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ISOLATION OF ANTIBIOTIC COMPOUND PRODUCING BACTERIA FROM THE SOIL RHIZOSPHERE AGAINST ESCHERICHIA COLI AND STREPTOCOCCUS AUREUS

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ABSTRACT

This research aims to isolate bacteria from the rhizosphere soil which are known to be good media for the growth of bacteria, such as Escherichia coli and Straphlococus aureus as new types of antibiotics. The method used by isolated the bacteria from rhizosphere soil of Rimpang Pacing and Guava rhizome plants and then do the cell morphology characterization, purification, propagation of antibiotic-producing bactrial isolates and continued testing the antibiotic activity of potential bacterial isolates. From the observations, it was found that the isolation of bacteria from the rhizosphere soil of the pacing rhizome had antibiotic compounds that were able to inhibit the growth of Escherichia coli and Straphlococcus aureus bacteria as indicated by the formation of a clear zone around the colony, which had an inhibitory index of 0.5 mm in Ec.2 and Bp.1 isolates is also 0.5 mm. From Both Ec.2 and Bp.1 isolates concluded that the rhizosphere soil produces an inhibitory zone that has the potential to be used as an antibiotic agent.

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1. Introduction

Antibiotics are active substances produced in low concentrations by microorganisms, particularly fungi and bacteria, especially fungi and bacteria, which are capable of killing or inhibiting the growth and metabolic activity of certain microorganisms, while their toxicity for humans is relatively small. Antibiotics can be used to treat infectious diseases. Those are classified as antibacterial, antifungal, anti-amoebic, and other antibiotics. Generally, the mechanism of antibiotics involves suppressing cell wall construction, interfering with protein synthesis, inhibiting cell membrane synthesis, destroying nucleic acids, and interfering with enzyme function (as competitive inhibitors). Antibiotics are classified as beta-lactams (penicillins and cephalosporins), macrolides, tetracylines, and aminoglycosides(Wahjudi et al. 2014; Arora et al. 2013).

Since the discovery of antibiotics, it has been feasible to conquer the problem of infection, which was once one of the world's leading causes of mortality. Infectious infections are a major issue all around the world. The advent of novel infectious diseases that necessitate the discovery and development of antibiotics in order to minimize death and morbidity rates. However, with the widespread manufacture and usage of antibiotics comes the growth of antibiotic-resistant microorganisms.The existence of antibiotic resistance has prompted research to produce new types of antibiotics that are more effective in killing infection-causing microorganisms. (Singer et al. 2003; Wajudi et al. 2014).

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Microorganisms that produce antibiotics can be found in soil, ocean, mud, compost, rumen contents, domestic waste, rotting food ingredients, and other places. The majority of antibiotic-producing microbes, such as Steptomyces, Actinomycetes, Micromonospora, and Bacillus, are acquired from soil microbes, which are mostly bacteria and fungi. (Sethi et al. 2013). Soil, particularly rhizosphere soil, is an excellent medium for the growth and development of numerous microorganisms. The rhizosphere is the area of the soil that surrounds plant roots and protects them from root infections from the outside. Microorganisms in the rhizosphere are usually more numerous and diverse than those found in non-rhizosphere soils. Pseudomonas, Agrobacterium, Azotobacter, Mycobacter, Flavobacter, Cellulomonas, Micrococcus and Bacillus have been found to be abundant in the rhizosphere (Mukamto et al. 2015).

On the other hands, efforts are ongoing to develop antibiotic compounds that are more diverse in terms of activity, mode of action, or chemical structure, and that can be used to compensate for pathogenic bacteria's high level of resistance (Cetina et al. 2010). One of them is to continue searching for new forms of possible antibiotics by isolating microorganisms of diverse types. Therefore, in this experiment, antibiotic-producing bacteria were isolated from the rhizosphere of pacing rhizome and guava rhizosphere soil.

2. Methods

The materials used in this experiment were sterile distilled water, Escherichia coli and Staphylococcus aureus bacteria, 0.85% physiological salt, ±6 mm sterile disc paper, NA (Nutrient Agar) medium, NB (Nutrient Broth) medium, 100 ppm chloramphenicol and samples from soil. rhizosphere of pacing rhizome and guava rhizosphere soil.

a. Technique Sampling

Soil samples were obtained using plastic from the rhizosphere of the paced rhizome and ± 200 g of guava rhizosphere soil. Afterwards, the sample was taken to the laboratory and used immediately in the next phase.

b. Isolation and Selection of Antibiotic-Producing Bacteria

The soil sample from the rhizosphere weighed up to 1 g. The weighed rhizosphere soil sample is then homogenized in 9 mL of sterile 0.85 percent physiological saline solution before being diluted in stages to a dilution of 10-5. The NA dish, which already contained 1 % E. coli and S. aureus bacteria (Appendix 1), then incubated at 27 C for 24 hours with the cup upside down, based on dilutions result 10-4, 10-5, 10-6, and then 0.1 mL in the agar medium (Appendix 1). Bacterial growth and the creation of a clear zone around the colonies were seen on the medium after 24 hours.

c. Cell Morphological Characterization, Purification and Propagation of Antibiotic-Producing Bacterial Isolates

The bacterial colonies that emerged from the isolation were then chosen based on the establishment of a clean zone around the colony and morphology (shape, elevation, margin, color, and surface). The colonies that grew after being characterized were then purified about 6-8 replicas (according to the isolates obtained) in NA medium and incubated at 270 C for 24-48 hours with the cup inverted. From each pure colony that grew and then stored in slanted NA media by taking 1 ose of isolate that had been pure and then streaked on slanted NA media. The colonies were then incubated at room temperature for 24 hours and stored in the refrigerator

d. Antibiotic Activity Test of Potential Bacterial Isolates

Potential bacterial isolates were inoculated at a rate of 1 ose per 5 mL of NB media and incubated for 24 hours at 28 °C in a shaking incubator set to 120 rpm.After 24 hours, 1 mL of bacterial isolate was taken and placed into an Eppendorf tube and then centrifuged at 6000 rpm for 20 minutes. Then, 20 L of the supernatant was taken and dripped onto sterile disc paper which had previously been placed on NA media containing 1% of E. coli and S. aureus bacteria in duplicate. For positive control, 100 ppm chloramphenicol was used, which was dripped with 20 L of sterile distilled water, which was dripped with 20 L. For negative control, it was used sterile distilled water, which was dripped with 20 L. The treatments were then incubated at 27°C for 24 hours. After 24 hours, the clear zone formed around the colony was

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observed and measured using a ruler and the inhibition index was calculated using the equation:

$$\text{Inhibition Index} = \frac{\text{Clear zone diameter (mm)} - \text{disc paper diameter (mm)}}{\text{disc paper diameter (mm)}} \quad (1)$$

3. Results

3.1 Sampling

Rhizosphere soil samples of pacing rhizome and guava rhizosphere soil samples were examined to look for prospective bacterial isolates that might be employed as manufacturers of novel antibiotics. Antibiotic-producing bacteria are expected to be abundant in samples from the selected rhizosphere soil. The rhizosphere is the soil around plant roots. Microorganisms in the rhizosphere are usually more numerous and diverse than those found in non-rhizosphere soils. Ferfinia (2010) claims that soil in the rhizosphere contains more bacteria, fungus, and Actinomycetes than soil without a root structure (without rhizosphere).

Fig 1. Rhizosphere soil sampling location ; a. Rhizosphere of Rimpang Pacing ; b. Rhizosphere of Guava

3.2 Isolation of Antibiotic Producing Bacteria

Rhizosphere soil samples were isolated using NA media. NA media is a rich complex mediumfor growing bacteria. At the isolation stage, to determine the initial ability of the isolate to inhibit the growth of pathogenic bacteria, NA media was added with 1% of pathogenic bacteria , namely E. coli and S. aureus. Both bacteria were used because of their pathogenic nature, representing the Gram-positive and gram-negative groups of bacteria. And according to Melander RJ and Melander C (2017), it has a high level of antibiotic resistance.

The selection of isolated is isolates was carried out based on isolates that were able to grow an form clear zone around them, between the bacterial colonies that grew around them (figure 2). The formation of this clear zone is due to the fact that these bacteria maintain their presence in their habitat by releasing secondary metabolites (including antibacterials) which will diffused into their surrounding, so that the sorrounding microorganism cannot grow. According to Wahjudi et al. (2014) that the formation of clear zone indicates that there is suppression of the growth of microorganisms by antimicrobial compounds.

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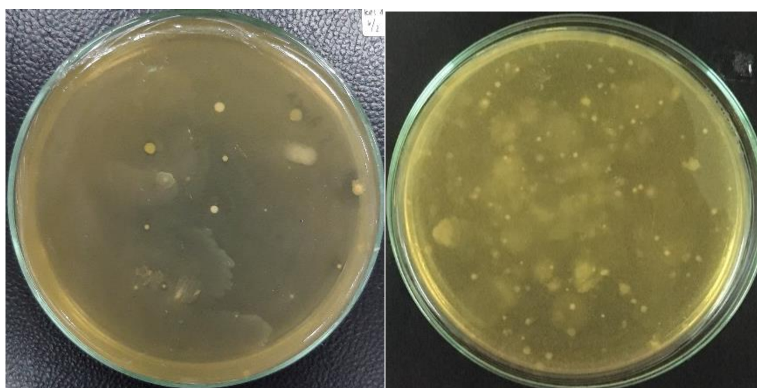

Fig 2. The results of isolation of antibiotic-producing bacteria that form a clear zone; a. Rimpang pacing dilution 10-6 in NA medium containing 1 % S. aureus; b. dilution 10-4 in NA medium containing 1% E. coli

The total isolates obtained and have the potential to produce antibiotic compounds were 13 isolates (Table 1). However, 8 isolates only grew and formed a clear zone on teh media with the addition of *E. coli* bacteria with guava rhizosphere soil samples at a dillution of 10^{-4}. Meanwhile, 5 isolates only grew and formed a clear zone on the media with the addition of *S. aureus* bacteria with rhizosphere soil samples of Rimpang Pacingrhizomes a dilution of 10^{-6}.

TABLE 1.
THE NUMBER OF BACTERIAL ISOLATES THAT GROW AND FORM A CLEARE ZONE

Pathogenic bacteria	Dilution 10^{-4}	Dilution 10^{-5}	dilution 10^{-6}
Eschericia coli	8	-	1
Staphylococcus aureus	-	-	5

Bacterial colonies that grew and formed a clear zone, in the the soil sample the rhizosphere of Rimpang Pacing rhizome only grew at a dilution level of 10-6 and guava rhizospheresoil only grew at adilution level of 10-4, whileat a dilution of 10-5 no colonies grew. The results of the isolation of antibiotic-producing bacteria isolates also showed that each soil sample produced a different number of isolates. Isolates that grew and formed a clear zone were mostly produced by soil samples taken from guava roots. According to Sulistiyani and Nunuk (2011, four different species will produce differnt numbers of microbial isolates even they are taken from the same type of plant roots. Differences in microbes may be influenced by differences in plant species.

3.3 Colony Morphological Characterization and Isolate Purification

A total of 14 isolates have been isolated from the soil samples of the rhizosphere of Rimpang Pacingrhizome and guava rhizosphere which are seen to form clear zone at dilutions 10-4 and 10-6. Each of these isolates had different colony morphological characters, but only 4 isolates were morphologically characterized, namely isolates with codes Ec.2, Ec.4, Bp.1 and Bp.2 (Table 1). Morphological characterization of these isolates was carried out prior to purification on the new NA medium. The morphological characterization of the colonies observed included colony shape, elevation, margin, opacity, color, surface nd colony abudance. The abudance of each type of colony for each isolate shhould be different. However, based on the characterization isolates which were isolated by forminga clear zone, they had the same abudance, which was only 1 isolate.

TABLE 2.
MORPHOLOGICAL CHARACTERISTICS OF ISOLATED BACTERIAL ISOLATES

No	Isolate code	Colony Morphology						Abudance
		Form	Elevation	Margin	Opacity	Color	Surface	(Colony)
1	Ec.2	Circular	Raised	Entire	Opaque	White bone	Smooth	1×10-4
2	Ec.4	Cirkular	Raised	Entire	Opaque	White	Smooth	1×10-4
3	Bp.1	Cirkular	Umbonate	Entire	Opaque	White	Rough	1×10-6
4	Bp.2	Cirkular	Raised	Entire	Opaque	White bone	Smooth	1×10-6

Purification of isolates of bacteria that produce antibiotics was carried out by trasferring them to a new NA agar medium. Colony purification is carried out to seperate one bacterium from another which is mixed into one colony. This is done in order to get one type of pure bacteria to be propagated. Purification was carried out on a patri dish containing NA media which had been

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divided in to 8 rectangular regions. According to Wahjudi et al. (2014) this stage was carried out to give isolates the opportunity to produce secondary metabolites, including antibiotics first (Figure 3).

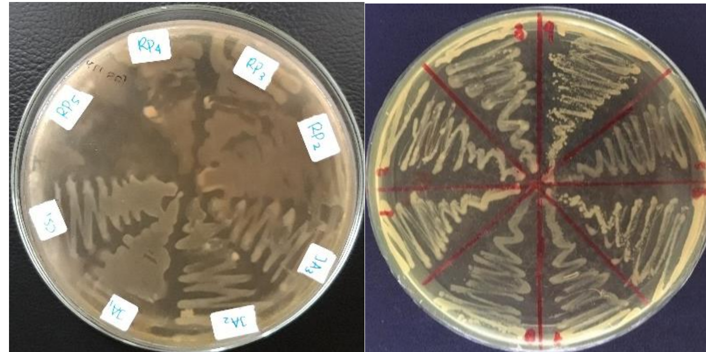

Fig 3. Purification of isolation results; a. Bacterial isolates from the Rimpang Pacing rhizosphere in NA medium containing 1 % S. aureus; b. Bacterial isolates from Guava Rhizosphere in NA medium containing 1% E. coli

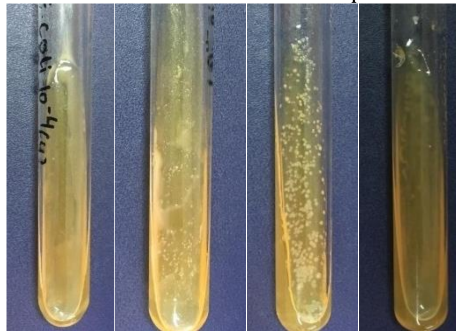

Fig 4. Propagation and storage (stock) on inclined NA medium; a. isolate of Ec.2; b. Ec.4; c. Bp.1; d. Bp.2.

Bacterial isolates that were purified, namely Ec.2, Ec.4, Bp.1 and Bp.2 isolates whose morphological characteristics were known werethen propagated by transferring them to an inclined NA agar medium (Figure 4). Colony propagation is carried out to store potential isolates so that they are not easily contaminated and can be used in the long term, but for storage of isolates it is better to use semi solid media in 1.5 mL Eppendorf tubes so that they can last up to 1-2 years.

3.4 Antibiotic Activity Test of Potential Bacterial Isolates

Testing the antibiotic activity of potential bacterial isolates in inhibiting the growth of E. coli and S. aureus bacteria using the paper disc method.. This method was chosen because the process is simple and produces distinct colonies and zones of inhibition compared to the spot method (dot). Potential bacterial isolates tested for their antibiotic activity were using 2 bacterial isolates from NA media containing E. coli bacteria with isolate codes Ec.4 and Ec.6. Meanwhile, isolates of potential bacteria from NA media containing S. aureus bacteria were also used 2 isolates with isolate codes Bp.1 and Bp.2. The four isolates were tested for their antibiotic activity but only 2 isolates had the ability to inhibit the growth of E. coli and S. aureus bacteria. The bacterial isolate used to test its activity was taken as much as 1 mL and then centrifuged. The purpose of centrifugation is to precipitate cells and also to separate cells with antibacterial substances released by these bacteria into NB media in the form of secondary metabolites. Ec.4 isolate was able to inhibit the growth of E. coli bacteria with an inhibitory index (IP) of 0.5 mm and Bp.1 isolate was able to inhibit the growth of S. aureus bacteria by having an inhibition index (IP) of 0.5 mm (Figure 5). While Ec.6 and Bp.2 isolates did not have an inhibitory index due to the absence of a clear zone around the colony, it was assumed that the Ec.6 and Bp.2 isolates were not able to inhibit the growth of E.Coli and S.areus bacteria (Table 3).

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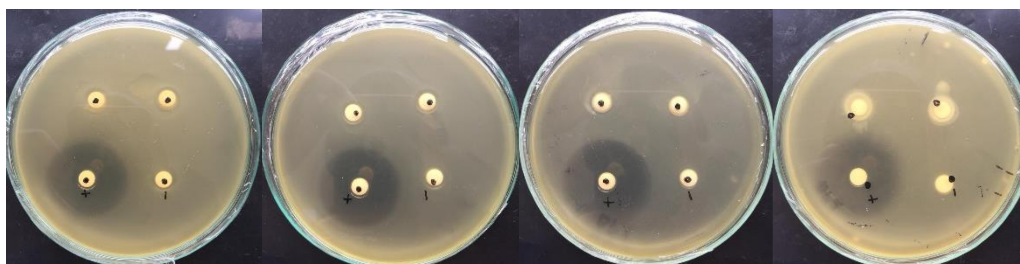

Fig 5. Antibiotic activity test of potential bacterial isolate against E. coli and S. aureus; a. isolate of Ec.4 and b. isolate of Ec.6 against E. coli; c. isolate of Bp.1 and d. Isolate of Bp.2 againts S. aureus (+; chloramphenicol and -; sterile distilled water)

E. coli is a gram negative bacteria while S. aureus is a gram positive bacterium. E. coli and S. aureus bacteria are pathogenic bacteria and are generally food-borne bacteria (Anggraini, 2019; Hardianti, 2019; Sukmawati and Hardianti, 2018; Sukmawati et al. 2018). The two bacteria can represent each group of bacteria based on the differences in the structure of the cell walls they contain. In general, the mechanism of antibiotics in inhibiting the growth of gram-positive and gram-negative bacteria has differences. The mechanism of specific antibiotics in inhibiting gram-positive bacteria by inhibiting the synthesis of peptidoglycan. . According to Martha (2015) beta lactam antibiotics work specifically to inhibit bacterial peptidoglycan synthesis. Beta lactams can inhibit transpeptidase and D-alanine carboxypeptidase which can catalyze the polymerization of the peptidoglycan chain (Suarez, 2009).

TABLE 3.
INHIBITION INDEX OF ANTIBIOTIC ACTIVITY OF POTENTIAL BACTERIAL ISOLATE

Isolate	Total diameter of isolate colony (mm)	**Diameter positive Control (mm)**	**Diameter negative control (mm)**	**Inhibition Index(mm)**		
				isolate	**+**	**-**
Ec.2				0.5	3.83	*
Ec.4	9	29	*	*	3.67	*
Bp.1	*	28	*	0.5	3.83	*
Bp.2	9	29	*	*	3.67	*

Information :
* No Clear zone formed
Control + = chloramphenicol 100ppm
Control - = sterile distilled water
Disc Diameter = 6 mm

4. Discussion

The results of measurement and calculation of the inhibition index on potential bacterial isolates showed that Ec.4 and Bp.1 1 isolates had the same inhibitory power of 0.5 mm (Table 3). The inhibitory power of the two isolates, both against E. coli and S. aureus had an inhibitory power that was not as large as the standard antibiotic activity of chloramphenicol (control +) which was 3.83 mm. This may be due to the low levels of compounds that act as antimicrobials in the supernatant and it is also possible that the E. coli and S. aureus bacteria have thick cell walls making it difficult for antibacterial metabolites to be penetrated by the Ec1 isolate. and Mr.2. According to Wahjudi et al. (2014) if the culture supernatant can provide growth inhibition on the test bacteria even though it is very small, it is possible that concentration will get greater activity. While the negative control using sterile distilled water did not show the formation of a clear zone around the colony. This means that there is no antibacterial activity in the negative control.

The high antibiotic activity was seen based on the high index value with the formation of a large clear zone. However, the small clear zone does not necessarily have a small antibiotic activity because it depends on the penetration ability, the number of antibiotic moleculles and the size and solubility of the antibiotich molecules in the media also affects the size of the inhibition time as well as the consistency of the media used. Therefore, although both Ec.2 and Bp.1 isolates producesd a small inhibition zone, both isolates still have the potential to be used as antibiotict-producing agents. However, further research is needed to test its inhibitory activity against E. coli and S. aureus and also to extract antibiotic compounds.

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5. Conclusions

Isolation of microbes from the soil rhizosphere of Rimpang Pacing and Guava rhizome has the ability to produce antibiotic compounds. . This was indicated by the formation of clear zone around the colony, which has inhibition index 0.5 mm for Ec.2 and Bp.1 also 0.5 mm. Both Ec.2 and Bp.1 isolates produced a small inhibition zone but both isolates still have the potential to be used as antibiotic-producingagents. Meanwhile, Ec.6 and Bp.2 Isolates did not have an inhibitory index due to the absence of clear zone the colony, so it assumed that Ec.6 and Bp.2 isolates were unable to inhibit the growth of E. coli and S. aureus bacteria.

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