PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.4667

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RESEARCH ARTICLE

Exploration of endophytic *Bacillus* -derived secondary metabolites from mosses through GC-MS profiling

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OPEN ACCESS

ARTICLE HISTORY

Received: 15 August 2024
Accepted: 30 January 2025

Available online
Version 1.0 : 25 June 2025

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
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Shivangi P, Saumya P, Afroz A. Exploration of endophytic *Bacillus* -derived secondary metabolites from mosses through GC-MS profiling. Plant Science Today (Early Access). https://doi.org/10.14719/pst.4667

Abstract

Bacterial metabolites produced by endophytic bacteria facilitate novel drug development for animals, humans and plants. This study aimed to identify the different secondary metabolites produced by moss-derived endophytic bacteria *Bacillus pumilus*. Endophytic bacteria were isolated from surface-sterilized mosses, screened for enzymatic activity and identified via 16S rDNA sequencing. Secondary metabolites were extracted, analyzed using FTIR for functional groups and characterized through GC-MS. Morphological, biochemical and molecular (16S rDNA) characterization was carried out for the isolates from three moss species *Hymenostylium recurvirostrum, Barbula viennealis* and *Plagiothecium cavifolium*. Each isolate can produce at least two industrially significant enzymes including esterase, cellulase, amylases and proteases. Gas chromatogram mass spectroscopy (GC-MS) of *B. pumilus* extract demonstrated the occurrence of the compounds such as Pentadecanoic acid, 13 methyl-, methyl ester, Benzoic acid, 2- amino-6- chloro-methyl ester, Molybdenum, tricarbonyl tris (trimethyl phosphite-P)-, 3'H-Cycloprop(1,2)-5-cholest-1-en-3-one, Benzaldehyde,4-methoxy N hexadecanoic acid (Palmitic acid), Tungsten, dicarbonyl-(ü-4-pinocarvone) [1,2-bis (dimethyl phosphine) ethane] Boronic acid, ethyl-, dimethyl ester Phenol, 2,6-bis (1,1-dimethyl ethyl)-, Stigmastan-6,22-dien, 3,5-dihydro, Phthalic acid, 2TMS derivative Prostaglandin D(2), O, O'-bis (trimethylsilyl)-, trimethylsilyl ester. These volatile organic compounds hold the potential as favorable candidates for advancing pharmaceutics and agriculture industries.

Keywords

Bacillus pumilus; endophytes; GC-MS; mosses; secondary metabolites

Introduction

Endophytic bacteria are ecologically important bacterial groups capable of producing a broad spectrum of secondary metabolites. Plant-associated endophytic bacteria exhibit diverse mechanisms to enhance growth, stress resistance and defense mechanisms and thus hold significant potential for application in agriculture, medicine and industry. Several reports suggest that the bryophytes present distinct diversity and composition of endophytic bacterial communities from those of higher plants due to their unique ecological niche. However, the nature of the mutualistic relationship between endophytic bacteria and bryophytes is still being explored (1).

These endophytes and their secondary metabolites serve dual purposes in fundamental and applied research. They emerged as a significant potential source for discovering novel compounds with antimicrobial, anticancer and other bioactive properties. Recent studies revealed that compounds from bryophyte endophytes are synthesized solely by endophytic organisms and resemble those found in endophytes of higher plants. No evidence links bryophyte endophytes to the biosynthesis of host-specific compounds, highlighting the need for further research (2).

Recently, endophytic *Bacillus* species have gained researchers' attention for their abundance in plants, broad-spectrum antimicrobial properties and ability to produce resilient endospores that withstand UV radiation, drought, extreme temperature, pH and salinity (3). Several studies have indicated that endophytic bacteria associated with bryophytes possess antimicrobial and antagonistic activities (4). Bacteriocins an antibiotic produced by *Bacillus* spp., play a pivotal role as precursors of antibiotic drugs, biocontrol agents and bio-preservatives for food and beverages (5). *Bacillus megaterium* exhibited antagonistic and bi-preservative properties in broth and solid medium (6). Bacilysin is a cyclic dipeptide antibiotic isolated from *B. amyloliquefaciens, B. pumilus* and *B. subtilis* strains. Its dual attributes as an antibiotic with antifungal and antibacterial properties and its role as a biocontrol agent against a range of phytopathogens like *Erwinia amylovora*, highlight its importance in various fields including agriculture and medicine (7). Recently, from bryophytes endophytic *B. thuringiensis* strain LLB6 carrying the novel cry2Ac5 gene was reported that demonstrated the highest toxicity towards *Aedes albopictus* mosquitoes (8).

Furthermore, bryophytes-associated endophytes belonging to the division proteobacteria and actinobacteria are excellent candidates for plant growth promotion (3, 9). These endophytes can promote plant growth by enriching the cytokinin's impact on bud development and the proliferation of protonema filament, nitrogen fixation, as well as aiding the *in-situ* conversion of methane to carbon dioxide (9). Also, endophytic strains from moss such as *Micromonospora chalcea* CMU55-4 possess numerous genes required for the synthesis of IAA and siderophores, cold and heat shock proteins, trehalose biosynthesis, carbohydrate metabolism and glycine-betaine production. These genes facilitate resilience in host plants to stress conditions (9).

Bacilli are Gram-positive bacteria in various environments and can produce abundant secondary metabolites. However, none of the research previously focused on extracting and analyzing metabolites produced by moss-associated endophytic *Bacillus* species. As a result, the current study aimed to conduct a phytochemical analysis of the metabolites present in moss-associated endophytic *Bacillus* species.

Materials and Methods

Collection and treatment of samples

Mosses from different ecological niches were collected and washed thoroughly under flowing tap water followed by drying (Table 1). The surface was sterilized using 75 % ethanol and tween followed by treatment with 2 % sodium hypochlorite and finally rinsing 5-6 times with sterile distilled water. Excess water was blotted off in a laminar airflow chamber and sterile water aliquots from the last rinsing step were plated onto R2A agar to confirm the disinfection process. Subsequently, the Petri plates were incubated for 2 to 3 days at 28 °C to foster the growth of bacteria.

Isolation of endophytic bacteria

The surface-sterilized 1 g mosses were ground in a sterile mortar pestle using 6 mL sterilized 0.85 % sodium chloride under aseptic conditions to get a homogenous paste and allowed to settle down for 20 min. The sample (≈100 µL) was spread on R2A agar plates and incubated for 2-3 days at 28 °C, bacterial colonies considered endophytes were characterized according to different visual observation parameters such as morphology, size and color were used for colony selection and finally purified using the streak plate technique. Further, the selected colonies were plated and preserved at 4 °C for later use (10).

Screening for enzyme production

Proteinase production : The bacterial strains' ability to produce proteinase was assessed using a protease casein medium containing R2A agar supplemented with 1 % casein. Aliquots (1 µL of) cultures were plated on the R2A agar medium for 2-3 days at 28 °C in the incubator. The plates were observed for halo/clear zone formation (11).

Esterase production : For determination of esterase activity the basal medium (pH 7.0) consisting of peptone (10.0 g/L), $CaCl_2.2H_2O$ (0.1 g/L), NaCl (5.0 g/L), agar (18.0 g/L) supplemented with 1 % Tween 80 was used. Bacterial isolates were cultured on the medium-containing plates for 2-3 days at 28 °C in the incubator. Plates having colonies with a zone of hydrolysis indicated esterase production (12).

Cellulase production : The activity was estimated using 1% carboxy methyl cellulose in the R2A agar medium. Followed by saturation with 0.5 % (w/v) Congo red solution after incubation at 28 °C for 2–3 days. Isolates with yellow-colored zones are indicative of cellulase production (13).

Amylase production

Bacterial isolates (1 µL) were inoculated unto the R2A agar medium supplemented with 1 g/L of starch for 2 to 3 days at 28 °C in the incubator. Following incubation, the cultured plates were saturated with Gram's dye. The colonies with clear zones were considered positive for amylase production (14).

Table 1. List of moss samples collected from different locations

S.No	Plant Name	Herbarium number	Samples source	Abbreviation for Locality
1	*Hymnostylium recurvirostre*	BURI 1409/2022	Moist Soil	MS
2	*Barbula vinealis* Brid.	BURI-1414/2022	Shaded rocks	SR
3	*Plagiothecium cavifolium* (Brid.) Z. Iwats.	BURI-1408/2022	Tree Roots	RT

Morphological characterization of endophytic bacteria

Gram staining techniques were used to investigate morphological physiognomies (gram stain, culture purity and shape) of pure culture isolates (15). The heat-fixed bacterial isolates slide was treated with a series of staining and decolorization steps and then viewed by a compound bright-field microscope at the magnification of 1000X.

Molecular identification of endophytic bacteria

DNA was extracted using a modified phenol-chloroform-isopropanol method. The amplification of the 16S rDNA sequence was performed through polymerase chain reaction (PCR) with forward and reverse primers U16SRT-F (5'-ACTCCTACGGGAGGCAGCAGT-3') and U16SRT-R (5'-TATTACCGCGGCTGCTGGC -3'), respectively. PCR products were separated by the electrophoresis technique using 1.5 % agarose gel. The desired bands (≈ 200 bp size) were excised and purified with Hiyield Gel/PCR DNA Mini Kit as per the manufacturer's instruction and sequenced by Eurofins Genomic India Pvt. Ltd., Bangalore (India). Further, alignment software followed by a BLAST was used to identify the closest bacterial species in GenBank. For phylogenetic analysis, BLAST was used to retrieve a similar sequence from NCBI. The alignment was performed with ClustalW and the UPGMA tree (Fig. 1 & 2) was constructed through MEGA version 4 (16).

Secondary metabolite extraction from bacterial isolate

The secondary metabolites were extracted using bacterial suspension (200 µL) inoculated into R2A broth followed by incubation for 2 to 3 days at 28 °C 130 rpm on a rotating shaker. After centrifugation at 10000 rpm for 15 min, an equal volume of methanol (500 mL) was added to the supernatant and incubated overnight at 4 °C. The extracted secondary metabolites obtained in the solvent phase were separated using a separating funnel. The crude metabolites obtained after evaporation were redissolved in methanol and preserved at 4 °C for further use (17).

Fourier Transform Infrared (FTIR) Spectroscopy

The analysis of different functional groups in the bioactive compounds present in the methanolic extract of the bacterial isolates was measured by FT-IR (Thermo Scientific Nicolet iS50) spectrometer in the transmittance mode at range 4000–400 cm^{-1} (18).

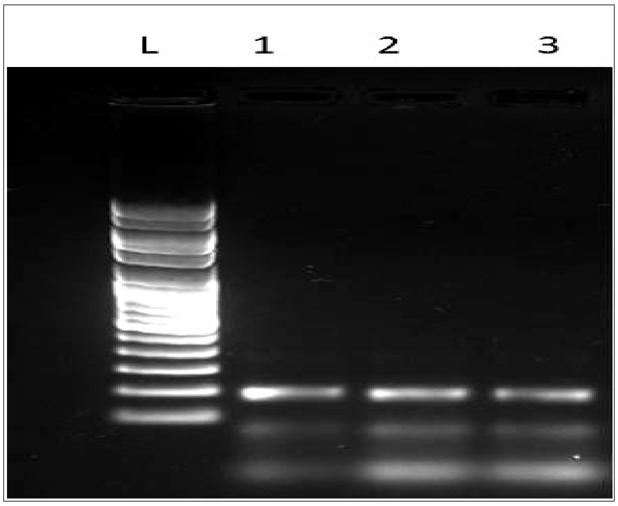

Fig. 1. Gel electrophoresis of 16S rDNA endophytes from mosses. L is a 3 kb DNA ladder as a marker.

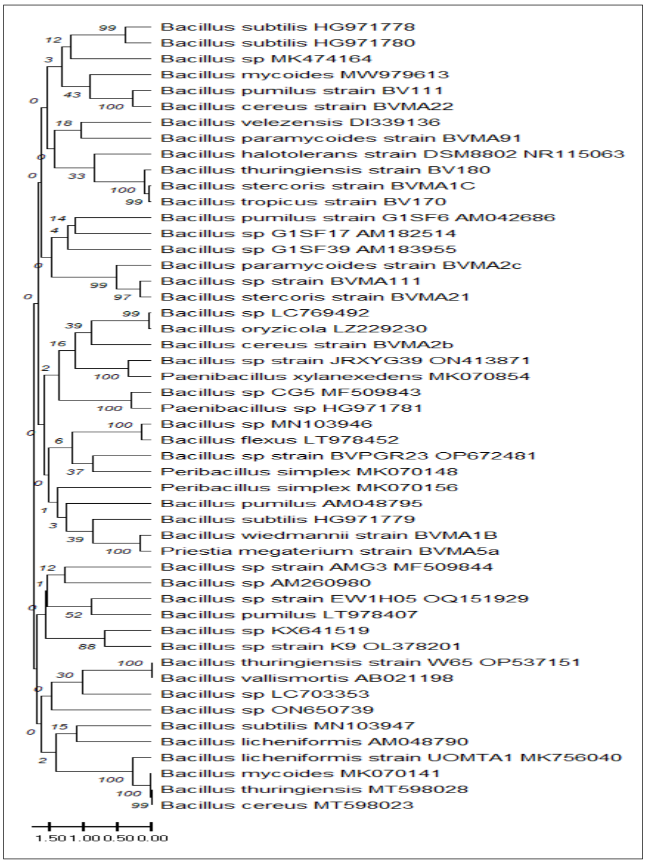

Fig. 2. Phylogenetic tree constructed based on the 16S rDNA sequences using ClustalW alignment tool and UPGMA clustering analysis within Mega 4 phylogenetic package.

GC-MS Analysis of Bacterial Metabolites

GC-MS analysis was performed in Thermo Scientific Triple quadruple GC-MS (trace 1300 GC, Tsq 8000 triple quadrupole MS) equipped with TG 5 MS (30 m × 0.25 mm, 0.25 µm) column 217 to identify the chemical compounds present in the bacterial extract. Carrier gas helium (99.99 %) was used in the constant flow mode (1 mL/min) with 2 µl of injection volume. The temperature of the injector and ion source was maintained at 250 °C and 230 °C, respectively. The oven temperature was raised to 280 °C with increase rate with an increase of 5 °C/min and maintained for 9 min at 280 °C. The ionization voltage was 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min and the total GC-MS running time was 36 min (17).

Results and Discussion

Most of the research till now has primarily focused on endophytic microorganisms inhabiting vascular plants, with a limited number of studies dedicated to endophytes found in non-vascular plants like bryophytes (2, 19). Most investigations primarily focused on the array of endophytic bacteria inhabiting their gametophytes, with limited attention given to analyzing metabolites these microorganisms produce and their potential properties (2).

Isolation and screening of endophytic bacteria

We have isolated 11 endophytic bacterial species exhibiting unique morphology from three mosses. Among these, nine bacterial isolates were Gram-positive and two were Gram-negative (Table 2). Based on morphological characterization

Table 2. Phylogenetic and morphological analysis of moss-associated endophytes

Isolates	Assigned bacterial Names	GenBank accession number	Similarities (%)	Shape of Cell	Gram reaction
1	*Bacillus pumilus*	BV111	100	Rod	+
2	*Bacillus paramycoids*	BVMA2c	92.27	Rod	+
3	*Bacillus wiedmannii*	BVMA1A	98	Rod	+
4	-	-	-	Rod	+
5	-	-	-	Rod	+
6	-	-	-	Rod	+
7	-	-	-	Rod	-
8	-	-	-	Rod	+
9	-	-	-	Rod	-
10	-	-	-	Rod	+
11	-	-	-	Rod	+

out of 11 bacterial isolates three were identified as species of the genus *Bacillus*. Endophytic bacteria belonging to the genus *Bacillus, Aeromonas* and *Pseudomonas* were reported from the bryophytes of Mount Abu (Rajasthan) (19). Various bacteria, including *Burkholderia* sp., *Hafnia* sp., *Methanobacterium* sp., *Methylobacterium* sp., *Pantoea* sp. and *Serratia* sp., are found on bryophytes in Japan (20, 21). The symbiotic associations of mosses with microorganisms enhance resilience to environmental stresses, allowing bryophytes to thrive in diverse habitats worldwide, from rocky terrains to polar regions and contribute significantly to ecosystem functioning (22).

Microorganisms are currently gaining increased attention as a reservoir of novel enzymes. This heightened interest arises due to the increased activity and stability of enzymes derived from microbes compared to enzymes derived from plants or animals. The 11 bacterial isolates were further qualitatively screened for four industrially important enzymes, namely esterase, cellulase, amylases and proteases on solid starch, cellulase, casein and tween 80 media. Each isolate demonstrated the potential to produce at least two of the analyzed enzymes. About 54 % of isolates produced proteinase and amylase, 72 % of isolates produced esterase and 63 % of the isolates produced cellulase. Most of the isolates were found to be enzyme producers (Table 3). Current findings are consistent with previous research that has shown endophytic bacteria such as *Bacillus sp.* can produce amylase, esterase, cellulose and proteinase extracellularly (23). These hydrolytic enzymes have a promising role in the promotion of plant growth and protection against phytopathogen. The bacteria amylase activity is also significant in promoting the seed germination process, which subsequently stimulates the enhanced growth of seedling roots and shoots (24). Like plant-associated microorganisms, cellulase activity can also improve the nutrient availability among endophytes (25). Six phosphate -solubilizing bacteria have been identified among 440 *Bacillus* isolates from various sources by earlier research (26). These strains also demonstrate a high capacity for synthesizing IAA (indole acetic acid), making them promising candidates for use as biofertilizers in agriculture. *Bacillus*-derived enzymes have been reported as promising candidates in agriculture and pharmaceutical industries, particularly in thrombolytic therapy and neurodegenerative treatment (27).

Table 3. Enzyme production test of endophytic bacteria isolated from mosses

Isolates	Enzyme production			
	Proteinase	Amylase	Esterase	Cellulase
1	+	+	+	+
2	+	+	+	+
3	+	+	-	+
4	-	-	+	+
5	+	-	-	+
6	-	+	+	-
7	+	-	+	+
8	-	+	+	-
9	-	+	-	+
10	+	-	+	-
11	+	-	+	-

Molecular characterization of endophytic bacteria

We conducted similarity analysis using 16S rDNA sequences for three bacterial isolates. The sequences with 16S sequences of endophytic bacteria available in GenBank databases enabled phylogenetically clustering of isolates within the genus *Bacillus.* More than 95 % similarity was reported between 3 individual isolated sequences. The isolates 1, 2 and 3 exhibited higher similarity indices with different stains of *Bacillus* namely, *B. pumilus, B. paramycoids and B. wiedmannii* (Table 2). However, *B. paramycoids* and *B. wiedmannii* species were included in *B. cereus* group (28). *B. pumilus* was included in the *B. subtilis species* complex (29). The reclassification of economically important strains is considered important as a plant protection product FZB 42 (DSM2311) was formerly identified as a *B. amyloliquefaciens* strain, based on molecular studies and is currently reclassified as *B. velezensis* (30). Among the species identified, they were phylogenetically clustered with other species such as *Bacillus cereus, B. mycoides, B. subtilis, B. megaterium* and *B. stercoris.* Furthermore, the diversity of endophytic bacteria is influenced by various factors encompassing cultivation conditions, species, plant age and geographical location.

Bacterial metabolite extraction and identification

The metabolites in plant endophytes can be produced by endophytic microorganisms alone or by plant-endophytes association (2). Endophytic *Bacillus* sp. has been identified as a source of a range of antifungal compounds such as isocoumarins, dehydrogenase, laccase, hydrolase, etc. and thus used as a biocontrol against a wide range of phytopathogens. Compared to other *Bacilli*, very little information is available about the secondary metabolites profile of *B. pumilus*. Thus, in the present study, FTIR and GC-MS analysis were conducted to determine secondary metabolites in *B. pumilus* extract (Fig. 3). FTIR of the metabolites detected 13 peaks for the metabolites produced by *B. pumilus*. These peaks corresponded to O-H stretching vibration, C-H stretching vibration of aliphatic Alkane, C-O stretching vibration of aliphatic amino, C-N stretching vibration for amines, S=O stretching vibration for sulfone, N–O stretching vibration for nitro-compound (Table 4). Secondary metabolite analysis by GC-MS in the *B. pumilus* unveiled the presence of the following- Paraldehyde, 2,2-Dimethylbutyl benzene, Pentadecanoic acid,13 methyl-, methyl ester, Benzoic acid, 2-

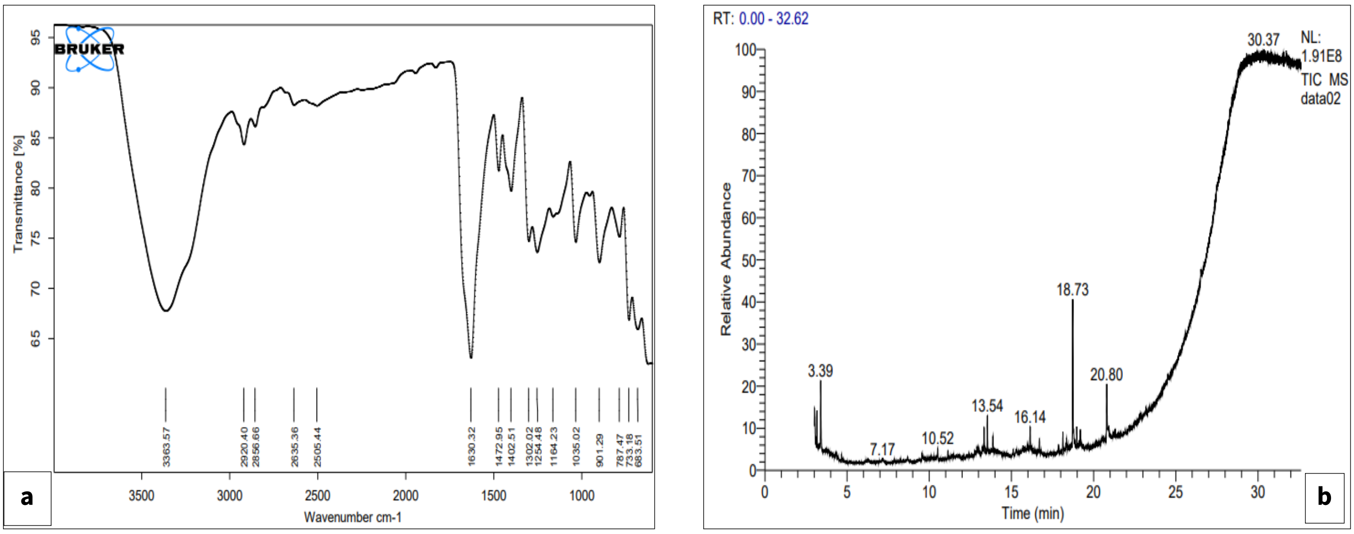

Fig. 3. Spectra of the secondary metabolites isolated from *Bacillus pumilus* using (a) FTIR; (b) GC-MS.

amino-6- chloro-methyl ester, Molybdenum, tricarbonyl tris (trimethyl phosphite-P)-, 3'H-Cycloprop(1,2)-5-cholest-1-en-3-one, Benzaldehyde,4-methoxy N hexadecanoic acid (Palmitic acid), Tungsten, dicarbonyl-(ü-4-pinocarvone) [1,2-bis (dimethyl phosphine) ethane] Boronic acid, ethyl-, dimethyl ester Phenol, 2,6-bis (1,1-dimethyl ethyl)-, Stigmastan-6,22-dien, 3,5-dihydro, Phthalic acid, 2TMS derivative Prostaglandin D(2), O, O'-bis (trimethylsilyl)-, trimethylsilyl ester (Table 5). Research has demonstrated the potential of *Bacillus*-derived bacitracin in inhibiting the growth of *Escherichia coli* and *Staphylococcus aureus* (31). Studies suggest that several *Bacillus* species produce a range of antimicrobial peptides each characterized by unique chemical structures including bacteriocins, iturin A and surfactin (32).

Bacillus species demonstrates the capacity to release a range of metabolites that can stimulate plant growth and provide protection against pathogenic infections. Based on GC–MS analysis, research has reported that the metabolites from *Bacillus* are lipopeptide derivatives (33). They reported a range of compounds including 1,2- Benzenedicarboxylic acid, phenol, 2,4-bis (1–1 dimethyl) and butyl 2-ethylhexyl ester, Hexadecanoic acid, Trioxoclane-2- octanoic acid 5 octyl, methyl ester and methyl ester, 9- Octadecenoic acid (Z)-methyl ester.

Also, twenty-nine compounds were identified from GC-MS analysis of metabolites extracted from *Bacillus* culture medium, predominantly 1,2-benzenedicarboxylic acid, 1,1-butoxy-1-isobutoxy-butane, 2-propanone, 3,3-ethoxy-carbonyl-5 -hydroxytetrahydropyran-2-one and 4,4-ethy-lenedioxy-1-pentylamine (34). A study reported similar compounds from *Bacillus* strains using GC-MS, highlighting the diverse properties of bacterial secondary metabolites, including antioxidant, anti-inflammatory and antimicrobial activities (35).

Table 4. Identified peaks in FTIR Spectra of the metabolite produced by the *Bacillus pumilus*

S. No.	Frequency	Group	Appearance	Compound
1.	787.47	Bending vibration of C=C	Medium	Alkene
2.	901.29	Bending vibration of C=C	Strong	Carboxylic acids
3.	1164.23	Stretching vibration of C-O	Strong	Aliphatic amines
4.	1254.48	Stretching vibration of C-N	Medium	Amine
5.	1302.02	Stretching vibration of S=O	Strong	Sulfone
6.	1402.51	Bending vibration of O-H	Medium	Phenol
7.	1472.95	Stretching vibration of S=O	Strong	sulfonyl chloride
8.	1630.32	Stretching vibration of N-O	Strong	Nitrocompound
9.	1725.89	Stretching vibration of C=O	Strong	conjugated acids
10.	2505.44	Stretching vibration of C-H	Strong	Aldehydes
11.	2856.66	Stretching vibration of C-H	Strong	Alkanes
12.	2920.40	Stretching vibration of C-H	Strong	Alkanes
13.	3363.57	Stretching vibration of O-H	Strong	Alcohol

Table 5. Compounds identified in the crude methyl alcohol extract of *Bacillus pumilus* by GC-MS analysis

S.No.	RT	Area	Names of the compound	Molecular formula
1.	3.39	45.67 %	Paraldehyde	$C_6H_{12}O_6$
2.	7.17	32.87 %	2,2-Dimethylbutyl benzene	$C_{12}H_{18}$
3.	13.06	13.67 %	Pentadecanoic acid,13 methyl-, methyl ester	$C_{17}H_{34}O_2$
4.	13.54	20.45 %	Benzoic acid, 2- amino-6- chloro-methyl ester	$C_8H_8ClNO_2$
5.	13.46	14.05 %	Molybdenum, tricarbonyltris(trimethyl phosphite-P)-	$C_{12}H_{27}MoO_{12}P3$
6.	16.14	12.43 %	3'H-Cycloprop(1,2)-5-cholest-1-en-3- one	$C_{32}H_{49}NO_3$
7.	18.73	16.56 %	Benzaldehyde,4-methoxy N hexadecanoic acid (Palmitic acid)	$C_8H_8O_2$ $C_{16}H_{32}O_2$
8.	20.80	10.45 %	Oleic acid	$C_{18}H_{34}O_2$
9.	27.89	11.97 %	Tungsten, dicarbonyl-(ü-4-pinocarvone)[1,2-bis(d imethylphosphino)ethane] Boronic acid, ethyl-, dimethyl ester Phenol, 2,6-bis(1,1-dimethylethyl)-	$C_{18}H_{30}O_3$ $C_4H_{11}BO_2$ $C_{14}H_{22}O$

Conclusion

The findings of this study demonstrated that bryophytes are the significant reservoir of a wide range of endophytic bacteria. However, the endophytic bacterial community structure and the metabolites produced by these bacteria have not yet been examined for these unexplored land plants. GC-MS analyses indicated that *Bacillus* spp. can produce many bioactive compounds, which are reported to have antimicrobial activity and these compounds have potential applicability in pharmaceutical and agricultural industries. However, these unexplored plants require the attention of researchers to identify and elucidate the composition of endophytes associated with bryophytes and their bioactive constituents with distinct biological activities that could be harnessed for the well-being of both humans and the environment.

Acknowledgements

The authors would like to thank Professor Ina Aditya Shastri, Vice-Chancellor, Banasthali Vidyapith for her encouragement and assistance. We are also grateful for DST networking assistance through the FIST programme at the Department of Bioscience and Biotechnology, as well as DBT support for the Bioinformatics Centre, which provided a facility to collect matter for this study.

Authors' contributions

The study was conceived and designed by AA. SP[1] conducted the experiments and drafted the manuscript. SP[2] analysed the data and results of the manuscript. All authors read and approved the final manuscript.

[SP[1] refers to Shivangi Pandey and SP[2] refers to Saumya Pandey]

Compliance with ethical standards

Conflict of interest: There is no competing interest stated by the authors.

Ethical issues: None

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