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Original Research Article

Isolation and Characterization of Cellulolytic Bacteria and Phosphate Solubilizing Bacteria from the Rhizosphere of Muli Banana Plants (Musa acuminata) in Labuhan Ratu District, Bandar Lampung

Eva Sofia El Kautsar[1], Kusuma Handayani[1*], Salman Alfarisi[1], Sumardi[1]

[1]Department of Biology, University of Lampung, Lampung, Indonesia

***Corresponding Author:** Kusuma Handayani
Department of Biology, University of Lampung, Lampung, Indonesia

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Abstract: Muli banana (*Musa acuminata*) from Lampung requires attention to the availability of phosphate in the soil, as phosphate is one of the most important elements for banana plant growth. To provide dissolved phosphate in the soil, phosphate-solubilizing bacteria (PSB) are needed. Cellulolytic bacteria can provide glucose as a carbon source for PSB. This research aims to isolate and characterize PSB and cellulolytic bacteria from the rhizosphere of Muli banana in Labuhan Ratu district, Bandar Lampung. This research was carried out using soil sampling, bacterial isolation, the selection of potential bacteria, and morphological, physiological, and biochemical characterization. The results showed that out of 5 isolated PSB colonies, BPF4 and BPF8 had the highest phosphate solubility index, reaching a value of 3. The characteristics of the BPF4 and BPF8 are different from each other. Among the 3 colonies with cellulolytic ability, the isolates with the highest cellulolytic activity index were isolates BS1 and BS7, which had an SAI value of 0.58. The morphological results of each cellulolytic bacterial colony varied. The cellulolytic bacteria showed coccus-shaped cells and were Gram-positive in BS1, whereas BS7 was Gram-negative. There was no spreading growth in the PSB motility test, whereas all cellulolytic bacteria exhibited motility. The glucose fermentation test only showed color change in isolates BPF2, BPF5, and BPF8, with no appearance of bubbles. Almost all isolates tested positive in the catalase test, with only isolate BS8 not showing any foam.

Keywords: Cellulolytic Bacteria, Phosphate-Solubilizing Bacteria, Rhizosphere, Banana Plants.

INTRODUCTION

Muli banana (*Musa acuminata*) is a typical banana from Lampung and is one of the frequently consumed fruits. The Muli banana plant has a smaller body and fruit size compared to other banana types and is tolerant to Fusarium wilt. The Muli banana plant requires important elements such as phosphate from the soil to carry out its metabolic processes, where phosphate is used as a component of protein and nucleic acids (Romadloni *et al*., 2024). Most of the phosphates in the soil come from rocks or the remains of plants and animals, so the form of phosphate is still bound to soil colloids and cannot be absorbed by plants. To provide phosphate supplies in the soil, phosphate solubilizing bacteria (PSB) are needed. PSB are regarded as plant growth-promoting rhizobacteria (PGPR) that can use carbon sources from root exudates or the metabolic byproducts of other bacteria to produce phosphate enzymes and organic acids that can dissolve bound phosphate to make it available for plants. Some types of phosphate solubilizing bacteria include *Bacillus mycoides*, *Pseudomonas mallei*, and *P. cepaceae* (Silva *et al*., 2023). Cellulolytic bacteria can produce carbon sources from the process of cellulose decomposition. Cellulose derived from plant cell walls is then broken down by cellulolytic bacteria into glucose and other compounds that can be used as an energy source for phosphate-decomposing microbes. The cellulase enzymes produced by cellulolytic bacteria play an important role in the carbon availability cycle in the soil. Some genera of cellulolytic bacteria are Cellulomonas, Cellvibrio, Pseudomonas, Bacillus, and Micrococcus (Khatoon *et al*., 2020). Both bacteria can be found in the root zone of plants (rhizosphere). Rhizosphere microorganisms will colonize the plant root system and

decompose organic matter in the soil, increasing soil nutrient availability, and promoting plant growth (Ma *et al*., 2022). Root exudates released will serve as a communication bridge between the roots and rhizosphere microbes through the exchange of chemical compounds. Root exudates can also attract and stimulate the growth of soil microbes or repel microbes that are pathogens to the plants (Widyawati, 2020). Labuhan Ratu District is one of the areas located in Bandar Lampung, Lampung Province. In this densely populated area, there are still environments planted with Muli banana trees. Therefore, this study aims to isolate and characterize bacteria from the rhizosphere of banana plants that have the ability to decompose cellulose and dissolve phosphates so that they can be absorbed by the soil and utilized by plants (Ilham *et al*., 2014).

RESEARCH METHOD

Sampling Collection

Samples were taken from 3 different places and the measurements of soil pH and temperature were conducted. The sampling location was cleared from debris before soil was dug to a depth of approximately 30 cm. A total of 50g per place was collected and placed in sterilized plastic clips, then put into an ice box and transported to the laboratory (Romadloni *et al*., 2024).

Serial Dilution

Dilution of bacterial suspension is performed in a Biological Safety Cabinet (BSC). A sample of 1 g of soil is placed into a NaCl solution and then homogenized using a vortex. Then, a serial dilution is performed by taking 1 ml of the previous solution into 9 ml of aquades in another test tube. This process is repeated until a dilution of 10^{-8} is achieved.

Isolation of Phosphate-Solubilizing Bacteria and Cellulolytic Bacteria

The isolation of phosphate-solubilizing bacteria was carried out using the pour plate method with selective Pikovskaya agar medium for phosphate-solubilizing bacteria, and selective CMC medium for cellulolytic bacteria. For the isolation of cellulolytic bacteria, suspensions were used from dilutions 10^{-3} to 10^{-8}, while for the isolation of phosphate-solubilizing bacteria, dilutions from 10^{-3} to 10^{-5} were used. A suspension of 0.1 ml was taken using a micropipette and placed into a sterile petri dish. Then, Pikovskaya and CMC agar media were poured into the dish and mixed by shaking the dish in an 8 shape to ensure that the suspension and media were combined. The isolation of bacteria was performed with two repetitions (duplicates) each. Subsequently, the petri dishes were incubated at room temperature for 7x24 hours for PSB (Romadloni *et al*., 2024), and at 36°C for 24 hours for cellulolytic bacteria.

Determination of Phosphate Solubility Index and Cellulolytic Activity Index

Isolates that have the potential to dissolve phosphate and decompose cellulose are re-selected and grown again on Pikovskaya medium and CMC agar using the spot method. The petri dishes are incubated at room temperature in an inverted position for 7x24 hours for phosphate solubilizing bacteria (PSB) and at 36°C for 24 hours for cellulolytic bacteria. This test is conducted to ensure that the selected bacteria indeed possess the ability to dissolve phosphate and decompose cellulose. Isolates that form clear zones are designated as phosphate solubilizing bacteria (PSB) and cellulolytic bacteria (BS). The clear zones and colonies are then measured, and the phosphate solubility index and cellulolytic activity index are calculated (Hidayat & Isnawati, 2021) (Oksana *et al*., 2020).

Characterization of Phosphate Solubilizing Bacteria and Cellulolytic Bacteria

Phosphate solubilizing bacteria and cellulolytic bacteria isolated were then characterized morphologically by observing the shape, color, margin, and elevation of the colonies. Furthermore, Gram staining and physiological tests were conducted, including glucose fermentation test, catalase test, and motility test.

RESULTS AND DISCUSSION

Results of pH and Soil Temperature Measurements

At each soil sampling point, environmental parameter tests were conducted, including tests for pH and soil temperature. The points are DR1, DR2, and DR3, with a depth of approximately 30 cm from the soil surface and close to the root system of the Muli banana plants. The test results from the examined rhizosphere areas indicate that the temperatures recorded are as follows: DR1 at 26.7°C, DR2 at 27.2°C, and DR3 at 26.2°C. Additionally, the pH values show 6 for DR3 and 7 for both DR1 and DR2. The calculated average pH across the sampled rhizosphere soil is 6.6, suggesting that the area is slightly acidic trending towards neutral. The ideal pH range for soil to promote the growth of banana plants lies between 5.0 and 7.5. Moreover, the average temperature found in the rhizosphere is 26.7°C, which is favorable for the development of banana plants. Monitoring soil pH and temperature is essential as these factors can significantly influence bacterial growth in the rhizosphere.

Table 1: Results of pH and Soil Temperature Measurements

Parameter	DR1	DR2	DR3	Average
pH	7	7	6	6,6
Temperature (°C)	26,7	27,2	26,2	26,7

Calculation of Phosphate Solubility Index

From the results obtained through serial dilution isolation from 10^{-3} to 10^{-8} on Pikovskaya medium using the pour method, it was observed that colonies only grew on the surface of the selective Pikovskaya medium at a 10^{-4} dilution. Among these colonies, eight were point-inoculated onto the Pikovskaya medium for phosphate solubilization testing, chosen based on distinct morphological differences. The isolation process ultimately identified five colonies capable of solubilizing phosphate, which were indicated by the presence of clear zones surrounding them. These clear zones were formed as a consequence of the metabolic activity of the phosphate-solubilizing bacteria, which produce organic acids that effectively convert bound phosphate into a soluble form.

From a total of 8 isolates that have been isolated, 5 colonies were selected based on differences in colony morphology and then re-inoculated to obtain isolates capable of dissolving phosphate. The calculation of phosphate solubility index values from the 5 colonies shows that the highest phosphate solubility index is held by colonies BKF4 and BKF 8, as seen in Table 2.

Table 2: Phosphate Solubility Index of Phosphate-Solubilizing Bacterial Isolates

Isolate Code	Phosphate Solubility Index Value
BPF2	2,7
BPF3	2,7
BPF4	3
BPF5	2,6
BPF8	3

Based on Table 2, there are 5 selected colonies that have the ability to dissolve phosphate.The phosphate solubility index shows the ability of each isolate to solubilize phosphate on Pikovskaya agar media. The formation of clear zones on the media indicates that the isolates can produce phosphatase enzymes and organic acids that will bind elements that bind phosphate, such as Ca in $Ca_3(PO_4)_2$, so that phosphate becomes available and turns into dissolved phosphate, creating clear zones (Tian *et al*., 2021). The ability to dissolve phosphate from isolates varies even though they come from the same ecosystem. The differences in phosphate solubilization ability are influenced by the genetic traits of each microbe that produces organic acids capable of dissolving phosphate (Nisa, 2018).

Calculation of Cellulolytic Activity Index

In the process of isolating cellulolytic bacteria, a serial dilution ranging from 10^{-2} to 10^{-5} was employed, followed by isolation through pour plating on selective CMC medium. From this procedure, the 10^{-3} dilution yielded eight colonies that developed on the surface of the selective CMC medium. Subsequently, these eight colonies were point-inoculated onto the CMC medium to evaluate their cellulolytic activity.

The eight colonies were then re-inoculated on CMC media for the cellulolytic activity test. From these 8 colonies, 3 colonies with cellulolytic activity were obtained, indicated by the presence of a clear zone after pouring 0.1% Congo red solution. The colonies were coded as isolates BS1, BS7, and BS8. Each of these colonies was then assessed for their cellulolytic activity index. These 3 colonies were then inoculated in a spot manner on selective CMC media. The results of this incubation yielded cellulolytic activity index values listed in Table 3.

Table 3: Cellulolytic Activity Index of Cellulolytic Bacterial Isolates

Isolate Code	Cellulolytic Activity Index
BS1	0.58
BS7	0.58
BS8	0.41

Based on Table 3, three isolates were obtained that produced clear zones. The cellulolytic activity index of BS1 and K8 is the same at 0.58, while B7 has an index value of 0.41. The index value of cellulolytic activity from these three isolates falls into the small category because the value is <1. This result indicates that their ability to degrade cellulose is very limited. Bacteria with such capabilities are considered less effective in rapidly decomposing lignocellulosic materials.

The calculation of clear zones was performed after determining the area of the clear zone using 0.1% Congo Red, which was then rinsed with a NaCl solution. The presence of a clear zone is due to the cellulolytic bacteria producing

cellulase enzymes that can break down cellulose in CMC media. The enzymes produced by cellulolytic bacteria consist of three types of enzymes that work together, namely exoglucanase (exo-1,4-β-D-glucanase), endoglucanase (endo-1,4-β-D-glucanase), and β-glucosidase. These three enzymes work simultaneously to degrade cellulose by hydrolyzing the β-1,4-glycosidic bonds in cellulose into simple sugars (glucose) (Namnuch *et al*., 2021).

Characterization of Phosphate Solubilizing Bacteria and Cellulolytic Bacteria

The morphological characterization performed on the eight bacterial isolates includes colony shape, colony color, colony edge shape, and colony elevation. Based on Table 3, the results of the morphological characterization of phosphate-solubilizing bacteria show that the shape is predominantly irregular, with raised edges, flat elevation, and a yellowish-white color. In cellulolytic bacteria, the cell morphology shows different results, with the same elevation being umbonate. The morphological characteristics of each colony from the highest results of PSI and CAI are shown in Table 4.

Table 4: Morphology of Phosphate Solubilizing Bacteria and Cellulolytic Bacteria Colony

Isolate Code	Colony Morphology			
	Shape	**Margin**	**Elevation**	**Color**
BPF2	Circular	Entire	Flat	Yellowish white
BPF3	Irregular	Irregular	Flat	Yellowish white
BPF4	Irregular	Entire	Flat	Yellow
BPF5	Irregular	Entire	Raised	Yellow
BPF8	Irregular	Entire	Raised	Yellowish white
BS1	Irregular	Undulate	Umbonate	White
BS7	Circular	Entire	Umbonate	Yellowish white
BS8	Irregular	Undulate	Umbonate	Yellowish white

The morphological characterization performed on the eight bacterial isolates includes colony shape, colony color, colony edge shape, and colony elevation. Based on Table 3, the results of the morphological characterization of phosphate-solubilizing bacteria show that the shape is predominantly irregular, with raised edges, flat elevation, and a yellowish-white color. In cellulolytic bacteria, the cell morphology shows different results, with the same elevation being umbonate.

Biochemical Tests of Phosphate-Solubilizing Bacteria and Cellulolytic Bacteria

All isolated colonies that have been tested for phosphate solubilization and cellulolytic activity were then Gram stained and re-examined to determine their biochemical characteristics. The biochemical tests conducted in this study included glucose fermentation test, catalase test, and motility test.

The results of the Gram staining observation of phosphate-solubilizing bacteria showed that isolates BPF2, BPF3, and BPF5 have the same cell shape, while isolates BPF4 and BPF8 have a coccus cell shape. Based on Figure 1, isolates BPF2 and BPF5 exhibit Gram-negative properties indicated by the red-colored bacteria cells, while isolates BPF3, BPF4, and BPF8 show Gram-positive properties characterized by the purple-colored cells. The results of Gram staining observations on cellulolytic bacteria showed that BS2 and BS8 have the same cell shape, which is rod-shape cell with Gram-positive characteristics, while isolate BS7 has a cocci-cell shape with negative Gram characteristics indicated by a red color. Nuraisya *et al*., (2020) found that phosphate-solubilizing bacteria isolated from soil mostly have the characteristics of Gram-negative bacteria. According to previous research, out of 12 bacterial isolates obtained from the rhizosphere of banana plants in Nanded, Maharashtra, India, it was shown that all 12 isolates are phosphate-solubilizing bacteria, 8 of which are Gram-negative, and the other 4 are Gram-positive (Apastambh *et al*., 2016).

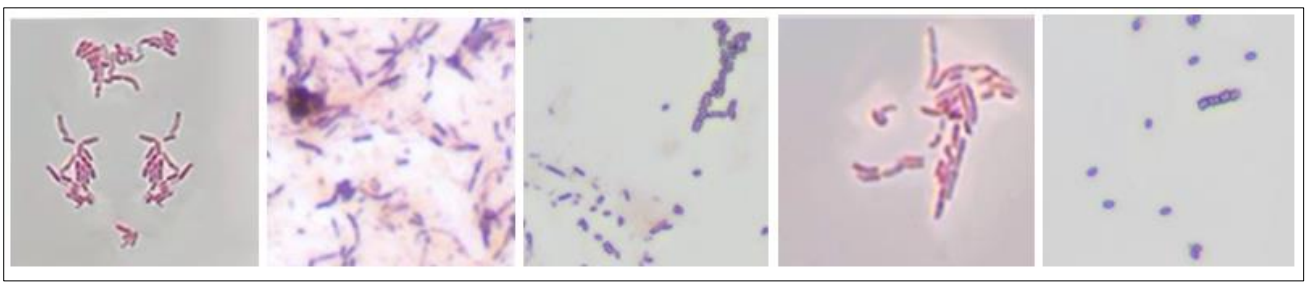

Figure 1: Gram staining in phosphate solubilizing bacteria, from left to right: BPF2, BPF3, BPF4, BPF5, and BPF8

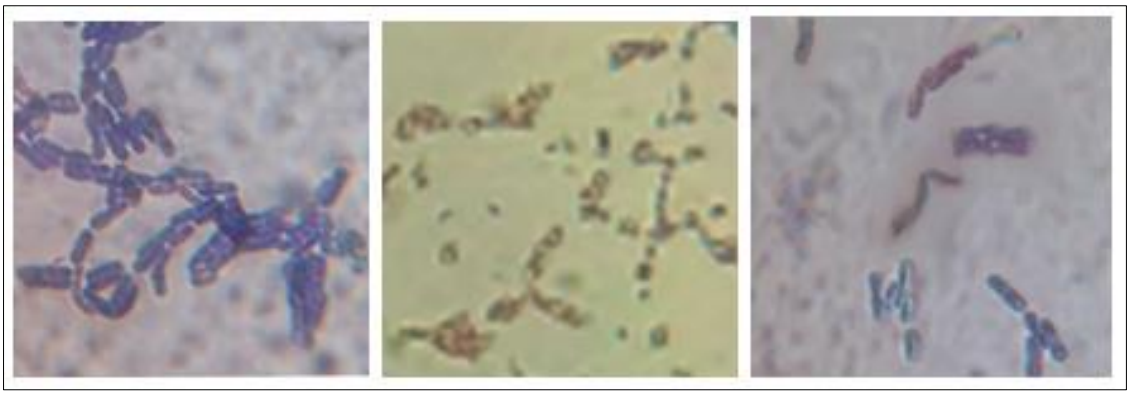

Figure 2: Gram staining on cellulolytic bacteria, from left to right: BS1, BS7, and BS8

Bacteria with Gram-positive properties have thick peptidoglycan cell walls that react with iodine and crystal violet, forming a combination that inhibits the entry of safranin into the bacterial cell. Bacteria with Gram-negative properties have a thin peptidoglycan layer on their cells and a high lipid content, allowing the primary dye to dissolve and the cell to be stained with safranin (Haswania *et al*., 2021). After the color is removed by the decolorizing solution, the solution interacts with the lipids present in the cell membrane and releases that layer from the cell, causing Gram-negative cells to leak and release crystal violet and iodine dye. In Gram-positive bacteria, the thick peptidoglycan layer is hydrated by the addition of the dye solvent, trapping the crystal violet and iodine complex within the cell and resulting in a purple color in the bacterial cell (Smith and Hussey, 2005).

Table 5: Characterization of Phosphate Solvent Bacteria and Cellulolytic Bacteria

Isolate Code	Character			
	Glucose Fermentation	**Catalase**	**Motility**	**Gram Staining**
BPF2	Yellow medium, no bubble	+	-	Rod, Gram-positive
BPF3	-	+	-	Rod, Gram-positive
BPF4	-	+	-	Coccus, Gram-positive
BPF5	Yellow medium, no bubble	+	-	Rod, Gram-negative
BPF8	Orange medium, no bubble	+	-	Coccus, Gram-positive
BS1	-	+	+	Rodl, Gram-positive
BS7	-	+	+	Coccus, Gram negative
BS8	-	-	+	Rod, Gram-positive

The results of the catalase test observations showed positive results in almost all isolates except for isolate BS8. A positive result in the catalase test is indicated by the formation of foam or bubbles when the bacterial isolate comes into contact with H_2O_2 (hydrogen peroxide) (Sianipar *et al*., 2020). Bacteria will release the enzyme catalase to protect their cells from hydrogen peroxide (H_2O_2) solution, which is toxic to microbes and can lyse cells if not broken down. The enzyme will decompose hydrogen peroxide (H_2O_2) into H_2O (water) and O_2 (oxygen) (Khatoon *et al*., 2022). As researched by Tatung and Deb (2023), out of 25 bacterial colonies isolated from the rhizosphere of wild bananas (Yunnan bananas or *Musa itinerans*), three of them have the ability to solubilize phosphate. These colonies exhibit different morphological characters and show positive results for catalase activity. According to a study conducted by Astriani (2017), the isolation of cellulolytic bacteria from the rhizosphere of banana plants in Palembang showed positive results in the catalase test.

Observations of motility tests on cellulolytic bacterial isolates showed positive results, marked by the spread of bacterial growth in the growth medium. All phosphate-solubilizing bacterial isolates were non-motile, with bacterial growth only occurring around the needle prick. Bacterial movement during the motility test occurs due to the presence of bacterial motility structures in the form of flagella (gliding motility), whereas bacteria without flagella will only grow around the needle prick (non-motile) (Panjaitan *et al*., 2020). Based on the research conducted by Reena *et al*., (2013), 3 phosphate-solubilizing bacteria isolates were obtained from the rhizosphere of banana plants from several areas in Bangalore, India. Of the three isolates, 1 isolate showed a negative result in the motility test, with a Gram-positive nature and coccus-shaped cells, while the other isolates had rod-shaped cells.

The results of the glucose fermentation tests showed that only BPF2, BPF5, and BPF8 were able to change the color of the phenol red glucose broth medium from red to yellow, but did not produce bubbles in the Durham tube. This color change is due to phosphate-solubilizing bacteria producing organic acids that can lower the pH of the medium and change its color. The final result of carbohydrate fermentation is acid or acid with gas. The common end products obtained from fermentation performed by bacteria are lactic acid, acetic acid, butyric acid, acetone, ethanol, carbon dioxide, and

hydrogen. The fermentation reaction is indicated by a change in color on the pH indicator when acid products are formed. This can be observed by adding a type of carbohydrate to the medium that contains a pH indicator. Because bacteria can also use pepton in the fermentation process and produce alkaline products, pH can change when acids are produced as a result of carbohydrate fermentation. Phenol red is used as a pH indicator in carbohydrate fermentation tests because the final product of carbohydrate utilization is generally organic acids (Reiner, 2012). Phenol red broth is used as a fermentation medium consisting of tryptone, NaCl, phenol red, and carbohydrates. Phenol red acts as a neutral pH indicator (pH 7). When bacteria are inoculated into the medium, bacteria that ferment sugars will produce acids that will change the color of phenol red to yellow (Borris, 2020). According to the research conducted by Marista *et al*., (2013), phosphate-solubilizing bacteria isolated from the rhizosphere of nipa palm bananas mostly cannot ferment glucose. Out of 30 isolates, 17 isolates could not ferment glucose. Based on the research by Haswania *et al*., (2021), out of 3 phosphate-solubilizing bacteria isolated from the rhizosphere of corn, 1 isolate could ferment glucose. In previous research conducted by Astriani (2017), the glucose fermentation tests on cellulolytic bacteria isolates from banana plantation soil in Palembang showed no color change and gas formation in Durham tubes.

CONCLUSION

The morphological characteristics of phosphate-solubilizing bacterial isolates with the highest phosphate solubility index are isolates BPF4 and BPF8 with a value of 3, while for cellulolytic bacteria, the isolates are BS1 and BS7 with a value of 0.58. The morphological characteristics of phosphate-solubilizing bacteria are dominated by irregular colony shapes, raised edges, flat colony elevations, and yellowish-white color. For the morphological characteristics, BPF4 and BPF8 share similarities in colony shape and edge, with an irregular shape and raised edge. The difference between the two isolates is that the elevation of BPF4 is flat and that of BPF8 is raised, with the color of BPF4 being yellow and BPF8 yellowish-white. The cell shape in BPF4 is coccus with a Gram-positive property, and the cell shape in BPF8 is also coccus with a Gram-positive property. Among the two isolates, only BPF8 is capable of changing color during the glucose fermentation test. Both isolates are non-motile and BPF4 can produce bubbles in the catalase test.

In cellulolytic bacteria, the morphological characteristics of BS1 and BS7 have the same elevation, which is umbonate, with different colony shapes, edges, and colors. The shape of the BS1 cell is rod with Gram-positive characteristics, while BS7 is a coccus cell with Gram-negative characteristics. All cellulolytic bacterial isolates cannot ferment glucose; are motile; and are positive in the catalase test.

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