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Isolation and Characterization of Endophytic Bacteria from The Roots of *Avicennia* sp. in The Mangrove Area of Gunung Anyar, Surabaya

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Abstract

Mangroves are unique ecosystems that support biodiversity, including endophytic microorganisms. Endophytic bacteria group is a group of microbes that have a mutualistic symbiosis to the host plant without harming the host, and can act as biocontrol agents and plant stimulants. Endophytic bacteria have a very wide host range. Each plant has an abundance and diversity of endophytic bacteria, depending on the internal and external factors that affect it. This study aims to determine the characteristics of endophytic bacteria and identify the diversity of endophytic bacteria found in Avicennia sp. Method Sampling from mangrove area of Gunung Anyar, Surabaya. Characterization of endophytic bacteria was done by Gram test, spore staining test, catalase test, oxidative-fermentative test, and selective media test (King's B and Yeast Dextrose Carbonate). The results showed the existence of several isolates of endophytic bacteria that have morphological diversity and different characteristics, such as shape, color, colony edge, and colony surface. Conclusions In this study has been successfully isolated some endophytic bacteria such as Lactobacillus sp., Corynebacterium sp., Bacillus sp., Xanthomonas sp., and there are also endophytic bacterial isolates that have not been identified. This study contributes to an early understanding of the diversity of microorganisms in mangrove ecosystems and opens up opportunities for further exploration of their potential applications, one of which is in agriculture.

Keywords: *Avicennia sp.; Bacteria; Endophytic; Mangrove*

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INTRODUCTION

Microbes are present in almost all ecosystems, including mangrove ecosystems. Mangrove ecosystem is an important ecosystem that supports vital life for coastal areas and oceans. Mangroves are unique ecosystems that play an important role in maintaining the balance of the coastal environment, including ecological functions, coastal protection, and habitat for various organisms. According to Matatula et al., (2019), mangrove ecosystems are also a challenging habitat for a variety of living organisms due to extreme environmental conditions, such as high salinity, large temperature fluctuations, and low oxygen levels. Behind these challenges, mangroves support a diversity of microorganisms, including bacteria that can survive and adapt to these conditions. One of the mangrove plants that have high ecological value is *Avicennia* sp., which is known as a pioneer plant in mangrove ecosystems (Alhaddad et al., 2019). In addition to its ecosystem benefits, this plant is also a host for various microorganisms, including endophytic bacteria.

Endophytic bacteria are microorganisms that live in plants for all or part of their life cycle, and do not cause disease for the host plant (Rori et al., 2020). Endophytic bacteria have great potential in various fields, such as in agriculture. According to Yanti et al., (2021), these endophytic bacteria have an important role in supporting the health and growth of the host plant through various mechanisms, such as protecting the host from pathogen attacks, inducing host resistance, nitrogen fixation, and being able to stimulate the growth of the host plant. The diversity of endophytic bacteria also depends on the plant species, geographical location, as well as environmental conditions (Harahap et al., 2023).

In a host plant, various types of endophytic bacteria can usually be found. In one host plant can consist of different genera and species of endophytic bacteria. According to Handayani et al., (2023), in some cases, mangroves of the same species do not necessarily have the same endophytic bacteria, even hundreds of species of endophytic bacteria can also be isolated from one type of host. The abundance and diversity of endophytic bacteria in the host plant is influenced by internal factors (biotic factors) in the form of plants, and external factors (abiotic factors) that include environmental factors (Dalimunthe et al., 2023). Both factors also determine the species of endophytic bacteria that colonize the host plant during its life cycle.

In the study of Ramadhanty et al., (2018) was found endophytic bacteria from mangrove *Avicennia marina* in the mangrove forest of Mangkang, Semarang, namely *Pseudomonas* sp., *Enterobacter* sp., and *Staphylococcus* sp., and each of those bacteria has antimicrobial capabilities. Research of Ntabo et al., (2018) about endophytic bacteria from the roots of *Avicennia marina* in the Kenya territory also managed to get 5 isolates, namely *Bacillus* sp., *B. Thuringiensis*, *B. Safensis*, *Myroides* sp., and *Streptomyces krainskii*. In India, endophytic bacteria from mangrove plant was reported as antibiotic producer, pectinase enzyme, protease, chitinase, lipase, phosphate provider, hormone-producing auxin, and able to fix nitrogen (Oktafiyanto et al., 2018).

Mangrove ecosystems have a high biodiversity, making it possible as a habitat for various endophytic bacteria. Roots of *Avicennia* sp. living in the extreme environment of mangrove areas, it is thought to have a unique diversity of endophytic bacteria. Mangrove environment that is saline and can create aerobic and anaerobic environmental conditions, providing natural selection for microorganisms that are able to adapt to these conditions. *Avicennia* sp. including one type of mangrove that is often found on the coast of Indonesia, such as in the area of Gunung Anyar, Surabaya. *Avicennia* sp. also has good conditions as a habitat for endophytic bacteria. Based on the amount of biodiversity in the mangrove area makes the prospect of research on endophytic bacteria from mangrove *Avicennia* sp., so it is important to do for such research. Research on endophytic bacteria at the root of *Avicennia* sp. in the mangrove area of Gunung Anyar, Surabaya, it is important to know the diversity and characteristics of these bacteria.

METHOD

Time and Place

This research time takes place from March to November 2024. This research was conducted at The Plant Health Laboratory of the Pembangunan Nasional "Veteran" Jawa Timur University.

Tools and Materials

In this study the tools used include petri dishes 15 x 100 mm, autoclaves, Laminar Air Flow (LAF), OSE needle, erlenmeyer 250 ml, beaker glass 250 ml and 500 ml, measuring glass 100 ml, analytical scale, stirrer, micropipettes 100-1000µ, microtips, vortex, bunsen, spray bottles, cover glass, object glass, microscope, test tube, stove, scalpel, tweezers, scissors, glass stirrer, cool box, camera cell phones, stationery, measuring devices. In this study the material used are the root sample *of Avicennia* sp., media Nutrient Agar (NA), media King's B, media Yeast Dextrose Carbonate (YDC), Oxidative – Fermentative media, plastic wrap, aquades, alcohol 70 %, crystal violet, iodine, safranin, KOH 3 %, Hydrogen Peroxide (H_2O_2) 3 %, malacite green, paraffin, spirtus, cotton, rubber, tissue, aluminumfoil, label paper, and plastic bags.

Procedure

Sampling

In this study the sample used is a healthy root sample of *Avicennia* sp. and comes from the Mangrove of Gunung Anyar, Surabaya. Root sampling is performed randomly, the samples were taken from 5 points and at one point 5 root samples were taken. The root sample that has been taken is then placed in a plastic bag and brought to the lab.

Isolation of Endophytic Bacteria

Before the isolation of bacteria, first sterilize the root sample by washing it thoroughly then sterilized with alcohol and rinsed with sterile aquades. Isolation of endophytic bacteria is carried out by direct planting technique, that is, by means of root sample pieces planted on sterile NA media. Then incubated for 24 - 48 hours at room temperature (Ginting et al., 2020). The colonies of bacteria are grown on media with different characteristics, then purified on other media.

Purification of Endophytic Bacteria

In colonies of endophytic bacteria with different characteristics, bacterial purification is carried out. At this stage, it is done by scraping a single colony on a new media using streaking techniques to separate other single colonies and incubated at room temperature for 24 - 48 hours. In mixed colonies that are still visible, it is necessary to repeat this process until a consistent single colony is obtained. Each endophytic bacterial isolates are also made two on angled NA, to be used as stock culture and working culture.

Characterization and Identification of Endophytic Bacteria

In this study the characterization and identification of endophytic bacteria is done by observing the morphological characterization of endophytic bacteria, and physiology and biochemistry characterization of endophytic bacteria. Morphological characterization of endophytic bacteria include colony shape, colony color, colony edge, and bacterial colony surface based on Kurniawan (2019), The color colony of the endophytic bacteria are also based on the Munsell classification of soil color chart. Physiological and biochemical characterization of endophytic endophytic bacteria include Gram test, spore staining test, catalase test, oxidative -fermentative test, and selective media test (King's B and yeast Dextrose carbonate).

Gram Test

Gram test consists of 2 stages, namely Gram test with KOH 3% and Gram staining test. KOH 3% Gram test is done by putting 1 OSE colony of endophytic bacteria on the glass object, then dripped a drop of KOH 3% solution on the glass object. The colony of endophytic bacteria and KOH solution are then stirred. According to Yatni et al., (2018), the result of Gram negative bacteria, that is, the solution becomes viscous and forms mucous threads when touched by the OSE needle. The result of Gram positive bacteria is that the solution remains liquid and does not form mucus.

Meanwhile, gram staining test is done by taking a small colony of bacteria and placed on a clean glass object. Then add a drop of sterile aquades, and make a thin smear, as well as dried with a bunsen. Next, dripped crystal violet (Gram A), let stand for 1 minute, and rinsed it with aquades. Then, add iodine (Gram B), let stand for 1 minute and rinse with aquades. Drip alcohol (Gram C) for decolorization for 30 seconds. Drip safranin (Gram D), let stand for 1 minute and rinse with aquades back. Dry the glass object and observe with a microscope, according to Aldina et al., (2023), Gram positive bacteria, bacterial cells are purple, while Gram negative bacteria, bacterial cells are red.

Spore Staining Test

This test is done by taking a small colony of bacteria and placed on a clean glass object and making a thin smear, and fixed with bunsen. Drip a solution of malachite green and let stand for 15 minutes, then rinse with aquades. Drip safranin solution on the smear for 1 minute to color the vegetative cells, and rinse again with aquades. Finally, observe under the microscope, according to Wulandari & Purwaningsih (2019), the endospore will be green (due to malachite green), while the vegetative cell will be red (due to safranin).

Catalase Test

Catalase test is done by putting 1 OSE colony of endophytic bacteria on the glass object, then dripped a drop of 3% H_2O_2 solution on the glass object. According to Pulungan & Tumangger (2018), the results of observations, namely the positive results are formation of oxygen bubbles (bacteria have catalase enzyme), while the negative results, no bubbles (bacteria do not have catalase enzyme).

Oxidative – Fermentative Test

The test was conducted by preparing two tubes of oxidative-fermentative media for each bacterial isolate. Inoculate the oxidative - fermentative media with bacterial cultures using sterile inoculation needles. In one of the tubes, paraffin oil is added (anaerobic condition), and in the other tube it is left without paraffiin oil (aerobic condition). According to Reimena et al., (2017), the results of observations in the form of isolates that can grow on media either existing or no paraffin oil means oxidative-fermentative, while isolates that only grow on media that are not closed paraffin oil only means oxidative.

Selective Media Test (King's B and YDC)

In the King's B media test, endophytic bacteria were inoculated on King's B media and incubated at room temperature for 24 - 48 hours. The results of the observations observed the growth of bacteria under a UV lamp. According to Febriani et al., (2022), a positive result in the form of bacterial colonies gives off yellowish-green fluorescence. Negative result of no fluorescence.

In the YDC (Yeast Dextrose Carbonate) media test, bacteria were inoculated on YDC media and incubated at room temperature for 24 - 48 hours. According to Karim et al., (2023), positive observation results in the form of bacterial colonies that can show yellow. Negative results were shown in colonies of bacteria that are not yellow in color on YDC media.

RESULT AND DISCUSSION

Morphological Characterization of Endophytic Bacteria

In this study obtained as many as 13 isolates of endophytic bacteria that will be identified further. The diversity and number of such endophytic bacteria is influenced by internal and external factors. These internal factors can be factors of the host plant, while external factors are environmental factors. According to Wilson et al., (2017), the differences in the number and character of isolates are influenced by host plants and their environmental conditions. Factors such as soil structure, plant age, environmental conditions and sampling time will affect the endophytic bacterial community in the host plant. Handayani et al., (2023) also added that factors such as salinity, temperature, pH, and soil nutrients also affect the diversity of these endophytic bacteria.

Table 1. Morphological characterization of selected endophytic bacterial isolates

Isolate Code	Morphological Characterization of Endophytic Bacteria			
	Colony Shape	**Colony Color**	**Colony Edge**	**Colony Surface**
A 2	Round	Whitish Beige	Equal	Flat
A 8	Round	Whitish Beige	Equal	Convex
A 10	Round	Whitish Beige	Equal	Flat
A 11	Round	Whitish Beige	Equal	Convex
A 14	Round	Yellowish White	Equal	Flat
A 17	Round	White	Equal	Flat
A 18	Round	White	Equal	Flat
A 24	Round	Pale Yellow	Equal	Convex
A 28	Round	Whitish Beige	Equal	Convex
A 29	Round	White	Equal	Convex
A 36	Round	White	Equal	Convex
A 37	Round	Whitish Beige	Equal	Flat
A 45	Irregular	Yellowish White	Wavy	Convex

The results of morphological characterization of selected endophytic bacteria showed mixed results (Table 1). Each of these endophytic bacterial isolates has different morphological characters, including shape, color, colony edge, and colony surface. Morphological observation is the first step to distinguish bacterial isolates and provide an overview of the types of endophytic bacteria involved. In Table 1. it can be seen that, for the shape of the bacterial colony is dominated by a round shape, but there is also 1 endophytic bacterial isolate with an irregular shape. The color of such endophytic bacterial colonies also consists of whitish-beige, yellowish-white, white, and pale yellow. Isolates of bacteria that have a whitish-beige color are as much as 6 isolates, based on the Munsell Soil Color Chart, those sixth isolates have a value of Hue: 2.5 Y, a Value: 8 and a Chroma: 4 (Pale brown). Yellowish-white bacterial isolates are as many as 2 isolates, based on the Munsell Soil Color Chart, the two bacterial isolates have a Hue value: 2.5 Y, a Value: 8 and a Chroma: 3 (Pale brown). White bacterial isolates are as much as 4 isolates, based on Munsell Soil Color Chart, the four bacterial isolates have Hue value: 2.5 Y, a Value: 8 and a Chroma: 1 (White). One isolates of bacteria are pale yellow, based on Munsell Soil Color Chart, bacterial isolates have a Hue value: 2.5 Y, aValue: 8 and a Chroma: 2 (Pale brown).

The edges of the bacterial colonies are mostly flat and 1 isolate is wavy. The surface of the colony is divided into flat and convex surfaces of bacteria. According to Handayani et al., (2023), states that each bacteria has a different ability to adapt to its environment, so that changes in the environment can also affect the morphological and physiological properties of a bacteria. The appearance of endophytic bacteria can be seen in Figure 1.

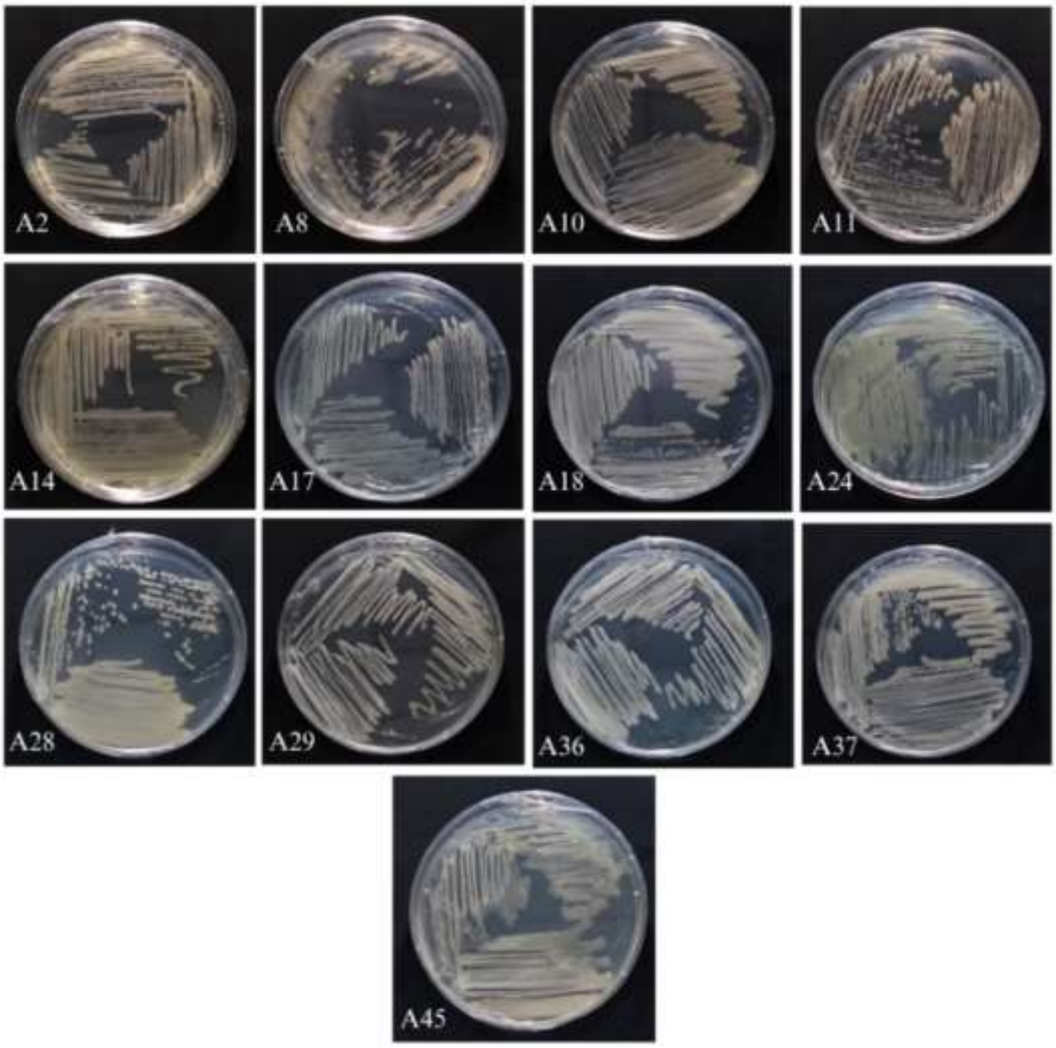

Figure 1. Morphological characterization of selected endophytic bacteria

Identification, Characterization of Physiology and Biochemical of Endophytic Bacteria

The results of physiological and biochemical characterization test of endophytic bacteria with different results on each bacterial isolate can be seen in Table 2. In the Gram test there were 3 isolates of Gram negative endophytic bacteria with cocci bacterial cell form, 1 isolate of Gram negative endophytic bacteria with bacilli bacterial cell form, and 9 isolates of Gram positive endophytic bacteria with bacilli bacterial cell form. In the spore staining test, there were 4 isolates of endophytic bacteria that had endospores and 9 other isolates did not have endospores. In the catalase test there are 7 isolates of endophytic bacteria that produce catalase enzymes and 6 isolates of other endophytic bacteria are not able to produce catalase enzymes. In the oxidative-fermentative test there are 3 isolates of endophytic bacteria that are fermentative and 10 other isolates are oxidative. In the YDC selective media test, there was only 1 bacterial isolate that showed yellow bacterial colonies on YDC media (positive results). In King's B selective media test, there were no endophytic bacterial isolates capable of producing fluorescent pigments.

Table 2. Identification and characterization of physiology and biochemical of endophytic bacteria

Isolate Code	Characterization of Physiology and Biochemistry Test of Endophytic Bacteria						Genus
	Gram Test	Spore Staining Test	Catalase Test	OF	YDC	KB	
A 2	+	-	-	F	-	-	*Lactobacillus*
A 8	+	-	+	O	-	-	*Corynebacterium*
A 10	+	+	+	O	-	-	*Bacillus*
A 11	+	+	+	O	-	-	*Bacillus*
A 14	+	+	+	O	-	-	*Bacillus*
A 17	+	-	-	F	-	-	*Lactobacillus*
A 18	+	+	+	O	-	-	*Bacillus*
A 24	-	-	+	O	+	-	*Xanthomonas*
A 28	-	-	-	O	-	-	Not identified
A 29	-	-	-	O	-	-	Not identified
A 36	+	-	+	O	-	-	*Corynebacterium*
A 37	+	-	-	F	-	-	*Lactobacillus*
A 45	-	-	-	O	-	-	Not identified

Note: In Gram test; (+) = Gram positive result, (-) Gram negative result, in spore staining & catalase test; (+) = positive reaction result, (-) negative reaction result, OF = Oxidative-Fermentative; YDC = Yeast Dextrose carbonate; KB = King's B

In Table 2. can also be seen the results of the identification of endophytic bacteria. The identification results showed that there were *Lactobacillus* bacteria as many as 3 isolates, *Corynebacterium* as many as 2 isolates, *Bacillus* as many as 4 isolates, and 1 *Xanthomonas* isolate. Isolates A 28, A 29, and A 45 have not been identified. These endophytic bacteria can be found in mangroves due to the condition of the uniqueness of mangrove *Avicennia* sp. The root condition of *Avicennia* sp. include rich in nutrients (Ridwan et al., 2018), has a complex root system (Pneumatophore) thus creating anaerobic and aerobic conditions, as well as salinity conditions, and diverse pH (Handayani et al., 2023). The presence of endophytic bacteria on the roots of *Avicennia* sp. also reflects the adaptation of these microorganisms to the typical environmental conditions of mangroves, such as high salinity, temperature fluctuations and low oxygen levels. These microorganisms also have the ability to help their host plants adapt to environmental stress conditions through mechanisms such as the production of enzymes, growth hormones, or antimicrobial compounds.

The identification results also found more Gram positive endophytic bacteria than Gram negative bacteria. This is because, according to Putri & Kusdiyantini (2018), Gram positive bacteria have a thicker cell wall, consisting of a strong peptidoglycan layer, than Gram negative bacteria that have thinner cell walls, thus helping Gram positive bacteria survive better. Gram positive endophytic bacteria also have a competitive advantage in the mangrove environment, because according to Ek-Ramos et al., (2019), some Gram positive endophytic bacteria are able to produce bioactive compounds such as antibiotics that can inhibit the growth of other microorganisms. Gram positive endophytic bacteria also form mutualistic interactions with their host plants, such as the production of growth hormones including auxins, nitrogen binding, or protection from pathogens (Ek-Ramos et al., 2019).

Isolates of endophytic bacteria with codes A 2, A 17, and A 37 based on morphological observations and physiological and biochemical characterization are suspected to have similarities with bacteria of the genus *Lactobacillus*. This is in accordance with Putrayana et al., (2023) which revealed that *Lactobacillus* sp., characterized by macroscopically white to cream color colonies, and microscopically Gram positive bacteria, bacilli cell forms, fermentative, and catalase negative. *Lactobacillus* bacteria are also commonly known as lactic acid bacteria (LAB), which are capable of fermenting carbohydrates into lactic acid. In mangrove ecosystems, *Lactobacillus* bacteria contribute to the decomposition of complex organic matter into organic acids that are beneficial to the host plant. *Lactobacillus* bacteria can also produce antimicrobial compounds such as bacteriocins, which work against plant pathogens. This is in accordance with the statement of Raman et al., (2022), which states that *Lactobacillus* bacteria can be a biocontrol, biostimulant, and biodegradation agents because *Lactobacillus* can improve soil health and fertility (biodegradation), encourage plant growth through stimulation of shoot and root growth (biostimulant), and produce antimicrobial metabolites in the form of bacteriocins (biocontrol). According to Suriawiria (1983), *Lactobacillus* bacteria are able to convert complex compounds into simpler compounds with the final result in the form of lactic acid. Lactic acid is able to produce a low pH on a substrate that creates acidic environmental conditions, so it can inhibit the growth of pathogenic microorganisms, *Lactobacillus* bacteria are also able to produce hydrogen peroxide which can act as an antibacterial.

Isolates of endophytic bacteria with codes A 8 and A 36 from morphological observations and physiological and biochemical characterization are suspected to have similarities with bacteria of the genus *Corynebacterium*. This is in accordance with Wibowo et al., (2020), which states that *Corynebacterium* sp. is characterized by being white or cream color, with a convex elevation shape, bacilli-shaped cell and Gram positive types. In mangrove ecosystems, *Corynebacterium* bacteria act as a bicontrol agent and decomposition agent, Corynebacterium bacteria are able to produce bioactive compounds such as antibiotics that can be used as biocontrol agents for plant protection. *Corynebacterium* bacteria can also help decompose organic matter in mangrove ecosystems. This is according to the statement of Megasari et al., (2017), where, the bacterium *Corynebacterium* sp. is a bacterial biological agent that is antagonistic to several pathogenic microorganisms, both fungi and bacteria, so it is able to control several types of plant diseases. Wardani & Pujiharti (2020), also added that *Corynebacterium* bacteria are also able to produce antibiotics and sideophores, so they can help plant growth.

Endophytic bacterial isolates with codes A 10, A 11, A 14, and A 18 based on morphological observations and physiological and biochemical characterization are thought to have similarities with bacteria of the genus *Bacillus*. This is according to

Holt et al., (1994), which states that, the characteristics of the bacterium Bacillus sp., that is, the bacterial colony color is whitish-beige, the shape of the colony is round and irregular, has the shape of bacilli cells, Gram positive, aerobic, and has endospores as a form of self-defense. In mangrove ecosystems, *Bacillus* bacteria can contribute as PGPR (Plant growth Promoting Rhizobacteria) bacteria and antagonist bacteria against pathogens. *Bacillus* bacteria are able to produce growth-stimulating hormones such as hormones Indole-3-acetic acid (IAA) of phytohormones natural auxin group (Sohibi et al., 2023). *Bacillus* bacteria as antagonist bacteria are able to inhibit the growth of pathogenic microbes through the mechanism of antibiosis, inter-space competition and nutrition, as well as the production of enzymes such as amylase, protease, pullunase, chitinase, xylanase and lipase (Prihatiningsih et al., 2017). *Bacillus* bacteria can also induce plant resistance by colonizing plant tissues and through increased production of secondary metabolite compounds such as salicylic acid, phenol, and peroxidase (Hartono et al., 2024). *Bacillus* bacteria can also help provide nutrients for plant roots. *Bacillus* bacteria when in the root area of plants are also able to utilize root exudate and dead plant residues as nutrients. *Bacillus* bacteria can also produce endospores in environmental conditions that are not suitable for the growth of the *Bacillus*, thus making the *Bacillus* to be able to survive in extreme conditions of high salinity in mangroves. *Bacillus* bacteria can also be bioremediating agents (Oktavia & Sumardi, 2022), because it is able to break down toxic compounds in mangrove soils, such as heavy metals and hydrocarbons, thus making *Bacillus* a potential candidate to improve polluted environments.

Isolates of endophytic bacteria with code A 24 from morphological observations and physiological and biochemical characterization are suspected to have similarities with bacteria of the genus *Xanthomonas*. This is in accordance with the Irpawa (2023) statement, *Xanthomonas* bacteria has the characteristics of yellow round colonies, Gram negative, catalase positive, and oxidative. In extreme conditions of mangrove environment, *Xanthomonas* bacteria can produce xanthan gum, which plays a role in forming biofilms for cell protection from high salinity conditions. This is in accordance with the statement of Hasan et al., (2018) which states that, *Xanthomonas* bacteria can produce biofilms as resistance structures of these bacteria. Most *Xanthomonas* species are generally known to be plant pathogens, but in mangrove ecosystems *Xanthomonas* bacteria can act as commensals by assisting in decomposing organic compounds into simpler forms. Zhang et al., (2022) Some bacteria of *Xanthomonas* species have the ability to produce various enzymes, such as cellulase and pectinase, which allow *Xanthomonas* bacteria to decompose organic compounds in the mangrove environment, thus obtaining a source of nutrients necessary for survival for microbes.

CONCLUSION

In this study were found several groups of endophytic bacteria from the roots of *Avicennia* sp. in the mangrove area of Gunung Anyar, Surabaya. The group of endophytic bacteria found were *Bacillus* bacteria group as many as 4 bacterial isolates, *Lactobacillus* bacteria as many as 3 bacterial isolates, *Corynebacterium* bacteria as many as 2 bacterial isolates, and *Xanthomonas* as many as 1 bacterial isolate. Meanwhile, 3 other endophytic bacterial isolates have not been identified, further identification can be done by molecular analysis. The diversity of endophytic bacteria is due to the unique condition of mangrove *Avicennia* sp.

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