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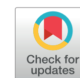
Research article

# Potential application of thermophilic bacterium *Aeribacillus pallidus* MRP280 for lead removal from aqueous solution



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ABSTRACT

Bacteria used for application of lead (Pb) removal is usually kept under suboptimal growth conditions. Certain application of Pb removal may be carried out under different condition, such as under aqueous and high temperature conditions. It is, therefore, of interest to examine the Pb removal capacity of the bacteria under adverse environmental conditions. In the present study, *Aeribacillus pallidus* MRP280, a lead-tolerant thermophilic bacterium was used as an absorbent for the removal of Pb from aqueous solution. The Pb removal and uptake capacity of living and non-living bacterial cells of *A. pallidus* MRP280 was investigated in 100 mg/L Pb solution. The optimum condition was examined based on several analytical parameters, including temperature, pH, contact time, and cell density. Biosorbent analysis and characterization was carried out using Fourier Transform Infrared (FT-IR) spectroscopy, Scanning Electron Microscope (SEM)-Energy Dispersive X-ray (EDX), and Transmission Electron Microscope (TEM). The results showed that the maximum Pb removal of 96.78 ± 0.19% and 88.64 ± 0.60% were obtained using living and non-living biomass, respectively at 55 °C, pH 6, $OD_{600}$0.5 for 100 min. Meanwhile, the maximum uptake capacity of 86.47 ± 1.32 mg/g and 85.31 ± 1.37 mg/g by living and non-living cells was reached at 55 °C, pH 6, $OD_{600}$0.25 for 60 min. Moreover, Pb removing activity was facilitated by the biosorption and bioaccumulation process. Overall, it is shown that *A. pallidus* MRP280 is effective when applied as biosorbent in removing Pb from contaminated wastewater at high temperatures.

## 1. Introduction

The massive release of lead (Pb) to the ecosystem raises a serious hazard to human beings and food safety. Remediation of lead from contaminated areas is considered a challenging task for the safety of the environment. To resolve the problem, several remediation approaches using chemical, physical, and biological methods have been implemented. The conventional physical and chemical techniques for recovery and cleaning-up of the contaminated sites have imposed a serious burden in cost, soil properties, and indigenous microflora alteration, as well as potential creation of secondary pollution problems. Therefore, novel biological approaches are needed to offer eco-friendly and highly selective bioremediation methods [1, 2, 3]. An efficient, nature-friendly, and value-effective biological method for the removal of Pb from the Pb-contaminated environments may be implemented by using microbial biosorbents. Microbes demonstrate several mechanisms of Pb sequestration that maintain greater capacities of Pb biosorption. Microbial biosorption is aimed at removing and/or recovering Pb from solutions, using biomass of living or non-living and their components [4, 5, 6]. Microorganism applications, specifically bacteria as biosorbent for the removal of Pb, have earned growing interest considering the high surface to volume ratio, huge availability, rapid kinetics of adsorption and desorption, and low cost [7].

Even though Pb remediation by bacteria has been quite extensively studied, yet the use of thermophilic bacteria in Pb removal is still limited. The use of thermophilic bacteria in Pb removal may offer a promising approach in bioremediation of high-temperature contaminated sites. Such group of bacteria can be used for the bioremediation of environments contaminated with extremely recalcitrant pollutants due to their stability and persistence under adverse environmental conditions, [8].

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Thermophilic bacteria, including *Anoxybacillus flavithermus* [9], *Geobacillus thermodenitrificans* [10], *G. thermocatenulatus* [11], *Stenotrophomonas maltophilia*, *Aeromonas veronii* and *Bacillus barbaricus* [12], *G. galactosidasius* sp nov [13], *Bacillus stearothermophilus* [14], and *Anoxybacillus rupiensis* 19S [15] have demonstrated pivotal role in Pb removal.

*Aeribacillus pallidus* MRP280 is a novel thermophilic bacteria isolated from solfatara of Mount Merapi, Indonesia, that demonstrated lead-tolerant. It has been observed that the bacteria thrive at 100 mg/L Pb. It is interesting to further explore the use of *A. pallidus* MRP280 as an approach for the bioremediation of Pb-contaminated sites through the removal of Pb from aqueous solution. This study aims at investigating the effect of cell condition (living and nonliving), temperature, pH, contact time, on Pb removal and uptake capacity of the lead-tolerant thermophilic bacterium *A. pallidus* MRP280. Characterization of this biosorbent by analyzing its ability to detoxify Pb was carried out using Fourier Transform Infra-Red spectroscopy (FTIR), Scanning Electron Microscopy with Energy Dispersive X-ray (SEM/EDX), Transmission Electron Microscopy (TEM), and Atomic Absorption Spectroscopy (AAS). The present study describes a novel approach in Pb bioremediation by employing lead-tolerant thermophilic bacteria isolated from solfatara of Mount Merapi, Indonesia.

## 2. Materials and methods

### 2.1. Bacterial strain and cultural condition

The biosorbent used in this study, *A. pallidus* MRP280 was isolated from solfatara of Mount Merapi located in Indonesia and deposited in the Laboratory of Microbiology, Department of Biology Education, Faculty of Mathematics and Natural Sciences, Universitas Negeri Yogyakarta, Indonesia with National Center for Biotechnology Information (NCBI) accession number of **MT422117**. The lead-tolerant thermophilic strain was able to grow at 100 mg/L of Pb and tolerates a growth temperature of 55 °C. The bacteria were routinely cultured at 55 °C in Luria Bertani (LB) containing (g/L): tryptone 10, yeast extract 5, and NaCl 10 at pH 7. on LB agar supplemented with 10 mg/L of Pb to maintain the Pb tolerance.

Pb(II) stock solutions were prepared by dissolving $Pb(NO_3)_2$ in aquabidest, followed by the addition of 1 mL/L of 0.1 mol/L $HNO_3$ to the solutions to avoid precipitation of Pb(II). The LB media and $Pb(NO_3)_2$ stock solution were sterilized by autoclaving at 121 °C for 15 min.

### 2.2. Preparation of living and non-living cells

The *A. pallidus* MRP280 was inoculated into LB medium at 55 °C in a reciprocating shaking water bath at 100 rpm. Pre-cultures were prepared by growing overnight in 500 mL shake-flasks filled with 200 mL of growth media at 55 °C in a reciprocating shaking water bath at 100 rpm. Bacterial pre-cultures were then transferred into 1000 mL of fresh media followed by incubation for 24 h at 55 °C. Living cells were harvested from the medium, whereas the non-living cells were harvested after autoclaving at 121 °C for 15 min [16]. Cells were harvested by centrifugation at 2,400 x *g* for 10 min. The supernatant was discarded, and then the cells were resuspended in sterilized aquabidest to eliminate the residual medium components and centrifuged again 3 times. Living and non-living cells were prepared as biosorbents for Pb.

### 2.3. Pb removal and uptake capacity evaluation

The Pb removal and uptake capacity evaluation of this strain was conducted in an aqueous solution containing 100 mg/L of Pb with continuous shaking of 100 rpm. The living and non-living cells were used as biosorbents. The experiments were conducted according to the *One Variable at One Time* (OVAT) method. The studies were divided into four parts, namely (I) the effect of temperature (45, 55, and 65 °C) with fixed pH 6.0, contact time 60 min, and cell density of $OD_{600}$ 0.5; (II) the effect of pH in the range of 3–7 with the fixed temperature at 55 °C, contact time 60 min, and cell density of $OD_{600}$ 0.5; (III) the effect of contact time in the range of 20–100 min with fixed temperature at 55 °C, pH 6, and cell density $OD_{600}$ 0.5; and (IV) the cell density effects in the range $OD_{600}$ 0,25-1,0 by with fixed temperature at 55 °C, pH 6, and contact time 60 min. All of the experiments were conducted in triplicates. The supernatant was collected after the removal of bacterial cells by centrifugation at 2,400 x *g* for 10 min. Pb concentration was measured using atomic absorption spectrophotometer (AAS 7000, Shimadzu, Japan). In addition, lead ion removal percentages and uptake capacity were calculated by using the following formula [17, 18]:

$$\text{Lead removal (\%)} = (\text{Co-Ct})/\text{Co} \times 100 \quad (1)$$

$$\text{Lead uptake capacity (mg/g)} = (\text{Co-Ct}) \times \text{V/m} \quad (2)$$

where Co and Ct are initial Pb concentration in the solution (mg/L) and the Pb concentration after time t in the solution (mg/L), respectively; V is the solution volume (mL) and m is the adsorbent mass (mg).

### 2.4. The desorption experiment

The desorption experiment was also performed by using a modified protocol [19]. 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), was used to wash the biomass after the biosorption experiment at a temperature of 55 °C, pH 6, $OD_{600}$0,5 for 60 min. The bacterial pellet was harvested by centrifugation and resuspended by each desorbent in an equal volume and incubated at 55 °C, 100 rpm, for 60 min. The suspension was centrifuged at 2,400 x *g* for 10 min. Analysis of Pb ion concentration in the supernatant was carried out by using the Atomic Absorption Spectrophotometer (AAS 7000, Shimadzu, Japan).

### 2.5. Biosorbent characterization

The use of living and non-living cells of *A. pallidus* MRP280 as biosorbent was applied in an aqueous solution containing 100 mg/L Pb at temperature of 55 °C, pH 6, $OD6_{00}$0,5 for 60 min. Fourier Transform InfraRed (FTIR) spectroscopy, Scanning Electron Microscopy integrated with Energy Dispersive X-Ray (SEM-EDX), and Transmission Electron Microscopy (TEM) were used to characterize the biosorbent.

### 2.6. FTIR spectroscopy

The alteration in the functional groups of biosorbents analyzed using Fourier Transform Infrared (FTIR) spectra in the frequency range of 4000–400 $cm^{-1}$ was used at a resolution of 1 $cm^{-1}$. Before and after Pb contact, biosorbent was centrifuged at 2,400 x *g* for 10 min, followed by washing three times with aquabidest for FTIR analysis. The pellets obtained were subsequently dried in an oven at 80 °C overnight. Cell pellets were then diluted to 5% KBr and casting them in disks for FTIR spectroscope analysis (8201PC, Shimadzu, Japan) [20].

### 2.7. SEM-EDX analysis

SEM-EDX analysis was carried out to determine the effect of Pb on the surface of the biosorbent. The changes on the surface structures of biosorbent can be observed directly by SEM. Biosorbent were centrifuged at 2,400 x *g* for 10 min, followed by washing three times with aquabidest for SEM analysis, then the pellets were applied onto a copper grid coated with carbon onto these pellets prior to observation under SEM-EDX (Hitachi SU 3500) [21].

### 2.8. TEM images observation

The cellular localization of Pb complexes formed by biosorbent was carried out using transmission electron microscopy (TEM) (JEM-1010

JEOL Japan). The living and non-living biosorbent were suspended in a 2.5 % glutaraldehyde (in 0.1 M cacodylate buffer and 3 % sucrose) fixative solution. The suspension was shaken overnight at 4 °C. The pellet was centrifuged at 3,000 x *g* for 3 min, followed by washing twice with 0.1M cacodylate buffer and 3 % sucrose, and then fixed in 2 % osmium tetroxide, 2.5 % $K_3Fe(CN)_6$, and 3 % sucrose for 2 h at 4 °C. The pellet was then centrifuged at 3,000 x *g* for 3 min, followed by washing twice with 0.1M cacodylate buffer and 3 % sucrose. Subsequently, to remove all water from the pellet, the pellet was subjected to a series of ethanol solutions, starting at 10 %, 50 %, 70 %, 80 %, 96 %, and ending with 100 % ethanol. The prepared pellet was infiltrated gradually with propylene oxide and immediately embedded in Spurr resin. Ultrathin sections of 50–100 nm thickness were prepared by cutting using an ultramicrotome. Thin sections were supported on copper grids and examined after staining with uranyl acetate and triple lead prior to the observation under a TEM [22].

### 2.9. Statistical analysis

Data for removal and uptake capacity of Pb by living and non-living cells at various temperatures, pH, contact time, and cell density were analyzed statistically using SPSS 22.00 (IBM Corporation, Armonk, NY, USA). All experiments were performed in triplicates, and the reported results demonstrated the mean of three values ± standard deviation. Mean variables were compared using Analysis of Variance (ANOVA) and Duncan's multiple range test (DMRT) in which the p value of $<0.05$ is considered as significant. Prior to the Two Way Analysis of Variance (ANOVA), all data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and Levene's test.

## 3. Results

### 3.1. Pb removal and uptake capacity experiment

Both living and non-living cells of *A. pallidus* MRP280 demonstrated promising Pb removal potential, with living cells (83.46–96.78 %) showing superior removal efficacy compared to non-living cells (79.45–88.64 %). For Pb uptake, however, living cells showed a slightly lower improvement (27.09–86.47 mg/g) compared to non-living cells (27.0–85.31 mg/g). The capability of this strain to absorb biosorb Pb related to temperature, pH, contact time, and cell density are presented in Tables 1, 2, 3, 4, 5, and 6 that shows the maximum removal and uptake of Pb achieved at 55 °C and pH 6 using living and non-living cells. In the case of cell density, maximum Pb removal was reached at $OD_{600}$0.5, while Pb uptake decreased according to the cell density. The results of this study demonstrated that the highest Pb removal process of 96.78 ± 0.19% and 88.64 ± 0.6% was achieved using living and non-living biomass, respectively at 55 °C, pH 6, $OD_{600}$0.5 for 100 min. Meanwhile, the highest uptake capacity of 86.47 ± 1.32 mg/g and 85.31 ± 1.37 mg/g biomass was obtained by using living and non-living cells at 55 °C, pH 6, $OD_{600}$0.25 for 60 min.

**Table 1.** Pb removal rate (%) by living and non-living cells of *A. pallidus* MRP280 at various temperatures, 100 mg/L Pb, pH 6, $OD_{600}$ 0.5 for 60 min contact time with Pb.

| Cell condition*temperature (°C) | Removal rate (%) |
|---|---|
| Living*45 | 87.81 ± 0.36$^{b}$ |
| Living*55 | 90.24 ± 0.63$^{a}$ |
| Living*65 | 87.01 ± 0.95$^{b,c}$ |
| Non-living*45 | 85.09 ± 2.22$^{c}$ |
| Non-living*55 | 86.51 ± 0.40$^{b,c}$ |
| Non-living*65 | 79.45 ± 1.80$^{d}$ |

Means sharing the same superscripted letters at each column are statistically non-significant at $p < 0.05$.

**Table 2.** Pb uptake capacity (mg/g) by living and non-living cells of *A. pallidus* MRP280 at various temperatures, 100 mg/L Pb, pH 6, $OD_{600}$ 0.5 for 60 min contact time with Pb.

| Temperature (°C) | Living cell | Non-living cell |
|---|---|---|
| 45 | 58.28 ± 0.21$^{a,b}$ | 54.22 ± 1.75$^{a}$ |
| 55 | 59.25 ± 0.95$^{a}$ | 56.19 ± 0.96$^{a}$ |
| 65 | 57.12 ± 0.76$^{b}$ | 53.06 ± 0.70$^{a}$ |

Means sharing the same superscripted letters at each column are statistically non-significant at $p < 0.05$.

**Table 3.** Pb removal rate (%) by living and non-living cells of *A. pallidus* MRP280 at various pH, 100 mg/L Pb, 55 °C, $OD_{600}$ 0.5 for 60 min.

| Cell condition*pH | Removal rate (%) |
|---|---|
| Living*3 | 85.99 ± 1.81$^{c}$ |
| Living*4 | 86.44 ± 0.57$^{b,c}$ |
| Living*5 | 90.85 ± 0.80$^{a}$ |
| Living*6 | 90.24 ± 0.63$^{a}$ |
| Living*7 | 87.92 ± 0.82$^{b}$ |
| Non-living*3 | 83.99 ± 0.72$^{d}$ |
| Non-living*4 | 85.81 ± 0.84$^{c}$ |
| Non-living*5 | 86.80 ± 0.46$^{b,c}$ |
| Non-living*6 | 86.51 ± 0.40$^{b,c}$ |
| Non-living*7 | 83.97 ± 0.54$^{d}$ |

Means sharing the same superscripted letters at each column are statistically non-significant at $p < 0.05$.

**Table 4.** Uptake capacity (mg/g) by living and non-living cells of *A. pallidus* MRP280 at various pH, 100 mg/L Pb, 55 °C, $OD_{600}$ 0.5 for 60 min.

| pH | Living cell | Non-living cell |
|---|---|---|
| 3 | 55.60 ± 1.36$^{a}$ | 54.21 ± 1.48$^{b}$ |
| 4 | 58.12 ± 1.38$^{a}$ | 55.28 ± 1.45$^{b}$ |
| 5 | 60.19 ± 1.81$^{a}$ | 57.77 ± 1.69$^{a}$ |
| 6 | 59.25 ± 0.95$^{a}$ | 56.19 ± 0.96$^{a,b}$ |
| 7 | 58.31 ± 2.63$^{a}$ | 54.76 ± 0.58$^{b}$ |

Means sharing the same superscripted letters at each column are statistically non-significant at $p < 0.05$.

**Table 5.** Pb removal rate (%) and uptake capacity (mg/g) by living and non-living cells of *A. pallidus* MRP280 at various contact times, 100 mg/L Pb, pH 6, 55 °C, and $OD_{600}$ 0.5

| Cell condition*contact time (min) | Removal rate (%) | Uptake capacity (mg/g) |
|---|---|---|
| Living*20 | 83.85 ± 0.61$^{e}$ | 54.72 ± 1.42$^{d}$ |
| Living*40 | 87.76 ± 0.48$^{c}$ | 57.02 ± 1.55$^{b,c,d}$ |
| Living*60 | 90.24 ± 0.63$^{b}$ | 59.25 ± 0.95$^{b}$ |
| Living*80 | 96.39 ± 0.27$^{a}$ | 63.01 ± 0.98$^{a}$ |
| Living*100 | 96.78 ± 0.19$^{a}$ | 63.16 ± 1.88$^{a}$ |
| Non-living*20 | 80.47 ± 0.63$^{f}$ | 52.26 ± 0.44$^{e}$ |
| Non-living*40 | 83.49 ± 1.16$^{e}$ | 55.05 ± 0.40$^{d}$ |
| Non-living*60 | 86.51 ± 0.40$^{d}$ | 56.19 ± 0.96$^{c,d}$ |
| Non-living*80 | 88.11 ± 1.26$^{c}$ | 56.14 ± 0.89$^{c,d}$ |
| Non-living*100 | 88.64 ± 0.60$^{c}$ | 58.11 ± 2.13$^{b,c}$ |

Means sharing the same superscripted letters at each column are statistically non-significant at $p < 0.05$.

### 3.2. Effects of temperature

The highest Pb removal and uptake capacity was obtained when living cells were used at 55 °C, while the lowest result was achieved by

**Table 6.** Pb removal rate (%) and uptake capacity (mg/g) by living and non-living cells of *A. pallidus* MRP280 at various cell densities, 100 mg/L Pb, pH 6, 55 °C, for 60 min.

| Cell condition*cell density ($OD_{600}$) | Removal rate (%) | Uptake capacity (mg/g) |
|---|---|---|
| Living*0.25 | $84.64 \pm 2.27^c$ | $86.47 \pm 1.32^a$ |
| Living*0.50 | $90.24 \pm 0.63^a$ | $59.25 \pm 0.95^b$ |
| Living*0.75 | $86.75 \pm 0.47^b$ | $42.53 \pm 0.62^d$ |
| Living*1.00 | $83.46 \pm 0.88^c$ | $27.09 \pm 0.79^e$ |
| Non-living*0.25 | $83.89 \pm 1.06^c$ | $85.31 \pm 1.37^a$ |
| Non-living*0.50 | $86.51 \pm 0.40^b$ | $56.31 \pm 1.18^c$ |
| Non-living*0.75 | $86.45 \pm 0.54^b$ | $42.31 \pm 0.63^d$ |
| Non-living*1.00 | $81.63 \pm 0.22^d$ | $27.00 \pm 0.28^e$ |

Means sharing the same superscripted letters at each column are statistically non-significant at $p < 0.05$.

non-living cells at 65 °C (Tables 1 and 2). Table 1 shows that the highest Pb removal (90.24 %) was achieved at 55 °C but decreased to 87.81 % and 87.01 % for living cells at 45 °C and 65 °C. In the case of non-living cells, the maximum removal of Pb was obtained at 55 °C (86.51 %), then decreased to 85.09 % at 45 °C and 79.45 % at 65 °C.

As shown in Table 2, the best uptake capacity of Pb (59.25 mg/g) was in living cells at 55 °C, but the uptake decreased to 58.28 mg/g at 45 °C, and to 57.12 mg/g at 65 °C. Pb uptake capacity by non-living cells, however, did not show significant difference ($p < 0.05$) at 45, 55, and 65 °C.

### 3.3. Effects of pH

The results indicate that Pb removal of living and non-living cells increased steadily with the increase of pH and reached a peak level at pH 5, followed by a slight decrease from pH 5 to 7 (Table 3). However, there is no significant difference ($p < 0.05$) observed between pH 5 and 6. The maximum Pb removal for living cells (90.85 %) and non-living cells (86.80 %) were observed at pH 5.0.

It was also found that the initial pH of Pb solutions influenced Pb uptake capacities of living and non-living cells (Table 4). The highest Pb uptake capacity by living cells was achieved at pH 5 (60.19 mg/g). Pb uptake capacity of living cells was found higher than non-living ones at all pH solutions (pH 3–7). In general, it was observed that Pb uptake capacity increased with the increase of pH values and reached the optimum level at pH 5, for both cell states. At pH 6 and 7 there was no further increase of Pb uptake at pH 6 and 7, and the level of uptake reduced at pH 6 and 7, instead. The living cell's Pb uptake capacity was also found non-dependent on culture medium pH (pH 3–7) at a level of significance of ($p < 0.05$).

### 3.4. Effects of contact time

The percentage of Pb removal rate and uptake capacity by this isolate at various contact times (20–100 min) are summarized in Table 5. Activities of cells at both states were significantly affected by cell condition and contact time ($p < 0.05$). The Pb removal and uptake capacity of living cells was more superior to non-living cells at all different contact times. As expected, contact time resulted in a significant increase in the final Pb removal and uptake values; the desired maximum values were achieved at the longest contact time (100 min). At 80 and 100 min contact time, however, there was no different significance ($p < 0.05$) of Pb removal and uptake.

### 3.5. Effects of cell density

The bacterial cell's density is calculated based on its optical density, using a spectrophotometer at 600 nm. The increase in bacterial cell density is directionally proportional to the increase in its optical density ($OD_{600}$). Value of Pb removal rate and uptake capacity at different cells condition and density ($OD_{600}$) are shown in Table 6. All treatments exhibited variance in their Pb removal and uptake capacity towards different cell density and demonstrated a significant difference at $p < 0.05$. For Pb removal activity, the maximum Pb removal rate (90.24 %) was obtained using $OD_{600}$0.5 of living cells.

The exposure of Pb to the bacterial cell at different densities shows that Pb uptake capacity is significantly affected. It was observed that cell density showed inversely proportional to Pb uptake capacity whereby the reduction of cell density ($OD_{600}$) was followed by the increase of Pb uptake capacity. The maximum decrease in Pb uptake capacity (86.47 mg/g) was observed at $OD_{600}$0.25 by living cells.

### 3.6. The desorption experiment

Appropriate desorption reagents (milli Q water, $NH_4NO_3$, and EDTA) can be used to determine Pb ion binding mechanisms to bacterial cells. Water can be used to desorb Pb ions adsorbed by physical entrapment. $NH_4NO_3$ can be used to desorb Pb ions, exchangeable for $K^+$, $Ca^{2+}$, $Na^+$, and $Mg^{2+}$ on cell wall polysaccharides. Meanwhile, EDTA can be used to desorb Pb ions complexes with cell wall functional groups such as carboxyl and phosphate groups but not by water or $NH_4NO_3$ [23], [24].

The desorption test as presented in Table 7 indicates that Pb ions binding mechanisms on living and non-living cells decreased in the following order: complexation > accumulation > ion exchange > physical entrapment. Meanwhile, physical entrapment, complexes with functional groups, and accumulation inside cells mechanisms adsorbed by different cell conditions are significantly different ($p < 0.05$). On the other case, ion exchange is not significantly different at $p < 0.05$.

### 3.7. Pb bioprecipitates characterization

FT-IR, SEM-EDX, and TEM were used for biosorbent characterization of living and non-living cells *A. pallidus* MRP280. Differences in functional groups on the biosorbent surface interacting with Pb ions were shown by FTIR analysis before and after the biosorption process. To obtain a more detailed surface characterization, FT-IR spectroscopy was also performed (Figure 1). In general, no shift of band peaks was observed, however, band peak shift occurred under Pb biosorption. Figure 2 present the infrared spectra of the bacterium under both cell conditions from 500 to 4000 $cm^{-1}$. The peak positions are: 3400-3500 $cm^{-1}$(O–H and N–H); 2600-2800 $cm^{-1}$ (C–H); 2300-2400 $cm^{-1}$ (C≡C and C≡N); 1500–1600 $cm^{-1}$ (C–N and N–H); 1300–1400 $cm^{-1}$ (C=O); 1000–1100 $cm^{-1}$ (oxygenated groups (CAO bonds) in alcohols, ethers, and carboxylic acids).

To identify and visualize the uptake of Pb (II) by *A. pallidus* MRP280, SEM/EDX was applied to the samples. Figure 2 presents how living and non-living cells after making contact with Pb. The morphology of the biosorbent surface after Pb uptake was visualized using SEM. Results of visualization showed considerable changes to the morphology of the biosorbent surface. The living cells demonstrated a smooth surface characterized by several puckers. The non-living biosorbent cells were

**Table 7.** T-test independent analysis of Pb distribution on living and non-living cell.

| Distribution | Cell condition | Mean | Standard deviation | Sig (2 tailed) | Remarks |
|---|---|---|---|---|---|
| Physically entrapped | Living<br>Non-living | 3.88<br>10.55 | 0.29<br>0.49 | 0.000 | Significant |
| Complexation | Living<br>Non-living | 55.90<br>45.19 | 1.64<br>2.09 | 0.002 | Significant |
| Ion exchange | Living<br>Non-living | 19.72<br>20.07 | 1.24<br>2.48 | 0.835 | Nonsignificant |
| Accumulation | Living<br>Non-living | 29.78<br>22.47 | 0.23<br>0.76 | 0.000 | Significant |

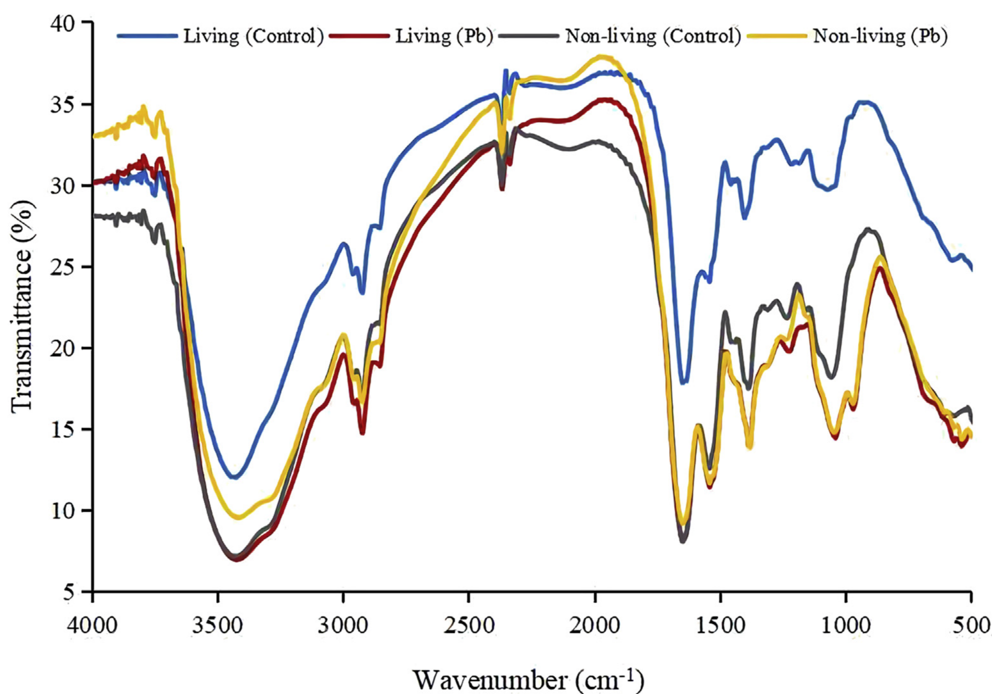



**Figure 1.** FTIR *A. pallidus* MRP280 living (a) and non-living (b) cell at $OD_{600}$ 0,5; 55 °C; pH 6 for 60 min contact time at 100 mg/L Pb.

torn apart, surrounded by numerous cell debris, with a wrinkled surface. According to the EDX result, the elemental composition weight of the living cells were C (46.55 %), N (21.62 %), O (29.74 %), and Pb (2.09 %), while non-living cells were composed of C (51.99 %), O (44.03 %), Na (2.14 %), P (0.93 %), and Pb (0.57 %). The EDX quantification results confirmed that the sorbent demonstrates the capability to retain Pb.

Figure 3 shows the image of TEM analysis of *A. pallidus MRP280* with accumulated Pb after 60 min incubation at pH 6 at 55 °C. In some Pb-loaded cells, it was observed that cell walls, plasma membrane, and internal cytoplasms underwent lysis. Electron dense particles were observed distributed outside the cell, on the cell wall and plasma membrane, and some of them were located within the cytoplasm, demonstrating the main interaction that occurred in the cell wall. The black dots in the living cells represent Pb that was distributed throughout the cell walls and concentrated on the cytoplasm.

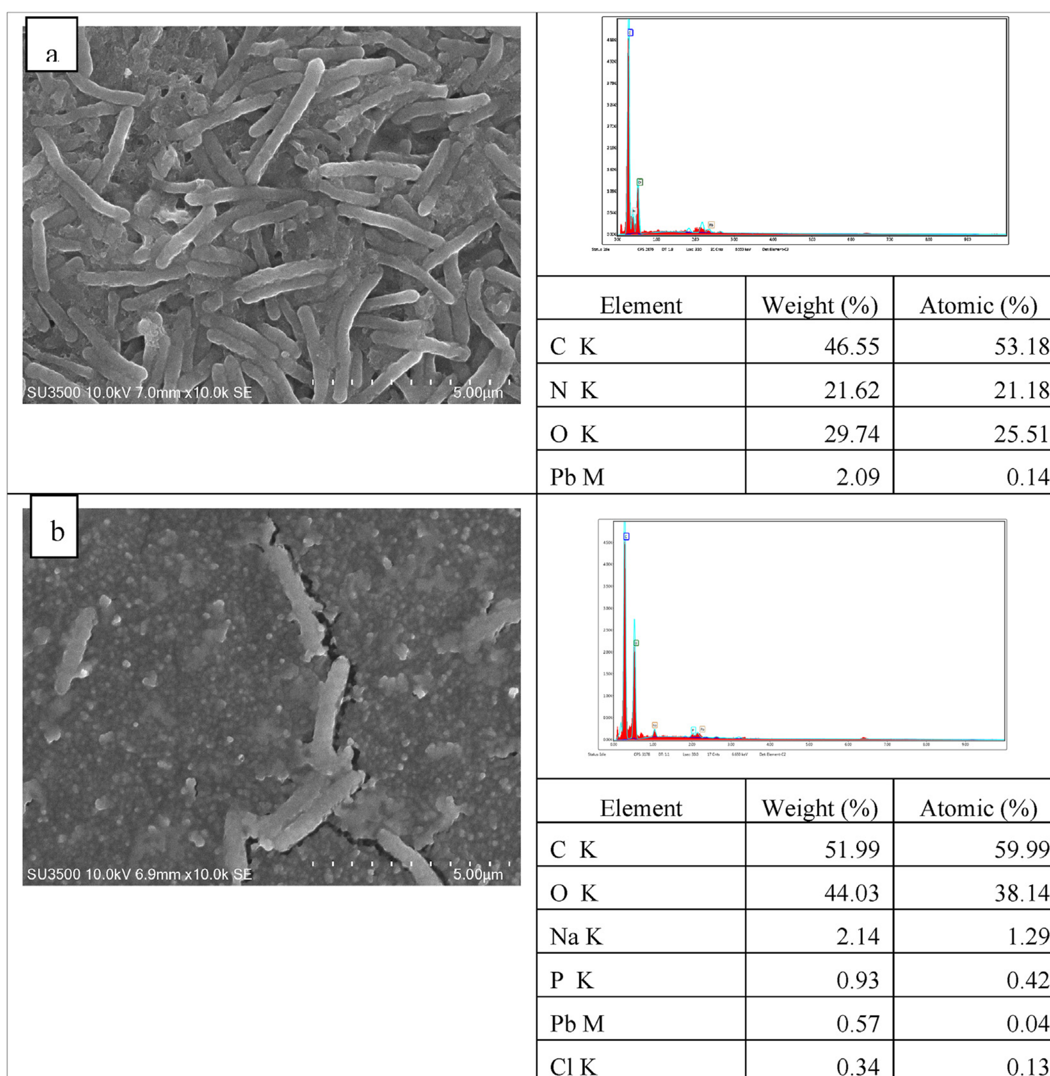
## 4. Discussion

Pb removal and capacity measurements were conducted on the thermophilic bacteria *A. pallidus* MRP280 at two different cell states, i.e. living and non-living biosorbents. The data show significant differences between the two investigated cell states. Living cells show a greater removal of Pb (II) capacity when compared to non-living cells. Non-living cells were disrupted, and as a result, the uptake capacity was lower than that of the living cells as previously suggested that not all non-living biomass from microorganisms demonstrate a good removal capacity [25]. These findings are consistent with previous study suggesting that the higher capacity of living cells in Pb biosorption than that of non-living cells might be contributed by the extra intracellular accumulation of Pb in the living cells, while the lower binding sites for Pb in the non-living cells was due to heat inactivation [26]. Bioaccumulation is a favorable process that improves metal removal together with biosorption (by living cells), and is believed more effective than the biosorption process alone by non-living cells [27].

The experimental maximum removal rate (96.78 ± 0.19%) and uptake capacity (86.47 ± 1.32 mg/g) observed in this study were also comparatively higher than other known biosorbents such as *B. cereus* NSPA8 with maximum biosorption of lead (87 %) [28], while *Bacillus* SS19 demonstrated 57 % efficiency [29], and Pb uptake at 95.9 % was achieved with *Aeromonas* sp. BDL2 [20]. In other cases, a lower uptake capacity was observed in *Bacillus* AS2 Pb which had a capacity of 74.5 mg/g (99.5 % of initial Pb) [30], *Methylobacterium hispanicum* EM2 showed Pb removal of 96 % and uptake capacity at 79.84 mg/g [31]. These different results may be attributed to differences in the experimental condition i.e. bacterial strain, cell condition, initial Pb concentration, temperature, pH, contact time, and cell density.

In the present study, the cell was incubated at various temperatures, pH values, contact time, and cell density to determine the optimum conditions of Pb removal and uptake. The optimum temperature for the maximum biosorption process in both living and non-living cells demonstrated comparable trends at 55 °C. This temperature was also found optimum for the growth of *A. pallidus* MRP280. The most ideal condition for microbial growth was the condition for maximum Pb removal. In essence, the effect of temperature on biological Pb removal depends on bacterial adaptation to high or low temperature [32, 33]. On the other hand, the lower adsorption capacity at higher temperatures (65 °C) indicated that biological mechanisms may contribute more to the adsorption than physicochemical adsorption [34].

The important factor for Pb removal and uptake process is pH value. The optimum pH for Pb removal and uptake capacity by bacterial isolates in the present study was pH 5. It was demonstrated that the pH solution gave stronger effect on the affinity of cationic species for the functional groups presents on the cellular surface [35]. However, it was observed that the trend in metal uptake in relation to pH reveals a lower value at acidic pH. The lower metal uptake at acidic pH range (pH 2.0–3.0) can be attributed to the higher solubility of the cations, supported by the predominantly protonated state of cellular binding groups and competition prompted by $H^+$ ions [36]. The metal acidities of lead are represented by the stability constant of the first hydroxyl-metal complex at pH 4.0. Such acidities corresponds with the order of binding-site affinities observed here, suggesting that metal acidity may increase the affinity for metal sorption to the bacterial cell. The binding-site affinities were found maximized in the range of pH 4.0 and 5.5, where, interestingly, the sum of the free-protons and complexed ions close to a minimum level for solutions containing lead [37]. Similarly, it has been demonstrated [38] that the increase in adsorption capacity from 9.3 to 9.9 occurred when




| Element | Weight (%) | Atomic (%) |
|---|---|---|
| C K | 46.55 | 53.18 |
| N K | 21.62 | 21.18 |
| O K | 29.74 | 25.51 |
| Pb M | 2.09 | 0.14 |

| Element | Weight (%) | Atomic (%) |
|---|---|---|
| C K | 51.99 | 59.99 |
| O K | 44.03 | 38.14 |
| Na K | 2.14 | 1.29 |
| P K | 0.93 | 0.42 |
| Pb M | 0.57 | 0.04 |
| Cl K | 0.34 | 0.13 |

**Figure 2.** SEM-EDX *A. pallidus* MRP280 living (a) and non-living (b) cell at 55 °C, pH 6 for 60 min contact time at 100 mg/L Pb.

the pH of solution increased from 5.0 to 5.7. At higher pH value (above 5.7), the insoluble metal hydroxides were found precipitated concomitant with the decrease of the adsorption capacity. It is known that at low pH, protonation of cell surface ligands may occur, which suggests interaction with metal cations as a result of repulsive force. At higher pH values, on the other hand, there will be more ligands such as amino, phosphate, and carboxyl groups become exposed, which in turn can attract more metal ions. However, it was observed in this study that the increase in pH, prompted the interaction between Pb (II) with the oxygen or hydroxyl ions which resulted in oxide or hydroxide precipitation, which impedes the process of adsorption. This value was similar to the previous study in which the biosorption rate of *Bacillus* sp PZ-1 [39], *Alcaligenes* BAPb.1 [40], and *Bacillus subtilis* [41] were found increased with the increase of pH and reached the peak at pH 5.0. The different values, however, were reported in which Pb maximum adsorption by thermophilic bacteria *Geobacillus thermodenitrificans* was achieved at pH 4.5 [10].

It is understood that one of the important parameters for a successful biosorbent application is contact time. The removal efficiency of lead ions increased gradually with the increase of contact times up to 80 min and almost constant at 100 min. Similarly, previous study also demonstrated that higher removal efficiency also increased with the increase in contact time [21]. It is also believed that the surface binding promoted fast initial metal biosorption rate while the following slower sorption was related to the interior penetration. At biomass surface, different kinds of functional groups, with different affinities to Pb ion, are usually present [42]. At initial stage it was assumed that the adsorption rate during the first time was very high but will decrease upon reaching the equilibrium state. At the equilibrium state, the value of lead adsorption onto the bacterial cell was in a state of dynamic equilibrium with the value of lead desorption from the adsorbent. It was suggested that the rapid lead adsorption at the initial level of the adsorption assay may be attributed to unoccupied adsorption positions present at a great number on the surface of the adsorbent. After a certain time, the adsorbed metal ions covered the empty positions which induced a repulsive force between the adsorbed molecules on the surface of the adsorbent and in bulk phase and, as a consequence reduced the adsorption rate [43, 44].

The interesting finding in this study was that differences between Pb removal rate and uptake capacity occurred when cell density was varied. In Pb removal determination, the number of Pb (II) removed at various cell densities ($OD_{600}$0.25-$OD_{600}$1.0) was non significantly distinct. It was also observed that the amount of Pb (II) uptake at lower cell density ($OD_{600}$0.25) resulted in more Pb taken and the value was significantly larger than the higher cell density ($OD_{600}$1.0). The present study indicated that the Pb removal rate was not in line with uptake capacity. A reasonable explanation for the increased Pb removal at lower cell density

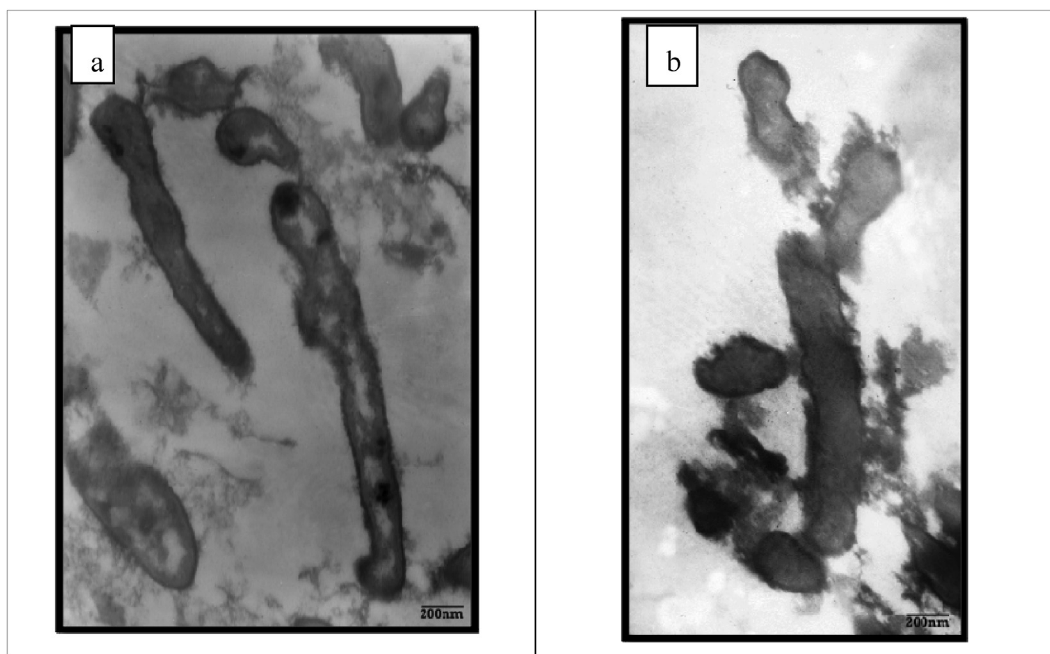



**Figure 3.** TEM *A. pallidus* MRP280 living (a) and non-living (b) cell at 55 °C, pH 6 for 60 min contact time at 100 mg/L Pb.

was that extracellular precipitation occurred during the experiment. In general, the uptake of Pb by bacteria occur via two main mechanisms: adsorption onto the cell surface, and an active uptake into the cytoplasm at a slower rate. The influence of biomass concentration was found significant on uptake or removal of Pb. The decrease of biomass concentration increased its sorption capacity, although absolute loss of metal increased with the increase of biomass concentration. The use of lower biomass concentration was found more effective for this reason [11]. It was suggested that agglomeration of biomasses occurred with the increase of the amount of biomass. The agglomeration of biomasses may also contribute to the lower homogenous suspension which resulted in a lower surface to volume ratio and in turn. Such condition, in turn, resulted in the decrease of absorption sites available for Pb adsorption [45]. Moreover, the increase in the number of biosorbents may induce the increase of the electrostatic interactions between biosorbents that resulted from an agglomeration [46].

It was apparent that the binding Pb mechanism of the living cells was more superior to non-living cells at complexation with functional groups and bioaccumulation. Meanwhile, ion exchange and physically entrapped binding mechanisms are predominantly shown by non-living cells than living cells. Different bacterial species may contribute to different performance of Pb distribution as it is known that different ways of Pb accumulation is a consequence of different mechanisms of bacterial resistance to Pb. It was stated [26], that in *Bacillus subtilus* DBM the dominant binding mechanisms for the total amount of Pb (II) were ion-exchange and intracellular accumulation in living cells. While for non-living cells, the dominant mechanism were ion-exchange and complexation with functional groups. However, the results from the previous studies, may not be directly comparable to the present study due to differences in experimental conditions i.e., bacterial strain, pH, temperature, initial Pb concentration, contact time, and biomass weight.

In this experiment, assays using Fourier-transform infrared spectroscopy (FTIR) analysis, scanning electron microscope (SEM) with energy dispersive X-ray spectra (EDX), and transmission electron microscope (TEM) were done to investigate the Pb precipitate characterization. FTIR analysis revealed that functional groups including amino, carboxyl, phosphate, and hydroxyl groups were participated in the biosorption process of the tested biosorbents, irrespective of the living or non-living states of the cell. It was observed that the peak transmittance in the loaded biomass was substantially lower than those in the original biomass. Pb-treated cells displayed similar changes in vibrational frequencies although with fewer new peaks appearing [27]. This results indicate that bond stretching occurs to a lesser degree due to the presence of metals, while the reduction of the following peak transmittance suggest that the main functional groups responsible for biosorption of heavy metals are carboxylic, hydroxyl, amine, and phosphate groups [47]. Furthermore, the presence of hydroxyl, carboxyl, amino, and amide group ligands with significant shifts after accumulation and biosorption experiment also suggests the interaction between these surface molecules and Pb ions [20, 38, 40].

SEM results showed that the morphology of the bacterial cell at different conditions was distinct, heat-pressure combined by autoclaving to obtain the non-living cells imposed detrimental effects. Autoclave treatment with a rapid temperature ramp and depressurization might result in the efficient rupture, heat denaturation of the cellular proteins, and heat-denatured materials (including proteins) which renders them to become less soluble [48]. Moreover, steam autoclaving also physically inactivates bacteria through denaturation of cellular enzymes, thereby inhibiting their activities [49].

The surface bacterial EDX analysis showed that the elemental compositions of living cells (C, N, O, Pb) and non-living cells (C, O, Na, P, Pb, Cl) after Pb biosorption were different. These results indicated that the mechanism of biosorption of the two cells states was dissimilar. Pb (II) could covalently bond with functional groups on bacterial cell surface containing C-, N-, O- and C-, O-, P- on the living and non-living cells, respectively. The existence of Na element after the non-living cells were mixed with Pb(II), suggesting the occurrence of exchange between Pb with Na on the cell surface. The Cl elemental presence on the non-living cell surface indicated that Pb ions were biosorped and precipitated to $PbCl_2$. The results this study indicated that the process of Pb(II) adsorption included surface adsorption and micro-precipitation. Similarly, previous report demonstrated that the interactions between Pb(II) and the surface of biosorbents resulted in irregular shape and topography of the cell. It was shown that autoclaving has reduced the number of types of active sorption sites on the surface of viable cells suggesting the presence of different mechanism of cation uptake between living and non-living bacteria [50].

TEM results showed that the cells were warped and aggregated, which increase the capacity of extracellular adsorption of Pb. Pb passed through the cell wall and periplasmic space to accumulate in the cytoplasm of living cells. Non-living cells, however, did not actively incorporate Pb through metabolic processes in the cytoplasm. In addition, the Pb distribution was regulated by adsorption and/or precipitation mechanisms on or within the bacterial cell walls. Overall, it was observed that the concentrations of metal inside the living biosorbents cell were higher than those in the non-living ones. The mechanisms of intracellular accumulation may provide explanation for the difference in biosorption between the living and non-living biosorbents [51].

However, in Pb removal applications, living cells have certain advantages over non-living cells in higher Pb removal and simpler installations. On the other hand, living cells may partially lose their binding capacity due to intoxication and a small amount of metal is subsequently released back into solution. It is important to ensure that a constant removal capacity in the growing cells is maintained after multiple bioaccumulation desorption cycles. In addition, optimizing essential operating conditions require a suitable method. On the contrary, the non-living cells give advantages over living cells in terms of operational cost, process maintenance, metal ions uptake, regeneration, and toxicant recovery. Furthermore, the use of living cells for heavy metal removal, may require genetic manipulation to enhance the metal tolerances of microbial strains. Therefore, more comprehensive studies are required to obtain a better understanding of heavy metal removal by bacteria under different physiological conditions [52].

## 5. Conclusions

This study has demonstrated that a lead-tolerant thermophilic bacterium *Aeribacillus pallidus* MRP280 exhibited a removal and uptake capacity of Pb (II). The capability of the bacterium to remove and uptake lead was demonstrated both in living and non-living cells. Such capability is of a paramount importance in terms of its potential use in the industrial waste management as well as in the environmental bioremediation of lead-contaminated sites, especially in lead-contaminated water. Data of this work have also demonstrated that lead bioremediation may be accomplished by using living or non-living cells at 55 °C, pH 6, $OD_{600}$0.25 for 60 min with the maximum uptake capacity of 86.47 mg/g and 85.31 mg/g by living and non-living cells, respectively. The use of thermophilic bacteria gives additional benefit of the approach for lead removal under industrial condition which may require high temperature during the industrial processes. Data also shows that the capacity of lead uptake by living cells were higher than the non-living cells, suggesting that physical state of the cells affected the removal of lead. Such condition, thus, provides an easier protocol in lead removal as there is no specific treatment required to the cell prior to their use as bioremediation agent as the cells can be used directly without having to physically disrupt the cells. Elaboration of the potential of thermophilic bacteria for industrial waste management and environmental bioremediation is, therefore, required to establish the applicable design and a more details parameter for lead removal.

## Declarations

### Author contribution statement

Anna Rakhmawati: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Endang Tri Wahyuni: Analyzed and interpreted the data; Wrote the paper.

Triwibowo Yuwono: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.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### Data availability statement

Data associated with this study has been deposited at NCBI under the accession number **MT422117**.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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