

Biodegradation Test of Rhodamine B by Mangrove Root Symbiont Bacteria Avicennia marina in Sea Panipahan, Muara Rokan, Panipahan Village, Riau, Indonesia

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Abstract

Rhodamine B is a major problem that arises as a result of the development of the textile industry. Rhodamine B is known to be difficult to degrade naturally thus causing a lot of damage, both to the environment and human health such as respiratory tract infection, skin irritation, digestive tract irritation, and eye infection. This study aims to find and determine the type of mangrove root symbiont bacteria Avicennia marina from Panipahan Village that can degrade Rhodamine B. This research is divided into several stages, namely sampling, isolation, purification, characterization of colony morphology, Rhodamine B biodegradation test through the overlay method, gram staining, biochemical tests, and molecular tests to determine the type of bacteria. The results obtained 16 isolates of symbiont bacteria that were successfully isolated from the roots of the Avicennia marina mangrove in Panipahan Village. Of the 16 isolates of symbiont bacteria, only 2 isolates have the potential to degrade Rhodamine B. The results of the biochemical activity test showed positive results in the Catalase, TSIA, motility, and O/F tests on RA5 symbiont bacterial isolates, while the citrate test showed negative results. For RA16 symbiont bacterial isolates, it is known that only the O/F test shows positive results, while the catalase, TSIA, citrate, and motility tests show negative results. The results of bacterial characteristics obtained are that the RA5 symbiont bacterial isolate belongs to the Bacillus circulans type and the RA16 symbiont bacterial isolate belongs to the Bacillus insolitus type. So, it was concluded that there were 2 bacterial isolates of Avicennia marina mangrove root symbionts in Panipahan Village, Riau that were able to degrade Rhodamine B.

Keywords: Rhodamine B, Avicennia marina, biodegradation

INTRODUCTION

Industrial development in the modern era has an important role in society. One industry that is experiencing rapid development is the textile and clothing industry. According to the Ministry of Investment, the growth of the Textile and Clothing Industry increased significantly by 8.73% from 2018 to 2019 (Ramiayu, 2022). The rapid growth of the textile industry harms the environment in the form of direct disposal of textile dye waste into the environment, in a colored state the waste will be difficult to degrade naturally (Ahmad et al., 2021).

This dye waste pollutes the environment which is characterized by changes in O2 and pH levels and inhibition of light penetration in aquatic ecosystems. As well as being toxic and mutagenic to aquatic flora and fauna (Jamee & Siddique, 2019). It is also toxic to humans because it can cause physical and mental disabilities (Sa'adah, 2020). The textile dye that is often used in this industry is Rhodamine B which is a dye that is easily soluble in water, alcohol, HCl, and NaOH, and belongs to the xanthenes dyes group with hazardous chemicals phthalic anhydride and diethyl aminophenol as raw materials (Lellis et al., 2019).

To deal with environmental pollution due to dye waste, biological treatment can be carried out using bacteria (Ahmad et al., 2021). Textile dyes can be decomposed by



microorganisms through two methods, either adsorption on microbial biomass or biodegradation of dyes by cells or enzymes. The use of biomass is particularly useful if the effluent is highly toxic and does not support the growth and maintenance of microbial cells. Adsorbents can include bacteria, microalgae, and fungi but adsorption does not degrade the dye into fragments. In contrast to biosorption, the original dye structure is disrupted in the biodegradation process, often completely decomposing. Thus, biodegradation is a more practical option (Lellis et al., 2019). Referring to the opinion of (Sa'adah, 2020) and Ahmad et al., (2021) that several bacteria can degrade Rhodamine B because they can produce an extracellular polymeric matrix, namely extracellular polymeric substance (EPS), in response to environmental stress.

Some of these bacteria can be found in mangrove ecosystems because they can adapt morphologically, biologically, ecologically, and physiologically to respond to extreme environments under conditions of high salinity, tides, high-temperature pressure, dirt, anaerobic soil, and severe wind pressure. One potential mangrove ecosystem area that has not received special attention is located in Panipahan Village, Pasir Limau Kapas Sub-district, Rokan Hilir Regency, Riau Province. Panipahan Village is a village affected by environmental pollution due to changes in the use and function of mangrove land (Suharni, 2021). Due to its strong resistance to pH stress, high salinity, weather stress, high temperature, tides, and osmotic stress, *Avicennia marina* is the dominant mangrove species in Panipahan Village (Yanti et al., 2021). Therefore, the purpose of this study was to examine the root part of *Avicennia marina* mangrove which contained potential symbiont bacteria as Rhodamine B degraders.

METHOD

Materials and Tools

The main materials used in this study were the roots of *Avicennia marina* mangrove breath obtained from Panipahan Village and Rhodamine B. While other supporting materials are distilled water, 70% alcohol, sterile seawater, sterile cotton, aluminum foil, plastic wrap, ice pack, ziplock, cotton bud, Zobell marine 2216E, Nutrient Broth (NB), Sulfide Indole Motility (SIM), Triple Sugar Iron Agar (TSIA), Fermentative Oxidase media (OF), Simmons Citrate Agar (SCA), Hydrogen Peroxide (H O22), crystal violet, lugol, safranin, and spirtus.

The equipment used is analytical scales, incubator, mortar, and pestle, ose needle, microscope, oven, tweezers, knife, tube rack, spatula, Erlenmeyer, vortex mixer, autoclave, cover glass, object glass, test tube, petri dish, measuring cup, drop pipette, ose needle, goblet, measuring cup, Bunsen, refractometer, pH meter, thermometer, and meter.

This research method is a laboratory experiment that is designed descriptively and through several stages, including isolation of bacteria on the Pneumatophore of mangrove *Avicennia marina*, characterization of bacterial isolates, and testing the ability of bacterial isolates to degrade Rhodamine B.



Figure 1. Research Flow Diagram



Figure 2. Mangrove root sampling points Avicennia marina (Source: Google Maps, 2022)

Sampling Point	Coordinates	Description
1	2°27'55.8"N100°20'18.4"E	Close to the beach
2	2°27'16.8"N100°20'26.7"E	Close to the river

Point Determination and Measurement of Sampling Parameters

Determination of sample points in this study was carried out based on the condition of *Avicennia marina* mangrove plants which were considered representative of the entire research area (Yasin, 2020). Parameter measurements were carried out in stages. First, temperature measurements were taken using a thermometer. The thermometer automatically records the temperature in these waters. Second, a pH meter can be used to measure the amount of acidity (pH). Finally, a refractometer measured salinity by applying a drop of water sample to the tip, and then recording the results given (Kholifi et al., 2021).

Sample Preparation

The purposive sampling method was used to conduct sampling, which is a technique based on the existence of certain objectives rather than strata, randomness, or regions (Sa'adah & Novitasari, 2022). By the sampling conditions, mangrove root samples were taken from 2 different *Avicennia marina* mangrove trees. Root samples were taken at the tip outside the sediment surface and the base was immersed in the sediment by cutting with a sterile knife 3-5 cm long. Mangrove roots were cleaned using sterile seawater by spraying, then put in a zip lock and stored in a cooler box before being transferred to the laboratory within \pm 24 hours to be stored at 4 C until the isolation procedure was carried out (Nursyam and Prihanto, 2018).

Avicennia marina was chosen based on previous research, such as research by Sa'adah (2020) which successfully isolated 10 isolates from the roots of *Avicennia* sp. and tested on crystal violet dye decoloration. Also, research by Sa'adah and Novitasari (2022) with 36 isolates on the roots of *Avicennia marina* and tested as antifouling. Then, *Avicennia marina* is the dominant mangrove commodity in Panipahan village which is affected by environmental pollution. This is due to its ability to survive by symbiosis with bacteria in its root system.

Isolation and Purification of Bacterial Isolates

Pneumatophore mangrove root samples were dried and then crushed using a sterile mortar until smooth (Sa'adah & Novitasari, 2022). The results of the grinding were weighed 1g and put into an Erlenmeyer containing 9 ml sterile seawater, then 1 ml of suspension was put into 9 ml sterile seawater to make a dilution of 10-1, dilutions were made up to 10-5



(Modification of Yanti et al., 2021). Each dilution was homogenized using a vortex and inoculated as much as 35μ L into Zobell 2216E agar media with the spread plate method. After that, the samples were wrapped in plastic wrap to prevent contamination and incubated for 2 to 7 days at 30°C. Bacterial colonies were observed for shape, color, and texture. Each colony of a different color and shape is separated and purified. Zobell Marina Agar 2216E is a selective medium for heterotrophic bacteria with a composition that mimics seawater with minerals, peptone, yeast extract, and salt to support the growth of marine bacteria. (Sa'adah, 2020).

Rhodamine B Biodegradation Test by Bacterial Isolates

The Rhodamine B biodegradation test was carried out by the overlay method. Symbiont bacteria were taken 1 ose and grown on Zobell 2216E agar media on a petri dish with 4 points of bacterial colonies grown on 1 petri dish and formed into a small sphere. Then incubated for 48 hours. After 48 hours, Rhodamine B dye with a concentration of 50 ppm was mixed into agar media with a volume of 200 mL. Zobell marine 2216e which has been mixed with Rhodamine B is poured into a Petri dish that contains bacterial culture that has been incubated 48 hours before, then incubated again for 48 hours and observed for 5×48 hours. If a clear zone is formed, then the bacterial isolate can degrade the Rhodamine B dye (Sa'adah, 2020).

Clear Zone Measurement

The clear zone around the bacterial isolate indicates that the bacterial isolate has the potential to degrade Rhodamine B. The clear zone is measured using a caliper with an accuracy of 0.05 mm (Sa'adah & Novitasari, 2022).

Macroscopic and Microscopic Characterization of Bacterial Isolates

Macroscopic observation is made by observing the color, size, shape, elevation, and edges of the growing colonies (Ramadhanty et al., 2021). Microscopic observation is the observation of cell shape and gram staining following the method of Prihanto et al., (2018) that has been modified. The purpose of this test is to distinguish between gram-positive and gram-negative bacteria, the bacteria are dissolved with sterile distilled water droplets on the object glass so that they form a thin layer and are fixed. Then it is dripped with crystal violet and allowed to stand for 1 minute, then washed with running water and dried in the wind. Next, it is dripped with Lugol and allowed to stand for 1 minute, washed with running water, and dried in the wind. Given a bleach solution that is 95% alcohol, drop by drop until the



purple dye is no longer visible, then wash in running water and dry in the wind. Then given safranin for 30 seconds, then washed and allowed to dry in the air. The red color formed indicates gram-negative bacteria and the purple color indicates gram-positive bacteria.

Physiological Characterization of Bacteria

The catalase test is carried out by applying bacterial isolates to the object glass using an ose needle, dripped with 3% hydrogen peroxide (H2O2) as much as 1-2 drops. The reaction results are positive if there are gas bubbles (O2).

The TSIA (Triple Sugar Iron Agar) test is carried out by inoculating bacterial isolates on the media with the prick and scratch method which is then incubated for 48 hours. The results of the TSIA (Triple Sugar Iron Agar) reaction are seen from 2 parts, namely the slant (sloping surface) and butt (base). Positive test results ferment glucose if the slant is red and butt yellow, ferment lactose and sucrose if the slant and butt are yellow.

Bacterial isolates were inoculated on SCA (Simmons Citrate Agar) media with the scratch method and then incubated for 48 hours for the citrate test. If the media changes color from green to blue, then the test is affirmative.

SIM media is used for motility test by sticking a straight ose needle containing bacterial isolates into a tube containing 10 ml of SIM (Sulfide Indole Motility) media, which is then incubated for 24 hours at 37°C. Bacteria are declared motile if the media becomes hard because the bacteria grow and spread away from the inoculation line.

The oxidative fermentation (O/F) test was carried out by inoculating bacterial isolates in 2 test tubes with paraffin-covered media. Positive bacteria metabolize carbohydrates fermentatively if the tube containing paraffin changes color from green to yellow, while positive bacteria metabolize carbohydrates oxidatively if the media without paraffin is yellow (Yanti et al., 2021).

The salinity test was carried out by making a bacterial suspension with distilled water equalized to 0.5 Mc Farland concentration. Then 1 ml of suspension was taken and NaCl was added with different levels, namely 0%, 10%, 20%, and 30%, each added to NB media and homogenized using a vortex mixer. Then, the NB media containing the culture media was incubated for 24 hours at room temperature.

The pH test was carried out by making a bacterial suspension with distilled water equalized to a concentration of 0.5 Mc Farland. Then 1 mL of bacterial suspension was taken and added to NB media that had been adjusted to pH 5, 6, 7, and 8. Next, it was homogenized using a vortex. Furthermore, NB media that has contained culture media is incubated for 24 hours at room temperature (Islamiah et al., 2017).



Bacterial Identification Test

Bacteria obtained from the breath roots of *Avicennia marina* plants were then identified by referring to Bergey's Manual of Determinative Bacteriology 7th ed and Bergey's Manual of Systematic Bacteriology 2nd ed, based on morphological and physiological characteristics of bacteria, including cell shape, grammatical characteristics of bacteria, biochemical tests, and physiological tests of colony shape, colony color, colony height, and colony boundaries.

Data Analysis

The research data were analyzed descriptively by displaying tables, images, and descriptions of research results based on morphological and physiological character data, as well as the ability to degrade Rhodamine B.

RESULTS AND DISCUSSION

In this study, a comparison of the incubation period for 10 days with a concentration of RhB B 5 ppm was carried out to see the development of bacterial potential in degrading Rhodamine B when the incubation period was added. The clear zone was measured using a caliper every two days to see the comparison of the clear zone produced at each incubation period, as shown in Figure 1, where there was an increase in the clear zone formed during the incubation period of 10x24 hours at 37°C with 4 replicates. The results obtained indicate that the *Avicennia marina* mangrove root symbiont bacteria that have the potential to degrade Rhodamine B have different abilities in the biodegradation process.



Figure 3. The potential of mangrove root symbiont bacteria A. marina to degrade Rh B.



In this study, results were obtained from the isolation of 18 isolates of the mangrove root symbiont bacteria *Avicennia marina*. Then the 18 isolates were characterized based on shape, edges, elevation, size, and color to be purified into single colonies using the quadrant streak plate method. Of the 18 isolates of symbiont bacteria, two isolates of the mangrove root symbiont bacteria *Avicennia marina* were obtained which showed the potential for biodegradation of Rhodamine B. The two isolates of symbiont bacteria were strongly suspected to be *Bacillus circulans* and *Bacillus insolitus* based on biochemical activity, physical resistance tests, and gram staining.

Sampling	Colony Morphology					
Point	Isolate Code	Shape	Edge	Elevation	Size	Color
1	RA5	Circular	Entire	Convex	Punctiform	Cream
2	RA16	Circular	Entire	Convex	Small	Cream

Table 3. Gram stain results					
Postarial Isolata Cada	Gram Staining of Bacteria				
Bacterial Isolate Code	Cell Shape	Color	Description		
RA5 RA16	Basil Coccobasil	Ungu Ungu	Positive Positive		

Table 4. Biochemical Test Results					
Pinchamical Tost	Bacterial Isolate Code				
biochennicai Test	RA5	RA16			
Catalase	+	-			
TSIA					
- Slant/Butt	A/K	K/K			
- Gas	-	-			
- H ₂ S	-	-			
Citrate	-	-			
Motility	+	-			
Oxidative/Fermentative	Fermentative	Fermentative			

Table 5. Physiological Test Results

Bacterial Isolate Code	Physiological Test							
	Salinity				pН			
	NaCl 0%	NaCl 10%	NaCl 20%	NaCl 30%	5	6	7	8
RA5	-	-	+	+	-	-	+	++
RA16	+	+	-	-	+	+	+	+



Based on Table 2, Table 3, Table 4, and Table 5 which are adapted to the determination results using Bergey's Manual of Systematic Bacteriology 2nd ed, it is strongly suspected that isolate RA5 is a halophilic bacterium and belongs to the species Bacillus circulans. The determination results are in line with the identification results by Alebouyeh et al., (2011) The identification results are in line with the results of identification by Alebouyeh et al (2011), who obtained bacteria with Bacillus form, gram-positive, positive TSIA (Triple Sugar Iron Agar) (A/K), positive catalase which means that these bacteria can produce the enzyme catalase to hydrolyze H2O2 compounds, negative citrate, motile, fermentative, grow well in salinity and high pH range, and have cream colony color, convex, with and smooth texture. The size of the bacterial colonies obtained was punctiform, with flat edges, so they were classified into facultative anaerobic bacteria Bacillus circulans.

By the bacterial identification test using Bergey's Manual of Determinative Bacteriology 7th and Bergey's Manual of Systematic Bacteriology 2nd, the RA16 isolate is strongly suspected to be a halophilic bacterium Bacillus insolitus based on the morphological and physiological characteristics of these bacteria, which have a coccus basil form in rows, gram-positive, negative catalase which means that these bacteria are facultative anaerobes, negative citrate, nonmotile, fermentative, halotolerant, grow well at pH 7.4-8.1. And has circular colonies, flat edges with convex elevations, and a small cream color (Whitman, 2009).

The data presented in the diagram shows that only 2 bacterial isolates were able to degrade Rhodamine B, while the rest did not show any Rhodamine B dye decolorization activity. According to Pearce et al (2003), this is related to the ability of bacteria to defend themselves by processing compounds in synthetic dyes into carbon and nutrient sources by changing the chemical structure of the dye.

The difference in the ability to degrade the dye is influenced by the ability of bacteria to carry out appropriate metabolic responses to degrade contaminants in predetermined dye concentrations. The efficiency of biodegradation by bacteria also depends on the ratio between bacterial colonies and the concentration of dye dissolved in the bacterial growth medium (Pinheiro et al., 2022). It is known that bacteria capable of degrading textile dyes have an extracellular matrix (Ahmad et al., 2021) and produce enzymes that can change the chemical structure of pollutants polluted with synthetic dyes to become less complex so that their toxicity level is reduced and become harmless metabolites (Purnamawati et al., 2015). Enzymes commonly produced by synthetic dye-degrading bacteria include azoreductase, laccase, and peroxidase. These enzymes can remodel the chemical structure of textile dyes



through certain mechanisms to produce simpler and colorless molecules (Januariawan et al., 2019).

Determination results showed that isolates RA5 and RA16 are bacteria from the genus Bacillus. Some previous studies classified Bacillus sp. bacteria into the category of lignolytic bacteria which are bacteria that degrade textile dye waste (Prakoso et al., 2022). According to Mamulak, (2018), lignolytic bacteria can damage textile colors due to the similarity of some chemical structures of lignin with dyes so that they match the active side of lignolytic enzymes produced by bacteria. Extracellular lignolytic enzymes can remodel dyes through oxidation-reduction reactions, which will oxidize carbon compounds into C2O and H2O.

CONCLUSION

Based on the results of the study, it can be concluded that there are *Avicennia marina* mangrove root symbiont bacteria from Panipahan Village that have the potential to degrade Rhodamine B as indicated by the increase in the clear zone during the biodegradation test of two isolates from two different locations, namely isolate RA5 from the estuary while RA16 from the seashore. And, the types of *Avicennia marina* mangrove root symbiont bacteria found in Panipahan Village as potential Rhodamine B degrading bacteria are *Bacillus circulans* and *Bacillus insolitus*.

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