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Original Research

Characterization of Antibacterial Compounds from Seaweed Against Pathogenic Bacteria

Wilis Ari Setyati[1]*, AB Susanto[1], Dony Bayu Putra Pamungkas[2], Dewi Basthika Makrima[2], Juwita Lesly Senduk[2], Dafit Ariyanto[3]

[1]Marine Science, Faculty of Fisheries and Marine Science, Diponegoro University, Central Java, 50275, Indonesia
[2]Department of Biology, Faculty of Biology, Jenderal Soedirman University. Jl. dr. Suparno 63 Grendeng, Purwokerto 53122, Central Java, Indonesia
[3]Research Center for Oceanography, National Research and Innovation Agency (BRIN), Jakarta,14430, Indonesia

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Abstract

Bacterial communities collaborating with seaweed play an important role in many aspects, especially in the production of secondary metabolites that help ecosystem function and have good prospects for pharmacological utilization. The objective of this study was to determine the antibacterial activity of associated bacteria isolated from seaweed in Awur Bay, Jepara, Indonesia. The antibacterial test uses the pathogenic bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*. Isolation of seaweed-associated bacteria obtained 4 potential isolates, which were closely related to *Vibrionaceae bacterium* PH25 (99.86%), *Vibrio alginolyticus* strain GS MYPK1 (99.65%), *Salinivibrio costicola* strain M318 (99.86%), and *Vibrio alginolyticu*s strain 2014V-1011 (99.93%). In disc diffusion, MIC, and MBC tests, four isolates showed potential antibacterial activity. TLC identified groups of compounds that were reported to have antibacterial activity, such as alkaloids, terpenoids, steroids, and flavonoids. Compounds that were reported to have antibacterial properties were identified from GCMS, one of which was the compound azulane (CAS).

Keywords: Antibacterial effect, Associated bacteria, Seaweed, *Vibrio* sp

Introduction

Seaweed is a type of photosynthetic organism that plays an important role in the maintenance of diverse microbial communities as well as important functions in the health and protection of their hosts. The bacterial communities that collaborate with seaweeds play critical roles in many aspects, particularly in the production of secondary metabolites that help these ecosystems function [1, 2], the immune response of fish [3], graded levels of dietary seaweed [4], and antimicrobial properties [5]. Secondary metabolites, such as halogenated compounds and polyphenols, are abundant in seaweeds. These compounds have various benefits and play a role in ecology by inhibiting the growth of other organisms. More importantly, one of the main forces driving coevolution in marine ecosystems is the interaction between seaweeds and microbes in the marine environment [6]. Some seaweed-associated bacterial strains are thought to play a role

*e-mail: wilisarisetyati@yahoo.co.id
Tel.: + 62 815-6504-390

in pathogen tolerance [7] and natural food preservatives [8].

The human skin is home to bacteria, fungi, and viruses that make up the skin microbiota. Microorganisms on the human skin play an important role in protecting the body from pathogens, supporting immune system formation, and breaking down natural compounds [9, 10]. The skin, as the largest organ in the human body, harbors microorganisms that provide benefits while also acting as a physical barrier to pathogen entry [11]. Disrupted skin can disrupt the balance of beneficial and pathogenic bacteria, leading to a variety of skin diseases and even systemic health issues [12]. The presence of *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Micrococcus luteus* bacteria has been linked to skin diseases [13-15].

Seaweed-associated bacteria are microorganisms that live alongside seaweed and can benefit both the seaweed and the bacteria. Associated bacterial communities produce substances that promote seaweed growth, quorum-sensing signaling molecules, biologically active compounds, and other molecules that contribute to the maintenance of seaweed morphology, development, and growth [16, 17]. The ability of associated bacteria to provide defense for seaweed is thought to produce antibacterial compounds. Natural antibacterial compounds could be one of the efforts to add to the pharmaceutical industry's collection of antibiotics. The objective of this study was to determine the antibacterial ability of seaweed-associated bacteria against pathogenic bacteria that cause skin diseases.

Experiments

Materials

Bacterial isolates were isolated from seaweeds, *Caulerpa racemosa* and *Halimeda opuntia,* collected in the waters of Awur Bay, Jepara, Indonesia. Bacterial isolates were isolated from seaweeds *Caulerpa racemosa* and *Halimeda opuntia* collected in the waters of Awur Bay, Jepara, Indonesia. Seaweed was collected from a depth of 1,5-3 meters, at a water temperature of 27ºC, and during the rainy season. *Vibrionaceae bacterium* PH25, *Vibrio alginolyticus* strain GS MYPK1, *Salinivibrio costicola* strain M318, and *Vibrio alginolyticus* strain 2014V-1011 were isolated from previous research [18]. In this study, test bacteria included *Staphylococcus aureus* F2-FNCC 0047, *Pseudomonas aeruginosa* F2-ATCC-IFO12689, and *Micrococcus luteus* F2-FNCC 0347.

Antibacterial Activity Test on Pathogenic Bacteria

Disc Diffusion Method

Associated bacteria were grown in 1 L of Nutrient Broth (NB) medium and incubated for 96 hours at 29±2ºC. The liquid culture was then extracted by maceration with an ethyl acetate solvent to extract bioactive compounds from the associated bacteria. A rotary evaporator will be used to concentrate the extract, which will then be used in further testing. The ethyl acetate crude extract from associated bacteria is diluted with DMSO organic solvent. In the next test, 15 l of the crude extract was dripped on a sterile paper disc with a diameter of 6 mm and placed in a sterile Petri dish. The disc was placed in a laminar airflow for 60 minutes to allow the extract to soak into the paper. *S. aureus*, *P. aeruginosa*, and *M. luteus* test bacteria cultured in NB medium will then be planted evenly on the MHA medium using a sterile cotton bud. Paper discs soaked in extracts will be placed on the culture [19]. As a negative control, DMSO organic solvent was used, and chloramphenicol was used as a positive control. Chloramphenicol is an antibiotic that is used as a positive control in gram-positive and gram-negative bacteria testing [20]. The inhibition zone will be measured using a caliper after 24 hours of incubation at 29±2ºC.

Minimum Inhibitory Concentration (MIC) Method

The MIC test was performed on a 96-well plate using the microdilution method. Crude extracts of associated bacteria were diluted to a concentration of 30 mg/mL in DMSO solvent. Each well in the plate received 100 µl of MHB media. 50 µl of MHB media were added to the first row. The positive control well was filled with 50 µl of chloramphenicol, and the negative control well was filled with 50 µl of DMSO. The remaining wells received 50 µl of bacterial extract. Serial dilutions were also performed, beginning with the first dilution at a concentration of 7500 µg/ml and ending with the 12th dilution. So that the quantity matches the other wells, the well at the 12th dilution will be discarded. Each well received a 100-l suspension of test bacteria with a cell density of 0.5 McFarland Standard. The plate was then incubated for 24 hours at 29±2ºC. The level of turbidity in the wells was used to assess antibacterial activity. Wells that remained clear indicated that the tested antibacterial substances inhibited the test bacteria [21]. The antibiotic chloramphenicol was used at concentration as a positive control, while DMSO was used as a negative control.

Minimum Bactericidal Concentration (MBC) Method

In the MBC test, the test bacteria (*S. aureus, P. aeruginosa,* and *M. luteus*) were inoculated on NA media in a Petri dish. NA media inoculated with test bacteria will be incubated for 24 hours at 29±2ºC. The MBC result will be the lowest concentration at which no growth of the test bacteria is observed [20].

Identification of Antibacterial Compounds

Thin Layer Chromatography (TLC) Method

The TLC model is used to separate a mixture of compounds using eluents with varying polarities [22]. Eluent ratios of 1: 6: 2 were used for methanol (polar), ethyl acetate (semi-polar), and n-hexane (non-polar). The crude extract of associated bacteria, weighing up to 5 mg, is mixed into 200 µL of ethyl acetate solvent, and then up to 2 µL is bottled on a TLC plate using a capillary pipette. The TLC plate was placed in an eluent-filled chamber and observed until the eluent reached the predetermined upper line limit. To determine the stains formed, the TLC plate was visualized with visible light and UV light at wavelengths of 254 nm and 365 nm, respectively. Rf = Distance traveled by the analyte / Distance traveled by the eluent was used to calculate the *Retention Factor* (Rf) value.

Spraying with 10% FeCl3 reagent (for flavonoid compounds), *Dragendorff* reagent (for alkaloid compounds), and *Liebermann Burchard* reagent (for terpenoid steroid compounds) was used to determine compound classes. The formation of orange or red spots indicates positive results for flavonoid compounds [23]. The formation of orange, orange, and brown-colored spots indicates positive results for the alkaloid compound [24]. Positive terpenoid compound testing results in the formation of red-violet spots indicating terpenoid compounds and reddish-brown colors indicating steroid compounds.

Gas Chromatography-Mass Spectrometry (GCMS) Method

The GCMS method combines gas chromatography (GC) and mass spectrometry (MS). GC is used to separate volatile and semi-volatile compounds, while MS is used to identify compounds separated in the GC method [25]. The ethyl acetate extract of associated bacteria was injected into the GCMS injector using an HP-5MS UI column (dimensions 0.25 mm x 30 m), following the conditions of the Carrier Gas tool using UHP Helium (He), injector temperature 250ºC, split flow 10 mL/min, split ratio 10, Front Inlet Flow 1.00 mL/min, MS transfer line temp 230°C, Ion Source temp 200ºC, Mass List Range (amu) 40-500, Purge Flow 3 mL/min, Gas Saver Flow 5 mL/min, Gas Saver Time 5 min.

Results and Discussion

Antibacterial Activity Test on Pathogenic Bacteria

The crude extracts of associated bacteria have varying antibacterial effects on the pathogenic bacteria *S. aureus*, *P. aeruginosa*, and *M. luteus*. Observe the diameter of the inhibition zone from the left edge to the right edge of the inhibition zone. Table 1 shows the results of the disc diffusion method tests. The MIC testing of crude extracts of associated bacteria against the test bacteria *S. aureus, P. aeruginosa,* and *M. luteus* yielded inconsistent results. The lowest concentration that inhibited *S. aureus* was 468.75 µg/mL, *P. aeruginosa* was 234.37 µg/mL, and *M. luteus* was 468.75 µg/mL, according to the MIC test results (Table 1).

MBC testing of associated bacteria crude extract against test bacteria *S. aureus*, *P. aeruginosa*, and *M. luteus* yielded varying results. According to the MBC test results, the lowest concentration that inhibited *S. aureus* was 1875 µg/m, *P. aeruginosa* was 937.5 µg/mL, and *M. luteus* was 937.5 µg/mL (Table 1).

Identification of Antibacterial Compounds

TLC analysis of antibacterial compounds in crude extracts of associated bacteria revealed 20 stains on a silica gel TLC plate. Stains were not visible in visible light visualization, but they were visible in ultraviolet s of *S. aureus*, 937.5 µg/mL capable of inhibiting the growth of *P. aeruginosa*, and 937.5 µg/mL capable of inhibiting the growth of *M. luteus* (Table 1). The MIC and MBC results are aligned because the MBC results have the same or higher concentration than the MIC results [26]. The ability to kill test bacteria necessitates a higher concentration than the concentration required to inhibit bacterial growth. The results of disc paper testing, MIC, and MBC all show a positive correlation. Antibacterial testing confirmed that the associated bacterial isolates could produce antibacterial compounds against the test bacteria *S. aureus, P. aeruginosa,* and *M. luteus*.

The presence of terpenoid compounds is indicated by the stain's reaction to the red color. The presence of reactive terpenoid compounds on Rf_{10} and Rf_{20} stains. The presence of flavonoid compounds is detected using a 10% AlCl3 reagent, which results in a color change reaction on the TLC silica gel plate, which is a red stain. Rf_{10} and Rf_{20} stains contain reactive flavonoid compounds (Fig. 1).

Alkaloids are a type of secondary metabolite compound with a wide range of structural variations. Nitrogen in these molecules causes changes in their biological activity [27]. Many alkaloid compounds isolated from marine organisms exhibit antibacterial activity [28, 29]. The antibacterial activity of the alkaloid chemical group is associated with a variety of mechanisms, including inhibition of bacterial cell wall synthesis, inhibition of bacterial metabolism, changes in cell membrane permeability, and inhibition of nucleic acid and protein synthesis [30].

Antibacterial activity has been demonstrated for terpenoids isolated from marine organisms [31]. The terpenoid chemical group acts as an antibacterial agent by damaging the target bacteria's cell wall. The bacterial cell wall is disrupted in this process by disrupting the peptidoglycan component in the bacterial

Table 1. The results of antibacterial testing using the disc diffusion method, MIC method, and MBC method.

Isolate	Inhibition zone diameter (mm)		
	S. aureus	*P. aeruginosa*	*M. luteus*
Disc diffusion method			
1	13	14	12
2	15	17	12
3	12	16	11
4	16	19	13
Minimum inhibitory concentration (MIC) Method			
1	1875	1875	1875
2	937	1875	937
3	1875	1875	1875
4	468	234	468
Minimum Bactericidal Concentration (MBC) method			
1	3750	3750	7500
2	3750	1875	1875
3	3750	3750	7500
4	1875	937	937

Note: The numerical code '1' corresponds to *Vibrionaceae bacterium* PH25, code '2' for *Vibrio alginolyticus* strain GS MYPK1, code '3' for *Salinivibrio costicola* strain M318, and code '4' for *Vibrio alginolyticus* strain 2014V-1011. The use of numerical codes as references is applied throughout subsequent testing.

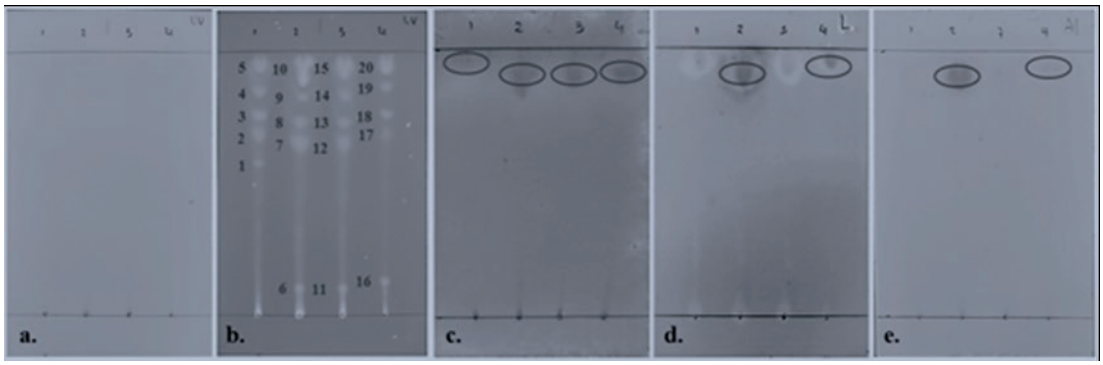

Fig. 1. The visualization results of TLC testing for crude extracts of seaweed-associated bacteria. a. TLC visualization under visible light, b. TLC visualization under UV light at a 381 wavelength of 365 nm, c. Alkaloid content test, d. Steroid/terpenoid content test, and e. Flavonoid content test.

cell, resulting in lysis cell damage to the target bacteria and eventually death [32].

Antibacterial activity has been demonstrated for flavonoids isolated from marine organisms [33]. The flavonoid chemical group is capable of directly damaging the envelopes of both gram-positive and gram-negative bacteria. Furthermore, flavonoids have the ability to act on specific molecular targets that are important to target microorganisms [34].

The GCMS identification of the crude extract of *Vibrionaceae bacterium* PH25 revealed several compounds with antibacterial activity (Table 2), including 2-Butoxyethyl acetate, Azulene (CAS), 1-TETRADECENE, 1-Pentadecene, 9-EICOSENE, (E)-, Hexadecanoic acid, methyl ester (CAS), Dibutyl phthalate, and 9-O- [35-38].

GCMS identification of the crude extract of *Vibrio alginolyticus* strain GS MYPK1 revealed several compounds with antibacterial activity, including 2-Butoxyethyl acetate, 1H-Indole (CAS), 1-Pentadecene, Hexadecanoic acid, methyl ester (CAS), 9-Octadecenoic acid, methyl ester, (E)-, and 1,2-Benzenedicarboxylic acid, 3-nitro- (CAS) [36, 39-41].

Table 2. The results of compound identification using GC-MS

Crude Extract	Compound identified using GC MS	Yield (%)
1	2-Butoxyethyl acetate	4.42
	Azulene (CAS)	2.34
	1-TETRADECENE	1.86
	PHENOL, 2,4-BIS(1,1-DIMETHYLETHYL)-	5.86
	1-Pentadecene	2.53
	9-EICOSENE, (E)-	2.09
	Hexadecanoic acid, methyl ester (CAS)	2.81
	Dibutyl phthalate	1.51
	9-Octadecenoic acid, methyl ester, (E)-	6.98
2	BENZENE, 1,2,4-TRIMETHYL-	3.28
	2-Butoxyethyl acetate	7.17
	1H-Indole (CAS)	8.84
	1-Pentadecene	1.31
	Hexadecanoic acid, methyl ester (CAS)	0.55
	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester (CAS)	2.72
	9-Octadecenoic acid, methyl ester, (E)-	1.48
	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester (CAS)	1.08
	1,2-Benzenedicarboxylic acid, 3-nitro- (CAS)	0.92
	3,4,5,6-TETRAHYDROXY-2-OXO-HEXANOIC	0.60
3	Benzene, 1,2,3-trimethyl- (CAS)	2.41
	BENZENE, 1,2,4-TRIMETHYL-	0.64
	2-Butoxyethyl acetate	7.02
	1-Pentadecene	0.55
	1-Hexadecene (CAS)	0.49
	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester (CAS)	2.31
	ETHYL DOCOSANOATE	1.40
	9-Octadecenoic acid, methyl ester, (E)-	0.44
	9-OCTADECENOIC ACID (Z)-, ETHYL ESTER	0.91
	1-Octadecanethiol (CAS)	0.66
4	2,3-Butanediol (CAS)	2.00
	2-Butoxyethyl acetate	7.11
	1-TETRADECENE	0.50
	9-OCTADECENE, (E)-	0.88
	Heptadecane (CAS)	0.51
	9-EICOSENE, (E)-	0.92
	Hexadecane (CAS)	0.46
	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester (CAS)	5.29
	ETHYL 9-OCTADECANOATE	1.29
	1-Octadecanethiol (CAS)	2.81
	1,2-Benzenedicarboxylic acid, 3-nitro- (CAS)	0.48

Note: The numerical code ‘1’ corresponds to *Vibrionaceae bacterium* PH25, code ‘2’ for *Vibrio alginolyticus* strain GS MYPK1, code ‘3’ for *Salinivibrio costicola* strain M318, and code ‘4’ for *Vibrio alginolyticus* strain 2014V-1011. The use of numerical codes as references is applied throughout subsequent testing.

GCMS identification of the crude extract of *Salinivibrio costicola* strain M318 showed several compounds that have been reported to have antibacterial activity, namely 2-Butoxyethyl acetate, 1-Pentadecene, 1-Hexadecene (CAS), ETHYL DOCOSANOATE, 9-Octadecenoic acid, methyl ester, (E)-, and 9-OCTADECENOIC ACID (Z)-, and ETHYL ESTER [36].

GCMS identification of the crude extract of *Vibrio alginolyticus* strain 2014V-1011 showed several compounds that have been reported to have antibacterial activity, namely 2-Butoxyethyl acetate, 1-TETRADECENE, 9-OCTADECENE, (E)-, 9-EICOSENE, (E)-, Hexadecane (CAS), ETHYL 9-OCTADECANOATE, and 1,2-Benzenedicarboxylic acid, 3-nitro- (CAS) [36, 37, 42].

Conclusions

The seaweed-associated bacteria *Caulerpa racemosa* and *Halimeda opuntia* have been proven to show antibacterial ability against bacteria that cause skin diseases, namely the pathogenic bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Micrococcus luteus*. The characterization of compounds in potential isolates identified the presence of groups of alkaloid, terpenoid, and flavonoid compounds in TLC identification. Identification using GCMS showed several compounds that had antibacterial properties in potential isolates, such as Azulene (CAS), 1,2-Benzenedicaboxylic acid, and 3-nitro- (CAS). Seaweed-associated bacteria have potential use in the development of antibiotics for the treatment of skin diseases caused by pathogenic bacteria.

Author Contributions

All authors have made an equal contribution and have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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