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The Potential Endophytic Bacteria Isolated from Rice (*Oryza sativa*) as Biofertilizer

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Abstract

Endophytic bacteria are beneficial microorganisms that interact with host plants without causing any interference or damage to plants. This research aimed to obtain endophytic bacteria isolated from the root tissue of rice plants (*Oryza sativa* L.) which have potential to produce IAA hormones,, identify the endophytic bacteria in morphologically and physiologically, and analysis of the selected isolate 16S rRNA genes. Based on the results of this research, there were six endophytic bacteria isolates obtained. They have high morphological diversity and differen ability producing IAA hormones. The highest concentration of IAA (425 ppm) was obtained isolates from EAP3. Isolate EAP3 also produce inhibit the growth of *Xanthomonas oryzae* with a 5.2 mm inhibition zone. Based on the biochemical test, EAP3 had 60% similarity with *Enterobacter asburiae*. Analysis of the 16S rRNA gene showed that EAP3 had the highest similarity with *Enterobacter asburiae* strain U4 by 99%. This research data is considered as new information about the potential of endophytic bacteria from the roots of rice plants (*Oryza sativa* L.) which is capable of producing IAA hormones and is able to inhibit pathogenic bacteria. This research provides information that can be used as a basis for developing endophytic bacteria as biological fertilizers.

How to Cite

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INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food source for the Indonesian people. According to Hermanto (2006), Indonesian people's rice comsumption every year is 139.5 kg/person/ year. Rice is planted throughout the year to meet the national food needs that continue to increase. Rice is cultivated in 114 countries and more than 50 countries have a minimum production of 100,000 tons/year. However rice production is declining due to several abiotic and biotic factors. Abiotic factors (climate) include rainfall, temperature, sunlight, and wind while biotic factors include the presence of pathogenic microbes that cause leaf midrib blight and blast disease in rice plants (Sari et al., 2017).

One of the efforts that is often carried out by farmers to prevent the attack of pests and microbial pathogens is by using chemical fertilizers. The use of chemical fertilizers results in disruption of the balance of ecosystems, the resistance of pathogenic bacteria and residues that left behind can also have a negative impact on humans and animals. In addition, chemical fertilizers can also reduce the number of soil microbes which are beneficial to plants (Tian et al., 2004). Alternative that can be used to replace the use of chemical fertilizers is utilizing endophytic bacteria (Momota et al., 2012). Endophytic bacteria are symbiotic microorganisms that live in plant tissues without negative effects on their host plants (Mano & Morisaki, 2008).

According to Tarabily et al. (2003), endophytic bacteria can be isolated from roots, stems, leaves, and seeds. Endophytic bacteria can also increase plant productivity by producing growth hormone namely Indol Acetic Acid (IAA) and as a biocontrol agent. According to Bolero et al. (2007), bacteria that are able to produce IAA can increase root growth and extension so that the root surface becomes wider and eventually the plant is able to absorb more nutrients from the soil. Endophytic bacteria can also be used as biocontrol agents because they are able to produce secondary metabolites to increase plant resistance to pathogenic infections (Bandara et al., 2006).

The study of the role and potential of endophytic bacteria producing IAA hormone in rice plants needs to be carried out to be able to optimally utilize endophytic bacteria in rice plants. In order to support this effort, it is necessary to isolate endophytic bacteria producing IAA from the roots of rice plants as biocontrol agents. This study was conducted to obtain endophytic bacterial isolates from the roots of rice plants (*Oryza sativa* L.) as well as to, characterize, the ability of bacteria to inhibit the growth of pathogenic bacteria, the highest producer of IAA hormones will be identified based on biochemical tests and 16S rRNA gene sequence analysis.

This study provides information about endophytic bacteria isolated from the roots of rice plants that have the potential to produce the highest IAA hormone concentration and can inhibit the growth of pathogenic bacteria, so that they can be used as biological agents.

METHOD

Plant Materials

This research was conducted at the Unsyiah Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, UPTD Balai Laboratorium Kesehatan Aceh and Biomolecular Laboratory of the Brackish Aquaculture Fisheries Center (BPBAP) Ujung Batee, Aceh. The rice used in this study were collected from the rice fields in Tengku Hasan on Bakoi, Aceh Besar.

Isolation of Endophytic Bacteria from Roots of Rice Plants (*Oryza sativa* L.)

Endophytic bacteria were isolated from the roots for about 85 days after rice plants obtained in the rice field area of Tengku Hasan in Bakoi, Aceh Besar. The stages of isolation of endophytic bacteria from plant roots begin with the surface sterilization process. The sample was washed with running water until it was clean, then dried and weighed as much as 1 gram. Furthermore, the sample was soaked for 6 minutes in 70% alcohol and continued with soaking into 1% sodium hypochlorite for 4 minutes. After that, the sample was rinsed again with running water 3 times. One gram of sample was taken then crushed until smooth and was grown on NA medium. The sample was then incubated at room temperature for 24-48 hours. Colonies that grew were subcultured on the new NA media until pure isolates were obtained (Strobel & Daisy, 2003).

Hypersensitivity Response Test

For testing the hypersensitivity response of endophytic bacterial isolates, they were grown in NB media stored in a shaking incubator for ± 24 hours at 100 rpm. After the incubation period was completed, 100 µL of endophytic bacterial isolate was taken with micropipette, then they were injected on the surface of the leaves of *Nicotiana tabacum* plants. The infected plants were then incubated at room temperature for 48 hours. Observations were made by looking at necrosis

reactions in *Nicotiana tabacum* leaves. Negative reaction of bacteria that did not show any symptoms of damage to *Nicotiana tabacum* leaves were used for further testing (Schaad et al., 2001).

Antagonistic Test of Endophytic Bacteria Against Pathogenic Bacteria

Endophytic bacterial isolates were tested by pathogenic *Xanthomonas oryzae* bacteria. Test microbes were taken using an ose needle and were made a suspension in 0.9% NaCl solution. Turbidity of the bacterial suspension test was adjusted to 0.5 McFarland's turbidity standard. Twenty-five µL of *X. oryzae* bacteria were grown on NA media using standardized cotton swabs with the scatter method. The endophytic bacterial isolates obtained were transferred by using cork borer (diameter = 7 mm) into NA media containing test bacteria. The media were then incubated for 24 hours at room temperature. Observations were made by measuring the inhibitory zone formed using a caliper. The inhibitory zone is an indication of bacterial sensitivity to the test material expressed by the diameter of the clear zone (Vandepitte, 2005).

Test of Ability to Produce IAA Hormones

Quantitative analysis of IAA was carried out by spectrophotometric method using Triptic Soy Broth (TSB) media. Isolates grown on TSB media were added with 1 g triptopan and then incubated at room temperature for 24 hours. The bacterial culture was then centrifuged at 3000 rpm for 20 minutes. One ml of supernatant was taken and added with 4 ml of Salkowski reagent (150 ml of H2SO4 + 1,351 g of FeCl3.6H2O 0.5M in 100 ml of distilled water) put in a test tube. The mixture was then incubated in a dark place for 30 minutes then the color change that occurred was observed. Color changes from yellow to reddish after incubation indicate that isolates were able to produce IAA. Measuring the IAA concentration produced by isolates was performed using a spectrophotometer at a wavelength of 535 nm. The IAA concentration of the sample was calculated based on a standard curve with pure IAA standards. Endophytic bacteria able to produce the highest IAA were continued to the identification test.

Morphological Characterization and Biochemical Tests

Observation of colony morphology was carried out macroscopically including the shape, color, edges, and elevation of bacterial colonies. Observations of cell morphology include cell shape and Gram staining was performed using a microscope at 1000x magnification. The biochemical test used was KIT DL-96E with a bacterial suspension made with 0.5 McFarland standard from pure bacterial colonies mixed into the diluent solution. One hundred µL of bacterial suspension was inserted into the well and incubated for 24 hours at 37°C. Test cart reading was done by using DL-96 II instrument.

Molecular Identification

The determination of the 16S rRNA gene sequence was carried out by sequencing nucleotides using the services of PT. Genetics Science that works with 1st Base. The base sequence resulted from the sequencing of the 16S rRNA gene from the two types of primers combined were then analyzed by the DNA Baserassembly program. The consensus from the results of merging sequences was stored in the FASTA format. Then the base sequences of each isolate were compared with other microorganisms found in GenBank using Basic Alignment Search Tools nucleotide (BLAST-N). BLAST-N can be accessed on the National Center for Biotechnology Information (NCBI) website http://blast.ncbi.nlm.nih.gov/Blast.cgi. Reconstruction of phylogenetic trees was performed using the MEGA 6.0 program.

RESULTS AND DISCUSSION

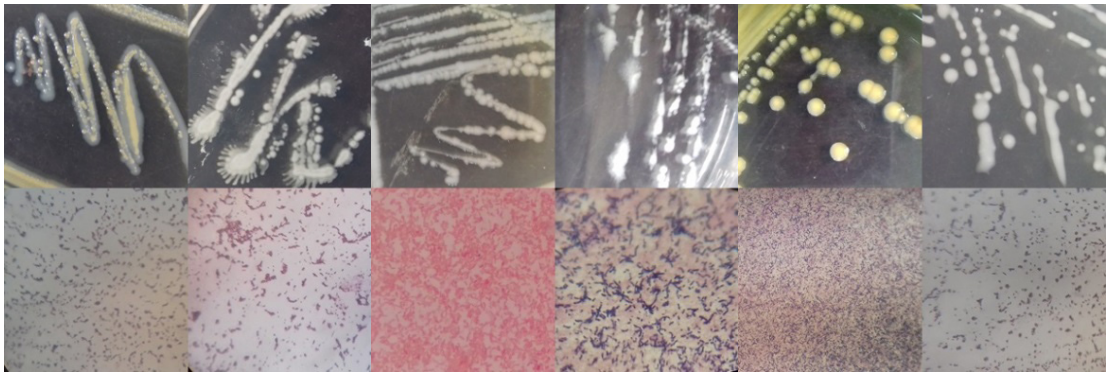
Characteristics of Endophytic Bacteria from the Roots of Rice Plants

Six isolates of endophytic bacteria had been isolated from the roots of rice (*Oryza sativa* L.). The six isolates showed various characteristics based on both morphological features and Gram staining status. Silitonga et al. (2015) reported that endophytic bacteria isolated from the roots of soybean plants had round-colored colonies (white, cream and yellow), bacil cell form and predominantly Gram negative. The results of the study of Anggara et al. (2014) which isolated endophytic bacteria from the roots of sweet potato plants produced 8 isolates with colony morphology dominated by rhizoid with white color. The morphological characterization of colonies and cells of endophytic bacteria isolated from the roots of rice plants can be seen in Table 1.

Table 1. Characterization of morphology of colonies and cells of endophytic bacteria in rice root

Isolat	Characteristics					
	Colony Morphology				Cell Morphology	
	Shape	Color	Elevation	Margin	Shape	Gram staining
EAP1	Circular	Cream	Convex	Smooth	Coccus	Positive
EAP2	Rhizoid	White	Convex	Filamentous	Coccus	Positive
EAP3	Circular	Cream	Convex	Smooth	Bacillus	Negative
EAP4	Circular	White	Convex	Filamentous	Bacillus	Positive
EAP5	Circular	Yellow	Convex	Smooth	Bacillus	Positive
EAP6	Circular	White	Convex	Smooth	Bacillus	Positive

Information: EAP1: Endophytic bacterial isolates from rice root



EAP1 EAP2 EAP3 EAP4 EAP5 EAP6

Figure 1. Morphology of 24 hour old rice root endophytic bacteria and cell colonies on NA media (100x magnification)

Based on the results of characterization with Gram staining, isolate EAP3 was Gram negative, whereas EAP1, EAP2, EAP4, EAP5 and EAP6 were Gram positive in the form of bacil and cocci colonies (Figure 1).

According to Sunatmo (2007) the differences in Gram staining reactions are based on bacterial cell wall composition. The cell wall of Gram positive bacteria contains 90% of peptidoglycan, whereas the cell wall of Gram negative bacteria consists of 5-20% peptidoglycan. Gram staining is useful for distinguishing Gram positive and negative bacteria.

Hypersensitivity of *Nicotiana tabacum* Leaf to Isolated Endophytic Bacteria

Hypersensitivity test results on *Nicotiana tabacum* leaves showed that the leaves injected with EAP1, EAP2, EAP3, EAP4, EAP5, EAP6 isolates and distilled water as negative controls showed no symptoms of necrosis during the 48 hour incubation period (Figure 2). Negative reactions on tobacco leaves showed that bacterial isolates were not pathogenic in plants, so that the six isolates could be applied in the next test. According to Zhu et al. (2000), hypersensitivity reactions are a process of rapid cell death. This reaction appears in plants infected with pathogenic bacteria.

Wulandari et al. (2012), said that the hypersensitivity test is a rapid defense reaction of plants facing pathogens accompanied by rapid cell death in tissues that have been injected with bacterial suspensions, but their presence does not affect the growth of host plants. Symptoms occur wihin 24 to 48 hours after inoculation indicated by green leaves that turn brown followed by drying the tissue. Hypersensitivity test can be used as part of the selection, to determine pure isolates of endophytic bacteria.

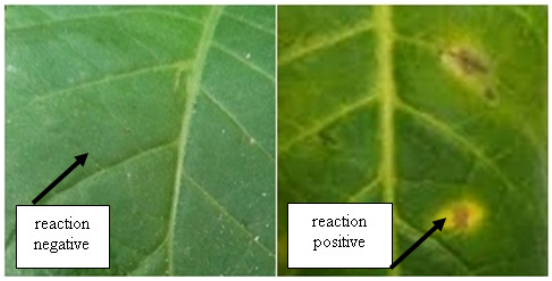


Figure 2. Hypersensitivity of *Nicotiana tabacum* leaf to Isolated Endophytic Bacteria after a 48-Hour Incubation Period

Antagonistic Test of Endophytic Bacteria against Plant Pathogen Bacteria

Based on the results of the antagonist test, it was seen that isolates EAP3 and EAP4 were able to inhibit the *X. oryzae* bacteria. However EAP1, EAP2, EAP5 and EAP6 were not able to inhibit the growth of *X. oryzae* bacteria. The different inhibition response are due to the difference in type of bacteria and secondary metabolites produced. The inhibitory activity is characterized by the formation of clear zones around bacterial isolates. Clear zones formed have varying sizes. EAP3 isolate has a moderate inhibiting ability to the growth of *X. oryzae* bacteria with clear zone diameter of 5.2 mm, whereas EAP4 have weak inhibiting ability with a diameter of only 2 mm. Morales et al. (2003) said that inhibitory zone activities were grouped into four categories: weak (<5mm), moderate (5–10 mm), strong (> 10-20 mm), very strong (> 20–30 mm). According to Kusmarwati and Ninoek (2008), bacterial inhibitory activity was expressed based on clear zones produced around bacterial isolates. The diameter of the inhibition zone of bacteria was measured in mm.

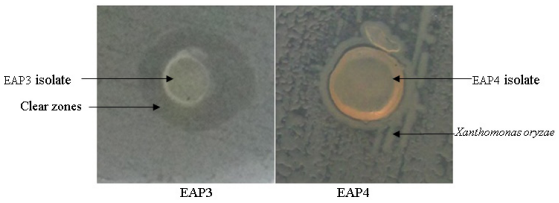


Figure 3. Endophytic bacteria

The ability test to produce IAA hormone

Based on the ability test to produce IAA hormone, it was seen that the six endophytic bacterial isolates were able to produce IAA hormones with varying concentrations. The results of this observation showed that isolates EAP1, EAP2, EAP3, EAP4, EAP5 and EAP6 were able to produce IAA concentrations of 246 ppm, 282 ppm, 425 ppm, 262 ppm, 283 ppm and 246 ppm respectively (Figure 4). The type and number of different bacterial colonies resulted in differences in IAA concentrations. Isolate EAP3 was able to produce the highest IAA concentration of 425 ppm. Based on the results of research from Akbariet al. (2007), endophytic bacteria isolated from the roots of wheat plants had an IAA concentration of 297, different from the results of root isolation of rice plants which showed higher IAA concentrations than the roots of wheat plants.Variations in the concentration of IAA produced by each isolate were probably caused bythe differences in the ability of endophytic bacteria to synthesize tryptophan to IAA.IAA is an endogenous hormone produced by endophytic bacteria which play a role in stimulating the growth of plants. IAA is found at the tip of roots, shoots, flowers.

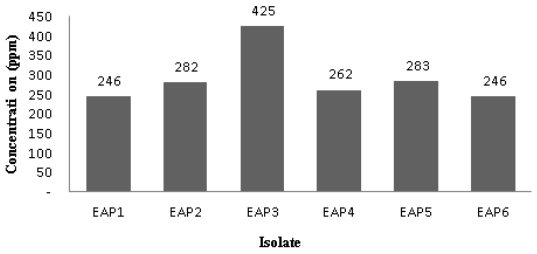


Figure 4. IAA concentration from endophytic bacterial isolates

According to Dewi et al. (2016) bacterial isolates capable of producing IAA will be pink in color because the IAA binds to Fe to form complex compounds. According to Joule and Mills (2000), indications of IAA are seen from the degree of red density that occurs due to the formation of an indole ring. The indole ring is formed after the supernatant of isolate is reacted with the Salkowski reagent. Salkowski is a coloring reagent that can be used to test the indole compound. The Salkowski reagent will oxidize the indole compound. IAA is one example of a compound that has an indole group so that its reaction with Salkowski will produce a pink color. The more concentrated the pink color shows the higher the content of IAA produced by bacteria. The higher the concentration of Salkowski used, the greater the potential for discoloration.

Results of Isolation and Amplification of Bacteria 16S rRNA Genes

Molecular identification was carried out on endophytic bacterial isolates which were able to produce the highest IAA concentrations, namely isolate EAP3. Based on the results of electrophoresis (Figure 5) thick DNA bands were seen. The results of isolate from EAP3 electrophoresis were found to have a parallel band with a marker of around 1,500 bp. Irmawati (2003) stated that thick DNA bands showed total DNA extracted in intact condition. Spread DNA bands indicate a bond between disconnected DNA molecules when the extraction process takes place, so that the DNA genome is cut into small sizes. According to Nugraha et al. (2014), the thickness or thinness resulted DNA band does not affect the ability of each genotype to express proteins because the molecules analyzed are DNA fragments. However the thickness of the DNA band influences the sequencing process.

A research from Nuroniyah and Putra (2012) also reported that the 16S rRNA gene produced DNA bands measuring 1,500 bp. The results of the amplification of the 16S rRNA gene from isolate EAP3 obtained the 16S rRNA gene with a size of 1500 bp which was continued for sequencing (Figure 5).

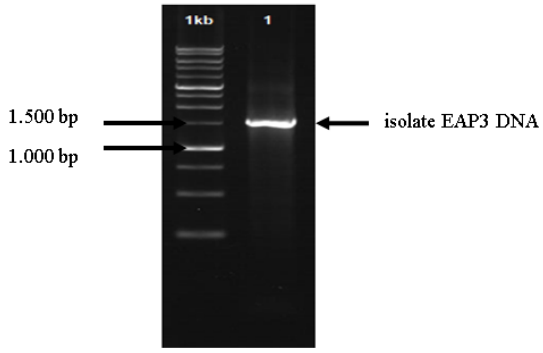


Figure 5. Results of 16S rRNA gene amplification from EAP3 isolates (bp = base pairs; M = marker 1 kb plus; 1 = EAP3 isolate)

Identification of Isolate EAP3

Based on the results of biochemical identification, it is showed that isolate EAP3 gave different responses to the biochemical tests carried out. Biochemical activity is very important in identifying a bacterium. Biochemical activities take place in the body of living things to sustain their lives. Biochemical tests of endophytic bacterial isolates using DL- 96E showed positive reaction for glucose, ornitine, arginine, citrate, arabinose, lactose, sucrose, melibiosa, rhamnosa, raffinosa, salicin, sorbitol, maltose and cellobiosa. The identification results using DL-96 E showed that EAP3 isolates had a similarity of 60% with *Enterobacter asburiae*.

This is in accordance with the results of the study of Brenner et al. (1986) which states that *Enterobacter asburiae* is Gram negative, with bacil cell form and not pigmented. The *Enterobacter asburiae* also have positive for the reaction of methyl red, citrate, hydrolysis of urea, ornitine, glucose, arabinose, selobiosa, galactose, lactose, mannitol, mannose, sorbitol, sucrose and trehalose. They also have negative reaction in proskauer voges, production of H2S, phenylalanine, lysine, gelatin hydrolysis, adonitol and raffinosa fermentation.

Molecular identification based on a partial sequence of 16S rRNA showed that isolate EAP3 had the highest similarity with *Enterobacter asburiae* strain U4 by 99% and the percentage of Query coverage reached 100%. According to Drancourt et al. (2000), the percentage of 16S rRNA sequence homology that is greater than 99% could represent the same species, while sequence homology of less than 97% only represents at the genus level. Based on the biochemical characterization and analysis of the 16S rRNA gene, it is known that isolate EAP3 that has the potential to produce the IAA hormone is members of the *Enterobacter* genus.

Based on BLAST analysis results, phylogenetic analysis is needed to describe the position and kinship between the sequence obtained from this research and sequences stored in the GenBank DNA Database. Phylogenetic trees of isolate EAP3 based on the 16S rRNA gene can be seen in (Figure 6). The results of the phylogeny tree construction showed that EAP3 isolate is in one cluster and has the closest kinship with *Enterobacter asburiae* strain U4. The bootstrapping value obtained at the branch formation is 77%. When reviewed on the BLAST analysis results, the isolate had a 100% Query cover.

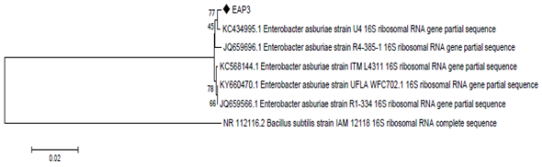


Figure 6. The results of phylogenetic tree reconstruction of isolate EAP3 with MEGA 6

Most bacteria with the *Enterobacter* genus have characteristics of rounded colon with flat edges, white color with flat and thick surfaces, bacterial cells in the form of Gram negative rods, have positive results for citric, motile, and produce gas from glucose fermenting lactose and sucrose but many from bacteria this also has a negative result in the H_2S test (Brooks et al., 2010). According to Ryan et al. (2008) several endophytic bacteria that play a role in the growth of plants such as *Pseudomonas* sp., *Enterobacter* sp., *Staphylococcus* sp., *Azotobacter* sp., and *Azospirilum* sp. This study provides information about endophytic bacteria isolated from the roots of rice plants to produce high hormone concentrations and can inhibit the growth of pathogenic bacteria, so that they can be used as biological agents.

CONCLUSION

Six isolates were obtained from the roots of rice plants (EAP1, EAP2, EAP3, EAP4, EAP5 and EAP6). Result of the analysis showed that there were no symptoms of hypersensitivity which indicated that the isolates were not pat-

hogenic. Based on the test results of EAP3 and EAP4 antagonists able to inhibit the growth of pathogenic bacteria. In hormone production testing, isolate EAP3 showed the highest activity in producing IAA hormone by 425 ppm, while the lowest activity was found in isolate EAP1 and EAP6 by 246 ppm. The results of identification with biochemical tests, EAP3 had similarities with *Enterobacter asburiae* by 60%. Analysis of the 16S rRNA gene showed that EAP3 endophytic bacteria had the highest similarity with *Enterobacter asburiae* strains U4 by 99%.

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