Exploration, isolation and characterization of indigenous rhizobacteria from patchouli rhizosphere as PGPR candidates in producing IAA and solubilizing phosphate

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Abstract. Microorganisms that are active and aggressive colonizing the rhizosphere are known as rhizobacteria. They are able to act as biofertilizers, bioprotectants, biostimulants and bioremediation. This study aims to identify and characterize groups of rhizobacteria present in the patchouli rhizosphere that can produce IAA compounds and have the ability to solubilize phosphate in the soil. Soil samples were taken from the patchouli rhizosphere at Purwosari Village, Nagan Raya, Aceh Province, Indonesia. This study used quantitative and qualitative descriptive analysis through serial dilutions to obtain rhizobacterial strains. Parameters observed were macroscopic and microscopic characteristics, gram test, IAA production and phosphate solubilization. The study obtained 37 isolates of rhizobacteria from Purwosari (PS), comprising 25 isolates of gram positive and 12 isolates of gram negative. The rhizobacteria PS 5/1 produced the lowest IAA at 21.66 ppm, whereas isolate 5/6 C produced the highest IAA at 83.38 ppm. Twenty-five isolates of rhizobacteria could solubilize phosphate while the remaining 12 isolates did not have this ability. The rhizobacteria PS 7/1 resulted in the highest PSI at 2.55 and isolates PS 8/7 produced the lowest PSI at 1.33. The rhizobacteria isolates that can produce IAA and phosphate solubilizing have the potential to be used as PGPR candidates.

1. Introduction
Patchouli (Pogostemon cablin Benth.) is one of the important export commodities from Aceh which includes patchouli oil, as the main fixative constituent in modern perfumery, and patchouli leaves. In 2018, the Agricultural Quarantine Agency recorded that nationally patchouli leaves were exported as many as 297.7 tons with a value of Rp 470 million, and in 2019 they increased to 1,227 tons with a value of Rp 1.3 billion. In 2020, however, there was a decrease in volume, reaching only 591.3 tons, and yet due to a surge of price, the economic value could reach Rp 6.8 billion [1]. As of 2021, the Aceh Jaya Department of Industry and Trade states that the average production of patchouli oil for farming communities in Aceh Jaya District ranges from 5-6 tons per day [2].

Due to its benefits, the optimization of patchouli cultivation is necessary to ensure that its production remains sustainable. One of the efforts to achieve this is by utilizing microorganisms in situ to support plant growth, such as rhizobacteria. Rhizobacteria known as Plant Growth Promoting Rhizobacteria (PGPR) are a group of microorganisms living in the rhizosphere that exert benefits for plants. PGPR activities provide advantages for plant growth through direct or indirect mechanisms. The direct effects
of PGPR in promoting plant growth occur through various mechanisms, including fixation of free nitrogen that is transferred into plants, production of siderophores that chelate iron (Fe) and make Fe unavailable to pathogens, and solubilization of minerals such as phosphorus and synthesis of phytohormones. Indirect effects in increasing plant growth, on the other hand, occur through suppression of phytopathogens [3].

The roles of rhizobacteria in the rhizospheric area consist of providing nutrients in the soil, decomposing organic matter, mineralizing organic matter, stimulating plant growth, and being a biological agent (controlling pests and plant diseases). Soil microbes have many important roles in the cycle of organic elements for life, such as producing the Indole Acetic Acid (IAA) hormone. IAA hormone is an endogenous auxin that plays a role in root development, inhibits the growth of side shoots, stimulates abscission, and plays a role in the formation of xylem and phloem tissues.

The ability to fix nitrogen, phosphate solubilizing, and produce siderophores, hydrogen cyanide (HCN), chitinases, proteases, and cellulases are characteristics of suitable rhizobacteria. Phosphate solubilizing bacteria can synthesize phytase and phosphatase enzymes that play a role in the hydrolysis of organic P to dissolve inorganic P and produce organic acids that help release metal-fixed P [4]. In addition, according to [5], the increase in plant growth by rhizobacteria can occur through one or more mechanisms related to the physiological characteristics of bacteria and conditions in the rhizosphere. Some rhizobacterial strains are able to synthesize IAA from precursors (basic materials) contained in root exudates as well as from organic matter (plant and animal residues), whereas in general plants they are unable to produce IAA in sufficient quantities for growth and development. Rhizobacteria that can produce IAA and phosphate solubilizing came from the genera Pseudomonas, Bacillus, and Serratia. The purpose of the research was to find out the rhizobacteria that produce IAA and phosphate solubilization.

2. Materials and methods
This study was conducted at the Laboratory of Seed Science and Technology, Faculty of Agriculture, Syiah Kuala University on October 2020 - April 2021. The initial stage was exploration aimed at taking soil samples from the rhizosphere of a healthy patchouli plant growing in Purwosari, Nagan Raya, Aceh Province, Indonesia.

2.1. Isolation and characterization
Isolation of rhizobacteria was carried out using the serial dilution method up to 10^-9. The incubation process was conducted at room temperature for 3-7 days. Soil samples were taken for a total of 10 kg from 10 different points in the area. Then, the soil was dried at room temperature (28 - 30°C). After 2x24 hours of drying, the soil was first sieved with a 9-mesh sieve shaker. Next, the soil was weighed at 1 gram and placed it in a test tube, and later added with 9 mL of distilled water, and then shaken until evenly distributed, hereinafter referred to as the first dilution of 10^-1. The 1 mL pipette from 10^-1 was afterward put into a test tube containing 9 mL of diluent solution, hereinafter referred to as the second dilution 10^-2, up to the final dilution 10^-9. Then, 1 mL at 10^-9, 10^-8 and 10^-7 dilutions was taken and poured into a petri dish by adding Sucrose Peptone Agar medium, which was still a liquid (±45°C), and then made a duplicate. Next, it was shaken homogeneously and waited until the medium solidified, after which the petri dish could be inverted and then incubated for 2 days (pour method). Afterward, observation and calculation for the number of colonies were done. Colonies can be distinguished by surface appearance (shiny or gloomy), by colour (white, yellow, red, brown, orange, blue, green, and purple), and by density (soft like slime, soft like butter, and some are hard and dry). Furthermore, identification of rhizobacteria was carried out to obtain pure cultures from local indigenous rhizobacteria. Parameters observed were the ability to produce IAA, phosphate solubilizing bacteria, and gram test.
2.2. IAA production test
IAA production test was conducted qualitatively and quantitatively. The isolates were tested qualitatively using Salkowski’s reagent. PGPR isolates were inoculated on flat Nutrient Agar media supplemented with tryptophan with a concentration of 100 ppm using the T streak technique [6]. Then, they were incubated at room temperature for 48 hours. Salkowski’s reagent was dripped onto PGPR isolates that had been grown in the Nutrient Agar media until evenly distributed. Furthermore, isolates that had been dripped with Salkowski’s reagent were stored in a dark room for 30 minutes. Positive results were indicated by a change in the colour of the isolate colony to light yellow.

2.3. Phosphate solubilization test
Rhizobacteria isolates were taken using a sterile needle and grown on Pikovskaya media aseptically. The medium was incubated for 7 x 24 hours at 37°C. Observation of colony growth capable of forming a clear zone was indicated as isolates capable of solubilizing phosphate. Colony diameter and clear zone were measured at 7 x 24 hours). The formula for measuring Phosphate Solubilization Index (PSI) was the ratio of total diameter (halo) and colony diameter. PSI category: 1.59 Low; 1.6 – 2.12 Medium; 2.15 – 2.59 High; 2.6 – 3 Very High [7].

3. Results and discussion
3.1. Isolation and identification of rhizobacteria from patchouli rhizosphere
The geographical position of indigenous rhizobacteria taken from the patchouli rhizosphere at Purwosari Village, Nagan Raya, Aceh Province, Indonesia. The exploration of rhizobacteria as PGPR candidates resulted in 37 isolates coded in PS. The performance of rhizobacteria isolates is shown in Figure 1.

![Figure 1](image)

**Figure 1.** Performance of several rhizobacteria isolates macroscopically (top) and microscopically (bottom).

Further, candidate isolates as PGPR were identified macroscopically to identify the shape, colour, edge, and elevation. The process of identifying rhizobacteria isolates was based on the key to the determination in the book “Bergey’s Manual of Systematic Bacteriology” [8] through the biochemical tests. The results of the identification of rhizobacteria isolates were presented in Table 1.
Table 1. Macroscopic characteristics and gram test of indigenous rhizobacteria isolates from patchouli rhizosphere.

| Isolate Code | Shape     | Colour               | Macroscopic          | Gram staining |
|--------------|-----------|----------------------|----------------------|---------------|
| PS 5/1       | Irregular | Greenish Brown       | Undulate             | Drop-like     |
| PS 5/2 C     | Irregular | Brown                | Irregular            | Flat          |
| PS 5/2 H     | Spindle   | Brown                | Undulate             | Flat          |
| PS 5/3       | Circular  | Greenish Dark Brown  | Undulate             | Flat          |
| PS 5/4       | Circular  | Brown                | Irregular            | Flat          |
| PS 5/5       | Spindle   | Brown                | Undulate             | Flat          |
| PS 5/6 C     | Circular  | Brown                | Entire               | Positive      |
| PS 5/6 PK    | Circular  | Brown                | Entire               | Positive      |
| PS 5/7       | Circular  | Yellowish Brown      | Entire               | Flat          |
| PS 6/1       | Circular  | Dark Brown           | Undulate             | Flat          |
| PS 6/2       | Circular  | Yellowish White      | Lobate               | Raised        |
| PS 6/3 A     | Irregular | Light Brown          | Undulate             | Raised        |
| PS 6/3 C     | Circular  | Yellowish White      | Entire               | Flat          |
| PS 6/4       | Circular  | Dark Brown           | Entire               | Flat          |
| PS 6/5       | Circular  | Light Brown          | Undulate             | Flat          |
| PS 4/1       | Circular  | Brown                | Undulate             | Flat          |
| PS 4/2       | Irregular | Brown + Yellow       | Undulate             | Convex        |
| PS 4/3       | Irregular | Greyish Brown        | Lobate               | Flat          |
| PS 4/4       | Circular  | Light Brown          | Entire               | Flat          |
| PS 4/5       | Circular  | Brown                | Entire               | Drop-like     |
| PS 4/6       | Spindle   | Brown                | Entire               | Flat          |
| PS 4/7       | Spindle   | Greyish Green        | Entire               | Flat          |
| PS 7/1       | Spindle   | Brownish Yellow      | Undulate             | Flat          |
| PS 8/1       | Spindle   | Purple               | Entire               | Raised        |
| PS 8/2       | Spindle   | Clear Yellow         | Entire               | Raised        |
| PS 8/3       | Circular  | Brown                | Entire               | Flat          |
| PS 8/4 K     | Irregular | Yellow + Orange      | Entire               | Convex        |
| PS 8/4 P     | Spindle   | White                | Entire               | Convex        |
| PS 8/4 U     | Spindle   | Whitish Purple       | Entire               | Convex        |
| PS 8/5       | Spindle   | Clear Yellow         | Entire               | Convex        |
| PS 8/6       | Circular  | Clear Yellow         | Undulate             | Raised        |
| PS 8/7       | Rhizoid   | Clear                | Entire               | Flat          |
| PS 8/8 C     | Irregular | Brown                | Entire               | Raised        |
| PS 8/8 K     | Spindle   | Brown                | Entire               | Raised        |
| PS 8/8 PK    | Irregular | Brown                | Entire               | Flat          |
| PS 8/9       | Rhizoid   | Yellow Orange        | Entire               | Convex        |
| PS 8/12      | Circular  | Clear Brown          | Entire               | Flat          |

Table 1 shows that the results of macroscopic identification reflect the diversity of rhizobacteria isolates obtained based on shape, colouration, surface or edge conditions, and elevation. The rhizobacterial isolate candidates that had been obtained were tested further to examine their ability to produce several compounds, namely growth stimulants (biostimulants) and nutrient providers (biofertilizers). Rhizobacteria are bacteria that colonize plant roots which produce phytohormones to support plant growth and development as well as induce plant resistance. In addition, its role is also a biocatalyst to support the availability of important organic acids for plants. The presence of rhizobacteria as an environmental conservation agent to maintain the biodiversity of root microbes helps support environmentally friendly agriculture that can increase agricultural yields.
The results of macroscopic tests on 37 isolates of rhizobacteria based on shape, colour, surface, edge and elevation can be presumed to belong to several groups of soil bacterial genera such as *Bacillus*, *Pseudomonas*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, and *Serratia* sp. The results of the gram staining test of rhizobacteria produced 25 isolates of gram-positive reaction rhizobacteria and 12 isolates of gram-negative reaction rhizobacteria (Table 1, Figure 2). The response of bacteria to gram stain reactions is an important property to help determine the group or type of bacteria. For example, the *Bacillus* group as one of the rhizosphere-dwelling bacteria can benefit plants with gram-positive reactions which form anaerobic and aerobic endospores [9]. *Pseudomonas* spp is a gram-negative straight or curved rod-shaped bacterium, measuring about 0.6x2 m, found singly, in pairs, and sometimes forming short chains, does not have spores, does not have a sheath, and has a flagellum. Certain *Pseudomonas* bacteria have two or three flagella, and so they are always active. The *Serratia* group of bacteria are gram-negative bacteria from the family *Enterobacteriaceae*. The research has indicated that the biological pigments produced by these bacteria have antifungal, immunosuppressive, and antiproliferative activities [10].

The analysis carried out on the identification of rhizobacteria isolates as PGPR included the ability of rhizobacteria to produce IAA compounds and the ability to solubilize phosphate qualitatively and quantitatively. The test results of the ability of rhizobacteria isolates to produce IAA and the ability to solubilize phosphate were presented in Table 2.

The results showed that 37 isolates of rhizobacteria were able to produce IAA as a whole. The PGPR candidate isolates of rhizobacteria was capable of producing the IAA hormone compound to stimulate plant growth. The rhizobacteria isolated from the exploration was able to produce IAA in the range of values of 21.66 – 83.38 ppm. The rhizobacteria isolate PS 5/1 produced the lowest concentration of IAA at 21.66 ppm with an absorbance value of 0.38 ml⁻¹ filtrate, while isolate PS 5/6C produced the highest concentration of IAA at 83.38 ppm with an absorbance value of 1.5 ml⁻¹ filtrate. IAA compounds released by rhizobacteria as exudates can be absorbed by plant roots which will be used to stimulate meristic cell division and initiation in plant physiological processes. Rhizobacteria are soil bacteria that aggressively colonize plant rhizosphere. The dynamic rhizosphere rich in energy sources from organic compounds released by plant roots (root exudates) is a habitat for various types of microbes to develop, and at the same time a place for microbial hub and competition [11]. Each plant secretes root

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**Figure 2.** Gram staining of several rhizobacteria isolates from patchouli rhizosphere.

**Figure 3.** Microscopic observation of the edges of several rhizobacterial isolates.
exudates with different compositions so that they also act as microbial selectors; its effects can increase the development of certain microbes and inhibit the development of other microbes [12][3]. The more root exudation is, the greater the number and diversity of microbes are. This condition will increase competition in the rhizosphere colonization process. Rhizobacteria are the most efficient competitor microbes that can shift from the position of native microbes in the rhizospheric zone to the middle age of the plant [13].

Table 2. Isolates of rhizobacteria that produce IAA and phosphate solubilizing.

| Isolate Code | Absorbance Value (μ/ml filtrate) | IAA Concentration (ppm) | Halo Diameter (cm) | Colony Diameter (cm) | Phosphate Solubility Index |
|--------------|---------------------------------|-------------------------|-------------------|---------------------|--------------------------|
| PS 4/1       | 0.51                            | 28.38                   | 1.47              | 0.73                | 2.01                     |
| PS 4/2       | 0.77                            | 43.16                   | 1.60              | 0.70                | 2.28                     |
| PS 4/3       | 0.69                            | 38.83                   | 1.00              | 0.70                | 1.42                     |
| PS 4/4       | 0.91                            | 50.61                   | 1.23              | 0.63                | 1.95                     |
| PS 4/5       | 0.93                            | 52.00                   | -                 | -                   | -                        |
| PS 4/6       | 0.58                            | 32.61                   | 1.67              | 0.80                | 2.08                     |
| PS 4/7       | 0.82                            | 45.61                   | -                 | -                   | -                        |
| PS 5/1       | 0.38                            | 21.66                   | -                 | -                   | -                        |
| PS 5/2C      | 0.50                            | 27.83                   | -                 | -                   | -                        |
| PS 5/2H      | 0.53                            | 29.83                   | -                 | -                   | -                        |
| PS 5/3       | 0.50                            | 28.16                   | 1.20              | 0.70                | 1.71                     |
| PS 5/4       | 0.84                            | 47.00                   | 1.50              | 0.77                | 1.94                     |
| PS 5/5       | 1.01                            | 56.16                   | 1.20              | 0.70                | 1.71                     |
| PS 5/6C      | 1.50                            | 83.38                   | 1.47              | 0.80                | 1.83                     |
| PS 5/6 PK    | 0.62                            | 34.94                   | 1.37              | 0.80                | 1.71                     |
| PS 5/7       | 0.72                            | 40.27                   | -                 | -                   | -                        |
| PS 6/1       | 0.61                            | 34.05                   | 1.57              | 0.70                | 2.24                     |
| PS 6/2       | 0.60                            | 33.38                   | 1.40              | 0.63                | 2.22                     |
| PS 6/3A      | 0.65                            | 36.50                   | 1.67              | 0.70                | 2.38                     |
| PS 6/3C      | 0.81                            | 45.33                   | 1.00              | 0.73                | 1.36                     |
| PS 6/4       | 0.62                            | 34.50                   | -                 | -                   | -                        |
| PS 6/5       | 0.67                            | 37.38                   | -                 | -                   | -                        |
| PS 7/1       | 0.64                            | 36.00                   | 1.97              | 0.77                | 2.55                     |
| PS 8/1       | 0.53                            | 29.61                   | -                 | -                   | -                        |
| PS 8/2       | 0.65                            | 36.16                   | 1.60              | 0.77                | 2.07                     |
| PS 8/3       | 0.54                            | 30.16                   | 1.67              | 0.80                | 2.08                     |
| PS 8/4K      | 0.57                            | 32.05                   | 1.90              | 0.80                | 2.37                     |
| PS 8/4P      | 0.48                            | 27.05                   | 1.10              | 0.80                | 1.37                     |
| PS 8/4U      | 0.46                            | 25.88                   | 1.93              | 0.83                | 2.32                     |
| PS 8/5       | 0.60                            | 33.50                   | 1.50              | 0.70                | 2.14                     |
| PS 8/6       | 0.69                            | 38.72                   | -                 | -                   | -                        |
| PS 8/7       | 0.46                            | 25.94                   | 1.03              | 0.77                | 1.33                     |
| PS 8/8C      | 0.60                            | 33.61                   | 1.63              | 0.80                | 2.03                     |
| PS 8/8K      | 0.41                            | 23.27                   | 1.43              | 0.80                | 1.78                     |
| PS 8/8 PK    | 0.69                            | 38.61                   | 1.97              | 0.80                | 2.46                     |
| PS 8/9       | 0.64                            | 35.83                   | -                 | -                   | -                        |
| PS 8/12      | 0.42                            | 23.83                   | 1.53              | 0.80                | 1.91                     |
IAA hormone is a growth regulator that plays a role in the process of plant growth. IAA produced by microbes in the rhizosphere can increase the number of root hairs and size and the number of adventitious roots of plants [14]. The IAA hormone is synthesized as a secondary metabolite produced under conditions of suboptimal bacterial growth or when the amino acid precursor tryptophan is available. It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage, and substrate availability. Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil [15][16].

In addition, the test results of 37 isolates of rhizobacteria differed in their ability to solubilize phosphate. There were 10 isolates that did not have the ability to solubilize phosphate (Table 2). The ability of rhizobacteria to solubilize phosphate is an indication that rhizobacteria can act as PGPR that can stimulate plant growth and release bounded-phosphate in the soil to become available to plants. The rhizobacteria isolates PS 7/1 produced the highest PSI of 2.55 and the isolates PS 8/7 produced the lowest PSI of 1.33.

Rhizobacteria isolates capable of phosphate solubilizing were characterized by the formation of a clear zone around the bacterial colonies when grown on solid Pikovskaya medium (Figure 5). The test for the ability of rhizobacteria to solubilize phosphate was marked by the formation of a clear zone around the bacterial colonies tested on Pikovskaya medium due to the breakdown of Ca₃(PO₄)₂ contained in the medium [17]. Rhizobacteria have different abilities in dissolving phosphate. Phosphate solubilizing rhizobacteria improve plant phosphorus nutrition by mobilizing inorganic and organic phosphates. Several mechanisms of phosphate solubilization have been reported. Phosphate solubilizing microbe produces different types and amounts of organic acids. Some groups of organic acids produced by rhizobacteria are citric acid, succinic acid, fumaric acid, gluconic acid, oxalic acid, malic acid, and gluconic acid [18]. Organic acids produced by microorganisms vary in quality and quantity in liberating phosphate. Different mechanisms of inorganic phosphate solubilization are acidification due to the production of organic acids and inorganic acids, as well as H⁺ excretion, exopolysaccharide production, and siderophore production [19]. Phosphate-solubilizing bacteria (PSB) have the ability to solubilize insoluble phosphorus (P) and release soluble P. Phosphate solubilizing rhizobacteria convert unavailable P to available P to meet the needs of plants through reshuflle and absorption [20].
Colony size does not always affect the formation of a clear zone around the colony because a large colony diameter does not always result in a large clear zone diameter. In principle, the clear zone area can show the ability of rhizobacteria to solubilize phosphate, but it cannot show the amount of phosphate concentration dissolved in the medium. Isolates of rhizobacteria that form clear zones faster and have a broad Phosphate Solubilization Index (PSI) value are phosphate solubilizing bacteria that have the potential as biofertilizers by solubilizing phosphate elements bound to other elements such as Fe, Al, Ca, and Mg, and thus, phosphate elements become available [21]. A biofertilizer consisting of a consortium of bacteria that has the ability to dissolve phosphate, dissolve potassium, and produce auxin can have a high potential for increasing growth and yields [22]. Isolated rhizobacteria with codes PS 4/1, PS 4/2, PS 4/4, PS 4/6, PS 5/3, PS 5/4, PS 5/5, PS 5/6C, PS 5/6K, PS 6/1, PS 6/2, PS 6/3A, PS 6/3C, PS 7/1, PS 7/2, PS 7/3, PS 8/4K, PS 8/4P, PS 8/4U, PS 8/5, PS 8/7, PS 8/8C, PS 8/8K, PS 8/8PK, and PS 8/12 were bacterial isolates that have the potential as biological fertilizers because of their ability to form clear zones compared to other isolates.

4. Conclusion

The results of exploration, identification, and characterization produced 37 isolates of indigenous rhizobacteria from patchouli rhizosphere located in Purwosari, Nagan Raya, Aceh Province, Indonesia. Thirty-seven rhizobacteria isolates had the ability to produce IAA. The rhizobacteria isolate PS 5/1 gained the lowest IAA at a concentration of 21.66 ppm, while isolate 5/6 C produced IAA at 83.38 ppm. Twenty-five rhizobacteria isolates could dissolve phosphate and the remaining 12 isolates did not have this ability. The rhizobacteria isolate PS 7/1 produced the highest PSI at 2.55 and the isolates PS 8/7 gained the lowest PSI at 1.33. Rhizobacteria isolates that produce IAA and able to solubilize phosphate have the potential to be used as PGPR candidates.

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