Characterization Partial and Lead (Pb) Resistant of Diazotrophs Bacteria

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Abstract. Heavy metals have toxic effects on animals, human health, plants, and the environment. Reduction of heavy metal pollution is needed one of which can use diazotrophs bacteria. The purpose of this study was to determine the characterization partial, the ability of diazotrophs bacteria NR 18, and NR 28 to excrete ammonium and their growth response to lead metal (Pb). The results of the study are known to each excretion ammonium for bacterial isolate NR 18 of 2.25 ppm and bacterial isolate NR 28 of 6.06 ppm. Bacteria NR18 and NR 28 were resistant in media supplement with lead (Pb) with a concentration of 300-600 ppm. In general, diazotrophs bacteria could be explored for bioremediation purposes.

1. Introduction
Heavy metals such as Zinc (Zn), Cuprum (Cu), and Lead (Pb) can accumulate to soils and aquatic ecosystems causing harmful impacts on human, and the environment [1] [2]. The other that, lead (Pb) can accumulate mainly in plant organs which can cause toxic to plants, disrupt plant of metabolism, and processes photosynthetic so that it will affect the growth of these plants [3].

Microbes have a role in the transformation, decomposition, and bioaccumulation of heavy metals. Bioaccumulation of heavy metals by bacteria and fungi has been the focus of bioremediation studies. Heavy metal toxicity has been eliminated using blocking functional mechanisms of biomolecules such as proteins and enzymes [4], and some bacteria have resistance to heavy metals through biofilm production or extracellular metabolites [5].

One way to overcome heavy metal toxicity to environmental recovery is by diazotrophs bacteria that have the potential to reduce heavy metal contaminants in the environment. Diazotroph bacterium is one that can convert nitrogen into ammonia without relying on other organisms [6]. This bacterium can fixation nitrogen from the air and can reduce heavy metals, such as Brevibacillus [7] and Azotobacter [8], Enterobacter, Sphingobacter, Pseudomonas, Rhizobium, Citrobacter, Bacillus, Athrobacter, and Fulvimonas [5].

Diazotrophs bacteria have the potential for nitrogen fixation to nitrate ions (NO3⁻) and ammonium (NH4⁺) which can be directly used by organisms [9] and can reduce heavy metals. Free-living bacteria one solutions are to supply nitrogen, increase crop production, and reduce the use of synthetic fertilizers. Also, [10] reported Azotobacter beijerinckii and Azotobacter vinelandii excreted of ammonium 0.192 mM and 0.63 mM, respectively. The test has been carried out on Burk's medium with a sucrose carbon source. Reported [7] that Brevibacillus has been known to produce Indole Acetic Acid (IAA) and to absorb Pb. This study aims to characterize partial, and knowing the Pb resistant of diazotroph bacteria NR 18 and NR 28.
2. Methodology

2.1 Sources and cultivation of bacteria
The bacterial NR 18 and NR 28 used in this study were diazotroph bacteria. The cultivation of single bacterial was carried out by growing on nutrient slant medium and incubated at room temperature for 24 hours. Cultures bacteria were maintained at 4°C until being used for farther analysis.

2.2 Preparation of media
Nitrogen Fixation of Bacteria Broth (NFB) consist of 0.4 gr KH₂PO₄; 0.2 gr MgSO₄·7H₂O; 0.1 gr NaCl; 0.026 gr CaCl₂H₂O; 0.017 gr FeCl₃·6H₂O; 2.0 gr Na₂MoO₄·2H₂O; 3.58 gr DL-malate acid; 18 gr agar, and 1 L H₂O [11].

2.3 Characterization colony and biochemical tests
The colony morphology bacterial NR 18 and NR 28 of on the nutrient agar (NA) medium were studied after 24 hours of incubation, such as their size, elevation, shape, and colour. The biochemical characteristics were observed of Gram staining, oxidase, catalase, and fermentation of carbohydrate.

2.4 Production of ammonium
Each bacteria N18 and NR28 of 1 ml isolate with a population of 10⁸ CFU/ml was inoculated into an Erlenmeyer containing 50 ml of liquid Ashby medium. Erlenmeyer incubated on shaker incubator at 150 rpm, 24 hours at 27 °C. Furthermore, each sample was centrifuged at 4000 rpm for 15 minutes. The supernatant was taken and adjust to pH 11 with the addition of NaOH (1N). The solution was calculated absorbance with a spectrophotometer at λ 435 nm [9].

2.5 Determination of tolerance for various Pb concentrations
Bacteria NR 18 and NR 28 were subject to study the ability to grow at various Pb concentrations. This study was conducted by using NFB media [11]. One Ose culture of each diazotroph bacteria was inoculated with streak quadrant technic on NFB agar containing Pb with concentration 300, 400, 500, and 600 ppm and incubated for five days in room temperature. Experiment for tolerance Pb concentration of the bacteria ended when inhibition of the growth complete. All treatments were performed in triplicate. The studied growth was divided into three levels: rapid growth (++), slow growth (+), and no growth (-).

2.6 Test Pb resistant by bacterial NR 18
100 ml of NBF broth and 600 ppm of Pb were added into Erlenmeyer and sterilized in the autoclave. The Erlenmeyer was inoculated bacteria NR 18 with 1 ml of 10⁸ CFU/ml. Erlenmeyer was incubated at 37 °C on the shaker at 150 rpm for five days. As a control, Erlenmeyer contains NBF medium without the addition of bacteria. The centrifuge at 6000 rpm for 30 minutes to obtain a supernatant. The supernatants are then measure using Atomic Absorption Spectroscopy (AAS) [12] has been modified. During period incubation was also observed growth of bacteria with measuring optical density (OD). All treatments were performed in triplicate.

3. Results and Discussion
Characteristics bacteria NR 18 and NR 28 has a distinct colony and biochemical character. Both bacteria showed Gram-negative, rod shape-cell, oxidase and catalase-negative and also potential nitrogen fixation Table 1. Qualitative tests use NBF agar media and bacteria diazotroph that can change the colour of NBF agar media from green to blue, as shown in Figure 1. The results demonstrated that both isolates of diazotroph.
Table 1. Morphological, microscopy and biochemical characterization of the bacteria

| Characteristic       | Isolate          | NR 18 | NR 28 |
|----------------------|------------------|-------|-------|
| Colony characteristic|                  |       |       |
| form                 | circular         | circular |
| margin               | entire           | entire |
| elevation            | convex           | convex |
| colour               | white            | white |
| size                 | small            | moderate |
| Morphology cell      | rod              | rod |
| Biochemical test     |                  |       |       |
| Gram stain           | negative         | negative |
| fermentative glucose | -                | +     |
| fermentative lactose | -                | -     |
| fermentative sucrose | -                | +     |
| Oxidase              | -                | -     |
| Catalase             | -                | -     |
| Reduction methylene blue | -              | -     |
| Test nitrogen fixation | +               | +     |
| Production ammonium  | 2.25±0.61 ppm    | 6.06±0.48 ppm |

Figure 1. Screening of bacteria diazotrophs on NFB medium (a): without inoculation (control),(b): reaction negative (c): reaction positive (diazotrophs bacteria)

Bacterial colonies isolated and grown in NBF agar with various concentrations of Pb supplement. Bacterial colonies NR 18 resistant until 600 ppm but bacterial NR 28 on 500 ppm. Different bacteria showed varied feedback to various concentration Pb, as observed in the study. This study showed an immense of Pb resistance for the bacterial NR 18 (Table 2 and Figure 2). Reported [13], microorganisms produce a variety of substances that can be inflaming, toxic, and immune sensitive when inhaled. Microorganisms have developed the mechanisms to cut off with a variety of toxic metals for their endurance in the environment enriched with such heavy metals [14]. Bacteria NR 18 has that potential in bioremediation and as a bioindicator of lead pollution.
Table 2. The tolerance of various Pb concentration

| Concentration of Pb (ppm) | Isolate     |
|---------------------------|-------------|
|                           | NR 18       | NR 28       |
| 300                       | ++          | ++          |
| 400                       | ++          | ++          |
| 500                       | ++          | +           |
| 600                       | ++          | -           |

Figure 2. The response of bacteria on NFB media supplement Pb 600 ppm. A. bacteria NR 18 B. bacteria NR 28

This experiment has shown that the bacteria NR 18 were particularly resistant to Pb. Growth of bacteria NR 18 was followed of biomass by aligning absorbance at 600 nm using a spectrophotometer. Figure 3 showed the bacteria NR 18 was biomass increase on NFB broth with supplement Pb on four days incubation but it biomass decrease on five days incubation. A reduction in the amount of biomass by bacteria whilst the toxic particularly is an effort to maintain cellular function. Similar observations with tolerance to Pb by bacteria B11 and B 20 have been reported [12].

Figure 3. The growth of the bacteria NR 18 in NFB medium with Pb supplementations

Figure 4 showed bacteria NR 18 cutback of 39.56% Pb concentration on NFB broth with supplement Pb (600 ppm), and the control was reduced to 8.55%. In [15] reports that bacteria A1 can reduce Pb for 81% and bacteria A2 for 66% using the nutrient broth for incubation of three days. This study was a possibility of tolerances for Pb resistant bacteria varies. The amount of Pb reduction is influenced by the strain bacteria, various concentration Pb, incubation time and type medium used. Research in [16] has been present such mechanisms of resistance based on metal conservation on the area of a cell, on intracellular conversion into less toxic forms, on the release of metal from a cell with
the help of polymers, and degradation the permeability of a cell membrane. Generally, bacterial resistant for toxic metals is encoded by plasmids.

![Figure 4. The reduction of Pb by bacteria NR-18 in NFB broth](image)

4. Conclusions
In this study, strain bacteria NR 18 and NR 28 are potential sources of bioremediation agents, especially NR18 bacteria which can absorb Pb of 38.6%. Bacteria NR 18 resistant to Pb concentrations of 600 ppm.

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