



Characterization of Phosphate Solubilizing Bacteria from Three Types of Rhizosphere and Their Potency to Increase Growth of Corn (*Zea mays*)

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Abstract

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The application of high doses of phosphate fertilizer is not in line with the availability of P in the soil because most of the P is bound by Al, Fe, and Ca, so it isn't obtainable to plants. Utilization of microbes as an effort to increase the availability of P that can be absorbed by plants. The purpose of this study was to isolate, characterize, and test the potency of P-solubilizing microbes isolated from three types of the rhizosphere. The study used a Completely Randomized Design (CRD) with one factorial. A series of characterizations through the hypersensitivity test, gram staining test, catalase test, oxidase test, oxygen demand test, motility test, ability to use different carbon sources (glucose, lactose, and sucrose), methyl red test, and growth test at various pH. Results showed that as many as 5 selected P solubilizing bacteria (Ca-Al-7, Ca-Al-8, Ca-Al-4, Ca-NF-1, and Ca-NF-3) weren't plant pathogens, with phosphate solubilization values sequentially 1.7; 2.9; 2.5; 3.1; 3.2 having a significant effect on the parameters of plant height, the number of leaves, and the fresh and dry biomass of *Zea mays* plants after 4 weeks of inoculation ($p < 0.05$).

Keywords: Acid soil, P availability, P-fertilizer, P-fixation, productivity

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INTRODUCTION

Indonesia has ± 107 ha of dry, acid land, which is generally spread outside Java Island, such as in Borneo ± 39 ha (36.42%), Sumatera ± 31 ha (28.81%), Papua ± 19 ha (18.03%), and Sulawesi ± 7 ha (6.95%) [1]. Acidic soil causes low land productivity, low pH, cation exchange capacity,

base saturation, low C-Organic, and poor biotic elements. However, this situation will trigger an increase in high Al, Fe, and Mn metal concentrations in the soil, resulting in high phosphate (PO_4) fixation. The P element (phosphorus) is one of the most important elements for plant growth, second only to nitrogen [2].



Phosphates play an important role in the physiological and biochemical activities of plants. Phosphate in the soil naturally occurs in organic and inorganic forms. Both of these forms are insoluble or slightly soluble forms of P, so their availability for soil biota is very limited. Inorganic phosphate minerals are generally bound as $\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ in acid soils and as $\text{Ca}_3(\text{PO}_4)_2$ (Calcium phosphate) in alkaline soils.

Phosphate fertilizer has an important role in increasing crop production. The application of high doses of P fertilizer is not in line with the availability of P in the soil because most of the P is bound by Al, Fe, and Ca, so it is not available to plants. This causes the use of P fertilizer to be inefficient. The utilization of P solubilizing microbes is one of the efforts to increase the availability of P, which can be absorbed by plants, to reduce the use of inorganic P fertilizers.

Phosphate Solubilizing Microorganisms (PSM) are microbes that can convert insoluble phosphate forms into soluble ones, consisting of phosphate-solubilizing bacteria (PSB) and phosphate-solubilizing fungi (PSF). These microorganisms have the potential to increase the availability of P and increase the absorption efficiency of inorganic P fertilizers by plants. Phosphate dissolving activity by soil microorganisms is a complex phenomenon that depends on many factors such as nutrition, physiology, and growing media conditions. The population of microorganisms in the soil is very complex, as are the phosphate-solubilizing microorganisms. Phosphate-solubilizing bacteria have a proportion of 1-50% while phosphate-solubilizing fungi only have a proportion of 0.1-0.5% [2].

The largest population of phosphate-solubilizing bacteria was found in conservative agricultural and soil environments [2]. The abundance of phosphate-solubilizing bacteria in the field is also greatly influenced by salinity, pH, humidity, temperature, and soil microclimatic conditions [3]. Phosphate-solubilizing bacteria can increase plant productivity, one of which is rice. [4] reported that treatment with inorganic phosphate fertilizer doses of 75% + PSM (bacteria and fungi) in the System of Rice Intensification (SRI) cultivation system resulted in higher P uptake of rice grains, a higher number of productive plants, and a higher yield than other treatments. A study [5] reported that the application of PSM isolates

increased the growth and productivity of soybean plants compared to controls, both on fertile and infertile soils.

Phosphate-solubilizing bacteria are often found in their natural habitats, such as in soil. Bacteria such as *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa*, *B. sircalmous*, *Pseudomonas striata*, and *Enterobacter* sp. can be named as the most important phosphate solubilizing strains. Among the soil bacterial community, ectorrhizospheric strains of *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers. Besides bacteria, molds such as *Penicillium* and *Aspergillus* are the strongest P solvents. These fungi convert unavailable phosphates into available forms, producing substances that promote growth and also protect against soil pathogens. Although fungi are known to be able to dissolve phosphate, bacteria are reported to be more effective in dissolving phosphate than fungi [6].

This research was conducted to isolate, characterize, and test the potency of microbial P solubilizers isolated from three types of rhizosphere, namely natural forest, oil palm agroforestry, and *Imperata*. A natural forest is a forest that grows naturally without human intervention. This forest contains various types, ages, and sizes of trees. Agroforestry is a land use system that combines woody plants (trees, shrubs, bamboo, rattan, and others) with non-woody plants or grasses to form ecological and economic interactions between woody plants and other components. In contrast to natural forests, during oil palm agroforestry harvesting, plant maintenance and fertilization are carried out periodically. *Imperata* land is land that is included in the category of degraded land, where this land is generally formed after land clearing that is not followed by plant cultivation activities. Reeds that grow suppress the growth of other plants by producing allelopathic substances.

METHODS

Soil sampling

Composite sampling is the technique used in sampling. Three random sampling points with a depth of 30 cm around the plant roots were taken at each location. The soil is then composited for each land type up to 1 kg and placed in plastic bags for laboratory analysis.



Phosphate-Solubilizing Bacteria (PSB) Isolation

A total of 10 g of soil sample was dissolved in 90 ml of distilled water as a 10⁻¹ dilution stock solution. The solution was homogenized by stirring with a shaker for 30 minutes to break up the lumps of soil. Then a dilution series of 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶ was made. From each dilution, 0.1 ml of solution was transferred to a petri dish that had been filled with Pikovskaya media. The solution was then spread evenly in a Petri dish using a glass rod spreader and incubated at 30 ± 1°C for 7 days. Colonies of bacteria that are capable of dissolving phosphate can be seen by the presence of a clear zone around the colony. The P solubility index measurement was carried out according to [7].

Furthermore, colonies that can form clear zones are purified, rejuvenated, and characterized. A total of 5 best phosphate solubilizing bacterial isolates were selected and used in a series of follow-up tests. The bacterial isolates obtained were then characterized morphologically through direct observation of colony shape and physiologically and biochemically characterization through staining tests such as the catalase test, oxidase test, oxygen demand test, motility test, the ability to use different carbon sources (glucose, lactose, and sucrose), methyl red test, and growth test at various pH as well as test the effectiveness of BPF administration to increase the growth of maize (*Zea mays*).

Physiology, morphology, and biochemical characterization

Hypersensitivity Test

The hypersensitivity reaction test was carried out by injecting a 24-hour-old bacterial suspension into 3 tobacco plant leaves. As much as 0.5 ml of the bacterial suspension was injected into the leaves using a sterilized syringe [8].

Gram staining

Microscopic observation was carried out through the Gram Staining test. The PSB culture, which had been cultivated on Nutrient Agar (NA), was removed and put on a glass slide for 24 hours. Afterward, it was sprayed with crystal violet dye for 20 seconds, rinsed with water, and then drip-iodized for a minute. Following that, rinse the medium for 10 to 20 seconds with 95% alcohol until it is clear, then rinse with water to halt the

alcohol reaction. With a 20-second safranin dye drip, the culture was washed with water. The bacteria's morphology was then examined under a microscope to see its size, shape, and color [9].

Catalase test

The catalase test was carried out to determine the nature of the bacteria involved in producing the catalase enzyme. The catalase enzyme is used by bacteria to catalyze the decomposition of hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen (O₂). Hydrogen peroxide is formed during aerobic metabolism, so bacteria that grow in an aerobic environment can decompose these toxic substances. A single loop of bacterial colonies was taken aseptically, then inoculated onto an object glass. The inoculant in the object glass is dripped with a sufficient 3% H₂O₂ solution. The presence of catalase can be seen from the formation of air bubbles around the colony after adding 3% H₂O₂ solution [7].

Oxidase test

The oxidase test was carried out to determine the nature of the bacteria involved in producing the oxidase enzyme, which was determined by the color change on the oxidase paper. Colonies that had been cultured for 24 hours were taken using a loop under sterile conditions, placed and rubbed on oxidase paper, and observed for changes in color. Changes in reaction are shown in the color change from color paper to blue within 1-20 seconds [7].

Oxygen demand test

Each isolate was inoculated on NB medium and incubated for 24-48 hours at 37°C. The characteristics of bacterial growth on NB medium were observed. Anaerobic bacteria will grow in clusters on the bottom of the medium, facultative anaerobic bacteria will grow scattered throughout the medium, microaerophilic bacteria will grow in clusters slightly below the surface of the medium, and aerobic bacteria will grow on the surface of the medium [10].

Motility test

The motility test was carried out to determine the ability of bacteria to move in bacteria. PSB isolates were pricked in semisolid sulfide-indole-motility media, incubated for 24 hours, and then observed for colony formation. A positive test is indicated by the presence of colony distribution,



which indicates that the bacteria can carry out movement [11]

The carbon utilization test

This test is performed to differentiate the abilities of bacteria based on their ability to ferment carbohydrates and reduce hydrogen sulphide. *Bsulfidewere* aseptically inoculated into a tube containing TSIA using an ose needle. Then incubated at 37°C for 24-48 hours. After 24-48 hours, observe the color of the slanted surface and the bottom of the agar.

Methyl Red and the Vogler-Prauskuer Test

Through this test, the ability of bacteria to oxidize glucose with the production and stabilization of high concentrations of acid end products is tested. Sterilized glucose-phosphate tubes were inoculated with the tested bacteria and incubated at $28 \pm 2^\circ\text{C}$ for 48 hours. After incubation, 5 drops of methyl red indicator were added to the tube and stirred gently. The red color has a positive meaning, and the yellow color indicates a negative meaning [12].

The effect of pH

The Nutrient Broth (NB) media used is conditioned to have a pH of 3, 5, 6, 7, and 9 [13]. The NB media is then sterilized. A total of 1 ml of liquid inoculum from the stock was put into 9 ml of nutrient broth and incubated for 24 hours. The turbidity that occurs is observed in NB media after incubation.

The production of starter

The isolate was grown on Pikovskaya broth medium and incubated for 7 days. The selected carrier medium is molasses [14]. A molasses solution was prepared as a carrier by mixing 10 ml of molasses with 90 ml of distilled water, then sterilizing it. As much as 3 ml of inoculum was taken from each inoculum stock, then transferred to 75 ml of carrier molasses that had been made. Incubated for 7 days. Before being transferred into the carrier, the bacterial population density was equalized to 10^8 CFU/ml. After incubation, the viability of the bacteria was checked using the Total Plate Count method.

BPF Effectiveness on Corn Growth (*Zea mays*)

There were 56 corn seedlings put in pot trays with sterile soil. Then, 4 replications of each treatment were inoculated with 0.05 ml of 5 chosen PSB inoculum isolates per plant. The observed

parameters were then measured 4 weeks after inoculation, including the addition of plant height, leaf number, fresh weight, and dry weight of plants.

Data analysis

The research design used one factorial completely randomized design, and the data were analyzed using the Analysis of Variance with a 5% confidence level and Duncan's follow-up test. The data were analyzed using the SAS version 9.0 program.

RESULTS AND DISCUSSION

Isolation of PSB and its Characterization

The results showed that the selected sources of PSB isolates were from natural forests and reed fields. No superior PSB candidates were obtained from oil palm plantations. Based on Table 2, 5 superior isolates that could dissolve P were available, namely CA-AL-7, CA-AL-8, CA-AL-4, CA-NF-1, and CA-NF-3. Based on the hypersensitivity test of the 5 bacteria, these bacteria are not plant pathogens. The characterization results can be seen in Table 1. The clear zone around the isolated PSB colony that appears on Pikovskaya media is the activity of PSB in dissolving bound P as a form of $\text{Ca}_3(\text{PO}_4)_2$ dissolving. The presence of a halo zone indicates that the type of BPF is effective in dissolving bound P in Pikovskaya media [6]. PSB's significant activity in dissolving element P from $\text{Ca}_3(\text{PO}_4)_2$ is also thought to be related to a decrease in pH and the secretion of organic acids. In the opinion of [15], this is due to the secretion of several organic acids in order from the highest to the lowest amount, such as pyruvic acid > tartaric acid > lactic acid > acetic acid > oxalic acid by 4 bacterial strains (P15, P13, P18, P7).

The P15 strain had the highest P solubilizing ability bound to $\text{Ca}_3(\text{PO}_4)_2$. Naturally dissolving natural P/rock phosphate takes longer than synthetic P but is more environmentally friendly. The presence of PSB in nature, although it takes a longer time to provide phosphate for plants, has a very positive impact on the environment.

The results of the characterization of the staining test showed that all isolates belonged to the group of gram-negative bacteria. Negative bacteria cannot retain the crystal violet dye during the gram staining process, so it will turn red when observed under a microscope. Researcher [16],



stated that the results of the characteristics of all PSBs applied to coffee plants were a group of Gram-negative bacteria, and their application could increase plant biomass. On microscopic observation, it was found that the shape of the

fifth BPF cell was in the form of a coccus. Based on Table 1, in the observation of the catalase test, isolates that show positive catalase will form air bubbles (Figure 1).

Table 1. Biochemical characterization of the five PSB isolates from the rhizosphere and *Imperata* fields

Isolates	Phosphate		Microscopy		Catalase
	Dissolving Index	Hypersensitivity	Cell shape	Gram	
CA-NF-1	3.1	-	Cocci	-	+
CA-NF-3	3.2	-	Cocci	-	++
CA-AL-4	2.5	-	Cocci	-	+
CA-AL-7	1.7	-	Cocci	-	+
CA-AL-8	2.9	-	Cocci	-	+

+ Enough, ++ Good

Bacteria can produce catalase enzymes that can break down H_2O_2 into H_2O and O_2 . H_2O_2 is a very dangerous compound for bacteria because it is a toxic gas that causes damage to bacterial cells. The catalase enzyme in bacteria can be detected by adding an H_2O_2 substrate. Of the five superior PSB isolates, all had positive catalase activity. It [17] stated that catalase is an antioxidant enzyme capable of degrading hydrogen peroxide into water and oxygen. Some pathogens produce catalase to defend against hydrogen peroxide attack, a weapon commonly used by the host's immune system, as well as oxidative stress. The formation of colonies on the surface of the

nutrient broth medium indicates that the five isolates are aerobic bacteria that require oxygen for their metabolism.

Meanwhile, based on Table 2, in the oxidase test, it was found that the five isolates were able to form a blue color on the oxidase reagent paper, giving positive results (Figure 1). Positive results indicated that the cytochrome oxidase enzyme was present in the five isolates when the test was carried out. Oxidase enzymes play an important role in the implementation of the electron transport system in aerobic respiration [18].

Table 2. Physiological characterization of the five PSBs from the rhizosphere of *Imperata* and natural forest

Isolates	Oxidase	Motility	C sources utilization	Methyl Red
CA-NF-1	+	Motile	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products	-
CA-NF-3	+	Motile	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products	-
CA-AL-4	+	Motile	Glucose and lactose and/or sucrose fermentation with acid accumulation in the slant and butt	-
CA-AL-7	+	Motile	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products	+
CA-AL-8	+	Motile	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products	-

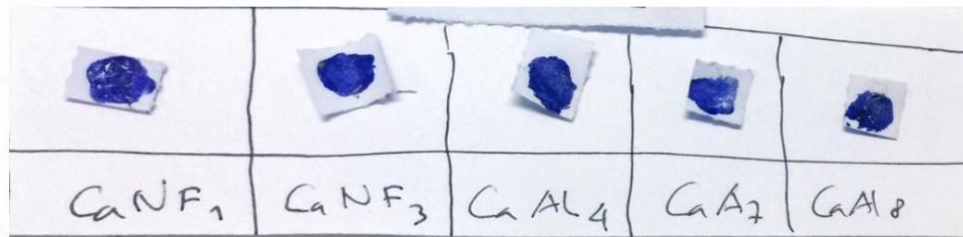


Figure 1. Positive oxidase test results for 5 selected isolates showed the appearance of blue color on the test paper

The test results showed that the five bacteria were motile, this was indicated by the formation of an inoculation line after the SIM media was inoculated with a straight loop of phosphate-containing bacterial colonies. The presence of growth away from the inoculated line indicates that the bacteria are motile [19].

The characterization also tested the ability of bacteria to use various carbon sources (sucrose, lactose, and glucose). A positive result is given if, after being scratched on the TSIA media, the media changes color from red to yellow (Figure 3). The yellow color is found on the slanted surface and the bottom of the TSIA agar [20]. BPF secretes some organic acids such as oxalic acid, succinic acid, tartaric acid, citric acid, and malic acid. The increase in organic acids is followed by a decrease in pH [21]. The results showed that only Ca-Al-4 isolates could use glucose, lactose, and sucrose as

C sources (Figure 2). The color is due to the accumulation of acid.

In the methyl red test, negative results were shown by four isolates Ca-Al-4, Ca-Al-8, Ca-NF-1, and Ca-NF-2. A positive test result occurs when the culture medium turns red after the addition of methyl red because the pH is at or below 4.4 during glucose fermentation. Only Ca-Al-7 isolates showed positive test results (Figure 3). In the negative methyl red test, the culture medium remains yellow because less acid is produced. The development of a steady red color surface of the medium indicates sufficient acid production to lower the pH to 4.4 and is a positive test. Other organisms can produce smaller amounts of acid from the test substrate, causing an orange between yellow and red to form, but this does not indicate a positive test [7].

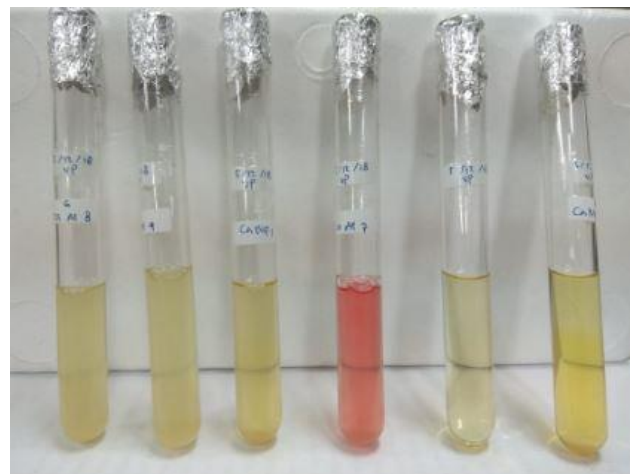


Figure 2. Test the test of 5 BPF isolates their ability to use various carbon sources (left), and a positive reaction to the methyl red test is indicated by a change in color to reddish (right)

Testing the survival ability of bacteria in the pH range showed that the five selected bacterial isolates showed different growth responses (Table 3). All isolates can grow in the pH ranges of 3, 5, 6, and 7 at room temperature. The higher the pH, the more concentrated the turbidity of the media. At pH 9, only Ca-NF-3 isolates could grow

at pH 9. BPF could grow under acidic, neutral, and alkaline conditions. In acid soils, the optimum BPF is at neutral pH, and the dominant BPF isolated from the soil rhizosphere can live at pH 4-10.6. For optimum phosphate solubilization fungi can grow at pH 5.5 [17].

Table 3. The result of pH testing on the survival ability of BPF bacteria

Isolate Code	pH 3	pH 5	pH 6	pH7	pH 9
Ca-AL-4	++	+++	+++	++++	-
Ca-AL-7	++	+++	+++	++++	-
Ca-AL-8	++	+++	+++	++++	-
Ca-NF-1	++	+++	+++	++++	+
Ca-NF-3	+	+++	+++	++++	-
Control	-	-	-	-	-

Turbidity level of liquid bacterial medium : (-)= no turbidity; (+) = very low turbidity; (++) = low turbidity; (+++) = moderate turbidity; (++++)= high turbidity

Table 4 Results of analysis of growth parameters of maize (*Zea mays*) 4 weeks after inoculation (MSI).

Treatment Code	Plant Height (cm)	Number of Leaves	Gross Weight (g)	Dry Weight (g)
K	6,75 ^b	3 ^b	1,237 ^e	0,500 ^f
KM	7,25 ^b	3 ^b	2,382 ^d	1,932 ^e
CA-NF-1	9,25 ^a	4 ^a	5,245 ^b	3,200 ^b
CA-NF-3	10,50 ^a	4 ^a	6,195 ^a	4,550 ^a
CA-AL-4	10,25 ^a	4 ^a	5,075 ^c	3,000 ^d
CA-AL-7	10,50 ^a	4 ^a	5,012 ^c	3,000 ^d
CA-AL-8	10,75 ^a	4 ^a	5,095 ^{bc}	3,055 ^c

Information: K = without treatment, KM = Pikovskaya medium without BPF, CA-NF-1 = Pikovskaya medium + BPF Natural Forest, CA-NF-3 = Pikovskaya medium + BPF Natural Forest, CA-AL-4 = Pikovskaya medium + BPF Imperata fields, CA-AL-7 = Pikovskaya medium + BPF Imperata fields, CA-AL-8 = Pikovskaya medium + BPF Imperata fields. *The numbers followed by the same letter in the same column are not significantly different based on the Duncan Multiple Range Test (DMRT) at the level of $\alpha \leq 5\%$

BPF Effectiveness on Corn Growth

Table 4 shows the administration of the five BPF isolates (CA-NF-1, CA-NF-3, CA-AL-4, CA-AL-7, and CA-AL-8) with a P dissolution index of 3.1, respectively: 3.2; 2.5; 1.7; 2.9. The five isolates effectively increased the growth of maize (*Zea mays*) compared to the control (K and KM) ($P < 0.05$). K code is a negative control (no treatment), while KM code is a positive control (Pikovskaya medium without BPF). These results are supported by [2], which states that BPF is a non-pathogenic soil bacterium included in the category of plant growth-promoting bacteria. Apart from acting as plant growth-promoting bacteria, BPF also produces vitamins and phytohormones that can improve plant root

growth and increase nutrient uptake [22]. These bacteria also play a role in the transfer of energy, nucleic acids, proteins, coenzymes, and other metabolic compounds that can increase P absorption activity in P-deficient plants [22].

CONCLUSION

In this study, 5 selected isolates of phosphate-solubilizing bacteria were obtained, including CA-AL-7, CA-AL-8, CA-AL-4, CA-NF-1, and CA-NF-3. The results of the characterization test Gram stain test, catalase test, oxidase test, oxygen demand test, motility test, ability test to use different carbon sources (glucose, lactose, and sucrose), methyl red test, and growth test at various pHs



showed that the isolates showed varied responses. Based on this series of tests, of the 5 selected isolates, 3 potential bacteria could dissolve P that was not available to become available in the soil, which had good cell activity and ability. The three isolates, namely Ca-Al-8, Ca-Al-4, and Ca-NF-3 had a significant effect on the parameters of plant height, number of leaves, and fresh and dry biomass of *Zea mays* after 4 weeks of inoculation ($p < 0.05$).

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