Isolation and characterization of endophytic bacteria from mangrove *Rhizophora mucronata* Lam. and antibacterial activity test against some pathogenic bacteria

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Abstract. Mangrove plants are widely used traditionally in treating various types of infectious diseases in the people of Lombok Island. This study aims to isolate and characterize endophytic bacteria from the roots, stems, leaves and fruits of mangrove *Rhizophora mucronata* Lam. which grow in coastal region of Lombok Island (Gili Sulat), and to determine their antibacterial activity against some pathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa*. Media used for isolation were NA and TSA. Antibacterial activity tests were carried out with well diffusion methods, and the level of antibacterial activity refers to the category according to Vasanthakumari. Characterization of endophytic bacteria includes their colony morphology, cell morphology, and physiology. Eighteen (18) endophytic bacteria isolates were obtained. Results showed that 12 isolates of endophytic bacteria had moderate (8-12mm) to strong category (Φ > 12mm) of their antibacterial activity against *B. cereus* and *P. aeruginosa*. Isolate of NBM2(1) gave the strongest antibacterial activity against *B. cereus*. It can be concluded that endophytic bacteria isolated from mangrove *R. mucronata* Lam. is highly potential to be developed as a new antibacterial source, especially against *B. cereus* pathogenic bacterium.

1. Introduction

Infection is one of the causes of death in the world, especially in the tropics, such as Indonesia. Treatment of infectious diseases caused by pathogenic bacteria is done through the use of antibiotics. However, the use of antibiotics in small doses and in a long time can cause resistance to pathogenic bacteria. This condition could occur because microorganisms can eliminate certain specific targets for drugs for several generations to become resistant [1]. One solution to this problem is to develop new antibacterials obtained from plants that have the potential for antibacterial activity.

Mangrove plants are one of the plants that are known to have great potential as medicines, insecticides and pesticides. *R. mucronata* Lam. has the potential for medical efficacy in treating febrifuge, hematoma, hepatitis and ulcers [2]. A study on the antibacterial activity of *R. mucronata* Lam leaf extract against the bacteria causing diarrhea and obtained the results that methanol extract of the leaves of *R. mucronata* Lam. able to inhibit the growth of *E. coli*, EPEC, *S. aureus*, *P. aeruginosa* and *S. typhimurium* bacteria [3].

The existence of medical benefits from plants in particular can come from a combination of secondary metabolites found in these plants such as alkaloids, steroids, tannins, and phenol components.
that synthesized and stored in plant-specific parts [4]. Leaf, stem, root, fruit and flower extracts of *R. mucronata* Lam. contains alkaloids, tannins, saponins, phenolics, flavonoids, terpenoids, steroids, and glycosides [5]. Furthermore, another research also found that *R. mucronata* Lam. leaf extract contains alkaloids, flavonoids and tannins [6].

Direct extraction of secondary metabolites from mangrove plants that have potential in antibacterial activity requires a large amount of biomass and this is potentially damage the mangrove community and their ecosystem. Endophytic bacteria that live in plant tissues can be used to obtain the bioactive compounds efficiently. Endophytic microbes are groups of organisms associated with various tissues and organs of several terrestrial and aquatic plants [7]. Some endophytic bacteria are known to produce secondary metabolites that are useful in pharmaceutical [8].

This study aimed to isolate and characterize endophytic bacteria from the roots, stems, leaves, and fruits of *R. mucronata* Lam. which grows in Gili Sulat Lombok and to determine its antibacterial activity against *E. coli, S. aureus, B. cereus* and *P. aeruginosa*.

2. Materials and methods

Mangrove plants samples were taken from Gili Sulat, Sambelia District, East Lombok Regency. The process of isolation, characterization and antibacterial testing of endophytic bacteria was carried out in the Microbiology Laboratory, Department of Science Education, Faculty of Teacher Training and Education, Mataram University.

2.1. Isolation of Endophytic Bacteria

Roots, stems, leaves and fruits of *R. mucronata* Lam. were taken in Gili Sulat and stored in a sterile box prior to transfer the laboratory. The root, stem, leaf and fruit materials of *R. mucronata* Lam. were washed and cut into ± 5 cm and sterilized in 70% ethanol solution for 30 seconds then it soaked in 4% NaOCl for 3-5 minutes to avoid surface bacterial contamination. Sterility test was done to ensure that the bacteria that grow on media were endophytic bacteria. Samples of roots, stems, leaves and fruits planted on NA medium, and then incubated for ± 24 hours at 34° C. If there were no epiphytic microbes or microbial contaminants that grow after the test, then it can be ascertained that the bacteria that grows in the sample are endophytic bacteria. Sample of the sterile roots, stems, leaves and fruits were cut in the middle about 1 cm then planted in NA media (dissolved by aquest and by sea water) and TSA (dissolved in aquest and in sea water), samples were then incubated at 34° C for 24-48 hours to allow the endophytic bacteria to grow on the media. The endophytic bacteria that grow on NA and TSA isolation media were then subcultured on fresh NA media and incubated at 34°C for 24-48 hours, until pure colonies were obtained.

2.2. Preparation of supernatant of Endophytic Bacterial Isolates

Endophytic bacterial fermentation is carried out by liquid fermentation using NB media. One ose of pure isolates was taken from stock culture of endophytic bacterial isolates and then planted on sterile NB media and incubated for 24-48 hours at 34° C. The medium containing endophytic bacterial isolates was cultured with shakers at 150 rpm for 24-72 hours. Furthermore, the isolates were centrifugated at 5000 rpm for 30 minutes. The supernatants were then tested for their antimicrobial activity against the growth of the pathogenic bacterial test.

2.3. Antibacterial test of Endophytic Bacteria

Antibacterial activity was tested by well diffusion methods. MHA media were inoculated with test bacteria by spread plate method. About 75µL of endophytic bacterial supernatant were dropped into 6 mm diameter well on the MHA media, then incubated for 24 hours at 34° C. Positive control used was 100 ppm of ciprofloxacin solution and sterile aquest used as negative control.

Antibacterial activity is indicated by the formation the clear zone around the well [9]. Data of antibacterial activity of endophytic bacteria isolated from roots, stems, leaves and fruits of *R. mucronata* Lam. against the growth of test bacteria were analyzed using standard clear zone diameters (Φ) category
according to Vasanthakumari [10], in which: Φ < 8 mm (resistant/weak effect), Φ = 8-12 mm intermediate, and Φ > 12 mm (susceptible/strong effect).

2.4. Characterization of Endophytic Bacteria
Characterization of endophytic bacteria that have antibacterial activity were carried out macroscopically, microscopically, and biochemical tests. Macroscopic characterization includes colony size, colony shape (form), edge shape (margin), color and surface shape (elevation). Microscopic characterization was carried out by Gram staining method. The biochemical test for physiological characterization of endophytic bacteria used included motility test, starch hydrolysis test, catalase test, carbohydrate fermentation test, TSI test, and Simon Citrate test.

3. Results and Discussion

3.1. Isolation and characterization of Endophytic Bacteria
Eighteen (18) isolates of endophytic bacteria were obtained from roots, stems, leaves, and fruit of mangrove R. mucronata Lam. from Gili Sulat. Most endophytic bacterial isolates were found in parts of the fruit, leaves and stems, while endophytic bacteria were not found in the roots as shown in Table 1. The distribution and population of endophytic bacteria can be influenced by several factors. The population density of endophytic bacteria that has been reported varies greatly depending on plant species, methodology and other factors [11]. The results of endophytic bacteria characterization shown in Table 2 that most of the isolates were Gram-positive bacteria which were characterized by purplish color on observation with a microscope. Thirteen isolates of endophytic bacteria were Gram positive and 5 isolates were Gram negative bacteria.

![Table 1. Distribution of endophytic bacteria isolated from R. mucronata Lam.](image)

The cell forms of endophytic bacteria are quite diverse. The most forms of endophytic bacteria were short rods (7 isolates) and coccus (6 isolates). Other forms of endophytic bacteria include 1 isolate oval (Coccobacillus), 2 isolates streptobacillus, 1 isolate (Bacillus), and 1 isolate (Vibrio). Endospores are only found in 3 isolates, 2 of them which have oval central endospores were TSMFB3 and TSMF11b', while NIDM1 has an oval subterminal endospores. The physiological characteristics of endophytic bacteria by biochemical tests also have varying in results as shown in Table 2.
Table 2. Characteristics of endophytic bacteria isolated from R. mucronata Lam.

| No. | Isolates Code | Oxygen requirement | Microscopic Characteristics (Cells Morphology) | Cells Physiology Characteristics |
|-----|---------------|--------------------|-----------------------------------------------|---------------------------------|
|     |               | requirement        | Form | Gram | Other | L | D | H2S | Gas | S | C | Mo | In | Cat | Carbohydrate Fermentation | HP |
| 1.  | TSMFB1        | Anaerob obligat    | Oval  | -    | Cell size 1.52μm-2μm | b | a | - | - | + | - | + | - | - | - | - |
| 2.  | TSMFB2        | Anaerob obligat    | Streptoba cillus | +    | Cell size 2.95 μm-4.85 μm | b | a | - | + | + | - | - | - | - | - | - |
| 3.  | TSMFB3        | Anaerob obligat    | Short rods | +    | Cell size 2.79 μm-3.18 μm | b | a | - | - | + | - | + | - | - | - | - |
| 4.  | TSMFB4        | Anaerob obligat    | Short rods | -    | Cell size 3.51 μm-4.37 μm | a | a | - | - | - | - | + | - | - | - | - |
| 5.  | TSMF11b       | Aerotolerant       | Streptoba cillus | +    | Cell size 1.405 μm-4.7 μm | a | a | - | - | - | - | + | - | - | - | - |
| 6.  | TSMF11b       | Aerotolerant       | Cococcus | +    | Cell size 1.48 μm-2.81 μm | b | a | - | - | + | - | + | - | - | - | - |
| 7.  | TSMF11c       | Microaeroph ilic | Short rods | +    | Cell size 2.27 μm-3.91 μm | b | a | - | - | - | - | + | - | - | - | - |
| 8.  | TSMF11c       | Anaerob facultative | (bacillus) | +    | Cell size 2.36 μm-2.63 μm | b | b | - | - | - | - | + | - | - | - | - |
| 9.  | NBM12a        | Anaerob facultative | Cococcus | -    | Cell size 1.2 μm-3.12 μm | b | b | - | - | - | - | + | - | - | - | - |
| 10. | NBM2(1)       | Aerob facultative | Short rods | +    | Cell size 2.1 μm-2.8 μm | b | a | - | - | + | - | + | - | - | - | - |
| 11. | NBM2(2)       | Aerotolerant       | Short rods | +    | Cell size 1.3 μm-2.78 μm | b | a | - | - | - | - | + | - | - | - | - |
| 12. | NBM22b        | Anaerob facultative | Short rods | -    | Cell size 2.32 μm-2.91 μm | a | a | - | - | + | - | + | - | - | - | - |
| 13. | NBM12a        | Anaerob facultative | Cococcus | -    | Cell size 1.25 μm-3.11 μm | b | a | - | - | - | - | + | - | - | - | - |
| 14. | NIDM1         | Anaerob facultative | Short rods | +    | Cell size 3.09 μm-4.14 μm | b | b | - | - | - | - | + | - | - | - | - |
| 15. | TSDM12b       | Anaerob facultative | Vibrio | +    | Cell size 1.30 μm-1.92 μm | b | a | - | - | + | - | - | - | - | - | - |
| 16. | TSDM12c       | Anaerob facultative | Cococcus | +    | Cell size 1.097 μm-1.12 μm | b | a | - | - | + | - | - | - | - | - | - |
| 17. | TSDM12a       | Anaerob facultative | Cococcus | +    | Cell size 1.31 μm-1.46 μm | b | b | - | - | - | - | + | - | - | - | - |
| 18. | TSDM12b       | Anaerob facultative | Cococcus | +    | Cell size 1.43 μm-1.60 μm | b | b | - | - | - | - | + | - | - | - | - |

Table description:
L: media slope | b: basic (reddish) | -: negative reaction
D: bottom | +: positive reaction
a: acidic (yellowish) | ++: very strong positive reaction

3.2. Antibacterial activity
Most endophytic bacterial isolates have antibacterial activity against test bacterial isolates with varied in their activity categories. Based on Table 3, it is indicated that from 18 isolates, 16 isolates showed antibacterial activity and 2 isolates (TSMFB1 and NBM12a) showed no antibacterial activity against all pathogenic bacterial test. Most endophytic bacterial isolates have antibacterial activity against B. cereus (14 isolates) and P. aeruginosa (13 isolates). While endophytic bacterial isolates that have antibacterial
activity against *S. aureus* were only 2 isolates and 4 isolates against *E. coli*. Endophytic bacterial isolates with the strongest activity was NBM2 (1) which was isolated from stem of *R. mucronata* Lam. against *B. cereus* with diameter 36 mm. Each microorganism has different levels of susceptibility to various antibiotics [12]. This depends on the molecular structure and metabolic pathways of bacterial strains, as well as the antibacterial mechanism of action.

The activity of antimicrobial compounds can have broad or narrow spectrum effects. Broad spectrum antibiotics can be used to treat infections that are not known yet the type of bacteria that causes the infection, to overcome some cases of superinfection (infections caused by several types of bacteria), to overcome infections due to pathogenic bacteria that have been resistant to narrow spectrum antibacterial (narrow spectrum), and as prophylaxis (a drug used to prevent infection when medical treatment such as surgery) [13].

Based on Table 3, most endophytic bacterial isolates have antibacterial activity in both gram positive and gram negative bacteria. TSlDM12a that isolated from leaves of *R. mucronata* showed an activity in all test bacteria with moderate effects on *S. aureus* and *E. coli* and has a strong effect on *B. cereus* and *P. aeruginosa*. While TSMFB3 and TSlDM12b which were isolated from fruit and leaf, repectively showed strong antibacterial effects on 3 types of test bacteria, such as *B. cereus*, *E. coli*, and *P. aeruginosa*. Eight other isolates (TSMFB2, TSMF11b, TSMF11b’, TSMF11c’, NBM2 (1), NlBM12a’, TSDM12b, and TSDM12c) have antibacterial activity on *B. cereus* and *P. aeruginosa* and NlDM1 isolates have activity against *B. cereus* and *E. coli* with various categories for each isolate.

![Figure 1. The average diameter of inhibitory zone of endophytic bacteria against *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa*.](image)

The inhibitory zone shows the influence of antibacterial effect of secondary metabolites produced by endophytic bacteria. Endophytic bacteria often produce metabolites similar to those produced by their host plants. Leaf extracts, stems, roots, fruits, and flowers of *R. mucronata* Lam. known contains secondary metabolites including alkaloids, tannins, saponins, phenolics, flavonoids, terpenoids, steroids, and glycosides [5].
Figure 2. The supernatant inhibition zone of NBM2 (1) against the growth of B. cereus. A. Negative control/aquadest); B. Supernatant of NBM2 (1) isolate; C. Positive control.

Endophytic bacterial isolate with the strongest activity was NBM2 (1) isolated from stem of R. mucronata Lam against B. cereus with diameter of 36 mm (Figure 2). Phytochemical screening using methanol extract, the stem of R. mucronata Lam. shows contain alkaloid, tannin, saponine, phenolic, flavonoids, triterpenoids and glicoside [5]. Flavonoids are widely known as secondary metabolites produced by plants that have antibacterial abilities in response to microbial infections [14]. Alkaloids, tannins, and phenolics are known can inhibit synthesis and damage the structure of bacterial cell walls.

Alkaloids have an antibacterial role by interfering the components of peptidoglycan in bacterial cells so the wall layer is not fully formed and causes cell death [15]. Tanin is known to alter membrane integrity and cell wall formation by binding directly to peptidoglycan acting as an inhibitor of β-lactamase enzymes [16]. The phenol compounds can denaturate cell proteins and affect the permeability of cell walls and cytoplasmic membranes so that cells become lysis [15].

4. Conclusion
Eighteen endophytic bacteria isolates were successfully isolated from mangrove Rhizophora mucronata Lam. Characteristics of colony morphology, cell morphology, and cell physiology of endophytic were very varied among the isolates, in which 15 isolates of endophytic bacteria were gram positive bacteria and 3 isolates were gram negative bacteria. Twelve endophytic bacteria isolates that had strong activity against B. cereus, 9 isolates against P. aeruginosa, 1 isolate against S. aureus and 2 isolates against E. coli. It can be concluded that endophytic bacteria isolated from mangrove R. mucronata Lam. is highly potential as a new antibacterial source to be developed in the future, especially against B. cereus pathogenic bacteria.

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