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Phosphate-Solubilizing and Indole Acetic Acid-Producing Actinomycetes from the Plant Rhizosphere in the Hungayono Karst, Gorontalo

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Abstract

Actinomycetes are soil microorganisms that play a crucial role in nutrient cycles and interactions, particularly in nutrient-limited environments such as karst ecosystems. This study aimed to evaluate the preliminary functional potential of actinomycetes isolated from the plant rhizosphere in the Hungayono karst ecosystem, Gorontalo, as phosphate solubilizers and producers of indole acetic acid (IAA). The study employed a quantitative descriptive approach with soil sampling at a depth of 10–15 cm using purposive sampling. Isolation was performed using the spread plate method on Starch Casein Agar medium, followed by qualitative and quantitative testing of phosphate solubilization and IAA production capabilities. Identification of selected isolates was conducted through 16S rRNA gene-based phylogenetic analysis. The results showed that 11 actinomycete isolates were obtained from 4 host plants, of which two isolates exhibited low-index qualitative phosphate solubilization activity. However, quantitative results indicated that some isolates that did not form clear zones still produced measurable amounts of soluble phosphate, suggesting differences in method sensitivity. The highest phosphate-solubilizing activity was exhibited by isolate RzPO-09. In addition, four isolates were capable of producing IAA at varying levels, with the highest production observed in isolate RzPH-07 at 0.896 ppm. Phylogenetic analysis showed that isolate RzPO-09 (PZ458677) is closely related to the genus Pseudonocardia, while RzPH-07 (PZ458434) is closely related to the genus Streptomyces. Overall, the isolates obtained show initial functional potential; however, further validation is required through optimization of culture conditions, genetic characterization, plant growth tests, and testing both in greenhouses and in the field to ensure their practical feasibility.

Keywords: Actinomycetes; IAA; Karst; Phosphate; Rhizosphere.

INTRODUCTION

Karst ecosystems generally have low soil fertility, in terms of both physical, chemical, and biological properties. The relatively extreme environmental conditions in these ecosystems act as limiting factors for the growth of living organisms, including plants and microorganisms (Mubarak *et al*., 2017). Karst soils are known to have low levels of essential nutrients such as nitrogen (N), phosphorus (P), potassium (K), and carbon (C), but they have high levels of calcium (Ca) and magnesium (Mg) as well as a pH that tends to be alkaline (Hao *et al*., 2015). These conditions limit the availability of nutrients, particularly phosphate, as they are bound by calcium in complex forms that are difficult for plant roots to absorb (Pan et al., 2018). This limitation drives plants in karst ecosystems to adapt, one of which is through interactions with microorganisms in the rhizosphere (Pakaya, 2025).

The rhizosphere is the zone surrounding plant roots where intensive interactions occur between the roots and soil microorganisms. This environment is rich in root exudates that support microbial activity, particularly that of aerobic microorganisms. One group of microorganisms that plays a crucial role in supporting plant growth in the rhizosphere is actinomycetes (Lanti, 2025). Actinomycetes are known for their high adaptability to various extreme environmental conditions, including dry, nutrient-poor soils such as those found in karst ecosystems.

Based on their functions, actinomycetes provide various mechanisms that support plant growth. These bacteria are capable of secreting exopolysaccharides when faced with drought, as well as producing the enzyme ACC deaminase, which reduces ethylene levels in plants (Silitonga and Nurwahyuni, 2013). Furthermore, actinomycetes are known to solubilize phosphate through the synthesis of organic acids, which ultimately makes phosphorus more readily available to the soil. The availability of phosphorus is crucial for plant growth because of its role in various metabolic pathways. Not only that, actinomycetes also play a role in producing indoleacetic acid (IAA), an auxin hormone that is essential for lateral root formation, cell division

and specialization, and shoot growth (Patma *et al*., 2013). With their combined ability to solubilize phosphate and produce IAA, Actinomycetes are highly promising microbes for supporting plant development.

The adaptability of actinomycetes has also been reported in various extreme environments, such as thermotolerant and thermophilic conditions (Prabhu *et al*., 2024), dry soil (Zucchi *et al*., 2012), compost (Yan *et al*., 2011), desert soil (Busarakam *et al*., 2016), geothermal soils (Jiao *et al*., 2015), hydrothermal vents (Zhang *et al*., 2017), hot spring sediments (Duan *et al*., 2014), and karst ecosystems (Retnowati, 2024). In Gorontalo Province, research on actinomycetes in karst ecosystems has been conducted by Matalauni (2025), who successfully isolated actinomycetes from the plant rhizosphere in the karst area of Kota Barat with the ability to produce IAA at a concentration of approximately 0.133 mg/L.

However, to date, no studies have examined the presence of actinomycetes in the Hungayono karst area. This area has distinctive environmental characteristics, including steep limestone cliffs, caves, underground rivers, and natural hot springs, indicating hydrothermal activity that could potentially influence soil chemistry. These conditions allow for the formation of a microbial community distinct from that found in other karst areas. Based on this, there remains a knowledge gap regarding the exploration of rhizosphere actinomycetes in the Hungayono karst region, particularly concerning their ability to solubilize phosphate and produce IAA. Therefore, this study aimed to explore and identify actinomycetes from the plant rhizosphere in the Hungayono karst area, Gorontalo, as well as to test their ability to solubilize phosphate and produce IAA through initial screening and molecular identification of selected isolates.

MATERIALS AND METHODS

Study area

Soil samples from the rhizosphere were collected from the Hungayono karst area, Tulabolo Village, East Suwawa Subdistrict, Bone Bolango Regency, Gorontalo, at three locations using the purposive sampling method. The selection of sampling points was based on differences in microhabitats, soil conditions, vegetation cover, and accessibility, ensuring they represent the environmental variation in the area. The selection of sampling points was based on an elevation gradient, with Point 1 at the bottom, Poin 2 in the middle, and Point 3 at the summit, while also considering soil conditions. The host plants selected were young, naturally growing plants. The plants at each point referred to different individual plants, for a total of 4 species. Rhizosphere soil samples were collected at a depth of 10–15 cm around the roots (Maulana et al., 2022), then placed in sterile plastic bags, labeled, and stored in a cool box (Retnowati et al., 2024). Environmental parameters, including temperature, pH, and soil moisture, were measured at each sampling point (Figure 1).

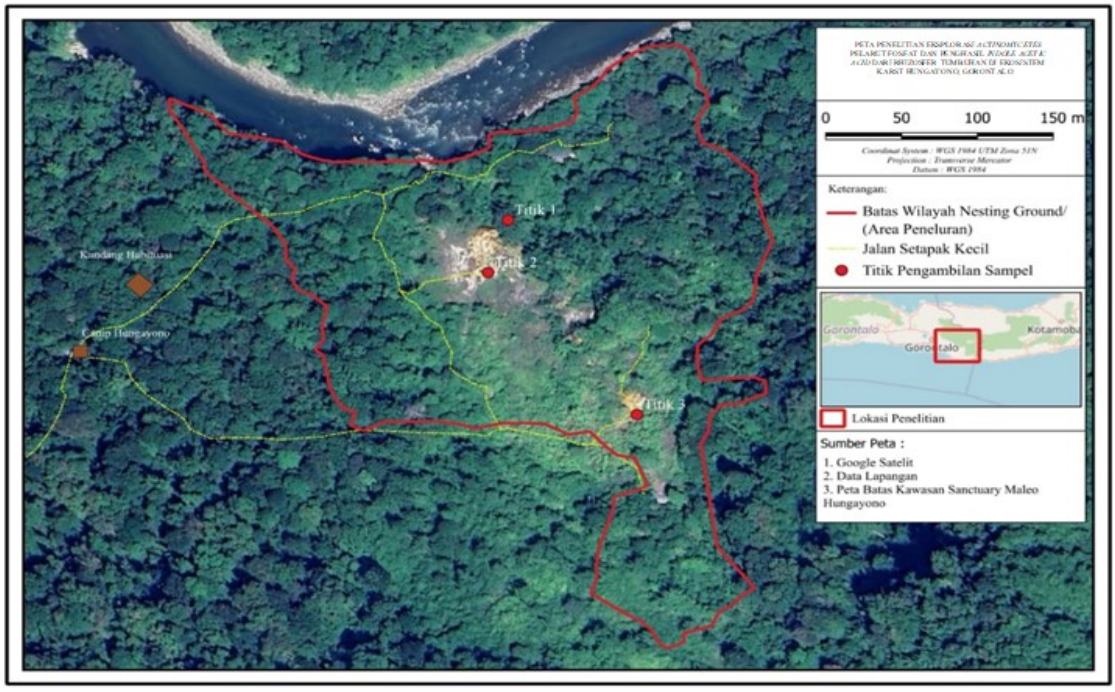

Figure 1. The Hungayono karst site in Gorontalo was selected as the location for collecting rhizosphere soil samples: Point 1 (0°30′19.4“N 123°17'29.5”E), Point 2 (0°30′18.13“N 123°17'29.08”E), Point 3 (0°30'14.7“N 123°17'32.25”E).

Procedures

Rhizosphere soil sampling from the Hungayono Karst Region, Gorontalo

This study is an exploratory screening study of actinomycetes from the rhizosphere of plants in the Hungayono karst area. Rhizosphere soil samples were collected at three sites using purposive sampling. Soil samples were collected from each plant at a depth of 10–15 cm using a small shovel (Maulana *et al*., 2022). Each soil sample from each plant and sampling point was considered an independent biological sample unit. The samples were then placed in sterile plastic bags, labeled according to the location/sampling point code and plant name, and stored in a cool box to maintain sample quality (Retnowati et al., 2024). Measurements of soil temperature, pH, and moisture were taken at each sampling point using a soil tester.

Isolation and Purification of Actinomycetes from the Hungayono Karst Region, Gorontalo

Hirteen plant rhizosphere samples were collected from three locations. The samples were ground using a mortar and pestle; then, 5 g of soil sample was suspended in 45 mL of sterile Ringer's solution and homogenized using a shaker incubator at 225 rpm until uniform. The soil suspension was then serially diluted from 10^{-1} to 10^{-5}. From dilution levels of 10^{-3}, 10^{-4}, and 10^{-5}, 100 µL of the suspension was inoculated onto Starch Casein Agar (SCA) medium using the spread plate method. Each soil sample was analyzed in 2 replicates at each dilution level. Thus, a total of 72 Petri dishes were used, derived from 3 dilution levels × 2 replicates × 12 rhizosphere soil samples from the 3 locations. Nystatin antibiotic was added to the SCA medium at a concentration of 25 µg/mL to inhibit the growth of fungi that could interfere with isolation (Aminnullah *et al*., 2020). The petri dishes were then incubated at 37°C for 14–28 days. Next, the colonies confirmed to be actinomycetes were isolated using SCA medium via the streaking technique.

Analysis of Phosphate Solubilizing Ability

The ability of actinomycetes to phosphate solubilize was tested using two methods: qualitative and quantitative. The qualitative method was used as an initial screening step to identify the presence of phosphate-solubilizing activity based on the formation of a clear zone (halo) around the colonies on Pikovskaya agar medium. Meanwhile, the quantitative method was used to more accurately measure the amount of dissolved phosphate produced, expressed in units of mg/L (ppm). Thus, the qualitative method indicates the presence of phosphate-solubilizing ability, while the quantitative method describes the level or efficiency of phosphate-solubilizing.

A qualitative test of the ability of actinomycetes to solubilize phosphate was conducted by taking 1-cm-long pieces from each purified actinomycete isolate colony, growing them on Pikovskaya medium, and incubating them for 7 days at 30°C (Asril & Lisafitri, 2020); each isolate had three replicates. The growth of phosphate-solubilizing bacterial colonies on Pikovskaya medium was indicated by the formation of a clear zone around the bacterial colonies (Rini *et al*., 2020). This clear zone was then measured to determine the ability of actinomycetes to dissolve phosphate. According to (Elias *et al*., 2016), the Phosphate Solubility Index (PSI) is measured as follows:

$$\text{PSI} = \frac{colony\ diameter + Diameter\ of\ the\ clear\ zone}{colony\ diameter}$$

According to Silva and Vidor (2000) as cited in Alfiansyah (2023), the phosphate solubility index is categorized as follows: if <1.00, the phosphate solubility index is very low; 1.00–2.00 indicates low; 2.00–3.00 indicates moderate; and if >3.00, it indicates high.

The quantitative assessment of actinomycetes ability to phosphate-solubilizing was conducted by incubating each actinomycetes culture in liquid Pikovskaya medium, with three replicates of each isolate culture, at 26°C and continuously shaking them in a shaking incubator at 130 rpm for 7 days. Each replicate of the isolates was then centrifuged at 4,000 rpm for 15 minutes (Septariana, 2015). A total of 1 mL of the supernatant produced from each replicate of each isolate was taken and added to 2.5 mL of 2,5% sodium molybdate solution and 1 mL of 0,3% hydrazine sulfate to initiate the reaction (Hartanto et al., 2023). The phosphate reagent was allowed to stand for 15 minutes. Spectrophotometric analysis was then performed at a wavelength of 830 nm (Alfiansyah *et al*., 2023). KH_2PO_4 solutions with concentrations of 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9; and 1.0 mg/L were used to prepare the standard curve. All absorbance values were corrected against a blank (distilled water without phosphate), so the values used in the analysis were the corrected absorbance values. Based on the measurement results, the following standard curve equation was obtained: $y = 1.7982x + 0.4318$ with a coefficient of determination $R^2 = 0.8987$. The phosphate concentration of the sample was calculated by substituting the corrected absorbance values into the regression equation; thus, the absorbance values obtained from the spectrophotometer were converted to phosphate concentration (ppm) based on the standard curve. The final quantitative result is the average value of three biological replicates for each isolate. "This study did not use either positive or negative controls, as the research objective focused primarily on exploring and characterizing the properties of the isolates obtained; therefore, the results were analyzed based on comparisons among the test isolates.

Analysis of the Capabilities of IAA-Producing

The IAA hormone production assay was conducted using Nutrient Broth (NB) medium enriched with L-tryptophan as a precursor. The medium was prepared by adding L-

tryptophan to achieve a final concentration of 0.1 g/L (100 mg/L), then homogenized thoroughly. A total of 60 mL of medium was added to each 100 mL Erlenmeyer flask, with a total of 15 flasks prepared in triplicate. The medium was then sterilized using an autoclave at 121°C, then cooled to room temperature before inoculation. Inoculation was performed by adding the inoculum to each flask containing 60 mL of medium. Subsequently, the cultures were incubated in a shaking incubator at 30°C with a shaking speed of 100 rpm for 35 days. Sampling was performed periodically every 7 days on days 7, 14, 21, 28, and 35 to monitor IAA production dynamics during the incubation period. According to Fernandes *et al*. (2021), Actinomycetes grow relatively slowly, and IAA production tends to increase during the stationary phase. Regular monitoring is necessary to determine the dynamics and peak IAA production of each isolate. At each observation time point, 2 mL of culture was collected and centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cell biomass (Yuniarti & Purwani, 2022). The supernatant was then used for a qualitative test to visually detect the presence of IAA. A total of 4 mL of supernatant was mixed with Salkowski's reagent, then incubated for 24 hours in the dark at room temperature. A color change in the solution to pink indicates the production of IAA by the tested isolate (Mawarti *et al*., 2027). Meanwhile, the quantitative test was performed by mixing 2 mL of supernatant with 2 mL of Salkowski's reagent, then incubating the mixture in the dark for 30 minutes. After that, the absorbance of the solution was measured using a spectrophotometer at a wavelength of 535 nm. The absorbance values obtained were then converted to IAA concentration using a standard curve, and the results were expressed in mg/L (ppm) (Hanafi & Raharjo, 2017).

The preparation of the IAA standard curve begins with the preparation of a 1 mg/mL IAA stock solution, obtained by dissolving 0.02 mg of IAA in 20 mL of distilled water. This stock solution is then diluted to produce standard solutions with the following concentrations: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/L. A 2 mL aliquot of each standard solution was mixed with 2 mL of Salkowski's reagent, homogenized using a vortex mixer, and incubated for 15–30 minutes in the dark (Fithah, 2015). The absorbance of the solutions was measured using a spectrophotometer at a wavelength of 535 nm (Sharma & Rai, 2015). The obtained data were then plotted on a graph with the X-axis representing IAA concentration (mg/L) and the Y-axis representing absorbance values. Based on the measurement results, the linear regression equation for the standard curve was obtained as $y = 1.857x + 0.7256$ with a coefficient of determination $R^2 = 0.8101$. This equation was used to calculate the IAA concentration in the samples based on the obtained absorbance values. "This study did not use either positive or negative controls, as the research objective focused primarily on exploring and characterizing the properties of the isolates obtained; therefore, the results were analyzed based on comparisons among the test isolates."

Analysis of the Phylogenetic Relationships Among Phosphate-Solubilizing and IAA-Producing Actinomycetes

The actinomycetes exhibiting the best activity were selected for molecular identification: isolate RzPO-09, which had the highest phosphate-solubilizing ability, and isolate RzPH-07, which produced the highest amount of IAA. Both isolates were then characterized based on molecular analysis of the 16S rRNA gene. The actinomycete isolates were cultured in Starch Casein Broth (SCB) and incubated for 7 days at 28–30°C in a shaking incubator at 200 rpm to obtain optimal cell biomass. The cultures were then centrifuged for 15 minutes at a centrifugal force of approximately 4,000–6,000 ×g (equivalent to ±5,000–6,000 rpm) to pellet the cells. The resulting cell pellet was subsequently used for genomic DNA extraction using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research). The 16S rRNA gene was amplified using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), with a PCR reaction mixture using MyTaq™ HS Red Mix 2× (Meridian Bioscience). PCR (Polymerase Chain Reaction) conditions were adjusted according to the kit's standard protocol, including an initial denaturation step, followed by cycles of denaturation, annealing, and extension. The PCR products were then purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) prior to sequencing.

Gene sequencing was performed in both directions (forward and reverse) to improve data accuracy; the sequencing results were then analyzed using the Basic Local Alignment Search Tool (BLAST) program against the NCBI (National Center for Biotechnology Information) database to determine the taxonomic relationships of the isolates. Next, phylogenetic tree reconstruction was performed using the neighbor-joining method in the MEGA XI (Molecular Evolutionary Genetics Analysis) software with 1.000 bootstrap repetitions to test the confidence level of the phylogenetic branches (Retnowati & Katili, 2023)

Data analysis

The ability of actinomycetes isolates to solubilize phosphate was analyzed descriptively based on the IKF value, with data presented in figures and tables, as well as tables showing the mean ± standard deviation (SD). The ability of IAA-producing actinomycetes was analyzed descriptively, and the data were presented in the form of tables of calculation results according to standard curves and tables of mean ± standard deviation (SD). The phylogenetic relationship of potential phosphate-

solubilizing and IAA-producing actinomycetes isolates was analyzed descriptively by comparing the 16S rRNA sequences of selected isolates with DNA sequences in the NCBI GenBank. Phylogenetic tree reconstruction was based on the neighbor-joining algorithm with 1.000 bootstrap repetitions.

RESULTS AND DISCUSSION

Description of the Research Location

The Hungayono karst area is located in Tulabolo Village, East Suwawa Subdistrict, Bone Bolango Regency. Environmental conditions within the Hungayono karst ecosystem tend to have a neutral pH, which is ideal for microbial growth, particularly that of actinomycetes, and temperatures in the Hungayono karst fall within the tolerance range of microorganisms. Dewi *et al*., 2024, state that most actinomycetes exhibit optimal growth up to mesophilic temperatures. Meanwhile, soil moisture levels are relatively low. According to Jagannathan *et al.* (2021), actinobacteria have the ability to adapt to dry conditions by forming spores, enabling them to survive and grow in environments with limited water availability (Table 1). This study did not include soil chemistry data (e.g., available phosphorus and organic matter), making it impossible to provide a comprehensive interpretation of phosphorus availability at the study site. Therefore, the results of this study focus on the initial screening of each isolate without directly linking them to soil fertility status.

Sampling was conducted at three different locations, yielding a total of 12 plant samples comprising 4 species. Of all the plant species found, four host plant species were associated with actinomycetes capable of dissolving phosphate (RzPO-09) and producing the highest levels of the growth hormone IAA (RzPH-07), namely *Anthocephalus macrophyllus*, *Acrostichum aureum* L, *Alstonia scholaris*, and *Pluchea indica* (Table 2).

Table 1. Environmental conditions of the Hungayono karst ecosystem, Gorontalo.

Location	Coordinate point	Environmental parameters		
		Temperatur	pH	Humidity
Point 1	0°30′19.4"N 123°17'29.5"E	29	6,8	20%
Point 2	0°30′18.13"N 123°17'29.08"E	31	6,8	10%
Point 3	0°30'14.7"N 123°17'32.25"E	37	7	10%

Table 2. Type of host plant.

Isolate code	Host Plant	Colony color		Point	Colony structure
		Substrate	Areal		
RzPP-02	*Anthocephalus macrophyllus*	White	White	1	Irregular
RzKK-03	*Anthocephalus macrophyllus*	Yellow	Yellow	3	Circular
RzPC-04	*Alstonia scholaris*	White	Brown	2	Irregular
RzOO-06	*Acrostichum aureum* L	Orange	Orange	3	Irregular
RzPH_07	*Anthocephalus macrophyllus*	White	Black	2	Irregular
RzPO-09	*Pluchea indica*	White	Orange	3	Circular
RzCC-11	*Alstonia scholaris*	White	Cream	3	Circular
RzAH-12	*Acrostichum aureum* L	Gray	Black	1	Irregular
RzPC-13	*Pluchea indica*	White	Cream	2	Circular
RzPC-14	*Acrostichum aureum* L	White	Cream	2	Circular
RzPP-15	*Pluchea indica*	White	White	1	Circular

Phosphate solubilizing of actinomycetes

The phosphate-solubilizing ability of 11 actinomycete isolates from the rhizosphere of four host plant species in the Hungayono karst region was tested. The qualitative results of this study indicate that 2 isolates (RzPO-09 and RzAH-12) possess phosphate-solubilizing ability, though with a very low phosphate solubility index (Table 3). Quantitative test results show that the 11 Actinomycetes isolates exhibited no phosphate-solubilizing activity on day 7. Activity began to emerge on day 14 and peaked on day 21, marked by an increase in solubility values in almost all isolates, with isolate RzPO-09 exhibiting the highest phosphate solubilization capacity among the other isolates. By day 28, a decline in activity occurred, indicating the onset of the stationary phase due to nutrient limitations and metabolite accumulation; eventually, by day 35, none of the isolates exhibited phosphate-solubilizing activity (Table 4).

This indicates that phosphate solubilization capacity is dynamic and influenced by the growth phase and differences in the metabolic potential of each isolate. Qualitative test results do not always align with quantitative tests, as some microorganisms can dissolve phosphate in liquid media without forming a clear zone

on solid media, due to differences in dissolution mechanisms and method sensitivity. In general, only a few isolates exhibit phosphate-dissolving activity at a relatively low level; therefore, these findings are more appropriate as an initial screening step.

Table 3. Results of the Qualitative Test for Phosphate-Solubilizing Actinomycetes.

Isolate code	Qualitative Testing (Phosphate Solubility Index)	Category
RzPP-02	0	0
RzKK-03	0	0
RzPC-04	0	0
RzOO-06	0	0
RzPH-07	0	0
RzPO-09	0,66	(Very Low)
RzCC-11	0	0
RzAH-12	0,61	(Very Low)
RzPC-13	0	0
RzPC-14	0	0
RzPP-15	0	0

Note: IKF <1.00 = very low; 1.00–2.00 = low; 2.00–3.00 = moderate; >3.00 = high

Table 4. Results of the Quantitative Test for Phosphate-Solubilizing Actinomycetes**.**

Isolate Name	The ability of actinomycetes to dissolve phosphate (ppm) on a quantitative basis (mean ± SD)				
	7 days	14 days	21 days	28 days	35 days
RzPP-02	0.000 ± 0.000	0.081 ± 0.012	0.094 ± 0.007	0.105 ± 0.010	0.000 ± 0.000
RzKK-03	0.000 ± 0.000	0.000 ± 0.000	0.125 ± 0.007	0.194 ± 0.007	0.000 ± 0.000
RzPC-04	0.000 ± 0.000	0.000 ± 0.000	0.180 ± 0.005	0.118 ± 0.007	0.000 ± 0.000
RzOO-06	0.000 ± 0.000	0.141 ± 0. 010	0.178 ± 0.007	0.193 ± 0.009	0.000 ± 0.000
RzPH-07	0.000 ± 0.000	0.101 ± 0. 012	0.178 ± 0.007	0.180 ± 0.007	0.000 ± 0.000
RzPO-09	0.000 ± 0.000	0.290 ± 0.009	0.431 ± 0.007	0.263 ± 0.007	0.000 ± 0.000
RzCC-11	0.000 ± 0.000	0.070 ± 0.012	0.112 ± 0.007	0.143 ± 0.007	0.000 ± 0.000
RzAH-12	0.000 ± 0.000	0.281 ± 0.012	0.419 ± 0.007	0.189 ± 0.007	0.000 ± 0.000
RzPC-13	0.000 ± 0.000	0.000 ± 0.000	0.155 ± 0.007	0.114 ± 0.004	0.000 ± 0.000
RzPC-14	0.000 ± 0.000	0.079 ± 0.006	0.192 ± 0.007	0.095 ± 0.007	0.000 ± 0.000
RzPP-15	0.000 ± 0.000	0.000 ± 0.000	0.116 ± 0.007	0.099 ± 0.010	0.000 ± 0.000

The ability of Actinomycetes to produce Indole acetic Acid (IAA)

Qualitatively, IAA-producing actinomycete isolates were identified by a red color change in the culture following the addition of Salkowsky's reagent. The results showed that out of 11 isolates, 4 isolates were capable of producing IAA qualitatively, namely RzOO-06, RzPH-07, RzCC-11, and RzAH-12 (Table 5). Meanwhile, quantitative testing showed that among the 11 Actinomycetes, there was significant variability in both quantity and optimal incubation time. Isolate RzPH-07 produced the highest IAA level of 0.896 ppm on day 21 compared to the other isolates; however, this value is quite low compared to previous studies, such as that by Alfian (2023), where the test isolates were able to produce IAA at 29.38 ppm. Most isolates showed a production pattern that increased gradually until reaching an optimal point between days 14 and 21, before eventually declining on day 35, likely due to a reduction in nutrients or the degradation of organic compounds in the medium. Meanwhile, some isolates, such as RzPO-09, RzPC-13, and RzPP-15, showed no IAA synthesis activity at all (Table 6).

Table 5. Qualitative results of the test of Actinomycetes' ability to produce IAA.

Isolate Name	Incubation period				
	7 Days	14 Days	21 Days	28 Days	35 Days
RzPP-02	-	-	-	-	-
RzKK-03	-	-	-	-	-
RzPC-04	-	-	-	-	-
RzOO-06	+	+	+	+	+
RzPH-07	+	+	+	+	+
RzPO-09	-	-	-	-	-

RzCC-11	+	+	+	+	+
RzAH-12	-	+	+	+	-
RzPC-13	-	-	-	-	-
RzPC-14	-	-	-	-	-
RzPP-15	-	-	-	-	-

Note: (+) indicates a color change/potential to produce IAA; (-) indicates no color change/no potential to produce IAA.

Table 6. Quantitative results of the test of Actinomycetes' ability to produce IAA.

Isolate Name	Quantitative assessment of the ability of actinomycetes to produce IAA (ppm) (mean ± SD)				
	7 Days	14 Days	21 Days	28 Days	35 Days
RzPP -02	0.000 ± 0.000	0.000 ± 0.000	0.071 ± 0.010	0.000 ± 0.000	0.000 ± 0.000
RzKK-03	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.159 ± 0.015	0.010 ± 0.030
RzPC-04	0.000 ± 0.000	0.008 ± 0.007	0.012 ± 0.013	0.010 ± 0.013	0.000 ± 0.000
RzOO-06	0.324 ± 0.013	0.573 ± 0.002	0.283 ± 0. 015	0.269 ± 0.015	0.128 ± 0.013
RzPH-07	0.356 ± 0.019	0.747 ± 0,007	0.896 ± 0.020	0.629 ± 0.019	0.449 ± 0.034
RzPO-09	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
RzCC-11	0.286 ± 0.013	0.358 ± 0.021	0.381 ± 0.013	0.238 ± 0.010	0,067 ± 0.007
RzAH-12	0.036 ± 0.018	0.040 ± 0.007	0.089 ± 0.020	0.100 ± 0.032	0.000 ± 0.000
RzPC-13	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
RzPC-14	0.000 ± 0.000	0.000 ± 0.000	0.028 ± 0.013	0.000 ± 0.000	0.000 ± 0.000
RzPP-15	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

Phylogenetic analysis of Phosphate-Solubilizing and Producers of Idole Acetic Acid (IAA) Actinomycetes

The phosphatesolubilizing actinomycetes (RzPO-09) and the highest IAA-producing actinomycetes (RzPH-07) were then examined macroscopically and microscopically. Macroscopically, both isolates appeared as powdery, cotton-like colonies, and microscopically, they were Gram-positive with filaments/hyphae characteristic of actinomycetes (Figure 2).

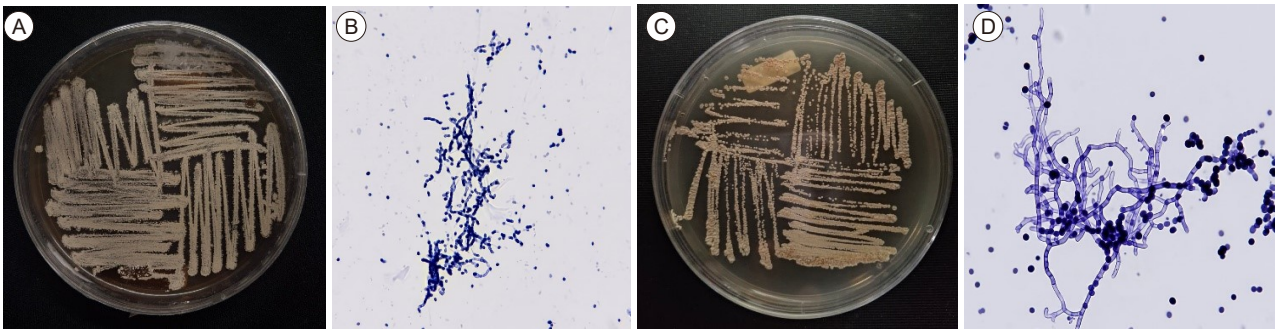

Figure 2. Morphology of the phosphate-solubilizing isolate (RzPO-09) and the IAA-producing isolate (RzPH-07) (PZ458434). (a) macroscopic morphology of the RzPH-07 isolate colony; (b) microscopic morphology/hyphae of the RzPH-07 isolate; (c) macroscopic morphology of the RzPO-09 isolate colony; (d) microscopic morphology/hyphae of the RzPO-09 isolate.

Phylogenetic analysis was based on the 16S rRNA molecular method, in which purified DNA from the isolates was extracted and its purity was qualitatively assessed using a spectrophotometer based on the 260/280 absorbance ratio. Subsequently, amplification of the 16S rRNA gene was performed using the universal primer pair 27F and 1492R. Subsequently, the 16S rRNA gene sequences from isolates RzPH-07 and RzPO-09 were compared using reference sequences from the NCBI database. BLAST results showed that isolate RzPH-07 shared sequence similarity with the genus *Streptomyces*, with identity percentages ranging from 96.19% to 98.29%. Meanwhile, isolate RzPO-09 showed similarity to the genus *Pseudonocardia* with identity percentages ranging from 99.28% to 99.71% (Table 4). A phylogenetic tree reconstructed using the neighbor-joining algorithm with 1,000 bootstrap repetitions showed that isolate RzPH-07 is closely related to the genus *Streptomyces* sp., specifically associated with *Streptomyces triticirhizae* strain NEAU-YY642, with a similarity percentage of 98.29%. Meanwhile, isolate RzPO-09 showed similarity to *Pseudonocardia alni* strain DSM 44104 with a similarity percentage of 99.71% (Figure 3).

Table 7. BLAST sequence results for the 16S rRNA genes of isolates RzPH-07 and RzPO-09.

Isolate	Description	Scientific name	Query cover	E-Velue	Percentage of identity	Accession number
RzPH-07 (PZ458434)	*Streptomyces triticirhizae* strain NEAU-YY642 16S ribosomal RNA partial sequence	*Streptomyces triticirhizae*	99%	0.0	98.29%	NR_180032.1
	Streptomyces sedi strain YIM 65188 16S ribosomal RNA. partial sequence	*Streptomyces sedi*	100%	0.0	97.65%	NR_044582.1
	Streptomyces mimosae strain 3MP-10 16S ribosomal RNA partial sequence	*Streptomyces mimosae*	99%	0.0	97.77%	NR_170412.1
	Streptomyces zhaozhouensis strain NEAU-LZS-5-16S ribosomal RNA partial sequence	*Streptomyces zhaozhouensis*	99%	0.0	97.43%	NR_133847.1
	Streptomyces mayten strain YIM 60475-16S ribosomal RNA partial sequenc	*Streptomyces mayten*	99%	0.0	96.19%	NR_044434.1
RzPO-09 (PZ458677)	*Pseudonocardia alni* strain DSM 44104 16S ribosomal RNA. partial sequence	*Pseudonocardia alni*	99%	0.0	99.71%	NR_117429.1
	Pseudonocardia alni subsp. carboxydivorans strain Y8 16S ribosomal RNA, partial sequence	*Pseudonocardia alni*	100%	0.0	99.42%	NR_044092.1
	Pseudonocardia alni strain IMSNU 20049 16S ribosomal RNA, partial sequence	*Pseudonocardia alni*	100%	0.0	99.35%	NR_042000.1
	i strain DSM 44104 16S ribosomal RNA. partial sequence	*Pseudonocardia alni*	100%	0.0	99.53%	NR_119240.1
	Pseudonocardia tropica strain YIM 61452 16S ribosomal RNA, partial sequence	*Pseudonocardia tripica*	100%	0.0	99.28%	NR_104541.1

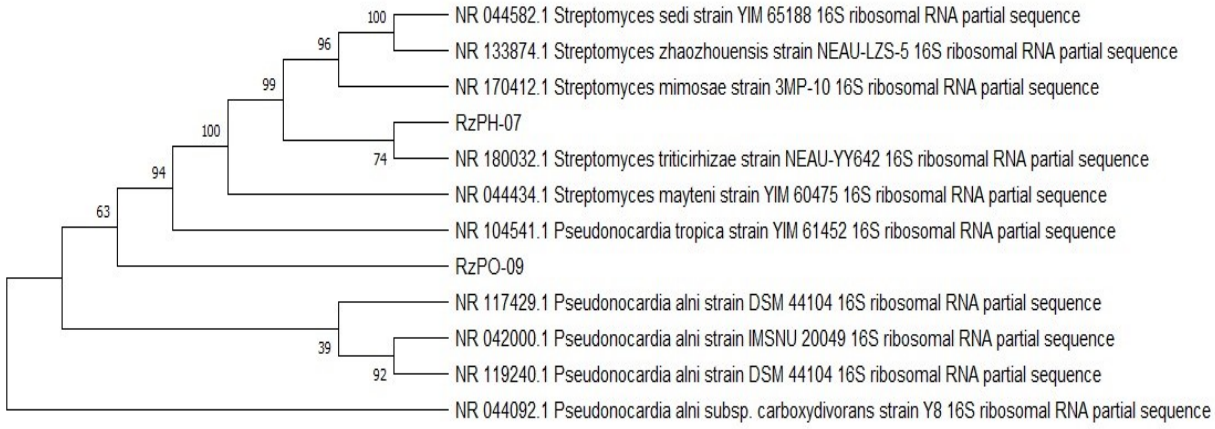

Figure 3. The phylogenetic trees for isolates RzPO-09 (PZ458677) and RzPH-07 (PZ458434) were reconstructed using the clustering algorithm with 1,000 bootstrap repetitions.

Discussion

This study shows that of the 11 actinomycete isolates obtained from the rhizosphere of four host plants in the Hungayono karst ecosystem, only a small fraction exhibited functional activity: two isolates were capable of dissolving phosphate, and four isolates were capable of producing IAA, though at relatively low activity levels. Quantitatively, isolate RzPO-09 showed the highest phosphate solubilization capacity of 0.430 ppm, while isolate RzPH-07 produced the highest IAA at 0.896 ppm compared to other isolates in this study. These values are lower compared to previous reports, where phosphate-solubilizing microorganisms generally produce dissolved phosphate in the range of ±10–500 ppm, and IAA production by rhizosphere bacteria can reach ±5 to >30 ppm under optimal conditions (Fitriatin *et al*., 2008; Alfin, 2023; Lata *et al*., 2024). This difference indicates that the functional capacity of the isolates in this study is still limited or not yet optimal.

The low phosphate solubilization capacity observed can be attributed to physiological and genetic variations among isolates, as it has been reported that phosphate solubilization efficiency is strongly influenced by the ability of microorganisms to produce organic acids and

phosphatase enzymes. In the literature, this mechanism is often associated with the presence of the pqq and gcd genes, which play a role in organic acid production, as well as the phoA and phoD genes, which encode phosphatase enzymes (Zhang *et al*., 2023; Chen *et al*., 2024). However, since genetic aspects were not analyzed in this study, this mechanism remains speculative and requires further verification. Previous studies have shown that IAA production generally occurs via tryptophan-based pathways, specifically the indole-3-pyruvate (IPyA) pathway involving the ipdC gene, and the indole-3-acetamide (IAM) pathway involving the iaaM and iaaH genes (Spaepen *et al*., 2007). However, since these biosynthetic pathways were not directly tested, the interpretation of the IAA production mechanism in the obtained isolates remains inferential.

Although the functional activity obtained was relatively low, the successful isolation of actinomycetes from the karst environment indicates that these microorganisms are capable of surviving in the extreme conditions of the karst environment, particularly in the Hungayono karst, which features hot springs. This finding is consistent with previous reports stating that rhizosphere microbes in extreme environments can still thrive through interactions with host plants and the utilization of root exudates as a nutrient source (Retnowati *et al*., 2024).

Molecular identification based on 16S rRNA gene sequences indicates that isolate RzPO-09 is closely related to the genus Pseudonocardia, specifically Pseudonocardia alni strain DSM 44104, with strong bootstrap support (92%), suggesting a reliable phylogenetic placement. Members of this genus have previously been reported to possess phosphate-solubilizing ability (Nurkanto, 2008). Meanwhile, isolate RzPH-07 belongs to the genus Streptomyces and is related to Streptomyces triticirhizae strain NEAU-YY642, although with moderate bootstrap support (74%), meaning this cannot yet be confirmed. This indicates that identification at the species level remains uncertain. However, the genus Streptomyces is widely known for its ability to produce plant growth-promoting compounds, including IAA (Alfin, 2023).

Overall, the findings of this study indicate that isolates RzPO-09 and RzPH-07 have potential as candidates for functional microorganisms, but cannot yet be classified as effective biological agents. The low activity values compared to the literature indicate the need for further optimization. Therefore, further research will be needed, including optimization of culture conditions, genomic screening for functional genes, plant inoculation tests, greenhouse tests, and testing for tolerance to environmental stress.

CONCLUSIONS

In conclusion, this study successfully isolated 11 actinomycete isolates from the rhizosphere of four host plants in the Hungayono karst ecosystem. Among these isolates, RzPO-09 exhibited the highest phosphate-solubilizing ability, while RzPH-07 exhibited the highest IAA production. Molecular analysis indicates that the selected isolates are related to the genera Pseudonocardia and Streptomyces. However, the results obtained still require further validation through additional testing.

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