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Endophytic bacteria inducing antibacterial synthesis of the bark of Raru (*Cotylelobium melanoxylo*n)

Idramsa^{1*}, Endang Sutariningsih Soetarto², L. Hartanto Nugroho², Rarastoeti Pratiwi² and
Eko Prasetya¹

¹Department of Biology, Faculty of Mathematics and Natural Sciences, the State University of Medan, Indonesia

²Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia

ABSTRACT

The bark of raru (*C. melanoxylo*n) has been utilized by local community as antidiarrheal medicine because of the content of active antibacterial compounds. The antibacterial activity of the bark of raru is induced by endophytic bacteria. The objectives of this study are to obtain, and elucidate and the role of endophytic bacteria in tissues of the bark of raru. The presence of endophytic bacteria in tissues of the bark of raru (5-10 years old) was observed microscopically using the slice of technique histological tissues, and the bacteria were directly isolated from the tissues by planting pour plate on Tryptic Soya Agar (TSA) medium. The antibacterial activity of endophytic bacteria isolates was examined using a dual cultural assay against *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 as a model bacterial. The characterization of endophytic bacteria isolates as the antibacterial synthetic inducer of the bark of raru was carry out through a cultivation experiment to elucidate the ability to grow and produce antibacterial compounds endophytic bacterial was identifying based on molecular morphology. Twenty six bacterial isolates were successfully isolated and only one strain BER-17 should on antibacterial activity similar to that of the bark of raru against the tested bacterial. The endophytic bacteria isolate of strain BER-17 was found in specific cells of phellem tissues and inhibitat the growth of *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923. Based on both cellular and molecular morphology analysis, the strain BER-17 revealed the characters with similarity of 97% to genus *Nocardiopsis* sp.

Keywords: Antibacteria, Endophytica bacteria, *Nocardiopsis* sp., *Cotylelobium melanoxylo*n.

INTRODUCTION

Endophytic microbes are microorganisms that colonize the parts of plant organs and interact with host plant without causing any symptoms or diseases in the host plant [1, 2, 3]. Endophytic microbes, including bacteria and fungi, can produce a variety of secondary metabolites that play important roles in agricultural, health, industrial, and biotechnological sectors.

Secondary metabolites are complex chemical compounds consisting of alkaloids, flavonoids, phenol, peptides, steroids, terpenoids, polyketone, benzopyranone, xanthone, tetralone, and so on [5,6]. Several types of endophytic bacteria are able to produce compounds such as taxol, cryptocin, cryptocandin, jesteron, oocydin, isopestacin, pseudomycin, and ambuis acid as the secondary metabolites produced at stationary growth phase [7]. The compounds have been utilized as anticancer, antimicrobial, antidiabetic, antiviral, and antioxidant compounds [8, 6].

Various efforts to found out antibacterial sources as new natural drugs have been performed. The bark of raru (*Cotylelobium melanoxylo*n) is one of the antibacterial sources potentially used as herbal medicines in a long period

of time by local community. The community in South Tapanuli Regency, North Sumatera Province, has utilized the barks and leaves of *raru* for treating some diseases such as diarrhea, malaria, and diabetes [9]. The barks of *raru* contain several compounds such as *ampelopsin* F., *isoampelopsin* F, ϵ -*viniferin*, *vaticanol* A, E, G, and *lyoniresinol* that are useful as antidiabetic medicines [10].

A search for the compounds of secondary metabolite from endophytic microbes in various plants is interesting and important to do [6]. This study is emphasized on the presence and role of endophytic bacteria. The role of endophytic bacteria in plants has not been recognized [11] particularly in association with the production of secondary metabolites. To date very little has been studied about the species of plants such as those in tropical zones for the presence of endophytic bacteria and its role in the formation of secondary metabolites [12]. The objectives of the study are to find out and examine the presence and role of endophytic bacteria in tissues of the bark of *raru*.

MATERIALS AND METHODS

Collection of the bark sample of *C. melanoxylon*

The bark sample was collected by skinning the bark of *raru* (2-10 years old) from the forest around Sitahuis Village, Sibunga Bunga Subdistrict, Central Tapanuli Regency, North Sumatera Province. The sample was cut into plate forms (15-20 cm in length x 3-5 cm in width x 1-2 cm) for further study in laboratory.

Endophytic bacterial isolates

The bark sample was washed to remove the waste particles using the flowing water. The sample was cut into the sizes of 1.0-1.5 cm x 0.5-1.0, placed on filter paper and dried by wind [13]. Furthermore, the bark sample was sterilized by Hallman method [14] modified by immersing it in 96% alcohol (for one minute) and hypochlorite sodium (for five minutes) and reimmersing it in 70% alcohol (for one minute) and passing it through flame. The sample was then washed using sterile aquadest for 3 times, 90 gr was taken and crushed using blender, and 10.0 mL of 0.85% sterile NaCl solution was added. The crushed sample was used as the source of endophytic bacteria.

Isolation of endophytic bacteria was performed by a serial dilution (10^{-2} to 10^{-4}). Each suspension was planted by pour plate technique into TSA media with addition of nystatin (100 mg/L), incubated at a temperature of 30 °C for 3-10 days or until the growth of endophytic bacteria colony occurred.

Selection isolates based on antibacterial activity

The endophytic bacterial isolates were selected based on the ability of inhibiting the growth of pathogenic bacteria such as *Escherichia coli* (ATCC 35218) and *Staphylococcus aureus* (ATCC 25923) by dual culture assay. Endophytic bacteria were grown in petri dish and the tested bacteria were grown in reaction tube using NA media. The bacteria were tested for 24 hours at a temperature of 30 °C and the endophytic bacteria were incubated for 3-5 days at a room temperature. The tested bacteria were diluted until 10^{-6} or in accordance with Mc Farland standard they were swabbed using cotton bud evenly on the surface of NA media. Endophytic colony was taken using a drill (5-7 mm in diameter) and placed in the media that was previously inoculated by the tested bacteria and incubated for 2-3 days. The inhibitory zone (clear zone) was measured and stated in millimeter. The isolates with the ability of inhibiting the growth of both gram-positive and gram-negative bacteria were used for further study.

Observation of the site of endophytic bacteria in tissues of the bark

Endophytic bacteria contained in tissues of the bark of *raru* were detected by using a microscope. The sterile bark sample was cut into small pieces using a sliding microtone in transversal and longitudinal manner with 10 μ m in thick. The preparations of bark slice were colored by Gram staining [18] and observed under a microscope.

Characterization of endophytic bacteria

Endophytic bacterial isolates with potential antibacterial activity were identified by using a standard identification method [15]. Morphological, physiological, and biochemical characters were identified based on the ability of the isolates to grow.

The molecular identification of endophytic bacteria isolates was carried out using 16A rRNA gene by Kumar [19]. DNA isolation was carried out using a specific GeneJET DNA isolation kit. The amplification of 16S rRNA gene of endophytic bacteria isolates was performed by two universal primers 27f (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r (5'-CGG TTA CCT TGT TAC GAC TT-3'). PCR was carried out by PCR machine of SensoQuest Labycycler by regulating an initial denaturation phase at 94°C for 5 minutes, and followed by the denaturation phase at 94 °C for one minute, annealing at 55 °C for one minute, extension at 70 °C for 2 minutes in 40 cycles and final extension at 72 °C for 10 minutes. Analysis of PCR results was carried out using 1.5% agarose gel, 100 ml of TBE buffer, 1 μ l of loading dye, 6 μ l of sample PCR product, 100 bp–1500 bp DNA markers with electrical voltage of

100 V for 45 minutes. The results of electrophoresis were observed by UV Light Gel Documentation System (Biostep, Felix 2040).

The nucleotide sequencing of 16S rRNA gene from endophytic bacteria isolates was compared by referring to the species of bacteria contained in National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) by Basic Local Alignment Search (BLAST) Program to determine the types of isolates from the GenBank database. Reconstruction of phylogenetic tree was done by using MEGA6 program by comparing some DNA sequences of the members of species obtained from the GenBank database of DNA in NCBI.

RESULTS AND DISCUSSION

Collection of the bark sample of *C. melanoxylon*

The bark sample used was *C. melanoxylon* plants collected from the forest in Sibunga-Bunga Village, Sitahuis Subdistrict, Central Tapanuli Regency, North Sumatera Province (01°50'45" N; 98°46'25" E). The plants grow at the altitudes of 750-800 with soil acidity (pH) of 6.4.

Isolation of endophytic bacteria

The results of the isolation of endophytic bacteria grown in TSA media were 26 isolates and the population density ranged from 1.3×10^{-3} – 6.0×10^{-4} CFU. The amount of population density in plant organs ranged from 10^4 to 10^6 individuals per gram of plant sample [16]. Difference in population density can be affected by several factors. Significant difference in population density of endophytic bacteria was associated with a season when the isolation was done, plant growth phase, and plant organs isolated [17].

The site of endophytic bacteria

The endophytic bacteria were generally contained in plants with vascular tissues. The site of endophytic bacteria in plant organs was generally found in intercellular spaces and xylem vessels [18, 19]. Endophytic bacteria in the bark of *raru* were located in phellem tissues with purple and pink color of colony (figure 1).



Figure 1. Transverse section of bark raru (*C. melanoxylon*), showing bacteria (a) colonizing the phellem tissue

Bioactivity assay of endophytic bacteria isolates

The tested bacteria used to select endophytic bacteria were gram-negative bacteria of *Escherichia coli* (ATCC 35218) and gram-positive bacteria of *Staphylococcus aureus* (ATCC 25923). From 26 isolates tested by pathogenic bacteria, one strain was selected based on the ability of inhibiting the growth of gram-positive and gram-negative bacteria as well as the width of inhibiting zone measured in figure 3. The results of the study indicate that the strain with the ability of inhibiting the growth of pathogenic bacteria had a code of BER-17.

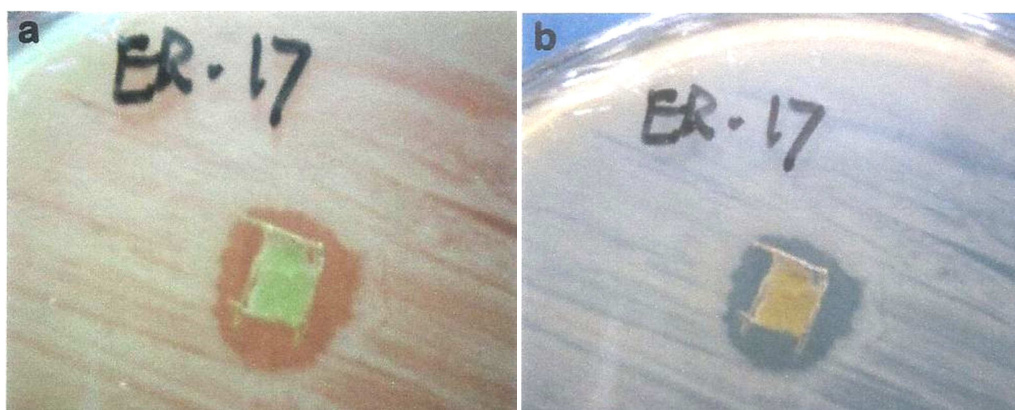


Figure 2. Endophytic bacteria (BER-17) showing antibacterial activity against of *E. coli* (a) dan *S.aureus* (b)

BER-17 Isolates were able to inhibit the growth of pathogenic bacteria because of activities by the secondary metabolites contained in the isolates. It is assumed that the ability of the compound synthesis was a result of both genetic exchange and recombination between endophytic microbes and host plants [5, 6]. The ability of BER-17 isolates in inhibiting gram-positive and gram-negative bacteria indicates that the isolates were able to produce metabolite categorized as broad spectrum antibacteria [22].

Table 1. Morfological, Fisiological and Biochemical Characteristics of Isolate Endophytic Bacteria BER-17

Characteristics	Result
Colony	
Form	Filamentous
Elevation	Raised
Margin	Filamentous
Appear	Dull
Optical proverty	Opaque
Pigmentation	Non pigmentation
Texture	Rough
Growth at temperature	
4 °C	-
10 °C	-
25 °C	+
30 °C	+
35 °C	+
40 °C	-
55 °C	-
Growth at pH	
5	+
6	+
7	+
8	+
9	+
10	+
Growth at NaCl (%)	
2.5	+
5.0	+
7.5	+
10.0	+
12.5	+
15.0	+
20.0	-
22.0	-

The morphological and physiological characters of endophytic bacteria

Endophytic bacterial colony slowly grown for 4-7 days in TSA media, has round gram-positive (0.5-3 mm), white color, non-motile character, filament at edge, and rough surface. The morphological, physiological, and biochemical characters of BER-17 endophytic bacteria isolates can be seen in Table 1.

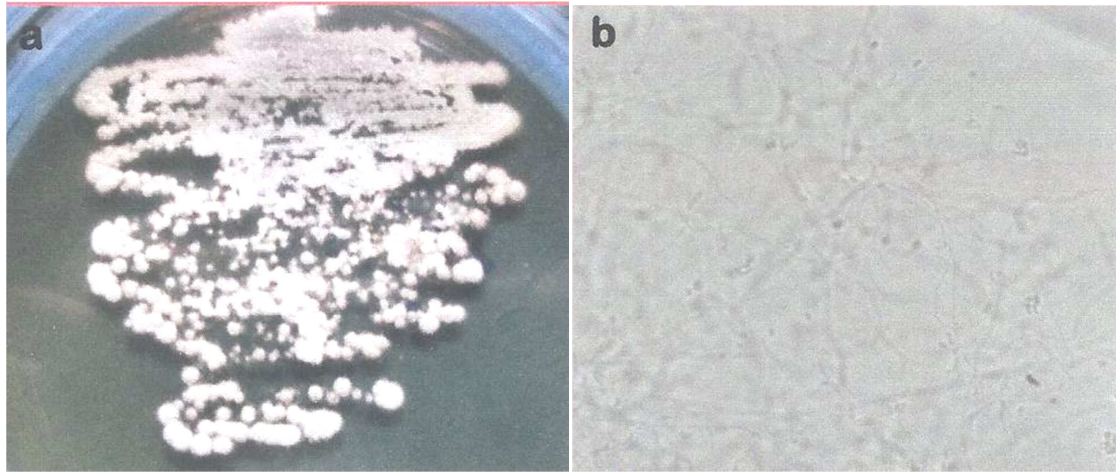


Figure 3. Shows morphological colony isolate endophytic bacteria (BER-17) (a), light micrograph of *Nocardioopsis* sp. (BER-17) grown on TSA plates for 14 days at 30 °C (b)

Genom DNA isolation and the amplification of 16S rRNA gene

Based on the molecular characters, results of the PCR amplification of 16S rRNA gene of BER-17 isolates by the colorization of Green SYBR at 1.5% agarose gel can be seen in Figure (4). Results of the PCR were visualized in tape of less more 1500 bp in length using the primers described above.

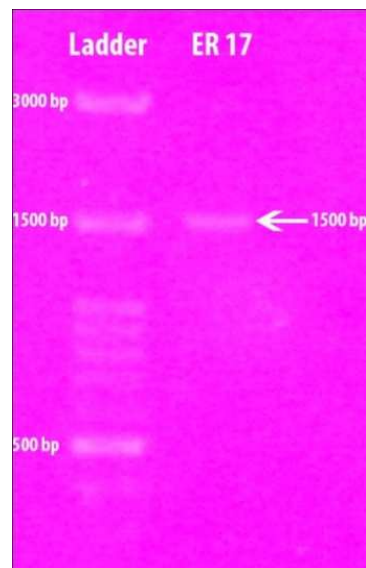


Figure 4. PCR Amplified and Electrophoresis 16S rDNA in 1.5% Agarose Gel

A partial analysis of the sequence of 16S rRNA gene of BER-17 isolates was done compared to those of all bacteria in the GenBank database by using Blast program that can be accessed through <http://www.ncbi.nlm.nih.gov/blast>. The identification of BER-17 isolates based on the sequences of 16S rRNA gene indicates that they had the closest genetic relationship with *Nocardioopsis* sp. (homology 97%).

A phylogenic analysis of the sequence of 16S rRNA gene was performed by using *CLUSTAL W* by Molecular Evolutionary Genetics Analysis (MEGA) program version 5. The phylogenic tree was reconstructed by using MEGA5 program with a neighbor-join algorithm. The topological construction of phylogenic tree was analyzed by the bootstraps of 1000 repetition times (Figure 5). The comparison of the sequences of 16S rRNA gene of BER-17 bacteria isolates showed that the organism formed phyletic lines different from other *Nocardioopsis* species.

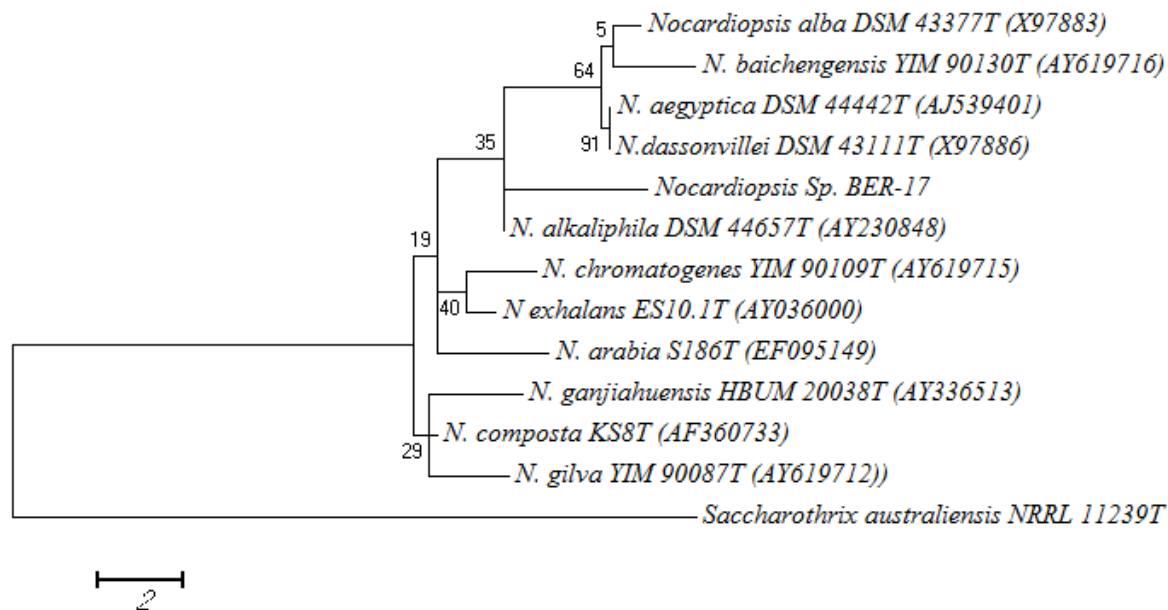


Figure 5. Phylogenetic tree showing the position of *Nocardiosis* sp. BER-17. Maximum Parsimony tree showing the position of strain BER-17 compared to related organisms in a 16S rRNA gene tree. Numbers at nodes indicate levels of bootstrap support (%) based on 1000 resampled datasets

CONCLUSION

Twenty six endophytic bacteria isolates of the bark of *raru* were successfully isolated and they were present in phellem tissues. Based on the selection using a dual culture assay, one isolate with the ability of inhibiting gram-negative bacteria *Escherichia coli* ATCC 35218 and gram-positive bacteria *Staphylococcus aureus* ATCC 25923 was selected. As the selected isolate, BER-17 had the mostly strong activity of inhibiting the gram-positive and gram-negative bacteria. The results of identification using the sequences of 16S rRNA gene showed that BER-17 isolate was *Nocardiosis* sp. (homology 97%).

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