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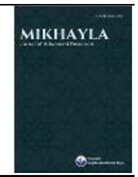

Isolation and Characterization of *Actinomycetes* from Plant *Rhizosphere* in Gorontalo Karst Area

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A B S T R A K
Kawasan karst merupakan bentang alam yang tersusun atas batuan karbonat dan kaya akan unsur kalsium dan magnesium sehingga membuat beberapa unsur hara makro tidak dapat diserap oleh tumbuhan secara langsung. Salah satu mikroorganisme tanah yang berasosiasi dengan tumbuhan di kawasan karst adalah Actinomycetes. Beberapa spesies Actinomycetes dikenal mampu menghasilkan hormon pertumbuhan seperti Indole Acetic Acid serta mampu melarutkan fosfat yang terikat dalam tanah. Actinomycetes juga berperan penting dalam ekosistem tanah, terutama pada area dengan kondisi lingkungan ekstrem seperti kawasan karst. Kawasan karst dikenal dengan keanekaragaman hayatinya yang unik dan potensi mikroorganisme yang belum sepenuhnya dieksplorasi. Kawasan karst Gorontalo menjadi lokasi yang potensial untuk eksplorasi mikroorganisme tersebut. Penelitian ini bertujuan untuk mengisolasi dan melakukan karakterisasi Actinomycetes yang terdapat pada rhizosfer tumbuhan di kawasan karst Gorontalo. Pengambilan sampel dilakukan pada 9 jenis tumbuhan yang terdapat pada dua lokasi yaitu Tanjung Kramat, Kecamatan Hulonthalangi dan Pegunungan Karst. Hasil karakterisasi makroskopik menunjukkan bahwan isolat Actinomycetes yang diperoleh memiliki 2 bentuk yakni Irregular (5 isolat) dan Circular (4 isolat) dengan warna miselium yang didominasi oleh warna hitam keabu-abuan – coklat, orange – orange, kuning – orange, abu-abu – kuning, dan putih – kuning. Karakterisasi mikroskopik menunjukkan hasil semua isolat yang didapatkan berwarna biru atau ungu dengan sel berbentuk filamen tipis yang menandakan isolat tersebut Actinomycetes.

A B S T R A C T
Karst areas are landscapes composed of carbonate rocks and are rich in calcium and magnesium elements, making some macronutrients unable to be absorbed by plants directly. One of the soil microorganisms associated with plants in karst areas is Actinomycetes. Some species of Actinomycetes are known to produce growth hormones such as Indole Acetic Acid and dissolve phosphate bound in the soil. Actinomycetes also play an important role in soil ecosystems, especially in areas with extreme environmental conditions such as karst areas. Karst areas are known for their unique biodiversity and the potential of microorganisms that have not been fully explored. Gorontalo karst area is a potential location for the exploration of these microorganisms. This study aims to isolate and characterise Actinomycetes found in the rhizosphere of plants in Gorontalo karst area. Sampling was carried out on 9 plant species found in two locations namely Tanjung Kramat, Hulonthalangi District and Karst Mountains. Macroscopic characterisation results showed that the Actinomycetes isolates obtained had 2 forms, namely Irregular (5 isolates) and Circular (4 isolates) with mycelium colour dominated by greyish black - brown, orange-orange, yellow-orange, grey-yellow, and white - yellow. Microscopic characterisation showed the results of all isolates obtained were blue or purple with thin filamentous cells indicating the isolates were Actinomycetes.

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1. INTRODUCTION

Karst areas are landscapes composed of carbonate rocks (such as limestone) that undergo a dissolution process (Aprilia *et al.,* 2021). Karst areas are characterized by soils that have high calcium (Ca) content and can affect the solubility of various macroelements that are important for plants and organisms in the soil as a component of karst areas (Fan *et al.,* 2019).

Karst area in Gorontalo Province is one of the landscapes that widely stretches in the southern region of Gorontalo. Based on the Geological map of North Sulawesi, Bolaang Mongondow and Gorontalo, the distribution of karst in Gorontalo is in the southern area of Gorontalo Regency, around Limboto Lake and the southern area of Bone Bolango Regency. Research that reveals the biodiversity of karst areas in Gorontalo is still lacking. Based on a report by Retnowati *et al.,* in 2024, the Gorontalo Karst area shows diverse soil physico-chemical conditions (pH, temperature, and humidity) and vegetation. It was further reported that microorganisms can grow and associate with plants in karst areas.

Plants living in karst areas have the ability to respond to conditions of high calcium (Ca) content. These conditions are carried out by plants in karst areas by developing physiological and morphological adaptations (Meng *et al.,* 2023). Some plants also develop interactions with various types of microbes in the root system (Lakshmanan *et al.,* 2014). Some types of microbes found in karst areas include fungi, bacteria, Actinomycetes, and algae (Mubarak *et al.,* 2017). According to (Mubarak *et al.,* 2017) the Actinomycetes group of bacteria is one of the microbes found interacting with the root system in karst areas or rhizosphere areas.

Rhizosphere is the area around plant roots that supports the development and activity of microorganisms, which play a role in helping plant growth and development (Putra *et al.,* 2020). Some microorganisms in the rhizosphere play a role in various aspects, such as nutrient cycling, soil formation processes, plant growth, the activity of other microorganisms, and function as biological control agents (Prayudyaningsih *et al.,* 2015). The presence and activity of rhizosphere microorganisms are influenced by exudates produced by plant roots (Dewi, 2018). Root exudates in each type of plant are different, so the presence of microorganisms in the soil is also different. This is proven by research (Syahril *et al.,* 2023) which shows the results that, the presence of microorganisms originating from the rhizosphere of plants is specifically distributed based on soil physico-chemical conditions.

One of the microorganisms that interact with plant rhizosphere is Actinomycetes. Actinomycetes are soil organisms that have properties commonly shared by bacteria and fungi but also have quite different characteristics. Unlike bacterial colonies in general that are clearly slimy and grow quickly, Actinomycetes colonies appear slowly showing a powdery consistency and tightly attached to the agar surface. Observations studied on a colony under a microscope that forms asexual spores for reproduction (Mutmainnah, 2013). Research related to the exploration of Actinomycetes in karst areas, especially the Gorontalo region, is still relatively small.

Gorontalo as an area that has high biodiversity, including unique flora and fauna, makes it an attractive location for microbiological research. The purpose of this research is to isolate and identify Actinomycetes from the rhizosphere of plants in Gorontalo karst area. By conducting this research, it is expected to find new strains that have the potential to be used in the development of biotechnology products, such as biological fertilizers or biocontrol agents.

2. METHOD

This research was conducted from June-October 2024. Rhizosphere soil sampling was carried out in two karst area locations, namely in Tanjung Kramat, Hulonthalangi District, and Karst Mountains, West Kota District, which was carried out in June-July 2024. Isolation and identification were carried out at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Gorontalo State University in August-October 2024.

Soil Sampling from Plant Rhizosphere in Gorontalo Karst Area

Rhizosphere soil sampling in karst areas was carried out by random sampling method in 2 locations of karst areas, namely in Tanjung Kramat, Hulonthalangi District, and Karst Mountains, West Kota District. Determination of sampling points using random sampling method (Tüfekci *et al.*, 2023). Rhizosphere soil samples were taken using a small shovel at several sampling points with + 20 cm sampling on each type of plant found. According to (Maulana *et al.*, 2022), sampling rhizosphere soil at a depth of 10-15 cm will maximize the soil containing Actinomycetes bacteria. Soil samples were then stored in sterile plastic and coded with location labels and stored in a coolbox to keep them safe (Katili & Retnowati, 2017). For mapping the research location, the GPS maps camera application was used to take coordinate points.

Isolation of Actinomycetes

Soil samples obtained from karst areas were pulverized using a mortar and pestle and then 5 grams were taken and mixed with 45 ml of sterile distilled water and vortexed to make it homogeneous using an incubator shaker with a rotation of 225 rpm until the sample was homogeneous. Then, the soil suspension was heated using a water bath at 60°C for 15 minutes (Mangamuri *et al.*, 2012; Retnowati *et al.*, 2017). Furthermore, the soil suspension was carried out a dilution series by taking 1 ml of the suspension into a test tube containing 9 sterile mlaquades or tube 10-1 then vortexed and took 1 ml from the first tube then transferred to tube 10-2 to test tube 10-5. Soil suspensions at dilution levels 10-3 - 10-5 were taken as much as 200 µl, and inoculated on the surface of the Starch Casein Agar (SCA) medium using the surface/spread plate technique with the duplo method. Petri dishes were then incubated at 37°C for 7-14 days. SCA medium was added cycloheximide or nystatin each 25µl/ml to prevent the growth of fungal contaminant colonies during incubation (Baskaran *et al.*, 2011).

Purification of Actinomycetes

Actinomycetes colonies that grew on SCA media were inoculated into new dishes containing SCA media to be purified based on the single cell colony method in streak plates and incubated for 7-14 days at 37°C.

Morphological Characterization of Actinomycetes

Microscopic observations of Actinomycetes morphology include cell shape and spores. Actinomycetes isolates that have been purified are observed macroscopically for colony morphology including colony shape, substrate mycelium color, and aerial mycelium color (Listiana, *et al.*, 2006; Ekowati and Achmad, 2008).

For microscopic observation, solid SCA media was cut from a petri dish using a scalpel with a size of ± 1 × 1 cm. The pieces of media were placed on a glass object that had been given a holding rod and filter paper that had been moistened with water. Spores from pure culture of Actinomycetes were taken using an ose needle and then placed on the surface of the media piece and to the four sides of the media piece on the glass object. Furthermore, the media piece is covered with a cover glass. Petri dish was closed and then incubated for 5-7 days (Hamdiyati *et* *al.,* 2010). The cover glass contained in the piece of media that has been overgrown with Actinomycetes is then dabbed with methylene blue. Actinomycetes morphology was observed using a microscope with a magnification of 10×100, then identified using Bergey's Manual of Determinative Bacteriology (Hamdiyati *et al.,* 2010; Armaida & Khotimah, 2016). Preparation was made for microscopic observation of Actinomycetes using slide culture technique (Sharma, 2014). Morphological observations of Actinomycetes microscopically include cell shape and spores.

Data Analysis

Actinomycetes characterization data were analyzed descriptively. Presentation of data is done using tables.

3. RESULT AND DISCUSSION

Description of Research Results

The environmental conditions at the two data collection sites show differences and can be seen in the table below.

Table 1. Research environment conditions

Location	Average pH	Average Moisture	Coordinate Points
Tanjung Kramat, Kecamatan Hulonthalangi	5,43	4,2%	0°30'20.0"N 123°03'02.8"E
Pegunungan Karst, Kecamatan Kota Barat	6,6	1,67%	0°32'37.5"N 123°01'57.3"E

Karst areas in the two research locations show specific vegetation in each location where in the first location namely Tanjung Kramat there are 8 plant samples namely *Catharanthus roseus, Mesosphaerum suaveolens, Indigofera tinctoria, Lantana montevidensis, Jatropha gossypifolia, Imperata cylindrica, Dolichos oliveri Schweinf., Leucaena leucocephala* and in the second location namely Karst Mountains there is 1 plant sample namely *Pneumatopteris pennigera*. Karst areas are unique ecosystems, consisting of a thin layer of soil made up of carbonate rock, which results in high levels of calcium and magnesium in the soil. This situation makes some macro-nutrients unavailable directly in the soil. The uniqueness of this area makes the types of vegetation that grow in it very specific (Retnowati *et al.,* 2024).

Morphological Characteristics of Actinomycetes

Isolation results from several types of plants, obtained 9 isolates of Actinomycetes bacteria originating from the rhizosphere of plants in the Gorontalo karst area. The macroscopic characteristics of each Actinomycetes isolate can be seen in Table 2.

Table 2. Macroscopic and microscopic characteristics of Actinomycetes bacteria isolated from plant rhizosphere in Gorontalo karst area on SCA medium

Code Isolate	Colony Shape	Areal Mycelium Colour	Susbtrate Mycelium Colour	Gram Positive/Negative
TD-a1	Circular	Greyish black	Brown	Positive
SV-a2	Irregular	Yellow	Orange	Positive
IF-a3	Irregular	Orange	Orange	Positive
JP-b1	Circular	Greyish black	Brown	Positive
IM-b2	Circular	Greyish black	Brown	Positive
IM-b3	Irregular	Grey	Yellow	Positive
DC-c1	Irregular	Orange	Orange	Positive
LC-c2	Circular	White	Yellow	Positive
P-c3	Irregular	Orange	Orange	Positive

Note: TD-a1: *Catharanthus roseus* titik a-1, SV-a2: *Mesosphaerum suaveolens* titik a-2, IF-a3: *Indigofera tinctoria* titik a-3, JP-b1: *Jatropha gossypifolia* titik b-1, IM-b2: *Imperata cylindrica* titik b-2, IM-b3: *Imperata cylindrica* titik b-3, DC-c1: *Dolichos oliveri Schweinf* titik c-1, LC-c2: *Leucaena leucocephala* titik c-2, P-c3: *Pneumatopteris pennigera* titik c-3.

The isolates observed have different substrate and areal mycelium colors. The results of macroscopic characterization of Actinomycetes colonies from several types of plant rhizosphere, the colony shape obtained in the form of Irregular originating from the rhizosphere of *Mesosphaerum suaveolens, Indigofera tinctoria, Imperata cylindrica, Dolichos oliveri Schweinf., Leucaena leucocephala* which is dominated by the color of mycelium in the form of yellow - orange, orange - orange, gray - yellow, white - yellow. Meanwhile, the circular colony shape comes from the rhizosphere of *Catharanthus roseus, Jatropha and gossypifolia, Imperata cylindrica,* and *Pneumatopteris pennigera* plants with mycelium colors dominated by grayish black - brown and orange - orange.

The results showed that Actinomycetes associated with rhizosphere plants in Gorontalo karst area were obtained as many as 9 isolates and have been identified using *Bergey's Manual of Determinative Bacteriology*, resulting in the genus Streptomyces and Non-Streptomyces (*rare actinomycetes*). Some Actinomycetes isolates, obtained from the rhizosphere of the same plant and distributed specifically in karst areas. In addition, environmental factors from the two research locations with acidic pH conditions and low humidity can affect the presence of Actinomycetes in the rhizosphere of plants in karst areas. In line with research (Retnowati *et al.*, 2024), plants that live in karst areas have adapted morphologically and physiologically. One form of adaptation is done by producing root exudates that can dissolve phosphate and Actinomycetes obtained in the study have the ability to dissolve phosphate. The composition and pattern of root exudates, plant type, and soil type affect the activity and population of rhizosphere microbes (Rante *et al.*, 2020).

Figure 1. Colony of Actinomycetes (a)TD-a1; (b)SV-a2; (c)IF-a3; (d)JP-b1; (e)IM-b2; (f)IM-b3; (g)DC-c1; (h)LC-c2; (i)P-c3

Then, from the Actinomycetes isolates obtained, and obtained the same colour of substrate mycelium and areal as greyish black - brown, orange-orange, white-yellow, grey-yellow, and yellow-orange, representative colonies were taken for purification. The purification picture can be seen below.

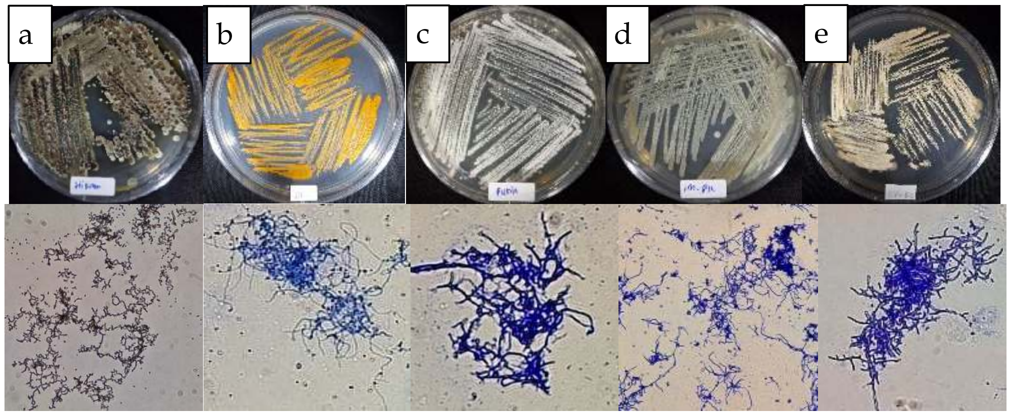

Figure 2. Purification and microscopic results of Actinomycetes colonies (a) greyish black - brown; (b) orange-orange; (c) white-yellow; (d) grey - yellow; (e) yellow-orange

The identification of microscopic characteristics of Actinomycetes bacteria was carried out through gram staining. Actinomycetes bacterial cells isolated from plant rhizosphere are thin filaments and mycelium similar to filamentous fungi. Isolates TD-a1, JP-b1, IM-b2, on macroscopic observation appeared greyish black mycelium areal with brown substrate mycelium, and round/circular colonies. Isolate with yellow - orange mycelium colour is SV-a2 with irregular colony shape. Isolates with orange mycelium colour are IF-a3, DC-c1, and P-c3 with irregular colony shape. Isolates with grey-yellow mycelium colour, namely IM-b3, have irregular colony shape. Isolate LC-c2 has white-yellow mycelium colour with circular colony shape. Colony morphology obtained showed a variety of actinomycetes. This is in line with research (Li *et al.*, 2016) which states that in general the colour of mycelium substrate from Actinomycetes varies, namely white, yellow, orange, grey, red, green, blue, and purple. Aerial mycelium is a hypha that proliferates from the substrate mycelium to a certain stage and grows into the air. In general, the colour of aerial mycelium from Actinomycetes varies, namely white, grey, red, green, blue, and purple (Sharma *et al.*, 2014). All Actinomycetes isolates showed microscopic morphology in the form of thin filaments and purplish blue in colour. This indicates that the Actinomycetes isolates are gram-positive bacteria. In line with the statement (Fauziah & Djide, 2022) where Actinomycetes are gram-positive bacteria and have mycelium like fungi.

4. CONCLUSION

This research shows that the rhizosphere of plants in the karst area of Gorontalo is one of the potential habitats for Actinomycetes which has a high adaptability to extreme environments such as soil in karst areas. The presence of Actinomycetes in plant rhizosphere varies such as colony shape, substrate mycelium colour and areal mycelium. This indicates the presence of endemic or specific species of Gorontalo karst area.

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