria isolated from leachate can be used as an environment-friendly biotechnology and an efficient bioremediation agent that can be manipulated to improve their performance. Bacterial resistance to heavy metals must be considered in developing bioremediation techniques because of its direct relationship to the survival and growth of bacterial species [57].

**Conclusions**

Heavy metals were not detected in non-active sanitary landfill leachate, except for Mn. Four bacteria were isolated from leachate and identified as *V. damsela*, *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens*. Heavy metal resistance test indicated that *V. damsela*, *P. aeruginosa*, *P. stutzeri*, and *P. fluorescens* exhibit high resistance to Fe, Mn, and Cu. *P. fluorescens* was the most inhibited among the four bacteria at low concentrations of Hg, Cd, Pb, Mg, and Zn. In addition, *P. aeruginosa* FZ-2 can also eliminate Hg at different salinity levels with a

**Table 5** Summary of studies related to Hg removal by bacteria

No.	Bacteria	Source	Heavy metal	Summary	References
1.	<i>Pseudomonas aeruginosa</i> strain FZ-2	Leachate	Hg <sup>2+</sup>	This research conducted heavy metal resistance bacteria and removal of Hg in difference salinity levels. The result showed that <i>P. aeruginosa</i> strain FZ-2 has high resistance to Fe, Mn, Cu, Cd, Pb, Mg, and Zn and is inhibited by Hg with 10 mg L <sup>-1</sup> concentration. Besides that, <i>P. aeruginosa</i> strain FZ-2 can remove Hg in 0, 10, 20, and 30‰ of salinity with removal efficiencies are 54, > 99, 94, and 89%, respectively. It was indicated that salinity exerts a significant positive effect on <i>P. aeruginosa</i> FZ-2 in removing Hg.	Current research
2.	<i>Pseudomonas</i> sp. B50A	Hg-contaminated soil from a land farming area	Hg <sup>2+</sup>	This research discussed Hg-resistant bacteria, the removal of Hg, and the characterization of Hg reductase by the new strain of <i>Pseudomonas</i> sp. B50A. The result showed that <i>Pseudomonas</i> sp. B50A can remove Hg <sup>2+</sup> up to 82 ± 2% with 10 μM of initial Hg concentration after 24 h at 30 ± 2 °C. In addition, <i>Pseudomonas</i> sp. B50A can produce Hg reductase, which plays a role in removing Hg.	[44]
3.	<i>Pseudomonas aeruginosa</i> KHY2	Water around a gold mining area	Hg <sup>2+</sup>	The purpose of this study was to determine the effect of co-enzyme addition on Hg bioremediation. The result showed that <i>P. aeruginosa</i> KHY2 can decrease Hg <sup>2+</sup> by more than 60% with the addition of co-enzyme (128 ppm of vitamin B12). The initial Hg concentration was 12 ppm.	[20]
4.	<i>Pseudomonas aeruginosa</i> CH07	Marine sediments	Hg	This research was conducted in fixed-bed continuous up-flow reactors by using Hg-resistant bacteria. The effect of salinity on performance was also investigated. The result showed that <i>P. aeruginosa</i> CH07 isolated from marine sediments can remove up to 95% Hg with 8 mg L <sup>-1</sup> of Hg and 8 g L <sup>-1</sup> of NaCl. The removal rate of <i>P. aeruginosa</i> CH07 for 1 mg L <sup>-1</sup> of Hg was 1.16 × 10 <sup>-6</sup> ng cell <sup>-1</sup> min <sup>-1</sup> .	[21]
5.	<i>Pseudomonas aeruginosa</i>	Soil sample from an industrial area	Hg <sup>2+</sup>	This study was conducted to determine the optimum condition for Hg removal by <i>P. aeruginosa</i> . The result showed that the optimum pH and temperature conditions for <i>P. aeruginosa</i> were 6 and 28 °C, respectively, with 62% of removal efficiency. Moreover, Hg reductase was detected during incubation time.	[45]
6.	<i>Pseudomonas</i> spp	Contaminated sediments from a lagoon	Hg <sup>2+</sup>	The objective of this research was to isolate Hg-resistant bacteria from the sediments of a lagoon and determine their capability to remove and transform Hg. The result showed that <i>Pseudomonas</i> spp. is resistant to Hg with a concentration of 0.075 mM. <i>Pseudomonas</i> spp. can convert volatile Hg <sup>2+</sup> into Hg <sup>0</sup> , and the removal of Hg <sup>2+</sup> can reach up to 99% with an initial concentration of 10 mg mL <sup>-1</sup> .	[46]

removal efficiency of more than 90%. Salinity exerts a significant positive effect on *P. aeruginosa* FZ-2 in removing Hg. Therefore, *P. aeruginosa* exhibits high potential in assisting in the bioremediation of heavy metal-polluted areas.

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42834-021-00088-6>.

**Additional file 1: Figure S1.** Overview of heavy metal resistance mechanisms evolved by bacteria. **Table S1.** Analysis of variance for DCZ value.

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### Authors' contributions

Muhammad Fauzul Imron: Conception and design of research; acquisition, analysis, and interpretation of data; drafted the manuscript or substantively

revised it. Setyo Budi Kurniawan: creation of new software used in the research, drafted the manuscript or substantively revised it. Siti Rozaimah Sheikh Abdullah: Conception and design of research, drafted the manuscript or substantively revised it. All the authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article or can be requested from the corresponding author.

### Declarations

#### Competing interests

The authors declare no competing interests.

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