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Predominance and bioactive potential studies of endophytic *Bacillus* Sps. from marine algae of Palkbay costal region, India

A.K. RAMYA*, R. DEVIKA*[2] AND K. SETHUMADHAVAN[3]

Department of Microbiology, Aarupadai Veedu Medical College and Hospitals, Vinayaka Mission's Research Foundation - DU, Puducherry, India

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ABSTRACT

The genus Bacillus is a widely distributed and prominent species that has been extensively studied for its numerous benefits in various industries, medical applications, and agriculture. In this study, endophytic Bacillus species were isolated from seven different algal species using various growth media such as Nutrient agar, starch casein agar, ISP 2, and ISP 4. Throughout the study period, a total of 36 bacterial strains with similar morphological characteristics were identified. Among the isolates, five bacterial strains were found to be predominant when grown on ISP 2 media. These five strains were further subjected to 16s RNA Sequencing to determine their taxonomic classification. The results revealed that all of these strains belonged to the genus Bacillus. However, the other endophytic strains could not be identified due to limited sequence homology with existing entries in the NCBI database. To gain further insights into the isolated Bacillus cultures, analytical studies were conducted. Firstly, the functional groups present in the cultures were identified using Fourier-transform infrared spectroscopy (FTIR). Additionally, the compounds present in the crude extracts of the cultures were analyzed using gas chromatography-mass spectrometry (GC-MS). The findings of this current research demonstrate the presence of Bacillus species among green and brown algae in the Palk Bay coastal range. Moreover, these Bacillus strains exhibit potential for various biotechnological applications. The study aims to establish the predominance of Bacillus species in the algae of the Palk Bay region in South India, thereby contributing to our understanding of the microbial ecology in this specific geographical area.

Keywords: Gram positive, antimicrobial, 16s RNA, FTIR, GC-MS

INTRODUCTION

The genus *Bacillus* was first identified in 1872 by Ferdinand Cohn and is composed of Gram-positive bacteria that are commonly found in soil, water, plant roots, and fermented foods (Almaary *et al.*, 2021a; Castro *et al.*, 2014; Duan *et al.*, 2013; Hussain *et al.*, 2015; Lobo, 2014; Saxena *et al.*, 2020). This genus encompasses approximately 390 species, many of which have the capability to produce a wide range of antimicrobial compounds (AMCs). One notable species within the genus *Bacillus* is *Bacillus subtilis*, which has been extensively studied for its ability to produce a diverse array of secondary metabolites with antimicrobial properties (Emanuel, 2012). These secondary metabolites often take the form of antimicrobial peptides, which are typically cyclic and contain hydrophobic intracellular thio ether bonds. In addition to AMCs, *Bacillus* species can also produce volatile metabolic compounds that play important functional roles. *Bacillus* strains have gained significant attention in various industries due to their potential for the production of enzymes, antibiotics, flavour enhancers, and insecticides (Janardhan and Vijayan, 2012). Different species of *Bacillus*, including *Bacillus cereus, Bacillus megaterium*, *Bacillus aquimaris*, and *Bacillus licheniformis*, have demonstrated the ability to fix atmospheric nitrogen, uptake phosphorus, and promote plant growth. For instance, *B megaterium* M510, isolated from the rhizosphere of maize in the Eastern Himalayan region, has been found to solubilize both aluminium phosphate and iron phosphate, thus aiding in nutrient availability for plants (Kumar, 2013). In recent years, several noteworthy endophytic metabolites have been reported, with approximately 51% of these isolates originating from novel endophytic fungi (Strobel and Daisy, 2003). These findings highlight the potential of endophytic *Bacillus* species in producing novel metabolites with diverse applications.

*[1]Research Scholar and [3]Professor, [2]Professor Cum RPMO, Department of Biotechnology, Aarupadai Veedu Institute of Technology, Vinayaka Mission's Research Foundation-DU, Paiyanoor, Chennai, India, ORCID ID: A K RAMYA,0000-0002-5680-2235 : DR R DEVIKA,0000-0002-1914-1378 : DR K SETHUMADHAVAN,0000-0002-7990-7689, *Corresponding Author: vineethdevika@gmail.com ; akramya@gmail.com*

Marine resources

The marine environment is a rich source of natural compounds that exhibit remarkable specificity and potency against targeted molecules. These compounds hold immense potential as drugs for human use. Due to the unique and diverse ecosystem of the marine environment, organisms such as marine plants, animals, and microorganisms have evolved to produce a wide array of bioactive compounds with exceptional pharmacological properties. Studies have revealed that natural compounds derived from marine sources possess distinct chemical structures and exhibit biological activities that make them highly attractive for drug development. These compounds have demonstrated significant efficacy in targeting specific molecular pathways involved in various diseases, including cancer, infectious diseases, inflammation, and neurodegenerative disorders. The marine environment offers a vast array of organisms, including sponges, corals, mollusks, algae, and microorganisms, each harbouring a wealth of potentially valuable bioactive compounds. Scientists have been exploring these resources through extensive research and have discovered numerous natural products with promising therapeutic applications. The development of drugs from marine natural compounds involves a rigorous process of isolation, purification, structural elucidation, and pharmacological evaluation. Advanced techniques such as high-throughput screening, combinatorial chemistry, and computer-aided drug design have facilitated the identification and optimization of lead compounds from marine sources. Furthermore, the sustainable harvesting and conservation of marine resources are of utmost importance to ensure the continued availability of these valuable natural compounds. Strict regulations and ethical practices are being implemented to protect marine ecosystems and maintain their biodiversity for future generations. The exploration of natural compounds from the marine environment holds great promise for the development of novel drugs with high specificity and therapeutic potential. Continued research in this field is vital to uncovering the full range of bioactive compounds and harnessing their benefits for human health.(Bajpai, 2016; Bhadury *et al.*, 2006; Magarvey *et al.*, 2004; Mayer *et al.*, 2007; Mayer and Hamann, 2005; Prudhomme *et al.*, Sipkema *et al.*, 2005)

Palk Bay costal region

The Palk Bay is a semi-closed shallow water body situated between the Southeast coast of India and Sri Lanka, covering an area of approximately 15,000 square kilometers. It is located between 80°50' and 100° north latitudes and 78°50' and 80°30' east longitudes. The Northeastern side of the Palk Bay is open to the Bay of Bengal, while the southern region is adjacent to Dhanush Kodi. This bay is known for its highly productive ecosystem, supporting a diverse range of marine life, including 302 species of marine algae, 580 species of fishes, five species of marine turtles, and 11 species of seagrass, along with a few species of mangroves.

MATERIALS AND METHODS

Collection of algal samples

Fresh samples of algae, including two species of brown algae and seven species of green algae, were collected from the Palk Bay region in South India. The samples were selected based on their distinct morphological appearances and colors. Care was taken to ensure the samples remained free from external contamination during collection and transportation. Prior to transportation, the algal samples were washed to remove any debris and dirt, and surface sterilization was performed using sea water. The samples were then washed with sterile water, air dried, and identified by an expert taxonomist. The identified samples were preserved in the herbarium for future reference.

Detection of pure surface sterilization evidence

Surface sterilization is a crucial step in the isolation of endophytes, ensuring the removal of epiphytic microorganisms from the outer surface of the algae (Almaary *et al.*, 2021b; Usha Nandhini *et al.*, 2018). To confirm the effectiveness of surface sterilization, the final wash water collected during the process was spread on nutrient agar plates and incubated at 28°C for 48 hours. This step helped to determine

the presence of endophytic microorganisms. The isolation of endophytes was carried out using a standard protocol based on established methodologies (Erbert *et al.*, 2012; Kjer *et al.*, 2010).

Surface sterilization

The dried algal samples were subjected to surface sterilization by treating them with 70% ethanol for 60 seconds, followed by 0.4% sodium hypochlorite for 30 seconds. This process effectively removed the epiphytic microorganisms from the outer surface of the algae. The sterilized algae were then washed with sterile distilled water, and the final wash water was collected for further analysis. The water-washed algae were placed on filter paper to remove excess water. Using a sterile blade, the algae were cut into small segments (2.0 cm) and pressed onto various growth media, including Potato Dextrose Agar (PDA), Nutrient Agar (NA), Actinomyces Agar (AA), ISP 2, and ISP 4. The growth media were prepared using sea water, with the addition of streptomycin to reduce bacterial growth in Potato Dextrose Agar and nystatin to suppress fungal isolates in starch casein agar. The plates were then incubated at appropriate temperatures and durations to facilitate the growth of colonies around the algal segments. The isolated colonies were subculture in slants for further studies, and pure endophyte cultures were preserved in glycerol and subjected to morphological analysis.

Molecular identification of endophytes (16S rRNA sequencing)

The isolated bacteria were identified through bacterial cultures were grown on Nutrient Agar slants at 28°C for 24 hours. DNA isolation was performed using an Expure Microbial DNA isolation kit. Subsequently, the 16S rRNA gene was amplified using specific primers (27F and 1492R). The PCR products were sent for Sanger sequencing at a regional facility for DNA fingerprinting. The obtained raw data in FASTA format were subjected to Basic Local Alignment Search Tool (BLAST) analysis for bacterial species identification. The identified endophytic bacteria were used to construct a phylogenetic tree using ClustalW in MEGA-X. The sequence data of the bacterial species were submitted to the NCBI database, and accession numbers were obtained (Leylaie and Zafari, 2018).

Preparation of bacterial crude extract

To evaluate the antimicrobial efficiency of the isolated bacterial strains, the cultures were inoculated in nutrient broth and PD broth, respectively. After incubation, the fermented broth cultures were centrifuged at 10,000 rpm for 15 minutes at 4°C. The resulting pellets were discarded, and the supernatants were collected using Whatman filter paper No. 1. The collected supernatants were treated with polar and nonpolar solvents such as hexane, ethanol, chloroform, ethyl acetate, and methanol (Lee *et al.*, 2017).

Identification of bioactive compounds by Thin Layer Chromatography (TLC)

TLC was employed to determine the qualitative presence of compounds in the bacterial crude extract. Silica gel was used as the stationary phase, while solvents such as hexane/ethyl acetate (1:1), hexane/ethyl acetate (7:3), and hexane/ethyl acetate (3:7) were used as the mobile phase. Different ratios of mobile phases were used to assess the efficiency of compound extraction. The retention factor (RF) of the samples was calculated by dividing the distance travelled by the sample with the distance travelled by the solvent.

Fourier Transform Infrared spectroscopy (FTIR) Analysis

FTIR analysis was conducted to identify the functional groups present in the dried powder of the bacterial crude extract. The samples were scanned from 400 to 4000 cm-1, and the absorption radiation was recorded using Fourier Transform Infrared spectroscopy (FTIR) (Shimadzu - QP2010 PLUS, Japan). The intensities of the transmittance peaks were analyzed to identify the functional groups.

Gas Chromatography Mass Spectroscopy (GCMS) Analysis

GCMS analysis was performed on the solvent-extracted and dried concentrated A.K.

bacterial samples. The samples were diluted in Carbinol and subjected to GC analysis. Parameters such as column oven temperature, injection temperature, injection mode, pressure, total flow, column flow, ion source temperature, interface temperature, start time, end time, ACQ mode, scan speed, etc., were set for the analysis.

RESULTS AND DISCUSSION

Identification and authentication of algal samples

The algal samples collected from Palk Bay were identified and authenticated with voucher Nos. and listed in Table 1.

Table 1: List of Identified and Authenticated algal Samples

S. No.	Voucher No.	Family	Binomial Nomenclature
1	PARC/2022/4830	Gigartinaceae	*Chondrus crispus* Stackh
2	PARC/ 2022/4829	Dictyotaceae	*Padina boergesenii* Allender & Kraft
3	PARC/2022/4828	Dictyotaceae	*Padina gymnospora* (Kuetzing) Vickers
4	PARC/2022/4827	Caulerpaceae	*Caulerpa racemose* (Forsskal) J Agardh
5	PARC/2022/4826	Caulerpaceae	*Caulerpa sertularioides* (S G Gmel) M Howe
6	PARC/2022/4825	Ulvaceae	*Ulva intestinalis* L.
7	PARC/2022/4824	Ulvaceae	*Ulva lactuca* L.
8	PARC/2022/4823	Caulerpaceae	*Caulerpa taxifolia* (M Vahl)C.Agardh
9	PARC/2022/4822	Ceratophyllaceae	*Ceratophyllum submersum* L.

Isolation of endophytic bacteria

Following the surface sterilization process, the dried algal samples were placed on different growth media, including Nutrient Agar, Actinomyces Agar, ISP 2, Potato Dextrose Agar (PDA), and ISP 4. Among these media, Nutrient Agar and ISP 2 exhibited rapid and robust growth of bacterial colonies around the algae on the plates. In total, 36 isolates were identified in the vicinity of the algal samples, with 15 isolates on Nutrient Agar, 7 isolates on PDA, 7 isolates on ISP 2, and 5 isolates on ISP 4. These isolates are depicted in Figure 1. Among them, 14 colonies exhibited distinct colony morphologies and were selected for further investigation.

Figure 1: Sub cultured isolated endophytes from Algae

Table 2: Macroscopic Morphological characterization

Name of the isolates	Colony morphology
3	Yellow stripped colonies.
4	Pale white with mucoid colonies
1	Translucent colonies
3	Flat rhizoid colonies
4	Rhizoid pale white colonies
2	Pale yellow with white colonies
3	Yellow mucoid colonies
2	White colonies
5	White mucoid translucent colonies
3 isolates	Rhizoid colonies
6 isolates	Pale white mucoid colonies.

Microscopic Characterization

Gram staining was employed to determine the Gram-positive and Gram-negative nature of the isolated organisms. Out of the identified isolates, approximately 3 organisms were found to be Gram-positive, while the rest were Gram-negative. Furthermore, endospore staining was conducted, and a few microorganisms exhibited positive staining for endospores. Based on phenotypic studies, it was established that the isolated cultures belonged to the genus Bacillus. These purified strains were preserved in slants at 4°C for further analysis. To validate the isolation of endophytes, the isolated

strains were cross-checked with plates inoculated with the last surface-sterilized water. No bacterial growth was observed on these plates, confirming that the endophyte isolates originated from the internal tissues of the algae. A total of 7 strains were selected for subsequent investigations at the conclusion of the phenotypic studies. The protocol employed in this study, as per the findings of (Jeewon *et al.*, 2019) ensured the isolation of efficient endophyte strains, and the surface sterilization procedure was optimized for these algae.

For the identification and authentication of the endophytic organisms, 16S rRNA sequencing was conducted. Bacterial colonies were initially identified based on colony morphology and Gram staining, followed by authentication through Sanger Sequencing Analysis of the 18S DNA. The BLAST analysis of the sequencing results involved a comparison with the National Centre for Biotechnological Information (NCBI) database. Among the 36 endophytes, 5 bacterial endophytes were identified and taxonomically categorized under the phylum Bacillota. The detailed identification and classification of these strains, including their closest matches in the NCBI database, are provided in Table 2 and depicted in Figure 2. It is worth noting that the remaining endophytes could not be identified due to limited sequence homology in the gene bank database. The percentage of identity was determined among the closest species, with Bac_01, Bac_02, Bac_03, Bac_04, and Bac_05 exhibiting 100% similarity with *Bacillus velezensis, Bacillus anthracis, Bacillus thuringiensis, Priestia megaterium, and Bacillus subtilis*, respectively, as illustrated in Figure 2.

Table 3: Six endophytic Bacterial strains identified by 16 s r RNA Sequencing

Isolates Name	Closest Relative[a]	Accession No[b]	% Identity[c]
BAC_01	*Bacillus velezensis*	OM541328.1	100%
BAC_02	*Bacillus anthracis*	ON063211.1	100%
BAC_03	*Bacillus thuringiensis*	ON063216.1	100%
BAC_04	*Priestia megaterium*	ON063226.1	100%
BAC_05	*Bacillus subtilis*	OP020696.1	100%

aClosest species which high % identity in BLAST Analysis, bNCBI Gene bank accession number in website (http://www.ncbi.nlm.nih.gov/pubmed),cGenBank accession no. of our strains deposited on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed),d % identiy of strain based on BLAST Analysis

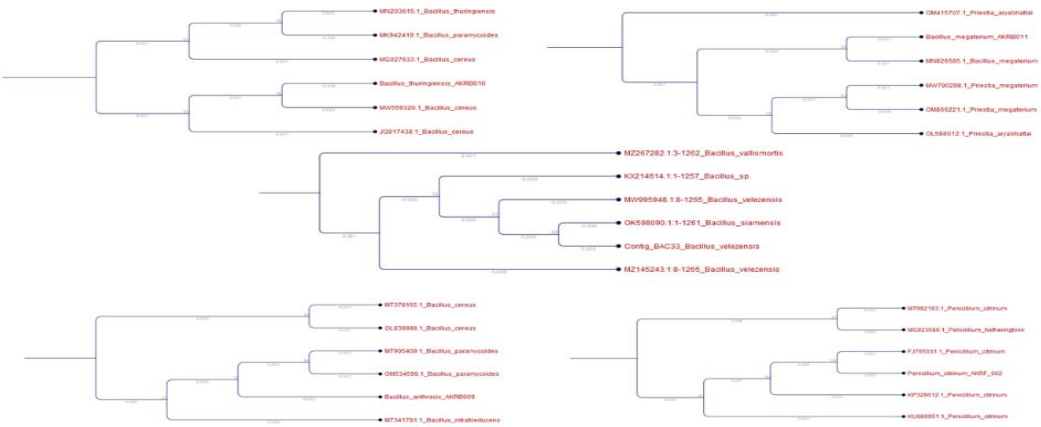

Figure 2: Phylogenetic tree of isolated endophytic Bacteria

FTIR Analysis

The FTIR results shown in figure show a prominent peak around 1736-1740 cm^{-1} that can be assigned to the stretching vibrations of the carbonyl group (C=O) in the ester functional group. Similarly, the peaks around 2980-2860 cm^{-1} are due to the C-H stretching vibration of alkyl groups present in ethyl acetate, and the peaks are broad in appearance shown in Figure 3. Similarly, the peaks at 1371 are attributed to the sp^3 C-H stretching, rocking, or bending vibration of alkyl groups. It can also be inferred that the absence of the -OH group in the

spectrum around 3200 cm^{-1} confirms that there was no water contamination in the compound. Peaks around 1710-1750 cm^{-1} shall be assigned to the stretching vibration of C=O ketone, carboxylic and ester groups, confirming the presence of secondary metabolites.(Li *et al.*, 2018; Xu *et al.*, 2011) The strong and weak peaks observed at 1445 cm^{-1} and 1640 cm^{-1} were due to the aromatic C=C which may be due to the metabolites. Similarly, the peak around 1040 cm-1 shall be assigned to the C-O stretching vibrations of ether groups, confirming the presence of secondary metabolites in the samples. The results confirmed the presence of secondary metabolites and the solvent from the FTIR results.

Table 4: FTIR results of Bacterial group of Bac 1

Sl No	Wave Number, Cm^{-1}	Bond	Functional Group
1	607.84 633.9	C -Br	Alkyl Halides
2	786.28 846.98	C -Cl	Alkyl Halides
3	936.55	OH	Carboxylic acid
4	1042.96	C-H	Alkyl Halides
5	1098.13 1232.52	C-N	Aliphatic amines
6	1371.89	N - 0	Nitro compounds
7	1445.77	C - H	Alkenes
8	1736.39	C=0	Alpha, beta , usaturated aldehydes, ketones
9	2984.56	C -H	Alkaes

Table 5: FTIR results of Bacterial group of Bac 2

Sl No	Wave Number, Cm^{-1}	Bond	Functional Group
1	607.91 633.76	- C ()C -H :CH	Alkynes
2	786.49 847.04	C -Cl	Alkyl Halides
3	935.5	OH	Carboxylic acid
4	1042.94 1099.23 1232.27	C-N	Aliphatic amines
5	1371.27 1445.77	C - H	Alkenes
6	1736.09	C=0	Saturated aliphatic, aldehydes, ketones
7	2984.56	C -H	Alkaes

Table 6: FTIR results of Bacterial group of Bac 3

Sl No	Wave Number, Cm^{-1}	Bond	Functional Group
1	786.35	C -Cl	Alkyl halides
2	847.01 936.11	NH	Primary and secondary amines
3	1043.01 1098.48 1232.32	C-N	Aliphatic amines
4	1371.46 1445.51	C - H	Alkenes
5	1736.42	C=0	Saturated aliphatic, aldehydes, ketones
6	2984.56	C -H	Alkenes

Table 7: FTIR results of Bacterial group of Bac 4

Sl No	Wave Number, Cm^{-1}	Bond	Functional Group
1	786.35 847.01	C -Cl	Alkyl halides
2	936.11	NH	Primary and secondary amines
3	1042.91 1098.48 1232.32	C-N	Aliphatic amines
4	1371.46 1445.51	C - H	Alkenes
5	1736.42	C=0	Saturated aliphatic, aldehydes, ketones

GC MS data analysis

After GC-MS analysis, the obtained spectrum was observed and tabulate it to investigate their *in-silico* medicinal property Table 8. The observed spectrum revealed that there is a present of 26 compounds and they are further used evaluated their activity using *in-silico* studies. The compounds obtained in crude were identified using NIST Library.

Table 8: GCMS Analysis and list of identified names using NIST Library

RT	Name	Area	Height	Mass	Formula	Area %
3.15	Propanamide, 2-hydroxy-	205334	27377	89	C3H7NO2	2.73
3.57	Tetrachloroethylene	141330	82142	163.9	C2Cl4	1.88
17.54	2-Decanol	34136	11117	158.2	C10H22O	0.45
17.91	Ethanamine, 1-(2-benzodioxanyl)-N-(2-benzodioxanylmethyl)-	36273	17469	327.1	C19H21NO4	0.48
18.56	Oxirane, 2-butyl-3-methyl-, cis-	33594	14009	114.1	C7H14O	0.45
20.68	2-Nonanol	47493	20679	144.2	C9H20O	0.63
20.80	2-Tetradecanol	73402	30016	214.2	C14H30O	0.98
21.02	4-Ethoxy-3-anisaldehyde	101207	24099	180.1	C10H12O3	1.35
23.64	N-[Dimethylaminomethyl]aziridine	59759	28333	100.1	C5H12N2	0.80
23.77	Dodecane, 1,1-dimethoxy-	439629	172922	230.2	C14H30O2	5.85
24.62	Phthalic acid, dodecyl 2-(2-methoxyethyl)hexyl ester	39757	13179	476.4	C29H48O5	0.53
25.09	Methoxyacetic acid, dodecyl ester	67826	25922	258.2	C15H30O3	0.90
25.85	2-Undecanol	30195	11836	172.2	C11H24O	0.40
26.11	5-(1-Iodo-1-methyl-ethyl)-3,3-dimethyl-dihydro-furan-2-one	438330	151346	282	C9H15IO2	5.84
26.39	4-Octadecenal	1739159	832133	266.3	C18H34O	23.15
26.52	cis-1,2-Cyclododecanediol	1160515	434395	200.2	C12H24O2	15.45
26.63	2-Octene, 1-(methoxymethoxy)-, (E)-	39919	14966	172.1	C10H20O2	0.53
27.38	Decanoic anhydride	605381	254306	326.3	C20H38O3	8.06
27.54	Fumaric acid, butyl pent-4-en-2-yl ester	1451006	562220	240.1	C13H20O4	19.32
27.81	Vinyl decanoate	71691	27979	198.2	C12H22O2	0.95
27.96	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	47960	13432	210.1	C11H18N2O2	0.64
28.86	2-Pentadecanol	28013	13593	228.2	C15H32O	0.37
28.99	2-Tridecanol	61153	13680	200.2	C13H28O	0.81
31.36	2-Hexanol	50447	17640	102.1	C6H14O	0.67
32.72	4-Nitrobenzoic acid, 2-butyl ester	147480	63105	223.1	C11H13NO4	1.96
34.19	N-cyclohexyl-3,4-methylenedioxyamphetamine	360689	101761	261.2	C16H23NO2	4.80

Discussion

Bacillus subtilis, isolated from *Sargassum myriocystum*, exhibited antimicrobial activity against Vibrio species. Additionally, the endophytes derived from this organism have been found to produce O-heterocyclic polyketide derivatives. Bacicyclin, a bioactive compound, is specifically produced by *Bacillus* species isolated from the mollusk *M. edulis*. A study conducted by Gray *et al.*, in 2013 reported that the presence of six fungal species, including *Penicillium chrysogenum* and *Aspergillus* species, in *Ulva intestinalis* and *Ulva lactuca*. Another study by Fasiku *et al.*, 2020 identified *Pseudomonas viridiflava* in terrestrial grass plants, which produces bioactive compounds such as ecomycin B and C, known for their antimicrobial properties.

In Wang *et al.*'s research, it was observed that approximately 80% of the bacteria isolated from soil samples belonged to the *Bacillus* species. *Bacillus* demonstrated a dominant presence compared to other genera in the soil samples. However, no studies have reported about the dominance of *Bacillus* species in marine algae. In the current study, a significant number of *Bacillus* strains were identified in marine algae. The prevalence of *Bacillus* species can be attributed to their ability to form endospores, which allows them to survive under harsh environmental conditions.

CONCLUSION

Studying endophytic microorganisms from marine organisms is essential for understanding their host relationship. These microorganisms offer significant biotechnological applications. The bioactive compounds produced by endophytes have the potential to enhance plant growth, serve as biocontrol agents, and find applications in the pharmaceutical and food industries. In this study, we aimed to explore the diversity of Bacillus species associated with various green and brown algae. By utilizing 16S rRNA sequencing, we identified a rich presence of endophytic Bacillus strains in both green and brown algae, belonging to the Bacillota family.

Our findings revealed promising novel bioactive compounds within these *Bacillus* strains, prompting further research on their extraction and purification. These compounds hold potential for anticancer, anti-inflammatory, and antimicrobial applications.

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Conflict interest

The authors declare that there is no conflict of interest.

Data availability statement

The datasets generated for this study can be found in the NCBI Bank, Accession numbers: OM541328.1; ON063211.1; ON063216.1; ON063226.1; OP020696.1

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