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Biodiversity and Antimicrobial Potential of Bacterial Endophytes from Halophyte *Salicornia brachiata*

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14 **ABSTRACT**

15 Untapped natural habitats like halophytes, marsh land, and marine environment are suitable arena for chemical ecology
16 between plants and microbes having environmental impact. Endophytes constitute an ecofriendly option for the
17 promotion of plant growth and to serve as sustainable resources of novel bioactive natural products. The present study
18 focusing on biodiversity of bacterial endophytes from *Salicornia brachiata,* led to isolation of around 350 bacterial
19 endophytes. Phylogenetic analysis of 63 endophytes revealed 13 genera with 29 different species, belonging to 3 major
20 groups: Firmicutes, Proteobacteria and Actinobacteria. 30% isolates belonging to various genera demonstrated broad-
21 spectrum antibacterial and antifungal activities against a panel of human, plant, and aquatic infectious agents. An
22 endophytic isolate *Bacillus amyloliquefaciens* 5NPA-1, exhibited strong in-vitro antibacterial activity against human
23 pathogen *S. aureus* and phytopathogen *X. campestris*. Investigation through LC-MS/MS-based molecular networking
24 and bioactivity-guided purification led to the identification of three bioactive compounds belonging to lipopeptide
25 class on the basis of ^{1}H- and ^{13}C-NMR and MS analysis. To our knowledge, this is the first report studying bacterial
26 endophytic biodiversity of *Salicornia brachiata* and isolation of bioactive compounds from its endophyte. Overall,
27 the present study provides insights into the diversity of endophytes associated with the plants from the extreme
28 environment as rich source of metabolites with remarkable agricultural applications and therapeutic properties.

29

30 **Keywords: *Salicornia brachiata*, Endophytes, Chemical ecology, Bioactive compounds, Lipopeptides**.

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32 **INTRODUCTION**

33 Ecological interactions are responsible for providing prime ecosystem services. Plant mediated interactions and
34 structure of natural communities go hand in hand as they potentially link organisms of different trophic levels and add
35 chemical complexity within communities leading to surging catalog of compounds. Endophytes, impart protection to
36 plants against various abiotic or biotic stress tolerance by production of plant hormones, bioavailability of nutrients,
37 and antagonistic action to phytopathogens in turn, sustaining on the nutrients provided by plants thereof [1].
38 Endophytic bacteria facilitates plant growth and developments through various processes including nitrogen fixation,
39 phosphate solubilization, production of hormones and siderophores, and decreasing ethylene concentration [2,3].
40 Along with host plant-growth cycle, endophytes also improvise their survival mechanisms during their continuous
41 efforts to live in the host tissues [4]. Biological control within host is mediated by endophytes that promotes plant
42 growth by protecting against the attack of phytopathogens, facilitated by the production of siderophores, antibiotics
43 or bacteriocins [5]. It has been proved that bioactive compounds derived from plants are mostly secondary metabolic
44 products of microbes inhabiting inside the plants symbiotically defined as endophytes [6]. Predominantly,
45 actinobacterial and bacterial endophytes contribute heavily in plant growth promotion and agriculture management
46 strategies via production of metabolites such as aromatic compounds, lipopeptides, plant hormones, polysaccharides,
47 and several enzymes linked to phenylpropanoid metabolism [7].

48 Halophytes are plants that thrive in saline environment with salinity upto 200 mM concentration [8]. Endophytic
49 bacteria and fungi isolated from halophytes aid hosts by altering plant hormone status and uptake of nutrient elements
50 and/or modulating the production of reactive oxygen species through different mechanisms [9,10]. Strains of *Bacillus*
51 *amyloliquefaciens* are reported to enhance plant growth promotion and also provide defense benefits against
52 phytopathogens like *Phytophthora parasitica var. nicotiana*, *Fusarium oxysporum* sp. , *F. graminearum, F. solani,*
53 *Alternaria alternate* etc. [11,12]. Beneficial metabolites produced due to ecological interactions between endophyte-
54 plant can be harnessed and employed for multifaceted applications in arena such as agriculture, medicine,
55 bioremediation, and biodegradation. Despite their beneficial characters, the research regarding endophytes from plants
56 inhabiting extreme environments is still at an early stage with respect to diversity of endophytes, their functional roles,
57 and bioprospecting for bioactive compounds.

58 *Salicornia brachiata* is a halophyte with medicinal properties having salty marsh lands as the natural habitat getting
59 exposed to extremities of salinity, heat, temperature, and humidity [13]. *S. brachiata* was reported to harbor plant-
60 growth promoting microorganisms [14,15]. It can be hypothesized that *S. brachiata* being a plant inhabiting in extreme
61 environment, must have evolved ways to harbor diverse kinds of endophytes apart from plant-growth promoters and
62 to employ those endophytes for protection from herbivores and pests including phytopathogenic fungi and insects,
63 competing for nutrients including trace elements. Motivated by this hypothesis, present study investigated diversity
64 of bacterial endophytes associated with the halophyte *S. brachiata* sampled at three distinct locations at Gujarat coast,
65 India. Further, their bioactive potential was also studied against a panel of human, plant, and aquatic infectious agents.
66 Repeated chromatographic separation resulted in isolation of three compounds from the endophyte *B.*

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67 *amyloliquefaciens* . This may be the first comprehensive study evaluating bacterial endophytic biodiversity of the
68 halophyte *Salicornia brachiata.*

69 METHODS

70 Collection of plant material and isolation of endophytes

71 Healthy and fresh plant samples of *S. brachiata* were collected randomly from three different sites i.e. New port (N
72 21° 45′ 15.7′′, E 072° 14′ 01.4′′), Sartanpar port (N 21° 17′ 52.1′′, E 072° 66′ 25.5′′), Victor port (N 20° 58′ 53.2′′, E
73 071° 33′ 21.2′′), located along Gujarat coast, India (**Figure 1**). Samples were placed in sample bags and stored on
74 icebox right after sampling to preserve microbial flora and then the plant material was transported to laboratory and
75 processed immediately.

76 The plant material was surface sterilized followed by different treatments to enhance the probability of maximum
77 number of novel bacterial or actinobacterial species. Surface sterilization of the samples was carried out by reported
78 method [16]. Sterilized samples were aseptically fragmented into small pieces and directly placed on eight selective
79 media prepared with cycloheximide and nystatin at concentration of 50 mg/ml to inhibit fungal growth. Sterilized
80 samples were given six different pre-treatments (**Table I**) and plates were incubated at 30 °C for 2 to 8 weeks. Control
81 plates inoculated with last wash of sterilization procedure was incubated to check effectiveness of surface sterilization
82 in triplicate. Periodic growth analysis and subsequent sub-culturing for purification of isolates was performed.
83 Glycerol stocks of purified strains were prepared and stored at -80 °C.

84 Identification of endophytes

85 DNA isolation was performed using reported protocol with some modification [17]. Briefly, 2 ml of 24 h grown
86 bacterial culture (48 h for slow growing bacteria) was centrifuged for 2 min at 13000 rpm. Cell pellet was resuspended
87 in 600 µl TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0). 100 µl of 10 mg/ml lysozyme was added and incubated
88 at 37 °C for 1 h followed by addition of 20 µl of 20 mg/ml Proteinase K and incubation at room temperature for further
89 1 h. After incubation, 200 µl of 10% SDS was added and kept at 55 °C for 1 h followed by purification through
90 extraction of aqueous phase with phenol : chloroform : isoamyl alcohol (24:24:1). DNA was precipitated using 3M
91 sodium acetate and chilled isopropanol and the obtained DNA pellet was washed by ethanol. 16S rRNA amplification
92 was done using universal primer sequences of FD_1 (5′– GAGTTTGATCCTGGCTCA-3′) and RP_2 (5′–
93 ACGGCTAACTTGTTACGACT-3′). Reaction mixture, consisting of DNA template – 1 µl (50 ng/µl), Primers – 0.5
94 µl of 10 µM, dNTP – 0.2 mM and Taq polymerase – 1.25 units, was prepared and the reaction was performed in
95 Thermocycler (Bio-Rad T100) with conditions: Initial denaturation – 95 °C for 5 min, 34 cycles of 95 °C for 30 s, 58
96 °C for 30 s and 55 °C for 1 min and final amplification for 5 min.

97 Amplification of 16S rRNA gene was confirmed by gel electrophoresis; subsequently PCR products were purified
98 with Qiagen PCR purification kit and sequenced by Macrogen Inc. Korea. The obtained sequences were trimmed to
99 align in BioEdit software (version 7.0.5.3) [18] and consensus sequence created was searched in NCBI GenBank
100 database using BLAST. Further, 16S rRNA sequences were aligned and used to construct maximum likelihood
101 phylogenetic tree using molecular evolutionary genetic analysis MEGA 6 software [19].

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102 **Statistical data analysis**

103 Simpson index of diversity and Shannon-Wiener's diversity index were calculated to determine endophytic diversity
104 obtained from samples of all the three locations [20].
105 Simpson's index of diversity (D) gives the probability that two individuals selected at random will belong to the same
106 species and was calculated using formula as given in the following equation:

107 $$D = 1 - \Sigma pi^2$$

108 Shannon-Wiener diversity index (H) determines actual diversity of the bacterial endophytes, and was calculated using
109 the following equation:

110 $$H = -\Sigma\, pi(lnpi)$$

111 where $p_i = n/N$;
112 n = total number of organisms of species 'i'.
113 N = total number of organisms of all species.
114 Shannon evenness (E) was calculated as H'/H_{max}, where $H_{max} = \ln(S)$,with S as the total number of species in the
115 sample. Further, to determine qualitative wealth species richness was also calculated by $S/\sqrt{N}$.

116 **Antimicrobial potential of endophytes**

117 A panel of 8 reference human, plant, and marine pathogens comprising of *Staphylococcus aureus* MCC 2043, *Bacillus*
118 *subtilis* MCC 2049, *Mycobacterium smegmatis* MTCC 6, *Escherichia coli* MCC 2412, *Candida albicans* MCC 1152,
119 *Xanthomonas campestris* NCIM 5028, *Fusarium oxysporum* NCIM 1008, *Alteromonas macleodii* NCIM 2815 was
120 employed to determine bioactive potential of all the endophytic isolates. 10 ml culture of endophytes in 50 ml tubes
121 was done for a period of 14 days at 30 °C, 180 rpm shaking condition, in-between assessing it at an interval of 7 days
122 for the antimicrobial activity. Whole cell culture and supernatant (obtained after centrifugation) were used to perform
123 well diffusion assay [21] for determination of bioactivity.

124 **Preparation of crude extracts**

125 In order to confirm endophytes play important role in survival of halophyte *S. brachiata* in extreme climate by
126 protecting against pathogens, crude extracts of isolates which were found bioactive in primary screening were
127 prepared. Briefly, 250 ml culture broth in 1000 ml Erlenmeyer flask in particular medium and incubation conditions
128 specific for an isolated strain was done. After incubation period, culture broths were extracted twice with equal volume
129 of ethyl acetate. Organic phase was collected over anhydrous sodium sulfate and concentrated under rotary evaporator
130 to yield dark-brown colored crude extracts. The activity of crude extracts of isolates bioactive against *S. aureus* MCC
131 2043 and *X. campestris* NCIM was confirmed through disk diffusion assay by loading 1 mg of extract on the disk
132 following Clinical and Laboratory Standards Institute (CLSI) protocols [22].

133 **LC-MS/MS based molecular networking**

134 LC-MS/MS data of the crude extract of *B. amyloliquefaciens* 5NPA-1 was studied with GNPS (Global natural products
135 social molecular networking) (https://gnps.ucsd.edu) [23]. Raw data received from Agilent 6545 Q-TOF LC/MS was
136 converted into GNPS compatible mzXML format using MSconvert application (version 3.0.19317-0ef6e44d0) in

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137 Proteowizard suit [24]. The converted file was uploaded to GNPS server (massive.ucsd.edu) using WinSCP FTP
138 client. Molecular networking was run as classical molecular networking work flow (METABOLOMICS-SNETS-V2)
139 with activated MS-Cluster. Parameters for input algorithm were set as: precursor ion mass tolerance 2.0 Da, fragment
140 ion mass tolerance 0.5 Da, minimum cosine score for an edge formation by two consensus MS/MS spectra as 0.7,
141 minimum number of common fragment ions as 6 (no. of fragment shared by two separate consensus MS/MS spectra
142 in order to be connected by an edge in the molecular network), minimum cluster size was 2, and edges between two
143 nodes were considered only if both nodes were within top 10 most similar nodes of each other. Molecular network
144 file was downloaded in GraphML format and visualized using Cytoscape (version 3.7.2) [25].

145 **TLC bioautography**

146 Ethyl acetate extract of endophyte *B. amyloliquefaciens* 5NPA-1 was subjected to thin layer chromatography (TLC)
147 analysis over analytical aluminium silica gel 60 TLC plate for separation of metabolites to obtain R_f value of active
148 fraction. The crude extract was dissolved in methanol to get a concentration of 10 mg ml^{-1} and 1 mg was loaded on
149 TLC plate which was developed with solvent system comprising of methanol and chloroform in the ratio 12:88.
150 Separate bands were observed under short wavelength UV light and their R_f values were calculated. To perform
151 bioautography, 1.5 ml of 1% mueller hinton agar (MHA) was spread on the developed TLC plate of size 10 × 2 cm
152 under sterile environment of biosafety cabinet and 50 μl of log phase culture of *S. aureus* MCC 2043 was spread with
153 the help of sterile spreader. After incubation for 24 h at 30 °C, the plate was visualized by spraying
154 iodonitrotetrazolium chloride solution (INT, 2 mg ml^{-1}) pink color on plates signified cell growth whereas bands with
155 clear zone indicated inhibition of cell growth. R_f values of the bands with clear zone were recorded [26].

156 **Isolation and identification of bioactive molecules**

157 Purification of compounds was carried out by separating 50 mg of crude extract on preparative TLC plates (Kieselgel
158 60 F_{254}, 25 mm, Merck) using the same solvent system as above. Based on TLC bioautography, the active portion was
159 selectively scrapped and dissolved in methanol. The supernatant was concentrated on rotary evaporator to obtain 15
160 mg of yellow, viscous oil (5-PTLC2), which was analyzed by HPLC. Further, ^{1}H-NMR spectrum (Bruker, Avance II
161 500 MHz in CD_3OD) of 5-PTLC2 was acquired in order to identify the class of compounds. Additionally, to purify
162 active compounds, the crude extract (2 g) was fractionated by MPLC (C18 RP-silica gel) eluting with aqueous MeOH
163 (30% to 100%) resulting in the collection of 18 fractions (fr. 1–fr. 18) which were evaluated for antimicrobial activity
164 against *S. aureus* MCC 2043. Fractions 11 and 13 containing bioactive compounds were further chromatographed on
165 semi-preparative HPLC (Dionex Ultimate 3000, Thermo Scientific) using gradient mixtures of acetonitrile–water (4:1
166 to 9:1) on YMC column (YMC-Triart C18, 5 μm, 250 × 10 mm I.D.) to yield three compounds (**1**–**3**). The three
167 compounds were further characterized by MALDI-MS and MALDI-MS/MS (Applied Biosystems 4800 MALDI
168 TOF–TOF analyzer) and NMR (JEOL 600 MHz).
169

170 # RESULTS

171 **Collection of plant material and isolation of endophytes**

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172 *S. brachiata* samples collected from three different sites along Gujarat coast (**Fig. 1**) were processed using eight
173 different media with six variable pretreatments resulting in isolation of 336 endophytes differentiated on the basis of
174 morphological characters (**Fig. S1**). The high number of isolates obtained were also found to be equally diversified.
175 Also, effectiveness of surface sterilization was established as no growth was observed on the negative control plate
176 even when incubated for a month long period. Maximum number of bacterial isolates were obtained on medium
177 supporting fast growing microbes i.e. 81 isolates collectively from NA, ZMA, and TSA; 52 from SCA; 52 from ISP2;
178 50 from AIA; 70 from ISP4; 31 from TWYA (**Fig. S2**). The diverse group of isolated actinobacteria preferred ISP4
179 media for their growth and pigment production.

180 **Identification of endophytes**

181 Out of 336 isolates, 63 isolates obtained from different media were identified on the basis of molecular phylogeny by
182 16S rRNA sequencing. Evolutionary relationships of different endophytes obtained from *S. brachiata* were inferred
183 from maximum likelihood phylogenetic tree (**Fig. 2**). On the basis of 16S rRNA sequencing results, the most
184 predominant as well as diverse genera identified was *Bacillus* being 76% of the isolates with 15 different species. The
185 remaining percentage accounted for various genera of actinomycetes or bacteria like *Isoptericola, Paenibacillus,*
186 *Nocardiopsis, Rhodococcus, Salinicola, Jonesia, Nitratireductor*, *Enterococcus, Streptomyces, Micromonospora,*
187 *Pseudomonas* and *Marinilactibacillus* (**Table II**).

188 **Statistical data analysis**

189 α-diversity indexes, i.e. Shannon- Wiener diversity index and Simpson's index of diversity and their components;
190 richness and evenness were used to determine diversity of the endophytic community isolated from *S. brachiata*
191 sampled from three different locations. Shannon-Wiener diversity index (H) was calculated as 3.028, indicating high
192 diversity within endophytes. Species richness indicating abundance of the species in a sample was found to be
193 3.615,viz., greater the value higher the richness. Shannon evenness measures relative abundance of different species
194 contributing to richness of a sample, was calculated as 0.899 signifying an even community structure [20]. In
195 agreement with aforementioned data, Simpson's index of diversity (D) of 0.931, demonstrate a high diversity of
196 endophytes harbored by the host halophyte (**Table III**).

197 **Antimicrobial potential of endophytes**

198 From the primary screening study, a total of 101 isolates were found to exhibit broad-spectrum antimicrobial activity
199 against one or the other pathogen from panel of pathogens tested. 8 isolates were found to be active against *M.*
200 *smegmatis* MTCC 6; 20 against *S. aureus* MCC 2043; 51 against *X. campestris* NCIM 5028; 42 against *A. macleodii*
201 NCIM 2815; 12 against *C. albicans* MCC 1152; 14 against *F. oxysporum* NCIM 1008; 19 against *B. subtilis* MCC
202 2049; 5 against *E. coli* MCC 2412 (**Fig 3a**). It was observed that some endophytes displayed inhibition activity against
203 two or more pathogens (**Table IV**).

204 **Preparation of crude extracts and**

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205 Crude extract of bioactive strains were prepared through solvent-solvent extraction method. Bioactivity of isolates
206 active against *S. aureus* MCC 2043 and *X. campestris* NCIM 5028 was confirmed by disk diffusion assay (**Fig. 3b**),
207 according to the guidelines of CLSI. The isolate *Bacillus amyloliquefaciens* 5NPA-1 exhibited a prominent zone of
208 inhibition of 29 mm and 14 mm against pathogens *S. aureus* MCC 2043 and *X. campestris* NCIM 5028 respectively,
209 serving as a potential isolate for isolation of bioactive compounds which are beneficial to plants as well as humans.

210 **LC-MS/MS based molecular networking**

211 Molecular networking of crude extract of *B. amyloliquefaciens* 5NPA-1 using the GNPS platform was found to consist
212 of 24 nodes grouped into 4 clusters. Largest cluster had 5 nodes, which was annotated as surfactin by automatic
213 dereplication using MS/MS spectral libraries available at GNPS (**Fig. 4**). The network resulted into identification of
214 3 types of surfactin with respect to variations in length of fatty acid chain. Further, no specific networks denoting other
215 class compounds were observed, supporting the idea that lipopeptides in *B. amyloliquefaciens* 5NPA-1 could be
216 responsible for the bioactivity.

217 **TLC Bioautography**

218 In order to identify bioactive metabolites, TLC plate was developed to obtain 7−8 bands as observed under short UV
219 radiations (254 nm wavelength). Developed TLC plate when overlaid with *S. aureus* MCC 2043 suspension on 1%
220 agar, displayed clear zone with no cell growth against pinkish background at R_f between 0.12 to 0.41 (**Fig. 5**).

221 **Isolation and identification of bioactive molecules**

222 The active middle band (5-PTLC-2) obtained from TLC-bioautography was scrapped in order to acquire ^{1}H-NMR
223 spectrum to identify the chemical class of the compounds. ^{1}H-NMR spectrum of PTLC-2 exhibited signals for a long
224 aliphatic alkyl chain at δ_H 1.29, CH_3 groups at δ_H 0.85−1.00, an oxy-methine at δ_H 5.31, seven α-H at δ_H 4.05−4.80,
225 indicating lipopeptide nature of compounds present in the fraction PTLC2 (**Fig. 6**). HPLC-DAD chromatogram of
226 PTLC-2 revealed three peaks suggesting it to be a mixture of three compounds (**Fig. S3**). Hence, for their targeted
227 separation, the crude ethyl acetate (EtOAc) extract was subjected to MPLC followed by HPLC (**Fig. S4**) resulting
228 three compounds **1**, **2**, and **3** (**Fig. 7a**). The MALDI-TOF-MS spectra of **1**, **2**, and **3** exhibited intense sodium adduct
229 $[M+Na]^+$ ion peaks at *m/z* 1031.2147, 1045.0100 and 1058.8456, respectively. Comparison of the MALDI-TOF-MS
230 data of **1**, **2**, and **3** with those available in literature revealed that the purified compounds belonged to surfactin class
231 with different number of CH_2 units in fatty acid chain and subsequently identified as C_{13}-surfactin (**1**), C_{14}-surfactin
232 (**2**), and C_{15}-surfactin (**3**) [27] (**Fig. S5,S6,S7**). To determine the sequence of the amino acids, the sodiated ions of **1**,
233 **2**, and **3** were separately subjected to tandem MS experiment giving rise to spectra with diagnostic ion series containing
234 C- and N-terminus. The MS/MS spectra of compounds **1**, **2**, and **3** displayed a major ion sequence of fragments *m/z*
235 707.4, 594.3, 481.2, 382.1, 267.1 corresponding to loss of (fatty acid)-Glu-Leu-Leu-Val-Asp-Leu-Leu from C-
236 terminal region. Similarly, another major fragment ion series of **1** [*m/z* 945.5, 832.5, 717.4, 618.4], **2** [*m/z* 931.5,
237 818.5, 702.4, 604.3], and **3** [*m/z* 917.5, 804.4, 688.4, 590.3] confirmed the presence of amino acid residues sequence
238 as Leu-Leu-Asp-Val-Leu-Leu-Glu-(fatty acid) from the N-terminal region. Taken together, the connection of the two
239 series suggested that the surfactins **1**, **2**, and **3** contained the same heptapeptide sequence (fatty acid)-Glu-Leu-Leu-

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240 Val-Asp-Leu-Leu (**Fig. S8,S9,S10**). ^{13}C NMR chemical shifts allowed to differentiate branching of the hydroxy fatty
241 acid side chain among the *normal* (δ_C 13.8, 22.0, 31.2), *iso* (δ_C 22.4, 22.4, 27.3, 38.4), and the *anteiso* (δ_C 11.1, and 19.0)
242 chain types [28]. Based on this approach, the β-hydroxyl fatty acid chains were found to be mixtures of *iso*-$C_{10}H_{21}$ and
243 *anteiso*-$C_{10}H_{21}$ in **1**; *n*-$C_{11}H_{23}$, *iso*-$C_{11}H_{23}$, and *anteiso*-$C_{11}H_{23}$ in **2**; and, *iso*-$C_{12}H_{25}$ and *anteiso*-$C_{12}H_{25}$ in **3**, respectively
244 (**Fig. 7b**). On the basis of the previous studies, absolute configurations of amino acid units from N- to C-terminal of **1**,
245 **2**, and **3** were assumed to be L-, L-, D-, L-, L-, D- and L-, respectively, and the C-3 configuration of fatty acid was
246 assumed to be as *R* [29].

247 **DISCUSSION**

248 Endophytes, plant associated symbionts, have emerged as an interesting source for natural products because of their
249 diversity in bioactive secondary metabolites. Halophytes are reported to overcome their abiotic and biotic stress with
250 the help of metabolites, regulators or enzymes released from endophytes [30]. In this study, diversity of bacterial
251 endophytes from *S. brachiata* was assessed and their probable role in host-plant interaction including identification of
252 metabolites produced by one of the endophyte *B. amyloliquefaciens*. All endophytes were screened for antimicrobial
253 potential against human, plant, and marine pathogens. To our knowledge this is a first comprehensive report regarding
254 diversity of bacterial endophytes from *Salicornia brachiata* from Gujarat coast of India and investigation of their
255 antimicrobial potential.

256 Surface sterilized plant material with six different pretreatments and eight different previously reported media resulted
257 into a total of 336 endophytes delivering a quantitative idea about the bacterial diversity within the halophyte. The
258 results indicated a mixed composition of the endophyte communities comprising majorly firmicutes followed by
259 actinobacteria and proteobacteria. Studies on endophytic bacteria from different parts of halophytes *Salicornia*
260 *europaea, Arthrocnemum macrostachyum* etc. have been performed previously and predominance of aforementioned
261 phyla was observed [31,32]. Out of 336 endophytes, 63 isolates identified in the study represented 13 genera with 29
262 different species. *Bacillus* as a dominant genus was observed with a diversity of around 15 different species. *Bacillus*
263 sp. due to their better resilience is usually the dominant firmicute isolated from saline environments [30,33]. The
264 statistical analysis showed a higher richness and evenness of species diversity among isolated endophytes. Amidst the
265 endophytes obtained in our study from *S. brachiata*, genera *Bacillus, Isoptericola, Streptomyces, Salinicola,*
266 *Rhodococcus* have already been reported from sister species of *Salicornia europaea* [32,34]. *Nocardiopsis, Jonesia,*
267 *Nitratireductor, Paenibacillus, Micromonospora* are some genera from *S. brachiata* we report in our study.

268 It is well established that endophytes support plant ecological progression through production of various metabolites.
269 Processes supported by these metabolites increases bioavailability of nutrients to host, tolerance against abiotic stress
270 and strength to fight against biotic stress including pests and phytopathogens. Among the isolated strains in the study
271 *Bacillus* sp. is predominantly reported to produce ACC deaminase enzymes to alleviate stress by ethylene, indole
272 acetic acid and gibberellic acids promoting cell division and growth [30], phosphate solubilization enzymes, biological
273 nitrogen fixation, siderophores and bioactive metabolites against phytopathogens [5,31,35]. Bioactive metabolites
274 from *Bacillus* sp. include polyketides bacillomycin, fengycin, iturin, lichenysin, mycosubtilin, plipastatin,
275 pumilacidin, and surfactin [36]. Hence, abundance of *Bacillus* endophytes can be validated due to its profuse chemical
276 interactions with host plant. The strains *Salinicola sp.* and *Rhodococcus sp.* also display ACC deaminase activity

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277 important for plant growth promotion events in stress conditions [34]. Moreover, endophytic *Salinicola* sp. isolated
278 from *Spartina maritima* was reported to be an excellent producer of siderophores and contain heavy metal tolerant
279 genes thereby supporting the plant to alleviate the toxic effect of heavy metals [37]. The actinobacterial genera of
280 *Nocardiopsis* and *Isoptericola* [38] thrive in the saline conditions. Genetic makeup of *Nocardiopsis* is filled with
281 megaplasmid genes encoding antibiotic productions like apoptolidin, lipopeptide biosurfactants, thiopeptides,
282 griseusin D etc., heavy metal resistance and stress response including osmoregulation benefitting their survival in
283 halophilic environment [39]. It can be said that the ecological stress within host plant stimulated production of such
284 bioactive metabolites in *Nocardiopsis*. *Jonesia denitrificans* as the name suggests is reported to perform denitrification
285 [40]. Various strains of *Streptomyces* are reported to exhibit phosphate solubilization property, ammonia production,
286 enzymes production for breakdown of organic matter, PKS and NRPS gene clusters for production of bioactive
287 compounds etc. which contributes to plant health either directly or indirectly [41]. These reports reflect direct plant-
288 microbe interactions of the isolates obtained in study, further supporting their endophytic origin from halophyte.
289 In-vitro screening of the isolates for bioactivity revealed one third of the population to be bioactive against one or
290 more reference pathogen. This aligns with the work of Verma *et al*. who reported 60% of the endophytic actinobacteria
291 isolates obtained from *Azadirachta indica* showed wide-spectrum antagonistic potential [42]. Given their metabolite
292 productions, a large number of isolates exhibited inhibition of growth of plant pathogens *X. campestris* NCIM 5028,
293 *F. oxysporum* NCIM 1008 and marine bacterial pathogen *A. macleodii* NCIM 2815. Inhibition of phytopathogens at
294 such enormous amount indicates role of endophytes in defense mechanisms of host plant. Some of the isolates were
295 found to inhibit *M. smegmatis* MTCC 6, *S. aureus* MCC 2043, etc., suggesting that the antimicrobial activity exhibited
296 by plant *S. brachiata* [13] can be attributed to the bioactive metabolites secreted by inhabiting endophytes. The genus
297 *Bacillus* was dominant in displaying activity against all indicator pathogens pertaining to its siderophore and bioactive
298 lipopeptide production potential. Production of siderophores was reported from halotolerant *Bacillus* isolated from
299 wheat seedlings , further it improve soil fertility increasing plant productivity in agriculture and also remediates toxic
300 metals from human body [43]. *Bacillus* sp. have been isolated as endophytes from ginger, turmeric etc. and shows
301 enormous antifungal properties and antibacterial properties due to the presence of cyclic lipopeptides [44].
302 In the present work, along with endophytic biodiversity, an emphasis was also laid on isolate displaying activity
303 against *S. aureus* MCC 2043, a common nosocomial pathogen and *X. campestris* NCIM 5028, a meticulous plant
304 pathogen. Crude extract of isolate *B. amyloliquefaciens* 5NPA-1 (MT459305) was found to be most potent with zone
305 of inhibition of 29 mm and 14 mm against *S. aureus* MCC 2043 and *X. campestris* NCIM 5028 respectively. Molecular
306 networking using LC-MS/MS data of crude extract gave an idea about the presence of secondary metabolites
307 encrypted in the strain *B. amyloliquefaciens* 5NPA-1 indicating surfactin type compounds. High potency of crude
308 extract served as a driving force for purification of bioactive compounds to identify its potential as strong antimicrobial
309 agents. Following that paradigm, purification and characterization of active compounds from *B. amyloliquefaciens*
310 5NPA-1 was also performed leading to compounds **1–3** belonging to lipopeptides class. Lipopeptides form an
311 important class of metabolites from endophytic bacteria, wherein serving as antibiotic and inducing plant systemic
312 resistance. *B. amyloliquefaciens* was recognized as a higher lipopeptide producer when isolated from different plants
313 including *Phaseolus vulgaris*, *Oryza sativa*, *Ophiopogon japonicus*, *Musa acuminata*, marine plants etc., meanwhile

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314 also secreting plant growth promoters, phyto-hormones, siderophores, antifungal, anticancer and antimicrobial agents
315 [36]. Diversity of endophytes within plant structures is proportional to various benefits of plant-microbe interactions.
316 Such interactions are of ecological importance as they improve adaptation capabilities of either species and improve
317 soil fertility and texture. An understanding of the chemical ecology of plants-microorganisms should enable the
318 development of new crop improvement strategies, the conservation of indigenous varieties, and definitely a source of
319 interesting pharmaceutical compounds.
320 To conclude, present study was the first attempt where endophytic bacterial community residing in stress-tolerant
321 halophyte *S. brachiata* was studied and examined for the production of antimicrobial compounds against pathogens
322 of various niches. Through identification of 20 % isolates, it was revealed that the plant harbors a rich bacterial
323 biodiversity accounting for 13 genera and 29 species with *Bacillus* being dominant and distinct actinobacteria
324 exhibiting different morphology, producing pigments, metabolites and polysaccharides which benefits the plant.
325 Metabolites from species inhabiting the plant have history in supporting the host plant through various chemical
326 interactions. It was also deciphered that surfactin class molecules produced by endophytic strain *B. amyloliquefaciens*
327 5NPA-1 possess high biocontrol properties against nosocomial pathogen and bacterial plant pathogen. *Bacillus*
328 *amyloliquefaciens* being an environmentally stable and fast replicating bacteria serves as an ideal source for extraction
329 of plethora of metabolites. Such enormous antimicrobial potencies displayed by several endophytes from *S. brachiata*
330 indicate their role in plant defense system, and serve as an example of plant microbe interaction. This diverse
331 population can be further explored for novel metabolites given that demand for novel bioactive agents is everlasting
332 and it may help us with better understanding the chemical ecology of an ecosystem.

333 **Data Availability Statement**

334 The datasets generated for this study are available on request to corresponding author.

335 **Conflict of Interest**

336 *The authors declare that the research was conducted in the absence of any commercial or financial relationships that*
337 *could be construed as a potential conflict of interest.*

338 **Author Contributions**

339 Sanju Singh, Vishal Ghadge, Pankaj Kumar, and Pramod B. Shinde designed and planned the research. Sanju Singh,
340 Vishal Ghadge, and Pankaj Kumar isolated and identified the bacterial strains. Sanju Singh and Doniya Elze Mathew
341 performed bioactivity. Pankaj Kumar performed molecular networking analysis. Sanju Singh, Asmita Dhimmar,
342 Pankaj Kumar, Harshal Sahastrabudhe, and Yedukondalu Nalli isolated and identified bioactive secondary
343 metabolites. All authors analyzed and interpreted the results and commented on the manuscript prepared by Sanju
344 Singh and Pramod B. Shinde.

345 **Funding**

346 This research was supported by Scientific and Engineering Research Board (SERB), Department of Science and
347 Technology [ECRA/2016/000788 and EEQ/2016/000268]; and Council of Scientific and Industrial Research
348 [MLP/0027].

349 **Acknowledgments**

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350 Sanju Singh, Doniya Elze Mathew, Asmita Dhimmar acknowledges CSIR-JRF fellowship from Council of Scientific
351 and Industrial Research (CSIR), Pankaj Kumar acknowledges DBT-JRF fellowship from Department of
352 Biotechnology (DBT) and Harshal Sahastrabudhe acknowledges GATE-JRF fellowship from Council of Scientific
353 and Industrial Research (CSIR). Yedukondalu Nalli acknowledges Scientific and Engineering Research Board
354 (SERB) for providing National Postdoctoral Fellowship (N-PDF).

355 **Supplementary Material**

356 A schematic diagrams of isolation of endophytes and compounds, HPLC chromatograms, fragmentation pattern, and
357 MS- and MS/MS-spectra of compounds **1–3** is available.

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470 **Table I** Location, Pre-treatments and Media used in the study.
471

Sites of sampling	Pre-treatments	Media
1. **New port** 2. **Sartanpar port** 3. **Victor port**	1. Sample grinded by mortar pestle, diluted and spread. 2. Moist incubation of grinded sample with $CaCO_3$ for 7-14 days and then spread. 3. Rehydration and centrifugation method. 4. Dry heating the plant at 80-100 °C. 5. Treatment with 1.5% (w/v) phenol. 6. Treatment with 0.01% potassium dichromate.	1. Nutrient Agar (NA) 2. Zobell Marine agar (ZMA) 3. Tryptic Soya agar (TSA) 4. Starch Casein Agar (SCA) 5. Yeast Malt Agar (ISP 2) 6. Actinomycetes isolation agar (AIA) 7. Inorganic Salts Starch agar 4 (ISP 4) 8. Tap water Yeast Extract Agar (TWYA)

472

473

474 **Table II** Identification and similarity values of 16S rDNA sequences retrieved from the endophytic bacteria from *S. brachiata*
475

S.no.	Isolates	Identification	Accession number	*Closest bacteria in database*	Similarities (%)
1	NPROOT-3	*Bacillus subtilis*	MT459325	*B. subtilis* NR_027552	99.86
2	NPB-5	*B. subtilis*	MT645716	*B. subtilis* NR_027552	99.93
3	NPA-10	*B. tequilensis*	MT645715	*B. tequilensis* NR_104919	99.86
4	NPA-4	*B. subtilis*	MT645714	*B. subtilis* NR_027552	99.93
5	1VPT1-5	*Pseudomonas parafulva*	MT645710	*Pseudomonas parafulva* NR_104280	99.86
6	1VPT5-7	*B. infantis*	MT645711	*B. infantis* NR_043267	99.63
7	1VPT5-10	*B. infantis*	MT645712	*B. infantis* NR_043267	99.63
8	2VPT5-4	*Pseudomonas cremoricolorata*	MT645713	*Pseudomonas cremoricolorata* NR113855	100
9	4NPA-2	*B. safensis*	MT645719	*B. safensis* NR_041794	99.79
10	4NPA-4	*Paenibacillus taichungensis*	MT645718	*Paenibacillus taichungensis* MH553940	99.29
11	4SPS-3	*Nocardiopsis aegyptia*	MT645720	*Nocardiopsis aegyptia* NR025889	99.71
12	4SPT4-10	*B. safensis*	MT645721	*B. safensis* NR_041794	99.78
13	4SPT4-14	*B. safensis*	MT645722	*B. safensis* NR_041794	99.93
14	4SPT4-16	*B. safensis*	MT645723	*B. safensis* NR_041794	99.79
15	4SPT6-1	*B. safensis*	MT645724	*B. safensis* NR_041794	100%
16	4SPT6-1A	*B. zhangzouensis*	MT645725	*B. safensis* NR_041794	99.79
17	4SPT8-2	*B. filamentosus*	MT645726	*B. filamentosus* NR_134701	99.64
18	4SPT8-6A	*B. subtilis*	MT645727	*B. subtilis* NR_113265	99.93
19	4SPT12-17	*B. aerius*	MT645728	*B. aerius* NR_118439	99.86
20	4VPT1-4	*Isoptericola chiayiensis*	MT645729	*Isoptericola chiayiensis* NR_116696	99.05
21	4VPT1-8	*Isoptericola chiayiensis*	MT645730	*Isoptericola chiayiensis* NR_116696	99.20
22	4VPT1-9	*B. subtilis*	MT645731	*B. subtilis* NR_027552	99.35
23	4VPT4-5	*B. tequilensis*	MT645732	*B. tequilensis* NR_104919	99.86
24	4VPT5-1	*B. pumilus*	MT645733	*B. pumilus* NR_043242	99.71
25	4VPT5-1A	*B. zhangzhouensis*	MT645717	*B. zhangzhouensis* NR_148786	99.35
26	5NPA-1	*B. amyloliquefaciens*	MT459305	*B. amyloliquefaciens* NR_041455	98.91
27	5SPRoot-1	*B. paralicheniformis*	MT645735	*B. paralicheniformis* MK517555	99.93
28	5NPB-5	*B. aerius*	MT645734	*B. aerius* NR_118439	99.78
29	5SPS-3	*Rhodococcus ruber*	MT645737	*Rhodococcus ruber* NR_118602	99.93
30	5SPT4-2	*B. tequilensis*	MT645738	*B. tequilensis* NR_104919	99.71
31	5SPT7-2	*B. paralicheniformis*	MT645740	*B. paralicheniformis* MF_321822	99.93
32	5SPROOT 3	*B. stratosphericus*	MT645736	*B. stratosphericus* NR_118441	99.93
33	5SPT4-2A	*B. paralicheniformis*	MT645739	*B. paralicheniformis* MK517555	99.86
34	5SPT7-7	*B. tequilensis*	MT645741	*B. tequilensis* NR_104919	99.86
35	5SPT8-5A	*B. tequilensis*	MT645742	*B. tequilensis* NR_104919	99.86

36	5VPT7-8	*B. paralicheniformis*	MT645746	*B. paralicheniformis* MF321822	99.93
37	5VPT11-1	*Bacillus endophyticus*	MT645748	*Bacillus endophyticus* KY194734	98.8%
38	5VPT1-11	*B. tequilensis*	MT645744	*B. tequilensis* NR_104919	99.86
39	5VPT4-11	*B. subtilis*	MT645745	*B. subtilis* NR_113265	99.86
40	5VPT7-10	*B. subtilis*	MT645747	*B. subtilis* NR_112629	99.93
41	5VPT1-13	*B. subtilis*	MT645743	*B. subtilis* NR_113265	99.72
42	6SPT2-1	*Marinilactibacillus piezotolerans*	MT645751	*Marinilactibacillus piezotolerans* NR_112661	99.56
43	6NPB-8	*Salinicola tamaricis*	MT645750	*Salinicola tamaricis* NR_157001	99.34
44	6SPT4-14	*B. subtilis*	MT645755	*B. subtilis* NR_113265	99.79
45	6SPT7-1	*B. safensis*	MT645752	*B. safensis* NR_041794	99.65
46	6SPT12-11	*Nitratireductor indicus*	MT645754	*Nitratireductor indicus* NR_117518	99.55
47	6VPT1-2	*B. safensis*	MT645756	*B. safensis* NR_041794	99.79
48	6VPT5-9	*B. zhangzouensis*	MT645758	*B. zhangzouensis* NR_148786	99.50
49	6NPA-7	*B. aerius*	MT645749	*B. aerius* NR_118439	99.64
50	6SPT8-3	*Nitratireductor indicus*	MT645753	*Nitratireductor indicus* NR_117518	99.70
51	6VPT1-8	*B. pumilus*	MT645757	*B. safensis* NR_112637	99.57
52	7NPA-13	*B. stratosphericus*	MT645759	*B. stratosphericus* NR_118441	99.93
53	7NPB-1	*B. stratosphericus*	MT645760	*B. stratosphericus* NR_042336	99.86
54	7NPB-2	*Salinicola tamaricis*	MT645761	*Salinicola tamaricis* NR_157001	98.80
55	7VPT5-5R	*Streptomyces hyderabadensis*	MT645769	*Streptomyces hyderabadensis* NR_116934	99.55
56	7NPSHOOT-4	*B. subtilis*	MT645765	*B. subtilis* NR_112629	100
57	7NPB-3B	*B. velezensis*	MT645763	*B. velezensis* NR_116240	99.78
58	7SPROOT-1	*Enterococcus faecalis*	MT645764	*E. faecalis* NR_115765	99.80
59	7SPT4-20	*Enterococcus faecalis*	MT645767	*E. faecalis* NR_115765	99.93
60	7SPT4-19	*Jonesia denitrificans*	MT645766	*Jonesia denitrificans* NR_119162	99.78
61	7NPROOT-4	*B. cereus*	MT645762	*B. cereus* NR_115526	99.51
62	7SPT5-13	*Micromonospora echinospora*	MT645768	*Micromonospora echinospora* NR_118843	99.41
63	4NPBL	*B. subtilis*	MT447880	*B. subtilis* NR_112116	99.86

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477
478 **Table III** Diversity indices of the 63 endophytes isolated from *S. brachiata*
479

Diversity indices	Values calculated	Range
Shannon-Wiener diversity index	3.028	0 onwards
Simpson's index of diversity	0.931	0-1
Species richness	3.615	0 onwards
Shannon evenness	0.899	0-1

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481 **Table IV** Results of primary screening of endophytic isolates against microbial pathogens.
482

S N	Isolates	Taxonomic identification	*MS*	*SA*	*BS*	*EC*	*CA*	*XC*	*FO*	*AM*
1	NPA-4	*Bacillus subtilis*								
2	NPA-10	*Bacillus tequilensis*								
3	NPB-5	*Bacillus subtilis*								
4	2VPT4-2									
5	NP ROOT-3	*Bacillus tequilensis*								
6	2VPT11-1									
7	4VPT1-8	*Isoptericola chiayiensis*								
8	4VPT1-9	*Bacillus subtilis*								
9	4VPT1-4	*Isoptericola chiayiensis*								
10	4SPT4-14	*Bacillus safensis*								
11	4SPT4-10	*Bacillus safensis*								
12	4NPA-2	*Bacillus safensis*								
13	4NPA-4	*Paenibacillus taichungensis*								
14	4NPB-1									
15	4NPB-4A									
16	4SPT4-16	*Bacillus safensis*								
17	4SPT4-12									
18	4SPT6-1A	*Bacillus zhangzouensis*								
19	4VPT4-5	*Bacillus tequilensis*						1		
20	4VPT5-1	*Bacillus pumilus*								
21	4SPT8-6A	*Bacillus subtilis*								
22	4SPT8-2	*Bacillus filamentosus*								
23	4SPS-1									
24	4SPS-3	*Nocardiopsis tangguensis*								
25	4SPR-4									
26	5VPT1-11	*Bacillus subtilis*								
27	5NPA-1	*Bacillus amyloliquefaciens*								
28	5NPB-5	*Bacillus aerius*								
29	5SPT4-9									
30	5SPT4-8									
31	5SPT4-1									
32	5SPT4-2	*Bacillus tequilensis*								
33	5SPT6-1									
34	5VPT1-13	*Bacillus subtilis*								
35	5VPT1-3									
36	5SPT7-2	*Bacillus paralichenfiormis*								
37	5SPT7-4									
38	5SPT7-7	*Bacillus tequilensis*								
39	5SPT8-5A	*Bacillus tequilensis*								
40	5SPT10-5									
41	5SP-Root-1	*Bacillus paralichenfiormis*								
42	5SP-ROOT-3	*Bacillus stratosphericus*								
43	5VPT4-11	*Bacillus subtilis*								
44	5VPT7-2	*Bacillus paralichenfiormis*								
45	5SP-SHOOT-3	*Rhodococcus ruber*								
46	5VPT7-8	*Bacillus haynesii*								
47	5VPT11-1	*Bacillus paralichenfiormis*								
48	6SPT2-1	*Marinilactibacillus peizotolerans*								
49	6NPA-7	*Bacillus aerius*								
50	6SPT4-2									
51	6SPT4-14	*Bacillus subtilis*								
52	6SPT6-3									

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53	6VPT1-2	*Bacillus safensis*
54	6VPT1-8	*Bacillus pumilus*
55	6SPT6-9	
56	6SPT7-1	*Bacillus safensis*
57	6SPT8-7	
58	6VPT4-3	
59	6SPT8-3	*Nitratireductor indicus*
60	6SPT12-10	
61	6SPT12-11	*Nitratireductor indicus*
62	6SPT12-7	
63	6VPT5-9	*Bacillus zhangzouensis*
64	7NPB-1	*Bacillus stratosphericus*
65	7SPT4-19	*Jonesia denitrificans*
66	7SPT4-20	*Enterococcus faecalis*
67	7SPT4-17	
68	7SPT5-1	
69	7SPT6-15	
70	7VPT1-1	
71	7VPT1-3	
72	7NPA-11	
73	7NPB-2	*Salinicola tamaricis*
74	7NPB-3B	*Bacillus velezensis*
75	7SPT9-4	
76	7SPT9-3	
77	7SPT10-3	
78	7VPT1-11	
79	7VPT4-2	
80	7VPT4-11	
81	7VPT4-8	
82	7VPT5-8	
83	7SPT12-10	
84	7SPR-1	*Enterococcus faecalis*
85	7SPTR-2	
86	7NPROOT-4	*Bacillus cereus*
87	7NPSHOOT-1	
88	7NPSHOOT-4	*Bacillus subtilis*
89	7VPT7-1	
90	7NPB-4	
91	7NPROOT-3	
92	7NPA-13	*Bacillus stratosphericus*
93	8NPA-8	
94	8NPB-1	
95	8NPB-6	
96	8SPT4-4	
97	8VPT5-6	
98	8VPT7-6	
99	8SPT4-2	
100	8VPT11-2	
101	3VPT4-4	*Bacillus sp.*

Where, *MS*: *Mycobacterium smegmatis* MTCC 6, *SA*: *Staphylococcus aureus* MCC 2043, *BS*: *Bacillus subtilis* MCC 2049, *EC*: *Escherichia coli* MCC 2412, *CA*: *Candida albicans* MCC 1152, *XC*: *Xanthomonas campestris* NCIM 5028, *FO*: *Fusarium oxysporum* NCIM 1008, *AM*: *Alteromonas macleodii* NCIM 2815.

Red boxes: *Inhibitory activity*, *Yellow boxes*: *No inhibitory activity*

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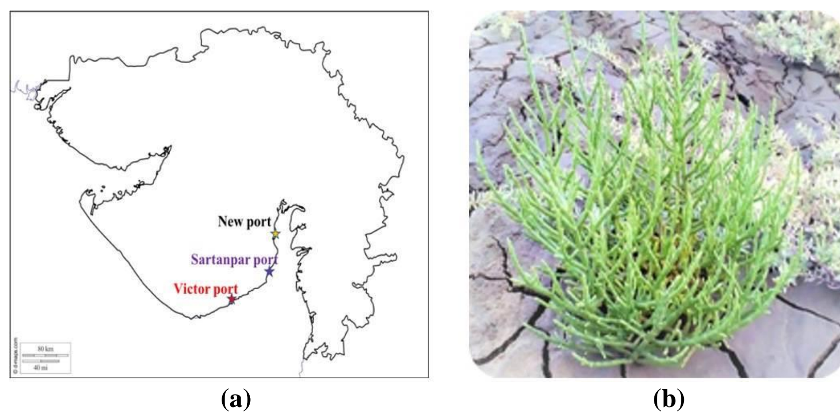

489 (a) (b)

490 **Fig. 1** Sampling location. (a) Location Map of Gujarat coast, India showing three sampling sites 1) New port, 2)
491 Sartanpar port, 3) Victor port. (b) Morphology of *Salicornia brachiata* plant in natural marsh habitat

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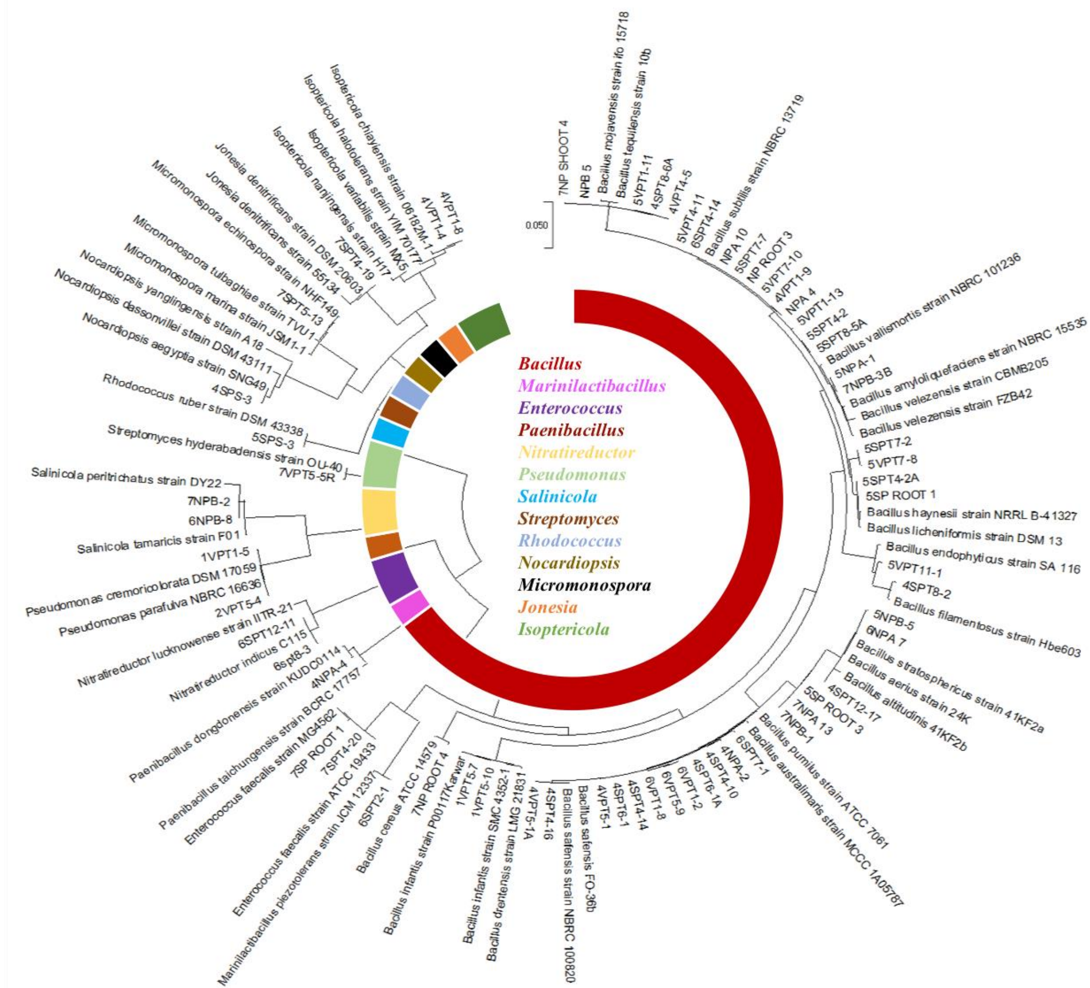

494
495 **Fig. 2** Identification of endophytes by 16S rRNA sequencing. Phylogenetic tree of 63 bioactive isolates obtained by
496 Maximum likelihood analysis constructed using Mega 6 software depicting their phylogeny with related genera as
497 well as pie graph displaying percentage distribution of genera obtained
498

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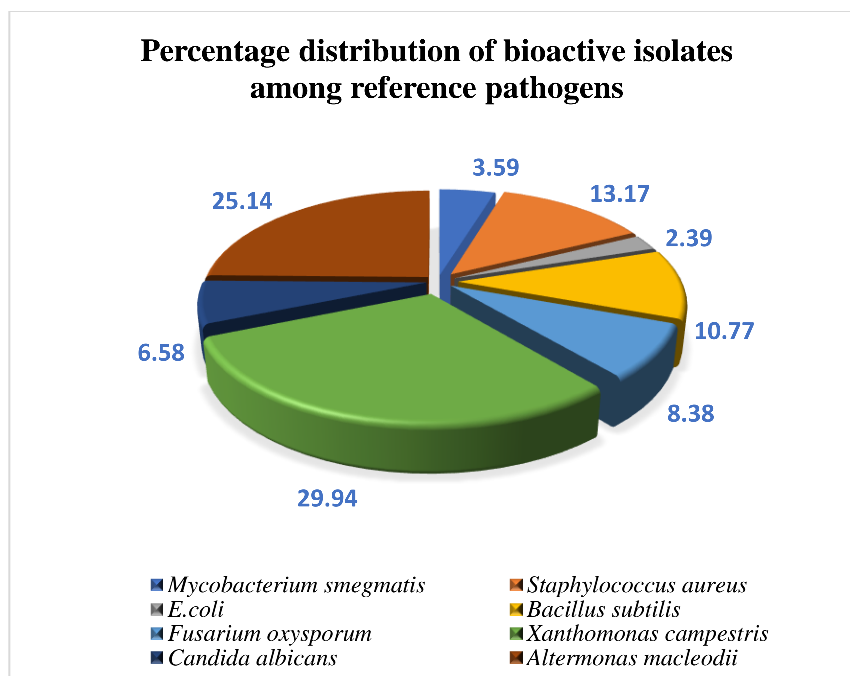
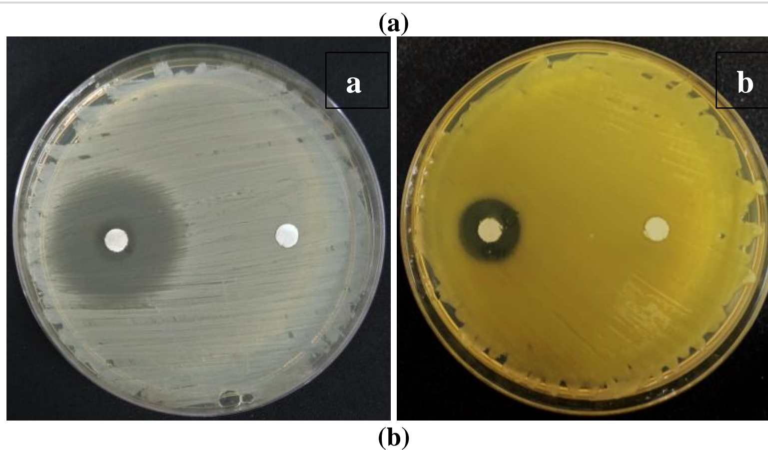


499
500 (a)



501
502 (b)
503 **Fig. 3** (a) Pie graph depicting distribution of isolates exhibiting bioactivity against various pathogens. (b) Bioactivity
504 of *B. amyloliquefaciens* 5NPA-1 against (a) *S. aureus* MCC 2043 and (b) *X. campestris* NCIM 5028
505
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507
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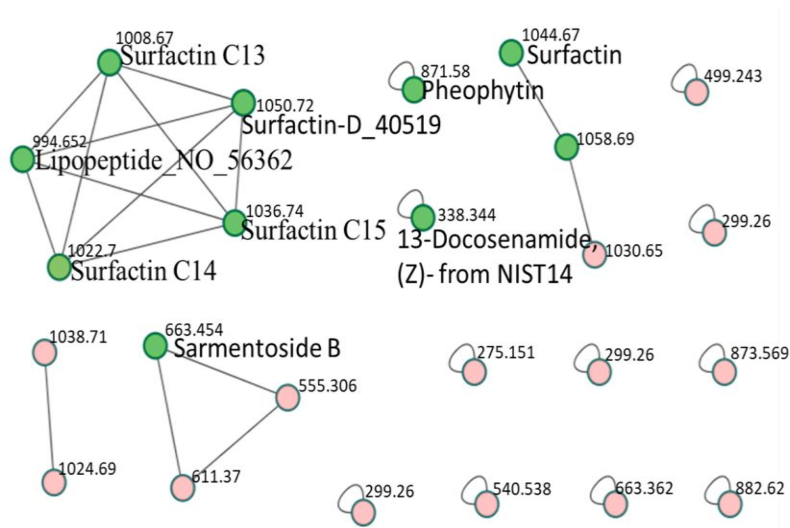

511
512
513 **Fig. 4** Molecular network of *B. amyloliquefaciens* 5NPA-1. Annotated molecular network (green) by GNPS for
514 crude extract identifies surfactin networks. Molecular weights beside nodes indicate mass of parent ions.
515 Unidentified nodes (pink) were not automatically detected.
516

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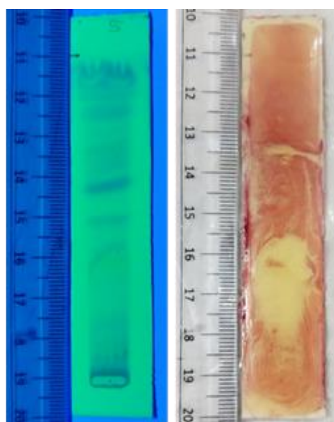

519
520 **Fig. 5** TLC bioautography plate of crude extract of *B. amyloliquefaciens* 5NPA-1. Pink area represents cell growth
521 and clear zone area depicts presence of bioactive compound at that region
522
523
524

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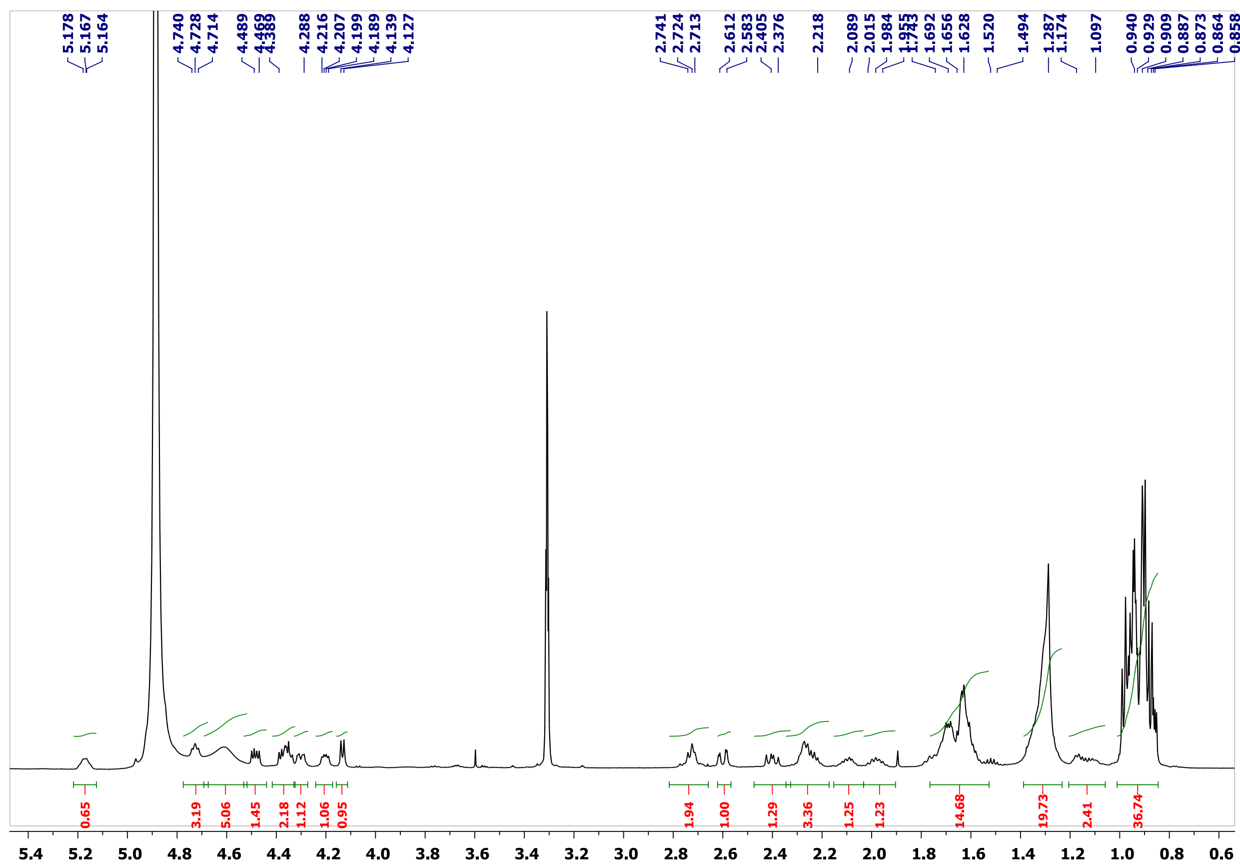

528
529
530 **Fig. 6** ^{1}H-NMR spectrum of 5-PTLC2 fraction obtained from preparative TLC of crude extract of *B.*
531 *amyloliquefaciens* 5NPA-1
532
533

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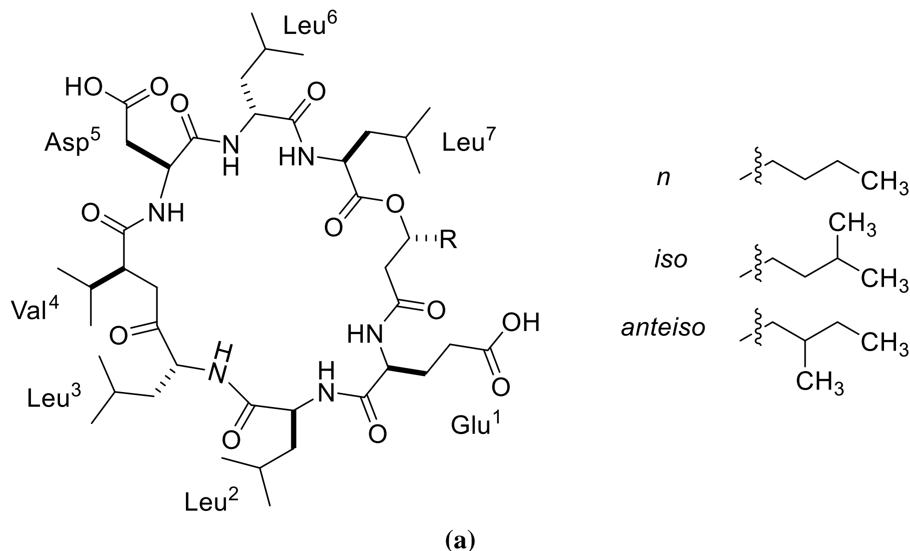
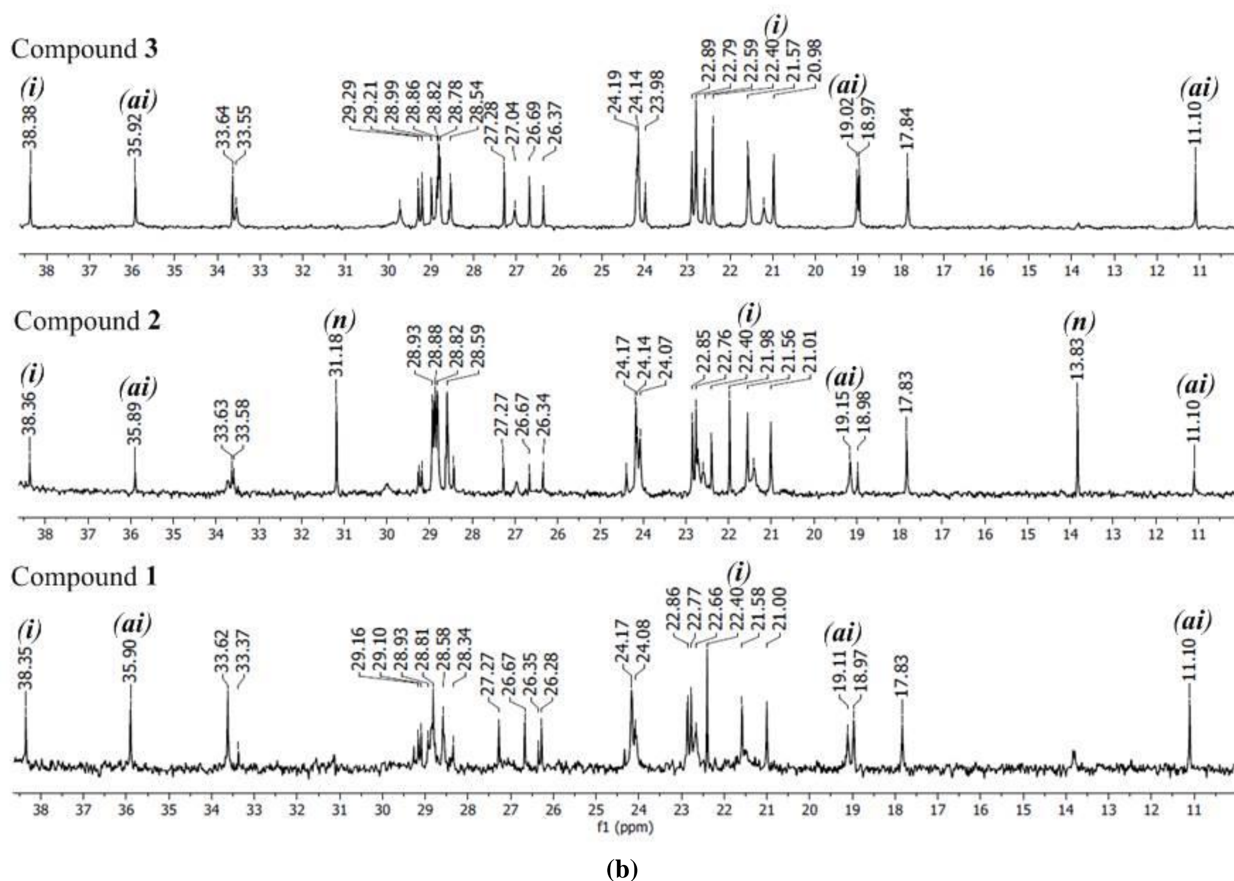


(a)



(b)

Fig. 7 **(a)** Structures of compounds **1** (R = mixture of *iso*-$C_{10}H_{21}$ and *anteiso*-$C_{10}H_{21}$ patterns), **2** (R = mixture of *n*-$C_{11}H_{23}$, *iso*-$C_{11}H_{23}$, and *anteiso*-$C_{11}H_{23}$ patterns), and **3** (R = mixture of *iso*-$C_{12}H_{25}$ and *anteiso*-$C_{12}H_{25}$ patterns). **(b)** Characteristic ^{13}C NMR signals differentiating branching of the hydroxy fatty acid side chains in compounds 1–3; **(n)**: normal (δ_C 13.8, 22.0, 31.2), **(i)**: iso (δ_C 22.4, 22.4, 27.3, 38.4) and the **(ai)**: anteiso (δ_C 11.1, and 19.0) chain type.