

Antibacterial Activity and Salinity Tolerance of Endophytic Bacteria Isolated from Marine Macroalgae

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Abstract. Marine organisms, especially macroalgae, are unique microhabitats for endophytic bacteria that have the potential to provide unique microhabitats for endophytic bacteria that can produce bioactive compounds with antimicrobial activity. However, the exploration of endophytic bacteria from marine macroalgae, especially those directly associated with host tissues, remains relatively limited. This study aims to isolate endophytic bacteria from various macroalgae and explore the antibacterial potential and salinity resistance of these endophytic isolates. This study aims to identify the potential isolates that produce antibacterial compounds at the molecular level. The antibacterial activity was qualitatively evaluated using the Kirby–Bauer disk diffusion method. The salinity tolerance of the bacterial isolates was assessed by growing them on media with varying NaCl concentrations. Molecular identification of the isolates was performed through 16S rRNA gene amplification and sequence analysis. The results showed that isolate H3 had the highest antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*, with inhibition zones of 28.9 ± 2.3 mm and 26.2 ± 4.36 mm, respectively. Isolate R1A showed the highest inhibitory activity on *S. aureus* and *Escherichia coli*, with inhibition zones of 25 ± 1.08 mm and 35 ± 2.9 mm. Salinity resistance test results showed that isolate R1A was able to grow well at a 10% (w/v) NaCl concentration. Molecular identification results with 16S rRNA gene analysis showed that isolate H3 has similarities with the species *Lysinibacillus fusiformis*, isolate R1A has similarities with *Bacillus mojavensis*, and R2C has similarities with *Bacillus stercosis*. This study suggests that endophytic bacteria from macroalgae represent promising and sustainable sources of novel antibacterial agents for pathogen control in high-salinity aquaculture systems. This study aligns with SDG 3 and SDG 14 by supporting human health initiatives and the sustainable utilization of marine biodiversity.

Keywords: endophytes; macroalgae; antibacterial; salinity

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INTRODUCTION

The ocean makes up two-thirds of the Earth, and with such a large area, the biodiversity within it is certainly more varied than life on land. Compared to the terrestrial environment, research on marine bioresources remains limited, particularly in physical, chemical, and biological research (Almaary et al., 2021). The large number of marine microbes that have not been fully studied offers opportunities to discover new bioactive compounds (Adnan et al., 2018). The bioactive chemical diversity of marine microbes is a beneficial source of antimicrobial compounds that can be applied in medicine, industry, and other biotechnology products (Shaaban, 2022).

Marine antimicrobial compounds are uniquely novel compared to compounds from terrestrial organisms (Beygmoradi & Homaei, 2017). Previous studies have identified bacteria originating from marine environments, such as the rhizosphere of mangrove plants (Ryandini et al., 2018) and bacteria associated with sponges (Santosa et al., 2023) that have the potential to produce antimicrobial substances (Rhimou et al., 2010).

Macroalgae play a crucial role as primary producers in marine ecosystems, supporting the diversity of aquatic organisms, including endophytic bacteria associated with macroalgae (Carlos et al., 2022). Active compound extracts from macroalgae are known as sources of

antimicrobial secondary metabolites such as phenolic compounds, flavonoids, alkaloids, and terpenoids that effectively inhibit the growth of pathogenic bacteria (Michalak & Chojnacka, 2014). Several macroalgae genera, such as *Chondracanthus*, *Caulerpa*, and *Ulva*, have been shown to have antibacterial activity against pathogenic bacteria *S. aureus*, *Streptococcus pyogenes*, *L. monocitogenes*, *Salmonella enterica*, *E. faecalis*, and *P. aeruginosa* (Rhimou et al., 2010); (Munoz-Ochoa et al., 2010); (Maria et al., 2023). The presence of endophytic bacteria in macroalgae tissue is also thought to be capable of synthesizing secondary metabolites similar to those produced by the macroalgae itself. The endophytic bacterium *Catenococcus thiocycli*, which originates from *Cystoeira myrica*, is equally capable of producing metabolites such as phenols, flavonoids, tannins, and saponins, and both species have the same bioactive activity as antioxidants, anti-inflammatories, and antibacterials (Hagaggi & Abdul-Raouf, 2022). This indicates that the presence of endophytic bacteria within macroalgae tissue has the same bioactive activity as the host plant.

Research on the potential of algal endophytic bacteria is being intensively carried out, given the limited knowledge about it. Research (Li et al., 2024) indicates that the endophytic bacteria in *Sargassum thunbergii* encompass various bacterial groups with the potential for nitrogen fixation, salt tolerance, pollutant degradation, antibacterial properties, and even pathogenicity. Various studies summarized by (Boonman et al., 2023) concluded that endophytic bacteria from the genus *Graciliaria* can effectively inhibit *Staphylococcus aureus*, *Streptococcus faecalis*, and *Escherichia coli*, but are not strong enough against *Enterobacter aerogenes*. Endophytic bacteria isolated from several macroalgae species in Peruvian waters exhibit antibacterial activity against several pathogenic bacteria (*Enterococcus faecalis*, *Staphylococcus epidermidis*, *S. aureus*, *E. coli*, *Salmonella enteric sv typhimurium*) and pathogenic fungi (*Candida albicans*, *C. tropicalis*, *Colletotrichum* sp., *Fusarium* sp., *Fusarium oxysporum*, and *Alternaria* sp.) (Vega-Portalatino et al., 2024).

Pathogenic microorganisms that cause infections in animals, humans, and plants have developed resistance to antimicrobial substances. Therefore, research on the search for antimicrobial compounds from various habitats has been conducted extensively, particularly in macroalgae endophytes. Isolating endophytic bacteria from

macroalgae that produce antimicrobial compounds is a more sustainable approach than direct extraction from host plants. Through culture and fermentation, bioactive compounds can be produced efficiently, in a controlled manner, and with minimal ecological impact on coastal ecosystems (Semenzato & Fani, 2023). However, studies that specifically explore the antibacterial potential of endophytic bacteria that live in macroalgae tissue are still relatively limited. This study aims to determine the potential of endophytic bacteria from macroalgae that have antibacterial activity and to characterize potential isolates molecularly. The findings provide insights into the ecological and functional relationships between macroalgae and their endophytic bacteria. Understanding these symbiotic interactions enhances scientific knowledge of microbial ecology, particularly how endophytes contribute to host defense mechanisms in marine environments.

METHODS

Macroalgae Sampling and Isolation of Algal Endophytic Bacteria

Samples of macroalgae *Hypnea* sp., *Chaetomorpha linum*, and *Rhizoclonium riparium* were taken from the coast of Kukup Beach, Gunung Kidul, Yogyakarta (-8.133605285714056, 110.55476958500039). Macroalgae were taken together with 70% ethanol. The macroalgae were then cut into smaller pieces and placed on Mueller-Hinton Agar media. After incubating for 48 hours at 37°C, colonies growing around the macroalgae tissue were purified onto fresh Mueller-Hinton (MH) medium.

Antibacterial Activity Test with Pour Plate Method

The antibacterial activity test was carried out using the Kirby-Bauer method with the pour plate technique. A total of 100 µl of liquid culture of test bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*) with a concentration of 10⁷ CFU/ml was mixed with 100 mL of sterile Mueller-Hinton (MH) Agar medium and then poured into a petri dish. Macroalgae endophytic bacterial isolates were grown on 10 mL of liquid MH medium and incubated for 24 hours at 37°C. Discs were dipped into the liquid culture when the cell count reached 10⁷ CFU/ml. Then, the disc was placed on MH agar medium that had been mixed with the test bacteria in four quadrants and

incubated for 24 hours at 37°C. Antibacterial activity can be seen by measuring the clear zone area that appears around the disc (Hudzicki, 2012).

Salinity Resistance Test

Salinity resistance was tested by modifying the composition of NaCl in Luria-Bertani (LB) Agar medium. Isolates were grown in LB agar with various salinity concentrations: 0%, 1%, 2.5%, 5%, 10%, 20%, and 50%. Isolates of macroalgae endophytic bacteria were inoculated on LB-NaCl medium and incubated for 24 hours at 37°C. Isolate growth on agar medium was observed qualitatively (Sharma et al., 2021).

Morphological Observations of Bacterial Colonies

Purified bacteria were identified morphologically by referring to characters based on colony size, pigment, elevation, margin, and texture (Breakwell et al., 2007). Morphological observations were made on isolates grown on LB agar medium.

Molecular Identification Using 16S rRNA Gene

The most promising isolates as producers of antibacterial compounds were identified molecularly to determine the species of these isolates. Molecular identification using 16S rRNA gene analysis. Bacterial DNA was isolated using a special bacterial isolation tool on the total genome that appears with the procedure according to the instructions listed in the manufacturer's instructions. PCR was performed using primers 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWTGTACAAGGC-3') for amplification of the 16S rRNA consensus gene of 1.300 bp (Marchesi et al., 1998). The PCR reaction was carried out with a volume of 40 µL composed of 20 µL DNA polymerase MyTaq™ HS Red Mix (Bioline), 2 µL of each primer, 12 µL nuclease-free water (NFW), and 2 µL DNA template. The PCR steps include pre-denaturation (95 °C, 4 minutes), denaturation (95 °C, 30 seconds), annealing (57 °C, 30 seconds), elongation (72 °C, 1 minute), and post-elongation

(72 °C, 7 minutes) for 30 cycles. The initial sequencing data results were trimmed and assembled using the BioEdit program and SeqTrace 0.9, then further analyzed using the BLAST tool from the National Center for Biotechnology Information (NCBI) (Fatmawati et al., 2019).

RESULT AND DISCUSSION

Endophytic Bacteria Isolated from Various Types of Macroalgae

Several species of macroalgae were sampled for endophytic bacteria. Based on the results of literature studies and their characteristics, the algae isolated in this study are *Hypnea* sp., *C. linum*, and *R. riparium*. as shown in Figure 1. After the antibacterial activity test, it was found that the results varied in each isolate. Figure 2 shows examples of antibacterial activity results on several isolate samples.

In this study, the algae isolated were *Hypnea* sp., *C. linum*, and *R. riparium*. Unlike the other two species that belong to Chlorophyta (green algae), *Hypnea* sp. is a member of Rhodophyta (red algae). The thallus of this alga can be membranous or cartilaginous, with a consistency that varies from soft to hard (Arianti et al., 2024). *C. linum* is characterized by its thallus in the form of strands, such as long hair, and is often found in twisted or clumped conditions (Ghazali et al., 2018). In *Hypnea* sp., there were 2 potential isolates were successfully isolated. In *C. linum*, there is only one potential isolate. Meanwhile, there are three potential isolates successfully isolated in *R. riparium*.

Antibacterial Activity Assay from Macroalga Endophytic Bacteria

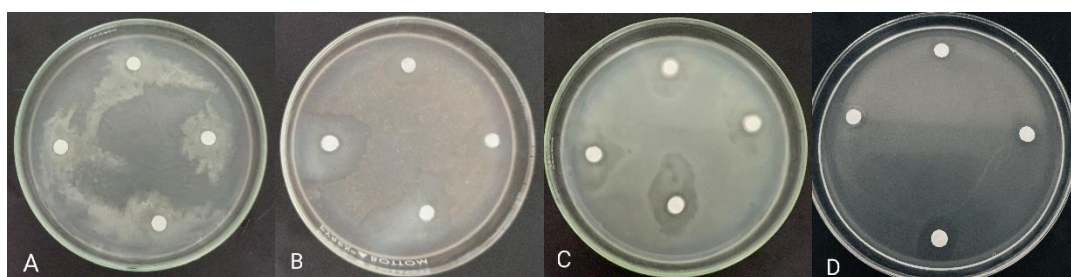
The six isolates have diverse antibacterial potential as listed in Table 1. The wider the clear zone produced, the greater the potential antibacterial activity of the isolate. Broadly speaking, the most potent isolate against the three test bacteria is isolate R1A. Meanwhile, when compared to other isolates, the least potent isolate against the three is isolate C3C.



Figure 1. Macroalgae species identified. Left to right: (A) *Hypnea* sp., (B) *C. linum*, and (C) *R. riparium*

Table 1. Antibacterial activity of endophytic bacteria from marine macroalgae using pour plate methods

No	Macroalgae Species	Isolate	Clear Zone Inhibition (mm)		
			<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>
1	<i>Hypnea sp.</i>	H2	21.45 ± 3.83	15.29 ± 5.63	14.66 ± 1.8
2	<i>Hypnea sp.</i>	H3	28.9 ± 2.3	26.2 ± 4.36	22.72 ± 6.82
3	<i>C. linum</i>	C3C	8.12 ± 2.4	7.74 ± 1.09	6.62 ± 1.65
4	<i>R. riparium</i>	R1A	18.23 ± 3.06	25.27 ± 1.08	35.1 ± 2.9
5	<i>R. riparium</i>	R1C	21.57 ± 1.37	22.92 ± 2.28	25.72 ± 1.43
6	<i>R. riparium</i>	R2C	13.23 ± 3.49	10.38 ± 2.49	12.16 ± 1.9


Figure 2. Antibacterial activity test results on isolates. Left to right: (A) isolate R1A against *S. aureus*, (B) isolate H3 against *E. coli*, (C), isolate H2 against *B. subtilis*, and (D) isolate R2C against *S. aureus*.

Based on Figure 2, a large, clear zone area is an indicator of the ability of a microbe to inhibit the growth of other bacteria. *R. riparium* algal endophytic bacteria have the highest antibacterial potential when compared to other algal endophytic bacteria. Endophytic bacteria from *Hypnea sp.* (H3) have antibacterial properties that are almost identical to those of endophytic isolates from *R. riparium* (R1C) against the test bacteria *B. subtilis*, *S. aureus*, and *E. coli*. Meanwhile, isolates from *C. linum* endophytes showed the weakest antibacterial activity against the three test bacteria. Differences in the ability of endophytic bacterial isolates to inhibit pathogenic bacteria *in vitro* may be due to several factors, including the ability of bacteria to colonize within the host (Hnamte et al., 2024), environmental factors (temperature,

humidity, nutrient availability, host type) (Digra & Nonzom, 2023), and the type of interaction between algae and endophytic bacteria in producing active compounds (Goecke et al., 2010).

Salinity Tolerance Assay of Endophytic Bacterial Isolates from Macroalgae

The isolate with the highest salinity resistance is R1A, which can reach 10% salt content. Isolates H2, H3, and R2C have salinity resistance below R1A, which is 5% salt content. In isolate H, it was found that both endophytes had the same salinity resistance. However, in isolate R, although derived from the same algae, the three isolates have different salinity resistance, as listed in Table 2.

Table 2. Salinity tolerance of endophytic bacteria from marine macroalgae in Luria Bertani Agar supplemented with sodium chloride

No	Isolate	Sodium Chloride Concentration (%)						
		0	1	2.5	5	10	20	50
1	H2	++	++	+	+	-	-	-
2	H3	++	++	+	+	-	-	-
3	C3C	++	++	-	-	-	-	-
4	R1A	++	++	++	++	+	-	-
5	R1C	+	+	-	-	-	-	-
6	R2C	++	++	+	+	-	-	-

Based on Table 2, different patterns of salinity tolerance were observed among the isolates. Isolate R1A showed the highest tolerance, maintaining growth up to 10% NaCl, but failed to grow at 20%. Meanwhile, isolates H2, H3, and R2C tolerated up to 5% NaCl (++)/(+) but failed at 10%. Isolates C3C and R1C showed the lowest tolerance (only growing at 0–1% and disappearing at 2.5% or higher). This pattern indicates physiological/biochemical variations between isolates regarding halotolerance mechanisms (Abdelrazek et al., 2024). Salt-tolerant endophytic bacteria (e.g., R1A, H2/H3/R2C) have the potential to function as bioinoculants to enhance macroalgae resistance to salinity fluctuations in marine aquaculture (e.g., tidal areas or intensive ponds subject to evaporation). Additionally, halotolerant bacteria can contribute to nutrient cycling (mineralization of organic matter, production of vitamins or enzymes) that aids in the recovery of host physiology under high salinity conditions (Saha et al., 2023). Microorganisms, such as bacteria, archaea, and fungi, that grow optimally at high salinity are capable of producing pharmaceutically interesting biomolecules for therapeutic applications. The existence of this potential can be used to overcome recent cases of increasing antibiotic resistance (Corral et al., 2020). Halophiles can be used in pharmaceuticals, drug delivery, agriculture, saline wastewater treatment, biodegradable plastic production, metal recovery, and biofuel energy generation (Dutta & Bandopadhyay, 2022).

Morphological Characterization of Endophytic Bacterial Isolates from Macroalgae

The characteristics of all isolates are more or less the same. Characteristics that vary only in terms of size. Colonies of isolates derived from specimen R1C, R2C, and R1A have a moderate

size. Isolates C3C and H2 have a small colony size, while isolate H3 has a large colony size (Table 3.)

The morphological characterization of endophytic bacterial isolates from marine macroalgae revealed a high degree of similarity among all isolates, with variations observed primarily in colony size (Table 3). Most isolates (R1C, R2C, and R1A) exhibited moderate colony size, while C3C and H2 showed smaller colonies and H3 formed a larger colony. Pigmentation (nonpigmented), elevation (flat), border (entire), and texture (mucoid) all showed consistent characteristics. Endophytic bacteria are frequently found to exhibit such homogeneity in colony shape (Li et al., 2024). It raises the possibility of functional convergence resulting from adaptation to the interior macroalgal environment, where host compatibility and persistent colonization features are favored over morphological variation due to selective pressures (Afzal et al., 2019).

In particular, a mucoid texture is frequently linked to the generation of extracellular polysaccharides (EPS), which are essential for biofilm formation, host adhesion, and defense against environmental stresses, such as salt and host-produced antimicrobial chemicals (Ibrahim et al., 2022). Instead of taxonomic divergence, variations in colony size may indicate variations in isolates' growth rates, metabolic activities, or physiological adaptations (Zhu et al., 2025). Crucially, endophytic bacteria with similar phenotypes may have different biosynthetic gene clusters that produce secondary metabolites, similar colony morphologies do not always imply the same antibacterial capability (Drozdynski et al., 2024). In order to completely clarify the functional variety and pharmacological potential of macroalgal endophytic bacteria, morphological characterisation is a necessary first step that must be supplemented by biochemical, molecular, and antibacterial experiments.

Table 3. Morphological colony features of endophytic bacteria from marine macroalgae

Feature	Endophytic bacteria isolate					
	R1C	R2C	R1A	C3C	H2	H3
Size	moderate	moderate	moderate	small	small	large
Pigmen	nonpigmen	nonpigmen	nonpigmen	nonpigmen	nonpigmen	nonpigmen
Elevation	flat	flat	flat	flat	flat	flat
Margin	entire	entire	entire	entire	entire	entire
Texture	mucoid	mucoid	mucoid	mucoid	mucoid	mucoid

Molecular identification of 16S rRNA genes of endophytic bacterial isolates from macroalgae

PCR results indicate that isolate R1A has a strong likelihood of belonging to the *Bacillus mojavensis* species, and isolate R1C belongs to the *Bacillus stercoris* species. Generally, *B. mojavensis* can be found as a soil bacteria or plant endophytic bacteria (Jasim et al., 2016), (Youcef-Ali et al., 2014). The antagonistic test results showed inhibition of the three test bacteria, *B. subtilis*, *S. aureus*, and *E. coli*, by isolate R1A. This is in line with the results of a study (Jasim et al., 2016) that isolated *B. mojavensis* from the traditional medicinal plant *Bacopa monnieri* (L), which showed broad-spectrum antimicrobial activity against both Gram-positive and gram-negative bacteria, as well as antagonism against several pathogenic fungi. *B. mojavensis* strain A21, originating from the marine environment, has been proven capable of producing biosurfactant lipopeptides that can be utilized in the medical, food, and biotechnology industries (Ayed et al., 2014) as well as for the bioremediation of soil contaminated by petroleum hydrocarbon waste and heavy metals (Singh & Cameotra, 2013). *B. mojavensis* produces the active compound mojavensin, which has broad-spectrum antifungal activity and is cytotoxic to leukemia (HL-60) cell lines (Ma et al., 2012).

The results of molecular identification of the 16S rRNA gene in isolate R1C showed similarities with *Bacillus stercoris*. In this study, *B. stercoris* was isolated from the endophyte *R. riparium*. The same species was also found as an endophyte in the alga *C. atenina*, and this bacterium also exhibits antibacterial activity against *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Das et al., 2025). *B. stercoris* was also found in estuarine habitats and also has antibacterial activity against the genera *Vibrio* and *Aeromonas* (Nair et al., 2021). Another strain of *B. stercoris* has been shown to have antifungal activity against plant pathogenic fungi such as *Magnaporthe oryzae* (Wang et al., 2021). Based on these studies, *B. stercoris* has the potential to be developed as a biocontrol agent in both aquatic

and terrestrial ecosystems.

Isolate H3 isolated from *Hypnea* sp. tissue was found to be similar to *Lysinibacillus fusiformis*. In this study, H3 also showed good antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli*. Previous research reported that *L. fusiformis* isolated from marine sponges exhibited strong antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *E. coli*, and *Salmonella typhimurium* (Mamangkey et al., 2025). *L. fusiformis* NR_042072.1 produces bacteriocins that very strongly inhibit the growth of *E. coli* and *Pseudomonas aeruginosa* (Adedayo & Abdulkareem, 2024). In addition to producing antibacterial compounds, *L. fusiformis* has also been reported to produce acetic acid, which can stimulate jasmonic acid (JA) signaling and inhibit Cd uptake in tomato roots as a form of plant defense against environmental stress caused by heavy metal contamination (Zhu et al., 2021). Furthermore, *L. fusiformis* is also utilized as a PGPB in plants to make them resistant to extreme salinity stress (Ahsan & Shimizu, 2021).

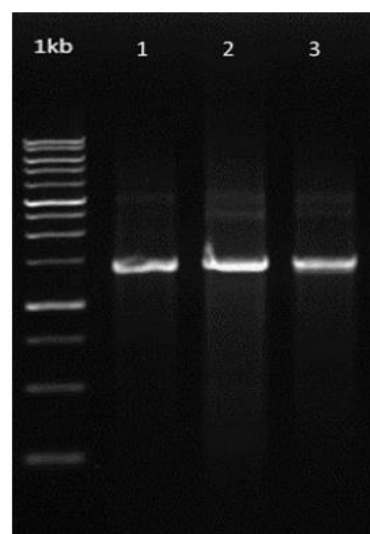


Figure 2. 16S rRNA gene electrophoresis results on agarose gel with a DNA size of 1,300 bp. Lane 1 is the H3 isolate, lane 2 is the R1A isolate, and lane 3 is the R1C isolate.

Table 4. BLAST results of isolate identification from GenBank

Isolates	Species Affiliation (GenBank)	Query Cover (%)	E Value	Similarity (%)	Accession Number
H3	<i>Lysinibacillus fusiformis</i>	67	0.0	83.09	NR_112628.1
R1A	<i>Bacillus mojavensis</i>	100	0.0	98.73	NR_118290.1
R1C	<i>Bacillus stercoris</i>	100	0.0	98.15	NR_181952.1

The novelty of the research findings, is the isolation of strain R1A (*B. mojavensis*), which not only exhibits strong antibacterial activity against both Gram-negative (*S. aureus* and *B. subtilis*) and Gram-positive (*E. coli*) bacteria but is also capable of growing well at high salt concentrations (10% NaCl). This study offers a new approach to the exploration of antibacterial candidates from macroalgal endophytes in a more sustainable manner. The halotolerant nature of these endophytic bacteria makes them potential candidates for development as antimicrobial agents in aquatic environments with high salt concentrations. . This study supports SDG 3 (Good Health and Well-Being) by contributing to the search for novel antibacterial agents derived from marine microorganisms and SDG 14 (Life Below Water) by promoting the sustainable utilization of marine biodiversity.

CONCLUSION

Several endophytic bacteria have been successfully isolated from marine macroalgae, *Hypnea* sp., *C. linum*, and *R. riparium*. Six isolates showed different antibacterial activity towards *Bacillus subtilis*, *E. coli*, and *Staphylococcus aureus*. All six isolates have good salinity resistance. Three isolates (H3, R1A, and R2C) were analyzed by PCR, which showed that the isolates were related or belonged to the species *L. fusiformis*, *B. mojavensis*, and *B. stercoris*. *B. mojavensis* (R1A) is an isolate that exhibits the strongest activity against both Gram-positive and Gram-negative test bacteria; it is also capable of growing under high salinity conditions (10% NaCl). Although this study successfully demonstrated the antibacterial activity and salinity tolerance of macroalgae-associated endophytic bacteria, several aspects remain unexplored and warrant further investigation, especially for the isolation and identification of bioactive compounds. Halophilic endophytic bacteria can be developed for pathogen biocontrol in aquaculture systems. The existence of these various potentials can be utilized as much as possible for the sustainability of human life and the surrounding nature.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

UF designed the research, collected data, and wrote the manuscript. MIF collected and analyzed the data, and wrote the manuscript. MIP helped analyze the data and wrote the manuscript.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

All authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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