Lead-resistant bacteria isolated from oil wastewater sample for bioremediation of lead

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ABSTRACT

Anthropogenic activities such as oil exploration have resulted in an environmental concern as they are comprised of residual hydrocarbons and metals. Following the hypothesis that endogenous bacterial communities have enhanced tolerance to heavy metals, we isolated and characterized culturable lead-resistant bacteria from an oil wastewater sample and determined whether they could reduce lead ions from the medium. The wastewater sample containing indigenous bacteria were taken out from a traditional oil field, Bojonegoro District, East Java, Indonesia, and bacteria were cultured *Halomonas* complex (HMC) medium containing lead (II) chloride ($PbCl_2$) with different concentrations. Bioaccumulation of lead by heavy-metals resistant bacteria was determined by using atomic absorption spectrophotometry (AAS). Our result found 21 bacterial strains that resist lead ions, of which one strain (RPb5-3) highly resisted to 10 mM. This bacterial strain also exhibited the highest accumulation of Pb, and it could grow at various temperatures, or more than their original environment. The bacterial strains could be used for bioremediation of lead toxicity, especially in oil pollutants.

Key words | bioremediation, lead-resistant bacteria, oil wastewater

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HIGHLIGHTS

- This study resulted in 21 bacterial strains that can be used to decontaminate lead pollution and toxicity from oil wastewater samples containing lead pollution.

INTRODUCTION

Petroleum is the primary energy source used in households, industry and transportation. Petroleum can be used as a source of fuel and a source of foreign exchange (Sayuti *et al.* 2016). In addition to producing fuel oil, basic materials for lubricants and other oil products, the processing of petroleum also produces waste. Petroleum waste can be in the form of solids or liquids carried during the oil drilling process. Petroleum waste also contains wax materials, hydrocarbon components, non-hydrocarbons and heavy metals.

Oil waste is a hazardous and toxic material (B3). Petroleum waste can endanger the environment, as well as the survival of humans and other living things (Lodungi *et al.* 2016). One of the impacts of petroleum mining activities is heavy metal pollution. According to Ozdemir *et al.* (2003), heavy metals are produced from various industrial wastes, one of which is oil mining. Heavy metals are sedentary in the environment because they cannot be degraded. Heavy metal pollution is a significant problem because toxic effects can accumulate in the food chain, causing severe ecological and environmental health problems.

One type of heavy metal contained in petroleum mining waste is lead (Pb). Crude oil, mining industry drilling fluid contains lead (Pb). Petroleum mining waste also contains high levels of lead (Pb) (Ali *et al.* 2009; Naik & Dubey 2013). Saeni (1997) explains that lead (Pb) is the second most dangerous heavy metal after mercury (Hg) because lead (Pb) is accumulative. More lead (Pb) is disposed of as waste compared to mercury (Hg). For example, disposal from the use of coal and petroleum for energy generates 2,835 tons/year of lead (Pb), while the amount of waste mercury (Hg) is 221 tons/year.

Lead (Pb) is the most dangerous heavy metal, even in small quantities. Hg^{2+}, Pb^{2+} and Cd^{2+} are the three metals that are included in the priority list of hazardous pollutants

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from the US Environmental Protection Agency (EPA). Hg^{2+}, Pb^{2+} and Cd^{2+} are serious concerns of the US EPA because they are nondegradable, highly toxic, and are in various waste streams that pollute the environment (Paul *et al.* 2012). Lead pollution (Pb) in waters causes death in aquatic biota, so it can disrupt the food chain in these waters (Lestari & Edward 2004). In humans, lead (Pb) can cause anaemia, nervous disorders, and impaired kidney function (Sudarmaji *et al.* 2006).

Traditional petroleum and natural gas mines or community mines in Kedewan Subdistrict, Bojonegoro Regency, consist of 74 wells covering Wonocolo Village, Hargomulyo Village and Beji Village. Wonocolo Village is one of the mining locations with the highest number of cutting wells, 35 wells. For every mining activity in the cutting well, there is an oil spill on the surrounding land due to the process of transporting oil, through pipes, transportation equipment, or spills due to the transfer process (Nugroho 2006). In addition to oil spills, the traditional oil mining process in Wonocolo Village also produces liquid waste from the oil drilling process. The liquid waste is in the form of water originating from the separation of crude oil and water from petroleum drilling. Liquid waste is usually discharged directly into the environment without being processed first.

Mining wastewater is a waste with extreme pH (acid or base), high salinity, contains SO_4^{2-}, and contains dangerous heavy metals (Kamika & Momba 2014). In general, heavy metals Cd, Pb, Ni, Cr, V and Zn are found in crude oil and drilling fluids in the oil mining industry (Fu *et al.* 2014).

Based on the results of the preliminary test, the Wonocolo oil mine liquid waste contains lead (Pb) up to 0.32 ppm (1.54 μM). The lead level (Pb) in the Wonocolo oil mine wastewater has exceeded the threshold. According to US Environmental Protection Agency (US EPA), the limit of lead (Pb) content in liquid waste is 0.1 ppm (0.48 μM) (Paul *et al.* 2012).

Processing of waste with biological methods is environmentally friendly. Biological methods can be carried out by utilizing the adaptive mechanisms of microorganisms to survive and grow in less favourable environments, such as industrial areas and mining areas. This adaptation is considered as a functioning tool in the processing of toxic waste.

The use of bacteria for heavy metal bioremediation processes from locations contaminated with mining waste and industrial waste is more effective than physical or chemical methods because it is more efficient and environmentally friendly. Bacteria that grow in the lead (Pb) polluted environments are often resistant to lead (Pb). Growing bacteria can be isolated, selected, identified and characterized. Isolation and characterization of lead-resistant bacteria (Pb) is essential to determine the ability of bacterial resistance to lead (Pb) and the ability to accumulate lead (Pb). Furthermore, the isolates obtained can be optimized and duplicated so that they can be used in the bioremediation process (Marzan *et al.* 2017). Bacteria isolated from an environment contaminated with heavy metals have strong potential to be used as bioremediation agents because bacteria have resistance and tolerance to heavy metals around them (Chojnacka 2010).

So far, there has been no information about lead-resistant bacteria (Pb), which were successfully isolated and tested for the ability to bioaccumulate lead (Pb) from Wonocolo oil mine wastewater. Research on petroleum waste in the Wonocolo oil mine is limited to bioremediation of hydrocarbon-contaminated soil and identification of Vascular Arbuscular mycorrhizal fungi (MVA) from petroleum-contaminated soil (Holifah *et al.* 2018). Therefore, it is necessary to do further research on the potential of lead-resistant (Pb) bacterial isolates from Wonocolo oil mine wastewater as a lead (Pb) bioaccumulator, so that the bacterial isolates obtained can be utilized for the bioremediation process and reduce the impact of lead pollution (Pb).

METHODS

Oil wastewater sample and bacterial isolation

Oil wastewater sample used in this study was collected from Wonocolo Village, Bojonegoro District, East Java. Aliquots (10 μl) of 10–2 to 10–4 dilutions were spread plated in the surfaces of 20 ml sterile *Halomonas* complex (HMC) medium consist of casamino acid 7.5 g/l, peptone 5 g/l, yeast extract 1 g/l, NaCl 50 g/l, $MgSO_4$ $7H_2O$ 20 g/l, sodium citrate 3 g/l, K_2HPO_4 0.5 g/l, $FeSO_4$ $(NH_4)2SO_4$ 0.05 g/l, and agar 20 g/l and containing 5 and 10 mM of $PbCl_2$ and incubated at 37 °C for 48 hours. Bacterial colonies growth in all medium were then selected for the following identification.

Identification of the most lead-resistant bacteria from oil wastewater sample by morphology and biochemistry

Bacterial isolates were grown on HMC, followed by colony morphological characterization including colony color and shape, gram staining and biochemical test. The lead-resistant bacterial isolates (Pb) obtained were identified using the Microbact Kit GNB 12A/12 E or 24E and refer to Bergey's Manual of Determination Bacteriology 9th book. Before being determined using 12 A/12E or 24 E, the bacterial colony is tested for oxidase. If the oxidase test results are negative, then the Microbact system 12A/12E only is used, while if the oxidative results are positive then 24E is used.

Growth characterization of isolate RPb5-3 at different concentrations of lead

The characteristics of the growth of lead-resistant bacteria (Pb) selected were carried out by growing the bacteria on the HMC broth media that was induced by $PbCl_2$. Testing of the effect of lead concentration (Pb) is done by adding 1 ose of bacterial culture to 3 ml HMC broth media with concentrations of 0 mM, 1 mM, 2 mM, 5 mM and 10 mM. Each treatment was carried out three times. For treatment at this stage, the bacteria were incubated in an incubator shaker for 48 hours at 37 °C and 150 rpm. The purpose of this treatment is to see differences in the growth density of lead-resistant bacteria (Pb) at different concentrations of lead (Pb). For each bacterium and each treatment the Optical Density (OD) value was measured with a wavelength of 600 nm using a spectrophotometer for every 4 hours of incubation time. The higher the OD value at different lead concentrations (Pb), the higher the bacterial density and the more resistant to lead concentration (Pb).

Bioaccumulation of lead by the most lead-resistant bacteria isolate RPb5-3

RPb5-3 bacterial isolates were grown on HMC broth media with lead concentrations (Pb) of 0 μM, 100 μM, and 200 μM. They were incubated in an incubator shaker at 37 °C, 150 rpm for 24 hours. The incubated isolate was transferred into an empty weighted tube. Then the samples were centrifuged at a speed of 5,000 rpm for 15 minutes, separated into pellets and supernatants and the weight of the pellets and tubes was weighed. The pellets were heated in the oven at 65 °C for 48 hours. After drying, the dry weight of the tube and pellet was weighed. Samples were added to 7 ml of 1N HNO_3 and heated again at 65 °C for 24 hours. Samples were centrifuged at 5,000 rpm for 15 minutes. Supernatants were used for the analysis of lead bioaccumulation (Pb) using atomic absorption spectrophotometers (AAS).

RESULTS AND DISCUSSION

Isolation of lead-resistant bacteria from oil wastewater samples

In this study, we performed an experiment for the isolation of bacteria in HMC medium containing some concentrations of lead chloride ranging from 0 to 10 mM. The average number of colonies grown on HMC media decreased with increasing lead concentration (Pb) in the bacterial growth media. We found at least 21 isolates from this study and further determination for their MIC (minimum inhibitory concentration) level found that only six bacterial isolates have the ability to resist to 10 mM of lead chloride (Table 1 and Figure 1). The research by Murthy *et al.* (2012) showed that an increase in lead (Pb) concentration (100, 200, 300, 400, 500 mg/l) in the test media could reduce the growth of *Bacillus cereus*. Lead pollution (Pb) can interfere with metabolism and microbial activity, such as respiration, transport and membrane synthesis, and ribosome activity that will cause cell death (Wani *et al.* 2015).

Minimum inhibitory concentration to determine the most resistant bacteria

The screening of lead-resistant bacteria (Pb) produced in RPb5-3 selected bacterial isolates that are lead resistant

Table 1 | Minimum inhibitory concentration (MIC) level of six lead-resistant bacterial isolates

	Lead concentration (mM)			
Bacterial code	0	2.5	5	10
RPb5-1	++++	++++	++	−
RPb5-2	++++	++++	+	−
RPb5-3	++++	++++	+++	+
RPb5-4	++++	++++	++	−
RPb5-5	++++	+++	++	−
RPb5-6	++++	++++	++	−

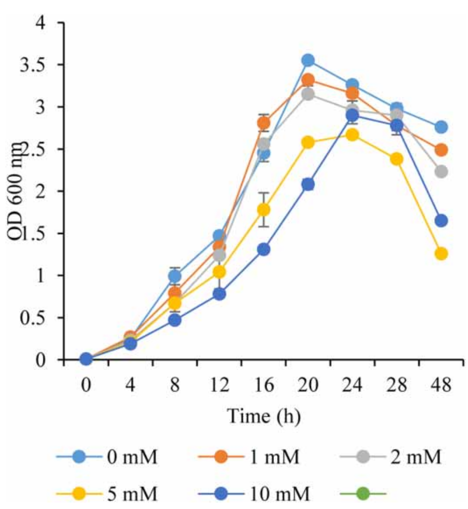

Figure 1 | The effect of concentration of metals on growth characteristic of lead-resistant bacteria, isolate RPb5-3.

(Pb) up to 10 mM. Bacterial resistance is characterized by brown colonies, which indicate the presence of lead (Pb) resistance by precipitation. Lead precipitation (Pb) is a mechanism of extracellular lead resistance that aims to limit the movement of lead (Pb) on bacterial cell walls (Bruins *et al.* 2000). Pb (II), which binds to negative groups and extracellular polymers, will be non-toxic because it changes to Pb (0) (Yani & Kurniasari 2008). Anions that can bind to Pb (II) include chloride, phosphate, and hydroxyl ions, which will change insoluble deposits (Levinson & Mahler 1998).

Mechanism of lead (Pb) resistance in *Citrobacter* sp. was through a precipitation process involving the hydrolysis of organic P by the enzyme phosphatase, followed by precipitation of $PbHPO_4$ on the cell surface, as indicated by brown colonies on agar media or brown pellets in liquid media (Park *et al.* 2011). *Providencia alcalifaciens* is resistant to lead (Pb), with the growth of brown colonies in the media. The SEM-EDX and X-ray diffraction spectroscopy (XRD) test results, brown dyes on the media, showed an extracellular lead (Pb) resistance mechanism to lead orthophosphate [Pb9 (PO_4) 6] as a result of enzyme phosphatase catalysis (Naik & Dubey 2013).

Identification of lead-resistant bacteria based on morphological and biochemical characters

The macroscopic characterization of bacterial colonies that grew at the highest concentration (5 mM) obtained six types of bacterial isolates dominated by bacteria that were white to yellowish, irregular shapes, with flat surfaces and wavy edges. Different forms of colonies indicate the six bacterial colonies, including various groups of bacteria. According to Sumarsih (2003), the colonies of a bacterium are influenced by adjustments to the environment and the physiological properties of the microbes themselves.

In this study, bacterial cell shape showed that the six lead (Pb) resistant bacterial isolates were included among the gram-positive bacteria. Bacteria are dominated by rod-shaped bacteria (five isolates), and one is shaped like cocci. Gram-positive bacteria are more resistant to environments contaminated with heavy metals, and this is related to the cell structure of the bacterial cell wall. The surface of the cell wall of positive salt bacteria has a higher capacity to bind metal ions than negative Gram bacteria. Gram-positive bacteria have the ability to absorb heavy metals larger than gram-negative bacteria. The cell wall of gram-positive bacteria consists of carboxyl groups which become agents of heavy metal uptake. The source of the carboxyl group is teichoic acid, which is associated with the peptidoglycan layer on the bacterial cell wall (Issazadeh *et al.* 2011; Murthy *et al.* 2012).

A previous study examined the isolation of lead-resistant bacteria (Pb) in the form of bacilli and including gram-positive bacteria originating from the genus *Bacillus*. According to Arinda *et al.* (2012), *Bacillus* is a bacterium that is abundant in nature. *Bacillus* can be isolated from air, soil, fresh or salty water, solid or liquid waste, from normal or extreme environments, including heavy metal contaminated environments. Kermanshahi *et al.* (2007) managed to isolate lead-resistant (Pb) bacteria from lead-contaminated soil (Pb). Bacteria from the genus *Bacillus* dominated the isolates obtained. Gurave *et al.* (2015) succeeded in isolating lead-resistant *B. thuringiensis* (Pb) from petroleum-contaminated soil with a resistance of up to 5 mM.

As a result of the identification of Microbact kit bacteria NB 12A and 12B, RPb5-3 bacteria were identified as *Bacillus cereus* with a similarity of 82% (Table 2). *Bacillus cereus*

Table 2 | Identification result of isolate RPb5 using biochemistry method (Microbact GNB 12 A/E and 12 B)

Kit Code	Character	Result
12A	Oxidase	+
	Motilitas	–
	Nitarte	+
	Lysine	–
	Ornithine	–
	H_2S	–
	Glucose	–
	Mannitol	–
	Xylose	–
	ONPG	–
	Indole	–
	Urease	–
	VP	+
	Citrat	–
	TDA	–
12B	Gelatin	–
	Malonate	–
	Inositol	–
	Sorbitol	–
	Rhamnose	–
	Sucrose	–
	Lactose	–
	Arabinose	–
	Adonitol	–
	Raffinose	–
	Salicine	–
	Arginine	–
	Gram	+
	Form	Basil
Species	*Bacillus cereus*	

is one of the biofilm-forming bacteria. The ability for lead bioaccumulation (Pb) is also supported by the morphology of bacterial colonies RPb5-3 (*Bacillus cereus*), which is sticky in the media and difficult to extract, showing one of the indicators of biofilm formation. With this ability, bacteria RPb5-3 (*Bacillus cereus*) can be used as a candidate for bioremediation agents, because lead (Pb) can be absorbed in biofilms and accumulates in the body of bacteria formed by RPb5-3 bacteria, so that the concentration of lead (Pb) in the environment decreases. RPb5-3 bacteria can be used as a candidate for a lead bio-accumulator (Pb) because it can accumulate lead (Pb) up to 54.54%.

Growth characterization of isolate RPb5-3 at different concentrations of lead

In order to obtain a good bacterial strain for application purposes, especially to recover Pb pollution, we also performed growth characteristics of lead-resistant bacteria isolate RPb5-3 in different concentrations of Pb ranging from 0, 1, 2, 5 and 10 mM. The result revealed that no significant difference between each treatment of lead concentration, indicating that the isolate RPb5-3 could survive even in a high concentration of Pb (or 10 mM). This study is the first report of lead-resistant growth exhibited by indigenous bacteria obtained from an oil wastewater sample. This study is also in opposition with the data reported previously on *Halomonas* sp. reported by Kamika & Momba (2013), in which the bacterial growth decreased with the increase of lead concentration indicated by the decrease in the optical density (OD).

Bioaccumulation of lead by the most lead-resistant bacteria isolate RPb5-3

The ability of bacteria to accumulate lead (Pb) increases with increasing lead (Pb) concentration. In the treatment of 100 μM bacteria, RPb5-3 was able to accumulate lead (Pb) to 28.62 μM. The bacteria RPb5-3 had the highest bioaccumulation ability in the 200 μM treatment, which is equal to 109.08 μM.

The percentage of bioaccumulation of lead (Pb) by bacteria RPb5-3 is included in the high category because it can accumulate lead (Pb) to a percentage of 28–54% of the given lead concentration (Table 3 and Figure 2). The study of Jaafar *et al.* (2016) showed that lead (Pb) concentrations of 5, 10, 25, 50 ppm (24 μM, 48 μM, 121 μM and 241 μM) were able to accumulate lead (Pb) by *Shewanella oneidensis* the higher the concentration of lead the ability to bioaccumulate lead (Pb) is higher. At 50 ppm (241 μM) treatment it was able to accumulate lead as large as three ppm (14.5 μM) or only about 6% of total lead (Pb) given to the test medium.

The ability to bioaccumulate lead (Pb) in bacteria varies depending on lead concentration (Pb), incubation time, and type of bacteria. Research by Arifiyanto *et al.* (2016) showed that *Bacillus* S1 and SS19 isolates with lead (Pb) induction at a concentration of 50 ppm (241 μM) were able to accumulate lead (Pb) up to 57% for S1 isolates and 51% for SS19 isolates. The ability to bioaccumulate lead (Pb) of *Bacillus* S1 and SS19 isolates decreased in line with the addition of lead concentration (Pb) in the test media. In the treatment of a lead concentration (Pb) of 75 ppm (362 μM) the ability to bioaccumulate lead (Pb) in S1 and SS19 isolates decreased, S1 isolates were able to accumulate lead (Pb) by 53% while the bioaccumulation ability of SS19 isolates was 37%.

Table 3 | Bioaccumulation of lead by lead-resistant bacteria, isolate RPb5-3, isolated from oil wastewater sample

Initial concentration of Pb	Pb accumulated (μM ± SE)	% accumulated
0 μM	2.73 ± 0.24 (a)	0%
100 μM	28.62 ± 5.01 (b)	28.62%
200 μM	109.08 ± 11.676 (c)	54.54%

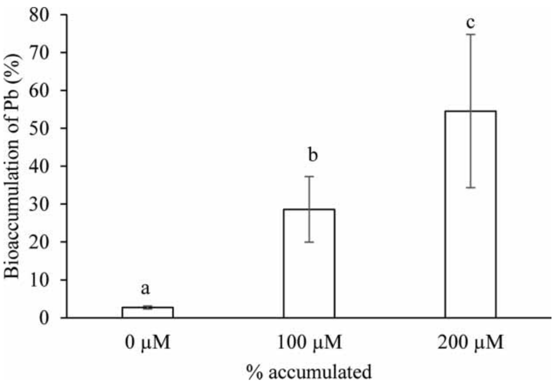

Figure 2 | Bioaccumulation result of lead by lead-resistant bacteria, isolate RPb5-3, isolated from an oil wastewater sample. The different notation indicates the significant difference in lead accumulation affected by different concentrations of lead (Sig. = 0,05).

CONCLUSIONS

In conclusion, the exhibited six bacterial strains are able to resist different concentrations of lead, of which strain/ isolate RPb5-3 is highly resistant to Pb up to 10 mM by further MIC test. A biochemical test using a microbact kit found that isolate RPb5-3 corresponds to *Bacillus cereus*. Isolate RPb5-3 also exhibited the highest accumulation of Pb, up to 54.54%. This study could be used in decontaminating lead pollution and toxicity from oil wastewater samples containing lead pollution.

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