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1 # Diversity of culturable actinobacteria producing protease inhibitors isolated from
2 # the intertidal zones of Maharashtra, India

3

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21 ABSTRACT

22 Phylogenetic diversity of culturable actinobacteria isolated from the intertidal regions of west
23 coast of Maharashtra, India was studied using 16S rRNA gene sequencing. Total of 140
24 actinobacterial isolates were obtained, which belonged to 14 genera, 10 families and 65 putative
25 species with *Streptomyces* being the most dominant (63%) genus followed by *Nocardiopsis*
26 and *Micromonospora*. They were screened for production of extracellular protease inhibitors
27 (PI) against three pure proteases viz. chymotrypsin, trypsin, subtilisin and one crude
28 extracellular protease from *Pseudomonas aeruginosa*. Eighty percent of the isolates showed PI
29 activity against at least one of the four proteases, majority of them belonged to genus
30 *Streptomyes*. Actinobacterial diversity from two sites Ade (17°52' N, 73°04' E) and Harnai
31 (17°48' N, 73°05' E) with varying degree of anthropological pressure showed that more putative
32 species diversity was obtained from site with lower human intervention i.e Ade (Shannon's H
33 3.45) than from Harnai (Shannon's H 2.83), a site with more human intervention. Further, in
34 Ade percentage of isolates not showing PI activity against any of the proteases was close to
35 21% and that in Harnai was close to 9%. Considering human activities in the coastal region
36 might be contributing to increasing the organic load and in turn increasing the presence of
37 extracellular enzymes in the intertidal environments it would be interesting to look at the
38 association of PI production and organic load in these habitats.

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40 INTRODUCTION

41 Proteases are enzymes that catalyse proteolytic reactions and are involved in variety of
42 biological processes like digestion, cell signalling or tumour formation (Groll et al., 2002;
43 Koblinski et al., 2000; Overall and Blobel, 2007; Roberts et al., 2012). They are ubiquitously
44 present across different plant, animal and microbial taxa. Two of the many roles of these
45 enzymes in bacteria is to aid in bacterial pathogenesis (Ingmer and Brøndsted, 2009; Maeda,
46 1996) and bacterial predation (Martin, 2002) where they secrete hydrolytic enzymes in order
47 to degrade prey species in the vicinity (Martin, 2002). Protease inhibitors bind reversibly or
48 irreversibly with the enzymes, rendering them inactive. Hence, protease inhibitors (PIs) play a
49 crucial role as defence molecules in intracellular or extracellular environment. They are
50 classified based on either the type of protease they inhibit (e.g. serine protease inhibitor, aspartic
51 protease inhibitor) or based on the mechanism of action (e.g. reversible or irreversible enzyme
52 inhibitors). Various plants, animals and microorganisms produce PIs in order to deter pests and
53 predators or secrete them as toxins for self-defence (Habib and Fazili, 2007; Hartl et al., 2011;
54 Mourão and Schwartz, 2013). Also, PIs have high commercial potential since they are used as
55 drugs against viral, bacterial and other parasitic infections (Karthik et al., 2014; Liu et al., 2012;
56 Sreedharan and Bhaskara Rao, 2017; Umezawa, 1976).

57 Marine intercostal community harbours a variety of sedentary invertebrates and
58 microorganisms. These organisms rely primarily on chemicals for self-defence (Engel et al.,
59 2002; Pawlik, 1993). Additionally, as stated by Hay *et al*. the "biggest challenge for marine
60 organisms is to obtain lunch without becoming lunch" (Hay, 2009). Protease inhibitors can
61 play a major role in these situations since they can inhibit the action of digestive proteases
62 present in the surroundings. Literature reports suggest that PIs are indeed produced by marine
63 intercostal communities (Covaleda et al., 2012; Karthik and Kirthi, 2015; Karthik et al., 2014;

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64 Mourão and Schwartz, 2013) considering these facts, it might be possible to detect PIs
65 synthesised by marine microorganisms for their defence.

66 Intertidal zones are exposed to air at low tides and are submerged at high tides. Thus they
67 experience varying dry and wet periods, fluctuating temperatures and atmospheric pressure.
68 Intertidal life forms adapt to these constantly changing environment by developing unique
69 features. Their adaptations for survival in dynamic environments might also increase the
70 chances of finding unique set of metabolites from them. . Moreover, it is expected that marine
71 microorganisms will have different set of defence molecules than their terrestrial counterparts
72 (Mourão and Schwartz, 2013; Xie et al., 2018).

73 Actinobacteria is one of the largest phyla in domain bacteria. These are gram positive organisms
74 with high guanine to cytosine ratio (Trujillo, 2016). They at large are known to produce
75 commercially important diverse metabolites. Some reports show that the marine environment
76 has become a prime source for discovery of novel actinobacteria and novel natural products
77 produced by them (Maldonado et al., 2005; Ward and Bora, 2006). There are few recent reports
78 of PI production by marine actinobacteria (Karthik et al., 2014; Sreedharan and Bhaskara Rao,
79 2017; Sun et al., 2014) yet, to our knowledge, there are limited reports on PIs from west coast
80 of India. Thus, in the current study, marine actinobacteria from intertidal zone were screened
81 for presence of PIs.

82 It has been reported that sponges and bacteria are two main sources of enzyme inhibitors from
83 marine environments (Ruocco et al., 2017). Some reports also suggest that many a times,
84 metabolites obtained from sponges are actually produced by the bacterial symbionts (Haygood
85 et al., 1999; Mehbub et al., 2014). Therefore, in the current study sponge, sediment and water
86 samples were collected from the coast of Maharashtra and Goa for isolation of actinobacteria.
87 Obtained isolates were subjected to molecular identification using 16S rRNA gene sequencing

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88 and isolates were screened for presence of protease inhibitors. Data was analysed to check
89 correlations between inhibitor molecules and environmental parameters and their probable role
90 as defence molecules.

91 MATERIALS AND METHODS

92

93 *Sampling*

94 Around 60 samples comprising of sponge, sediment and sea water were collected from the
95 intertidal rock pools from seven different locations along the coast of Ratnagiri district from
96 Velas, Kelshi, Ade, Anjarle, Harnai, Murud and Aare Ware. (17°57'04.9"N 73°01'43.0"E to
97 17°04'35.9"N 73°17'17.5"E). From Ade and Harnai, sampling was carried out in different
98 seasons viz. pre monsoon (Mar, Apr, May), Monsoon (Early Sep), post-monsoon (Oct) and
99 winter season (Dec, Jan, Feb). Whereas, a single sampling was done from rest of the sites. Sub-
100 tidal sampling of sediment was also done in a single event at a place off shore to Goa
101 (15°21'08.4"N 73°46'41.8"E). The depths of collection for this sampling were 9m, 11m, 12m
102 and 16m. (Figure 1).

103 Small tissue samples of sponge were collected without damaging the sponge colonies or their
104 habitat. Samples were rinsed in sterile Poor Ravan Saline (PRS) broth to remove loosely bound
105 particles and debris and were stored in sterile collection tubes containing the same medium.
106 Sediment and water samples in the vicinity of sponge were collected in sterile collection tubes.
107 All the samples were transported to the laboratories in ice box and were processed fresh for
108 microbial isolations.

109 *Sample Processing, Selective Isolation and Culture Maintenance*

110 All the samples were given heat treatment at 60°C for 15 mins to reduce the load of non-
111 sporulating bacteria. Sponge samples were homogenised in Poor Ravan Saline (PRS) medium

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112 (Watve et al., 2000) and diluted serially with 10 fold dilutions up to 10^{-5}. Sediment samples
113 were vigorously shaken for two minutes and diluted up to 10^{-5} dilutions. Sea water samples
114 were also diluted up to 10^{-5} dilutions. 0.1 ml of undiluted, 10^{-3} and 10^{-5} dilutions were spread
115 plated in triplicates on different growth media and incubated at room temperature for up to 21
116 days.

117 Four different growth media Sponge agar (1% macerated sponge colonies collected from the
118 site, 50% sea water and 2.5% agar), Sea Water Agar (50% sea water and 2.5% agar), ZOBELL
119 Marine Agar (ZOBELL, 1941) and Modified poor ravan medium (Watve et al., 2000)) were
120 used to obtain maximum culture dependent diversity of actinobacteria. Nalidixic acid and
121 Cyclohexamide were added to the culture medium in 25 μg ml^{-1} concentration to inhibit the
122 growth of Gram-negative bacteria and fungi (Magarvey et al., 2004). Plates were incubated at
123 30°C and monitored daily for 21 days. Isolated colonies showing resemblance to typical
124 actinobacterial colony morphology were picked up and subcultured several times for obtaining
125 pure cultures. All the pure cultures were later stored on modified (1:4 diluted) ZOBELL marine
126 agar at 4°C for further use.

127 *DNA Sequencing and Diversity Analysis*

128 16S rRNA sequences from previous study of (Baig et al., 2020) were used to make maximum
129 likelihood phylogenetic tree of actinobacteria used in the study. Maximum likelihood tree was
130 constructed in IQtree (Nguyen et al., 2015). Best nucleotide substitution model was determined
131 in model finder (Kalyaanamoorthy et al., 2017). Note support was examined using bootstrap
132 values of 1000 iterations and *Bacillus sp.* were used as out-group. Shannon's diversity index to
133 measure species diversity of actinobacteria was calculated using Past (version 4.0) (Hammer,
134 2001).

135 *Screening for Protease Inhibitors (PI) using spot assay*

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136 ***Sample preparation***

137 Detection of extracellular PIs from cell free supernatant was done as follows. Pure cultures of
138 actinobacteria were inoculated (single colony in 150 ml broth) in ZOBELL Marine broth and
139 incubated for 5 days at 30°C on incubator shaker with 100 rpm speed. After incubation, the
140 cultures were centrifuged at 4000 rpm for 20 mins and cell free supernatants (CFS) were
141 collected. 1 ml of each of these CFSs were kept in hot water bath of 70°C for 15 mins for
142 denaturation of proteases produced by the bacteria. Heat treated CFSs were used for detection
143 of PIs. Unprocessed X-ray films coated with gelatine were used to detect PIs. (Gelatine is
144 degraded by various proteolytic enzymes, so, upon action of the proteases, clear zones are
145 observed on unprocessed x-ray films at the point of contact of enzymes. On the other hand, if
146 the gelatine layer remains intact, no clearance is observed. PIs inactivate proteases and thus,
147 presence of protease inhibitors is marked by no clearance on the x-ray films as shown in Figure
148 3).

149 Proteases used for the assay included Trypsin (SRL Pvt. Ltd, Cat no.- 60484) and α-
150 Chymotrypsin (SRL Pvt. Ltd, Cat no.- 35085) of bovine origin and Subtilisin (Sigma-Aldrich,
151 Cat no.- P5380) and crude protease obtained in the laboratory from cell free supernatant of
152 *Pseudomonas aeruginosa* (NCBI Accession number - MN044759) of bacterial origin were
153 used to detect PIs. Amongst these enzymes, trypsin and chymotrypsin were of bovine origin.
154 Additionally, Protease inhibitor cocktail (Sigma-Aldrich, Cat no.- P5380) was used as a
155 positive control and un-inoculated culture broth incubated with the enzyme was used as a
156 negative control.

157 ***Spot assay***

158 Standardization of enzyme concentration was done as described by Tripathi et al.,
159 (2011).Various dilutions of pure enzyme were spotted on gelatine coated X-ray films. Lowest

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160 dilution that gave complete clearance, in turn indicating complete digestion of gelatine, was
161 used in the assay.

162 Spot assay as described by (Cheung et al., 1991) for detection of PIs was carried out as follows.
163 10 µl of pure enzyme (100 µg ml^{-1}) was incubated with 10 µl of heat treated CFS for 10 mins
164 and then transferred to untreated X-ray-Fuji Medical X-ray, HRU grade-films. The assembly
165 was kept undisturbed for 15 mins at room temperature to allow the enzyme substrate reaction
166 to take place. Films were washed with running tap water and allowed to dry before recording
167 the results.

168 RESULTS

169

170 *Sample Collection*

171

172 A single sampling of sediment and water was carried out from intertidal and sub-tidal regions
173 of Aare Ware and Goa respectively. Similarly, one time sampling was carried out from
174 intertidal zones of Velas, Kelshi, Anjarle and Murud however, at the time of sampling, sponge
175 colonies could not be located on any of these sites and thus only sediment and water samples
176 were collected. Whereas, at Harnai and Ade four to five distinct morphotypes of sponges were
177 recorded. Thus, sponge sediment and water samples were collected from these sites. These
178 sites were also selected for sampling in various seasons throughout the year. Harnai, one of the
179 busy ports on the western coast is used for various activities like, auction of fishes on a daily
180 basis, recreational space for the villagers or tourist hang out spot. Certain parts of intertidal rock
181 patch in the vicinity are used as open defecation sites by the villagers and few other parts of the
182 rock patch are used for clamp collection and fishing from the rock pools. Conversely Ade, is
183 hardly used for any of the above purposes except for occasional fishing from the rock pools.

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184 Thus, intertidal regions of the above sites faced varying degree of anthropological disturbance
185 with Harnai being the most and Ade being the least used site with respect to human activities.

186 *Distribution, Identification and Phylogeny of Actinobacteria from Sponge, Sediment and Sea*
187 *Water Samples*

188 We obtained 140 actinobacterial isolates from sponge, sediment and sea water samples. They
189 showed high phylogenetic diversity wherein there were 65 putative species belonging to 14
190 genera of 10 different families. Most abundant genus amongst the isolates was *Streptomyces*
191 *sp.* (~63%) followed by *Nocardiopsis sp.* (~22%) and *Micromonospora sp.* (~4%).
192 Approximately 11% isolates belonged to other genera (Table 1).

193

194 **Table 1:** Counts of putative species obtained from sponge, sediment and sea water samples

Family	Genus	Putative species identity	Isolates
Actinomycetaceae	*Streptomyces*	*Streptomyces albidoflavus*	6
		Streptomyces albogriseolus	8
		Streptomyces atrovirens	1
		Streptomyces aurantiogriseus	1
		Streptomyces aureofaciens	1
		Streptomyces cellulosae	1
		Streptomyces champavatii	1
		Streptomyces coeruleofuscus	1
		Streptomyces collinus	1
		Streptomyces diastaticus	3
		Streptomyces euryhalinus	4
		Streptomyces fradiae	6
		Streptomyces geysiriensis	1
		Streptomyces graminearus	1
		Streptomyces griseorubens	3
		Streptomyces koyangensis	1
		Streptomyces longispororuber	4
		Streptomyces maritimus	6
		Streptomyces nigra	1
		Streptomyces olivaceus	4
		Streptomyces prasinosporus	1
		Streptomyces pseudogriseolus	4

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		Streptomyces redgersensis	1
		Streptomyces rochei	2
		Streptomyces sampsonii	3
		Streptomyces smyrnaeus	1
		Streptomyces sp. 102H11-4	1
		Streptomyces sp. 13650C	8
		Streptomyces sp. CNS-753	1
		Streptomyces sp. FZ42	1
		Streptomyces sp. OAct 12	1
		Streptomyces sp. OAct 89	1
		Streptomyces tempisquensis	2
		Streptomyces tendae	2
		Streptomyces thermocarboxydus	1
		Streptomyces variabilis	1
		Streptomyces viridobrunneus	1
		Streptomyces xylophagus	1
Nocardiopsaceae	*Nocardiopsis*	*Nocardiopsis alba*	21
		Nocardiopsis dasonvelli	2
		Nocardiopsis fildesensis	1
		Nocardiopsis metallicus	3
		Nocardiopsis salina	1
		Nocardiopsis synnematoformis	3
Micromonosporaceae	*Micromonospora*	*Micromonospora chalcea*	2
		Micromonospora maritima	1
		Micromonospora sp	1
		Micromonospora tulbaghiae	1
Nocardiaceae	*Rhodococcus*	*Rhodococcus aetherivorans*	1
		Rhodococcus cory	1
		Rhodococcus rhodochrous	1
		Rhodococcus zopfii	1
Pseudonocardiaceae	*Actinomycetospora*	*Actinomycetospora chiangmaiensis*	1
		Actinomycetospora straminia	1
	Pseudonocardia	*Pseudonocardia kongjuensis*	1
Micrococcaceae	*Glutamicibacter*	*Arthrobacter mysoreus*	1
	Kocuria	*Kocuria rosea*	1
	Micrococcus	*Micrococcus aloeverae*	1
	Rothia	*Rothia terrae*	1
Brevibacteriaceae	*Brevibacterium*	*Brevibacterium leteolum*	2
Intrasporangiaceae	*Kytococcus*	*Kytococcus sedenteris*	1
Jonesiaceae	*Jonesia*	*Jonesia denitrificans*	1
Microbacteriaceae	*Agrococcus*	*Agrococcus carbonis*	1

195

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197 From sponges, 53 actinobacterial isolates were obtained which belonged to 7 genera and 7
198 families. Whereas, 70 isolates were obtained from sediments that belonged to 7 different genera
199 and 7 families. 17 isolates belonging to 8 genera of 7 families were isolated from sea water
200 (Table 2). Even though, more number of isolates were obtained from sediment, Shannon's
201 diversity index showed higher putative species diversity in isolates obtained from sponge
202 (Shannon's H 3.27) than sediment (Shannon's H 2.97) and water (Shannon's H 2.76).

203 **Table 2:** Number of isolates belonging to different families obtained from various sources

Families	Sediment	Water	Sponge	Total
Actinomycetaceae	47	9	32	**88**
Brevibacteriaceae	1	1	-	**2**
Intrasporangiaceae	1	-	-	**1**
Microbacteriaceae	-	1	-	**1**
Micrococcaceae	1	1	2	**4**
Micromonosporaceae	1	-	4	**5**
Nocardiaceae	1	1	2	**4**
Nocardiopsaceae	18	2	11	**31**
Pseudonocardiaceae	-	2	1	**3**
Jonesiaceae	-	-	1	**1**
Grand total	**70**	**17**	**53**	**140**

204

205 More number of isolates (67) and more species diversity (10 genera with 43 putative species)
206 were obtained from anthropologically less disturbed site Ade. Whereas, from Harnai which is
207 a site with higher disturbance 47 isolates belonging to 25 putative species of 9 genera could be
208 isolated. (Figure 2). Shannon's diversity index (H) value for Ade was 3.45 and that for Harnai
209 was 2.83. This indicates that the putative species diversity at Ade is more than diversity at
210 Harnai.

211 *Production of Protease inhibitors*

212

213 Results of PI production were recorded using spot assay as shown in Figure 3. It was observed
214 that, PIs retained their activity even after heat treatment at 70°C for 15 mins. Out of 140 isolates

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215 used for screening of PIs, 113 isolates showed activity against at least one of the three pure
216 proteases used for the study (viz. chymotrypsin, trypsin and subtilisin), whereas 27 isolates
217 showed no PI production at all against any of these enzymes. Out of 113 isolates, 37 showed
218 some degree of activity against all pure enzymes, of which 8 showed strong positive activity.
219 Total of 17, 7 and 9 isolates showed PI activity exclusively against chymotrypsin, trypsin and
220 subtilisin respectively (Figure 3). Majority of isolates which showed strong positive activity
221 against enzyme chymotrypsin, also showed strong positive activity against subtilisin and
222 trypsin. Further, even though 20% of the *Streptomyces* members showed complete absence of
223 protease inhibitors, all the isolates showing strong positive activity against all enzymes
224 belonged entirely to *Streptomyces* sp. Very few isolates of *Nocardiopsis* sp. showed PI activity
225 against subtilisin. Regarding the source, highest number of strong positive isolates (7 out of 8)
226 were obtained from the sediments and only 1 was obtained from the sponge. (Figure 2).

227 Out of 113 PI producers, strong positive (++) activity was shown by 10, 54, and 27 isolates
228 against chymotrypsin, trypsin and subtilisin respectively (Table 3 A). A subset of 90 isolates
229 belonging to most common genera *viz. Streptomyces sp.*, *Nocardiopsis sp.* and
230 *Micromonospora sp.* were chosen to check the activity against another extracellular bacterial
231 protease which was obtained from cell free supernatant of *P. aeruginosa* , 36 isolates gave some
232 degree of activity against this crude bacterial protease (Table 3B).

233 **Table 3A:** Genus-wise counts of PI producers active against pure proteases

234

Genus	Chymotrypsin			Trypsin			Subtilisin			Total
	-	+	++	-	+	++	-	+	++	
Actinomycetospora	0	2	0	1	1		2	0	0	**2**
Agrococcus	0	1	0	1	0	0	1	0	0	**1**
Brevibacterium	1	1	0	1	1	0	2	0	0	**2**
Glutamicibacter	0	1	0	0	0	1	1	0	0	**1**
Jonesia	0	1	0	1	0	0	1	0	0	**1**

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Kocuria	0	1	0	1	0	0	1	0	0	**1**
Kytococcus	0	1	0	0	1	0	1	0	0	**1**
Micrococcus	0	1	0	0	1	0	1	0	0	**1**
Micromonospora	2	3	0	2	1	2	4	1	0	**5**
Nocardiopsis	6	23	2	17	3	11	27	4	0	**31**
Pseudonocardia	0	1	0	1	0	0	1	0	0	**1**
Rhodococcus	0	4	0	2	1	1	4	0	0	**4**
Rothia	0	1	0	0	0	1	1	0	0	**1**
Streptomyces	43	37	8	31	19	38	33	28	27	**88**
Total	**52**	**78**	**10**	**58**	**28**	**54**	**80**	**33**	**27**	**140**

Table 3B: Genus-wise counts of PI producers active against crude protease

Genus	**Crude *P. aeruginosa* Protease**			**Total**
	-	+	++	
Micromonospora	2	1	0	3
Nocardiopsis	3	4	0	7
Streptomyces	49	29	2	80
Grand Total	**54**	**34**	**2**	**90**

Effect of Season and Anthropological Activities on Production of Protease Inhibitors

Least number of actinobacterial isolates were obtained from monsoon and post-monsoon seasons and the number of isolates obtained from each sampling increased towards the month of May i.e. the pre-monsoon season. Majority of actinobacterial isolates were obtained from pre-monsoon season (Figure 4A). From Ade and Harnai, over all, occurrence of inhibitors against chymotrypsin and trypsin was more as compared to that against subtilisin (Figure 4B). Proportion of isolates not giving PI activity against any of the enzymes was higher in Ade (14 out of 67 isolates i.e. approximately 21%) compared to that in Harnai (4 isolates out of 47 i.e. approximately 19%) (Figure 4C). Thus to summarize, more number of putative species were obtained from Ade however, proportion of PI producers from that site was less compared to Harnai.

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250

251 DISCUSSION:

252 Actinobacteria are widespread in terrestrial and aquatic environments. Many of them also
253 produce external spores which are resistant to dehydration (Trujillo, 2016). Various reports say
254 that marine actinobacteria have a high potential for production of biomolecules (Jose and Jha,
255 2017; Solanki et al., 2008) however, the number of actinobacterial compounds discovered so
256 far are limited not by the number of compounds produced by them but by the amount of
257 screening efforts put in (Watve et al., 2001). Many reports have highlighted the need for
258 studying marine actinomycete diversity and their potential use for obtaining novel metabolites
259 (Dharmaraj, 2010; Jensen et al., 2005; Lam, 2006; Subramani and Aalbersberg, 2012).
260 Researchers from India have also isolated actinobacteria from coastal regions of West Bengal
261 (Peela et al., 2005; Ramesh and Mathivanan, 2009), Gujarat (Jose and Jha, 2017), Tamil Nadu
262 (Raja et al., 2010; Valli et al., 2012) and Andaman and Nicobar (Karthik et al., 2014) to name
263 a few for bioprospecting studies. However, there are very few reports of isolation of
264 actinobacteria and still fewer reports on PI producing actinobacteria from the coast of
265 Maharashtra. Thus considering these facts, current study focused on isolation of actinobacteria
266 from coast of Maharashtra and their potential as producers of PIs.

267 *Phylogenetics of Actinobacteria from Intertidal Regions of Maharashtra*

268 From 7 different sites including Ade and Harnai used in the study, high diversity of
269 actinobacterial species was obtained. Highest number of isolates were obtained from sediment
270 samples followed by sponge and water samples. Majority of isolates belonged to *Streptomyces*
271 *sp.* followed by *Nocardiopsis sp.* and *Micromonispora sp.* The observations coincided with
272 observations of (Jose and Jha, 2017) who worked on the samples obtained from coast of Gujarat
273 which is situated north of Maharashtra. These findings might suggest that members of above
274 three genera have adapted well to live in marine environments and show a widespread

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275 distribution along the western coast of India. Few earlier reports strongly support the existence
276 of sponge-specific microbes (Simister et al., 2012; Taylor et al., 2007). In the current study,
277 higher Shannon index values for sponge associated actinobacteria likely suggests their sponge
278 specific nature.

279 *PI Production by Marine Actinobacteria*

280 Marine actinobacteria are known to produce large number of bioactive molecules like
281 antimicrobial and anticancer compounds intra- as well as extracellularly (Subramani and
282 Aalbersberg, 2012), however, it is difficult to understand the strategy of producing extracellular
283 molecules in the marine ecosystems, since, the molecules secreted outside can be easily diluted
284 in the surroundings. Endopeptidases like chymotrypsin and trypsin are also secreted
285 extracellularly by bacteria and invertebrates in the marine environments (Holmström and
286 Kjelleberg, 1999; Thao et al., 2015). Moreover, reports have shown that coastal waters contain
287 significant amounts of trypsin-type and chymotrypsin-type endopeptidases (Obayashi and
288 Suzuki, 2005). Interestingly, results of current study demonstrated that high number of
289 actinobacteria produced PIs against chymotrypsin and trypsin. Further, more than 80% of
290 cultured actinobacteria produced extracellular inhibitors against at least one of the four
291 enzymes and almost 20% isolates showed inhibition against all the enzymes used in the study.

292 The presence of PIs suggests that they are needed for the defence of organisms against exo-
293 and endoproteases present in the surrounding environment. Moreover, they might be used as
294 defence molecules against proteases secreted by protists and bacteria.

295 *Micro-ecological Dynamics in PI Producing Actinobacteria*

296 In this study, PI producing actinobacteria were obtained from all locations and from samples
297 collected in all seasons. However, there was a remarkable difference in proportions of PI
298 producing actinobacteria obtained from sites with varying human disturbance. As seen from

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299 Shannon's diversity index, comparatively less disturbed site Ade showed more species
300 diversity, majority of non-producers of PI were reported from this site. On the contrary, almost
301 80% isolates obtained from Harnai (which has more human interference and thus chances of
302 containing higher organic load) were producing PIs. Organic load and presence of extracellular
303 proteases in the surroundings might be a driving force for production of extracellular PIs.
304 Therefore, these results can be used as a lead for carrying out studies on a larger expanse to
305 check correlation of organic load and production of PIs by the marine actinobacteria.

306 Actinobacteria are involved in bacterial predation at oligophilic conditions (Kumbhar et al.,
307 2014), they might need to produce proteases for killing prey species. Hence, one of the
308 possibilities of finding high number of PI producers amongst actinobacterial isolates might be
309 to achieve self-protection so that their own proteases are rendered ineffective against their own
310 selves. Hence, it would be interesting to check if there is any significant correlation between
311 production of PI and bacterial predation, (Baig et al., 2020).

312 Through this study we show that extracellular protease inhibitor producing actinobacteria are
313 abundant in the intertidal zones of west coast of Maharashtra. We hypothesize that this might
314 have a correlation with extracellular proteases and bacterial load in marine environment.
315 Understanding the need for production of protease inhibitors by actinomycetes can give
316 interesting insights into marine microbial ecology and clues for production of novel protease
317 inhibitors.

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324 COMPETING INTERESTS:

325 The authors declare no competing interests.

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Fig 1: Map of west coast of India (A) illustrating sampling sites along the coast of Maharashtra and Goa (B). Corresponding positions are shown in the inset (C & D). The maps were generated using QGIS software (http://qgis.osgeo.org) and Google maps.

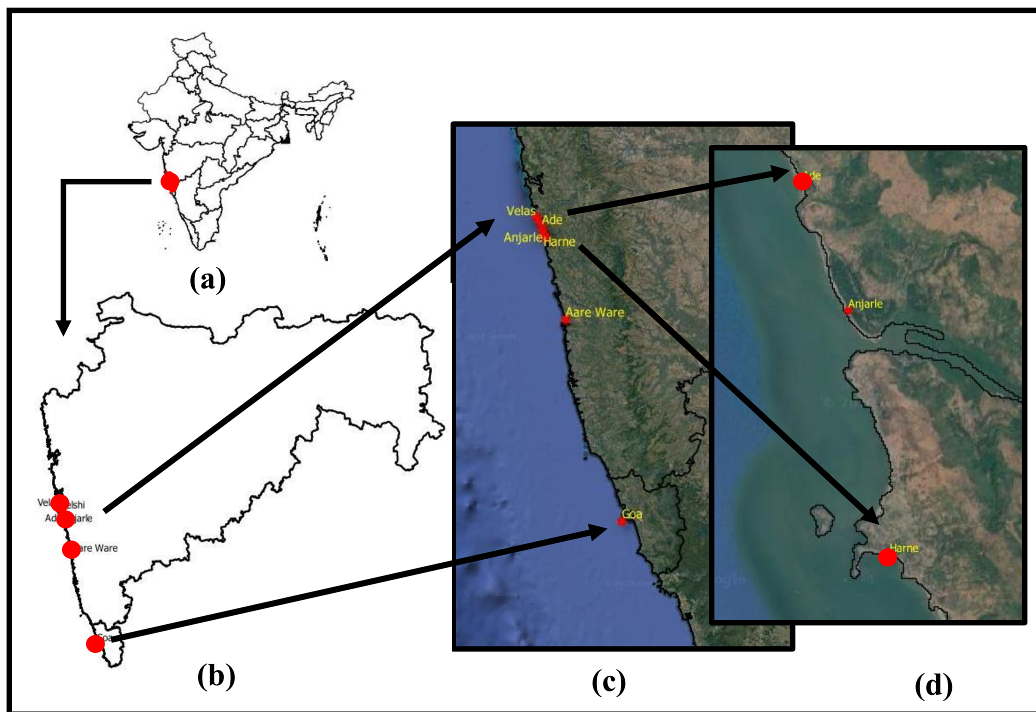

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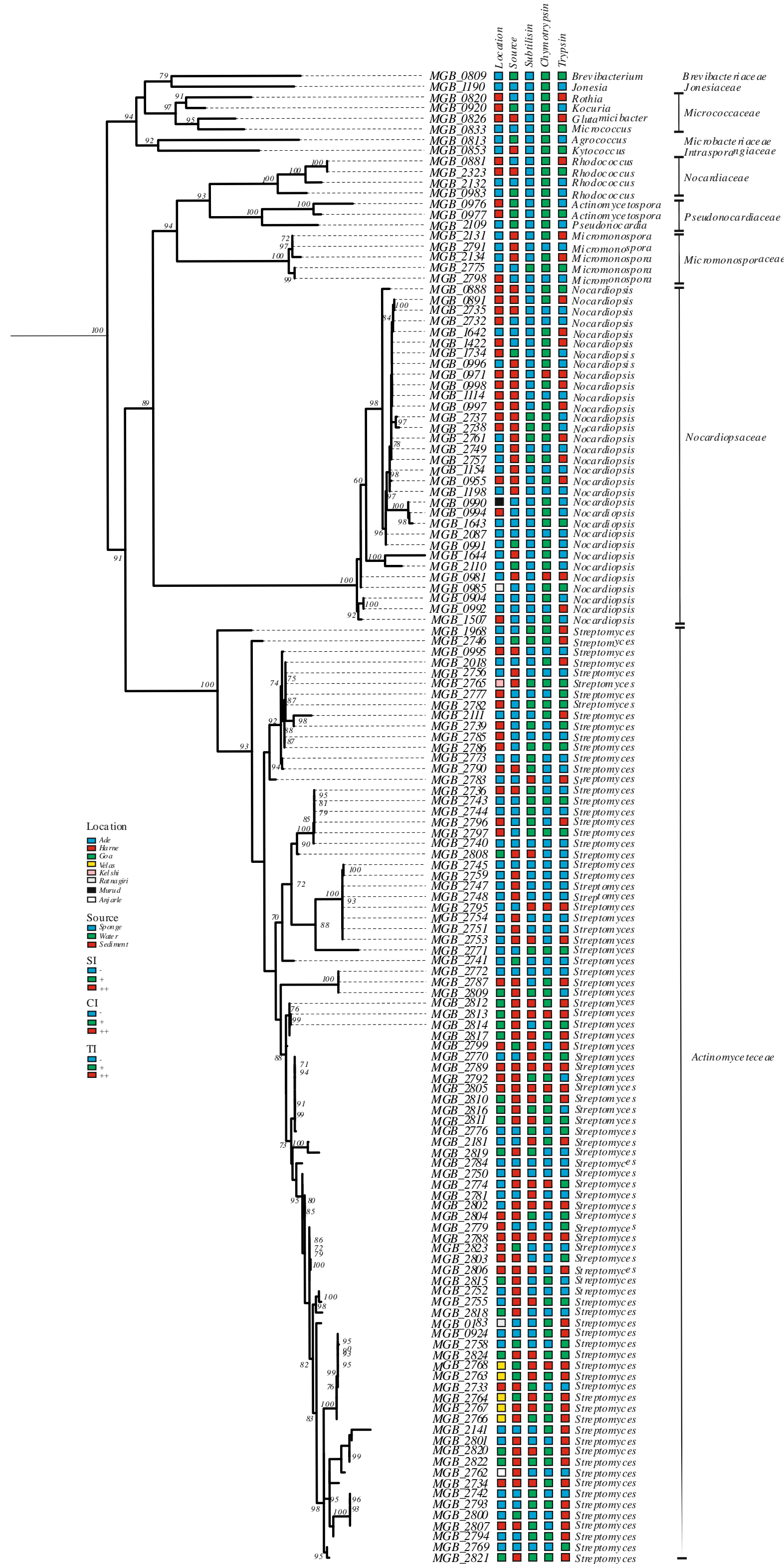
Fig 2: Phylogenetic relatedness of isolates in the study along with location, source of isolation and PI profile against different enzymes. Given is a maximum likelihood tree of actinobacteria used for screening of PIs. Outgroup taxa (*Bacillus* sp.) not shown. Percent bootstrap values of 1000 iterations are provided along the nodes. The figure also depicts location and source of isolation of the isolates along with their ability for production of PIs against subtilisin (SI), chymotrypsin (CI) and trypsin (TI) where (- : negative, + : positive and ++ : strong positive)



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490
491 **Fig 3:** Spot assay representing PI activity against trypsin (A); and Venn diagram showing
492 overlap of number of isolates producing subtilisin, chymotrypsin and trypsin inhibitors (B).
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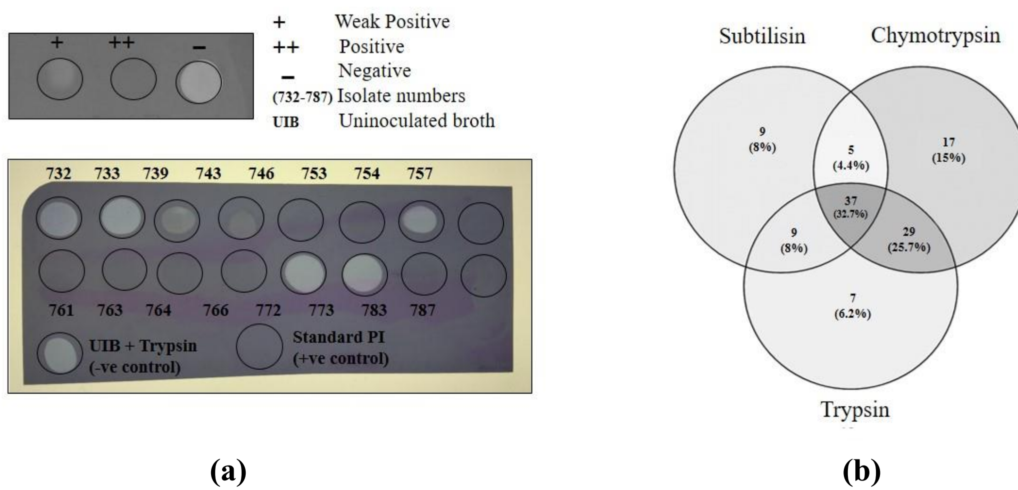

(a) (b)

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504

505 **Fig 4:** Graphs showing number of actinobacterial isolates obtained from Ade and Harnai in different
506 seasons (A) , number of isolates showing inhibitory activity against subtilisin (SI), chymotrypsin
507 (CI) and trypsin (TI) is shown in (B) and number of isolates negative for PI production are shown
508 in (C).

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