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Research Article

Isolation and Identification of Marine Bacteria in Raja Ampat Islands West Papua Producing Antibacterial Against *Salmonella typhi* and *Staphylococcus aureus*

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Abstract

Indonesia's vast archipelago harbors a wealth of natural resources, including marine bacteria with potential antibacterial properties. Given the increasing prevalence of antibiotic resistance, particularly against *Salmonella typhi* and *Staphylococcus aureus*, there is a pressing need to explore alternative antimicrobial agents. This study aimed to isolate and characterize marine bacteria with antibacterial activity and evaluate their efficacy against *S. typhi* and *S. aureus*. Isolation of marine bacteria was conducted using the spread plate method. Antibacterial activity screening of the secondary metabolites was performed using the well diffusion method. Minimum inhibitory concentration (MIC) was determined using the dilution method, while minimum bactericidal concentration (MBC) was determined using the spread plate method. Seven bacterial isolates were obtained, all identified as Gram-negative bacilli. The secondary metabolites of these marine bacteria demonstrated antibacterial activity against both S. *typhi* and *S. aureus*, with inhibition zones of 8.50 mm and 8.46 mm, respectively. The MIC for both bacteria was determined to be 1500 µg/mL. Statistical analysis revealed a significant difference in antibacterial activity between the isolates (Kruskal-Wallis Test, p-value = 0.007) and between *S. typhi* and *S. aureus* (Mann-Whitney Test, p-value = 0.025). While the secondary metabolites exhibited antibacterial activity against both bacteria, they did not demonstrate bactericidal activity as measured by the MBC test.

Keywords:
Marine bacteria
Minimum inhibitory concentration
Salmonella typhi
Secondary metabolites
Staphylococcus aureus

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INTRODUCTION

Indonesia, an archipelago nation, possesses a vast maritime domain, its sea area exceeding its landmass by a factor of 2.5. This expansive marine environment presents immense potential, offering a wealth of natural resources and crucial environmental services that can significantly contribute to economic growth at local, regional, and national levels[1]. Among the myriad of Indonesian seas, the Raja Ampat Islands in Papua stand out as a region of exceptional marine biodiversity and ecological significance.

The Raja Ampat Islands, a renowned archipelago situated in the western part of Papua Island, Indonesia, comprise four major island groups: Waigeo, Misool, Salawati, and Batanta. This region, renowned for its breathtaking underwater scenery, has captivated divers and researchers alike[2]. Importantly, the Raja Ampat Islands lie within the Coral Triangle, recognized globally as a center of marine biodiversity. This exceptional biodiversity extends to the microbial realm, with the region

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harboring a rich diversity of marine bacteria. Given the potential for these bacteria to produce novel bioactive compounds, including secondary metabolites, exploring the microbial diversity of the Raja Ampat Islands presents a significant opportunity for scientific discovery and potential applications in various fields, such as medicine and biotechnology[3].

Marine environments offer a unique and diverse source of novel bioactive compounds, particularly from bacterial secondary metabolites. These metabolites often exhibit distinctive structures and potent bioactivities, driven by the complex and challenging conditions of their marine habitats[4]. Previous studies have demonstrated the potential of marine bacteria, such as those associated with *Japis* sp., to produce bioactive compounds, including protease inhibitors[5]. Notably, these protease inhibitors have shown significant antibacterial activity, offering a promising avenue for the development of novel antibiotics. The increasing prevalence of antibiotic resistance poses a significant global health threat. According to the Centers for Disease Control and Prevention (CDC), antibiotic resistance contributes to approximately 2 million infections and 23,000 deaths annually in the United States alone, with *Salmonella typhi* and *Staphylococcus aureus* being major concerns[6]. The development of new antibacterial agents, such as those derived from marine bacterial secondary metabolites, is crucial to combat this growing public health challenge[7]. This research aims to investigate the potential of marine bacterial isolates from the coast of Waigeo Regency, Raja Ampat Islands, West Papua, Indonesia, to produce antibacterial compounds with activity against *S. typhi* and *S. aureus*.

MATERIALS AND METHODS

Materials

This study utilized several laboratory materials, including Petri dishes, micropipettes (Accumax pro fix), pycnometer (Pyrex), magnetic stirrer (Oem), Erlenmeyer flasks (Pyrex and Herma), analytical balance (Shimadzu Corporation), autoclave, incubator, hot plate (Thermo Scientific), laminar air flow (LAF) cabinet, and test tubes (Pyrex). Biological materials used included marine bacteria isolated from the Raja Ampat Islands, pure cultures of *S. typhi* ATCC 13311 and *S. aureus* ATCC 25923, nutrient agar (NA), nutrient broth (NB), sterile cotton, 10% NaCl solution, distilled water, and Gentian Violet dye.

Methods

Bacterial isolation

Water samples were collected from coastal areas within Waigeo Regency, Raja Ampat Islands, West Papua, Indonesia. Serial dilutions of each water sample were prepared, ranging from 10^{-1} to 10^{-5}. Subsequently, 20 mL of each diluted sample was aseptically transferred onto NA plates. The plates were then spread evenly using a sterile glass rod and incubated at 37°C for 24-48 hours. Following incubation, bacterial colonies were observed and differentiated based on their morphological characteristics, including colony color, shape, elevation, and edge[8].

Bacterial morphological identification

Bacterial morphological identification aimed to characterize the physical appearance of the isolated bacterial colonies. This involved a visual examination of colony characteristics, including color, shape, elevation, and edge morphology. The observed characteristics were then recorded in a table to facilitate further analysis. Bacterial colony morphology, particularly its shape, provides valuable initial clues for bacterial identification[9].

Antibacterial activity screening

Antibacterial activity of the marine bacterial supernatants was evaluated using the well diffusion method. Briefly, 20 µL of bacterial suspensions of *S. typhi* and *S. aureus*, standardized to 0.5 McFarland turbidity, were inoculated onto Mueller-Hinton agar (MHA) plates and spread evenly using a sterile cotton swab. Three wells, each with a diameter of 6 mm, were then created in the agar using a sterile cork borer. Subsequently, 50 µL of each marine bacterial supernatant was carefully added to the respective wells. Control wells received 50 µL of sterile distilled water. The plates were then incubated at 37°C for 18-24 hours. After incubation, the plates were examined for the presence of zones of inhibition around the wells, indicating antibacterial activity. The diameters of the inhibition zones were measured using a caliper and recorded.

Determination of minimum inhibitory concentration

To determine the minimum inhibitory concentration (MIC) of the marine bacterial supernatant against pathogenic bacteria, serial two-fold dilutions were prepared in NB. The supernatant was added to sterile test tubes containing NB at concentrations of 500 µL, 700 µL, 1000 µL, and 1500 µL. Subsequently, each test tube was inoculated with 100 µL of standardized suspensions of *S. typhi* and *S. aureus*, adjusted to 0.5 McFarland turbidity standards. The inoculated tubes were then incubated at 37°C for 24 hours. The MIC was determined as the lowest concentration of the marine bacterial supernatant that exhibited no visible turbidity, indicating the absence of bacterial growth.

Determination of minimum bactericidal concentration

To determine the minimum bactericidal concentration (MBC), 20 µL of each bacterial culture from the MIC broth dilutions exhibiting no visible growth were transferred to sterile NA plates. The bacterial suspensions were then spread evenly over the agar surface using sterile L-shaped glass rods. The inoculated plates were incubated at 37°C for 24 hours. After incubation, the plates were visually inspected for the presence of bacterial colonies. The MBC was defined as the lowest concentration of the test substance that resulted in no visible bacterial growth after subculturing on solid media.

Data analysis

Data analysis in this study employed a descriptive approach. To determine significant differences among multiple groups, the One-Way ANOVA was utilized, a parametric test suitable for comparing means across three or more groups. The prerequisites for employing ANOVA are normality and homogeneity of variances within the groups. Normality of data distribution was assessed using the Shapiro-Wilk test, while homogeneity of variances was evaluated using Levene's test. If the data violated the assumptions of normality or homogeneity, the non-parametric Kruskal-Wallis test was employed as an alternative. Subsequently, if significant differences were detected by the Kruskal-Wallis test, pairwise comparisons were performed using the non-parametric Mann-Whitney U test to identify specific groups that differed significantly from each other.

RESULTS AND DISCUSSION

Bacterial isolation

Bacterial isolation from seawater samples collected from the Raja Ampat Islands, West Papua, yielded seven distinct bacterial isolates (MB 1 to MB 7; **Figure 1**). The density of bacterial colonies observed on the agar plates decreased progressively with increasing dilution factors. At the 10^{-1} dilution, a high density of bacterial colonies was observed, indicating a significant bacterial load in the original seawater sample. This trend is expected, as higher dilutions result in a significant reduction in the number of viable bacterial cells present in the sample[10]. These findings demonstrate the presence of a diverse bacterial community within the seawater of the Raja Ampat Islands.

Bacterial morphological identification

Morphological characterization of the marine bacterial isolates revealed a predominance of rod-shaped (*bacillus*) morphology. Seven isolates exhibited this characteristic, with variations in colony color observed (**Table I**). Isolate 1, 4, and 5 displayed a cream color, while isolates 2, 3, and 6 were clear white, and isolate 7 exhibited a milky white coloration. All isolates shared common morphological features, including a flat elevation and flat edges. These findings are consistent with previous studies conducted by Marzuki *et al.*[11], which reported that marine bacterial isolates from Melawai Beach, Balikpapan, predominantly exhibited rod-shaped morphology and displayed a range of colors, including cream, milky white, and clear white. Furthermore, Sariadji[12] as well as Sabdaningsih *et al.*[13] also reported the presence of flat edges in their respective studies on marine bacterial isolates.

Antibacterial activity screening

Antibacterial activity screening was conducted using the disc diffusion method. Isolate 7, designated as MB 7, isolated from marine bacteria in the Raja Ampat Islands, West Papua, exhibited antibacterial activity against the test organism (**Table II**). The average diameter of the inhibition zones produced by MB 7 against *S. typhi* and *S. aureus* were 8.5 and 8.46 mm, respectively, which falls within the category of moderate inhibition according to the classification system defined by

Prijatmoko *et al*[14]. This classification system categorizes inhibition zones as weak (diameter ≤5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (≥20 mm)[15]. It is noteworthy that marine bacteria in symbiosis with other organisms, such as sponges, have been reported to produce a significantly higher diversity of secondary metabolites compared to free-living marine bacteria[16]. Since MB 7 is a free-living marine bacterium, the observed moderate antibacterial activity aligns with this expectation. According to Kusuma *et al.*[17], bacteria with high antibacterial activity often exhibit specific morphological characteristics, such as rod shape, milky white color, and flat edges. Interestingly, MB 7 displays these characteristics, further supporting its observed antibacterial potential.

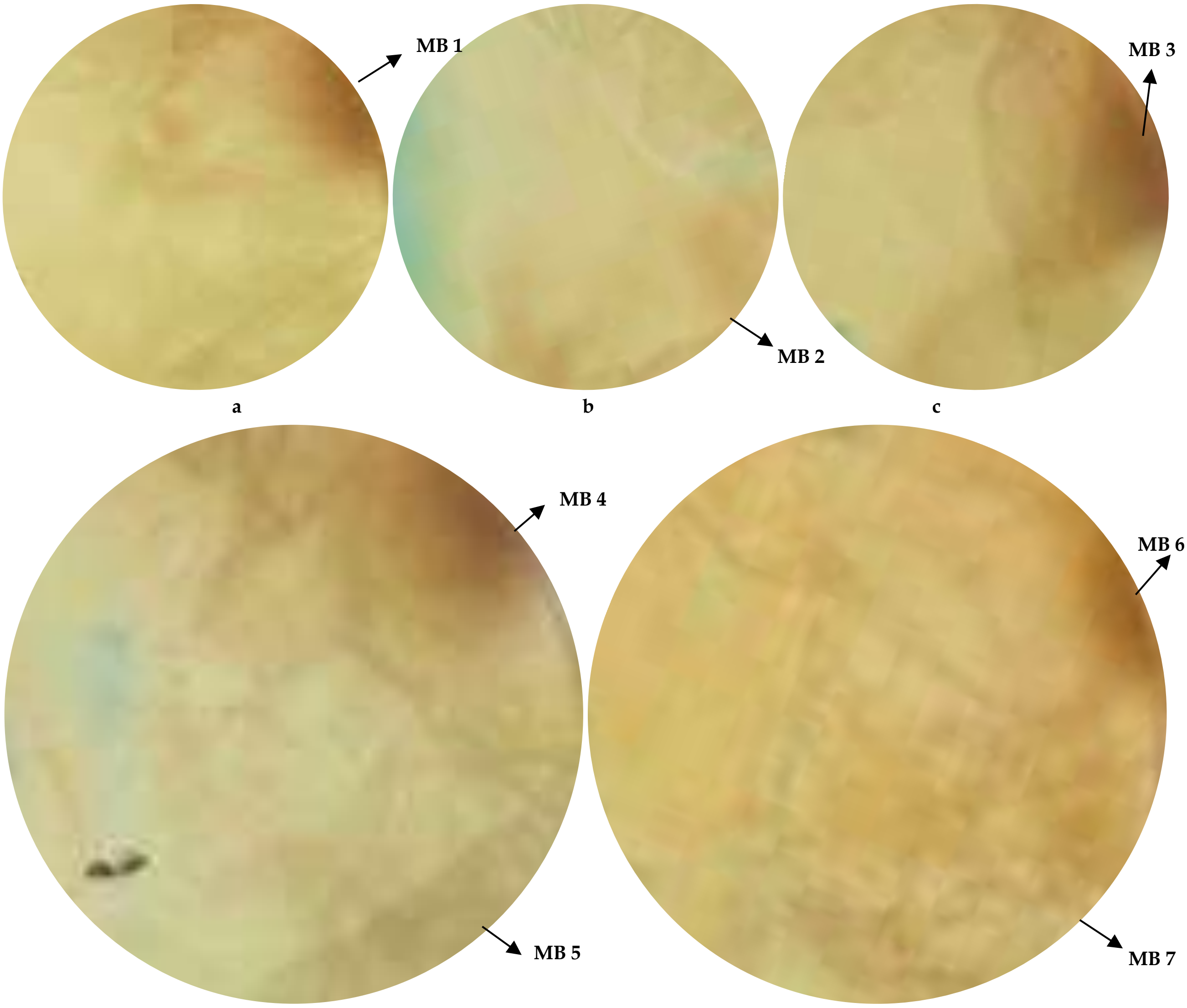
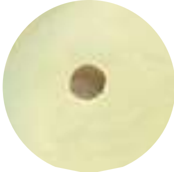
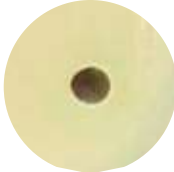
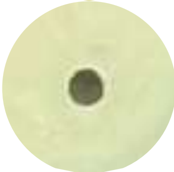
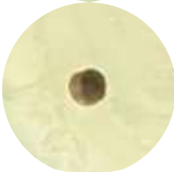
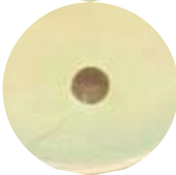
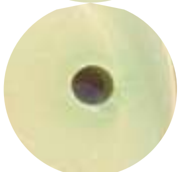
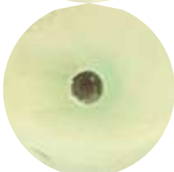


Figure 1. Marine bacterial isolation from seawater samples of the Raja Ampat Islands, West Papua. The dilution factors are **(a)** 10^{-1}, **(b)** 10^{-2}, **(c)** 10^{-3}, **(d)** 10^{-4}, and **(e)** 10^{-5}.

Table I. Characteristics of marine bacterial from seawater samples of the Raja Ampat Islands, West Papua.

Isolate	Characteristics of marine bacteria			
	Shape	Color	Elevation	Edge
MB 1	Rod	Cream	Flat	Even
MB 2	Rod	Clear White	Flat	Even
MB 3	Rod	Clear white	Flat	Even
MB 4	Rod	Cream	Flat	Even
MB 5	Rod	Cream	Flat	Even
MB 6	Rod	Clear white	Flat	Even
MB 7	Rod	Milky white	Flat	Even

Table II. Antibacterial activity screening of marine bacterial isolation from seawater samples of the Raja Ampat Islands, West Papua.

Isolates	*Salmonella typhi*		*Staphylococcus aureus*	
	Average	Figures	Average	Figures
MB 1	8.13		8.13	
MB 2	8.16		8.16	
MB 3	8.2		8.2	
MB 4	8.2		8.2	
MB 5	8.23		8.23	
MB 6	8.36		8.36	
MB 7	8.5		8.46	

Determination of minimum inhibitory concentration

The MIC of MB 7 against *S. typhi* and *S. aureus* was determined to be 1500 µg/mL, as indicated by the absence of turbidity in the test tubes at this concentration in both cases (**Table III**). While the MIC value was the same for both bacterial species, a clearer visual observation of bacterial inhibition was noted in the *S. typhi* cultures. This observation aligns with the general principle that bacteriocins produced by Gram-positive bacteria are typically more effective against other Gram-positive bacteria, and *vice versa* for Gram-negative bacteria[18]. This phenomenon is attributed to the specific mechanisms of bacteriocin action and the differences in the cell wall structures of Gram-positive and Gram-negative bacteria. Furthermore, bacterial communication systems, such as quorum sensing, play a crucial role in regulating bacteriocin production. Quorum sensing allows bacteria to coordinate gene expression and release compounds that inhibit the growth of competing microorganisms, thereby establishing a competitive advantage within their environment[19].

Statistical analysis using the Kruskal-Wallis test revealed significant differences ($p < 0.007$) in the antibacterial activity of various concentrations of marine bacterial secondary metabolites from the Raja Ampat Islands against *S. typhi* and *S. aureus*. To further investigate these differences, post-hoc Mann-Whitney tests were conducted to compare each concentration with the negative control. The results demonstrated that the 1500 µg/mL concentration exhibited significant antibacterial activity against both bacterial strains (p <0.025), while lower concentrations (500, 700, and 1000 µg/mL) did not show significant differences compared to the control (p >0.05). These findings suggest that higher concentrations of secondary metabolites extracted from marine bacteria in the Raja Ampat Islands exhibit significant antibacterial activity against *S. typhi* and *S. aureus*.

Table III. Minimum inhibitory concentration of MB 7 against *S. typhi* and *S. aureus*.

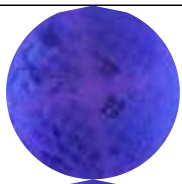
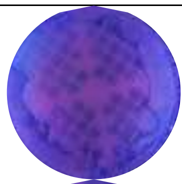
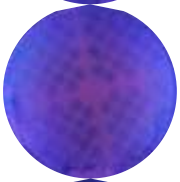
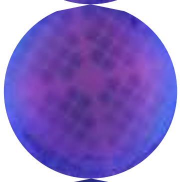
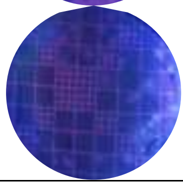
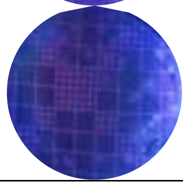
Concentration (µg/mL)	*Salmonella typhi*			*Staphylococcus aureus*		
	Turbidity	p-value	Figures	Turbidity	p-value	Figures
500	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
700	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
1000	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
1500	Slightly cloudy	0,007[a] 0,025[b]		Slightly cloudy	0,007[a] 0,025[b]	
Bacterial suspension (negative control)	Cloudy	0,007[a] 1,000[b]		Cloudy	0,007[a] 1,000[b]	
Chloramphenicol (positive control)	Clear	0,007[a] 0,025[b]		Clear	0,007[a] 0,025[b]	

Notes: [a]: the significance value of the Kruskal-Wallis Test; [b] : the significance value of the Mann-Whitney Test

Determination of minimum bactericidal concentration

To further evaluate the antibacterial activity of MB 7, the MBC was determined. The MBC, which assesses the concentration of the compound required to kill the bacteria, was evaluated by subculturing samples from the MIC test onto NA plates[20]. The MBC is defined as the lowest concentration of the compound that results in no visible bacterial growth on the NA plates[21]. Our results, presented in **Table IV**, demonstrated that MB 7 from the Raja Ampat Islands of West Papua did not exhibit any bactericidal activity against either *S. typhi* or *S. aureus*. This was evidenced by the observation of bacterial growth on the NA plates at all tested concentrations of MB 7, indicating the absence of a discernible MBC value. These findings are consistent with previous studies[22,23] that also reported the lack of an MBC for certain compounds against specific bacterial strains. This suggests that while MB 7 may inhibit the growth of these bacteria at certain concentrations (as indicated by the MIC values), it may not necessarily kill them[24,25].

Table IV. Minimum bactericidal concentration of MB 7 against *S. typhi* and *S. aureus*.

Concentration (µg/mL)	*Salmonella typhi*				*Staphylococcus aureus*			
	Replication			Figures	Replication			Figures
	I	II	III		I	II	III	
1500	Grow colony	Grow colony	Grow colony		Grow colony	Grow colony	Grow colony	
Bacterial suspension (negative control)	Grow colony	Grow colony	Grow colony		Grow colony	Grow colony	Grow colony	
Chloramphenicol (positive control)	Clear	Clear	Clear		Clear	Clear	Clear	

CONCLUSION

This study successfully isolated and identified seven bacterial strains from seawater samples collected from the Raja Ampat Islands, West Papua. All isolates exhibited a rod-shaped morphology. Among these isolates, strain MB 7 demonstrated significant antibacterial activity against both *S. typhi* and *S. aureus* with moderate inhibition zones. Further analysis revealed MIC of 1500 µg/mL for both bacterial pathogens. Statistical analysis using the Kruskal-Wallis and Mann-Whitney tests confirmed the significant antibacterial activity of MB 7 secondary metabolites. However, no MBC was observed, suggesting that the observed antibacterial activity may be bacteriostatic in nature.

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AUTHORS' CONTRIBUTION

Conceptualization: Dede Mahdiyah
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Formal analysis: Dede Mahdiyah, Muhammad Rifqi Dharmawan, Noval
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Investigation: Muhammad Rifqi Dharmawan
Methodology: Dede Mahdiyah, Noval
Project administration: Dede Mahdiyah, Noval
Resources: Dede Mahdiyah
Software: -
Supervision: Dede Mahdiyah, Noval
Validation: Dede Mahdiyah, Noval
Visualization: Muhammad Rifqi Dharmawan
Writing - original draft: Muhammad Rifqi Dharmawan

Writing - review & editing: Dede Mahdiyah, Noval

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this study.

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