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Thermophilic bacteria isolated from Mount Merapi, Java, Indonesia as a potential lead bioremediation agent

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Abstract. *Rakhmawati A, Wahyuni ET, Yuwono T. 2021. Thermophilic bacteria isolated from Mount Merapi, Java, Indonesia as a potential lead bioremediation agent. Biodiversitas 22: 3101-3110.* Contamination by lead (Pb) has become a serious health and environmental problem, that has to be urgently prevented. Bioremediation is one of the environmentally friendly methods for eliminating Pb from the contaminated environment. This study was aimed to investigate the potency of lead-tolerant thermophilic bacteria isolated from solfatara Mount Merapi, Indonesia. A total of 340 isolates of thermophilic bacteria were screened for lead tolerance at 55 °C. Five bacterial isolates were found to show tolerance to 100 mg/L Pb (II), and then were further evaluated and identified as *Aeribacillus pallidus* strains MRP112, MRP148, MRP272, MRP278, and MRP280 based on 16S rRNA gene sequences analysis. Among the five isolates, *A. pallidus* MRP 280 showed highest activity in removing Pb at pH 6, 55°C for 24 h. The analysis of scanning and transmission electron microscopy, biofilm formation, and siderophore production, demonstrated that lead tolerance of *A. pallidus* MRP 280 strain was also accompanied by morphological changes, bioaccumulation, biosorption, biofilm, and siderophore assembly. In conclusion *A. pallidus* MRP 280 was demonstrated as one of the most potential bacterial strains, can be recommended as an agent for high-temperature lead bioremediation.

Keywords: *Aeribacillus pallidus*, biosorption, lead tolerance, thermophilic bacteria

INTRODUCTION

Lead (Pb) has been regarded as one of the most hazardous heavy metal pollutants. Geogenic and anthropogenic activities are the lead sources in the environment. The smaller levels of lead are hazardous to people's health, the physiological role of lead in the organism has not been well known. Lead is also highly persistent and accumulated, thus it becomes a serious environmental problem (Wani et al. 2015, Vigneri et al. 2017, Kushwaha et al. 2018).

Several techniques such as chemical precipitation (Akinterinwa and Adibayo 2018), electrocoagulation (Abbas and Ali 2018), adsorption (Ghasemi et al. 2014), ion exchange (Bezzina et al. 2019), and membrane filtration (Wang et al. 2018) have been implemented for removal of lead However, these technologies have many limitations such as high cost for energy and maintenance, and the emergence of toxic byproducts. On the other hand, biological agents such as microorganisms offer easy, eco-friendly, cost-effective, and efficient methods for Pb remediation (Gupta and Joia 2016; Ayangbenro and Babalola 2017; Jacob et al. 2018; Sharma et al. 2018). Therefore, microbe-based approaches have several advantages over traditional physical and chemical methods including (i) higher specificity, (ii) suitability for *in situ* techniques, and (iii) potential for improvement by genetic engineering (Tiquia 2018).

Lead tolerant bacteria used as Pb bioremediation agents are urgently required. In general, bacteria that are tolerant to toxic metals have extreme properties such as tolerance to high temperatures up to 75 °C. Multiple lead tolerant thermophilic bacterial species have been identified from various parts of the world such as *Geobacillus thermodenitrificans* (Chatterjee et al. 2010), *G. thermodenitrificans* and *G. thermocatenulatus* (Babák et al. 2012), *Stenotrophomonas maltophilia*, *Aeromonas veronii*, and *Bacillus barbaricus* (Sen et al. 2014), *Thermus thermophilus* (Nicolaus et al. 2016), *G. galactosidasius* sp (Özdemir et al. 2016); *Pseudomonas* sp. (Kalita and Joshi 2017), and *B. stearothermophilus* (Al-Khafaji et al. 2018).

This study focused on characterization of lead-tolerant thermophilic bacteria isolated from volcano mud of Merapi, Indonesia. The selected isolates that demonstrated tolerance to high concentrations of Pb were further investigated to determine the Minimum Inhibitory Concentration (MIC), Pb removal ability, growth profile, and Pb tolerance mechanisms. Biofilm formation, siderophore production, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Fourier Transform Infra-Red spectroscopy (FTIR), and Atomic Absorption Spectroscopy (AAS), as well as analysis of

ability to detoxify Pb were used for characterization of the selected isolates. The present results, thus, are promising to be a foundation to develop a method of bioremediation at high temperatures by using indigenous microorganisms.

MATERIALS AND METHODS

Bacterial strain, media, and chemicals

Thermophilic bacteria were originally isolated from solfatara of volcano Mount Merapi at Gendol Atas River, Indonesia (7°39'20.1"S 110°27'49.9"E). The bacterial isolates were deposited in Laboratory of Microbiology, Department of Biology Education, Faculty of Mathematics and Natural Sciences, Universitas Negeri Yogyakarta, Indonesia. All media and chemicals used in the study were of analytical grade. Luria Bertani (LB) was prepared by using following composition (g/L): tryptone 10, yeast extract 5, and NaCl 10, and agar 15 to prepare agar plate. Stock solutions of Pb(II) (1000 mg/L) were prepared by dissolving $Pb(NO_3)_2$ in aquabidest and 1 mL/L of 0.1 mol/L HNO_3 was added to the solutions to prevent the precipitation of Pb(II) by hydrolysis. All glassware used during the Pb (II) analysis were washed in 30% HNO_3 and rinsed several times in aquabidest prior to use.

The medium used for screening was a solution containing NaCl at 5 g/L concentration to prevent $PbCl_2$ precipitation as suggested by Peens et al. (2018). The LB media and $Pb(NO_3)_2$ stock solution were autoclaved at 121 °C for 15 minutes separately.

Screening of lead-tolerant bacteria

The lead tolerance assay was performed by streak plate method as reported by Chaudhary et al. (2017). Luria Bertani (LB) agar medium was supplemented with 25, 50, 75, and 100 mg/L of $Pb(NO_3)_2$ at pH 7. Duplicated plates were prepared for each Pb concentration and incubated at 55 °C for 72 h. Each plate was checked for the appearance of visible growth which indicated the tolerance towards Pb is present in the medium. Several bacterial strains that demonstrated tolerance up to 100 mg/L of Pb were further investigated.

Bacterial identification using 16S rRNA

The selected lead-tolerant bacterial isolates were identified by using 16S rRNA sequencing technique (Kumar et al. 2018; Penaloza-Vazquez et al. 2019). The bacterial isolates were cultured in LB broth in 100 r/min shaking incubator at 55 °C for 24 h. Bacterial cells were harvested and genomic DNA was extracted using Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The 16S rDNA amplification was carried out by using universal bacterial primers MRPs 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-TACGGTTACCCTTACGACTT-3') and amplified with MyTaq HS Red Mix (Bioline, BIO-25047). The amplification cycle was as follows: an initial denaturation step at 95 °C for 1 min; 35 cycles of denaturation at 95 °C for 15 s, annealing at 52 °C for 15 s, and extension at 68 °C for 45 s. The amplification product was then purified by using Zymoclean Gel DNA Recovery Kit (Zymo Research) for sequencing. Sequence analysis was carried out by comparing with the Gene Bank database using BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST) using nearest neighbor of sequences method. In addition, Mega X software was used to construct a phylogenetic tree based on 16S rDNA of selected bacterial isolates.

Minimum Inhibitory Concentration

The selected bacterial strains were also tested for their Minimal Inhibitory Concentration (MIC). In the present study, both solid and liquid media were used to determine the MIC and metal mobility (bioavailability) of those bacterial strains. The combined approach gave a better understanding of exact concentration of metals that inhibit bacterial growth (Neethu et al. 2015). The MICs to Pb(II) for a series of concentrations (0, 25, 50, 75, 100, 125, and 150 mg/L) were tested both in LB broth and LB agar for 24 h. Cultures with an OD_{600} of 0.8, were inoculated into medium solutions for each treatment, in triplicates. Bacterial cultures were incubated at 55 °C for 24 h, with agitation at 100 r/min. Bacterial growth was measured by using spectrophotometric method at OD_{600}. LB culture medium without Pb addition was used as positive control, while the non-inoculated LB culture medium was used as negative control.

Pb removal assay

The Pb removal assay on five selected strains was carried out according to a modified protocol of Li et al. (2013) and Abdi and Kazemi (2015). The isolates were firstly cultivated at least for 24 h to be used as inoculum. Three 100 mL Erlenmeyer flasks containing 30 mL of autoclaved LB medium were inoculated with 3 mL (OD_{600} 0.8) of inoculum and supplemented with 100 mg/L of Pb. Cultures were then incubated at 55 °C pH 5, 6, and 7 under 100 r/min for 24 h. LB media supplemented with corresponding concentrations of Pb in each treatment was used as the blank control. Following the removal of bacterial cells by centrifugation at 5000 r/min for 10 min, supernatant was collected and Pb concentration was measured using atomic absorption spectrophotometer (AAS 7000, Shimadzu, Japan). In addition, lead removal rate (R) was calculated by using the following formula (Jiang et al. 2016):

$$R = (1-C/C_0) \times 100\% \quad (1)$$

Where; R, C, and Co are Pb removal rate (%), final Pb concentration (mg/L), and initial Pb concentration (mg/L), respectively.

Biofilm formation

Evaluation of biofilm formation at different Pb concentrations (0, 25, 50, 75, and 100 mg/L) by five isolates was performed by using the protocol of Giovanella et al. (2017) with modification. The bacterial isolates were grown in 200 µL LB with various concentrations of Pb at 55 ºC and pH 7 without agitation. After 72 h of incubation, the culture medium was aspirated and washed three times

with 250 µL of 0.9% NaCl solution and left to dry at 40 ºC for 40 min. The adhered cells were dyed with 1% gentian violet for 10 min and the excess dye was removed. The biofilm was washed with 0.9% NaCl solution five times and lastly washed with 95% ethanol. The biofilm was quantified by reading the absorbance using an ultraviolet-visible spectrophotometer GENESYS 10S (ThermoFisher Scientific) at 595 nm. Non-inoculated LB culture medium was used as negative control, while LB culture medium without metals was used as positive control.

Siderophores production

The selected five isolates were evaluated for siderophore production after 24 h of incubation at 55 ºC and at pH 7. The bacterial cells were prepared following the procedure as previously described by Arora and Verma (2017) and Giovanella et al. (2017) with some modifications. LB medium was used to quantify siderophore production with the addition of 0, 25, 50, 75, and 100 mg/L of Pb. The positive control was the inoculated nutrient broth without Pb addition and the negative control was uninoculated nutrient broth without Pb addition.

Quantitative siderophore production was determined by CAS assay (Schwyn and Neilands 1987) with modifications. The chromeazurol S (CAS) solution was prepared as follows: six mL 10 mM HDTMA was added to a 100 mL volumetric flask and diluted with ultrapure water. A mixture of 1.5 mL iron III (1 mM Fe_3Cl_3 in 10 mM HCl) and 7.5 mL CAS solution were then added to the flask. Anhydrous piperazine (4.307 g) was dissolved in ultrapure water. This solution (at pH 5.6) was added to the volumetric flask to complete the 100 mL.

Two mL aliquots of the culture were centrifuged at 10,000 r/min for 10 min, and 1 mL of the supernatant was then removed prior to the addition of 1 mL of the CAS solution. After 10 min of incubation at room temperature, the absorbance was read using a UV-Vis spectrophotometer GENESYS 10S (ThermoFisher Scientific) at 630 nm. Culture medium without the addition of metals was used as the blank. Siderophore production was calculated as follows: A- Ab (A=absorbance of the sample; Ab=absorbance of the blank).

Growth profile of *Aeribacillus pallidus* MRP280

Aeribacillus pallidus MRP280 that showed the highest activity in removing Pb, biofilm, and siderophore assembly was further investigated. *A. pallidus* MRP280 growth profile was determined by measuring the cell density at OD_{600} every 3 hours for 48 h at 55 °C under different Pb concentrations (0, 50, 100 mg/L) and pH (5, 6, 7). All experiments were carried out using LB media and bacterial inoculum size of OD_{600} 0.8 in a shaking incubator at 100 r/min.

Bioaccumulation and biosorption assay

Lead bioaccumulation and biosorption assay in bacterial cell was conducted on *A. pallidus* MRP 280 strain with the highest level of Pb removal, biofilm formation, and siderophore synthesis, following the assay procedure of Bai et al. (2014) with some modifications. After Pb removal experiment conducted at 100 mg/L pH 6 for 24 h, the *A. pallidus* MRP 280 suspension was centrifuged and the biomass was resuspended in one of three desorption reagents: milli-Q water, 1.0 mol/L NH_4NO_3 (AR grade, Merck, Germany) or 0.1 mol/L EDTA-Na_2 (AR grade, Merck, Germany). The suspension was centrifuged at 5000 r/min for 10 min. The Pb ion concentration in the supernatant was analyzed by the Atomic Absorption Spectrophotometer (AAS 7000, Shimadzu, Japan).

Characterization of the Pb bioprecipitates

Lead (Pb) bioprecipitation by *A. pallidus* MRP280 was investigated in media containing 100 mg/L Pb after incubation up to mid-exponential phase (15 h). The characterization of the precipitates was carried out by Fourier Transform InfraRed (FTIR) spectroscopy, Scanning Electron Microscopy which were integrated with Energy Dispersive X-Ray (SEM-EDX), and Transmission Electron Microscopy (TEM).

FTIR spectroscopy

FTIR spectrum was obtained to describe the changes in the bacterial cell functional groups due to Pb precipitation. For FTIR analysis, the control and Pb treated cell biomass were centrifuged at 5000 r/min for 10 min, washed three times with aquabidest, and the pellets obtained were then oven-dried at 80 °C overnight. Samples were prepared by diluting cell pellets to 5% KBr and casting them in disks for FTIR spectroscope analysis (8201PC, Shimadzu, Japan).

SEM-EDX analysis

This analysis was performed to detect the cell surface morphology and elemental composition (Ren et al. 2015) of samples before and after applying Pb. For SEM analysis, the control and Pb-treated cell biomass was centrifuged at 5000 r/min for 10 min, washed three times with aquabidest. The samples then were loaded pellets a copper grid coated with carbon onto these pellets for observation under SEM-EDX (JSM-6510LA, Japan).

TEM images observation

Lead accumulation in bacterial cells was investigated by TEM based on Building (2010), with modification for sample preparation. Two mL aliquots of the culture were centrifuged for 3 min at 3000 r/min, fixed in 2.5% glutaraldehyde (in 0.1M cacodylate buffer and 3% sucrose), and stored at 4 °C with shaking condition overnight. Next, the cells were centrifuged at 3000 r/min for 3 min and washed twice with 0.1M cacodylate buffer and 3% sucrose, followed by fixation in 2 % osmium tetroxide, 2.5% $K_3Fe(CN)_6$, and 3% sucrose for 2 h at 4 °C. The bacterial pellets were then washed three times with cacodylate buffer. A graded ethanol series (10%, 50%, 70%, 80%, 96%, and 100% ethanol) were used to dehydrate the samples. After dehydration, the prepared biomass samples were infiltrated gradually with propylene oxide and immediately embedded in Spurr resin. Ultrathin sections of 50-100 nm thickness were cut using an

ultramicrotome and taken on copper grids, and stained with uranyl acetate and triple lead before the observation under a transmission electron microscopy (JEM1010, JEOL, Japan).

Statistical analysis

Data for Pb removal, biofilm formation, and siderophore production for each selected bacterium were subjected to statistical analysis using SPSS 22.00 (IBM Corporation, Armonk, NY, USA). Mean variables were compared using Duncan's multiple range test (DMRT) with $p < 0.05$ was considered as significant data.

RESULTS AND DISCUSSION

Lead-tolerant bacteria screening

A total of 340 thermophilic bacteria have been isolated from solfatara Mount Merapi, Indonesia, which were then screened for lead tolerance. The numbers of bacterial isolates that demonstrated growth at 25; 50; 75; and 100 mg/L Pb were 140; 39; 12; and 5 respectively, demonstrating that only 5 bacterial isolates showed tolerance to a very high Pb concentration (100 mg/L). The five isolates, designated as isolates MRP112, MRP148, MRP272, MRP278, and MRP280 were further investigated (Table 1).

Phylogenetic analysis

The results of the phylogenetic analysis of MRP112, MRP148, MRP272, MRP278, and MRP280 on 16S rRNA gene sequences are displayed in Figure 1. It is clear that those five isolates belong to species *Aeribacillus pallidus* with 99-100% of identity. The 16S rRNA gene sequences of these five isolates were then submitted to NCBI under these accession numbers (Table 1).

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations represent the lowest Pb concentration that completely inhibited bacterial growth. The MIC both in solid and liquid medium has been shown the same results. Figure 2 represents the MIC data of *A. pallidus* MRP112, MRP148, MRP272, MRP278, and MRP280 in LB broth. As shown in Figure 2, MIC test indicated that the increase of Pb in the liquid media from 25-50 mg/L, slightly reduced bacterial growth, compared to the control without Pb (0 ppm). It was observed that all isolates did not show any growth when exposed to 125 mg/L Pb.

Table 1. The comparison of the 16S rRNA gene sequences of the selected lead-tolerant bacterial isolates

Strain ID	GenBank acc. number
MRP112	MT422124
MRP148	MT422122
MRP272	MT422121
MRP278	MT422120
MRP280	MT422117

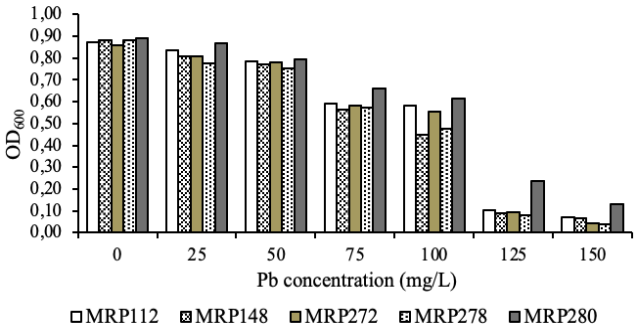

Figure 2. Minimum Inhibitory Concentration (MIC) of *Aeribacillus pallidus* MRP112, MRP148, MRP272, MRP278 and MRP280

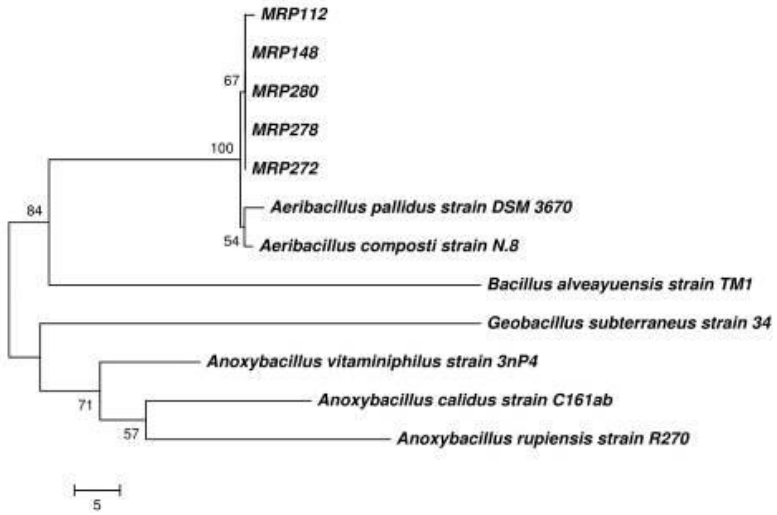

Figure 1. Phylogenetic tree of *Aeribacillus pallidus* isolates based on 16S rRNA gene sequences

Pb removal assay

Percentage of Pb removal rate by all selected isolates is summarized in Table 2. Among five selected isolates, *A. pallidus* MRP280 showed the highest Pb removal capability. The Pb removal capacity of all the selected isolates was highly dependent on the pH culture medium ($p<0.05$).

Biofilm formation

Biofilm formation assigns the capability of binding significant quantities of metals under natural conditions, and serves as matrices for insoluble mineral phase precipitation (Fomina and Gadd 2014). Table 3 showed that the addition of Pb had a significantly positive response on the production of biofilms ($p< 0.05$). It was also found that the increase of Pb concentrations in medium, resulted in biofilm by all selected isolates.

Siderophores production

Determination of siderophores production depends on the binding of Pb ion outside bacterial cell. Enhancing siderophores production is one of bacterial detoxification Pb mechanism (Naik and Dubey 2013). Moreover, siderophore secreted by bacterial cells had beneficial impacts on the Pb removal (Ren et al. 2015). It was found that Pb influenced the increase in siderophore production compared to the control ($p<0.05$) (Table 4). This table shows that all tested isolates gave positive response to siderophore assembly. It was also observed that the increase in Pb concentration correlated with the ability to produce siderophore by all the selected isolates.

Growth profile of *Aeribacillus pallidus* MRP280

The impact of Pb stress and pH on growth potential of bacteria was observed by bacterial growth curve assessment (Figures 3-5). *A. pallidus* MRP280 exhibited different growth patterns in the presence of Pb over an experimental time period of 3 to 24 h until the growth of bacteria reached stationary phase. The major effect of Pb stress (50 and 100 mg/L) on this bacterium was manifested in longer stationary phase (6 h) than the control (3h). An increase in lag phase due to the toxic levels of Pb caused the strain requires some time to acclimatize to the environment (Singh et al. 2019). However, the growth curve pattern suggested that these bacteria exhibited tolerance towards Pb stress. A lower value of optical density indicated that biomass growth of these bacterial isolates was highly affected by the presence of Pb in the growth medium.

Table 2. Pb removal rate (%) by selected bacterial isolates, *Aeribacillus pallidus* at 100 mg/L Pb, 55 °C for 24 h

pH	MRP112	MRP148	MRP272	MRP278	MRP280
5	85.45 ± 0.48^{c}	78.35 ± 0.27^{b}	75.87 ± 0.43^{c}	78.71 ± 1.02^{b}	91.85 ± 0.39^{b}
6	91.03 ± 0.87^{a}	80.48 ± 0.20^{a}	87.23 ± 0.69^{a}	85.10 ± 0.15^{a}	93.53 ± 0.22^{a}
7	87.59 ± 0.78^{b}	79.06 ± 0.62^{b}	82.52 ± 0.17^{b}	80.48 ± 1.20^{b}	92.43 ± 0.47^{b}

Note: * Means sharing the same superscripted letters at each bacterial isolates are statistically non-significant at $p < 0.05$.

Table 3. Biofilm formation (OD_{595}) by selected bacterial isolates, *Aeribacillus pallidus* at 55 °C for 72 h

Pb conc. (mg/L)	MRP112	MRP148	MRP272	MRP278	MRP280
0	0.067 ± 0.005^{c}	0.066 ± 0.006^{d}	0.061 ± 0.003^{d}	0.067 ± 0.002^{d}	0.078 ± 0.006^{e}
25	0.074 ± 0.004^{c}	0.085 ± 0.009^{c}	0.074 ± 0.004^{c}	$0.071 \pm 0.005^{c,d}$	0.090 ± 0.001^{d}
50	0.087 ± 0.010^{b}	$0.079 \pm 0.004^{b,c}$	0.077 ± 0.004^{c}	$0.080 \pm 0.001^{b,c}$	0.104 ± 0.005^{c}
75	0.103 ± 0.006^{a}	0.111 ± 0.008^{a}	0.088 ± 0.009^{b}	0.076 ± 0.001^{b}	0.117 ± 0.004^{b}
100	0.100 ± 0.001^{a}	0.098 ± 0.006^{b}	0.113 ± 0.005^{a}	0.110 ± 0.002^{a}	0.134 ± 0.006^{a}

Note: * Means sharing the same letters at each bacterial isolates are statistically non-significant at $p < 0.05$.

Table 4. Units siderophore production (%) by selected bacterial isolates, *Aeribacillus pallidus* at 55 °C for 24 h

Pb conc. (mg/L)	MRP112	MRP148	MRP272	MRP278	MRP280
0	4.10 ± 0.56^{d}	7.70 ± 0.10^{d}	6.67 ± 0.65^{c}	3.97 ± 0.21^{e}	7.60 ± 0.10^{e}
25	7.30 ± 0.53^{c}	13.27 ± 1.96^{c}	10.50 ± 0.44^{c}	7.37 ± 0.55^{d}	12.40 ± 1.17^{d}
50	19.80 ± 1.21^{b}	25.13 ± 0.32^{b}	23.60 ± 6.06^{b}	20.40 ± 0.10^{c}	31.20 ± 0.40^{c}
75	20.67 ± 0.15^{b}	28.33 ± 0.57^{a}	27.60 ± 0.20^{b}	22.53 ± 0.38^{b}	31.77 ± 0.38^{b}
100	27.33 ± 1.24^{a}	28.97 ± 0.06^{a}	29.43 ± 0.45^{a}	25.97 ± 0.35^{a}	38.63 ± 2.28^{a}

Note: * Means sharing the same letters at each bacterial isolates are statistically non-significant at $p < 0.05$

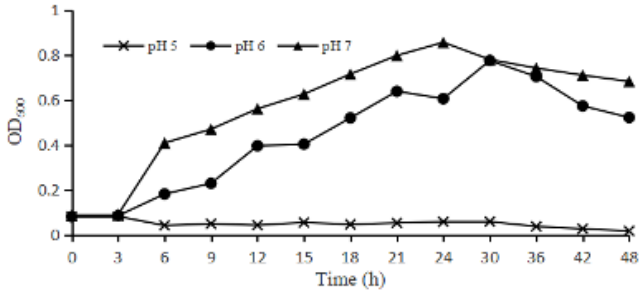

Figure 3. *Aeribacillus pallidus* MRP280 growth profile without Pb at 55° C

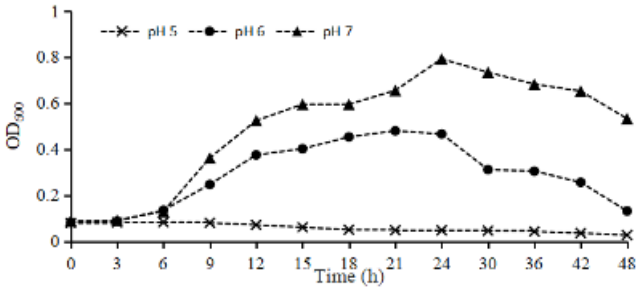

Figure 4. *Aeribacillus pallidus* MRP280 growth profile with addition 50 ppm Pb at 55° C

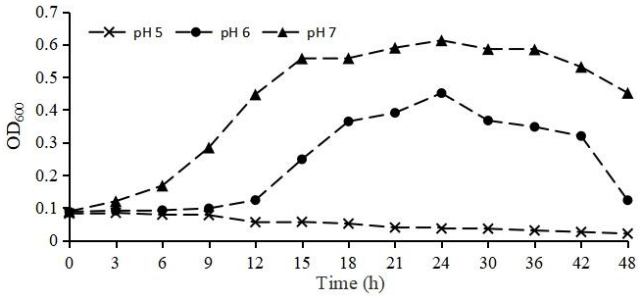

Figure 5. *Aeribacillus pallidus* MRP280 growth profile with addition 100 ppm Pb at 55° C

The effect of pH on *A. pallidus* MRP280 growth is shown in Figures 3-5. It was observed that the isolate demonstrated growth at pH 6 and pH 7, while at pH 5, no growth was observed. A higher optical density value at pH 7 than pH 6 indicated that pH 7 was found optimum for *A. pallidus* MRP280. The results of this study confirm previous report that the optimum growth of *A. pallidus* was obtained at pH 7-8 (Yasawong et al. 2011). The growth patterns of the bacteria at pH 6 and 7 with concentrations Pb 0 and 50 mg/L were relatively similar. However, at concentration Pb 100 mg/L the growth curves were slightly different. The lag phase for bacterial growth at pH 6 was found extended up 9 has compared to growth at pH 7 which lasted for 6 h.

The amounts of Pb desorbed by milli-Q water, NH_4NO_3, and EDTA-Na_2 were 4.7%, 11.19%, and 41.82% respectively. Several mechanisms responsible for the binding of Pb ions to bacterial cells can be quantitatively assessed using appropriate desorption reagents. When Pb ions come into contact with bacterial cells, some ions enter into the cell wall mesh structure and are adsorbed by physical entrapment. This fraction is bound weakly and can be desorbed by water. A second fraction that is exchangeable with K^+, Ca^{2+}, Na^+, and Mg^{2+} on cell wall polysaccharides can be desorbed by NH_4NO_3. A third fraction that complexes with cell wall functional groups such as carboxyl and phosphate groups can be desorbed by EDTA but not by water or NH_4NO_3 (Y. Li, Yue, and Gao 2010; Fang et al. 2011). These results revealed that the Pb ions adsorbed by *A. pallidus* MRP280 cell, 4.7% was physically entrapped, 11.19% was held by ion exchange, 41.82% was complexed with functional groups, and 42.3% was accumulated inside cells (Figure 6).

Pb precipitates characterization

FT-IR analysis was carried out to investigate whether the interaction of lead with bacterial cells was due to a biosorption process of lead ions with functional groups of cell surface. Comparison of *A. pallidus* MRP280 cells spectra loaded with Pb metal ions is shown in Figure 7. The zone range of 2900-3400 cm^{-1}, 1350-1450 cm^{-1}, and 700-800 cm^{-1} showed remarkable changes in the shape and intensity of peaks, indicating bacterial cell exhibited considerable changes in N-H, C-H, C=O, and C-C groups and thus supporting the adsorption of Pb. Lead ions interact with functional groups of proteins and phosphate groups on the cell surface.

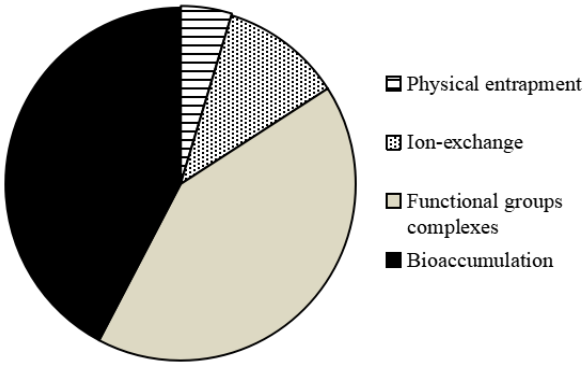

Figure 6. Pb distribution in *Aeribacillus pallidus* MRP280 cell at pH 6 after 24 hours

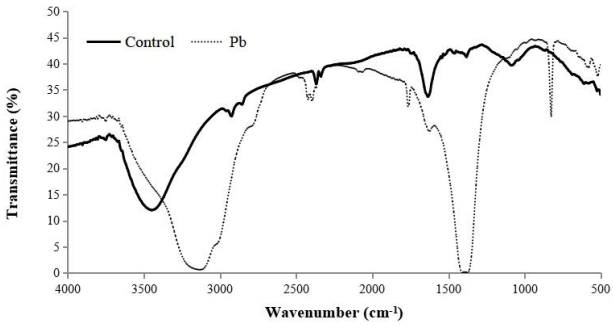

Figure 7. FTIR analysis of *Aeribacillus pallidus* MRP280 cell with (Pb) and without Pb (control)

Bioaccumulation and biosorption assay

The morphological differences of *A. pallidus* MRP280 in the presence of Pb were examined by SEM-EDX (Figure 8). It is clearly demonstrated that bacteria grew in an aggregated formation and as intact morphological features in the medium without heavy metals (Figure 8.A). Under heavy metals, however, cells showed an aggregated form, disrupted cell wall, and highly distorted morphological features (Figure 8.B). To investigate further the intracellular accumulation and extracellular absorption of heavy metals, a TEM analysis was conducted. Figure 9 showed an obviously closed region with a double-layer border in each TEM picture representing cell wall in the cross-section of cell. The dark spots in the external region indicated that Pb was distributed uniformly in the periplasmic space and cell surface, while the dark spots in the internal region showed Pb precipitation, suggesting the possibility of Pb bioaccumulation inside the cell.

Discussion

Five isolates isolated from Mount Merapi, Indonesia, designated as isolates MRP112, MRP148, MRP272, MRP278, and MRP280 showed tolerance to Pb at very high concentrations (100 mg/L). BLAST analysis of all isolates revealed highest sequence similarity (100%) with *Aeribacillus pallidus*. Despite the fact that *A. pallidus* have been obtained from various high-temperature environment, no lead tolerant strain has been recorded so far (Adigüzel et al. 2011; Yasawong et al. 2011; Mnif et al. 2014; Muhammad and Ahmed 2015; Aanniz et al. 2015, Filippidou et al. 2015; Bose and Satyanarayana 2016; Poltaraus et al. 2016; Mechri et al. 2017; Baltaci et al. 2017; Arab et al. 2019; Ma et al. 2019; Mehetre et al. 2019; Ktata et al. 2020).

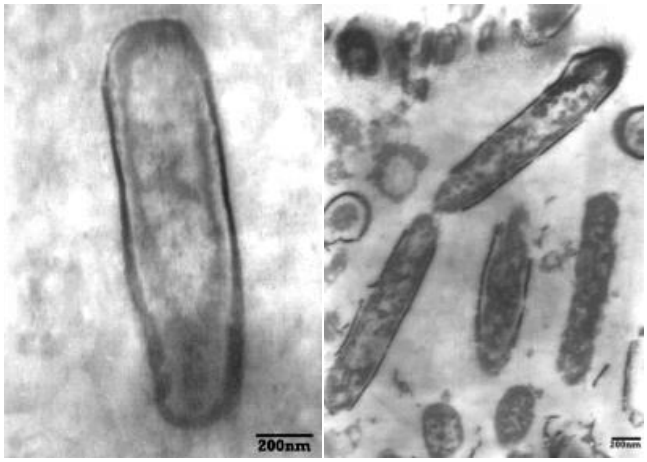

Figure 9. TEM analysis of *Aeribacillus pallidus* MRP280 (A) without Pb (B) 100 mg/L Pb

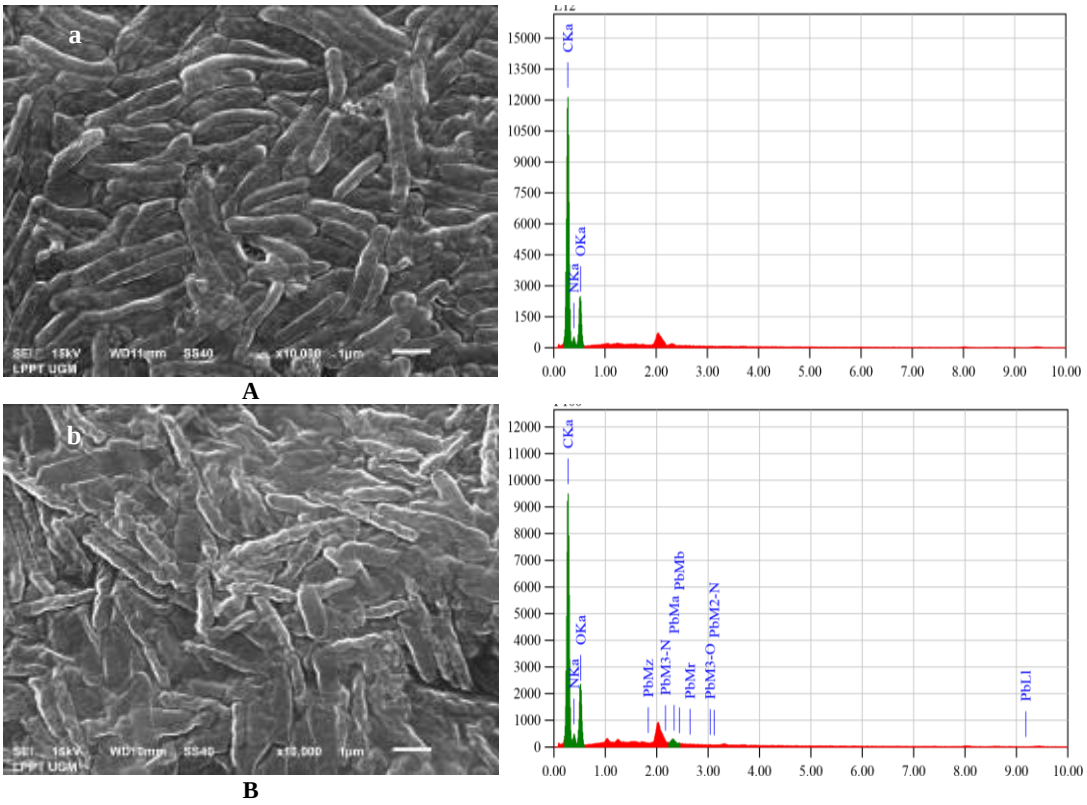

Figure 8. SEM-EDX analysis of *Aeribacillus pallidus* MRP280 (A) without Pb (B) 100 mg/L Pb

The order of cell growth for all five isolates was found to be *A. Pallidus* MRP280>MRP112>MRP272>MRP278> MRP148 at 100 mg/L of Pb solution, suggesting strong Pb tolerance potential of *A. pallidus* MRP280. The major factor determining the toxicity of Pb to bacteria is probably the extent to which they penetrate cytoplasm (Aljerf and AlMasri 2018), that damages the bacterial cell membranes, and destroys DNA structure (Igiri et al. 2018). This study demonstrated different findings in comparison with previous study. A marked negative effect of Pb on thermophilic bacteria, *Thermus thermophilus* growth was only observed at 200 and 300 mg/L (Nicolaus et al. 2016) which might be attributed to the differences in the strains, observed growth stage, growth media composition, and temperature.

The removal of Pb in the bacterial cell was greatly enhanced and reached the maximum value at pH 6, related to the availability of free binding sites depending on the pH. The anionic functional groups present in the cell envelop were the components primarily responsible for the metal-binding capability of the bacterium (Chakravarty and Banerjee 2012). At lower pH (pH 5), more H^+ was present that can protonate the functional groups in the bacterial cell reducing the negative surface, meanwhile, most of Pb(II) is present as Pb^{2+}. This condition inhibited the interaction of Pb on the bacterial cell. In addition, bacteria cannot grow at pH 5 as seen in Figures 3-5 which suggested that sufficient number of cells was not achieved at pH 5, compared to pH 6 and 7. The increase of pH (pH 6) resulted in the reduction of the H^+ number, which further decreased the protonation which gave negative surface of the cell favorable for the biosorption of Pb ions by *A. pallidus* MRP280. Higher pH (pH 7) also significantly reduced the solubility of Pb, forming Pb hydroxides, which collides and thus impedes the biosorption. These pH values are different from those previously reported that Pb maximum adsorption by thermophilic bacteria non-living biomass *Geobacillus thermodenitrificans* was observed at pH 4.5 (Chatterjee et al. 2010). In this study, Pb adsorption was determined by living and non-living bacterial cells in LB medium, thus, an overestimation of pH values must be considered.

Among all isolates, it was found that *A. pallidus* MRP280 formed the thickest biofilm at 100 mg/L of Pb after 24 h. It indicated that this isolate was the most Pb tolerant among all isolates. Villegas et al. (2018) reported that bacterial cell which formed the thickest biofilm was believed as the heaviest metals tolerant bacteria. Several studies showed that Pb-tolerant bacteria could produce biofilm as reported by numerous studies on *Klebsiella* sp. 3S1 (Muñoz et al. 2012); *Bacillus pumilus* (Pepi et al. 2016); *Aeribacillus pallidus* E334 (Kilic et al. 2017); *Pseudomonas pseudoalcaligenes* NP103 (Kumari et al. 2017); *Rhodococcus equi*, *R. opacus*, *Pseudomonas aeruginosa*, *Bacillus megaterium*, *Solibacillus silvestris*, *Enterococcus faecium*, and *Bacillus* sp. (Villegas et al. 2018).

Aeribacillus pallidus MRP280 was identified as the highest siderophore producer at 100mg/L of Pb. It is, therefore, proposed that this isolate may be elaborated further as a candidate for Pb bioremediator agent. Tiquia (2018) stated that the siderophore's ability to reduce the mobility of Pb in the environment by forming stable metal-ligand complexes makes them an ideal strategy for Pb remediation. Similarly, siderophore-producing bacteria in the presence Pb ion have been studied on *Kluyvera ascorbata* SUD165 (Burd et al. 2000); *Pseudomonas putida* KNP9 (Tripathi et al. 2005); *Pseudomonas aeruginosa* 4EA (Naik and Dubey 2011); *Bacillus amyloliquefaciens* NAR38.1 (Gaonkar and Bhosle 2013); *Bacillus* sp. PZ-1 (Yu et al. 2017); *Pseudomonas* sp. B50D (Giovanella et al. 2017).

Bioaccumulation and biosorption assay revealed that the Pb ions adsorbed by *A. pallidus* MRP280 cell, was 4.7% physically entrapped, 11.19% was held by ion exchange, 41.82% was complexed with functional groups, and 42.3% was accumulated inside cells. According to Bai et al. (2014), the dominant binding mechanisms of the total amount of Pb (II) adsorbed by *Bacillus subtilus* DBM, were ion-exchange and intracellular accumulation (43.4% and 38.5%, respectively) for living cells. For non-living cells, on the other hand, ion exchange and complexation with functional groups were the dominant mechanisms (34.9% and 34.3%, respectively). The results from these studies, however, may not be directly comparable to the present study as there were experimental conditions differences i.e., bacterial strain, pH, temperature, initial Pb concentration, contact time, biomass weight, and cell condition (living or non-living cells).

As a result of the addition of Pb, *A. pallidus* MRP280 cells became twisted with stretched cell size and surrounded by numerous cell debris in comparison to the control. The feature of Pb bioprecipitation showed distinct morphological alterations, deformation, and severe membrane damage, indicating Pb toxicity to bacteria. Additionally, cell debris could be a matrix of exopolysaccharides which was secreted to protect the cell from metals or to retain metals by adsorption and prevent accumulation into the cells (Shao et al. 2019).

Thermophilic bacteria isolated from solfatara Mount Merapi demonstrated their ability to tolerate relatively higher concentrations of Pb 100 mg/L and thus suggesting their potential for Pb removal. One of the isolates, *A. pallidus* MRP280 demonstrated the highest MIC and Pb removal capacity. Moreover, it was also clearly demonstrated that lead tolerance was facilitated by morphological changes, bioaccumulation, biosorption, biofilm, and siderophore assembly. Further investigations are necessary to explore the capability of this thermophilic bacteria to remove Pb from contaminated environments.

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