Phosphate solubilizing bacteria isolated from Tuban mangrove soil, Indonesia

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Abstract. This research aims to find Indonesian local isolates of Phosphate Solubilizing Bacteria (PSB) originated from Tuban Mangrove Center. The composted soil samples was taken by purposive sampling method. The isolation technique was done by serial dilution on pikovskaya media (10⁻¹⁻¹⁰⁻⁸ CFU/g soils). Bacteria was show clear zones around colonies indicated to solubilize phosphate. The potential bacteria were identified by morphological, physiological and molecular (16SrRNA gene). A total of 19 isolates were shown phosphate solubilizing. One isolate was namely 4a has highest phosphate solubilization index (2.36 ± 0.07mm) with similarity 97.28% belong to Klebsiella variicola based on 16S rRNA gene analysis.

1. Introduction

Phosphorus (P) is one of the macronutrients for the metabolism of plants to form adenosine triphosphate (ATP) [1]. Phosphorus content in the soil is relatively low, ranging from 500 to 10,000 kg at a depth of 50 cm from 1 ha soil [2]. The lack of phosphorus availability in the soil with approximately 95–99% is present in the form of insoluble phosphates caused the plants are unable to utilize it properly [3]. Plants difficult to absorb bounded phosphorus, therefore it must be turned into a soluble form [4]. To increase the availability of phosphorus for plants, large amounts of fertilizer are used on a regular basis. But after application, a large proportion of fertilizer phosphorus is quickly transformed to the insoluble form [5]. However, chemical fertilizers might have negative effects such as include water pollution, soil acidification, and mineral depletion. To reduce the harmful effects of chemical fertilizers on human health and environment, nowadays there is increasing trend on the utilization of biofertilizer.

Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms [6]. One of the roles of microorganism in biofertilizer is phosphate solubilizer. Phosphate Solubilizer are a group of microorganisms capable of turning phosphate from an insoluble form into a soluble form for plants to utilize by releasing a phosphorus element through the organic acids secretion process [7]. Inorganic phosphate is hydrolyzed enzymatically by using a phosphatase enzyme into
organic phosphate available for plants [8]. There are many microorganisms in soil to solubilize phosphorus include yeasts [9], mold [10], and bacteria [11-13]. Therefore, this research aims to find Indonesian local isolates of phosphate solubilizing bacteria (PSB) as biofertilizer candidate.

2. Materials and Methods

2.1 The Phosphate Solubilizing Bacteria isolation

The soil samples were taken by using purposive sampling around the mangrove rhizosphere. Sampling locations are divided in two places (Figure 1) where Location 1 is former fishpond and Location 2 is near the sea. In Location 1, the soil samples was taken in 10 points, while in Location 2, the soil samples was taken in 8 points. Approximately 250 g soil samples was collected using cylindrical crop at a depth of 20cm from the soil surface. The collected sampled was analyzed for physicochemical parameters including humidity (70-82%), temperature (27-28°C), salinity (0-15%), and soil pH (4-6.4).

![Figure 1. Sampling location, Red flag is location 1, yellow flag is location 2](image)

The soil samples were composited based on the pH similarity. Then, 25 g of the samples put into a 500 ml culture bottle which had been filled with 225 ml of sterile physiological saline solution. The soil suspension was homogenized by using a shaker for ± 30 minutes, then diluted to $10^{-8}$. Approximately 1 ml of aliquote of each serial dilution ($10^{-1}$, $10^{-5}$, and $10^{-8}$) CFU/g soils were poured onto Pikovskaya media, respectively. After that, the plates were incubated at room temperature, for 3-5 days. Phosphate Solubilizing Bacteria were exhibited the ability to solubilize phosphorus on the Pikovskaya medium by showing the clear zone formation around colonies. The colonies were purified on nutrient agar medium. Subsequently, all colonies stored at 4°C for further study.

2.2 The Phosphate Solubilizing Bacteria potential test

The ability of isolates to solubilize phosphate was evaluated on Pikovskaya agar medium by using the spot inoculation method. The clear zone around the isolates were measured. Subsequently, the data were analyzed and expressed as Phosphate Solubilization Index (PSI) according to formula:

$$\text{PSI} = \frac{\text{Clear zone diameter (mm)}}{\text{Colony diameter (mm)}}$$  \hfill (1)
The isolate with the highest PSI value were characterized morphologically (microscopic and macroscopic), physiological and molecular by using 16SrRNA gene.

3. Result and Discussion

Table 1 shows the data of 19 isolates of PSB that were successfully recovered on a selective Pikovskaya agar medium.

Table 1. Phosphate Solubilizing Bacteria isolates from the soil samples of Tuban Mangrove Center

| No. | Location | Isolate Code | Total Isolate |
|-----|----------|--------------|---------------|
| 1   | L1\(^a\) (pH 6) | 1             |               |
|     |          | 2             |               |
|     |          | 3             |               |
|     |          | 4a            |               |
|     |          | 4b            |               |
|     |          | 5             | 11            |
|     |          | 7             |               |
|     |          | 9             |               |
|     |          | 12            |               |
|     |          | 17            |               |
|     |          | 20            |               |
| 2   | L2\(^b\) (pH 6) | 6             |               |
|     |          | 10            |               |
|     |          | 11            |               |
|     |          | 14            | 7             |
|     |          | 15            |               |
|     |          | 18            |               |
|     |          | 19            |               |
| 3   | L1\(^a\) (pH 4) | 16            | 1             |
| 4   | L2\(^b\) (pH 4) | -             | -             |

\(^a\) L1 : Location 1  
\(^b\) L2 : Location 2

The sample location is divided into two groups, which are Location 1 with the pH level of 6 (nearly neutral) and the pH level of 4 (acid) – similar to Location 2. There are 11 isolates were found in Location 1 (pH 6), 1 isolate in Location 1 (pH 4), and 7 isolates in Location 2 (pH 6) that have potency to dissolve phosphate. The test result of the 19 isolates on the Pikovskaya medium is presented in Figure 2.
Figure 2. The results for Phosphate Solubilizing Bacteria assay: number 1–20 shows the isolate codes; yellow arrow indicates bacterial colonies; red arrow exhibiting the clear zone formed.
The bacterial isolates that capable to solubilize phosphate showed clear zone around colonies. Diameter of clear zone formed showed the isolate potency to dissolve Ca$_3$(PO$_4$)$_2$ in medium. Table 2 presents Phosphate Solubilization Index (PSI) value from each isolate bacteria.

**Table 2.** The Phosphate Solubilization Index (PSI) of Bacterial isolates from Tuban Mangrove Center

| Isolate Code | Phosphate Solubilization Index (PSI) |
|--------------|-------------------------------------|
| 1            | 1.03 ± 0.33                         |
| 2            | 1.46 ± 0.75                         |
| 3            | 1.05 ± 0.36                         |
| 4a           | 2.36 ± 0.07$^a$                     |
| 4b           | 1.55 ± 0.06                         |
| 5            | 1.09 ± 0.05                         |
| 6            | 1.26 ± 0.12                         |
| 7            | 1.39 ± 0.09                         |
| 9            | 1.22 ± 0.11                         |
| 10           | 1.29 ± 0.11                         |
| 11           | 1.02 ± 0.21                         |
| 12           | 2.03 ± 0.43                         |
| 14           | 1.38 ± 0.15                         |
| 15           | 1.46 ± 0.33                         |
| 16           | 1.99 ± 0.29                         |
| 17           | 1.03 ± 0.73                         |
| 18           | 1.23 ± 0.10                         |
| 19           | 1.79 ± 0.24                         |
| 20           | 2.11 ± 0.06                         |

$^a$highest PSI

These bacteria have potency to dissolve phosphate because they form clear zone around it’s colony when grown on Pikovskaya agar medium. Pikovskaya agar medium contains insoluble phosphate, Ca$_3$(PO$_4$)$_2$. The compound is bound by bacteria and released H$_3$PO$_4$ ion. The releasing ions causes clearance zone formed around colony [14]. The clear zone formed because of the phosphatase enzyme activity. Besides, [15] also revealed that the clear zone occurs because of pH decreasing in a medium as the result of the phosphatase enzyme activity. These microbes also produce organic acids which can decrease pH and react with P-binding agents such as Al$^{3+}$, Fe$^{3+}$, Ca$^{2+}$, and Mg$^{2+}$ to form organic chelates so it can release free phosphate ions (PO$_4^{3-}$) [16]. The 4a isolate has the highest potency compared to the other isolates in terms of solubilizing Ca$_3$(PO$_4$)$_2$ phosphate on Pikovskaya agar medium by solubilization index 2.36 ± 0.07mm.
The result of phosphate-solubilizing bacteria identification

Characteristic of 19 PSB isolates based on macroscopic colony and microscopic cells are showed in Table 3.

**Table 3.** Macroscopic and microscopic characteristics of the Phosphate Solubilizing Bacteria isolates from Tuban Mangrove Center

| Isolate code | Colony morphology (Macroscopic) | Cell characteristic |
|--------------|---------------------------------|---------------------|
|              | Size | Color | Shape | Edge | Elevation | Shape | Gram* |
| 1             | Large | White | Circular | Entire | Convex | Coccus | +     |
| 2             | Small | White | Circular | Entire | Umbonate | Rod | +     |
| 3             | Small | Cream | Irregular | Irregular | Flat | Coccus | +     |
| 4a            | Large | Cream | Circular | Undulate | Convex | Rod | -     |
| 4b            | Small | Yellow | Circular | Undulate | Umbonate | Coccus | +     |
| 5             | Large | White | Irregular | Serrate | Raised | Coccus | +     |
| 6             | Large | Cream | Irregular | Irregular | Raised | Coccus | +     |
| 7             | Small | White | Circular | Entire | Raised | Coccus | +     |
| 9             | Moderate | White | Irregular | Undulate | Umbonate | Rod | +     |
| 10            | Moderate | White | Irregular | Undulate | Umbonate | Coccus | -     |
| 11            | Moderate | Yellow | Circular | Entire | Raised | Coccus | +     |
| 12            | Large | White | Circular | Entire | Convex | Rod | -     |
| 14            | Small | Yellow | Circular | Entire | Raised | Coccus | -     |
| 15            | Small | Yellow | Irregular | Undulate | Flat | Rod | +     |
| 16            | Large | Cream | Circular | Entire | Raised | Coccus | +     |
| 17            | Moderate | Cream | Irregular | Serrate | Flat | Rod | +     |
| 18            | Large | Cream | Circular | Entire | Convex | Strepto-Rod | -     |
| 19            | Moderate | Yellow | Circular | Serrate | Flat | Coccus | +     |
| 20            | Moderate | Yellow | Circular | Entire | Convex | Coccus | +     |

*+ : Gram positive bacteria (violet), - : Gram negative bacteria (red)

**Biochemical test**

Based on Table 4, 4a isolate is non-motile, positive oxidase and catalase, and it has an ability to reduce nitrate. 4a isolate and *Klebsiella variicola* have similarity as much as 93% in morphological and physiological characteristics (based on Jaccard Index calculation) [17] and has 97.28% similarity with *Klebsiella variicola* DBBP1 (based on 16S rRNA gene analysis). The 4a isolate bacteria can be prospectively developed in agriculture to increase the availability of nutrients, especially soluble phosphate for plants. Organic P in soil is hydrolyzed by PSB enzymatically by using phosphatase enzyme to form organic P which then can be utilized by plants.
Table 4. Biochemical test result of 4a isolate

| Test                      | 4a isolate | Comparative isolate (Klebsiella variicola) |
|---------------------------|------------|------------------------------------------|
| Gram                      | -          | -                                        |
| Catalase                  | +          | +                                        |
| Lysine                    | +          | +                                        |
| Ornithine                 | -          | -                                        |
| H₂S                       | -          | -                                        |
| Glucosa                   | +          | +                                        |
| Mannitol                  | +          | +                                        |
| Xylose                    | +          | +                                        |
| ONPG                      | +          | +                                        |
| Indole                    | -          | -                                        |
| Urease                    | +          | +                                        |
| VP                        | +          | +                                        |
| Citrate                   | +          | +                                        |
| TDA                       | -          | -                                        |
| Gelatin                   | -          | -                                        |
| Malonate                  | +          | +                                        |
| Inositol                  | +          | +                                        |
| Sorbitol                  | +          | +                                        |
| Rhamnose                  | +          | +                                        |
| Sucrose                   | +          | -                                        |
| Lactose                   | +          | +                                        |
| Arabinose                 | +          | +                                        |
| Adonitol                  | -          | +                                        |
| Raffinose                 | +          | +                                        |
| Salicine                  | +          | +                                        |

4. Conclusion
Based on the results, it can be concluded that the Phosphate-Solubilizing Bacteria isolated from Mangrove Center Tuban Rhizosphere soil were 19 isolates. The 4a isolate has the highest potential among the others. This isolate was identified as Klebsiella variicola 4a with 97.28% similarity to Klebsiella variicola DBBP1.

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