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Secondary Metabolites from Symbiotic Bacteria of Seagrass and Molluscs as A Reference for Natural Food Preservatives

Delianis Pringgenies*, Nirwani Soenardjo, Wilis Ari Setyati and Rini Pramesti

Department of Marine Science, Faculty of Fisheries and Marine Science, Diponegoro University, Jawa Tengah 50275, Indonesia

(*Corresponding author's e-mail: delianispringgenies@lecturer.undip.ac.id)

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Abstract

Symbiotic bacteria from seagrass and molluscs have the potential to be a source of secondary metabolites. Compounds from these secondary metabolites, such as natural food preservatives, can be used in the bioindustry sector. This research aims to identify the potential secondary metabolites from symbiotic bacteria in molluscs and seagrass that can support the Sustainable Development Goals (SDG) public health program. The samples used in this study were symbiotic bacteria of mollusc from seagrass environments, symbiotic bacteria from seagrass, and seagrass samples. The seagrass samples were analysed phytochemically, while the symbiotic bacteria samples from molluscs and seagrass were tested for their antibacterial activity against food spoilage pathogens such as *Staphylococcus aureus* and *Escherichia coli* using the disc diffusion method. Bacteria that showed potential antibacterial activity were identified using molecular methods, and the content of active compounds was analysed using gas chromatography-mass spectrometry. The phytochemical analysis revealed that seagrass contains flavones, tannins, steroids, and compounds with potential antibacterial properties. GC-MS analysis indicated that seagrass is predominantly composed of unsaturated fatty acids, which have antimicrobial effects by damaging bacterial cell membranes and disrupting the function of enzymes essential for growth and reproduction. Ten symbiotic bacteria isolates demonstrated proteolytic, cellulolytic, and lipolytic enzyme activity, suggesting their potential as antibacterial agents due to their ability to break down cell walls. Molecular identification of selected bacteria revealed the presence of mollusc symbionts, including *Vibrio owensii*, *Bacillus paramycoides*, and *Pseudoalteromonas flavipulchra*. The symbiotic bacteria found in seagrass in this study included *Bacillus velezensis*, *Bacillus paramycoides*, *Nocardiopsis alba*, *Nocardiopsis alba*, *Bacillus tropicus*, *Lysinibacillus fusiformis*, and *Bacillus tropicus*. GC-MS analysis of selected symbiotic bacteria from seagrass showed that the dominant compound components were phthalic acid, bis(7-methyl octyl) ester (25.18, 43.84, 35.55 %), and trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone (36.36, 47.71, 64.48 %). In conclusion, seagrass and its symbiotic bacteria, as well as symbiotic bacteria from molluscs living in seagrass, have the potential to serve as antibacterial agents and can be used as references for food preservatives.

Keywords: Antibacterial, Enzymatic, Natural food preservative, Seagrass, Symbiotic bacteria

Introduction

Studies on marine environments have focused chiefly on mangrove and coral ecosystems. However, seagrass ecosystems are also coastal environments with great potential and produce secondary metabolites with antimicrobial and other important properties, making them suitable for food preservation. These secondary metabolites have been found to inhibit the growth of various pathogenic bacteria, thus extending the shelf life of foods that are not widely known. Seagrass plants require sunlight for photosynthesis, so they are primarily found in shallow waters. Despite this, seagrass can be found along most of Indonesia's coastlines. It offers many environmental and health benefits [1], such as increasing oxygen levels and serving as a food source for marine organisms such as turtles, molluscs, and fish [2]. Additionally, seagrass acts as a sediment trap, positively affecting water quality. It can also be used by communities for weaving, such as making baskets, as roofing material, and even for composting purposes. This research explores the potential of secondary metabolites derived from seagrasses, symbiotic bacteria of seagrasses, and molluscs living in seagrasses that have great potential as natural preservatives to support the Sustainable Development Goals (SDGs) and public health programs. Seagrasses are a group of high-level flowering plants (Angiospermae) that have adapted to live submerged in shallow seawater with sand and mud.

The ecological function of seagrass beds is to serve as nursery areas, spawning grounds, foraging areas, and shelter areas for various marine organisms such as fish, crustaceans, molluscs, and echinoderms. Seagrass and the symbiotic bacteria living in seagrass have been proven to have active substances with antibacterial and antifungal properties [3,4]. They can even be potentially used as antibacterial agents against multidrug-resistant (MDR) pathogens [5]. Research has shown the presence of various molluscs in seagrass [6]. Furthermore, it has been discovered that protease enzymes, which are helpful in the food industry, are found in bacteria associated with sponges in seagrass [7].

These findings have prompted researchers to conduct further studies to obtain secondary metabolites from seagrass symbiotic bacteria and test the potential of secondary metabolites from seagrass symbiotic bacteria as reference natural preservatives.

The results of this research have promising implications for the health field, such as being used as natural preservatives and in the industrial sector. It is noteworthy that seagrass holds potential as a source of secondary metabolites for natural therapy, which could be utilized to prevent or inhibit human diseases [8]. Furthermore, the compounds found in symbionts are related to the compounds found in their hosts [9], suggesting that the compounds found in molluscs in seagrass are similar to the compounds from their symbiont bacteria and have potential health benefits. Targeting microorganisms will also help maintain environmental conservation by not damaging the flora and fauna.

The biological compounds found in seagrass include 4 main classes: Steroids, fatty acids, terpenes, and (mainly) polyphenols, including flavonoids, catechins, chalcones, and phenyl Mari [8]. These bioactive compounds are highly beneficial to human health. Symbiotic bacteria in molluscs living in seagrass also have promising prospects. Active substances from the secondary metabolites of symbiotic bacteria from various molluscs have shown antibiotic [10], antifungal, antiviral [11], and antioxidant properties [12]. Selected bacteria can be further developed into bacterial consortia for use as natural food preservatives. This study aims to identify seagrass species, determine the potential of symbiotic bacteria that produce antibacterial compounds and demonstrate enzymatic activity, and determine the symbiotic bacteria of molluscs as the producers of these antibacterial compounds and the enzymatic activity. Furthermore, the study aims to determine which bacteria can produce antibacterial and enzymatic compounds and also determine the contents of these compounds using chromatography.

Materials and methods

Research sites

The research was conducted from February to June 2021, and 3 types of samples were collected: Seagrass samples, seagrass bacteria isolation, and mollusc bacteria isolation. The mollusc samples were obtained from the seagrass ecosystem in the waters of Bangka, Indonesia. Meanwhile, the seagrass samples were obtained from the seagrass ecosystem in the waters of Jepara, Indonesia. Bangka waters and Jepara waters are still influenced by the Java Sea monsoon current system and the Karimata Strait. During the West Monsoon, currents move from the Karimata Strait towards the Java Sea. And during the East Monsoon, the current moves in the opposite direction from the Java Sea towards the Karimata Strait [13].The collected mollusc and seagrass samples were placed in plastic Ziplock bags and in an icebox. Seagrass sample used was *Cymodocea serrulata* and mollusc (*Rochia maxima*, Gastropod) samples live in a seagrass environment.

Sample preparation

The seagrass was cleaned, removing adhering dirt, and air dried, protected from sunlight, until completely dry, and blended into powder. Subsequently, a seagrass sample was extracted using maceration with n-hexane, ethyl acetate, and methanol solvents (1:5 w/v) (grammes of solute present in a volume of liquid solvent) each for 24 h [14]. The extraction results were tested using thin layer chromatography (TLC) [15] and phytochemical analysis. Furthermore, seagrass extract samples were tested for seagrass using gas chromatography-mass spectrometry (GC-MS) [15].

Symbiont bacterial isolation

Bacterial symbiont isolation was conducted separately on the collection organs of molluscs and seagrass samples. Bacterial purification was performed using the streak method until pure colonies were obtained. Macroscopic morphology observations included several characteristics such as texture, elevation, colony edges, colour, and colony shape [16].

Enzymatic quantitative test

Enzymatic testing was conducted on symbiotic bacteria found in seagrass and mollusc symbionts to assess their ability to degrade polysaccharide compounds. The activity of protease, amylase, and cellulase enzymes was measured using this approach. A protease enzyme activity test was conducted with the results of liquid culture isolates obtained from isolation, and the activity would be indicated by a clear zone around the paper disc with a white background. The activity test for amylase enzyme production was carried out on Zobell 2216 E medium supplemented with starch (1 %) and 1 % iodine. Lugol's solution was added on top of the culture, and if there was amylase enzyme activity, a clear zone formed around the paper disc with a dark blue background. A cellulase assay was performed on an agar medium supplemented with CMC and Congo Red solution added to the culture. The results indicated clear zones around the paper disc with a pink background [17]. Furthermore, the symbiotic bacteria isolated from seagrass and molluscs were tested against the pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* [18].

DNA extraction, PCR, and sequencing

Isolation of DNA from symbiotic bacterial isolates was performed using the Presto™ Mini gDNA Bacteria Kit (Geneaid Ltd., Taiwan). A total of 1.5 mL Zobell Broth containing the isolated culture (1×10^9 cells/mL) was transferred to a 1.5 mL microtube and centrifuged for 1 min at a speed of 14,000 - 16,000 rpm. The supernatant was discarded, leaving the pellet in the microtube. Buffer GT (200 µL) and lysozyme (0.8 mg/mL) were added to the microtube containing the pellet and homogenized using a vortex. Proteinase K (20 µL) was added, and the solution was incubated for 10 min at 60 °C The isolation of DNA from symbiotic bacterial isolates was conducted using the Presto™ Mini gDNA Bacteria Kit (Geneaid Ltd., Taiwan). First, a total of 1.5 mL of Zobell Broth containing the isolated culture, with a concentration of 1×10^9 cells/mL, was transferred to a 1.5 mL microtube. The microtube was then centrifuged at a speed of 14,000 - 16,000 rpm for 1 min. After centrifugation, the supernatant was discarded, leaving only the pellet in the microtube.

Next, 200 µL of Buffer GT and lysozyme at a concentration of 0.8 mg/mL were added to the microtube containing the pellet. The contents were mixed thoroughly using a vortex. Then, 20 µL of Proteinase K was added to the solution, and the mixture was incubated at 60 °C for 10 min [14].

Identify bacteria and analyse their active compounds

The cell lysis process involves the addition of GT buffer (200 µL) to the sample, followed by 10 s of cell homogenization and incubation at 70 °C for 10 min. The cell binding process involves the addition of absolute ethanol (200 µL), followed by homogenization. The GD column is transferred to a 2 mL microtube and rotated at a speed of 14,000 - 16,000 rpm for 2 min, and the supernatant is discarded. Then, the GD column is transferred to a new microtube. The washing process involves the addition of W1 buffer (400 µL) and centrifugation for 3 min, after which the supernatant is discarded. Wash buffer (600 µL) and ethanol mixture are added, and the solution is centrifuged at a speed of 14,000 - 16,000 rpm for 3 min, and the supernatant is discarded. The GD column is transferred to a new 2 mL tube, and elution buffer (30 µL) is added (heated to 70 °C). The solution is incubated at room temperature (27 - 29 °C) for 3 - 5 min and centrifuged at a speed of 14,000 - 16,000 rpm for 1 min. The microtube containing DNA is stored at −20 °C.

One microliter genomic DNA sample (template) is inserted into a PCR test tube that has been filled with PCR cocktail. After all the reagents are mixed in the PCR tube, the solution is homogenized using a vortex. The PCR process is carried out using a Thermal Cycle machine with the appropriate settings. The electrophoresis method used refers to the study [19], where an agarose gel is prepared first. The agarose gel concentration for this detection process is 1 %, which means that 1 g of agarose gel is needed in 100 mL of TBE solvent. The agarose gel, which has been added TBE, is microwaved until completely dissolved and waited for it to cool down before pouring it into the agar mold. The electrophoresis chamber is filled with TBE buffer, and the solidified agarose gel is placed inside it, ensuring that it is submerged in the TBE solvent.A total of 2.5 µl DNA ladder is added to the end well. As much as 7.5 µl of the sample is added to the well using a pipette. Electrophoresis is run at 100 V for 40 min. Next, the agarose gel is soaked in an EtBr (Etidium Bromide) solution for 10 min. It is then rinsed with distilled water. The DNA band is visualized using a UV-Transilluminator. A positive result is obtained when there is a band at around ± 1,400 bp. The PCR results containing the amplified DNA bands are sent to Genetic Science for nucleotide base sequence reading or sequencing. The sequencing results are processed using MEGA 11 software to align the obtained nucleotide base sequences. The alignment results are saved in FASTA format. The bacterial species are determined by matching the obtained nucleotide bases with the database in GenBank on the NCBI website (www.ncbi.nlm.nih.gov) using the BLAST feature.

The result of maceration was evaporated using a rotary evaporator. Gas Chromatography-Mass. Spectrometry (GC-MS) was performed using GCMS-QP2010 SE: SHIMADZU equipment. The identification of types and amounts of compounds in the extract can be seen based on the number of peaks in the chromatogram [20].

Results and discussion

Based on thin layer chromatography (TLC) results, 3 spots were produced in visible light and one in UV light. The spot with the highest retention factor (Rf) was Rf3 (0.25), followed by Rf2 (0.15) and Rf4 (0.075). Phytochemical screening of the seagrass sample showed that it contained flavonoids in ethyl acetate maceration. In addition, the piece macerated with methanol included tannins. The model macerated with methanol had medium levels of steroids, while this test did not detect the presence of saponins and terpenoids. Furthermore, the GC-MS analysis of seagrass extract revealed that the most significant content was 9-Octadecenoic acid (Z) (26.73 %), Hexadecanoic acid (15.91 %), Oleic acid (15.90 %), Hexadecanoic acid (15.57 %), Tridecanedial (6.26 %), Hexadecanoic acid (5.48 %), and Octadecanoic acid (5.01 %), as shown in **Table 1**.

Table 1 Results of GC-MS analysis of seagrass extract.

Peak#	R.Time	Area	Area %	Height	Name
1	23.220	621392687	15.57	104481116	Hexadecanoic acid, methyl ester (CAS)
2	23.997	635101708	15.91	67875856	n-Hexadecanoic acid
3	25.084	1067188313	26.73	142283000	9-Octadecenoic acid (Z)-, methyl ester (CAS)
4	25.249	199951396	5.01	45404792	Octadecanoic acid, methyl ester (CAS)
5	25.510	146153691	3.66	16731904	Tricyclo[20.8.0.0(7,16) triacontane, 1(22),7(16)-diepoxy-
6	25.750	634593121	15.90	65075179	Oleic Acid
7	25.880	161340223	4.04	28997233	9,12-Octadecadienoic acid (Z,Z)-
8	26.137	218635089	5.48	33762724	Hexadecanoic acid, 2-hydroxy-1,3-propanediyl ester (CAS)
9	27.819	57942356	1.45	9694395	9-Tetradecenal, (Z)-
10	28.471	249944551	6.26	31294515	Tridecanedial
		3992243135	100.00	545600714	

The findings indicated the morphological characteristics of the seagrass, with slender leaves measuring 12 cm in length and 6 mm in width. The tips of the leaves were oval-shaped and serrated. The leaf surface was smooth and slippery, with slightly filled rhizomes measuring 3 mm in diameter. Based on the observations, the identified species of seagrass was *Cymodocea serrulata*. *Rochia maxima* belongs to the class Gastropoda, order Trochida, and family Tegulidae. It measures around 110 - 120 mm in diameter and 115 - 140 mm in height. The identified mollusc was *Rochia maxima*, which has a relatively flat shell surface, numerous spirals at the base, and a thinner and lighter body. The isolation of symbiotic seagrass bacteria had identified 7 bacterial isolates with small, moderate, large, and punctiform sizes. These isolates also exhibited white and cream colours with flat elevations. The forms of the isolates were irregular, filamentous, irregular, and circular, with various types of margins (**Table 2**).

Table 2 Characterization of seagrass symbiont bacteria isolates.

No.	Isolate code	Morphological characterization				
		Size	Colour	Elevation	Form	Margin
1.	DL11	Small	White	Flat	Irregular	Undulate
2.	DL13	Moderate	Cream	Flat	Filamentous	Rhizoid

No.	Isolate code	Morphological characterization				
		Size	Colour	Elevation	Form	Margin
3.	DL14	Large	Cream	Flat	Irregular	Entire
4.	DL15	Large	Cream	Flat	Irregular	Entire
5.	DL21	Moderate	White	Flat	Circular	Entire
6.	DL22	Large	White	Flat	Circular	Entire
7.	DL23	Punctiform	White	Flat	Circular	Entire

The enzymatic assay of symbiotic seagrass bacteria isolates showed that the isolate with the largest clear zone in the proteolytic assay at each observation hour (18, 24, and 30 h) was DL21. The isolate with the highest enzymatic activity against amylase at each observation hour was DL11, with 5.8 ± 1.56 mm at 24-hour observation and 12.25 ± 5.16 mm at 48-hour observation. The highest enzyme activity of bacteria in breaking down cellulase was found in isolate DL11 (12.15 ± 7.42 mm) at 24-hour observation and DL21 (12.4 ± 1.98 mm) at 48-hour observation (**Table 3**).

Table 3 Enzymatic test of seagrass symbiont bacteria.

No.	Isolate code	Inhibition zone (mm)						
		Proteolytic			Amylolytic		Cellulolytic	
		18 h	24 h	30 h	24 h	48 h	24 h	48 h
1.	DL11	7.55 ± 0.07	10.00 ± 0.14	13.48 ± 0.04	5.8 ± 1.56	12.25 ± 5.16	12.15 ± 7.42	10.55 ± 7.14
2.	DL13	7.10 ± 2.26	7.80 ± 2.97	9.05 ± 3.61	1.00 ± 0.28	1.35 ± 0.07	8.05 ± 5.87	8.60 ± 3.25
3.	DL14	4.82 ± 0.02	6.11 ± 0.01	8.81 ± 0.01	0.30 ± 0.28	1.45 ± 0.49	2.00 ± 1.13	4.55 ± 1.20
4.	DL15	5.50 ± 4.24	7.35 ± 2.62	9.35 ± 2.05	0.15 ± 0.07	3.05 ± 0.21	2.55 ± 1.34	5.3 ± 0.85
5.	DL21	9.45 ± 0.21	12.50 ± 1.70	15.85 ± 1.06	0.9 ± 0.42	3.5 ± 0.57	9.65 ± 1.06	12.4 ± 1.98
6.	DL22	4.65 ± 0.07	6.55 ± 0.07	9.10 ± 1.13	1.00 ± 1.27	1.75 ± 1.63	1.85 ± 0.78	6.05 ± 3.75
7.	DL23	4.60 ± 1.70	5.20 ± 0.99	5.70 ± 0.71	0.35 ± 0.35	0.3 ± 0.28	3.85 ± 0.35	8.05 ± 1.63

Next, to isolate symbiotic bacteria from *R. maxima* Mollusc, 3 isolates with minor and moderate sizes, with white and yellow colours, were found. The elevation was flat and convex, with irregular and circular forms, as shown in **Table 4**. The results of the antibacterial activity of the 7 symbiotic bacteria isolates above showed that they did not possess antibacterial activity against the pathogenic bacteria *S. aureus* and *E. coli.*

Table 4 Characterization of mollusk symbiont bacteria isolates.

No.	Isolate code	Morphological characterization				
		Size	Colour	Elevation	Form	Margin
1.	LP31	Small	White	Flat	Irregular	Entire
2.	LP22	Moderate	White	Flat	Circular	Entire
3.	LK11	Small	Yellow	Convex	Circular	Entire

Enzyme activity testing of bacterial isolates showed that the most considerable proteolytic ability against Symbiont Lola bacteria was exhibited by LK11, with a diameter of 0.65 ± 0.212 mm at 18 h of observation and 0.725 ± 0.064 mm at 24 h. However, all 3 isolates could not break down the protease substance after 30 h of observation. The most considerable amylolytic enzyme activity was shown by LP31, with a diameter of 0.9775 ± 0.82 mm at 24 h and 1.235 ± 0.375 mm at 48 h.

The most considerable cellulolytic enzyme activity was found in the LP22 isolate, which was 0.485 ± 0.332 mm at 24 h and 0.595± 0.148 mm at 48 h, as seen in **Table 5** and **Figure 1**.

Table 5 Enzymatic test of mollusces.

No	Isolate code	Inhibition zone (mm)						
		Proteolytic			Amylolytic		Cellulolytic	
		18 H	24 H	30 H	24 H	48 H	24 H	48 H
1.	LP31	0 ± 0	0 ± 0	0 ± 0	0.9775 ± 0.82	1.235 ± 0.375	0 ± 0	0.23 ± 0.325
2.	LP22	0.02 ± 0.028	0 ± 0	0 ± 0	0.4625 ± 0.467	0.495 ± 0.7	0.485 ± 0.332	0.595 ± 0.148
3.	LK11	0.65 ± 0.212	0.725 ± 0.064	0 ± 0	0.41 ± 0.58	0.615 ± 0.46	0.23 ± 0.325	0.25 ± 0.354

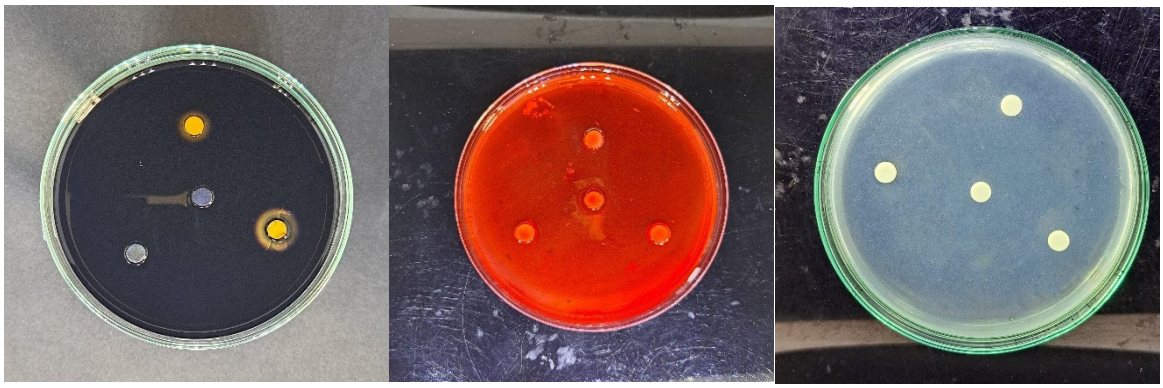

Figure 1 Enzymatic test of amylolytic molluscs (a) Cellulolytic, (b) Proteolytic, and (c) Molluscs LP22 isolate does not have antibacterial activity against the *S. aureus* pathogen, but it was observed that it has activity against the *E. coli* pathogen at 72 h of observation (1.21 ± 1.711 mm). LK11 showed the highest antibacterial activity against both *S. aureus* and *E. coli* pathogens, as shown in **Table 6**.

Table 6 Antibacterial test of mollusk symbiont bacteria isolates.

No.	Isolate code	Inhibition zone (mm)					
		S. aureus			*E. coli*		
		24 h	48 h	72 h	24 h	48 h	72 h
1.	LP31	0 ± 0	1.45 ± 1.051	0.65 ± 0.65	0 ± 0	0 ± 0	1.8 ± 2.546
2.	LP22	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.21 ± 1.711
3.	LK11	0 ± 0	1.7 ± 0.141	1.61 ± 0.919	0 ± 0	1.2 ± 0.283	2.625 ± 2.411

Identification of seagrass and mollusc symbiont bacteria

The sequencing results of 2 types of symbiotic bacterial isolates, namely 7 isolates of *Oceana serrulate* seagrass symbiotic bacteria and 3 isolates of *R. maxima* mollusc symbiotic bacteria, showed a band size of 1,500 bp (**Figure 2**).

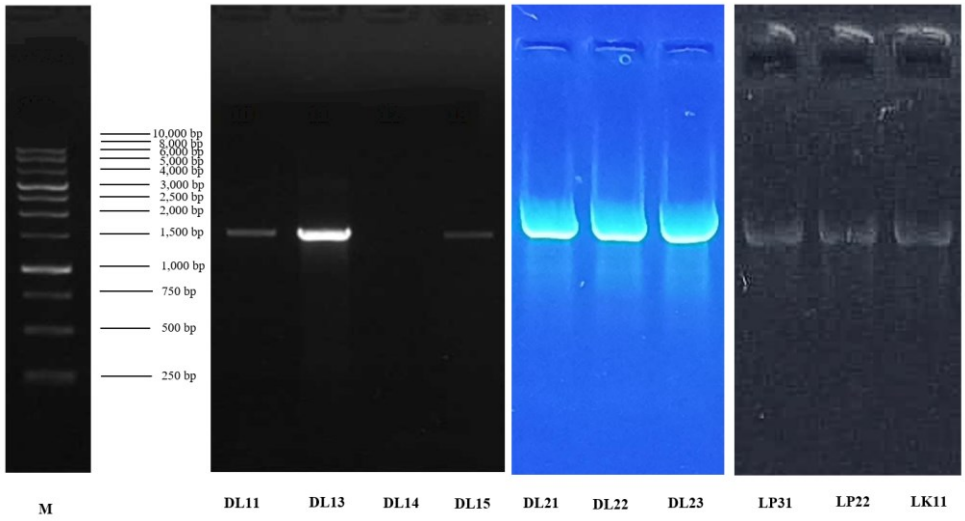

Figure 2 Electrophoresis results of bacterial species seagrass and mollusc symbiotic symbiotic (M, DL11, DL 13, DL 14, DL 15, DL21, DL 22, DL23, DL31 = seagrass simbiont bacteria and LP 31, LP22, LK 11 = Molluc simbiont bacteria).

Identification results of seagrass symbiont bacteria that have 7 isolates were *B. velezensis* (DL11, 100.00 %), *B. paramycoides* (DL13, 99.71 %), *Nocardiopsis alba* DSM 43377 (DL14, 100 %), *N. alba* DSM 43377 (DL15, 00 %), *B. tropicus* (DL21, 100 %), *L. fusiformis* (DL22, 100 %), and *B. tropicus* (DL23, 100 %), as stated in **Table 7**.

Table 7 Identification results of seagrass symbiotic bacteria.

Code	Description	Scientific name	Query cover	Percent identify	Acc number
DL11	*Bacillus velezensis* strain FZB42 16S ribosomal RNA, complete sequence	*Bacillus velezensis*	100 %	100.00 %	OR251863
DL13	*Bacillus paramycoides* strain MCCC 1A04098 16S ribosomal RNA, partial sequence	*Bacillus paramycoides*	99 %	99.71 %	OR251864
DL14	*Nocardiopsis alba* DSM 43377 16S ribosomal RNA, partial sequence	*Nocardiopsis alba* DSM 43377	100 %	98.85 %	OR251865
DL15	*Nocardiopsis alba* DSM 43377 16S ribosomal RNA, partial sequence	*Nocardiopsis alba* DSM 43377	100 %	100.00 %	OR251866
DL21	*Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence	*Bacillus tropicus*	100 %	100.00 %	OR251867
DL22	*Lysinibacillus fusiformis* strain DSM 2898 16S ribosomal RNA, partial sequence	*Lysinibacillus fusiformis*	100 %	99.93 %	OR251868
DL23	*Bacillus tropicus* strain MCCC 1A01406 16S ribosomal RNA, partial sequence	*Bacillus tropicus*	100 %	100.00 %	OR251869

The identification results of the symbiotic bacteria in the mollusc showed 3 isolates: *Vibrio owensii* CAIM 1854 = LMG 25443 strain DY05 (LP31, 99.79 %), *Bacillus paramycoides* strain MCCC 1A04098 (LP22, 100.00 %), and *P. flavipulchra* strain NCIMB 2033 (LK11, 100 %), as shown in **Table 8**.

Table 8 Identification results of mollusc symbiont bacteria.

Code	Description	Scientific name	Query cover	Percent identify	Accession number
LP31	*Vibrio owensii* CAIM 1854 = LMG 25443 strain DY05 16S ribosomal RNA, partial sequence	*Vibrio owensii* CAIM 1854 = LMG 25443 strain DY05	99 %	99.79 %	OR251860
LP22	*Bacillus paramycoides* strain MCCC 1A04098 16S ribosomal RNA, partial sequence	*Bacillus paramycoides* strain MCCC 1A04098	100 %	99.90 %	OR251861
LK11	*Pseudoalteromonas flavipulchra* strain NCIMB 2033 16S ribosomal RNA, partial sequence	*Pseudoalteromonas flavipulchra* strain NCIMB 2033	100 %	100.00 %	OR251862

The analysis of active compounds extracted from the bacterial symbiont of molluscs (isolates LD11, LD13, and LD14) using gas chromatography-mass spectrometer (GC-MS) identified phthalic acid, bis(7-methyl octyl) ester (25.18, 43.84, and 35.55 %, respectively), and trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone (36.36, 36.85, and 47.71 to 64.48 %, respectively) as the dominant compounds. These compounds belong to the phthalate compound class, as shown in **Figure 3**. Another compound, benzeneacetic acid, alpha,3,4-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester, was only found in isolates LD11 and LD13 (20.05 and 5.29 %, respectively). Isolate LD11 also had the compound 2H-1,4-Benzodiazepin-2-one, 7-chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl)-3-[(trimethyl, 2, 46 %). The seagrass extract at the research site was *O. serrulata* (formerly *C. serrulata*). The GC-MS analysis revealed that the primary compounds in the section were 9-Octadecenoic acid (Z) (26.73 %), Oleic acid (15.90 %), Hexadecanoic acid (15.57 %), Hexadecanoic acid (5.48), and Octadecanoic acid (5.01 %), all of which belong to unsaturated fatty acids. The presence of these fatty acids suggests that they may have antibacterial properties because some fatty acids possess antiseptic properties that help prevent bacterial growth or development. Fatty acids can block the bacterial cell wall, preventing nutrients from penetrating the cell and thus inhibiting the growth of harmful microorganisms. Several fatty acids found in organisms, including oleic acid, have been shown to act as antibacterial agents [21]. Fatty acids as promising antibacterial agents that disrupt bacterial cell membranes and cause direct and indirect inhibitory effects. The results of isolating bacterial symbionts from *C. serrulata* seagrass revealed 7 bacterial isolates. Antibacterial tests against *S. aureus* and *E. coli* bacteria showed that the bacterial isolates did not have antibacterial activity. However, they have not been tested against other pathogenic bacteria. Enzymatic tests of the bacterial isolates from seagrass symbionts showed that DL21 had the largest clear zone in proteolytic testing at 18, 24, and 36 h. Further identification of the bacteria revealed that the DL21 isolate were similar to *Bacillus tropicus*. The isolate with the most considerable enzymatic activity in amylolytic testing at each observation time was DL11, with measurements of 5.8 ± 1.56 mm (24-hour observation) and 12.25 ± 5.16 mm (48-hour observation). The isolate with the most significant enzyme activity in cellulase breakdown was DL11 (12.15 ± 7.42 mm) at 24-hour observation and DL21 (12.4 ± 1.98 mm) at 48-hour observation. DL11 was identified as *Bacillus velezensis,* and DL21 was identified as *B. tropicus*, with a 100 % similarity. Bacillus is found in soil, water, and the sea. This bacterium is Gram-negative, meaning it has a more complex cell wall structure and contains more lipid components than Gram-positive bacteria, which strengthens the cell wall of this type of bacterium [20]. This condition indicates that the bacterium *B. tropicus* does not have antibacterial activity. *B. tropicus* can be used for various purposes, the most important of which is as a raw material for biological products useful in multiple industries, including the food processing, chemical, pharmaceutical, and agricultural sectors. In addition, this bacterium can also be used to improve composting processes and land restoration due to its ability to break down organic matter. It can also be used in biotechnology industry to produce enzymes and organic chemicals. Lastly, it can also be used for detecting heavy metals in the environment and for biological research [22]. *B. velezensis* is a beneficial soil bacteria that produces an alkDH enzyme, a chlorhexidine gluconate inhibitor. This enzyme can be used in water

treatment production. It is also helpful in fighting pathogens that can trigger certain pathogenic bacteria, such as *Escherichia coli*. Additionally, *B. velezensis* can enhance the nutrient content of the soil, allowing plants to grow well [23]. *B. paramycoides* belongs to the Paramycetes group and is commonly found in aquatic environments like rivers, lakes, and ponds. It has a similar shape to other bacteria, either rod-shaped or round. Another bacterium found in the symbiotic bacteria of seagrass is *Nocardiopsis alba* (isolate DL 15 and isolate DL 21). The benefits of this bacterium are as follows: It can produce enzymes beneficial to industries, such as amylase, protease, and lipase enzymes. It is also used for pest control and decomposing organic materials such as agricultural and industrial waste, thereby assisting in recycling processes and waste management. Additionally, it can produce bioactive compounds with antimicrobial and antitumor activities, thus having potential in the development of pharmaceuticals and soil remediation, which involves cleaning soil contaminated by organic pollutants [24,25]. *L. fusiformis* is a Gram-positive, rod-shaped bacterium from the genus *Lysinibacillus*. It is known to have enzyme activity and functions in pest control, producing effective antimicrobial compounds for inhibiting pest growth. In addition, it functions in bioremediation by having the ability to degrade harmful compounds such as organic pollutants, heavy metals, and toxic chemicals, and it can enhance nutrient availability. *Vibrio owensii* is a Gram-negative bacterial species from the Vibrionaceae family. They are rod-shaped and move using flagella. They can live in various environmental conditions, including seawater, freshwater, and sediments. They can be used as a food source as they are found in fish and shellfish, and humans can consume some species. They have the potential to produce enzymes that can be applied in the biotechnology industry. Some of the enzymes produced by these bacteria can be used in the food, pharmaceutical, and biotechnology industries due to their potential against multidrug-resistant bacteria [26]. Research has revealed that food supplements containing the *B. paramycoides* strains can enhance specific growth rates and disease resistance against *A. hydrophila*. This condition demonstrates the potential of these bacteria as antibacterial agents beneficial as food preservatives [27].

P. flavipulchra is a species of the Pseudoalteromonas genus known to have potential as an antibacterial agent. The genus *Pseudoalteromonas* hosts 41 species, among which 16 are antimicrobial metabolite producers. Sixty-nine antimicrobial compounds belonging to 18 families have been documented [28]. Research shows that *P. flavipulchra* bacteria have antibacterial activity and, therefore, have potential as a food ingredient. GCMS analysis of the seagrass symbiotic bacteria (LD11, LD13, and LD14) showed the presence of phthalate compounds. Phthalate compounds were also found in the gut consortium of sea cucumbers [29]. It exhibited antifungal activity against *Candida albicans* and antibacterial activity against the Gram-positive bacterium *Sarcina lutea*. DEHP also showed cytotoxic activity against several carcinoma cell lines [30].

Conclusions

The research concludes that extracts from the seagrass species *Oceana serrulate* and 7 symbiotic bacterial isolates from seagrass, as well as symbiotic bacterial isolates from the mollusc *Rochia maxima* that live in seagrass, have the potential to be used as food preservatives due to their antibacterial and antifungal properties. This means seagrass and its symbiotic bacteria and the symbiotic bacteria of molluscs living in seagrass can inhibit the growth and development of pathogenic bacteria and effectively stop the growth of bacteria in food as a preservative. Several studies have shown that some symbiotic bacteria have strong antibacterial properties. The study showed that symbiotic bacteria isolated from seagrass and molluscs that live in seagrass have antibacterial activity against *S. aureus* and *E. coli* bacteria, as well as enzymatic activities such as proteolytic, amylolytic, and cellulolytic activities. On the other hand, symbiotic bacteria also have enzymatic activities that are needed in the food industry.

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