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ANTIBACTERIAL ACTIVITY SCREENING OF BACTERIAL ISOLATES ASSOCIATED WITH SEAWEEDS Caulerpa Lentillifera

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Abstract

Background: Caulerpa lentillifera is widely cultivated in Indonesia for its nutritional and health benefits. This seaweed is strongly associated with its bacterial community, essential in synthesizing various secondary metabolites, including antibacterial activity.
Objective: This research aimed to screen for antibacterial activity from bacteria associated with C. lentillifera.
Methods: Bacterial cultivation was performed on three different media, namely nutrient agar, plate count agar, and Zobell marine agar. The obtained bacterial isolates were identified morphologically and subsequently were screened for their antibacterial activity based on the perpendicular cross streak method against Staphylococcus aureus ATCC 25923, Streptococcus mutans FNCC 0405, Escherichia coli ATCC 25922, and Klebsiella pneumoniae ATCC 700603.
Results: 22 out of 28 bacterial isolates inhibited at least one of the bacterial targets. Bacterial isolate encoded as NAR9 showed as the most promising bacterial isolates with length inhibition of 19 mm against S. mutans FNCC 0405. Sanger sequencing of the amplified 16S rRNA gene fragment from bacterial isolate NAR9 revealed its phylogenetic relatedness to Marinobacter hydrocarbonoclasticus.
Conclusions: Overall, this research provides a preliminary result of the untapped potential of bacteria associated with C. lentillifera as a source of antibacterial activity.

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INTRODUCTION

Caulerpa lentillifera, commonly known as sea grapes, is a green macroalga that has garnered attention for its rich array of bioactive compounds and potential health benefits. This species is particularly noted for its high nutritional value, containing essential nutrients such as polyunsaturated fatty acids, vitamins, and minerals, which contribute to its popularity in culinary applications across Southeast Asia (1). The bioactive components of C. lentillifera include a variety of secondary metabolites such as flavonoids, terpenoids, phenolic compounds, and sulfated polysaccharides, which have been associated with numerous health-promoting properties (2-4).

Research has highlighted the antioxidant and antibacterial activities of C. lentillifera, suggesting its potential as a natural preservative in food products (5, 6). For instance, studies have indicated that extracts from C. lentillifera can inhibit the growth of bacteria isolated from infected wounds in fish, suggesting its potential as a natural antibacterial agent in aquaculture settings (7). Moreover, C. lentillifera is known to harbor diverse bacterial communities that may contribute to its antibacterial effects. Research has shown that the bacterial symbionts associated with C. lentillifera can possess enzymatic and antibacterial abilities, enhancing the overall antimicrobial activity of the seaweed (8, 9). The presence of specific bacterial strains, such as Pseudoalteromonas arabiensis and Pseudoalteromonas piscicida, has been linked to the production of bioactive compounds that further bolster the antibacterial potential of C. lentillifera (8).

In the past decade, a number of bacterial isolates have been purified from seaweeds, and many of these isolates have been shown to possess arrays of secondary metabolites, including antibacterial activity (5, 10-12). For instance, the epiphytic bacteria from the brown seaweed Padina pavonica have demonstrated broad-spectrum antibacterial effects, suggesting that these bacteria are capable of synthesizing compounds that inhibit pathogenic bacteria (5). Similarly, Bacillus amyloliquefaciens, derived from Hypnea valentiae, has been reported to produce novel antibiotics effective against Staphylococcus aureus (13). In addition, research has indicated that these bacteria not only support the health of their algal hosts but also produce secondary metabolites with significant antibacterial activity against skin disease agents like Propionibacterium acnes and Staphylococcus epidermidis (14, 15).

While a number of studies have been done to isolate bacteria from seaweeds, however, a rather limited study has been reported regarding bacterial isolates from C. lentillifera. Understanding these bacterial communities from C. lentillifera can reveal specific bacteria that may possess antibacterial properties, which could be harnessed for therapeutic applications. Therefore, this study was designed to isolate bacteria associated with C. lentillifera and to screen for potential antibacterial produced by the obtained isolates. It was expected as well to isolate a prolific bacterial isolate with strong antibacterial activities to be further studied.

MATERIALS AND METHODS

Sampling C. lentillifera

The seaweed C. lentillifera was collected from aquaculture ponds in Musi Village, Gerokgak District, Buleleng Regency, Bali, Indonesia. Upon arrival in the laboratory, the C. lentillifera samples were stored in refrigerator at 4oC until further processed.

Bacteria cultivation and morphological observation

Three agar media, namely Zobell Marine Agar (ZA), Nutrient Agar (NA), and Plate Count Agar (PCA), were prepared following the manufacturer's instructions. The aim to use 3 different agar was to obtain a diverse bacterial culture. In addition to keep the osmotic pressure of the NA and PCA media to resembles that of seawater condition, these two media were dissolved in 1L of artificial seawater (16) (33gr/L), which composed of Ca 450 – 490 ppm, Mg 1300 - 1400 ppm, Specific Gravity 1.023 - 1.026 as described by the manufacturer (asw salt mix, monsterlaut, Indonesia). The seaweed samples were divided into small pieces so that the interior and exterior parts were evenly mixed and washed with artificial seawater for 10 grams of sample C. lentillifera. Subsequently, 25 mL of sterile artificial seawater was added after homogenizing sample C. lentillifera by pounding it with a mortar and pestle. The sample was diluted by adding 1 mL of C. lentillifera to 9 mL of sterile artificial seawater, gradually diluting it (101 to 106). One-hundred microliter of 103 to 105 dilution was taken and plated on each corresponding agar medium and incubated for two weeks at 28oC. Observation of colonies that grew on each agar media was performed every three days to count the number of colonies on each agar medium. Subsequently, morphological appearance of each bacterial colony was determined based the previously described of the colony morphology code guidelines (17). Only distinct and unique bacterial colonies were picked from each agar media and each colony was purified further on Zobell marine agar media.

Gram staining

Each bacterial colony was stained following the Gram staining procedure. Moreover, cell morphology was observed under a light microscope (Leica, Germany) with 1000 times magnification.

Antibacterial screening

Antibacterial activity testing was done using perpendicular streak method as previously described (18). Briefly, the bacterial isolates were streaked perpendicularly on the LB agar medium and grown there for 48 hours at a temperature of 27°C until the colonies were thick enough to form on the agar. Subsequently, these four bacterial targets namely S. aureus ATCC 25923, S. mutans FNCC 0405, E. coli ATCC 25922 and, K. pneumoniae ATCC 700603 were horizontally streaked 5 cm away from the isolate colony. Finally, the agar medium was incubated at 37°C for 48 hours. Antibacterial activity of each isolate was measured by the distance that was formed from each of bacterial test against the screened isolate. The longer the distance indicated the stronger antibacterial activity of the isolate.

Molecular Analysis

DNA isolation and polymerase chain reactions

The bacterial isolate with the strongest antibacterial activity selected for molecular analysis. Initially, cell mass was grown in 1.5 mL Zobell Marine Broth prior to DNA isolation. DNA isolation was performed using BacteriaDNA Preparation Kit (Jena

Bioscience, Germany). The quality of DNA was measured using a nanodrop at a wavelength of 260/280 nm with the ideal DNA ratio is 1.8 (19). The obtained DNA was used for polymerase chain reactions by targeting 16S rRNA gene fragment following the previous described protocol and PCR setting (10, 17). The PCR product was run for electrophoresis on 1% agarose stained with SYBR safe and further was visualized under UV light using a GelDoc. Finally, PCR product was sequenced using Sanger sequencing method.

Phylogenetic Analysis

The sequencing result was analyzed using nucleotide BLAST (BLASTn) on the NCBI database. A phylogenetic tree was constructed using MEGA X software.

RESULTS AND DISCUSSIONS

Different media and dilution levels are utilized to gain access to a variety of bacterial isolates and decrease the intensity of bacterial isolates. In this research, C. lentillifera-associated bacterial isolates were isolated utilizing three media with dilution levels ranging from 103 - 105. As shown in Figure 1, the isolation of bacteria linked to C. lentillifera on three agar media revealed a range of colonies. On the three-agar media, a total number of colonies that could not be counted (>300 colonies) were obtained at a dilution level of 103. NA agar resulted in total colonies of 3x104 cfu/ml and 4x105 cfu/ml, respectively, at dilution levels of 104 and 105. At 104 and 105 dilution levels, the total colonies obtained in ZMA media were 4x104 CFU/ml and 8x105 CFU/ml (Table 1).

The total number of bacterial colonies on C. lentillifera against all media at a dilution level of 10-3 resulted in an insufficient sample size (>300 colonies) that prevented counting. Consequently, the number of colonies was calculated at dilution levels of 104 and 105. The difference in total bacterial colonies is due to the necessity of a synthetically appropriate environment to replace their natural state for the growth and development of bacteria (20). Due to the different nutrient needs of the cultured bacteria, the compositional precision of the media is dependent on the total colony yields, which are also varied.

Table 1. Total bacteria colonies of C. lentillifera at various dilution levels

Media	*C. lentillifera* (cfu/ml)		
	10^{-3}	10^{-4}	10^{-5}
NA	ND	$3x10^4$	$4x10^5$
PCA	ND	ND	0
ZMA	ND	$4x10^4$	$8x10^5$

ND: Not Determined (Colony >300).

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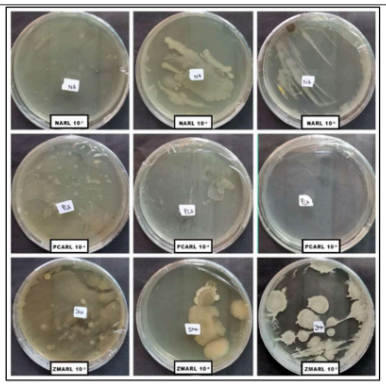

Figure 1. Bacterial isolates associated with *C. lentillifera*

Colony growth on agar was highly variable, and several colonies appeared identical. Consequently, bacterial colonies were chosen using the Colony Morphology Code method by categorizing them according to their morphological appearance. There were a total of 28 bacterial isolates, each of which represented a different type of bacterial isolate. It was impossible to isolate all of the bacteria associated with C. lentillifera, despite the wide variety of colonies obtained. This is a Great Plate Count Anomaly relating to the disparity between the number of colonies of samples cultured artificially in the laboratory and the number of colonies observed in natural environment (21)

In the antibacterial activity test outcomes for C. lentillifera isolates, 22 isolates demonstrated inhibitory activity against the indicator bacteria, evidenced by the formation of inhibition zones (Table 2). The diverse responses observed, indicated by distinct inhibition zone sizes, are attributed to variations in the type and concentration of antibacterial compounds produced by the isolates, as well as their diffusion rates in the agar medium (22). The perpendicular streak method (Figure 2) was selected as the antibacterial activity test because it is remarkably simple to execute and can be directly applied to 4-6 test bacteria (17). Those bacterial isolates with a high potential for synthesising active antibacterial molecules will separate themselves from the test bacteria indicators. The greater the distance between the test bacteria and the bacterial isolates, the greater the antibacterial activity of the isolates (18).

Table 2. The result of antibacterial screening from bacterial isolates of *C. lentillifera* against the test bacteria *S. aureus* ATCC 25923, *S. mutans* FNCC 0405, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603

NO.	Sample Code	Antibacterial Activity (mm)			
		S. aureus atcc 25923	*S. mutans* FNCC 0405	*E. coli* atcc 25922	*K. pneumoniae* atcc 700603
1.	NAR 1	5	4	3.5	2
2.	NAR 2	5	1	2	3
3.	NAR 3	-	-	-	-
4.	NAR 4	5	4	3	5
5.	NAR 5	5	4	4	2
6.	NAR 6	-	7	-	-
7.	NAR 7	2	12	3.5	5
8.	NAR 8	-	-	-	5
9.	NAR 9	6.5	19	4	5
10.	PCAR 1	5	3	3	1
11.	PCAR 2	-	-	-	-
12.	PCAR 3	4.5	1	-	4
13.	PCAR 4	3.5	2	1	1
14.	PCAR 5	3	-	1	2
15.	PCAR 6	0.5	0.5	-	-
16.	PCAR 7	-	-	-	-
17.	PCAR 8	-	-	-	-
18.	PCAR 9	-	-	-	-
19.	ZMAR 1	5	1	5	5
20.	ZMAR 2	-	2	-	-
21.	ZMAR 3	-	2	-	-
22.	ZMAR 4	-	1	-	1
23.	ZMAR 5	-	-	-	-
24.	ZMAR 6	-	0.5	-	-
25.	ZMAR 7	4	5	5	3
26.	ZMAR 8	3	2.5	1.5	2
27.	ZMAR 9	0.5	2	-	0.5
28.	ZMAR 10	1	5	0.5	1

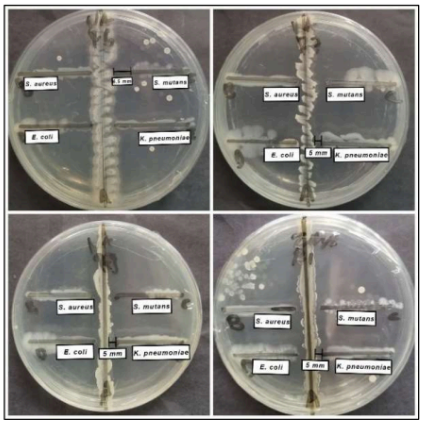

Figure 2. Perpendicular streak method of antibacterial activity of NAR6, NAR7, NAR9, and ZMAR1 isolates against bacterial targets.

The antibacterial activity test results showed that isolate NAR9 was the most effective against the tested bacteria. NAR9 exhibited a large inhibition zone of 19 mm against S. mutans FNCC 0405 and smaller zones ranging from 4-6.5 mm against the other bacteria tested. Molecular identification of NAR9 was performed by searching the NCBI BLAST database, which yielded the 10 most similar bacterial sequences listed in Table 3. A phylogenetic tree (Figure 3) constructed from these BLAST results identified the Marinobacter genus as the most prominent group. Genera sharing morphological characteristics within the same cluster often suggest a close genetic relationship (23).

This research showed that the isolate NAR9, with antibacterial activity and molecular identification, is a bacterium of M. hydrocarbonoclasticus. This species is a Gram-negative bacterium with a rod-shaped cell, a cell width between 0,3-0,6 µm, a cell length between 2-3µm, monotrichous flagella, and polar and motile characteristics (24). Primarily found in marine environments, M. hydrocarbonoclasticus is ubiquitous in seawater and marine sediments globally (24). It is also a halotolerant to halophilic organism, capable of growing across a wide range of salt concentrations, and has been isolated from diverse saline habitats including salt-affected soils, salt lakes, and even oil/gas wells. It can also be associated with marine life such as algae and copepods (25).

A key characteristic of M. hydrocarbonoclasticus is its metabolic versatility, particularly its notable ability to degrade and utilize hydrocarbons as a sole source of carbon

and energy (26). This gives the species ecological significance in the remediation of hydrocarbon-contaminated environments. It is an aerobic bacterium that can also utilize nitrate or nitrite as electron acceptors when oxygen is limited, providing metabolic flexibility (25). M. hydrocarbonoclasticus is known to form biofilms on hydrophobic surfaces, which facilitates the degradation of hydrocarbons.

Table 3. The top ten BLAST-n result

No	*Related species*	*Accession no.*	*Max Score*	*Query Cover*	*E-Value*	*Max Ident*	*Sequence fragment (bp)*
1	*Marinobacter hydrocarbonoclasticus* strain NMRL51	MT192574.1	1783	97%	0	96,99%	1498
2	*Marinobacter hydrocarbonoclasticus* strain P210(9)	GU370082.2	1783	97%	0	96,99%	1513
3	*Marinobacter hydrocarbonoclasticus* strain EP27-5-1	KY457439.1	1783	97%	0	96,99%	1507
4	*Marinobacter hydrocarbonoclasticus* strain EP27-4-12	KY457432.1	1783	97%	0	96,99%	1485
5	*Marinobacter hydrocarbonoclasticus* strain EP27-3-9	KY457431.1	1783	97%	0	96,99%	1506
6	*Marinobacter hydrocarbonoclasticus* strain EP27-1-5	KY457415.1	1783	97%	0	96,99%	1508
7	*Marinobacter hydrocarbonoclasticus* strain ss20	JN160761.1	1783	97%	0	96,99%	1487
8	*Marinobacter hydrocarbonoclasticus* strain ss19	JN160760.1	1783	97%	0	96,99%	1490
9	*Marinobacter hydrocarbonoclasticus* strain ss18	JN160759.1	1783	97%	0	96,99%	1485
10	*Marinobacter hydrocarbonoclasticus* strain ss17	JN160758.1	1783	97%	0	96,99%	1496

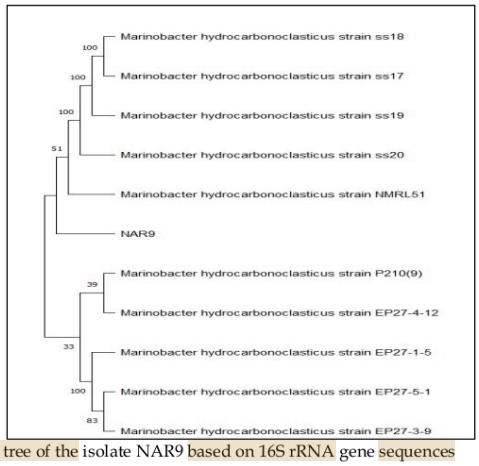

Figure 3. Phylogenetic tree of the isolate NAR9 based on 16S rRNA gene sequences

CLINICAL IMPLICATION

The current study focusing on screening the antibacterial activity of bacterial isolates associated with the seaweed Caulerpa lentillifera holds significant clinical implications. The discovery of bacteria from marine environments, such as those found in association with seaweeds, that produce compounds with inhibitory effects against other bacteria is crucial in the ongoing search for new antimicrobial agents. Given the increasing global challenge of antibiotic resistance in human pathogens, novel sources of antibacterial compounds are urgently needed. Findings from this research could potentially lead to the isolation, identification, and development of new drug candidates effective against resistant bacterial infections, offering potential new therapeutic strategies for clinical use.

LIMITATIONS

This study has two main limitations. First, the antibacterial screening was performed using a qualitative perpendicular streak method. While this approach provided a preliminary indication of antibacterial activity, it is a qualitative method and therefore does not provide quantitative data on the potency of the observed activity. Additionally, the absence of a positive control (e.g., a standard antibiotic) limits the direct comparison and strength of the interpretation of our results. Future work should involve quantitative assays such as Minimum Inhibitory Concentration (MIC) to determine the precise antibacterial efficacy, and explore extract-based approaches for further verification. Second, the antibacterial screening was primarily focused on non-resistant bacterial strains. Screening against multidrug-resistant bacterial strains in future studies may yield different outcomes and provide a more comprehensive understanding of the antibacterial potential.

CONCLUSIONS

From the seaweed C. lentillifera, a total of 28 bacterial isolates were obtained using three types of media: NA, PCA, and ZMA. General morphological characteristics observed among the isolates included a dull surface, opaque color, and raised colony elevation. Of the 28 isolates recovered from C. lentillifera, twenty-two bacteria showed antibacterial activity against at least one of the bacterial targets. The isolate designated NAR9 displayed the highest level of antibacterial activity, especially against S. mutans FNCC 0405. Subsequent molecular identification using 16S rRNA gene amplification indicated a strong association between the NAR9 isolate and Marinobacter hydrocarbonoclasticus, with a maximum sequence identity of 96.99%.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

AUTOR CONTRIBUTIONS

Sunthio Barran Cia AR performed the experiment and wrote the initial manuscript draft, Anak Agung Gede Indraningrat designed the experiment, provided consumables and supervised the experiment, Ni Wayan Widhidewi supervised the experiment and analyzed antibacterial data, Made Dharmesti Wijaya performed molecular analysis, Kadek Lila Antara provided C. lentillifera samples, Daegeun Choe contributed in data analysis, formatting and proof reading of the manuscript.

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