

Antibacterial Activity of Endophyte Bacteria Isolation from Mangrove Leaves *Scyphiphora hydrophyllacea*

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Abstract. Antibacterial activity testing is a fundamental method for evaluating the ability of bacterial isolates to inhibit the growth of pathogenic microorganisms. This study aimed to identify and describe endophytic organisms by isolating bacteria from the mangrove leaves of *Scyphiphora hydrophyllacea* Gaertn, and to assess their antibacterial potential against *Staphylococcus aureus* and *Escherichia coli*. Antibacterial assays were conducted using metabolites extracted from the endophytic isolates, employing both Kirby-Bauer test and Minimum Inhibitory Concentration (MIC) methodologies. Of the 12 bacterial isolates obtained, seven exhibited inhibitory activity against *S. aureus* and *E. coli*. Notably, isolates SH1 and SH2 demonstrated the most pronounced inhibitory zones, varying from 10 mm to 12 mm. MIC analysis revealed that both SH1 and SH2 effectively suppressed *E. coli* growth at a concentration of 1.56 µg/ml, as indicated by the reduced absorbance levels. For *S. aureus*, the MIC values were determined to be 12.5 µg/ml SH1 and 3.125 µg/ml SH2. These findings suggest that endophytic bacteria isolated from *S. hydrophyllacea* possess promising antibacterial properties and warrant further investigation for their potential therapeutic applications. Based on the identification results, bacterial isolates SH1, SH2, and SH7 were identified as *Amphibacillus* sp., *Sporolactobacillus* sp., and *Bacillus* sp.

1 Introduction

Endophytic bacteria are microbes that colonize plant tissues internally and remain non-pathogenic to the host. Their presence is often associated with protective functions, notably through the biosynthesis of bioactive compounds that exhibit antagonistic activity against external pathogens [1]. These antagonistic properties primarily manifest as antibacterial activities capable of suppressing the growth of pathogenic bacteria. For instance, [2] successfully isolated endophytic microbes from the mangrove species *Scyphiphora hydrophyllacea*, and demonstrated a modest inhibitory effect against *Staphylococcus aureus*.

Endophytic microorganisms contribute significantly to the biosynthesis of bioactive compounds beneficial to host plants and various biotechnological applications. Mangrove vegetation in Lake Wasti Sowi, Manokwari, is dominated by species from the Rhizophoraceae and Combretaceae families [3]. Previous studies on the mangrove species *Scyphiphora hydrophyllacea* have primarily explored its cytotoxic potential against cancer cells. Notably, several bioactive compounds,—including scyphiphorins A and B, geniposidic acid, cyclopenta derivatives, oleanolic acid, and stigmasterol-D-glucoside,—were isolated for the first time from the bark of *S. hydrophyllacea* [4]. According to [5] [6], the utilization of endophytic bacteria offers an efficient and sustainable alternative to direct plant extraction, as it reduces processing time, enhances the potential for scalable production of

bioactive compounds, and has antimicrobial activity capabilities.

Research on the antibacterial properties of *Scyphiphora hydrophyllacea* mangrove leaves remains limited, with only a few published studies addressing the potential of their associated endophytic bacteria. Therefore, it is essential to investigate the antibacterial activity of endophytic bacterial isolates derived from *S. hydrophyllacea* leaves. This study was designed to isolate and examine the antibacterial activity of these endophytes against two clinically relevant pathogenic bacteria: *Escherichia coli* and *Staphylococcus aureus*.

2 Methods

2.1 Study Area

This study was conducted between March and May 2022. Leaf samples of the mangrove species *Scyphiphora hydrophyllacea* were collected from Rendani Beach, Manokwari, and West Papua. Only healthy, uncontaminated leaves were selected, as determined by morphological assessments. All laboratory analyses were performed in the Laboratory of Microbiology, Faculty of Mathematics and Natural Sciences, Papua University.

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2.2 Isolation and Purification of Endophytic Bacteria

Mangrove leaf samples were sterilized by thoroughly washing with running water, followed by immersion treatment was carried out using 70% ethanol for a duration of 1 minute. The samples were then soaked in 5.3% sodium hypochlorite for 2 min, re-immersed in 70% ethanol for an additional 2 min, and rinsed three times with sterile distilled water. The sterilized leaves were dried using sterile tissue paper [7][1]. The dried samples were ground and weighed to obtain 10 grams, which were then suspended in Erlenmeyer flasks and subjected to serial dilution using 0.85% NaCl solution up to a dilution factor of 10^{-4} . From each dilution, 0.1 mL were pipetted into sterile Petri dishes and overlaid with nutrient agar (NA) medium maintained at 40–50°C. The plates were incubated at 28–30 °C for 24–48 h. Colonies exhibiting distinct morphological characteristics were subsequently purified on NA medium using the streak plate method, followed by incubation at 28–30 °C for 24 hours [8]. The purified isolates were preserved as stock cultures on NA slants and nutrient broth (NB).

2.3 Production of Metabolite Compounds from Endophytic Bacteria

Metabolite production was performed through fermentation. One milliliter of bacterial isolate cultured in nutrient broth (NB) was 9 transferred into fresh NB medium (mL) and maintained at 30 °C on a rotary shaker set to 130 rpm for 72 h. Following incubation, the culture was performed at 3,800 rpm for 15 min to separate the biomass fractions. The resulting pellet and cell-free supernatant were then processed using 0.22 µm Whatman filter paper. The pellet was discarded, and the filtered supernatant was retained for subsequent antibacterial activity assays.

2.4 Antimicrobial Susceptibility Testing (AST)

The AST assay was used to evaluate the antibacterial potency of endophytic bacterial metabolites using a serial dilution method. Susceptibility testing determines the effectiveness of antibiotics against specific microbial pathogens. In this study, serial dilutions of the cell-free supernatant from endophytic bacterial cultures were prepared in 2 mL of nutrient broth (NB), ranging from undiluted to a dilution factor of 10^{-7} . A volume of 50 µL of *Staphylococcus aureus* and *Escherichia coli* cultures (50 µL) were added to each dilution tube. The mixtures were then incubated at 35–37°C for 18–24 h.

Antimicrobial Susceptibility Testing (AST) is a standard laboratory procedure used to evaluate the effectiveness of antimicrobial agents, including antibiotics, in inhibiting the growth of bacterial pathogens. The primary objective of AST is to support evidence-based selection of appropriate antimicrobial therapies for the treatment of infectious diseases. AST approaches can be categorized into qualitative methods, such as the Kirby–Bauer disk diffusion assay, which assesses antimicrobial activity based on inhibition zone

diameters, and quantitative methods, including the determination of the minimum inhibitory concentration (MIC), which provides a precise measure of antimicrobial potency

Bacterial growth inhibition was assessed by comparing the turbidity and clarity of each test tube against control tubes containing only pathogenic bacteria (*S. aureus* and *E. coli*) without supernatant treatment. Quantitative determination of bacterial growth was performed using a UV–Vis spectrophotometer set at 600 nm. The AST value was identified as the lowest concentration of supernatant that resulted in the lowest absorbance, indicating the effective inhibition of bacterial growth.

The pathogenic bacterial strains employed in this study included *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative). Each strain was inoculated, using a single loopful, into 9 mL of nutrient broth (NB) and incubated at 28–30 °C for 24 hours with agitation at 130 rpm on a rotary shaker [7][1]. The antibacterial activity was assessed using two methods: disc diffusion and MIC. For the disc diffusion assay, 0.1 mL of each pathogenic bacterial suspension was evenly spread onto solid nutrient agar (NA) plates, chloramphenicol (positive control) and DMSO (negative control). Sterile paper discs were immersed in cell-free supernatants of endophytic bacterial isolates for 15 min and then placed onto inoculated NA plates. The plates were then incubated at 28–30°C for 48 h. The zones of inhibition were subsequently measured using the following formula:

$$\text{Inhibition zone diameter} = \frac{(1+2+3+4)}{4} [1].$$

2.5 Identification of Endophytic Bacteria

The endophytic bacteria were identified based on their morphological and physiological characteristics, including colony shape, color, elevation, and margin, as well as cellular morphology and Gram reaction. In addition, biochemical and physiological assays were performed in accordance with the procedures described in Bergey’s Manual of Determinative Bacteriology.

3 Results And Discussion

3.1 Isolation and Purification of Endophytic Bacteria

A total of 12 endophytic bacterial isolates were successfully obtained from mangrove leaves of *Scyphiphora hydrophyllacea*. Successful isolation was

Table 1. The macroscopic and microscopic observations of endophytic bacterial colonies

Isolate code	Forms	Margins	Elevation	Gram	NB Culture	Oxygen Requirements
SH1	Circular	Entire	Convex	positive	Pellicle	Aerob
SH2	Circular	Undulate	Raised	positive	Pellicle	Aerob
SH3	Circular	Undulate	Umbo-nate	positive	Pellicle	Aerob
SH5	Circular	Entire	Raised	positive	Turbidity	Aerob
SH7	Circular	Filamen	Convex	positive	Turbidity	Aerob
SH8	Irregular	Lobate	Convex	positive	Pellicle	Aerob
SH11	Circular	Serate	Raised	positive	Pellicle	Aerob
SH12	Circular	Undulate	Umbo-nate	positive	Pellicle	Aerob
SH13	Irregular	Entire	Umbo-nate	positive	Pellicle	Aerob
SH14	Circular	Entire	Raised	positive	Turbidity	Aerob
SH16	Irregular	Serate	Raised	positive	Sediment	Anaerob
SH18	Irregular	Serate	Umbo-nate	positive	Pellicle	Aerob

confirmed by the emergence of bacterial colonies on nutrient agar (NA) plates.

These isolates were subsequently purified using the streak plate method to obtain single, morphologically distinct colonies. As presented in Table 1, all the purified isolates exhibited white pigmentation. This observation is consistent with findings of [1], who reported seven white-pigmented isolates from *Avicennia marina* leaves, and [10], who identified six white-dominant isolates from *Acrostichum aureum* L. leaves using NA medium.

3.2 Kirby-Bauer Test

The Kirby–Bauer disk diffusion assay was used to evaluate the antibacterial activity of cell-free supernatants derived from endophytic bacterial isolates. These supernatants were obtained through fermentation in nutrient broth (NB) medium, allowing bacteria to produce and release extracellular metabolites. According to [11], bacterial fermentation results in the secretion of extracellular proteins into the culture medium. The fermented broth was subsequently centrifuged to remove cellular debris and extracellular proteins, yielding cell-free supernatant containing bioactive compounds.

Based on the disc diffusion assay, seven isolates,—SH1, SH2, SH3, SH5, SH8, and SH14,—exhibited varying inhibition zone diameters against the test pathogens. These isolates demonstrated broad-spectrum antibacterial activity, effectively restricting the proliferation of the gram-negative pathogen *Escherichia coli* and the gram-positive pathogen *Staphylococcus aureus*.

Table 2. The antimicrobial activity of bacteria isolated from mangrove leaves *Scyphiphora hydrophyllacea*

Isolate	Zone of clearing (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
SH1	10	12,3
SH2	12	11
SH3	9	7,7
SH5	8,7	7,3
SH7	10	10
SH8	9	8
SH14	9	9
positive control	21	20
negative control	0	0

Based on Table 2, the largest inhibitory zone area belongs to SH1 at 10 mm against *E. coli* and 12,3 mm in *S. aureus*. According to [13], the area of the inhibitory zone is divided into 4, namely weak inhibition ≤ 5 mm, medium 5-10 mm, strong 10-20 mm and very strong inhibition, ≥ 20 mm. As shown in Table 2, the SH1, SH2 and SH7 isolates tested against *S. aureus* and *E. coli* showed strong inhibition. Isolates SH3, SH5, SH8 and SH14 had medium inhibitory zone areas.

Based on the identification results, bacterial isolates SH1, SH2, and SH7 were identified as *Amphibacillus* sp., *Sporolactobacillus* sp., and *Bacillus* sp., respectively. All three isolates exhibited the ability

produced antimicrobial compounds against the tested bacterial strains and were classified within the genus *Bacillus*. These findings were consistent with those reported by [12], who demonstrated that the endophytic bacterium *Bacillus* sp. RAR_M1_45, isolated from *Rhizophora apiculata* mangroves produces three bioactive compounds,—2-(2-heptenyl)-3-methyl-4-quinolinone, 3-methyl-2-(2-nonenyl)-4-quinolinone, and 2-phenylacetic acid,—which have been shown to effectively inhibit the growth of pathogenic bacteria.

3.3 Antimicrobial Susceptibility Testing (AST) Assay of Endophytic Bacterial Metabolites Against Pathogenic Bacteria

AST is a diagnostic laboratory technique used to assess which antibiotics inhibit or eliminate particular infectious bacteria or fungi and was used to evaluate the lowest concentration of antibacterial substances capable of suppressing microbial growth compounds—derived from endophytic bacterial isolates,—capable of inhibiting the growth of pathogenic bacteria. The assay involved serial dilutions of cell-free supernatants in seven test tubes, each containing 2 mL nutrient broth (NB) medium. Following inoculation with 50 μ L of either *Staphylococcus aureus* or *Escherichia coli*, the tubes were incubated at 37 °C for 18–24 h, and bacterial growth was examined by observing turbidity and measuring absorbance.

The MIC was determined as the lowest concentration at which no observable bacterial growth occurred, indicating inhibition of bacterial growth. Quantitative measurements were performed using a UV–Vis spectrophotometer operating at 600 nm. This method offers rapid and reliable detection, because the absorbance value is directly proportional to the concentration of bacterial cells in the medium. The 600 nm wavelength is particularly suitable for this application, because it does not exert bactericidal effects [13].

Table 3. The results of the AST test using cell-free supernatants of endophytic bacteria against pathogenic bacteria *E. coli* and *S. aureus*.

Supernatant Concentration (ug/ml)	<i>E. coli</i> Absorbance value			Absorbance value of <i>S. aureus</i>		
	SH1	SH2	SH7	SH1	SH2	SH7
50	0.885	0.697	1.142	0.833	1.091	1.073
25	1	0.736	1.583	1.237	1.489	1.293
12.5	1.106	1.156	1.839	1.076	1.595	1.372
6.25	1.257	1.154	1.803	1.464	1.587	1.301
3.125	1.296	1.281	1.842	1.444	1.536	1.368
1.5625	1.252	1.211	1.359	1.388	1.55	1.331
0.78125	1.02	1.104	1.699	1.135	1.295	1.084
<i>S. aureus</i>				1.854		
<i>E. coli</i>				1.633		
Negatif/NB				0.34		

Description: Bolded values indicate reduced absorbance, corresponding to the MIC—the lowest concentration suppressing bacterial growth.

Table 3 presents the MIC test results for two endophytic bacterial isolates, SH1 and SH2, highlighting the variations in antibacterial activity based on pathogen type and metabolite concentration. Both isolates exhibited inhibitory effects against *Escherichia coli* at a concentration of 1.56 ug/ml, which was identified as AST. UV-Vis spectrophotometric analysis revealed that absorbance values increased with decreasing concentrations of the antibacterial compound, peaking at 3.12 ug/ml with absorbance

values of 1.296 for SH1 and 1.281 for SH2. A subsequent decrease in the absorbance was observed at 1.56 ug/ml, with values of 1.252 for SH1 and 1.211 for SH2, indicating effective bacterial inhibition. For comparison, the positive control (*E. coli*) exhibited an absorbance of 1.633.

In contrast, MIC testing against *Staphylococcus aureus* revealed distinct inhibitory concentrations for each of the isolates. SH1 demonstrated AST at 12.5 ug/ml, with an absorbance reduction to 1.076, compared to the positive control value of 1.854. Interestingly, the absorbance for SH1 increased at 25 ug/ml, decreased at 12.5%, and rose again at 6.25 ug/ml, suggesting a non-linear response. SH2 exhibited AST at a lower concentration of 3.125 ug/ml, with an absorbance of 1.536, followed by a slight increase to 1.550 at 1.56 ug/ml, indicating a threshold effect in antibacterial activity. Antibacterial activity assays revealed that the endophytic bacterial isolates obtained from *Scyphiphora hydrophyllacea* leaves exhibited a diverse spectrum of activity against the two tested pathogenic bacteria. Among the isolates, SH1, SH2, and SH7 demonstrated the strongest antibacterial effects against *Escherichia coli* and *Staphylococcus aureus*, with an AST of 12.5 µg/mL. According to the classification proposed by [14], this AST value indicates a very strong antibacterial activity, as it is below 100 µg/mL.

Based on the present study, secondary metabolite compounds produced by both the tested isolates contained bioactive substances with antibacterial properties. [2] identified a novel compound, scyphiphin, from the leaf-derived endophytic fungus *Scyphiphora hydrophyllacea*, which exhibited modest inhibitory activity against *Staphylococcus aureus*. Similarly, [15] reported that five fungal endophyte isolates from *S. hydrophyllacea* demonstrated inhibitory effects against *S. aureus*, *Escherichia coli*, and *Candida albicans* when extracted with ethyl acetate. These results indicated that endophytic fungi may serve as promising sources of antimicrobial and anticancer compounds. Previous research has predominantly examined fungal endophytes, and the current research highlights that endophytic bacteria isolated from *S. hydrophyllacea* leaves also produce secondary metabolites with promising antibacterial activity.

Results of the present study indicate that endophytic bacterial isolates associated with *S. hydrophyllacea* leaves possess significant inhibitory activity against *Escherichia coli* and *Staphylococcus aureus*, underscoring their potential therapeutic value as sources of antimicrobial agents. However, the specific chemical identity of the active compounds responsible for this inhibition remains unclear.

4 Conclusion

Twelve endophytic bacterial isolates were successfully recovered from the leaves of *Scyphiphora hydrophyllacea*, seven of which exhibited antibacterial activity. Among these, isolates SH1 and SH2 demonstrated the most potent inhibitory effects against

both *Staphylococcus aureus* and *Escherichia coli*, as evidenced by the formation of clear zones of inhibition. Minimum Inhibitory Concentration (MIC) assays revealed that both isolates effectively inhibited *E. coli* at a concentration of 1.56 ug/ml, while *S. aureus* was inhibited at concentrations of 12.5 ug/ml, 3.125 ug/ml, and 6.25 ug/ml, depending on the isolate. These findings suggest that endophytic bacteria from *S. hydrophyllacea* possess promising antibacterial potential and warrant further investigation into their bioactive compounds. Based on the identification results, bacterial isolates SH1, SH2, and SH7 were identified as *Amphibacillus* sp., *Sporolactobacillus* sp., and *Bacillus* sp.

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