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Isolation and Identification of Seaweed-Associated Bacteria and Their Antibacterial Activity against Skin Disease Agents

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Abstract

Bacteria associated with marine organisms have become the focus of worldwide research for their potential to generate bioactive substances, such as antibacterial compounds. This study aimed to identify the antibacterial activity of seaweed-associated bacteria in Teluk Awur Jepara, Indonesia, against *Propionibacterium acnes* and *Staphylococcus epidermidis*. Three seaweed species, *Caulerpa racemosa*, *Padina minor*, and *Halimeda opuntia*, were sampled in this study. Their isolates were selected tested for antibacterial activity against pathogenic bacteria using the paper disc diffusion and Minimum Inhibitory Concentration methods and were molecularly identified based on the 16s RNA gene (27F-1492R). Twenty-one isolates were isolated from the 3 seaweed species: 11 from *C. racemosa*, 6 from *P. minor*, and 4 from *H. opuntia*. Further testing revealed potential isolates (C2a, C2c, C2d, and H2d) with antibacterial activity against *P. acnes* F2 ATCC 6919 and *S. epidermidis* FNCC-0048. Gene-based identification using 16s RNA (27F-1492R) demonstrated the occurrence of 4 bacterial species, namely *Vibrionaceae bacterium* PH25 (99.86 %), *Vibrio alginolyticus* strain GS MYPK1 (99.65 %), *Salinivibrio costicola* strain M318 (99.86 %), and *V. alginolyticus* strain 2014V-1011 (99.93 %).

Keywords: Antibacterial, Association, Seaweed, Skin, *Vibrio* sp.

Introduction

Microorganisms are crucial in mediating global biogeochemical cycles in the marine environment [1]. Marine natural resources are being explored for vital beneficial prospects for human life. In recent years, research has focused on microorganisms as agents in bioactive compounds, such as antibacterial compounds [2]. The presence of microorganisms in the environment, such as bacteria, plays an extraordinary role. Several marine bacteria have high potential of application in human life [4]. Marine bacteria, for example, produce bioactive compounds with numerous properties, such as antibacterial, antineoplastic, antiviral, antifungal, antidiabetic, immunosuppressive, and antimalarial [5]. Furthermore, by identifying marine bacteria, numerous new antibiotics have been discovered [6,7], the immune response [8].

Associated bacteria is a bacterial community characterised by direct or indirect interactions with their host [9]. According to Hollants *et al.* [3], bacteria show highly diverse interaction with brown, green, and red seaweeds. According to Singh and Reddy [10], bacteria associated with seaweed have a crucial role in growth, morphogenesis, and protection. They produce plant growth substances, bioactive compounds, and quorum-sensing signal molecules that aid seaweed growth and development. Furthermore, bioactive compounds produced by associated bacteria determine the presence of other bacteria and protect the host against environmental threats.

According to Hollants *et al.* [3], *Vibrio* sp. was the most prevalent bacteria genus observed in red, green, and brown seaweed. The pathogen *Vibrio* sp. and several seaweed-associated bacillus species are efficient producers of active compounds, which act as antisettlement and quorum-sensing (QS) inhibitors, thus protecting the seaweed surface from pathogens, herbivores, and fouling organisms [11].

Bacteria in the human body frequently have both detrimental and beneficial effects. According to Alghamdi *et al.* [12], bacteria found in human hair account for nearly 85 % of the population of bacteria living in the surface layer of the skin and hair follicles. As a result, human hair is constantly exposed to pollutants and bacterial contamination. A study by Saxena *et al.* [13] identified several bacteria species in human hair, such as *Propionibacterium acnes* and *Staphylococcus epidermidis*. These bacterial species are most common on the scalp of dandruff individuals. Chessa *et al.* [14] also commonly observed *P. acnes* and *S. epidermidis* causing infections on the human skin.

The world seeks antibacterial chemicals obtained from natural resources. As a result, exploring Indonesia's marine resources may aid in acquiring natural antibacterial compounds that could act against bacteria causing scalp diseases. *Caulerpa racemosa* (Försskal) J. Agard, *Padina minor* Yamada, and *Halimeda opuntia* (Linnaeus) J. V. Lamouroux were found to be the dominant seaweed species in the Jepara sea in August 2022. Therefore, this study aimed to determine the antibacterial activity of seaweed-associated bacteria *C. racemosa*, *P. minor*, and *H. Opuntia* in Teluk Awur, Jepara, Indonesia, against bacterial species associated with scalp diseases, *P. Acnes* and *S. epidermidis*.

Material and methods

Material

C. racemosa, *P. minor*, and *H. opuntia* were collected from Teluk Awur, Jepara, Indonesia (**Figure 1**), in August 2022. At the time of sampling, these 3 species were growing and dominant. Identification of seaweed samples was carried out by observing their morphology and matching it with the key determination and literature [15-17]. The seaweed-associated bacteria were isolated in the microbiology laboratory of Diponegoro University. *P. acnes* F2 ATCC 6919 and *S. epidermidis* FNCC-0048 were employed as test bacteria in this study.

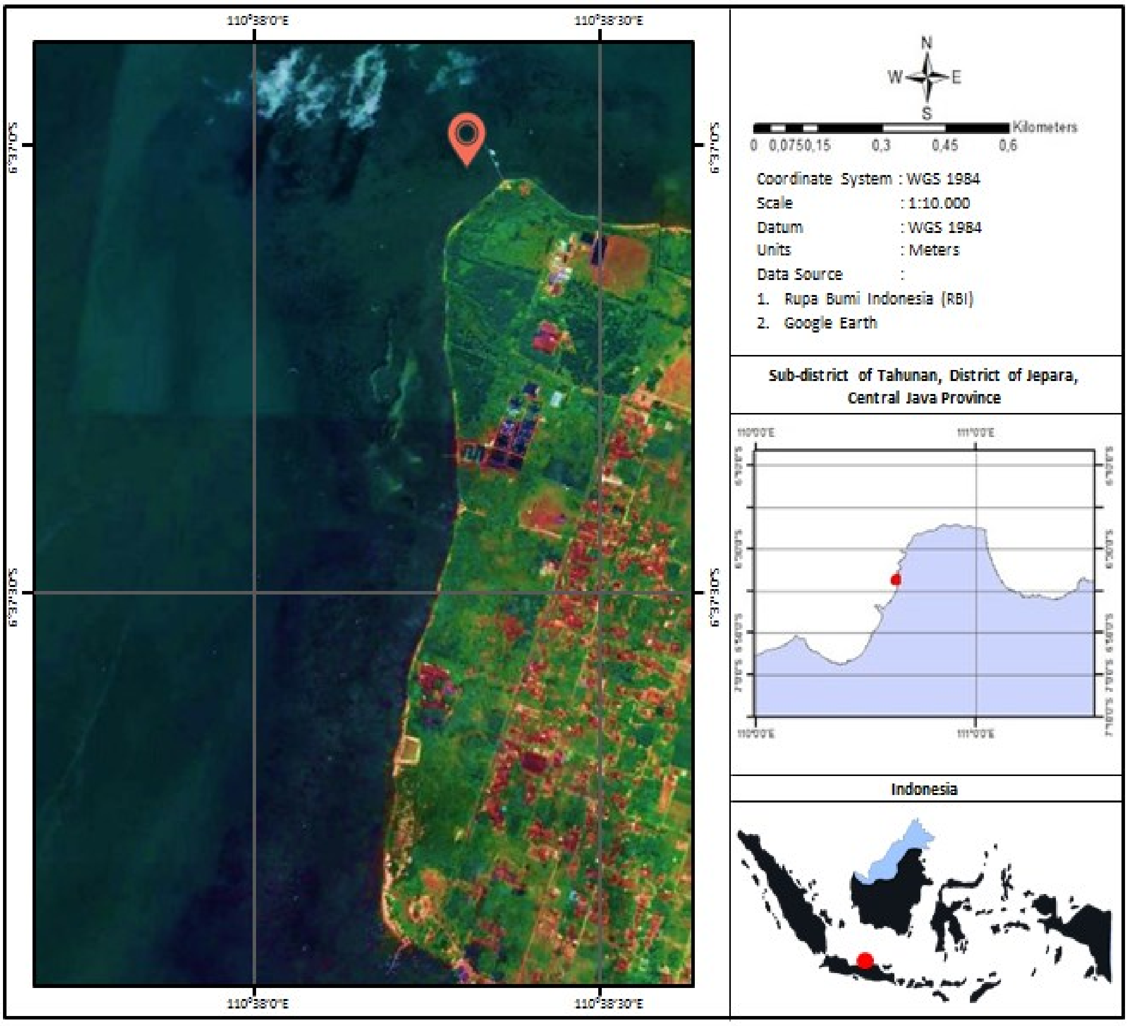

Figure 1 Location of Awur Bay, Jepara, Indonesia; where seaweed sampling was conducted (*Caulerpa racemosa*, *Padina minor*, and *Halimeda opuntia*); red point (6º37'2.04"S, 110º38'18.20"T).

Seaweed sampling

Samples of *C. racemosa*, *P. minor*, and *H. opuntia* were collected from Jepara, Indonesia. Seaweed samples were taken as a whole (thallus and holdfast). A sample of 500 g of each seaweed was collected and stored in 200 mL seawater. Samples were packaged and stored in a cool box with ice as 1:1 (w/w).

Isolation and purification of associated bacteria

Samples of *C. racemosa*, *P. minor*, and *H. Opuntia* were washed or rinsed in sterile running water for 15 min followed by being cut into 5 cm pieces. Further, they were sterilised with 70 % ethanol solution for 1 min, then 5.25 % NaClO for an additional 1 min, and 3 washes with 70 % ethanol. The sterilised samples were crushed with a mortar and pestle and planted on Zobell Marine Agar (ZMA) media at dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, then incubated at 30 ± 2 °C for 48 h [14]. In order to isolate a single colony, bacterial isolates that displayed different colony characteristics, including form, margin, elevation, size, and pigmentation, were purified [18,19].

Selection of associated bacteria exhibiting antibacterial activity

The direct challenge test was used to identify the antibacterial activity of associated bacteria against *P. acnes* and *S. epidermidis*. The test bacteria were inoculated on Mueller-Hinton Agar (MHA) using the pour plate method. A direct challenge test was carried out by adding 20 µL liquid culture of associated bacteria into 6 mm paper discs, then placing them on the surface of the *P. acnes* and *S. epidermidis* cultures in MHA medium. The cultures were incubated at 35 ± 2 °C for 24×3 h. Associated bacterial isolates that created an inhibition zone (4 isolates with the widest inhibition zone as measured by caliper) had this zone area measured and were followed for further testing [20].

Antibacterial activity test against *P. acnes* and *S. epidermidis*

Four bacterial isolates associated with the widest inhibition zone were tested with the diffusion method using paper discs and the Minimum Inhibitory Concentration (MIC) method. Before testing, associated bacteria grown in Nutrient Broth (NB) medium were incubated at 35 ± 2 °C for 96 h. Isolates on the NB medium were extracted with ethyl acetate solvent to obtain crude extracts from the associated bacteria and then concentrated using a rotary evaporator. The concentrated extract was weighed and used in further tests [21].

Screening of associated bacteria by diffusion method (paper disc)

The bacteria were screened using a concentrated extract that was injected into 6-mm paper discs, which were then placed directly on the surface of MHA medium with the test bacteria *P. acnes* and *S. epidermidis*. The antibacterial activity was seen after 24×2 h of incubation at 35 ± 2 °C. The formation of an inhibition zone around the paper discs indicated a successful outcome.

Minimum Inhibitory Concentration (MIC) method

The Minimum Inhibitory Concentration (MIC) method was used to determine the lowest concentration of the extract in which antibacterial activity was still prevalent. This study uses the dimethyl sulfoxide (DMSO) solvent to gradually dilute the extract [22]. The concentrated extract was diluted to several concentration levels, namely 2000, 1000, 500, 250 and 125 ppm. These concentrates were injected into 6-mm paper discs, which were then placed directly on the surface of MHA medium prepared with *P. acnes* and *S. epidermidis* test bacteria. Antibacterial activity was detectable after 24×2 h of incubation at 35 ± 2 °C if a clean zone is formed around the paper discs.

Molecular identification of potential isolates

Four pure isolates (single colonies) were transported to the Genetics Science Laboratory in Indonesia using Zobell Marine agar slanted media for molecular identification of the 16S rRNA gene (27F-1492R). According to Palkova *et al*. [23], the stages of molecular identification are the isolation of bacterial DNA, DNA amplification using 27F-1492R primers, purification of PCR products by electrophoresis and labelling of optimal bands, sequencing, and data processing. The data analysis of the base sequence used the BioEdit 7.0.5 software, and matches were checked NCBI database. Alignment and building of the phylogenetic tree were performed with Mega11 and the Neighbour-Joining method (NJ). The phylogenetic tree demonstrates the genetic relationships between the acquired bacteria. *Escherichia coli* strain EC1 OP364884.1 was used as an outgroup.

Results

Seaweed sampling

Three seaweeds prevalent in Teluk Awur, Jepara, Indonesia, were sampled in August 2022. These seaweeds grow on dead corals in the subtidal zone on open beaches. Based on the key determination of [15, 24]. The results of sampling were *C. racemosa*, *P. minor*, and *H. opuntia* (**Table 1**).

Table 1 Morphological identification of seaweed samples.

No.	Seaweed type	Description
1.		This seaweed belongs to the group of green macroalgae (Chlorophyta). Coenocytic plants are organised to form large stolons and upright branches to be included in the genus *Caulerpa*. There are short-stemmed round ramuli, arising on the stolons. Based on its morphological characteristics, the seaweed is called *Caulerpa racemosa*. *C. racemosa* seaweed was collected from the sea of Jepara [24-26].
2		This seaweed belongs to the group of brown macroalgae (Phaeophyta). This plant has a sheet-shaped thallus that resembles a fan, light brown to white in color. Radial lines 4 - 7 cm wide form a partition on each sheet and Habitat from a distance of 2 - 20 m from the shoreline. It is constantly inundated with water and attaches to a rock substrate. The morphology matches to *Padina minor*. *P. minor* seaweed was collected from Awur Bay, Jepara [25].
3.		This seaweed belongs to the group of green macroalgae (Chlorophyta). The plant is erect and forms calcified flat segments, so it belongs to genus *Halimeda.* Based on its morphological characteristics, the seaweed is called *Halimeda opuntia*. *H. opuntia* seaweed was collected from Bandengan Beach and Awur Bay, Jepara [25,27,28].

Isolation and purification of associated bacteria

The isolates of associated bacteria on ZMA medium grew optimally at 10^{-2} and 10^{-3} dilutions, with various morphological characteristics (**Figure 2**). The isolates were purified by identifying their key morphological features, yielding 21 isolates, 11 from *C. racemosa*, 6 from *P. minor*, and 4 from *H. opuntia* (**Table 2**).

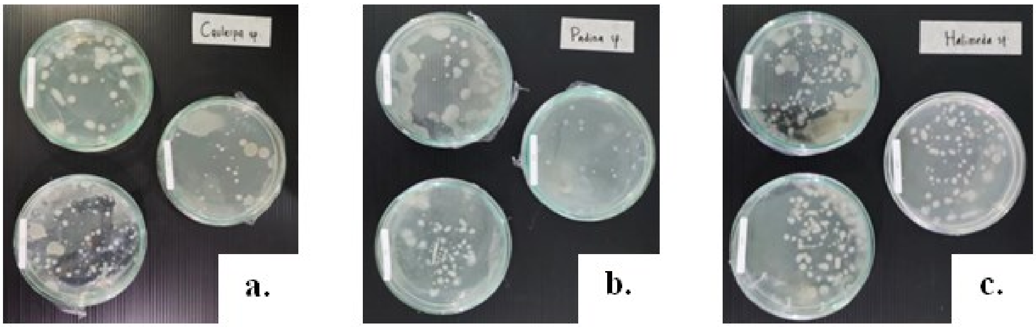

Figure 2 The results of the isolation of associated bacteria on (a) *Caulerpa racemosa*, (b) *Padina minor,* and (c) *Halimeda opuntia* with 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}.

Table 2 Morphological determination results.

Isolate	Form	Margin	Elevation	Size	Pigmentation
C2a	Circular	Curled	Raised	Large	Non pig.
C2b	Circular	Entire	Convex	Moderate	Non pig.
C2c	Circular	Rhizoid	Convex	Moderate	Non pig.
C2d	Circular	Entire	Flat	Moderate	Non pig.
C2e	Circular	Entire	Raised	Large	Non pig.
C3f	Irreguler	Entire	Flat	Large	Non pig.
C2g	Filamentous	Filamentous	Convex	Small	Non pig.
C2h	Spindle	Entire	Flat	Punctiform	Non pig.
C2i	Irreguler	Filamentous	Raised	Large	Non pig.
C2j	Irreguler	Undulate	Pulvinate	Small	Pigmented
C2k	Circular	Curled	Raised	Moderate	Pigmented
P3a	Irreguler	Filamentous	Flat	Large	Non pig.
P3b	Circular	Entire	Flat	Moderate	Non pig.
P3c	Circular	Entire	Raised	Moderate	Non pig.
P2d	Circular	Rhizoid	Umbonate	Moderate	Non pig.
P2e	Circular	Undulate	Raised	Moderate	Non pig.
P3f	Irreguler	Entire	Flat	Large	Non pig.
H3a	Circular	Entire	Raised	Moderate	Non pig.
H3b	Irreguler	Entire	Raised	Large	Non pig.
H2c	Circular	Rhizoid	Raised	Moderate	Non pig.
H2d	Circular	Undulate	Umbonate	Moderate	Non pig.

Note: Capital letter code ‘C’ indicates the isolate comes from *Caulerpa racemosa*, ‘P’ stands for *Padina minor* and ‘H’ for *Halimeda opuntia*. The code number refers to the origin of the isolate dilution, 2 for the 10^{-2} dilution and 3 for the 10^{-3} dilution. Lowercase code marks isolates originating from the same seaweed.

Selection of association bacteria exhibiting antibacterial activity

The isolates of associated bacteria were directly exposed to the test bacteria *P. acnes* and *S. epidermidis* to determine the possible antibacterial activity. Results showed that the antibacterial activity of 21 isolates varied (**Table 3**). Four of the 21 isolates with the widest inhibition zone and the most heterogenous colony characteristics (to minimise the same bacterial species) were investigated further, namely isolates C2a, C2c, C2d, and H2d.

Table 3 The activity of associated bacteria in a direct challenge test on *Propionibacterium acnes* and *Staphylococcus epidermidis* bacteria.

Isolate	inhibition zone diameter (mm)					
	24 h		48 h		72 h	
	S. epidermidis	*P. acnes*	*S. epidermidis*	*P. acnes*	*S. epidermidis*	*P. acnes*
C2a	7	11	7	11	8	12
C2b	8	9	9	13	7	12
C2c	7	21	7	24	8	23
C2d	10	13	8	17	8	14
C2e	11	7	8	12	8	18
C3f	8	8	8	13	7	10
C2g	7	14	7	7	9	9
C2h	8	14	8	11	9	12
C2i	9	12	8	12	9	13
C2j	8	18	8	9	9	11
C2k	7	9	8	9	9	9
P3a	7	17	7	-	7	-
P3b	7	7	8	-	7	-
P3c	9	13	8	-	8	-
P2d	7	17	7	17	7	-
P2e	7	-	7	-	7	-
P3f	7	13	7	11	7	-
H3a	9	9	7	19	8	11
H3b	8	8	7	13	8	11
H2c	8	9	7	13	8	-
H2d	8	9	7	26	-	25

Note: Unit in millimetres, the diameter of the paper discs (6 mm) is included, code '–' for the inhibition zone is lost.

Antibacterial activity test against *P. acnes* and *S. epidermidis*

Screening of associated bacteria by diffusion method (paper disc)

Isolates C2a, C2c, C2d, and H2d were selected because they showed antibacterial activity (inhibition zone). Additional tests were performed using crude extracts of associated bacteria against the test bacteria *P. Acnes* and *S. epidermidis*. The inhibition zones established by the 4 isolates varied (**Figure 3**).

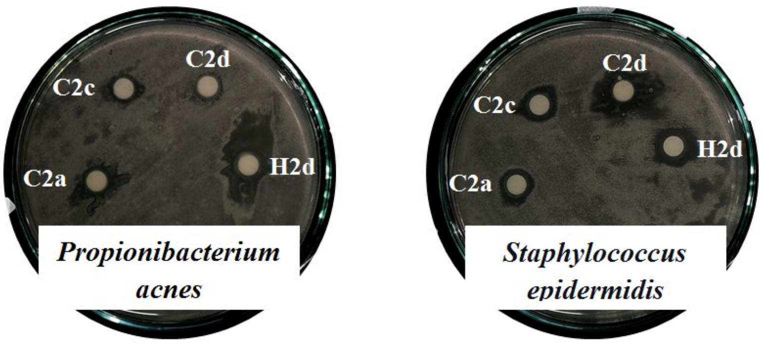

Figure 3 The results of the antibacterial activity of the isolates of associated bacteria against test bacteria *Propionibacterium acnes* and *Staphylococcus epidermidis*.

Minimum Inhibitory Concentration (MIC) method

The minimum inhibitory concentration was observed to be 1000 ppm for *P. Acnes* (**Table 4**) and 125 ppm for *S. Epidermidis* (**Table 5**).

Table 4 MIC results of bacterial isolates associated with the test bacteria *Propionibacterium acnes*.

Isolate	Concentration (ppm)				
	2000	1000	500	250	125
C2a	+	+	-	-	-
C2c	+	+	-	-	-
C2d	+	+	-	-	-
H2d	+	+	+	-	-

Note: Code '+' indicates antibacterial activity at that concentration, and code '–' indicates no antibacterial activity at that concentration.

Table 5 MIC results of bacterial isolates associated with the test bacteria *Staphylococcus epidermidis*.

Isolate	Concentration (ppm)				
	2000	1000	500	250	125
C2a	+	+	+	+	+
C2c	+	+	+	+	+
C2d	+	+	+	+	+
H2d	+	+	+	+	+

Note: Code '+' indicates antibacterial activity at that concentration, and code '–' indicates no antibacterial activity at that concentration.

Molecular identification of potential isolates

Molecular identification with the 16s RNA gene (27F-1492R) is highly effective in demonstrating the relationship of bacterial isolates. Therefore, this method was used to molecularly identify the species of the 4 selected associated bacteria isolates, coded C2a, C2c, C2d, and H2d. The phylogenic relationship of associated bacteria and the nearest species of bacteria was established based on 16S rRNA gene sequence and using Neighbour-Joining (NJ) methods. The closest matches for the isolate H2d were *Vibrio neocaledonicus* strains EP27-3-2 and KY457428.1, whereas for the isolate C2c, it was *Vibrio alginolyticus* strain SC9 MK308586.1. *Salinovibrio costicola* strain Pb-WC11147JX913853.1, *Vibrionaceae bacterium* PH25 AF513466.1, *Salinovibrio costicola* strain Pb-WC11129 JX913851.1, *Salinovibrio costicola* strain Pb-WC11018 JX913848.1 were the closest matches for the isolates C2a dan C2d (**Figure 4**). Using the NJ method, the cladogram forms 2 in-groups and 1 outgroup. The in-group is divided into 2 clades: Clade 1 consists of 18 species, and Clade 2 of 17 species. Species united in 1 clade have sequences that are close or similar. The NJ method is based on branch length. Isolates C2a and C2d have short branches, the same as isolates C2c and H2d. However, isolates C2a and C2d have long branches with isolates C2c and H2d, which indicate a long evolutionary process, and changes in nucleotide bases. The bootstrap results on the cladogram have a tree branch value of 100 for each clad. This value indicates a very low chance of changing the clade arrangement. Species identified from the 4 isolates were *Vibrionaceae bacterium* PH25 (99.86 %), *V. alginolyticus* strain GS MYPK1 (99.65 %), *Salinivibrio costicola* strain M318 (99.86 %), and *V. alginolyticus* strain 2014V-1011 (99.93 %) (**Table 6**).

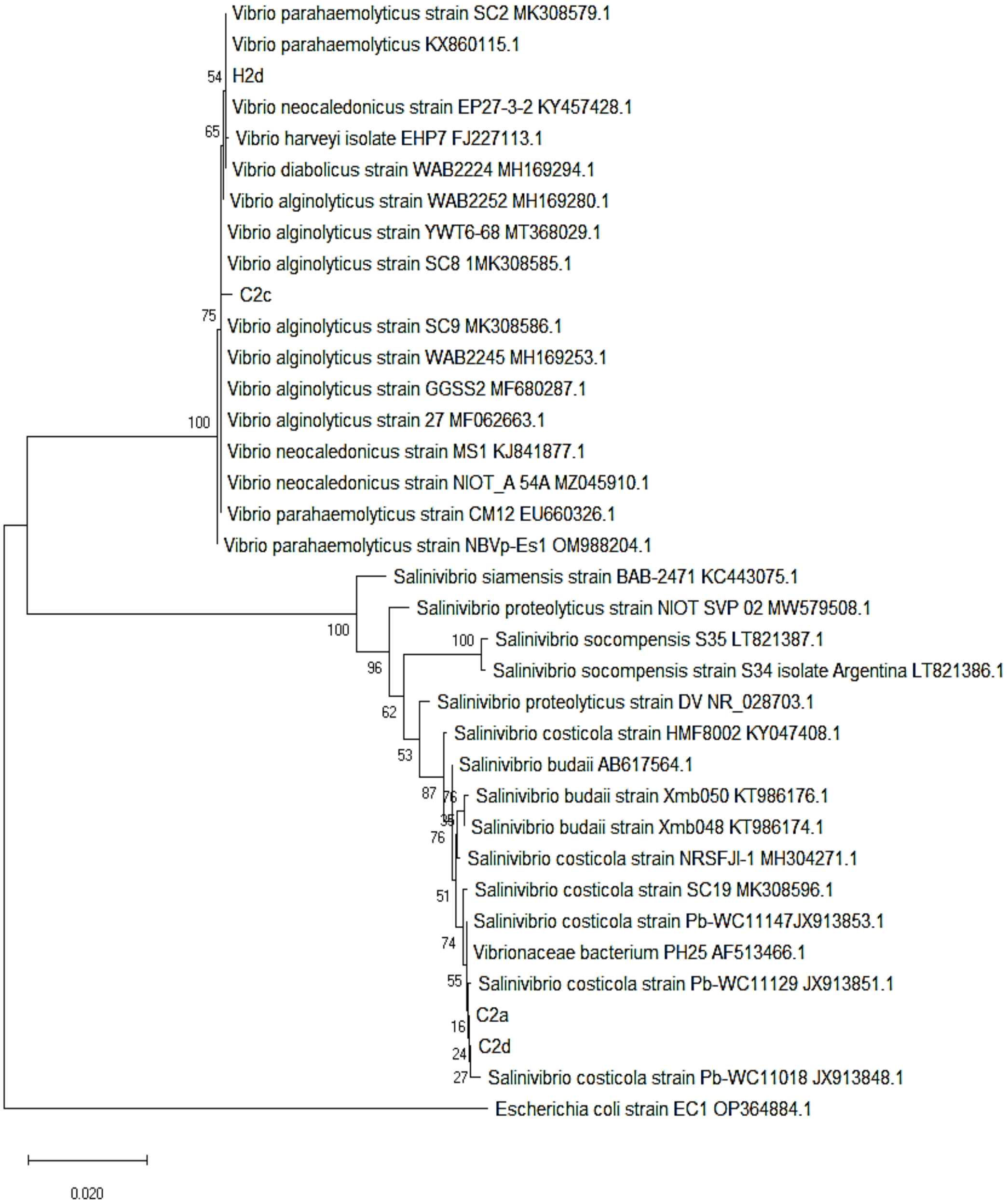

Figure 4 Phylogenic tree of the potential associated bacteria based on 16S rRNA gene sequence by Neighbour Joining (NJ) method. Isolates H2d, C2c, C2a, and C2d are the potential associated bacteria.

Table 6 Similarity of the 16s RNA (27F-1492R) gene sequences from the potential associated bacteria using BLAST-N at the NCBI.

Isolate code	Nearest species	% Similarity	Accession
C2a	*Vibrionaceae bacterium* PH25	99.86 %	AF513466.1
C2c	*Vibrio alginolyticus* strain GS MYPK1	99.65 %	CP054700.1
C2d	*Salinivibrio costicola* strain M318	99.86 %	CP050266.1
H2d	*Vibrio alginolyticus* strain 2014V-1011	99.93 %	CP046772.1

Discussion

Associated bacteria isolated from *C. racemosa*, *P. minor*, and *H. opuntia* showed good antibacterial activity against the test bacteria *P. acnes* and *S. epidermidis*. A total of 14 isolates had bactericidal activity, and 6 showed bacteriostatic activity. Bacteriostatic activity only inhibits the growth of bacteria, while bactericidal activity can kill bacteria. The isolates with bactericidal activity were marked with permanent inhibition zones, while 6 isolates with bacteriostatic activity were characterised by inhibition zones that disappeared over time. The bactericidal activity of associative bacterial isolates demonstrates possible employment in natural antibacterial products. This provides promising results, given that *P. acnes* and *S. epidermidis* are primarily found in people with dandruff [12]. Furthermore, results from antimicrobial testing suggest the possibility of producing powerful antibacterial medications to treat dandruff in humans.

A distinct correlation was found between the high antibacterial activity of associated bacterial isolates derived from *C. racemosa* seaweed. High dominance of *C. racemosa* seaweed was observed in Teluk Awur, Jepara, Indonesia, when sampling was done, as opposed to *P. minor*, and *H. opuntia*. The high dominance of *Caulerpa* sp. is considered unique as it is assumed that the presence of associated bacteria supports its dominance. According to Hollants *et al*. [3], bacteria associated with macroalgae can enhance their host's defence potential, morphogenic effects, growth-promoting effects, and photosynthetic activity. However, more research is required to confirm this unique association.

Identification of the 4 isolates indicated that they belonged to the genus *Vibrio sp.*, supporting the claim by Hollants *et al.* [3] that *Vibrio* sp. is the most prominent genus associated with brown, green, and red seaweed. Although the ecological relationships between *Vibrio* sp. and macroalgae are not fully understood, previous research has revealed that *Vibrio* sp. can have both beneficial and detrimental effects. *Vibrio* sp. exhibited adverse effects such as white rot disease and green spot rotting in macroalgae [29]. However, they have shown beneficial activity with macroalgae. According to Goecke *et al.* [11], the seaweed-associated *Vibrio* sp. is an efficient producer of chemicals that operate as antisettlement and QS inhibitors, protecting the seaweed's surface against diseases, herbivores, and fouling organisms. As a result, seaweed serves as a habitat that promotes survival of *Vibrio* sp. Conversely, *Vibrio* sp.'s ability to produce antibacterial compounds benefits seaweed as it increases its environmental survival by outgrowing parasites or other invading microorganisms, as the seaweed surface is a breeding ground for numerous organisms [30]. This would explain the interaction of the genus *Vibrio* sp. with macroalgae.

The isolate of the associated bacterium *V. alginolyticus* develops antibiotic substances against various bacterial and fungal infections. *V. alginolyticus* bacteria showed antibacterial activity against *E. coli*, *Staphylococcus aureus*, *Salmonella thypi*, MDR Bacillus cereus, MDR *E. coli*, Methicillin-Sensitive, *S. aureus* (MSSA), and Methicillin-Resistant *S. aureus* (MRSA) [31-33]. This demonstrates that the isolates of *V. alginolyticus* can produce effective antibiotic compounds. Furthermore, in addition to antibacterial activity, *Vibrio* sp. display antifungal action against *Candida albicans* and *Malassezia furfur* [34].

The antibacterial activity of the associated bacterium isolates against *S. epidermidis* was consistent with previous studies in which seaweed was harvested from Jepara. According to Siregar *et al.* [24], the crude extract of the *C. racemosa* seaweed had antibacterial properties against *S. epidermidis*. Additionally, Kaaria *et al.* [35] suggest that microbes associated with seaweed can create metabolites that act as antibacterials similar to their hosts and associated bacteria can produce the same secondary metabolites [36].

Conclusions

Bacteria associated with *C. racemosa*, *P. minor*, and *H. opuntia* can show strong antibacterial properties capable of mitigating the growth of *P. acnes* F2 ATCC 6919 and *S. epidermidis* FNCC-0048. As these are the major bacteria species on the scalps of those suffering from dandruff, we believe these isolates may be useful for dandruff therapy.

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