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Bioremediation of batik wastewater by Rhizobacteria isolated from iron sand soils tolerant of Pb and Zn

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Abstract. *Oedjijono, Lestari S, Samsudin LS, Hermilia. 2021. Bioremediation of batik wastewater by Rhizobacteria isolated from iron sand soils tolerant of Pb and Zn. Biodiversitas 23: 299-305.* Rhizobacteria originated from Iron sand soils are thought to be resistant to heavy metals because they are adapted to metal-contaminated environments. Batik industry produces pollutants containing organic substances, solid suspension, and heavy metals such as Lead (Pb) and Zinc (Zn). The objectives of this study were to select and determine the identity of Rhizobacterial isolates tolerant of Pb and Zn metals, to investigate their ability and the optimum pH in tolerating and reducing the concentrations of Pb and Zn in batik wastewater. The results found five Rhizobacterial isolates of Rb2, Rb6, Rb7, Rb8, and Rb12 showing high tolerance to Pb and Zn metals. The adsorption capacity of selected Rhizobacterial isolates of Rb2, Rb8, and Rb12 on Pb was significant at pH 5.5-6.0 with the mean concentration of Pb reduced was 2.836 mg/L (80%), while against Zn by isolates of Rb6, rb7, and Rb12 was high at pH 5.5 with a reduced concentration of 0.8761 mg/L (43.2%). The selected Rhizobacterial isolates of Rb2 and Rb6 were identified as members of the genus *Pseudomonas*, Rb7 and Rb8 belonged to the genus *Lactobacillus*, while Rb12 was a species member of the genus *Staphylococcus*.

Keywords: Adsorption capacity, iron sand soils, Pb and Zn bioremediation, Rhizobacteria

INTRODUCTION

Batik is an Indonesian technique of wax-resist dyeing applied to the whole cloth originating from the island of Java. Coloring agents used in the batik cloth belong to acids, bases, and others, e.g., remazol red, indigo, and golden yellow. Most of the dyes used in the batik industry are resistant to decolorization and have toxic effects on aquatic organisms (Tian et al. 2013). Liquid waste can cause environmental pollution and affect the condition of living organisms, including humans. Dyes can cause skin irritation, allergic dermatitis, and some of them were reported to be carcinogenic and mutagenic (Gong et al. 2005). The wastewater of batik industry also contains organic substances, solid suspension, and heavy metals such as Zinc (Zn), Cadmium (Cd), Chromium (Cr), and Lead (Pb) (Susilowati et al. 2018). Lead is a highly toxic metal in aquatic environment and its accumulation in fish tissues causes oxidative stress, neurotoxicity, and immune alterations (Lee et al. 2019). Zinc is an essential trace element for aquatic organisms and humans (Wang et al. 2001). It plays a biological role in catalytic and structural processes in the enzymes of oxidoreductase, transferase, hydrolase, lyase, isomerase, and ligase (Hafeez et al. 2013). However, Zn metal is toxic when entering the body in high amounts.

Pollution caused by batik wastewater, especially the content of heavy metals, is an important issue that requires significant efforts to recover the polluted habitat. Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency, and eco-friendly nature (Rajendran et al. 2003). On the contrary, remediation using conventional physical and chemical methods is uneconomical and produces a large volume of chemical waste (Tarekegn et al. 2020). Bioavailability of metals in the soil increases at high pH due to its free ionic species, and on the contrary, at low pH decreases due to insoluble metal mineral phosphate and carbonate formation. Microbes immobilize metals naturally through cellular sequestration and accumulation or extracellular precipitation (Rajendran et al. 2003). Several studies have reported the ability of microorganisms in the remediation of batik wastewater such as *Bacillus* sp. FS5 (Siddiquia et al. 2011), *B. subtilis* HAU-KK01 (Sarim et al. 2019), *Ganoderma weberianum* (Tian et al. 2013), *G. lucidum* (Pratiwi et al. 2017), *Chlorella* sp. (Kassim et al. 2018), *Aspergillus* spp. (Dewi et al. 2018a; Dewi et al. 2018b), and a bacterial consortium (Muchtasjar et al. 2019). Indigenous bacteria isolated from heavy metal contaminated sites had resistance to heavy metal toxicity and could be used for heavy metal removal (Irawati et al. 2017). A study regarding the bioremediation of heavy metals contained in batik wastewater, especially by Rhizobacteria is little.

Rhizobacteria are a group of soil bacteria in the rhizosphere that are able to increase plant growth (Klopper and Schroth 1978; Curl and Truelove 2012), such as *Azospirillum*, *Azotobacter*, *Enterobacter*, *Pseudomonas*, and *Bacillus* (Tilak et al. 2010). Rhizobacteria may have the remedial ability to metal-contaminated ecosystems because they can change the availability of metals in the environment to be easily absorbed. An extreme environment such as Iron sand soils may a source of microbes tolerant to heavy metals contaminated soils. Iron sand soil conditions have soil physical and chemical properties that are not supportive for plant growth, due to sandy soil texture, organic matter content, and low cation exchange capacity (Oedjijono et al. 2014). The low plant population in Iron sand soils resulted in limiting the growth and development of soil microorganisms such as bacteria. Rhizobacteria isolated from iron sand soils were thought to be resistant to heavy metal levels because they are adapted to a metal-polluted environment. Lestari et al. (2019) found 11 isolates of Rhizobacteria from the rhizosphere of *Ipomoea* sp. grown in Iron sand soils, and they were known to be tolerant of Cr heavy metal at a concentration of 114 $mg.L^{-1}$. Pal and Sengupta (2019) reported that Rhizobacteria (*Lysinibacillus varians* and *Pseudomonas putida*) isolated from Indian agricultural soils could tolerate Pb metal up to 450 ppm and 500 ppm. Singh et al. (2015) showed that *Pseudomonas* sp. and *Bacillus* sp. are tolerant to Pb metal at a concentration of 110 ppm. Darokar and Shanware (2015) found two Rhizobacterial isolates of the genus *Pseudomonas* were able to tolerate 220 ppm and 270 ppm of Zn metal. Several Rhizobacteria isolated from Iron sand soils were known to be potential in bioremediation of chromium (Cr) heavy metal (Lestari et al. 2019), but the tolerance and identity of the bacteria against Pb and Zn metals were not yet known.

The objectives of the present research were to select Rhizobacterial isolates that were tolerant of Zn and Pb metals *in vitro*, to know the ability of selected Rhizobacterial isolates and the optimum pH in reducing the concentrations of Pb and Zn in batik wastewater, and to know the identity of selected Rhizobacterial isolates tolerance of Pb and Zn metals.

MATERIALS AND METHODS

Procedures

Isolation and reculturing of Rhizobacterial isolates

Total 11 Rhizobacterial isolates (Rb1, Rb2, Rb3, Rb4, Rb5, Rb6, Rb7, Rb8, Rb10, Rb11, Rb12) isolated from iron sand soils (Lestari et al. 2019) were re-cultured on nutrient agar (NA) medium slant, and incubated for 24 hours at room temperature. Furthermore, the grown isolates were inoculated into nutrient broth (NB) medium, and incubated for 48 hours at room temperature. The culture was used to assay the tolerant of the Rhizobacteria toward Zn and Pb metals.

Screening of Pb and Zn tolerance of Rhizobacterial isolates

The ability of the eleven Rhizobacterial isolates tolerance to Pb and Zn was done in NB medium added with either $PbNO_3$ or $ZnSO_4$ (Darokar and Shanware 2015). Amount of 0.1 mL of each Rhizobacterial culture containing 10^8 cfu/mL was taken and inoculated into 5 mL of NB medium added with 110 µg/mL of $PbNO_3$ or into 5 mL of NB medium added with 270 µg/mL of ZnSO4. The control treatment was 5 mL NB medium without the addition of heavy metal. Each treatment was made repeated in triplicates. All experimental cultures were incubated for 24 hours at room temperature. To observe the viability of bacterial growth, each experimental culture was measured for its turbidity by using a spectrophotometer at a wavelength of 600 nm. Cultures showing high absorbance values indicated high turbidity value, thus, indicating that the Rhizobacterial isolates were tolerant of Pb or Zn metals. Three isolates showing higher tolerance of Pb or Zn were applied to reduce the metals contain in batik wastewater *in vitro*.

Adsorption capacity of Pb and Zn

The ability of selected Rhizobacterial isolates in reducing either Pb or Zn was done experimentally using a completely random sampling design and arranged in a split-plot. The main plot was the three selected Rhizobacterial isolates (Rb2, Rb8, Rb12) that tolerance of either Pb or Zn. The subplot was pH variations (pH 5, pH 5.5, pH 6). Each treatment was made repeated in triplicates. The batik wastewater was taken from the Sokaraja Batik Center, Banyumas Regency, Central Java of Indonesia. The batik wastewater used was the residue of the coloring and dyeing processes. The amount of one litre of the wastewater was put into a container, and the pH was adjusted to 5; 5.5; and 6 by adding NaOH or HCl solution. Laboratory scale bioremediation experiments were carried out by preparing 250 mL Erlenmeyer flasks, and each was filled with 90 mL of the batik wastewater with the pH has been determined. The flask was then added with 10 mL of a bacterial culture with a density of 10^8 cfu/mL and the solution was homogenized manually. The flasks were incubated in a shaker incubator at a speed of 120 rpm at room temperature for 30 days. Furthermore, the cultures were filtered using a membrane filter of 0.22 µm for separating the liquid and debris including the bacterial cells. Measurement of the metal concentrations of Pb or Zn was done by taking 50 mL of the filtrate and heating on a hotplate at a temperature of 180ºC until the liquid remaining of 10-20 mL. An amount of 5 mL of concentrated HNO_3 and 2 mL of 20% HCl was added and the solution was heated until the liquid become clear. The solution was filtered using a Whatman paper no. 42, and then diluted with distilled water to a volume of 50 mL. The Pb absorbance value of the filtrate was measured using Atomic Absorption Spectroscopy (AAS) at a wavelength of 283.3 nm, while, for Zn at a wavelength of 213.9 nm. Preparation of standard curve of either Pb or Zn concentration referred to APHA (2012). The capacity of a metal uptake by the Rhizobacteria was measured by entering the value of the metal concentration from the results of AAS analysis into the formula of q : V(Ce - Ceq)/v (q: absorption capacity [mg/L], V: volume of solution [L], v: volume of culture [L], Ce: initial metal concentration [$mg.L^{-1}$], Ceq: final metal concentration [mg/L]) (Lestari et al. 2019). Measurement of the population of bacteria was carried out before and after the bioremediation process using the TPC method. An amount of 1 mL of the culture was inoculated into 9 mL of sterile distilled water and then made serial dilutions. One mL of the last two serial dilutions of 10^{-5} and 10^{-6} was inoculated onto NA medium by a pour plate method. The plates were incubated for 24hr at room temperature and the number of colonies was counted using the TPC formula. Measurement of pH of the culture was conducted before and after the bioremediation process using pH meter (Thermo Scientific™).

Identification of Rhizobacteria*l isolates*

Rhizobacterial isolates with high tolerance to Pb and Zn metals were identified to the genus level. Characterization of the Rhizobacterial isolates was carried out based on phenetic properties including colony and cell morphology, biochemical, physiological, and nutritional characteristics conducted by conventional methods (Smibert and Krieg 1981).

RESULTS AND DISCUSSION

Selection of Rhizobacterial isolates tolerance to Pb and Zn heavy metals based on the bacterial growth

The selection of Rhizobacteria tolerant of Pb and Zn was based on the growth differences between isolates after exposure to the metals compared to the control. The higher tolerance of the Rhizobacteria against Pb was shown by Rb2, Rb8, and Rb12 with the difference absorbance values of 0.150, 0.212, and 0.102 respectively and toward Zn was by isolates of Rb6, Rb7, and Rb12 with the difference absorbance values of 0.163, 0.154, and 0.222 respectively. The result also showed that isolates of Rb6 and Rb12 tended to be tolerant to both Pb and Zn; while, isolates of Rb2, Rb8, Rb10, and Rb11 were only tolerance to Pb, and isolated Rb7 only to Zn metal (Table 1.). The isolates that showed a high tolerance against Pb and Zn were further selected and tested for the ability to reduce the heavy metal content in the batik wastewater. Isolate Rb12 was applied in the bioremediation of Pb and Zn because of its high capability of tolerating the two heavy metals.

Bioremediation of Pb and Zn in batik wastewater by selected Rhizobacterial isolates

Based on the result of analysis of variance, the ability of selected Rhizobacterial isolates of Rb2, Rb8, and Rb12 in the adsorption capacity of Pb showed that the type of isolates was not significantly different at P: 0.05, however, the pH variations significantly affected the Rhizobacteria in the adsorption of Pb. This result was also similar to the ability of selected isolates of Rb6, Rb7, and Rb12 at different pH in the adsorption capacity of Zn. The results indicated that pH showed a high effect on the bioremediation process of heavy metals by the Rhizobacterial isolates. The adsorption capacity of Pb by isolates Rb2, Rb8, and Rb12 tended to increase at pH 5.5 and pH 6 compared to at pH 5, except for isolate Rb8 (Table 2). The highest reduction of Pb in the batik wastewater as the amount of 2.955 mg/L was shown by isolating Rb8 at pH 5.5.

The adsorption capacity of Zn by isolates Rb6, Rb7, and Rb12 at different pH also showed that the pH significantly affected the ability of the Rhizobacteria in the reduction of Zn concentration contained in the batik wastewater based on the analysis of variance at P: 0.05. The pH 5.5 showed the best result for the ability of the Rhizobacteria in the reduction of Zn contained in the batik wastewater (Table 3.), on the contrary, their adsorption capacity decreased at pH 6.0. The highest adsorption capacity of Zn was demonstrated by isolates Rb7 at pH 5.5 in 1.086 mg/L.

Table 1. Mean of the difference between control and treatment of bacterial growth on the selection of Rhizobacterial isolates in tolerating Pb and Zn *in vitro*

Rhizobacteria	Average of bacterial growth (at 600 nm)				The difference in the final absorbance value of control and treatment*	
	Zn		Pb			
	Control	Treatment	Control	Treatment	Zn	Pb
Isolate Rb1	0.584±0.048	0.247±0.064	0.584±0.048	0.478±0.021	-0.336	-0.106
Isolate Rb2	1.553±0.151	0.517±0.074	1.055±0.151	1.205±0.039	-0.538	0.150
Isolate Rb3	0.716±0.027	0.440±0.297	0.716±0.027	0.482±0.099	-0.276	-0.234
Isolate Rb4	0.883±0.212	0.222±0.038	0.883±0.212	0.810±0.172	-0.660	-0.073
Isolate Rb5	1.245±0.129	0.220±0.075	1.245±0.129	0.936±0.087	-1,024	-0.309
Isolate Rb6	0.494±0.044	0.331±0.021	0.494±0.044	0.542±0.011	-0.163	0.048
Isolate Rb7	0.343±0.120	0.189±0.020	0.343±0.120	0.213±0.047	-0.154	-0.130
Isolate Rb8	0.876±0.072	0.357±0.149	0.876±0.072	1.088±0.067	-0.519	0.212
Isolate Rb10	1.396±0.038	0.635±0.047	1.396±0.038	1.459±0.013	-0.761	0.063
Isolate Rb11	1.372±0.082	0.528±0.017	1.372±0.082	1.393±0.169	-0.844	0.021
Isolate Rb12	1.228±0.019	1.004±0.090	1.227±0.019	1.329±0.091	-0.222	0.102

Note: *The negative (-) results indicated that the growth of the Rhizobacteria was decreased

Table 2. A mean of the adsorption capacity of Pb (mg/L) by selected Rhizobacterial isolates at different pH measured by AAS

Isolate code	pH	Initial concentration	End concentration	Adsorption capacity
Rb2	5	0.333	0.124	1.880
	5.5	0.408	0.105	2.731
	6	0.433	0.129	2.732
Rb8	5	0.333	0.134	1.791
	5.5	0.408	0.080	2.955
	6	0.433	0.114	2.866
Rb12	5	0.333	0.194	1.254
	5.5	0.408	0.114	2.642
	6	0.433	0.109	2.911

Table 3. A mean of the adsorption capacity of Zn (mg/L)by selected Rhizobacterial isolates at different pH measured by AAS

Isolate code	pH	Initial concentration	End concentration	Adsorption capacity (mg/L)
Rb6	5	0.273	0.23	0.384
	5.5	0.279	0.213	0.594
	6	0.175	0.108	0.567
Rb7	5	0.273	0.179	0.843
	5.5	0.279	0.159	1.086
	6	0.175	0.155	0.177
Rb12	5	0.273	0.179	0.84
	5.5	0.279	0.174	0.948
	6	0.175	0.089	0.777

Based on the analysis of the Duncan Multiple Range Test at P: 0.05, the mean of the reduction of adsorption capacity by the selected Rhizobacterial isolates on Pb was the highest at the pH of 6.0, while, for Zn was at the pH 5.5. The mean concentration of Pb reduced by the selected Rhizobacteria at pH 6.0 was 2.836 mg/L, while against Zn at pH 5.5 was 0.8761 mg/L.

The average number of selected Rhizobacterial population before bioremediation process at pH 5, 5.5, and 6 was 1.9 x 10^7 cfu/mL, 1.1 x 10^7 cfu/mL, and 4.0 x 10^7 cfu/mL, respectively, and the number at the end of bioremediation process was 9.5 x 10^6 cfu/mL, 1.25 x 10^7 cfu/mL, and 4.66 x 10^6 cfu/mL, respectively. The results showed that the selected Rhizobacteria could maintain their viability in batik wastewater containing heavy metals.

Identification of selected Rhizobacteria tolerant of heavy metals

Based on the capability of tolerate Pb or Zn heavy metals, five isolates of Rb2, Rb6, Rb7, Rb8, and Rb12 were selected to be characterized for identification. Results of characterization (Table 4.) showed that Rhizobacterial isolates of Rb2 and Rb 6 were Gram-negative, while, Rb7, Rb8, and Rb12 isolates were Gram-positive. Cells of isolates Rb2, Rb6, Rb7, and Rb8 were rod and motile, while, isolate Rb12 was coccus and non-motile. All Rhizobacterial isolates did not form endospore, positive in catalase and oxidative-fermentative tests. Isolates of Rb2, Rb 6, Rb7, and Rb8 were able to produce acid from mannitol and did not produce H_2S, while, isolate Rb12 was unable to produce acid from mannitol but able to produce H_2S. All Rhizobacterial isolates were able to grow at 4°C to room temperature, however, the Rb2, Rb7, and Rb8 isolates could grow at 4°C to 55°C. Nutritional requirements assay of the Rhizobacteria showed that all isolates used mannitol, fructose, sucrose, lactose, and galactose as a sole carbon source for their growth.

Based on a result of characterization (Table 4.) and referred to the Bergey's Manual of Determinative Bacteriology, the selected Rhizobacterial isolates that are tolerant of Zn and Pb metals could be identified as follows. Isolates of Rb2 and Rb6 belonged to species member of the genus *Pseudomonas*, isolates of Rb7 and Rb8 were species member of the genus *Lactobacillus*, and Rb12 isolate belonged to species member of the genus *Staphylococcus*.

Discussion

Rhizobacterial isolates of Rb1, Rb2, Rb3, Rb4, Rb6, Rb7, Rb8, Rb10, Rb11, and Rb12 isolated from Iron sand soils were also reported to be tolerant of Cr by Lestari et al. (2019). Selection of Rhizobacteria isolates tolerant to Pb and Zn was valuable to screen their ability and potential as heavy metal bioremediation agents. The ability of the bacterial tolerance to heavy metals was measured based on the difference of absorbance values of the control cultures (without the addition of metal) and the treatment cultures (with the addition of metal) by using a spectrophotometer at a wavelength of 600 nm. The tolerance ability of Rhizobacterial isolates to Pb and Zn heavy metals in an NB medium containing 110 µg/mL of PbNO3 and 270 µg/mL of ZnSO4, respectively could be seen from the value of the smallest absorbance difference. The difference in the average absorbance values of the control and the treatment showed that the isolates could be tolerant and able to grow in a medium containing heavy metals. This result also indicated that the bacterial isolates could detoxicate and reduce the heavy metals of Pb and Zn contained in the batik wastewater.

The ability of Rhizobacteria in decreasing heavy metals concentration could be indicated by the levels of their tolerance to the metals. Irawati et al. (2017) demonstrated that eight heavy metal-resistant bacteria isolated from Kemisan had minimum inhibitory concentration ranging from 7 mM to 11 mM against Cu, Pb, and Zn. Abidin et al. (2019) reported that *Acinetobacter* sp. P2, *Bacillus subtilis* 3KP, *Micrococcus* sp. LII61, and *Pseudomonas putida* T1 were resistant to heavy metals of Cr^{6+}, Cu^{2+}, and Zn^{2+} in either media of Mueller-Hinton Agar (MHA) or Synthetic Mineral Water Agar. *Micrococcus* sp. LII61 showed the highest tolerance compared to the other three bacteria, which grew at Zn^{+2} 1000 ppm in both media. Pal and Sengupta (2019) found *Lysinibacillus varians* and *Pseudomonas putida* isolated from agricultural soils in India showed tolerance against Pb at concentrations of 450 ppm and 500 ppm. Fahruddin et al. (2020) reported that maximal tolerance of each marine bacterium against Pb could be indicated based on the difference in the growth of bacteria. Microorganisms respond to heavy metal stress are through either enzymatic or non-enzymatic mechanisms. The strategies of microorganisms to protect against heavy metal toxicity are either by reduced uptake/active efflux or by the formation of complexes outside the cell and sequestration, reduction of free ions in the cytosol either by a synthesis of ligands to achieve intracellular chelation or by compartmentalization (Rajendran et al. 2003).

It has been observed that Rhizobacteria isolated from Iron sand soils were able to reduce Pb and Zn contained in the batik wastewater. Their ability was significantly affected by pH, in which the adsorption capacity of the selected Rhizobacterial isolates against Pb and Zn was high at pH 5.5, with the concentration of the metals reduced was 2.955 mg/L and 1.086 mg/L or 80% and 43.2 %, respectively. The pH is known to have a crucial role in microbial biosorption, and optimum pH is often different for each microorganism (Jin et al. 2018). This is due to the fact that pH affects the activity of enzymes thereby affecting the rate of heavy metals metabolism, affects the surface charge thereby affecting its adsorption of heavy metal ions in microorganisms, and affects the hydration and mobility of many metal ions in the soil. *Bacillus* sp. FS5 isolated from a batik textile dye effluent "Natural Batik Village" Kuantan, Malaysia (Siddiquia et al. 2011) and eight bacteria namely species of *Mesophilobacter, Methylococcus, Agrobacterium, Neisseria, Xanthobacter, Deinococcus, Sporosarcina,* and *Bacillus* isolated from a waste batik industry, Indonesia (Muchtasjar et al. 2019) were reported have the ability to decrease physicochemical pollutant from batik industry effluents.

Table 4. A characterization of selected Rhizobacteria isolated from Iron sand soils tolerant of Pb and Zn

Characteristic	Rb2	Rb6	Rb7	Rb8	Rb12
Colony morphology					
Optics	Translucent	Opaque	Opaque	Opaque	Opaque
Elevation	Flat	Flat	Flat	Raised	Flat
Size	Big	Big	Moderate	Moderate	Big
Form	Irregular	Irregular	Irregular	Circular	Irregular
Edge	Undulate	Undulate	Undulate	Flat	Undulate
Color	Dull white	Creamy white	White	White	Beige
Cell morphology					
Gram	-	-	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Coccus
Motility	+	+	+	+	-
Endospores	-	-	-	-	-
Biochemistry					
Catalase	+	+	+	+	+
Oxidase	+	+	+	-	+
Oxidative-fermentative	+ / +	+ / +	+ / +	+ / +	+ / +
Acid from					
Mannitol	+	+	+	+	-
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Lactose	+	+	+	+	-
Galactose	+	+	+	+	+
H_2S production	-	-	-	-	+
Physiology					
Temperature					
4 ℃	+	+	+	+	+
Room temperature	+	+	+	+	+
55 ℃	+	-	+	+	-
Salinity					
3%	+	+	+	+	+
5%	+	+	+	+	+
7%	+	+	+	+	+
pH					
3	+	-	+	+	+
7	+	+	+	+	+
11	+	+	+	+	+
Nutritional: *Carbohydrate as a sole carbon source*					
Mannitol	+	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Lactose	+	+	+	+	+
Galactose	+	+	+	+	+

Singh et al. (2015) reported that *Pseudomonas* sp. and *Bacillus* sp. tolerant of Pb at 110 ppm were able to reduce the concentration of Pb in amounts of 46.60-77.76% and 53.02-89.19%. Wierzba (2015) showed that the removal rate of heavy metals by bacteria increases with an increase in the pH over a limited range, but the removal rate begins to decrease after the pH rises to a certain limit. The maximum biosorption capacity of *Stenotrophomonas maltophilia* was 71.4 $mg.g^{-1}$ for Pb (II) and 29.8 $mg.g^{-1}$ for Zn(II) at pH 5.0 while *B. subtilis* gave 78.8 and 30.0 $mg.g^{-1}$, respectively. George et al. (2012) reported the ability of *A. niger* Tiegh in removing heavy metals of Pb and Hg as amounts of 91.1% and 97.1% from monometal solutions and of 89.3% and 96.9% from bimetal solutions, respectively.

The ability of the selected Rhizobacteria to decrease Pb and Zn in the batik wastewater was expressed by their population number to remain high. The high viability of the selected Rhizobacteria indicated that they were capable of utilizing substances as nutrition for the growth and also adsorbed heavy metals contained in batik wastewater. The results showed that the mean concentration of Pb reduced by the three selected Rhizobacteria (Rb6, Rb7, Rb12) at pH 6.0 was 2.836 mg/L, while against Zn at pH 5.5 was 0.8761 mg/L. The capability of the Rhizobacterial isolates in the tolerance and reduce Pb and Zn might due to their ability to accumulate the metal ions in either inner (absorption) or outer cells (adsorption). In addition, a number of heavy metals are required as micronutrients and for enzyme catalysis, nutrient transport, protein structure, charge neutralization, and control of osmotic pressure (Rajendran et al. 2003). Jain et al. (2020) reported that Zinc tolerant plant growth promoting bacteria alleviates phytotoxic effects of Zinc on maize through zinc immobilization.

Rhizobacteria isolated from iron sand soils of Rb2 and Rb6, Rb7 and Rb8, and Rb12 presumably belonged to the genera *Pseudomonas*, *Lactobacillus*, and *Staphylococcus*, respectively. The main characteristics of the genus *Pseudomonas* among others have cells of straight or slightly curved rods form, Gram-negative, motile by one or several polar flagella, aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor, oxidase-positive or negative, and catalase-positive. Most species fail to grow under acid (pH 4.5 or lower) conditions (Palleroni 2005). The members of this genus are also known as a versatile microorganisms capable of utilizing a variety of organic compounds as a sole source of carbon and energy for its growth. The main characteristics of the genus *Lactobacillus* are, cells vary from long and slender, sometimes bent rods to short, often coryneform coccobacilli. Cells are motile, Gram-positive, do not produce endospores, do not produce H_2S, and aciduric. Catalase and cytochrome negative, however, a few strains in several species decompose peroxide by a pseudocatalase or by true catalase. Its metabolism is fermentative and obligate saccharoclastic. Growth temperatures range are 2-53ºC and optimum generally at 30-40ºC (Hammes and Hertel 2005). Key characteristics of the genus *Staphylococcus* are cells spherical, Gram-positive, non-motile, and do not produce resting stages. In addition, they are facultative anaerobes, usually catalase-positive and oxidase-negative, most strains grow in the presence of 10% NaCl and the temperatures for growth are 18-40ºC. A variety of carbohydrates may be utilized aerobically with the production of acid (Schleifer and Bell 2005).

Although, the genera *Pseudomonas*, *Staphylococcus*, *and Lactobacillus* have been known to have tolerance against heavy metals, however, the research about the tolerance of the bacteria isolated from Iron sand soils toward heavy metals was very little. Giwa and Ibitoye (2017) showed that *Pseudomonas aeruginosa* was effective in the bioremediation of Lead, Copper, Zinc, and Cadmium. The ability of the bacterium to bioremediate soil polluted heavy metal in crude oil was associated with the presence of genes responsible for alkane monooxygenase enzymes. Kumar et al. (2011) reported that *Staphylococcus* sp. was able to absorb Pb with the bioremediation potential in the removal of Lead-containing waste was 87%. The bacterium accumulated the metal as an intracellular Lead-phosphate (Rehan and Alsohim 2019). Biosorption capacity of *Lactobacillus plantarum* MF042018 towards Cd^{2+} and Pb^{2+} was 0.18 $mgCd.h^{-1}$ mg^{-1} and 0.07 mgPb/h/mg, respectively at pH 2.0 (Ameen et al. 2020). Bioaccumulation of heavy metals by *L. plantarum* MF042018 observed using TEM micrographs revealed obvious changes in the cell surface morphology of heavy metals treated cells compared to the control cells, with the appearance of dense metal deposits uniformly adsorbed to the outer cell surface and accumulated inside the cells. Biosorption of Zn and Cu by *L. fermentum* showed that the removal of Zinc ions was more efficient than Copper with the highest removal rate of Zinc reaching 84% (Kargar and Shirazi 2020).

In conclusion, Rhizobacterial isolates having a high tolerance to Zn and Pb metals were Rb2, Rb6, Rb7, Rb8, and Rb12. The ability of selected Rhizobacteria in reducing heavy metals contained in the batik wastewater was optimal at pH 5.5 with adsorption capacity 80% for Pb and 43.2% for Zn. The identity of the selected Rhizobacterial isolates of Rb2 and Rb6, Rb7 and Rb8, and Rb12 was species member of the genera *Pseudomonas*, *Lactobacillus*, and *Staphylococcus*, respectively.

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