Lab-scale experimental investigation concerning ex-situ bioremediation of mercury (Hg) contaminated soil by local bacterial isolated from Bombana mining area

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Abstract. Hg Contamination is a most common occurrence in the industrial area, caused by several industrial activity wich release Hg into environment, like industrial activity of gold mining in Bombana Regency Southeast Sulawesi, Indonesia. So that it need effort to controll Hg contamination, one of wich is bioremediation techniques using bacterial. The aims of this study were to know the effect of local bacterial and incubation time to reduce the Hg content at post-gold mining soil of Bombana Regency. This study was experimental research atlab-scale (ex-situ bioremediation) using Completely Random Design (RAL) with two factorial. The 1st factor was the kind of bacterial inoculum namely Pseudomonas sp. strain LIIC (Ps), Bacillus sp. strain LIIC (Bc), and mixed inoculum both of these bacterial species (PB). The 2nd factor was incubation time consist of 1st week until 8th weeks incubation. Soil substrate contaminated by Hg was inoculated of bacteria inoculum as much as 100 mL/kg and incubated at room temperature for 8 weeks. Hg content was measured by AAS (Atomic Absorption Spectrophotometer).Data was analysed by software SAS. The result of inoculated of local bacteria and incubation times affect to the Hg content on post-gold mining soil. The highest reduction of Hg content at the treatment of Pseudomonas sp. LIIC inoculated was 98,89% with the optimum incubation time in the second weeks.

1. Introduction
Southeast Sulawesi Province is one of the mining area in Indonesia, especially for gold and nickel mining. Therefore, the potency of those area to be polluted by waste of mining industry wich contain heavy metal elements such as Mercury (Hg) is quite high. The presence of Hg contamination in the environment is naturally through volcanic activity, as well as sourced from anthropogenic activity such as industrial waste, coal combustion activities, catalyst industry, using fungicides wich contain Hg also mining activity [1,2]. Nowadays, Hg contamination is increase every year and become a global environmental issue due to its high toxicity [3,4,5]. According to [6], Hg content in the Bombana post-gold mining area reached 6.5 ppm. Its caused by the rest of Hg wich used in the mining industry process to separate gold and other materials was wasted in the environment. This was supported by
Ericson [7], gold mining activities were considered as one of the causes of pollution with large impacts. Exposed organisms has interacted with Hg due to the high mobility of Hg [8]. Heavy metal contamination have risk for environmental and human health [9], even in small concentrations, heavy metals such as Hg have risk for human health [8].

According to [10], the normal concentration of Hg in the soil is 0.03 ppm and the critical concentration was ranges from 0.3-0.5 ppm. Based on the results of previous study conducted by [11] in the post gold mining area of PT. Panca Logam Bombana obtain Hg content was 0.4-0.57 ppm. Based on these data, the concentration of Hg at the Bombana post-gold mining has exceeded the threshold. Therefore, need an effort to bioremediate to control Hg pollution. One of the efforts to bioremediate Hg contamination in post-gold mining areas by utilizing resistant bacterial [12]. Bacterial were one of microorganisms which found in the environment even in polluted land. Several studies have found that some bacterial has potency to reduce pollutants in the environment. The [13] reported that \textit{P. Aeruginosa} B237 and \textit{C. Taiwanensis} E324 bacteria were tolerant to Cd and Zn metals. The [14] reported that 17 types of potential bacteria to reduce heavy metal Hg from PT. Coal Mine, Bukit Asam, South Sumatra. Previous study which conducted by [11], has isolate \textit{Bacillus} sp. Strain LIIC and \textit{Pseudomonas} sp. Strain LIIC from the post-mining area of Bombana Regency which Hg resistance up to 1000 ppm and could reduce Hg in broth media more than 50%. However, further study about potency of both of bacteria (\textit{Bacillus} sp. and \textit{Pseudomonas} sp.) reduce Hg on post-mining soil media was still not done. Therefore, this study aims to determine the ability of both local bacterial to remediate of Hg soil contaminated.

2. Material and Methods

2.1. Study Site
Collection of post-gold mining soil from PT. Panca Logam, Bombana Regency and continued to ex-situ bioremediation process in the Microbiology Laboratory. Faculty of Mathematic and Natural Science, Halu Oleo University.

2.2. Research Procedures

2.2.1. Preparation of Media and Equipment
NA media (Nutrient Agar) was used to growth media of bacterial isolated from post-gold mining, made by 8 g NB, 8 g Agar and 1 L of akuades, heated and homogenized by magnetic stirer, and sterilized by autoclave with 121 °C constant temperature for 15 minutes. NB media which used as growth bacterial media was broth media and made by 0.5 g pepton, 0.3 g beef extract, akuades 1 L and enriched by HgCl$_2$ 10 ppm, with the same process as NA media. All the equipment was sterilized by autoclave with 121 °C for 15 minutes.

2.2.2. Sample Collection and Preparation
Soil which contaminated by Hg was collected from the field with 10-20 cm from surface and assumed in these soil layer, the soil was not has flashing by rainfall. The soil sample was brought to the laboratory for next process. The soil substrate was sterilized using autoclave with 121°C for 30 minutes.

2.2.3. Rejuvenation of Bacterial Isolate
The isolate of \textit{Bacillus} sp. LIIC and \textit{Pseudomonas} sp. LIIC bacterial which isolated from post-gold mining obtained from previous study, conducted by [11] to rejuvenation. The rejuvenation process of bacterial was done by streak method on NA media which enriched by HgCl$_2$ 10 ppm, and incubated for 24h.
2.2.4. Preparation of Bacterial isolates
Bacterial isolate which has rejuvenated on the NA media was taken as much as 2 ose and suspended into 50 mL of NB media, enriched by 10 ppm HgCl$_2$ then incubated using shaker incubator with speed of 150 rpm for 48h. The starter consist of Bacillus sp., Pseudomonas sp., as well as mixing both type of bacterial inoculum.

2.2.5. Preparation of Inoculum Bacterial
Bacterial inoculum that has been grown in 50 mL NB media, was inoculated into 450 mL NB media which enriched by HgCl$_2$ and was incubated for 48h. The calculation of bacterial cell was done by the direct count method using haemocytometer to obtain $10^7$ sel/mL. These bacterial inoculum were inoculated at post-gold mining soil’s substrate which Hg contaminated.

2.2.6. Bioremediation Process
Bioremediation process was done by 100 mL bacterial was inoculated into 1 kg of sterile soiland homogenized. The treatment of bacterial inoculation consist of Bacillus sp., Pseudomonas sp., as well as mixture of both bacterial. Each treatment consist 3 replication. Then all the treatment was incubated for 8weeks. During incubation process, each treatments were watered with 50 ml of sterile distilled water to maintain soil moisture every two days. Sampling was done every weeks to determined Hg content and bacterial cells count.

2.2.7. Measurement of Hg Content
The soil substrate of post-gold mining which contamined by Hg were measured for Hg content before treatment of bacterial inoculation to calculate the initial Hg content also Hg content were measured after treatment of bacterial inoculation. The measurment of Hg content was carried out every weeks for 8 weeks. 0.3 g soil substrate was mashed up and dissolved into 10 mL of HNO$_3$ and homogenyzed and heated by hotplate. Then the sample was filtered by filter papper. The filtrat was analyzed using done by AAS (Atomic Absorbtion Spectrophotometer) [15].

2.2.8. Calculation of Bacterial Cells
Bacterial which were inoculated in the soil substrate then were measured cells using TPC (Total Plate Count) method to determine the growth curve of bacterial.

2.2.9. Determination of Decrease Percentage of Hg Content
The determination of percentage (%) decrease of Hg content was done by Csuros method [16].

\[
R = \frac{C_0 - C_{eq}}{C_0} \times 100\%
\]

$R$ = The efficiency of bioaccumulation by bacterial (%)

$C_0$ = Initial Hg concentration in the substrate (mg/L)

$C_{eq}$ = Final Hg concentration in the substrate (mg/L)
2.3. Data Analysis
The data was analyzed by ANAVA multifactorial to knows the effect of treatment to the result. Then continued with further tests if the treatment shows a real effect. Data analysis using done by SAS software (Statistical Analysis System).

3. Result and Discussion

3.1. Growth Curve Bacterial in the Post-gold Mining Soil
The growth of local bacterial cells at the post-gold mining soil during incubation was measured based on the count of cells which has been growth at media using TPC (Total Plate Count) methods. The growth curve of bacterial are presented in Figure 1.

![Figure 1. Bacterial cell number in the post-gold mining soil](image)

The log of *Pseudomonas* sp. LIIC (Ps) and *Bacillus* sp. LIIIC (Bc) cells growth at Figure 1 shows that the growth cells increased at the 1st week until 6th weeks of incubation time, then was decrease at the 7th weeks until 8th weeks of incubation time. While the log of mixed bacterial cell growth (PB) was increase at the 1st week until 5th weeks, and decrease at the 6th weeks until 8th weeks of incubation time.

The log of initial cell number of *Pseudomonas* sp. LIIC (Ps) cell inoculum was 9,728 cfu/g, and increase to 9,447 cfu/g at 1st week. The cell number has been increase up to 6th weeks was 10,478 cfu/g and 8th weeks was 9,973 cfu/g. The log of initial cell number of *Bacillus* sp. (Bc) was 9,042 cfu/g, and increase to 9,176 cfu/g at 1st week up to 5th weeks of incubation was 10,579 cfu/g. The decrease of cell number log at 6th weeks 10,382 cfu/g up to 8th weeks was 10,117 cfu/g. The log of initial cell number of mixed inoculum (PB) was 9,255 cfu/g, has been increase to 10,204 cfu/g at 1st week up to 5th weeks was 10,654 cfu/g. The decrease of cell number was at 6th weeks of incubation time was 10,484 cfu/g to 8th weeks of incubation time was 9,991 cfu/g.

The increasing of cell number’s log also occurs at control or without addition of bacterial inoculum (C) was 8,0 cfu/g at 1st incubation, than increase was 8,954 cfu/g at 2nd weeks. These increase occurs to 5th weeks of incubation time was 9,579 cfu/g, than decrease at the 6th weeks was 9,361 cfu/g up to 8th weeks of incubation time was 8,03 cfu/g. The presence of bacterial growth at control (C) likely caused by contamination from air as well as from the equipment or other thing during incubation. The research conducted by Rehman et al. [17], also reported the growth of bacteria in the control treatment (without the addition of a bacterial inoculum), which is suspected of bacterial contaminants
Based on those growth curve at Figure 1, shows that bacterial cell number on each treatment has different. This was likely caused by different response, tolerance and resistance of bacterial to Hg. The toxic environment condition spur the bacterial to adjust through metabolic reaction through increase of enzyme which could reform the hazard pollutant become harmless and the bacterial become tolerant [5,17]. Dash & Das [2] states that the difference of bacterial resistance have relation to bacterial response to Hg through operon resistance system on the plasmid to induce of Hg reduction. According to Silver & Phung [18], detoxification of Hg bacterial resistance occurs because these bacterial has Hg resistance gene. These bacterial has mer-operone on these genome, so that it could tolerance Hg contamination even change it to non-toxic form [2] The Hg resistance mechanism of Gram negative bacterial by Hg reductase (merA) catalyzes the reduction of Hg$^{2+}$ become Hg$^0$ion [2].

The decrease of bacterial cell number commonly occurs in the 6th incubation time. This probably caused by nutrient deficiency in the substrate which cause the decrease of cell number. The nutrient deficiency on the substrate could lead the death phase of bacterial [19].

### 3.2. The Decrease of Hg Content During Incubation Time

Based on the Hg reduction graph in Figure 2, it shows that there was a significant decrease in Hg content in the post-mining substrate in all treatments during incubation. The treatment of Bacillus sp. inoculum (figure 2a) has decrease of initial Hg content was 1,428 ppm become 0.00016 ppm at 8th weeks. As well as treatment of Pseudomonas sp. inoculum (Figure 2b) has decrease of initial Hg content was 1,781 ppm become 0.00012 ppm at the 8th weeks. The treatment of mixed inoculum between Pseudomonas sp. and Bacillus sp. (Fig. 2c) also has decrease on the soil substrate with the initial content of Hg was 1,836 ppm become 0.00013 ppm at the 8th weeks. While without bacterial inoculum treatment (figure 2d) also has decrease of Hg content on the soil substrat which not significant. The initial Hg content was 1,734 ppm was decrease to 1,499 ppm at the 8th weeks. This was indicate that both bacterial inoculum has ability to reduce Hg content at post-mining soil substrat. The same result by [19], reported that the Pseudomonas sp. bacterial which isolated from the sea has Hg resistance ability. In the [17] reported that the Bacillus subtilis and Pseudomonas sp. bacterial which has Hg resistance ability and there were around plants root. In the [12] also reported that 3 bacterial species in the post-gold mining area of Mandor Regency, West Kalimantan which has resistance to extreeme environment were Bacillus subtilis, Burkholderia cepacia dan Burkholderia cenosepacia.

According to the Figure 2, shows that incubation time has affect the Hg content. The longest periods of incubation time has the biggest decrease of Hg content. This was supported by Petrus et al [20], stated that the longest incubation time could decrease Hg content.

### 3.3. Reduction of Hg in the post-gold mining soil during Incubation Time

During incubation process, reduction Hg content on each treatment has different. The reduction of Hg in the post-gold mining soil on each treatment was shown in Figure 3.

According to the data of percentage reduction of Hg content at Figure 3, showed that differences of reduction of Hg during incubation time. The reduction of Hg in the 2nd weeks of incubation time was 98.8994% on the Pseudomonas sp. (Ps) inoculum. While the reduction of Hg in the same incubation time on Bacillus sp. (Bc) treatment was 3,081232%, while on mixed inoculum treatment (PB) was 45,90959% and control was 2.3644%. In the final incubation (8th weeks), the reduction of Hg on Pseudomonas sp. (Ps) inoculum treatment was 99.9932%, the treatment of Bacillus sp. (Bc) inoculum was 99.988%, the treatment of mixed inoculum between Pseudomonas sp. and Bacillus sp. (PB) was 99.9931%, while the control (C) was 41.8108%.

The results at Figure 3 shows that the addition of bacterial inoculum to the Hg contaminated soil could reduce Hg in the soil. The treatment of Pseudomonas sp. (Ps) as a Gram negative bacteria has a higher ability of Hg reduction than Bacillus sp. (Bc) as a Gram positive bacteria (Figure 3). In the [21], stated that Gram negative bacterial tend to be more tolerant to heavy metals than Gram positive bacterial. This caused by the cells walls of Gram negative bacterial more complex and could bind and
mobilize the metal ions. The cell wall of Gram positive bacterial which could bind the metals ion was carboxyl group on peptidoglycan while Gram negative bacterial was phosphate group [22]. The treatment of Pseudomonas sp. inoculum was the highest Hg reduction ability (Fig. 3), so that probably better for bioremediation process of post-gold mining soil. This was supported by [5], that reported Pseudomonas sp. Bacterial has potency as bacterial which applicable on the bioremediation process of Hg contamination.

![Figure 2](image_url)

**Figure 2.** Reduction of Hg content in the post-gold mining soil by bacterial inoculum during incubation; (a) Bacillus sp. inoculum, (b) Pseudomonas sp. inoculum, (c) the mixed bacterial inoculum, (d) without adding bacterial inoculum (control (C))
4. Conclusion

According to this result, it could been concluded that the local bacterial inoculum has affect to the Hg content in the post-gold mining soil. The *Pseudomonas* sp. bacterial inoculum was the best of bacterial inoculum has a Hg reduction ability in the post-gold mining soil, also allows it to be applied to the bioremediation effort of post-gold mining soil. Beside it, the incubation time also has effect to the decrease of Hg content in the post-gold mining soil.

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