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ISOLATION OF ENDOPHYTIC BACTERIA AND FUNGI FROM SOURSOP (*Annona muricata* L.) AND BIOACTIVITY TEST AS ANTIMICROBIAL AGAINST *Eschericia coli, Staphylococcus epidermidis,* and *Candida albicans*

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Abstract: This study aimed to test the antimicrobial activity of endophytic bacteria and fungi isolates of soursop (*Annona muricata* L.) in inhibiting the growth of *E.coli*, *S. epidermidis*, and *C. albicans*. The stages of the research were the isolation of endophytic bacteria, antimicrobial assay, and characterization of potential endophytic bacteria and fungi. Endophytic microbial was isolated from leaves, bark, and root of soursop taken from Praya, Central Lombok. The agar diffusion method was used in the antimicrobial activity assay. Characterization was carried out on endophytic microbes with antimicrobial activity based on colony morphology, Gram staining, and biochemical tests. The antimicrobial activity test results showed that none of the endophytic bacteria and fungi isolates had intense activity inhibiting the test microbes' growth. Isolates with code DN_3, DNX, and AKRX had medium activity against *E. coli*, isolates with code AKR_1 had medium activity against *S. epidermidis*, and isolates with code DN_2 AKRX had medium activity against *C.albicans*. The research results showed that the endophytic bacteria from soursop plants could inhibit the growth of test microbes in the weak to moderate.

Keywords: Endophytic Microbia, Antimicrobial, *A. muricata* L.

INTRODUCTION

Soursop plants are included in the Annonaceae family, which has long been used as antibacterial, anthelmintic, fever, and dysentery medicine because of the acetogenin content spread in plant parts such as leaves, stems, roots, and seeds [1]. It is believed that the presence of endophytic microbia in these plants is closely related to the bioactive substances discovered in soursop leaves [2]. Endophytic bacteria and the plants they live on have a mutualistic symbiotic interaction that enables endophytic bacteria to create the same bioactive substances as the plant [3].

Fungi are one of the species that can produce antibacterial compounds. Fungi have contributed to the development of medicine. The earliest source of penicillin antibiotics is a fungus called Penicillium. Fumigatin and Aspergilin are antibiotics made by the Aspergillus fungus [4]. The capacity of endophytic fungi to produce secondary metabolites is most likely the result of genetic recombination from their host plants into endophytic fungi with bioactivities comparable to those of secondary metabolites produced by their host [5].

One of the organisms that can create antimicrobial compounds, in addition to fungi, is bacteria. It is recognized that some endophytic bacteria can create antibiotic-like active substances [6]. It takes a lot of biomass to extract the bioactive chemicals from a plant and consume them directly. Therefore, bioactive substances might be obtained by employing particular endophytic microorganisms in these plants to increase their effectiveness [7]. Endophytic bacteria from seaweed were isolated by Zulkifli et al. (2016), and it was found that these bacteria could inhibit the growth of pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Shygella dysentriae*, and *Staphylococcus aureus*) [8].

The leaves of *A. muricata* are one of the medicinal plants that can be used as a source of endophytic microbes. A plant known as soursop has medicinal properties. The leaf of the soursop plant is the portion most frequently utilized for medical purposes. Saponins, flavonoids, coumarins, alkaloids, and tannins can all be found in soursop leaves. Moreover, only one of the three endophytic bacteria isolates from soursop leaves exhibited antibacterial activity against *Bacillus subtilis* and *Salmonella typhi* [9].

This study used *E. coli, S. epidermidis*, and *C. albicans* as experiment microbes. Staphylococcus is a normal human body flora commonly found on the skin and mucous membranes. *Staphylococcus epidermidis* is a species frequently isolated from human epithelium [10]. *E. coli* bacteria are normal flora in the human colon. Normal flora usually does not cause losses for the host [11]. *C. albicans* is a microorganism that grows in the human body as normal flora in the digestive tract, respiratory tract, and female genital tract. *C. albicans* can cause candidiasis disease that can attack the mouth, skin, nails, bronchi, and lungs and also can attack humans at all age levels, both women and men [12]. Additionally, some endophytic bacteria can dissolve phosphate and

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produce HCN gas. It is also known that endophytic bacteria can bind to free nitrogen in the air, dissolve phosphate, and maintain the equilibrium of plant biomass under stressful environmental conditions [13]. Based on the description above, this research was conducted to determine the antimicrobial potential of the endophytic microbial from soursop (*A. muricata* L.) against micrrobial *E. coli, S. epidermidis*, and *C. albicans.*

RESEARCH METHODS

This research was conducted from September to January 2022 at the Biology Laboratory, Faculty of Teacher and Training Education, Mataram University. The roots, bark, and leaves of soursop plants were cut, and the leaves were then thoroughly washed under running water to isolate bacteria and endophytic fungi. Explants are then sterilized by soaking in 70% alcohol for 1 minute and a hypochlorite solution (NaClO) 5.2% for 2 minutes, after being rinsed in a sterile aqua dish until the sodium hypochlorite smell has disappeared and they are dried. Furthermore, explants are placed on NA and PDA medium, and each is incubated for 24 hours at 32ºC and then for seven days at room temperature to obtain pure isolates.

The endophytic bacterial culture was inoculated on a sterile NB medium and incubated at room temperature for two days on a shaker at 120 rpm. It was then centrifuged at a speed of 5500 rpm for 20 minutes, and taken its supernatant as a material to test for antimicrobial activity.

Endophytic fungal cultures were inoculated on PDY medium and incubated at room temperature on a shaker at a20 rpm for seven days, then centrifuged at 5500 rpm for 20 min. Then the supernatant is taken as a test material for antimicrobial activity.

Antimicrobial activity test is carried out by the method of diffusion of agar. The tested microbes are *E. coli, S. epidermidis,* and *C. albicans*. The inhibition zone criteria used are based on the classification of the inhibitory response of bacterial growth according to Suriawiria [14].

Table 1 Classification of inhibitory responses to bacterial growth (Suriawiria, 2005)

Growth inhibition response	Inhibitory zone diameter (mm)
Weak	<5
Moderate	5-10
Strong	>10-19
Very strong	≥20

Characterization was carried out on isolates of bacteria and fungi that had inhibitory activity against the growth of test microbes. Characterization is carried out by observing macroscopic and microscopic structures. The macroscopic structure of endophytic bacteria includes the colony's shape, color, surface, and edges; microscopic structures include Gram staining and biochemical tests (Simon's citrate test, TSI test, Indole test, carbohydrate fermentation test, and motility test). As for endophytic fungi, the macroscopic structure observed includes the color, shape, and surface of fungal colonies, and microscopic structures include hyphae and spores.

RESULTS AND DISCUSSION

Antimicrobial activity tests of all isolates of endophytic bacteria and fungi against *E. coli*, *S. epidermidis*, and *C. albicans* showed varying activity. More details can be seen in figure 1 and figure 2. The category of inhibitory strength is determined through an inhibitory zone formed around the wellhole. The nine isolates of endophytic bacteria and 3 isolates of endophytic fungi obtained have moderate to weak inhibitory activity. Endophytic microbes can enter plant tissues generally through the roots. However, parts of plants that are directly exposed to the air, such as flowers, stems, and leaves (through stomata), can also be the entry point for endophytic microbes. Microbes that have entered can grow at one particular point and spread to all parts of the plant [15].

In this study, it was found that the highest inhibitory zone diameter was 9 mm in AKR_3 against the growth of *S. epidermidis* and AKRX against the growth of *C. albicans* which were classified as moderate activity. In previous studies, Fibonacci and Hulyadi (2018) have tested antimicrobial activity on *Bacillus subtilis* and *Eschericia coli* using soursop leaf extract with successive inhibition zone results of 12 mm and 10 mm [16]. Silitonga (2019) also researched the effectiveness of soursop leaf extract in ethanol and ethyl acetate solvents. One of the test bacteria used in the study was *S. epidermidis* which showed that at a concentration of 500 mg/ml of soursop leaf extract was able to inhibit test bacteria with an inhibitory zone diameter of 13.13 mm for ethanol solvents and 14.26 mm for ethyl acetate solvents [17].

Meanwhile, in this study, five endophytic isolates were obtained that had inhibitory activity against the three test microbes, specifically DN_2 isolates weak against *E. coli* and *S. epidermidis*. They had moderate activity against *C. albicans*, DN_3 had moderate inhibitory activity against *E. coli* and was weak against *S. epidermidis* and *C. albicans*, DN_4 and AKR_1 had weak activity against the three test microbes, and AKR_3 which had weak activity against *E. coli* and *C. albicans* also has moderate activity against *S. epidermidis*. As for endophytic fungi, two isolates show inhibitory

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activity against the three test microbes: KBX isolates, which are classified as weak against all test microbes, and AKRX, which is weak against *S. epidermidis* and classified as moderate against *E. coli* and *C. albicans*.

Table 2. Results of measurements of the inhibitory zone of soursop (*A. muricata* L.) endophytic bacteria and fungi isolates

Isolate code	Average of the inhibitory zone diameter (mm)		
	E. coli	*S. epidermidis*	*C. albicans*
Endophytic Bacteria			
DN_1	0 (na)	0 (na)	3.3 (w)
DN_2	3 (w)	1.3 (w)	5 (m)
DN_3	3.3 (w)	1.3 (w)	1.6 (w)
DN_4	2 (w)	2.6 (w)	1 (w)
KB_1	0 (na)	3.6 (w)	3.6 (w)
KB_2	1.6 (w)	0 (na)	1.3 (w)
AKR_1	2 (w)	1.3 (w)	1 (w)
AKR_2	0 (na)	0.6 (w)	2.6 (w)
AKR_3	2 (w)	9 (m)	2.3 (w)
Endophytic fungi			
DNX	6.3 (m)	0 (na)	0 (na)
KBX	5 (w)	2 (w)	3 (w)
AKRX	6.3 (m)	2.6(w)	9 (m)

Description: Inhibition activity, no activity (na); weak (w); medium (m)

The presence of an inhibitory zone indicates the content of chemical compounds produced by endophytic bacteria, which is antibacterial [18]. The ability of endophytic bacteria to inhibit microbial growth is due to the presence of metabolite compounds belonging to isolates of endophytic bacteria [19]. Compounds produced by endophytes are secondary metabolite compounds that make up bioactive compounds that can serve to kill pathogens [20]. Antimicrobial substances produced by endophytes must have high toxicity properties but not affect hosts [21]. Endophytic bacteria is symbiotic with soursop leaves. The bacterial isolate has diplobacillus form, and it is a gram-positive bacterium [22]. The result secondary metabolites of such endophytic microbes are alkaloids and terpenoids. Isolated endophytic bacteria from the bark of the srikaya (*Annona squamosa*) and found that 13 colonies of endophytic bacteria have been isolated. Four types of endophytic bacteria are able to inhibit the growth of pathogenic bacteria, that are *B. Brevis*, *B. Latesporus*, *Virgibacillus pantothenticus*, and *B. Circulans*[23].

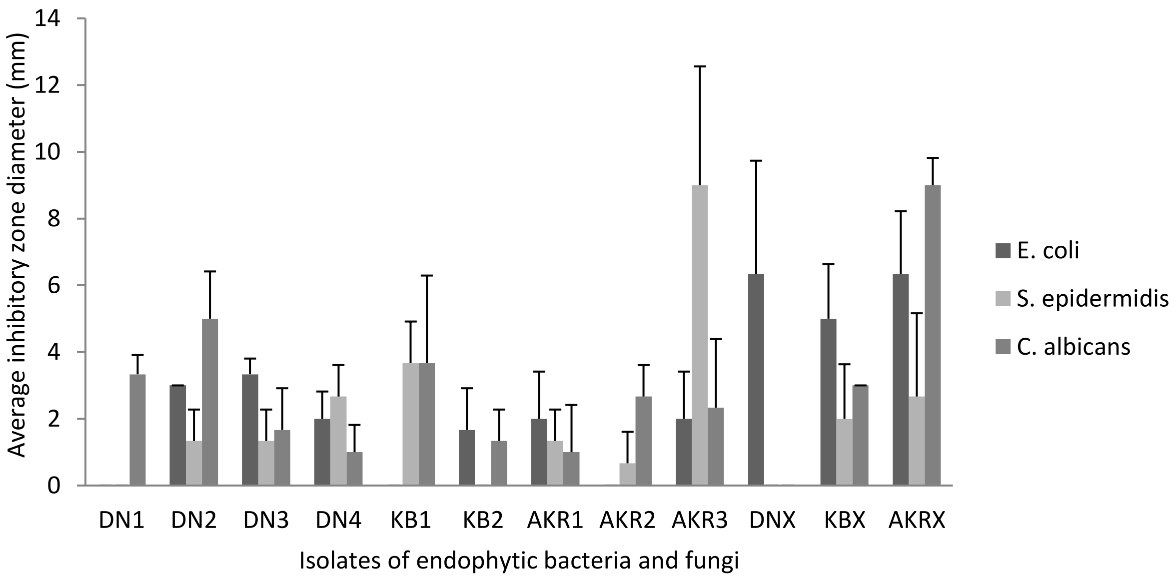


Figure 1. Average of the inhibition zone diameter of microbial growth assayed by endophytic bacteria and fungi

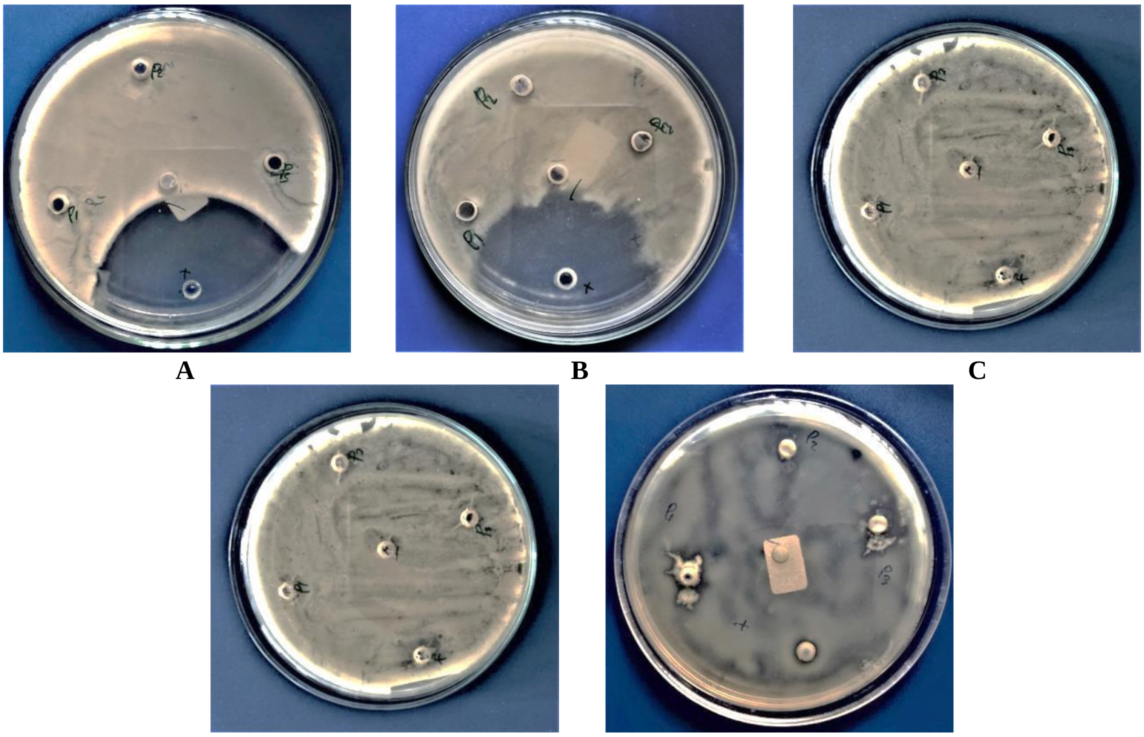


Figure 2. Antimicrobial activity test of endophytic bacteria and fungi: (A) isolate AKR_3 against *S. epidermidis*; (B) isolates DN_3, (C) isolates DNX against *E. coli*; (D) isolates DN2, (E) isolates AKRX against *C. albicans* repetition (P), positive control (+), negative control (-)

The colony morphology of the endophytic bacteria obtained has a circular and irregular shape with the color of the whole colony white. Colony elevations are flat and umbonate, with entire and undulate edges. Two isolates are Gram-negative, and four isolates have different physiological properties. More detail can be seen in table 2. The morphology of colonies of endophytic fungi varies. The shape of fungi's colony is round; some are irregular with different colors; some are dark green, white, and yellowish-white. Microscopically, observations of endophytic fungi are carried out by looking at the spores and their hyphae, two isolates of endophytic fungi do not have spores, and two isolates of endophytic fungi also have hyphae that are aseptatic. More details can be seen in Table 3. Endophytic bacteria in one host plant usually consist of several genera and species. The diversity of endophytic bacteria in a plant is influenced by the growing conditions of the plant, in particular, the soil conditions [24].

Table 3. Morphology of cell colonies and biochemical assays of endophytic bacteria of soursop plants (*Annona muricata* L.)

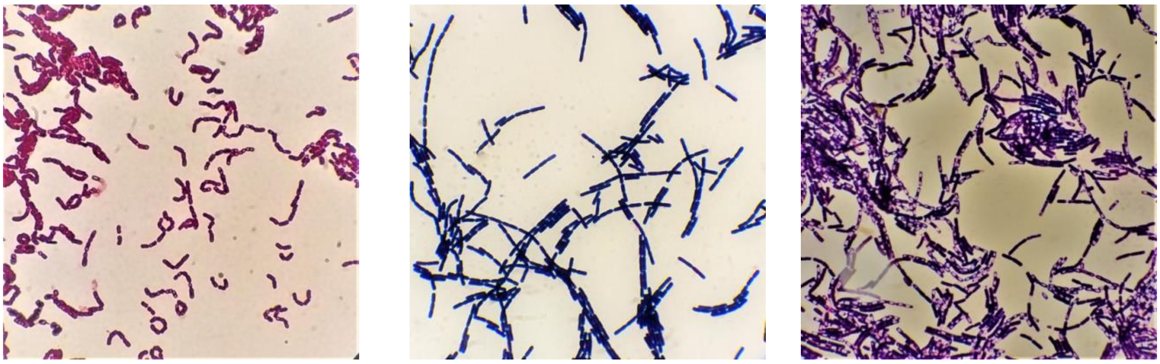
Isolate Characteristic	Bakteri endofit								
	DN_1	DN_2	DN_3	DN_4	KB_1	KB_2	AKR_1	AKR_2	AKR_3
Shape	Circular	Irregular	Circular	Irregular	Irregular	Circular	Irregular	Irregular	Irregular
Color	White	White	White	White	White	White	White	White	White
Elevation	Flat	Flat	Flat	Flat	Umbunate	Flat	Umbunate	Umbunate	Flat
Margin	Entire	Undulate	Entire	Undulate	Undulate	Entire	Undulate	Undulate	Undulate
Gram stain	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Negative
Cell type	Bacill	Coccus	Bacill	Bacill	Bacill	Bacill	Bacill	Bacill	Coccus
TSI	+	+	+	+	+	+	+	+	+
SC	-	-	-	-	-	-	+	+	+
Indole	-	-	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	_	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+

Description: Triple Sugar Iron (TSI) Test, Simon's Citrate Test (SC), negative (-), positive (+)

Table 4. Morphology of Endophytic Fungi of Soursop Plants (*Annona muricata* L.)

No	Isolate Characteristic	Endophytic Fungi		
		DNX	KBX	AKRX
1.	Shape	Irregular	Circular	Thin round
2.	Color	Dark green	White to brass	White
3.	Surface	Coarse as powder	Smooth fibrous	Smooth filaments
4.	Spore	None	None	Ascospores
5.	Spore	Aseptate	Aseptate	Septate

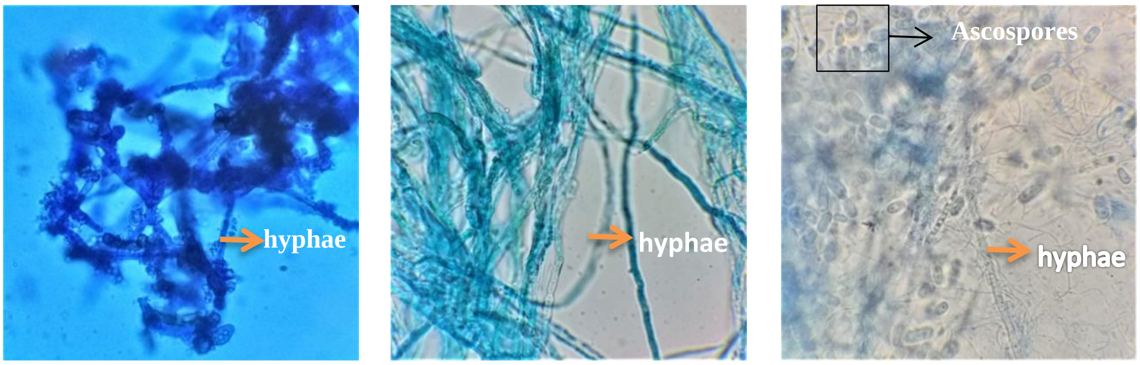
Microscopic characterization of endophytic bacteria is seen from the results of Gram staining. Isolates of endophytic bacteria that have been successfully isolated, 2 of which are Gram-negative bacteria with the form of coccus cells (DN_2 and AKR_3), and the other seven isolates are Gram-positive bacteria with bacillus cell forms (DN_1, DN_3, DN_4, KB_1, KB_2, AKR_1, and AKR_2) in full can be seen in figure 3.



A B C

Figure 3. Gram-staining isolates of endophytic bacteria. A). DN_2 are Gram-negative bacteria, and coccus cell form. B). KB_1 are Gram-positive bacteria and bacill cell form. C). AKR_1 are Gram-positive bacteria and bacill cell form.

As for isolates of endophytic fungi, microscopically, it is known that two isolates (DNX and KBX) do not have spores, and their hyphae are aseptates. AKRX isolates are known to have a type of spores, namely ascospores with septate hyphae (can be seen in figure 4).



A B C

Figure 4. Microscopic observations of isolates of endophytic fungi DNX (A), KBX (B), and AKRX (C)

CONCLUSION

The antimicrobial activity of all endophytic bacteria and fungi isolates against test microbes obtained different results. Endophytic isolates that have moderate activity against inhibition of *E. coli* growth are DN_3, DNX, and AKRX, isolates with a moderate activity that inhibit the growth of *S. epidermidi*s, namely AKR_1 and endophytic isolates that have moderate activity in inhibiting the growth of *C. albicans*, are DN_2 and AKRX isolates.

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