Biosaintifika 10 (3) (2018) 546-552

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Endophytic Bacteria from Faloak Plant Seed (*Sterculia comosa*) as Antibacterial Agent

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DOI: http://dx.doi.org/10.15294/biosaintifika.v10i3.15361

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History Article

Received 6 August 2018
Approved 19 September 2018
Published 31 December 2018

Keywords

Endophytic bacteria; Antimicrobial; Molecular Identification; Faloak

Abstract

Endophytic bacteria isolated from some various kind of plants are able to yield some active compounds which have a role as an antibacterial compound. This work aimed to isolate and to screen the Endophytic bacteria from Faloak seed in its charge in inhibiting two kinds of pathogenic bacteria, *Staphylococcus aureus* and *Escherichia coli*. There were six isolates of Endophytic bacteria isolated in this work. According to the screening result, one isolate which had the most potential antibacterial activity (marked by the formation of inhibition zone) against *S. aureus* and *E. coli*. That most potential isolate was then tested and identified for both biochemical properties and molecular 16S rRNA gene. The result of this study showed that the endophytic bacteria isolate of Faloak seed with the code of S1 had the similarity with *Enterobacter xiangfangensis* strain 10-17 by 93 %. The research about endophytic bacteria of Faloak plants was never conducted before. Thus this research was expected to give information about the potential of antimicrobial ability Faloak plants which can be utilized in the discovery of new antibiotic compounds which in the future are expected to overcome the problem of microorganism resistance to antibiotics. The use of endophytic bacteria is expected to prevents the extinction of Faloak plants due to excessive use.

How to Cite

Moi, M. Y., Kusdiyantini, E., & Pujiyanto, S. (2018). Endophytic Bacteria from Faloak Plant Seed (*Sterculia comosa*) as Antibacterial Agent. *Biosaintifika: Journal of Biology & Biology Education*, 10(3), 546-552.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

The use of plants as a source of herbal medicine increases along the terracing of infection-cause bacteria's resistance against some various antibiotic drugs. Meanwhile, the use of the herbs which are directly taken off from their parent plant, is feared could reduce the population of that plant itself. An alternative is clearly necessary to maintain the plant's sustainability, therefore its population can live longer and can be far from extinction.

One of the alternatives which can be implemented in the search of cheaper and environmentally friendly bioactive compounds is by utilizing endophytic microbes. Endophytic microbes live in the plant tissue without affecting its host plants. The endophytic microbes which is isolated from a herbal plant can produce the identical secondary metabolites like the host plants's, even its amount will be higher when the fermentation process is applied. Therefore, we do not need to cut the host plant off just to take the simplicial (Radji, 2005; Simarmata, 2007; Izza, 2011). Furthermore, compared to the method of the active compounds bacteria-extraction, it needs more complicated process and requires such a long time to gain the active compounds directly from the plant (Purwanto, 2014).

Faloak is a kind of tree that is classified also as an endemic tree found in the Timor Island, East Nusa Tenggara (Ranta, 2011). Faloak is expected as one of 20.900 endemic species or 56% out of 38.000 species found in Indonesia. Faloak is a high-level plants which belongs to genus *Sterculia*. Genus *Sterculia* plant is the biggest genes in the family of *Sterculieaceae* which is distributed in tropical and sub-tropical land (Budiana *et al.*, 2011). A number of research report that the *Sterculia* genus has such amount of active compounds activity. *Sterculia cordata* Bl. and *Sterculia cocciniea* Jack var. seeds contain alkaloids. Active compounds of *Sterculia guttata* seed extract is larvacidal to *Aedes aegypti* and *Culex quinquefascilatus* (Katade *et al.*, 2006). *Sterculia foetida* seed contains alkaloids, flavonoids, and saponins which are very useful in pharmacy. Beside that, active substances of *Sterculia foetida* and *Sterculia urceolata* Smith leaves have antimicrobials activity (Vital *et al.*, 2010; Budiana *et al.*, 2011). Active extract compounds of *Sterculia comosa* Wallich's leaf, seed, and stem bark also has antimicrobials activity on some pathogenic bacteria (Ranta, 2011).

The people of Timor Island use the stem bark of Faloak to cure some diseases like tifus, maag, live, hepatitis. Moreover, it also can be consumed to enhance stamina or to remove pain after hard work. It is believed that stem bark of Faloak is good for recovering after pregnan for woman. The efficacy of herbal medicine from Faloak can not be separated from the active compounds content, which also has a strong connection with the presence of endophytic microbes that live in the plants tissue. The study on Faloak's endophytic microbes is essential to be done so that the potential use of the bacteria live in that plants can be developed, especially for the development of a novel antibacterial drugs. The phenomenon that found these days showed that picking up stem bark of a plant which exceeds the carrying capacity of its tree could die the plant itself (Ranta, 2011). The use of some parts of Faloak which is picked up directly from its tree could give an impact in decreasing the population and even vanishing the Falaok itself. Recently, there is not yet any research concern in the isolation of endophytic microbes from Faloak along with the test of the active compounds that are produced by Faloak's endophytic microbes.

Based on the background above, this research aimed to find out and explore the existence of endophytic bacteria contained in Faloak seed in which its can produce the antimicrobial compounds and to determine endophytic bacterial species use 16S rRNA sequence analysis. This research was expected to give some information about the potential use of endophytic bacteria as antimicrobial agents in the effort to find out bioactive compounds which are efficacious for antibiotics. This research was also expected to inform the society to be able to maintain the presence of Faloak plants in order to prevent decreased population as well as extinction of the plants.

METHODS

Endophytic Bacteria Isolation

Fresh Falaok seeds were collected from Alak, Kota Kupang, NTT. Initially, the seeds were washed and separated from the shell. The samples were sterilized by maserasing in alcohol 70 % for about 5 minutes and in NaOCl 5, 25 % for 5 mins. They were then washed by using alcohol 70 % for 30 seconds and washed again using sterile distilled for 3 times for 5 seconds. The sterile samples were planted in nutrient agar which has been added with nistatin before and incubated on 37 °C for 48 hours. During this process, the grow of each colony was observed thoroughly. The growing isolated bacteria were then purified one by one and were classified based on its colony using Streak Plate method (Purwanto

et al., 2014).

Antagonistic test of Isolated Endophytic bacteria

The isolated endophytic bacteria were inoculated in nutrient broth medium, then incubated in incubator shaker with 150 rpm of velocity on 30 °C for 48 hours. After that, they were sentrifugated on 3000 ppm for 15 mins to separate the pellet and supernatant. The yielded supernatant was tested on *Staphylococcus aureus* and *Escherichia coli* as the target bacteria. Those two test bacteria were prepared through planting an ose of 24-hour-old test bacteria isolate into 10 ml nutrient broth medium and incubated within 18 hours on the temperature of 30 °C. The suspension of the target bacteria was diluted using NaCL 0,9 % and its turbidity was standardized with CFU 10^5.

The test of the antibacterial activity of endophytic bacteria was conducted by using *paper disc diffusion* method. 40 µL of the endophytic bacteria culture was dropped on to the sterile paper disc, then that paper disc was picked up using a sterile pinset and placed on the medium surface which contained target bacteria. It was incubated for 24 hours in 37 °C. The observation was done by measuring the inhibition zone formed around the paper disc. The clean zone formed show that there was an activity of antibacterial compounds produced by endophytic bacteria (Rokhana, 2016).

Biochemical activity test

This test consisted of some parts such as the katalase test, TSIA, Simmon citrate test, ureum hydrolysis test, motilitas test, indol test, H2S, methyl red test, voges proskauer test and koagulase test.

Molecular Identification of Potential Endophytic Bacteria Isolate

The Endophytic Bacteria Isolate which showed the widest inhibition zone against the target bacteria was then identified its molecular properties. The DNA extraction was finished using *Chelex* method (Sutrisno *et al.*, 2013), where two oses of Endophytic Bacteria Isolate were inoculated in 100 µl ddH_2O then were added with 1 mL 0,5 % saponins in phosphate buffered saline (PBS), and were incubated overnight on 4^0C. The endophytic bacteria solution was sentrifugated in 12.000 rpm for 10 mins. The pellet produced from the sentrifugation was added with 100 µl and 60 µl of ddH_2O and *Chelex* 20%, respectively. It was the boiled for 10 mins and vortexed for 5 mins. The sample was sentrifugaed again on 12.000 rpm within 10 minutes and the supernatant which contained DNA were replaced in a new sterile tube.

Furthermore, the DNA suspension was amplified using PCR 16S rRNA. The primer used for PCR 16S rRNA was universal prime bacteria for 27F (5'-TACGGYTACCTTGTTACGACTT- 3') and specific primer *eubacteria* 1492R (5'-AGAGTTTGATCCTGGCTCAG-3'). The initial condition of PCR was set on 94 ^{0}C of temperature along 3 minutes, then followed by 35 cycles which consisted of denaturation on the temperature of 94 ^{0}C for 3 minutes, annealing on 54 ^{0}C for 60 seconds, elongation on 72 ^{0}C for 1, 5 minutes and final elongation on 72^0 C for 8 minutes. The DNA amplification results's visualization was conducted through agarose 1 % gel electrophoresis with the voltage of 100 V within 40 minutes. The outcome was the captured by gelDoc to see the location and the size of the sample's DNA band which was then compared with DNA maker.

The PCR product was then sequenced at 1stBase Asia through PT Genetika Science Jakarta. The sequencing product would be in the form of the DNA base order which was aligned using MEGA6 software. The result encountered further analysis using BLAST on NCBI site ((http://www.ncbi.nlm.nih.gov) to determine the species of the potential endophytic bacteria while the the reconstruction of phylogenetic tree was done by applying *Neighbor Joining* method.

RESULTS AND DISCUSSION

Six isolates of endophytic bacteria were isolated from Faloak Plant Seed from Kupang City, East Nusa Tenggara Province. The isolates were tested for antibacterial activity against testing bacteria *Staphylococcus aureus* and *Escherichia coli*. The isolates of Endophytic bacteria showed diversity in terms of colony morphology (Table 1).

Table 1. The Morphology of Endophytic Bacte-

ria Isolates of Faloak Plant Seed

Isolate Code	Colony Morphology		
	Optical	Color	Surface
S1	Opaque	Broken white	Wavy
S2	Opaque	Yellow pale	Wavy
S3	Opaque	White	Jagged
S4	Opaque	White	Flat
S5	Opaque	White	Wavy
S6	Opaque	Yellowish white	Wavy

Table 2. Diameter of Inhibitory Zone of Endophytic Bacteria from Faloak Plant Seed

Endophytic Bacteria Isolates	Diameter of Inhibitory Zone (mm)	
	Staphylococcus aureus	*Escherichia coli*
S1	9.05	V
S2	8.5	V
S3	6.98	V
S4	7.53	V
S5	7.5	V
S6	6.55	V

Remarks: S = Endophytic Bacteria Isolates from Faloak Plant Seed; V = inhibitory zone was observed but very small and difficult to measure

The results of endophytic bacteria isolates inhibition test from plant seed of Faloak (S) showed that of 6 isolates of endophytic bacteria there was one isolate with S1 code which had the greatest inhibitory and could inhibit both types of target bacteria of *Staphylococcus aureus* and *Escherichia coli* (Table 2).

The formed of inhibition shows the activity of secondary metabolite compounds capable of killing or at least inhibiting the growth of pathogenic bacteria (Purwanto *et al.*, 2014). Based on the measurement of the inhibitory zone diameter (shown in Table 2). The endophytic bacteria from Faloak seed have the potential to produce medium (sensitive) antibacterial compounds in inhibiting target bacteria. Zone of inhibition with a diameter of 20 mm and above shows a very strong antibacterial potential, diamteter of 10-19 mm shows a strong antibacterial potential (sensitive), diameter of 5-9 mm shows an intermediate antibacterial potential, and diameter less than 5 mm shows a weak antibacterial potential (resistant) (Sipriyadi *et al.*, 2016). The antibacterial potential produced by endophytic bacteria isolates from Faloak plant seed belongs to the medium category because they produced 6-9 mm inhibitory zone diameter of zone of inhibition.

Based on the results above, the endophytic bacteria from Faloak plant seed is bactericidal to *Staphylococcus aureus* and is bacteriostatic against *Escherichia coli*. The result shows that

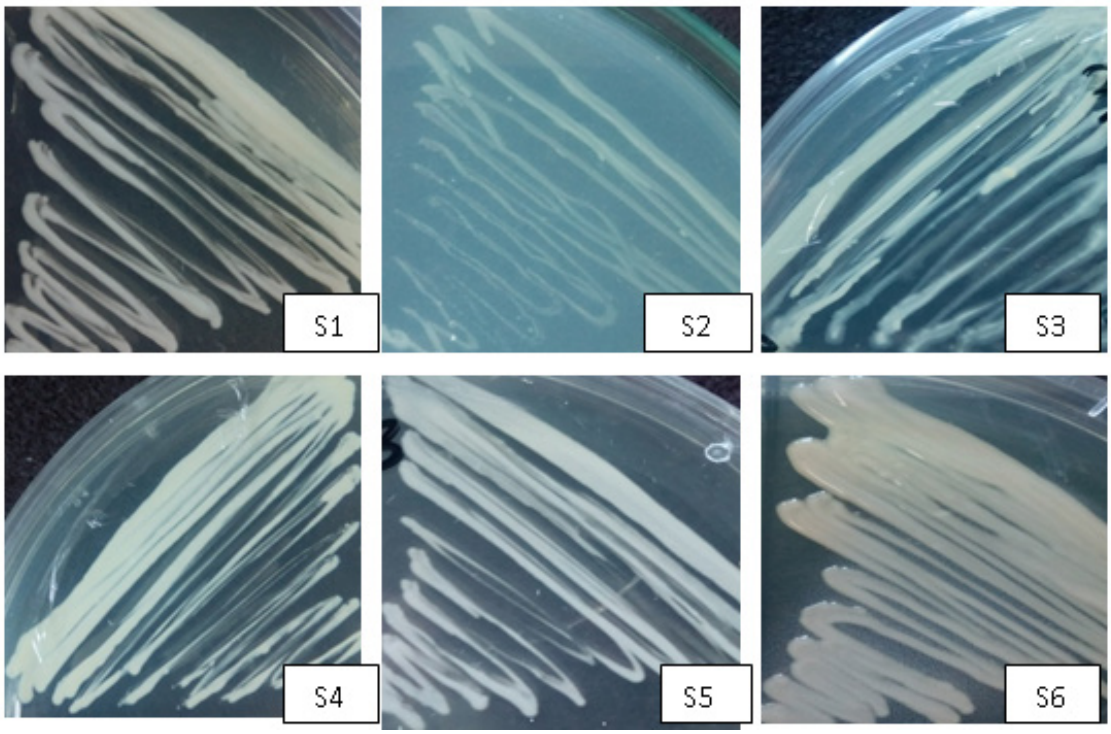

Figure 1. The Morphology of Endophytic Bacteria Colony of Faloak Plant Seeds on nutrient agar media

Gram-positive bacteria were more susceptible to antibacterial. Gram-positive bacteria are more sensitive to antibacterial components because of their simpler cell wall structures (Pelczar & Chan, 2008). Gram-positive bacteria's cell walls are single-layered that makes it easier for antibacterial compounds to enter cells and find targets to work. Meanwhile, Gram-negative bacteria are more resistant to antibacterial compounds because the structure of the bacterial cell wall is more complex that consists of three layers. An antibacterial compound enters the cell and destroys the cell membrane by reacting with phosphate and phospholipid in bacterial cell membrane phospholipids and sterol structure, thus affecting the selective permeability of the membrane (Vital *et al.*, 2010).

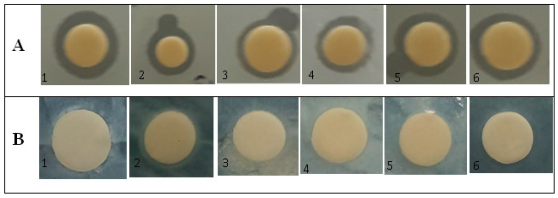

Figure 2. The inhibitory zone of endophytic bacteria isolates to pathogenic bacteria (incubation for 24 hours with temperature of 370 C). *Staphylococcus aureus* (A), *Escherichia coli* (B), (1 – 6) Endophytic bacteria isolates.

Table 3. The result of morphology observation and biochemical test of S1 on endophytic bacteria isolates from Faloak plant seed.

Observation and Biochemical Test	Observation Result
Gram	-
Shape of cell	Coccus
TSIA:	
Slope	a/a
H_2S/Gas	-/+
Simnon citrate	+
Urea	-
Motility	Motil
Coagulation	-
MR/VP	-/-
Indol	-
Catalase	+

Remark: a = acid, MR = *Metil Red*, VP = *VogesProskauer*

Based on the results of antibacterial activity test, endophytic bacteria isolates with the isolate code of S1 was chosen as the most potential isolate for further test (biochemical test and molecular identification) to determine the type of endophytic bacteria isolated from the seed of Faloak plant. The results of morphological observations and biochemical tests of endophytic bacteria S1 isolates are presented in Table 3.

Based on observation of cell morphology with gram staining of the most potential endophytic bacteria (isolates S1), it was known that the bacteria was Gram negative bacteria, basil, motile, facultative anaerobe, white colony color, opaque, and positive to catalase test and O-F test.

The results of the 16S rRNA gene amplification, the endophytic bacteria of S1 produced DNA bands with a base size of ± 1500 bp compared to the DNA marker (100 bp DNA lader) Clarridge (2004) describes the length of the 16S rRNA gene base is 1500-1550 bp. The PCR 16S rRNA visualization results can be seen in Figure 3.

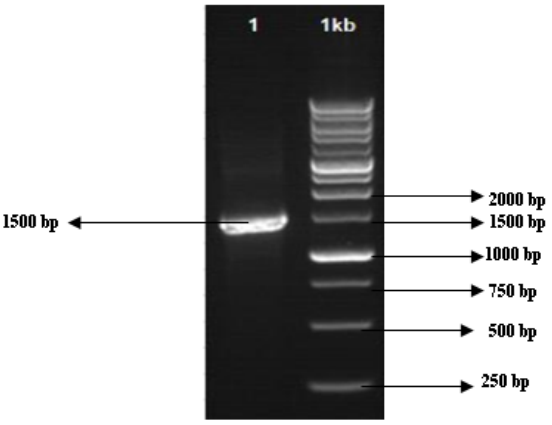

Figure 3. The visualization of the result of PCR 16S rRNA Isolate S1. Remark: 1 = Isolate S1, 1kb = DNA marker

The sequencing results were analyzed by comparing the sequence endophytic bacteria of isolate S1 with data contained in GenBank accessible via the National Center for Biotechnology Information (NCBI). The result of sequence analysis of 16S rRNA using BLAST program showed that S1 isolate had similarity with *Enterobacter xiangfangensis* strain 10-17 with homology similarity of 93% (Table 4). Isolates that have a homogeneous sequence of 16S rRNAs by 93% - 97% may represent an identification up to the genus level, but differ at the species level. Whereas if the sequence equations with a sequence homology level higher than 97%, it can represent the same species (Sing *et al*, 2011). Based on the partial sequence of 16S rRNA, isolate S1 had a homology level with a benchmark species below 97%. It is possible for endophytic bacteria isolate

of S1 as a new genus of γ-class Proteobactria (Drancourt *et al*, 2000). However, to be defined as a new genus, it requires further molecular and phenotypic characterization test, which is beyond the scope of this study.

Table 4. The result of Homology Searching on BLAST for endophytic bacteria of isolate S1

Sample-Code	Length of Sequence (bp)	Name of BLAST result	Accession Number	Homology (%)
S1	1479	*Enterobacter xiangfangensis*strain 10-17	NR_126208	93

The results of phylogenetic tree analysis of isolate S1 showed that the closest genetic relationship between endophytic bacteria with equivalent species were based on partial gene sequences of 16S rRNA. The phylogenetic tree was prepared by using the MEGA 6 program to show the relationship of the isolate sample with the obtained sequence. Phylogenetic tree was made by using Neighbor Joining method with bootstrap value 1000 times presented in figure 4.

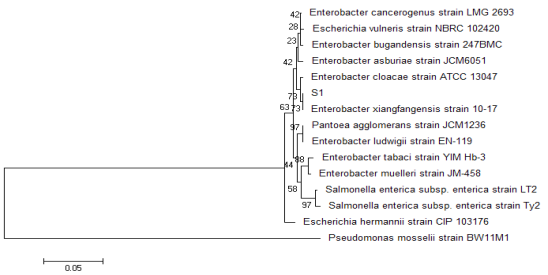

Figure 4. Phylogenetic Tree based on Gen Sequence of 16S rRNA of isolate S1

The genetic distance and phylogenetic tree of endophytic bacteria of isolate S1 molecularly showed that isolates S1 consisted of *Enterobacter* genus and have a genetic relationship with *Enterobacter xiangfangensis* strain 10-17.

The results showed that endophytic bacterial isolates from Faloak seeds have the ability to inhibit *Staphylococcus aureus* and *Escherichia coli* bacteria. The ability of endophytic bacteria in inhibiting pathogenic bacteria is caused by secondary metabolite compounds such as alkaloids, steroids, flavonoids and phenols, which mostly have potential as antibacterial compounds (Tan & Zou, 2001). In addition, endophytic bacteria are able to control some pathogenic microbes due to their ability to live on surfaces and enter into host tissues and they are also able to degrade cell wall components of pathogenic microbes in the presence of hydrolytic enzymes (Jose & Christy, 2013).

A study by Yuwantiningsih *et al.*, (2013) regarding isolated endophytic bacteria from national parks in Java, successfully identified the same endophytic bacteria, that was *Enterobacter xiangfangensis* strain 10-17 which was also known to inhibit the growth of *Fusarium oxysporum* pathogenic fungi. *Enterobacter xiangfangensis* strains 10-17 are known to produce compounds of classes of steroids that may be used as antibiotics. It also produces large amounts of fatty acids, acid phosphatase, alkaline phosphatase, and some enzymes such as esterase (C4), esterase lipase (C8), lipase (C14), trypsin, α-galactosidase, β-galactosidasee, α-glucosidase, β-glucosidase and leucine arylamidase (Gu Tao *et al.*, 2015).

There was not yet any research concern in the isolation of endophytic microbes from Faloak along with the test of the active compounds that are produced by Faloak's endophytic microbes. This research is a primary screening as the first step of innovation of new antibiotic compounds produced by endophytic bacteria in Faloak seeds by knowing the types of endophytic bacteria an for further study can be used to finding a new drugs. The utilization of endophytic microbes with various biotechnology techniques can produce the bioactive compounds to be more efficient without cut down the host plants into simplicia by large quantities, thus the existence and sustainability of the Faloak plants can be maintained, without any degradation or even extinction.

CONCLUSION

Endophytic bacteria isolated from Faloak plant seeds have inhibition ability against the target bacteria *Staphylococcus aureus* and *Escherichia coli.* From a total of 6 isolates, the endophytic bacteria isolated with the code S1 are the most potential isolates to produce antibacterial compounds because has the widest diameter of zone of inhibition. The endophytic bacteria of isolate S1 is known to have genetic relationship with the *Enterobacter xiangfangensis* strain 10-17 bacteria.

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