Isolation and Identification of Rhizosphere Bacteria Associated with Taro (*Colocasia esculenta* L. Schott) as Plant Growth Promoting Rhizobacteria

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Abstract

Taro is one of the food crops that has the potential to maintain food security. It has a high carbohydrate content and nutrients with a low glycemic index value. Taro is widely cultivated with intercropping techniques, especially in Kedungkandang District, Malang City. However, this technique caused a decrease in talas tubers production with only 7-10 tons ha^{-1}. This number was lower than the total optimal production with 20.7 tons ha^{-1} of taro tubers. Plant Growth Promoting Rhizobacteria (PGPR), one of the most potential biofertilizers, can solve this problem with abilities such as phosphate-solubilizing, production of IAA, and nitrogen fixation. This research aimed to analyze the potency and identify the species of taro rhizosphere bacteria that had the best ability as PGPR agents. Taro rhizosphere bacteria were isolated using Pikovskaya medium and TSA (Tryptic Soy Agar) respectively. The PGPR abilities were evaluated on the phosphate-solubilizing, production of IAA, and nitrogen fixation. A total of 12 isolates for phosphate-solubilizing bacteria and ten isolates of IAA-producing bacteria. The highest concentration of phosphate solubilization was P1 isolate, with the concentration of 6.8 µg.mL^{-1}; while I4 isolate had the highest potency for IAA production with the concentration of 23.11 µg.mL^{-1}. Isolates P1 and I4 were selected for the nitrogen fixation ability test. P1 isolate shows the highest ammonia concentration of 2.52 µg.mL^{-1}, and it was identified as *Stenotrophomonas rhizophila* with a similarity of 99.91% with *Stenotrophomonas rhizophila* R2A2 67. The potential isolates can be used as PGPR agents or biofertilizers to increase the production of taro plants.

Keywords: *Colocasia esculenta*, IAA, Nitrogen, PGPR, Phosphate.

INTRODUCTION

In Indonesia, agricultural sector plays a crucial role to fulfill food consumption for national economic resilience [1]. Food production is required to support population growth, where in the last 20 years, Indonesia's population has increased by 63.9 million people, this number equivalent to 26.9% of the total population in 2010 [2]. The increasing number of the population every year also increases the country's food needs [3].

The government encourages national food security with several strategies such as increasing agricultural production, optimizing and expanding land, as well as developing food diversity other than rice [4]. The taro commodities are one of the potential food contributing to food security in terms of their production potential [5]. Taro is a highly nutritious source of carbohydrates with a lower glycemic index value of 54 compared to other tuber crops such as potatoes and rice.

Kedungkandang District, which is the location of the sampling area, generally uses the intercropping technique, namely the technique of planting several types of plants in one area with a predetermined plant distance. The intercropping technique causes a decrease in plant growth due to limited nutrients and water, which results in a decrease in tuber productivity [6,7]. The optimal production potency of taro tubers can reach 20.7 tons ha^{-1}, but observations show that production only reaches about 7-10 tons ha^{-1} [8].

Biofertilizer is a fertilizer that contains microorganisms and is practical for increasing soil fertility and crop production [9]. One of the biofertilizers that are often used and have the potency for agricultural resilience can help ensure the availability of potential nutrients for plants and increase the use of nutrients more efficiently, namely Plant Growth Promoting Rhizobacteria (PGPR) [10].

PGPR has abilities, such as phosphate solubilization, which is required in all major metabolic processes in plants, as well as energy transfer, signal transduction, photosynthesis, and respiration [11]. Another ability for nitrogen fixation, which is the most important nutritional element for plants after phosphate and the production of phytohormones IAA, is a hormone that is very influential on plant growth [12]. Based on research from Soubeih and El-Sayed

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[13] on potato and garlic plants, using an intercropping technique with biofertilizer agents, namely *Azotobacter chrococcum*, *Bacillus megaterium*, *Thiobacillus thioparus*, showed increased growth from potato sprouting to height and weight of plants, leaf width, also increased garlic and production potato tuber.

Combination treatment among biofertilizer agents and minerals showed significant results in the growth and yield of tubers and cormels on taro plants [14]. The use of biofertilizer agents such as *Azotobacter* with nitrogen fixation potential, Vesicular Arbuscular Mycorrhizae (VAM), and Phosphate solubilizing bacteria (PSB) can minimize the use of inorganic fertilizers as well as increase the harvest index and dry matter content of roots, corms and leave of taro plants [15]. Therefore, this research aims to analyze the potential and identify the species of taro rhizosphere bacteria that have the best ability as PGPR agents.

MATERIAL AND METHOD
Taro Rhizosphere Soil Sampling

Soil samples were obtained from taro agricultural land in Tlogowaru Village, Kedungkandang District, Malang, East Java, Indonesia at 582 m.a.s.l (08'01'38.4"S 112' 40' 16.3"E). Samples were taken compositely at one location from three sampling areas, each area consisting of three plants. The sample had taken at 3-5 points per plant in the soil around the root area. Soil samples were taken using plastic slips, then stored in a cool box and brought to the Microbiology Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University. Physicochemical measurements include the C/N ratio, pH, organic matter, and water content. The C/N ratio and organic matter were analyzed in the Soil Laboratory of the Faculty of Agriculture, Brawijaya University.

Isolation of Taro Rhizosphere Bacteria

A total of 25 g of soil sample was diluted into 225 mL of 0.85% NaCl solution [16]. The serial dilution was made from $10^{-1} - 10^{-6}$. A total of 0.1 mL aliquot of the sample suspension was poured into a Petri dish containing Pikovskaya agar medium for isolation of phosphate solubilizing bacteria and TSA medium for isolation of IAA producing bacteria, respectively. The Pikovskaya agar consists of (g.L^{-1}) Glucose 5, CaHPO$_4$ 2.5, KCl 0.1, (NH$_4$)$_2$SO$_4$ 0.25, NaCl 0.1, MgSO$_4$.7H$_2$O 0.025, MnSO$_4$.H$_2$O 0.25, FeSO$_4$.7H$_2$O 0.25, yeast extract 0.25 and agar 10. The TSA medium consisted of TSA 40 g.L^{-1}, L-tryptophan 200 μg.mL^{-1}. The

culture was incubated at 30°C for 48-72 hours. Each isolates that grown on TSA and Pikovskaya agar media was observed and enumerated based on the Total Plate Count (TPC) formula and was purified using the spread plate method.

Quantitative Assay of Phosphate Solubilizing Bacteria

The ability of bacteria to solubilize phosphate was carried out by a quantitative test based on Ahmad *et al.,* [17] with minor modification. A total of one loop of bacterial culture were inoculated into 15 mL of Pikovskaya broth medium (pH 7) and then incubated in a shaker at 120 rpm, at 30°C, for 48 hours, then the OD was equalized. A total of 2 mL cultures were inoculated into 20 mL Pikovskaya broth medium (pH 7) then incubated in a shaker at 120 rpm, temperature of 30°C for 72 hours. Then 2 mL of culture were centrifugated at 10.000 rpm for 20 minutes, cultures were taken at 0, 24, 48, 72 hours. A total of 1 mL supernatant was homogenized with 10 mL of Molybdate reagent and 40 mL of distilled water, then added 0.1 mL of SnCl$_2$. The suspension was incubated for 10 minutes at 30°C until the color changed to blue. The absorbance value was measured using a spectrophotometer at 660 nm. The concentration of phosphate solubilizing concentration was calculated.

Quantitative Assay of IAA-Producing Bacteria

The ability of bacteria to produce IAA was carried out using a quantitative test [18]. A total of one loop of bacterial culture was inoculated into 25 mL of Tryptic Soy Broth (TSB) medium with 2% L-Tryptophan added, then incubated in a shaker at 120 rpm at 30°C for 48 hours and then the OD was equalized. Four milliliters of cultures were inoculated in 40 mL TSB with 2% L-Tryptophan and incubated in a 120 rpm shaker at 30°C for 72 hours. Three milliliters of culture were centrifuged at 0, 24, 48, and 72 hours at 10,000 rpm for 10 minutes. Two-milliliter supernatant was homogenized with 4 mL of Salkowsky's reagent. The suspension was incubated in a dark room for 30 minutes or until the suspension color turns pink. The absorbance value was measured using a spectrophotometer at 530 nm.

Quantitative Assay of Nitrogen Fixation Bacteria

Isolates that showed the best ability in the previous test were used for the nitrogen fixation test. One loop of culture was inoculated on semisolid N-free media with the addition of

bromothymol blue and incubated at 30°C for 72 hours. Isolates in the medium that changed color from green to bluish were used for quantitative testing based on Setia *et al.* [19] with minor modification. One loop of bacterial culture was inoculated into 10 mL of N-free (without Bromothymol Blue) and incubated in a shaker at 120 rpm at 30°C for 24 hours. Ten milliliters of culture were inoculated into 90 mL of N-free medium and incubated in a 120 rpm shaker at 30°C for 72 hours. Fifteen milliliters of culture at 0, 24, 48, and 72 hours incubation were centrifuged at 10.000 rpm for 10 minutes. Ten milliliters of the supernatant were added with the sera ammonia test kit, homogenized, and incubated for 5 minutes at room temperature 28-30°C, and the color changed to green. The absorbance of suspension was measured using a spectrophotometer at 700 nm. Ammonia concentration values were calculated based on the standard ammonia curve.

Identification of Bacteria Based on 16S rDNA

Potential PGPR bacterial chromosomal DNA was extracted using the Quick-DNA™Fungal/Bacterial Miniprep Kit (ZYMO RESEARCH, USA). Amplification of 16S rDNA sequences using universal primers 27f (5'-GAG AGT TTG CTG GCT ATC CAG-3') and 1492r (5'-CTA CGG CTA TGT CCT TAC GA-3'). The composition of the PCR mix was 94°C (5 minutes) followed by denaturation (94°C; 0.5 minutes), annealing (55°C; 0.5 minutes), extension (72°C; 1.5 minutes) to 35 cycles and post-extension (72°C, 7 min) [19]. The 16S rDNA amplicon was sequenced at First Base Malaysia. The 16S rDNA sequences were aligned with reference strains from the GenBank database. Construction of phylogeny tree using MEGA 11 program, according to Neighbor-Joining algorithm with 1000 bootstraps.

Data analysis

The quantitative data of phosphate-solubilizing, IAA production, and nitrogen-fixing bacteria was analyzed based on a Two-Way ANOVA. It was followed by a Tukey test using the SPSS 26 programs trial edition.

RESULT AND DISCUSSION
Soil Physicochemical Environmental Parameter Analysis

The results of soil environmental parameters (Table 1) were assessed based on criteria that referred to data from the Soil Research Center (Balai Penelitian Tanah) [20]. Soil pH can indicate nutrient content in the soil and can have an impact on the existence of microorganisms in the soil [21]. The pH of the soil was categorized as neutral, while the C-organic and N-total content were in a low category, and the organic matter content was classified on average. From these results, it can conclude that the acidity of the soil is not optimal for taro growth. According to Setiawan *et al.* [22], soil with low pH or acidic has an impact on stunted plant growth. But this can be solved by providing phosphate solubilizing bacteria that can produce organic acids, so it can increase soil alkalinity and provide dissolved phosphate for plant growth [23].

Table 1. Soil physicochemcial and environmental parameters

No.	Parameters	Value
1	Light intensity (×100 lux)	132.000
2	Humidity (%)	73.000
3	Soil temperature	27.100
4	C - organic matter (%)	1.135
5	N total (%)	0.116
6	C/N Ratio	9.330
7	Organic matter (%)	1.920
8	Moisture content (%)	56.150
9	Soil pH	7.530
10	Density of phosphate-solubilizing bacteria (10^5 CFU.g^{-1})	36.800
11	Density of bacteria producing IAA (10^5 CFU.g^{-1})	121.260

Carbon is an important element that indicates soil fertility, plays a major role as a food source for microorganisms, and can affect the abundance of phosphate and nitrogen. The elements decomposed by microorganisms. C-organic and N-total are categorized as less than optimal to support plant growth. The low carbon element in the soil causes a lack of food sources for microorganisms resulting in less decomposition of nitrogen elements [24]. The content of organic matter is categorized as average so that it's able to support plant growth. Organic matter has an impact on increasing the abundance of microorganisms and the growth of plant roots [25].

Quantitative Test of Plant-growth Promoting Potential
Phosphate Solubilizing Bacteria

A total of 12 isolates with different morphology showed different phosphate concentrations at each incubation time (Fig. 1). P1 isolate has the highest phosphate solubilizing potency with a concentration of 6.8 µg.mL^{-1} at 48 hours, compared to other isolates. The isolates

such as P1, P8, P9, P12 also showed the highest concentration at 48 hours. The other isolate with the highest concentration was P12 with a concentration of 5.43 μg.mL^{-1} at 48 hours, followed by P8 at 5.41 μg.mL^{-1} at 48 hours. In this study, P1 which was identified as *Stenotrophomonas rhizophila* had a higher phosphate solubilization concentration 6.8 μg.mL^{-1} than *Stenotrophomonas maltophilia* IMP289 with a concentration of 0.13 μg.mL^{-1} and *Stenotrophomonas maltophilia* 46 0.43 μg.mL^{-1} and *Stenotrophomonas maltophilia* μg.mL^{-1}[26]. Variations results in phosphate concentrations from bacteria are influenced by different types of microorganisms caused by the influence of pH, humidity, and soil temperature so that the population and diversity of microorganisms are also more diverse [12].

IAA Production Bacteria

A total of 10 isolates with different morphology showed different IAA production abilities (Figure 2). Isolate I4 had the highest IAA production with a concentration of 23.11 μg.mL^{-1} at 48 hours and 14.43 μg.mL^{-1} at 72 hours, while isolates I7 had the highest IAA concentration of 10.06 μg.mL^{-1} at 48 hours and 8.24 μg.mL^{-1} at 72 hours. A decrease in the IAA concentration of isolates I4 and I7 occurred at 72 hours of incubation, then followed by isolate I9 with 8.87 μg.mL^{-1} concentration at 24 hours. The decrease in concentration could be due to the bacteria having passed the optimal phase and the lack of nutrients in the medium, so the bacteria remodel the hormones produced to be used in the growth process [27].
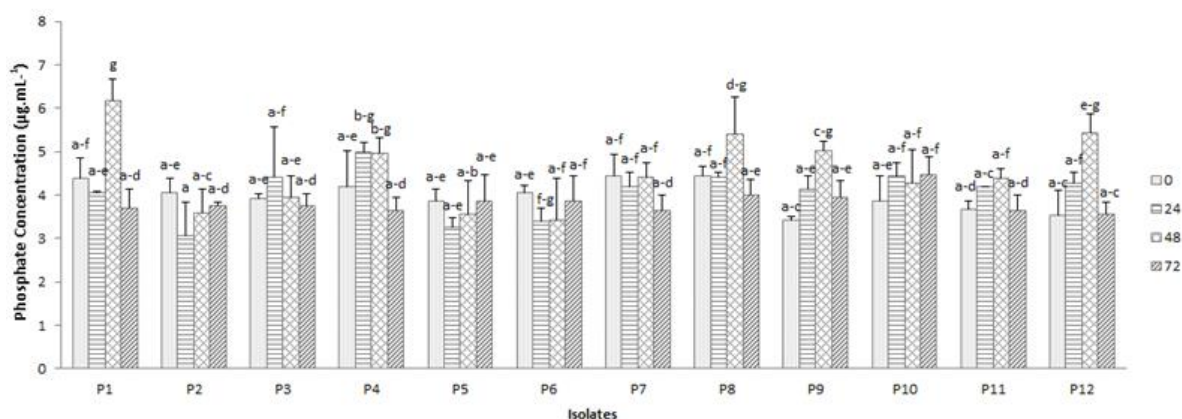

Figure 1. The concentration of dissolved phosphate by bacteria at different incubation times
*Data were analyzed using two way ANOVA at α= 0.05. It describe the difference in phosphate-solubilizing among isolates and incubation time (p<0.05).
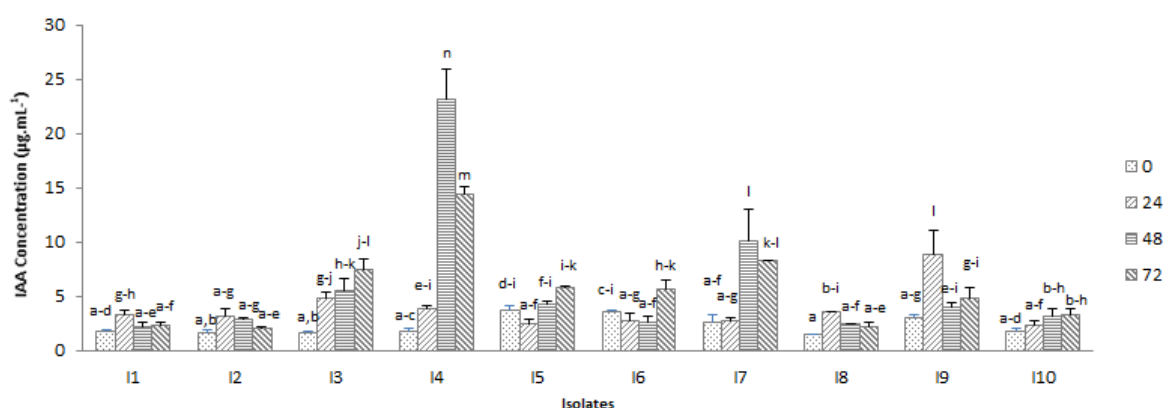

Figure 2. The concentration of IAA hormone production by bacteria at different incubation times
*Data were analyzed using two way ANOVA α= 0.05. It describe the difference in IAA production among isolates and incubation time (p<0.05).

This result was higher than some plant rhizosphere bacteria such as isolate S7.3 with a concentration of 7.944 μg.mL^{-1} in suren plants [28] and star fruit isolates at 16.71 μg.mL^{-1} [29]

much higher than isolates from corn plant-bacteria with a concentration of 0.9361 µg.mL^{-1} [30]. The addition of L-Tryptophan to liquid media according to Mohite [31] is considered as IAA precursor because it can increase the production of IAA in culture, media with the addition of L-Tryptophan has a higher production than that which is not added. Some examples of potential IAA-producing bacteria such as *Pseudomonas* sp., *Bacillus* sp., *Azotobacter* sp. Several strains showed different concentrations of production, and even strains in the genus *Bacillus* produced different IAA concentrations. IAA production is influenced by culture conditions, growth phases, and substrate availability [32].

Nitrogen Fixation Bacteria

Bacterial isolates that had the highest ability in phosphate solubilizing and IAA production were selected for the nitrogen fixation test. I4 and P1 isolates showed a blue color change on Nitrogen Free Bromothymol Blue (Nfb) medium. Thus, both isolates were selected for the quantitative test of nitrogen fixation ability.
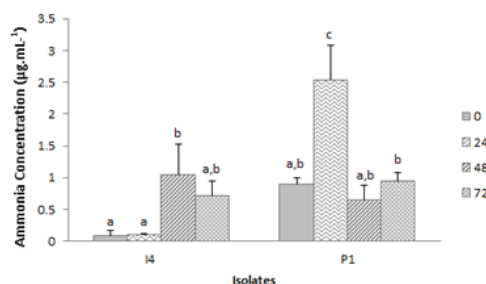
Figure 3. Ammonia concentration by bacteria at variant incubation times

*Data were analyzed using two way ANOVA at α= 0.05. It describe the difference in ammonia production among isolates and incubation time (p<0.05).

Isolate P1 with the highest concentration of 2.52 µg.mL^{-1} at 24 hours and decreased at 48 hours to 0.65 µg.mL^{-1}. Isolate I4 with the highest concentration of 1.04 µg.mL^{-1} at 48 hours and decreased to 0.72 µg.mL^{-1} at 72 hours of incubation. From these results, it was concluded that isolate P1 had a higher nitrogen fixation ability than isolate I4 (Fig. 3). The comparison of the ability to produce ammonia will be different for each type of microorganisms and can be caused by the variation of bacteria type, oxygen content in the growth medium, as well as the variation in carbon sources that can affect the ability of bacterial cells to excrete ammonium [33].

Phylogenetic tree of Rhizosphere Bacterial Species Based on 16S rDNA

Three isolates with the best ability were selected in each test, isolates P1, P8, P12 with the ability of phosphate-solubilizing and isolates I4, I7, I9 with the ability of IAA producing. The six isolates were tested for pathogenicity using blood agar media. Isolate P1 and I7 did not show lysis on blood agar media, so the isolate was concluded as non-pathogenic. Isolate P1 was chosen as the potential PGPR isolate, with the ability to solubilize phosphate and nitrogen fixation, was constructed using a phylogenetic tree based on 16S rDNA and compared with the reference strain. The P1 was identified as *Stenotrophomonas* at 99.91% similarity with *S. rhizophila* R2A2 67 (Fig. 4). *Stenotrophomonas rhizophila* is a rhizosphere bacteria and plant endosphere bacteria in all plant phylogenies. These bacteria produce osmoprotective substances and provide protection from pathogens, either bacteria or fungi [26].
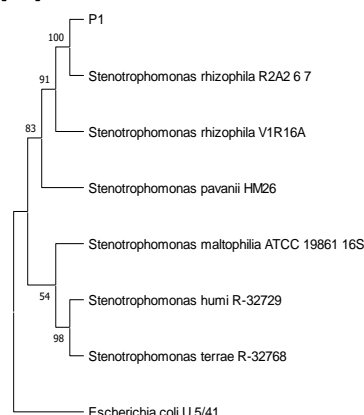
Figure 4. Phylogeny tree of potential PGPR isolates P1 and reference strains according Neighbor-Joining algorithm with 1000 bootstraps, using MEGA 11 program

The *S. rhizophila* was rarely studied for PGPR, especially for Phosphate-Solubilizing Bacteria (PSB) and Nitrigex Fixing Bacteria (NFB). However, the type of *S. maltophilia* has been confirmed to have the presence of the *nifH* gene [34]. According to Kumar and Audipudi [35], *S. maltophilia* AVP27 from chili plant rhizosphere has the ability to produce 80 µg.mL^{-1} of ammonia and confirm to dissolve phosphate [36]. However, *S. maltophilia* has pathogenic characteristics caused by its lower optimum growth temperature [37] than *S. rhizophila*.

CONCLUSIONS

P1 isolates from the rhizosphere of the taro plant in Tlogowaru Village showed the highest potential for phosphate solubilizing with the concentration of 6.8 µg.mL-^{1} at 48 hours and nitrogen fixation of 2.52 µg.mL-^{1} at 24 hours. P1 isolate was identified as *Stenotrophomonas rhizophila* with similarity (99.91%). P1 isolate has the ability to be used as a biofertilizer agent for the cultivation of taro plants.

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