Isolation of phosphate solubilizing bacteria from the rhizosphere of local aromatic rice in Bada Valley Central Sulawesi, Indonesia

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Abstract. Phosphate Solubilizing Bacteria (PSB) can be used to increase the efficiency and availability of P in the soil as it can help the process of releasing the bound of P elements so that they become available to the plants. This study aims to isolate and determine the morphological characteristics of the PSB colonies in the rhizosphere of the local aromatic rice plant, analyze the phosphate solubility of each isolate. A total of eleven rhizospheres bacterial isolates were successfully isolated from the local aromatic rice rhizosphere. The morphological characteristics, including the size, edge, shape, elevation, and color of the colony, were obtained vary. The bacteria found were gram-positive and gram-negative, which are 81.81% and 18.18%, respectively. The result of the catalase reaction test showed 72.72% positive and 27.27% negative catalase. Furthermore, eight bacterial isolates formed a clear zone with an area of 0.84–2.66 cm. The Phosphate solubility was 116.67–133.00, and the Phosphate dissolving index was 2.17–2.33 at acidic pH between 4.27–5.67. The concentration of dissolved phosphate was 5.152 mg L\(^{-1}\)–9.382 mg L\(^{-1}\). The results showed that the PSB has a potential being an alternative way to be developed as a biological fertilizer agent in supporting sustainable agriculture.

1. Introduction
Phosphorus is an important nutrient after large amounts of nitrogen needed by plants as it plays a role in all processes of plant metabolism, cell division, activation/inactivation of enzymes and carbohydrate metabolism [1]. Its presence in abundant soil in the form of both P-organic and P-inorganic [2] is mostly in an insoluble form (around 95-99%) so that it becomes unavailable for plants to be absorbed [3].

Plants can absorb phosphate in the form of H\(_2\)PO\(_4^-\) and HPO\(_4^{2-}\) ions. To dissolve phosphate into an available form, it needs assistance from soil microorganisms, one of them is PSB. The mechanism between PSB and plant roots results in phosphatase enzymes (in case if the availability of phosphate in the soil is low), and the more dominant is the phosphatase produced by microorganisms. The
phosphatase enzyme plays a role in releasing phosphates bound by organic compounds (Citric Acid, Glutamate, Succinate, Lactate, Oxalate, Glycooolsalat, Fumarate, Tartaric and Alpha-Ketobutiric Acid) into forms that are available and can be absorbed by plant roots. PSB not only dissolves and releases P from insoluble compounds but also releases other nutrients [3].

Sources of the food supply in the form of rice, especially upland rice (> 700 m above sea level), have an important role for people who live in unspoiled mountain areas such as the people in Bada Valley Poso Regency, Central Sulawesi. This region’s rice is known to have a distinctive aroma (fragrant) that has a fluffier taste, clean white color, and good storability after being cooked into the rice so that it remains cultivated by the local community. Both fluffier taste and its fragrant aroma are the advantages of these aromatic rice types. The local aromatic rice "Kamba" especially in Bada Valley, is generally cultivated with the traditional cultivation system following the customs of the local community based on local wisdom without the use of fertilizers, pesticides, and other chemicals. This rice plant can survive even though it is cultivated by a traditional cultivation system. With environmental conditions that are still awake and natural, it will certainly create an environment of growth and biodiversity of abundant microorganisms in the soil.

The PSB is one of the microorganisms that can be used as an alternative of P-availability in the soil so that it is very potential to be developed as biological fertilizer by inoculating with the soil directly [4] given directly to the seeds seed coating [5] or by adding PSB isolates to carrier media such as composted organic waste, for example, worm compost [6] cow dung, phosphate rock, and biogas mud [7]. Some studies showed the PSB utilization in various cases such as PSB isolated from heavy metal contaminated soil and the combination of PSB with biochar as heavy metal remediation [8]. PSB also can be inoculated with rocks phosphate, animal bone waste, eggshells, tea pulp, and pig bones as fertilizer [9]. PSB has the potential as a biopesticide, bioinoculant, and biosurfactant [10,11], in which a combination of PSB with silicon [12] can be mixed with nitrogen fixation bacteria [13], hydroxylapatite clay mineral (HAp), and montmorillonite (Mt.) [14]. Besides, the use of PSB (both fungi and bacteria) combined with several other fertilizers can substitute the use of chemical fertilizers [15]. Types of PSB have been identified, including Enterobacter sp [16,17] Bacillus sp, Enterococcus sp, and Serratia sp, Staphylococcus haemolyticus [18], Aneurinibacillus aneurinilyticus [19] Paenibacillus sp, Paenibacillus polymyxa [20] Pseudomonas spp, Pseudomonas aeruginosa [10], Alcaligenes aquatilis, Burkholderia cep. Sp [21], Pantoea cyripedi [22], Virgibacillus sp., Leclercia adecarboxylata [8], Gluconacetobacter sp [23], Acinetobacter sp, Sinorhizobium sp, Staphylococcus sp [24] and others. Thus, the potential of PSB (both fungi or bacteria) as biological fertilizers or biological agents is the most effective and efficient to increase crop production, support the sustainable management of biological resources and agriculture as it is able to substitute the use of chemical fertilizers and is environmentally friendly [14].

2. Materials and Methods

2.1. Sampling
The composite sampling method was adopted to get a soil sample [25]. These samples were taken around the rooting area (rhizosphere) of healthy local aromatic Kamba rice plants from five random sampling points. Sampling locations in Bakekau and Lelio Village in Bada Valley Poso Regency, Central Sulawesi. The plant was pulled out slowly, then the soil attached to the plant roots was taken in a composite up to 300 g, then was put in a sterile envelope, stored in a cooler box a while until it reached to the Laboratory.

2.2. The isolation and morphological characterization of the PSB
The isolates were carried out using a dilution method, 1 gram of soil sample was put into a fresh tube and then dissolved with 1 ml of sterile water then homogenized using a vortex. 1 mL of the solution was put in 9 mL of sterile water in the fresh tube so that a dilution rate obtained was $10^3$. The same procedures were performed until dilution $10^{-5}$. 0.1 mL resulting from the dilution of $10^{-5}$ to $10^{-6}$ was
spread on the Pikovskaya media and incubated at 28°C for 24 hours. Growing bacteria were selected and purified using the zig-zag scratch method and then were morphologically characterized by observing the size, shape, edges, elevation, and color of the colony.

The gram reaction analysis was adopted using a method by [26]. One loop of the pure culture of a single colony that has been cultivated was taken using an ose needle and rubbed to the glass object that has been added with two drops of 3% Potassium Hydroxide solution then was stirred clockwise repeatedly and removed gently. The Gram-negative (-) was detected with the appearance of the slimy colony, while the Gram-positive (+) was not.

The catalase reaction test was done by adding one loop (full loop) of pure single colony culture and then rubbed on the glass object that has been given two drops of 3% Hydrogen Peroxide solution. The appearance of gas bubbles showed that the reaction was positive, while the negative reaction was not.

2.3. The Ability of Phosphate Solubilizing Bacteria

The ability of bacteria to dissolve phosphate was determined by the formation of clear zones around the colony which can be analyzed using Pikovskaya media referring to [27] modified with the addition of 0.01 g L\(^{-1}\) bromophenol blue into Pikovskaya media then bacterial isolates were grown with the spot inoculation method and incubated for three days at 28°C. The qualitative testing was done by observing the growing colonies that are able to form a clear zone around the colony. This showed that the isolates are able to dissolve the phosphate. The measurement of the phosphate solubilization efficiency (PSE) and phosphate solubilization index (PSI) used these following formula:

\[
PSE = \frac{\text{The diameter of clear zone}}{\text{The colony diameter}} \times 100
\]

\[
PSI = \frac{\text{The colony diameter} + \text{The diameter of clear zone}}{\text{The colony diameter}}
\]

The other analysis to determine the ability of the isolates to dissolve phosphate was using liquid Pikovskaya media by adding Tricalcium Phosphate Ca\(_3\)(PO\(_4\))\(_2\), then was incubated for seven days at 28°C. After seven days, the bacterial suspension 1.5 mL was centrifuged for 15 minutes at 10,000 pm. 5 mL of supernatant was taken and added with 0.5 mL of P reagent concentrated, then shook gently for a few minutes and let stand for 30 minutes. The quantitative analysis was done by measuring the absorbance level of phosphate dissolution concentration with a UV-VIS spectrophotometer at 693 nm. The acidity (pH) was measured before and after cultivation. The phosphate concentration was measured using (standard Titrisol curve (PO\(_4\)) made from the dilution with Titrisol concentration ranging from 0 to 2.5 mg L\(^{-1}\) with a regression equation \(Y = 0.1911x + 0.0265\) where \(R2 = 0.9659\) (See Figure 1).
3. Results and Discussion

3.1. Isolation and Morphological Characterization of PSB Isolates

The isolation and selection of bacterial colonies from local aromatic rice at Poso, Central Sulawesi produce eleven pure isolates with varying characteristics (See Table 1). These isolates have a circular shape, including nine with the flat edge, one serrate, and one undulate. The size of the colonies varies from small, moderate, and large, which are dominated by seven small colonies. While the elevation resulted are six flat, four raised, and one umbonate. The color of colonies observed in five cream, four yellow, and two white isolates (See Table 1).

| Isolate Code | Size      | Colony Form | Colony Edge | Elevation | Colony Colour | Gram (+/-) | Catalase (+/-) |
|--------------|-----------|-------------|-------------|-----------|---------------|------------|--------------|
| KBA3         | Moderate  | Circular    | Entire      | Flat      | Cream         | (+)        | (-)          |
| KBA6         | Small     | Circular    | Entire      | Umbonate  | Yellow        | (+)        | (+)          |
| KBA11        | Small     | Circular    | Entire      | Raised    | White         | (+)        | (-)          |
| KBA12        | Large     | Circular    | Serrate     | Flat      | Cream         | (+)        | (+)          |
| KBA13        | Large     | Circular    | Undulate    | Flat      | White         | (+)        | (+)          |
| KBA15        | Small     | Circular    | Entire      | Raised    | Yellow        | (+)        | (-)          |
| KBA16        | Small     | Circular    | Entire      | Flat      | Cream         | (+)        | (+)          |
| KBA17        | Small     | Circular    | Entire      | Flat      | Cream         | (+)        | (+)          |
| KBA19        | Moderate  | Circular    | Entire      | Flat      | Cream         | (+)        | (+)          |
| KLE2         | Small     | Circular    | Entire      | Raised    | Yellow        | (-)        | (+)          |
| KLE4         | Small     | Circular    | Entire      | Raised    | Yellow        | (-)        | (+)          |

The characteristics differences among isolates colony are due to the expression of genes derived from different types of bacteria. Furthermore, the colony color differences that appear in (Table 1) show that bacterial isolates have different pigments to produce different colors. Carotenoid pigments in bacteria will result in red and yellow color; Melanin gives brown, black, and orange color, and Tripirilmethenes pigment results from orange, yellow, dark orange, and orange-red color.
The result of the Gram reaction test indicates it varies the amount of gram-positive and gram-negative, which are 81.81% and 18.18%, respectively. The catalase reaction test is dominated by positive catalase reactions of 72.72% and a negative catalase of 27.27%. Almost all living things that are exposed by oxygen will produce a catalase enzyme that can break down hydrogen peroxide into water and oxygen [28].

3.2. The phosphate dissolution ability in bacterial isolates
3.2.1. The result of the qualitative analysis of PSB isolates. The result of the qualitative analysis of PSB isolate is presented in Figure 3. The bacterial isolates that are grown on the Tricalcium Phosphate media then added with Bromophenol blue show different phosphate solubility ability. The clear zone formed around the colony is an indicator of the presence or absence of phosphate dissolution by bacterial isolates on the media. The best phosphate solvent bacteria are able to produce the largest area of the halo zone diameter compared to other colonies. In contrast, isolates that do not form clear zones are unable to dissolve phosphate.
Figure 3. (a) Control (Tricalcium Phosphate agar media without bacteria), (b,c,d) Bacterial isolates which do not form clear zones, (e,f,g,h,i,j,k,l) Bacterial isolates which form clear zones.

Results show that only eight bacterial isolates which are able to form the halo zone with different areas. KBA12 forms the largest area of the halo zone and can be indicated as a superior phosphate solvent isolate, while the smallest is KLE4. Whilst KBA6, KBA17, and KLE2 isolate do not form the halo zone. Thus, these isolates do not result in an index or efficiency of phosphate dissolution (See Figure 3). The variation in the width of the halo zone produced by bacterial isolates is due to the differences ability of each isolate to secrete organic acid extracellular. Whereas the changes in the medium around the colony from turbid to clear are due to the decreasing of pH on the medium used [29].

3.2.2. The result of the quantitative analysis of phosphate dissolution ability in bacterial isolates. The ability of PSB quantitatively is presented in Table 2 and Figure 4. The result of PSI measurement shows eight from eleven bacterial isolates are able to dissolve phosphate, which varies from 2.17 to 2.33. The highest PSI is resulted by KBA12, which is 2.33 with PSE 133.00 at pH 5.19. At the same time, the lowest PSI resulted in KLE4, which is 2.17, with PSE of 116.67 at pH 5.50. The pH value used in this study varies between (4.27–5.83) on Pikovskaya media, which is categorized as acidic pH (See Table 2).

Table 2. The quantitative analysis of phosphate dissolution ability in bacterial isolates.

| Isolate code | pH   | The diameter of the clear zone (cm) | Colony diameter (cm) | Phosphate Solubilization Efficiency (PSE) | Phosphate Solubilization Index (PSI) |
|--------------|------|-------------------------------------|----------------------|------------------------------------------|-------------------------------------|
| KBA3         | 5.02 | 1.15                                | 0.95                 | 121.05                                   | 2.21                                |
| KBA11        | 5.09 | 0.98                                | 0.76                 | 128.95                                   | 2.29                                |
| KBA12        | 5.19 | 2.66                                | 2.00                 | 133.00                                   | 2.33                                |
| KBA13        | 4.27 | 1.05                                | 0.85                 | 123.53                                   | 2.24                                |
| KBA15        | 5.35 | 1.00                                | 0.83                 | 120.48                                   | 2.20                                |
| KBA16        | 4.88 | 0.91                                | 0.76                 | 128.95                                   | 2.29                                |
| KBA19        | 5.67 | 0.91                                | 0.76                 | 119.74                                   | 2.20                                |
| KLE4         | 5.50 | 0.84                                | 0.72                 | 116.67                                   | 2.17                                |

The PSI of each bacterial isolate shows its ability to dissolve phosphate. The higher the value of PSI showed, the stronger the activity of the enzyme phosphatase in releasing P from organic compounds, and the wider clear zone will be obtained as the insoluble phosphate was processed into a soluble form by PSB.
The result shows that the clear zone diameter, PSE, and PSI from bacterial isolates cannot be used as indicators for the level of phosphate solubility concentration as there are isolates with large clear zone diameters and a high value of both PSE and PSI but results in a low dissolved phosphate concentration (See Figure 4).

The quantitative analysis of phosphate dissolution ability on PSB isolates is determined by growing a pure culture of bacterial isolates on the liquid Pikovskaya media. The dissolved P-content contained in the liquid media is measured using a UV-VIS 693 nm spectrophotometer (See Figure 4). The result shows that the PSB result varies the amount of soluble phosphate concentration with the highest value obtained by KBA15 then followed by KLE4, KBA19, and KBA3, which are 9.382 mg L$^{-1}$, 9.099 mg L$^{-1}$, 9.010 mg L$^{-1}$ dan 9.000 mg L$^{-1}$ respectively. The lowest phosphate solubility concentration is resulted by KBA13 bacterial isolate with a value of 5.152 mg L$^{-1}$.

![Figure 4. The quantitative analysis of phosphate dissolution ability on PSB isolates using Spectrophotometer UV-VIS 693 nm.](image)

The different concentrations of PSB are influenced by the type of bacterial strain and environmental condition. The study done by [30] stated that rhizobacterial isolates from chili [31], sugar cane and rice plant had a concentration of inorganic phosphate dissolution ranging from 50.07 - 717.99 ppm while the PSI of the rhizosphere soil of *Cicer arietinum*, *Vigna radiata*, *Zea mays*, *Oryza sativa*, *Colocasia esculenta*, *Allium cepa* ranges from 5.3 to 7.43 [32].

4. **Conclusion**
The research we have done by isolating phosphate solubilizing bacteria from the rhizosphere of local aromatic rice at Central Sulawesi can produce various phosphate solubility activities among isolate tested. KBA12 is superior bacterial isolates through the parameter of both clear zone diameter and PSI as it forms the largest clear zone area of 2.66 cm and the highest PSI value of 2.33. However, KBA15 also shows the highest concentration value of phosphate dissolution, which is 9.382 mg L$^{-1}$. It can be concluded that these PSB isolates have the potential to be developed and utilized thus further research is still needed to explore the potential of these PSB isolates as biological agents of biological fertilizers on increasing the plant growth and production to support sustainable agriculture and genetic stability of local aromatic rice at Central Sulawesi.

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