

## ISOLATION AND CHARACTERISTIC OF NITROGEN-FIXING BACTERIA AND PHOSPHATE-SOLUBILIZING BACTERIA FROM SOIL HIGH IN MERCURY IN TAILINGS AND COMPOST AREAS OF ARTISANAL GOLD MINE

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### ABSTRACT

The research was conducted at Brawijaya University and West Nusa Tenggara, from March 2013 to October 2013. The tailings areas of the gold mine contains high mercury (Hg) as much as 1,090 ppm, and living microbes (resistance) exist in a small number in such a condition. Microbial P solvents encountered came from the genus *Bacillus* with a population of  $23 \times 10^3$  cfu g<sup>-1</sup> and N-fixing bacteria encountered are of the genus *Bacillus*, with a population of  $4 \times 10^3$  cfu g<sup>-1</sup>. Identification of species using Becton Dickinson Phoenix test, both species belong to *Bacillus megaterium* and *Bacillus pumilus*. While the waste corn and peanuts that has been composted for 4 weeks acquired P-solubilizing bacteria, such as *Enterobacter*, *Bacillus* and *Pseudomonas*, and N-fixing bacteria found was *Pseudomonas* and *Azotobacter*. While testing the activity and antagonism of N-fixing and P-solubilizing bacteria, the result on agar media did not show antagonism in its growth. *Bacillus pumilus* and *Bacillus megaterium* effective at 5 % molasses medium with the number of  $0.15 \times 10^{12}$  on seven days of incubation.

Keywords: N-fixing bacteria, P-solubilizing bacteria, mercury, phytoremediation

### INTRODUCTION

Mining on various types of minerals in various countries are now common, including in Indonesia. In Indonesia, there are currently five metal mines and four large-scale coal mines. Since the economic crisis, only one new mine is opened, namely Newmont's gold and copper

mine. Freeport gold and copper mine has doubled, and the nickel mine Indo (Sulawesi) has embarked on the expansion of its operations. Although re-vegetation efforts on the tailings area have been done, none of the mines disposed of tailings correctly. So that the potential long-term impact of these activities cannot be certainly known (McMahon *et al.*, 2000).

Mine tailings are usually characterized by a high content of heavy metals due to the use of heavy metals in the process of separating the gold from the rich solution. In addition, it is also characterized by the content of organic matter, nitrogen and phosphorus which are very low, neutral to acidic, and highly potential for generating acid. Tailing heaps generally do not have the hard soil structure and overgrown plants (Krzaklewski and Pietrzykowski, 2002). Tailings cause stress on the microbial community. Ryan *et al.* (2013) implied that the main species found are of the genera *Acidithiobacillus* and *Acidiphilium*, while the previously dominant *Legionella* becomes completely undetectable. The results of the study reported by several investigators show that mine waste containing mercury has negative effect on seedling, growth, biochemical properties and the results of some crops (Munzuroglu and Geekil, 2002; Sekhar *et al.*, 2005).

Permanent vegetation cover is known as effective and environmentally friendly way to turn mine tailings and to trigger the process of soil formation (Moreno *et al.*, 2004).

Especially in terms of action phytoremediation, the plants are established and function primarily to accumulate metals occurring in the root tissue or to precipitate in

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the root zone (Rascio and Navari-Izzo, 2011). The use of native plants (indigenous) is the focus of this phyto-stabilization technology because native plants are generally tolerant to local environmental conditions and provide a foundation for the natural ecological viability.

In addition to re-vegetation technique to support reclamation or mining heap in the gold mine, the role of N-fixing bacteria and P solubilizing bacteria after phytoremediation of mercury in the tailing areas of gold mine was proposed in this study. The use of indigenous species and strains that have been adapted with local soil and climatic conditions is highly recommended. Utilization of local plants and indigenous bacteria is suggested so that the growth of vegetation and microbes can help restore and stabilize the environment. In the long term, it is expected that the microclimate can be improved, the biodiversity can be restored, and improve soil conditions towards more productive. N-non symbiotic fixing bacteria (*Azospirillum sp*, *Azotobacter sp*, *Aeromonas sp* and *Aspergillus sp*) can also function as soil aggregate stability. Strain bacteria isolation and selection on acid soil *Azotobacter* is also able to produce growth hormone regulation besides its N-Fixing potential (Widayati, 1998). P solubilizing bacteria can produce enzyme phosphatase, as well as organic acid which produces citrate, oxalate, and succinate (Paul and Clark, 1989). Species microorganism P solubilizing as like *Aspergillus sp*, *Penicillium*, and *Pseudomonas sp* (Mikanova and Novaka, 2002).

The purpose of this study was to isolate the non-symbiotic N-fixing bacteria and P solubilizing bacteria from local compost and to determine the effect of various propagation culture media of bacteria. Besides, this research also studied the effect of the application of non-symbiotic N-fixing bacteria and P-solubilizing bacteria after phytoremediation mercury in gold mine tailings using indicator plant growth and microbial biodiversity.

## MATERIALS AND METHODS

### Research Sites

This study was conducted from March 2013 to October 2013 in the Laboratory of Soil Biology, Microbiology Laboratory, and Greenhouse of Brawijaya University, Malang, combined with the sampling taken in tailing

areas of Artisanal gold mine, Lombok, West Nusa Tenggara.

### Composting

Compost material used in this research was the rest of the legumes crop, corn and cattle manure (Table 1). Each crop residue was obtained from 500 kg of agricultural land in the vicinity in Senggigi, Lombok, West Nusa Tenggara, Indonesia. The rest of the plants was collected and chopped into the size of  $\pm 2$  cm. Each type of crop residue was then mixed with cow dung with a ratio of 20:1 (5% cow dung). Then 250 kg of each mixture was dissolved in 50 L of water. Once dissolved, the mixture was dumped and covered with thick plastic for 30 days. During the period of composting, the temperature was observed to maintain the temperature below 50°C. If the temperature reached more than 50°C and the mixture was stirred again. Composting process was stopped when the temperature had stabilized at 28°C and the color turned into black.

### Bacterial Isolation, Selection and Identification

Isolation, selection and identification to obtain bacterial isolates of N-fixing and P-solubilizing used legume compost, corn compost and tailings, from which 100 gr was taken and shaken with 90 ml of 0.85% NaCl for 30 minutes. Furthermore, the clear solution was then dissolved into seven concentrations ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ) ml ml<sup>-1</sup> (v/v). Isolation of non-symbiotic N fixing bacteria was performed using N fix semisolid media. Microbial population was determined using Total Plate Count on N fix semisolid medium, followed by further observations on colony morphology. Observation of cell morphology was performed with gram staining. N-fixing was then measured on the N fix semisolid medium.

Bacterial identifications were performed using microbiological tests (morphology and biochemistry). Samples of bacteria were isolated and cultured in a nutrient medium and medium specific order. Identification of biochemical tests was performed with system identification automatically name the Becton Dickinson Phoenix Instrument Test, commonly known as BD Phoenix Test.

### Observation

Observation on the soil tailing included all

parameter soil and bacteria N fixing and P-solubilizing. Observations on the compost included pH, C/N ratio, and total N Fixing bacteria, P-solubilizing bacteria and total microorganisms. Further test activity to find out bacteria that had been selected was done by using Picovskaya (pour plate) and N-semisolid medium (MPN).

## RESULTS AND DISCUSSION

### Analysis of Soil Hg in Senggigi Region

The results of the soil analysis showed that the content of Hg contaminated soil was quite high, 1,090 (ppm) (Table 1(b)). Soil pH was neutral but tended to be alkali (base), C-organic low and low CEC. It is very dangerous for the health of animals or humans when they enter the food chain obtained from water or any food contaminated with the Hg. Thus, the high Hg levels need to be lowered to the normal range (Table 1(a)). This can be done by using a heavy metal accumulator plants (phyto-remediation), which has been proven to reduce or absorb Hg soil. However, it is known there are microorganisms and organic material that can reduce the levels of heavy metals or decompose into harmless compounds. The use of beneficial microorganisms (potential) and compost can degrade heavy metals as well as adding nutrients, especially N and P required.

Table 1. Results of physical and chemical analysis of soil and tailings in people gold mine locations district of Senggigi, West Lombok

| Soil Properties and Unit | Un-contaminated Soil by Tailing (a) | Contaminated Soil by Tailing (b) |
|--------------------------|-------------------------------------|----------------------------------|
| Texture                  | Silty loam                          | Sandy loam                       |
| pH                       | 6.40                                | 7.70                             |
| C organic (%)            | 0.95                                | 1.19                             |
| N total (%)              | 0.10                                | 0.001                            |
| P-available (ppm)        | 0.98                                | 2.89                             |
| S (ppm)                  | 8.92                                | 0                                |
| CEC (cmol/kg)            | 14.25                               | 11.57                            |
| K-dd (cmol/kg)           | 3.25                                | 0.001                            |
| Ca-exchangable (cmol/kg) | 3.04                                | 1.99                             |
| Mg-exchangable (cmol/kg) | 1.26                                | 0.84                             |
| Na-exchangable (cmol/kg) | 0.89                                | 0.64                             |
| Cu (ppm)                 | Td                                  | 792                              |
| Cd (ppm)                 | Td                                  | 4.0                              |
| Hg (ppm)                 | Td                                  | 1,090                            |
| Au (ppm)                 | Td                                  | 11.68                            |
| Pb (ppm)                 | Td                                  | 530                              |
| Fe (ppm)                 | Td                                  | 3,810                            |
| Mn (ppm)                 | Td                                  | 4,840                            |
| Zn (ppm)                 | Td                                  | 3,760                            |

Remarks: td = not detected (0)

Table 2. Chemical characteristic of analyzed corn and legume waste

| Analysis              | Method                             | Corn Waste | Legume Waste |
|-----------------------|------------------------------------|------------|--------------|
| pH (H <sub>2</sub> O) | H <sub>2</sub> O                   | 7.20       | 8.00         |
| pH (KCl)              | KCl                                | 7.00       | 7.90         |
| C-Organik (%)         | Walkey Black                       | 34.28      | 22.83        |
| N-total (%)           | Kjeldahl                           | 1.83       | 2.10         |
| C/N                   | Kjeldahl dan Walkey Black          | 19.00      | 11.00        |
| P-total (%)           | HNO <sub>3</sub> +HCO <sub>4</sub> | 0.21       | 0.81         |
| K-total (%)           | HNO <sub>3</sub> +HCO <sub>4</sub> | 1.06       | 1.73         |

Table 3. Compost characteristic of analyzed corn and legume

| Compost | pH               |      | C/N | N-available (ppm) |                 | P-available (ppm) | Total Microorganism (cfu/ml 10 <sup>7</sup> ) |
|---------|------------------|------|-----|-------------------|-----------------|-------------------|---|
|         | H <sub>2</sub> O | KCl  |     | NH <sub>4</sub>   | NH <sub>3</sub> |                   |   |
| Corn    | 8.20             | 8.10 | 8   | 562               | 176             | 830               | 40  |
| Legume  | 7.80             | 7.80 | 6   | 241               | 143             | 2,938             | 150   |

### Microbial Analysis of Soil Tailing

Analysis of soil taken from a gold mine disposal process that contained mercury (Hg) was found high for its Hg level (1,090 ppm) (Table 1). In such a condition, N-Fixing and P-solubilizing bacteria were found but only in a small number (Table 6). N-fixing bacteria after purification was obtained only 1 species and quantity of  $4 \times 10^3$  cfu/g soil while P-solubilizing bacteria although the number was the same but the total population was larger,  $23 \times 10^3$  cfu/g soil (Table 4). The lack of bacteria because of higher mercury could stress the growth of microbacteria especially the growth of N fixing and P solubilizing (Thangavel and Subhuram, 2004).

Table 4. Bacterial characteristic of Hg contaminated soil (tailing)

| No | Bacterial Type | Microbial Total (cfu g <sup>-1</sup> ) | Cell Form       | Gram Staining |
|----|----------------|--|-----------------|---------------|
| 1  | N-fixing       | $4 \times 10^3$                        | <i>Bacillus</i> | positive      |
| 2  | P-solubilizing | $23 \times 10^3$                       | <i>Bacillus</i> | positive      |

### Bacterial Capability in N Fixation and dissolved P

Bacterial activity of dissolved P and N fixation activity was listed in Table 5. Each bacterium had different activity in the dissolved P and N fixation. Dissolved P were characterized by the formation of a clear zone around the colonies of bacteria and it could quantify the amount of dissolved P and N fixed bacteria.

Clear zone was detected because the solubilizing Ca-P with organic acid was produced and measured by the clear zone index from diameter colony and size of colony. Expression of the dissolution of P could be measured by solubilizing P. Nitrogen fixation expressed amount of N fixing (N<sub>2</sub>), which showed bacteria's ability to fix Nitrogen in the air (Husen *et al.*, 2007).

Table 5. Bacterial capability in fixed N and dissolved P

| No | Isolate | Dissolved P      |                   | Fixed N (ppm) |
|----|---------|------------------|-------------------|---------------|
|    |         | Clear Zone Index | Dissolved P (ppm) |               |
| 1  | L2P1 1  | 1.4              | 3.7               | -             |
| 2  | L2P2 1  | 1.8              | 5.3               | -             |
| 3  | L2P2 2  | 2.8              | 0.4               | -             |
| 4  | M2P1 2  | 1.5              | 5.0               | -             |
| 5  | M2P3 1  | 1.0              | 0.1               | -             |
| 6  | M2P3 2  | 1.3              | 3.5               | -             |
| 7  | L2N1    | -                | -                 | 8             |
| 8  | L2N2    | -                | -                 | 94            |
| 9  | M2N1    | -                | -                 | 90            |
| 10 | M2N2    | -                | -                 | 32            |

Remarks: - = not detected

Table 6. The result of bacterial screening process from Hg-contaminated Soil (Tailing)

| Soil Sample         | Parameter                                   | Unit                 | Microbial Total  | Method                     |
|---------------------|---|----------------------|------------------|----------------------------|
| Tailing from Lombok | Non-symbiotic N Fixing bacteria (N1 and N2) | cfu gr <sup>-1</sup> | $4 \times 10^3$  | MPN (Most Probable Number) |
|                     | P Solubilizing bacteria (P1, P2 and P3)     | cfu gr <sup>-1</sup> | $23 \times 10^3$ | Pour plate                 |



### Bacterial Selection and Identification

Selected and indentified P-solubilizing and N-fixing bacteria were taken from soil contaminated with Hg. Two isolates of N1 and N2 were obtained from Non-symbiotic N fixing on MPN medium (Table 6). Of P-solubilizing bacterial were obtained 3 isolates and named P1, P2 and P3 (Table 6). N1 and P2 were then selected to be used in further study. Bacterial identification using Becton Dickinson Phoenix test showed that N1 belong to *Bacillus pumilus* (Table 7) and P2 belong to *Bacillus megaterium* (Table 8).

### Microbial Activity Test in Phosphate Dissolution and N Fixation

P dissolution activity test of bacteria isolated performed clear zone around bacterial colonies. The wider the clear zone provided the more P ( $H_2PO_4$ ) that can be used by plants. P<sub>2</sub> was the highest in dissolved P and has been identified as *Bacillus megaterium*. N fixing bacteria was marked by a color change from green to blue to N-fix semisolid medium, in which N1 was the highest in N-fixation and had been identified as *Bacillus pumilus*.

### Antagonist Test of Isolated Bacteria

Bacteria isolated from soil contaminated with Hg (Tailling) and have the ability in N fixation was N1 and N2 and the P dissolution. Three isolates namely P1, P2, P3 were taken from P dissolution. They were then put together on Nutrient Agar to determine the interaction between the bacterial antagonisms. No inhibition zone was found among the five bacteria. Instead, the bacteria were proven to grow synergistically. Furthermore, they could be applied simultaneously as mixed culture.

### Effect of Liquid Carrier on The Growth of Bacteria

The application of N fixing bacteria and P solubilizing bacteria selected (N1 and P2) were then tested for their ability to grow in a liquid carrier medium (Nutrient Broth) and molasses in concentration of 5% and 10% as shown in Table 9.

After 7 days of incubation, all liquid carrier media showed good results. Both media could be used as bacterial inoculum propagation material for application of biological fertilizer. However, 5% molasses were econo-

mically more efficient because it produced bacterial population of  $0.15 \times 10^{12}$  cfu/g. This amount was almost equal to the 10% molasses and NB. Molasses was used as a source of energy C for microbes to grow.

Table 9. Bacterial growth in liquid culture media (Molasses and Nutrient Broth)

| No | Incubation (Day) | Total Ammount of Bacteria Colony (cfu/g) | Media      |
|----|------------------|--|------------|
| 1  | 0                | $7 \times 10^5$                          | NB         |
|    | 3                | $11.15 \times 10^9$                      | NB         |
|    | 7                | $2.14 \times 10^{12}$                    | NB         |
| 2  | 0                | $4 \times 10^5$                          | Molase 10% |
|    | 3                | $1.67 \times 10^{10}$                    | Molase 10% |
|    | 7                | $2.88 \times 10^{12}$                    | Molase 10% |
| 3  | 0                | $9 \times 10^5$                          | Molase 5%  |
|    | 3                | $8 \times 10^9$                          | Molase 5%  |
|    | 7                | $0.15 \times 10^{12}$                    | Molase 5%  |

### CONCLUSION

Mercury polluted areas in the gold mine showed fairly high levels (1,090 ppm) and these need to be reduced to a non-dangerous level. Still there were microbes that were tolerant in spite of their small numbers. N fixing bacterium obtained was *Bacillus pumilus* with total amount of  $4 \times 10^3$  cfu g<sup>-1</sup> soil, and P solubilizing bacteria obtained was *Bacillus megaterium* with a population greater than that of N fixing bacteria ( $23 \times 10^3$  cfu g<sup>-1</sup>).

Nitrogen fixing bacteria populations of corn and legume compost have a larger population. It was expected that mixing bacteria and compost reduced Hg concentration bound by carboxyl groups and phenolic of compost. P would be available in the orthophosphate, able to bind Hg and increase P nutrient supply. N fixing bacteria could increase the fertility of soil contaminated with Hg. Antagonist interaction did not occur between the bacteria. The optimum population of mixture of *Bacillus pumilus* and *Bacillus megaterium* was  $0.5 \times 10^{12}$ , found on 5% moles.

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