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Abstract

Phylogenetic diversity of culturable actinobacteria isolated from the intertidal regions of west coast of Maharashtra, India was studied using 16S rRNA gene sequencing. Total of 140 actinobacterial isolates were obtained, which belonged to 14 genera, 10 families and 65 putative species with *Streptomyces* being the most dominant (63%) genus followed by *Nocardiopsis* and *Micromonospora*. Isolates were screened for production of extracellular protease inhibitors (PI) against three pure proteases viz. chymotrypsin, trypsin, subtilisin and a crude extracellular protease from *Pseudomonas aeruginosa*. Eighty percent of the isolates showed PI activity against at least one of the four proteases, majority of these belonged to genus *Streptomyces*. Actinobacterial diversity from two sites Ade (17° 52′ N, 73° 04′ E) and Harnai (17° 48′ N, 73° 05′ E) with varying anthropological pressure showed that more putative species diversity was obtained from site with lower human intervention i.e. Ade (Shannon's H 3.45) than from Harnai (Shannon's H 2.83), a site with more human intervention. However, in Ade, percentage of isolates not showing PI activity against any of the proteases was close to 21% and that in Harnai was close to 9%. In other words, percentage of PI producers was lower at a site with lesser human intervention.

Introduction

Proteases are enzymes that catalyse proteolytic reactions and are involved in variety of biological processes like digestion, cell signalling or tumour formation [1–4]. They are ubiquitously present across different plant, animal and microbial taxa. Two of the many roles of these enzymes in bacteria are to aid in bacterial pathogenesis [5, 6] and bacterial predation where they secrete hydrolytic enzymes in order to degrade prey species in the vicinity [7]. Protease inhibitors bind reversibly or irreversibly with the enzymes, rendering them inactive. Hence, protease inhibitors (PIs) play a crucial role as defence molecules in intracellular or extracellular environment. They are classified based on either the type of protease they inhibit (e.g. serine protease inhibitor, aspartic protease inhibitor) or based on the mechanism of action (e.g. reversible or irreversible enzyme inhibitors). Various plants, animals and microorganisms produce PIs in order to deter pests and predators or secrete them as toxins for self-defence [8–10]. Also, PIs have high commercial potential since they are used as drugs against viral, bacterial and other parasitic infections [11–13].

Marine intercostal community harbours a variety of sedentary invertebrates and microorganisms. These organisms rely primarily on chemicals for self-defence [14, 15]. Additionally, as stated by Hay et al. the "biggest challenge for marine organisms is to obtain lunch without becoming lunch" [16]. Protease inhibitors can play a major role in these situations since they can inhibit the action of digestive

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proteases present in the surroundings. Literature reports suggest that PIs are indeed produced by marine intercostal communities [10, 11, 17]. Considering these facts, it might be possible to detect PIs synthesized by marine microorganisms for their defence.

Intertidal zones are exposed to air at low tides and are submerged at high tides. Thus they experience varying dry and wet periods, fluctuating temperatures and atmospheric pressure. Intertidal life forms adapt to these constantly changing environment by developing unique features. Their adaptations for survival in dynamic environments might also increase the chances of finding unique set of metabolites from them. Moreover, it is expected that marine microorganisms will have different set of defence molecules than their terrestrial counterparts [10, 18].

Actinobacteria is one of the largest phyla in domain bacteria. These are gram positive organisms with high guanine to cytosine ratio [19]. They at large are known to produce commercially important diverse metabolites. Some reports show that the marine environment has become a prime source for discovery of novel actinobacteria and novel natural products produced by them [20, 21]. There are few recent reports of PI production by marine actinobacteria [11, 13] yet, to our knowledge, there are limited reports on PIs from west coast of India. Thus, in the current study, marine actinobacteria from intertidal zones of selected locations on west coast were screened for presence of PIs.

It has been reported that sponges and bacteria are two main sources of enzyme inhibitors from marine environments [22]. Some reports also suggest that many a times, metabolites obtained from sponges are actually produced by the bacterial symbionts [23, 24]. In the current study sponge, sediment and water samples were collected from the coast of Maharashtra and Goa for isolation of actinobacteria. Obtained isolates were subjected to molecular identification using 16S rRNA gene sequencing and isolates were screened for presence of protease inhibitors. Data was analysed to check correlations between inhibitor molecules and environmental parameters and their probable role as defence molecules.

Harnai, one of the busy ports on the western coast is used for various activities like, auction of fishes on a daily basis, recreational space for the villagers or tourist hang out spot. Throughout the day, thousands of humans pay visit to the beach for various purposes. Certain parts of intertidal rock patch on the coast are used as open defecation sites by the villagers and few other parts of the rock patch are used for clamp collection and fishing from the rock pools. Stray cattle inhabit certain parts and human settlement is also seen just adjacent to the sandy and rocky parts of the coast. All the faecal matter of humans and cattle mixes with sea water. Also, the waste and by-catch of fishing are dumped on the coast which eventually mix with sea water. There are occasional engine oil spills from the anchored fishing vessels. The tourists litter the coast with rubber and plastic wastes which sometimes remain stuck or entangled in rocky patches. Based on these observations, it can be assumed that the human activities result in increasing the organic load in the intertidal habitats of Harnai. Conversely, Ade is hardly used for any of the above purposes except for occasional fishing from the rock pools. Thus, it can be assumed that the intertidal regions of the above sites might be varying in the amount of organic load added. This assumption was used in the current study to check if there is any difference in the percentage of PI producers amongst the above sites. Sampling from these two sites was done for one seasonal cycle to check the seasonal variations in diversity and proportion of PI producers.

Materials and Methods

Sampling

Around 60 samples comprising of sponge, sediment and sea water were collected from the intertidal rock pools from seven different locations along the coast of Ratnagiri district from Velas, Kelshi, Ade, Anjarle, Harnai, Murud and Aare Ware (17° 57′ 04.9″ N, 73° 01′ 43.0″ E to 17° 04′ 35.9″ N, 73° 17′ 17.5″ E). A single subtidal sampling of sediment and water was carried out from a place off shore to the coast of Goa (15° 21′ 08.4″ N, 73° 46′ 41.8″ E) (Supplementary Fig. 1). The depths of collection for these sampling were 9 m, 11 m, 12 m and 16 m. Single sampling was also carried out from intertidal zones of Velas, Kelshi, Anjarle and Murud however, at the time of sampling, sponge colonies could not be located on any of these sites and thus only sediment and water samples were collected. Whereas, at Harnai and Ade four to five distinct morphotypes of sponges were recorded. Thus, sponge, sediment and water samples were collected from these sites. Further, the sample collection from Harnai and Ade was carried out in different seasons viz. pre-monsoon (Mar, Apr, May), Monsoon (Early Sep), post-monsoon (Oct) and winter season (Dec, Jan, Feb).

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

Sample Processing, Selective Isolation and Culture Maintenance

All the samples were given heat treatment at 60 °C for 15 min to reduce the load of non-sporulating bacteria. Sponge samples were homogenized in Poor Ravan Saline (PRS) medium [25] and diluted serially with tenfold dilutions up to 10^{-5}. Sediment samples were vigorously shaken for two minutes and diluted up to 10^{-5} dilutions. Sea water samples were also diluted up to 10^{-5} dilutions. 0.1 ml of undiluted, 10^{-3} and 10^{-5} dilutions were spread plated in triplicates on different growth media and incubated at 30 °C for up to 21 days.

As the current study focused only on the isolation of actinobacteria, growth media used for isolation were aimed at obtaining maximum culture dependent diversity. For this purpose, four different media, Sponge agar (1% macerated sponge colonies collected from the site, 50% sea water and 2.5% agar), Sea Water Agar (50% sea water and 2.5% agar), ZOBELL Marine Agar [26] and Poor Ravan saline medium were used to obtain the isolates. Sponge agar and sea water agar were designed to mimic nutrient composition close to the natural habitats. Poor ravan medium is an oligophilic medium developed to isolate oligophilic bacteria [25]. In order to isolate marine oligotrophic actinobacteria slight modifications were made in the medium composition by adjusting the salinity of the medium to 2%. The salinity was adjusted in terms of NaCl concentration and the medium was called as Poor Ravan Saline (PRS) medium. This medium was also used for transportation of samples. Nalidixic acid and Cyclohexamide were added to the culture medium in 25 µg ml^{-1} concentration to inhibit the growth of Gram-negative bacteria and fungi [27]. Plates were incubated at 30 °C and monitored daily for 21 days. Isolated colonies showing resemblance to typical actinobacterial colony morphology were picked up and subcultured several times for obtaining pure cultures. All the pure cultures were later stored on modified (1:4 diluted) ZOBELL marine agar at 4 °C for further use.

DNA Sequencing and Diversity Analysis

16S rRNA sequences from earlier study [28] were used to make maximum likelihood phylogenetic tree of actinobacteria. The tree was constructed in IQ-Tree version 1.6.12 [29]. Best nucleotide substitution model was determined in model finder [30]. Note support was examined using bootstrap values of 1000 iterations and *Bacillus* sp. were used as out-group. All the isolates were identified up to genus level. For this study, known species were assigned to these isolates based on BLAST search with sequence identity more than 99% [28]. Shannon's diversity index to measure species diversity of actinobacteria was calculated using Past (version 4.0).

Screening for Protease Inhibitors (PI) Using Spot Assay

Sample Preparation

Detection of extracellular PIs from cell free supernatant was done as described [31]. Pure cultures of actinobacteria were inoculated (single colony in 150 ml broth) in ZOBELL Marine broth and incubated for 5 days at 30 °C on incubator shaker with 100 rpm speed. After incubation, the cultures were centrifuged at 4000 rpm for 20 min and cell free supernatants (CFS) were collected. 1 ml of each of these CFSs were kept in hot water bath of 70 °C for 15 min for denaturation of proteases produced by the bacteria. Heat treated CFSs were used for detection of PIs. Unprocessed X-ray films coated with gelatine were used to detect PIs. (Gelatine is degraded by various proteolytic enzymes, so, upon action of the proteases, clear zones are observed on unprocessed X-ray films at the point of contact of enzymes. On the other hand, if the gelatine layer remains intact, no clearance is observed. PIs inactivate proteases and thus, presence of protease inhibitors is marked by no clearance on the X-ray films.

Proteases used for the assay included Trypsin (SRL Pvt. Ltd, Cat no.- 60484) and α-Chymotrypsin (SRL Pvt. Ltd, Cat no.- 35085) of bovine origin and Subtilisin (Sigma-Aldrich, Cat no.- P5380) and crude protease obtained in the laboratory from cell free supernatant of *Pseudomonas aeruginosa* (NCBI Accession number—MN044759) were of bacterial origin. Additionally, Protease inhibitor cocktail (Sigma-Aldrich, Cat no.- P5380) was used as a positive control and un-inoculated culture broth was used as a negative control.

Spot Assay

Standardization of enzyme concentration was done as described earlier [32]. Various dilutions of pure enzyme in triplicates were spotted on gelatine coated X-ray films. Lowest enzyme dilution that gave complete clearance, in turn indicating complete digestion of gelatine, was used for the assay.

Spot assay described previously [33] for detection of PIs was carried out as follows. 10 µl of pure enzyme (100 µg ml^{-1}) was incubated with 10 µl of heat treated CFS for 10 min and then transferred to untreated X-ray-Fuji Medical X-ray, HRU grade-film. The assembly was kept undisturbed for 15 min at room temperature to allow the enzyme substrate reaction to take place. Films were washed with running tap water and allowed to dry before recording the results. As elaborated in Fig. 2, the results were recorded

as strong positive (++), positive (+) and negative (−) with respect to the controls used. Those samples which showed no clearance at all (0% clearance) were demarcated as strong positive (++), the ones with complete clearance (100% clearance) were considered negative (−) and samples which gave partial clearance (between 1 and 99% clearance) were noted as positive (+) for PI production. All the assays were carried out in duplicates and results were recorded based on following criteria:

	Result of replicate 1	Result of replicate 2	Final result recorded
Case 1	Strong positive (++)	Strong positive (++)	Strong positive (++)
Case 2	Negative (−)	Negative (−)	Negative (−)
Case 3	Positive (+)	Positive (+)	Positive (+)
Case 4	Strong positive (++)	Positive (+)	Positive (+)
Case 5	Negative (−)	Positive (+)/ Strong positive (++)	Negative (−)

Results

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

We obtained 140 actinobacterial isolates from sponge, sediment and sea water samples. They showed high phylogenetic diversity wherein there were 65 putative species belonging to 14 genera of 10 different families. Most abundant genus amongst the isolates was *Streptomyces* (~63%) followed by *Nocardiopsis* (~22%) and *Micromonospora.* (~4%). Approximately 11% isolates belonged to other genera (Supplementary table S2).

From sponges, 50 actinobacterial isolates were obtained which belonged to 7 genera and 7 families. Whereas, 71 isolates were obtained from sediments that belonged to 6 different genera and 6 families. 19 isolates belonging to 8 genera of 7 families were isolated from sea water (Table 1). Even though, more number of isolates were obtained from sediment, Shannon's diversity index showed higher putative species diversity in isolates obtained from sponge (Shannon's H 3.34) than sediment (Shannon's H 3.00) and water (Shannon's H 2.69).

More number of isolates (67) and more species diversity (10 genera with 43 putative species) were obtained from anthropologically less disturbed site Ade. Whereas, from Harnai which is a site with higher disturbance 47 isolates belonging to 25 putative species of 9 genera could be isolated. (Fig. 1). Shannon's diversity index (H) value for Ade was 3.45 and that for Harnai was 2.83. This indicates that the putative species diversity at Ade is more than diversity at Harnai.

Production of Protease Inhibitors

Results of PI production were recorded using spot assay as shown in Fig. 2. It was observed that, PIs retained their activity even after heat treatment at 70 °C for 15 min. Out of 140 isolates used for screening of PIs, 113 isolates showed activity against at least one of the three pure proteases used for the study (viz. chymotrypsin, trypsin and subtilisin), whereas 27 isolates showed no PI production at all against any of these enzymes. Out of 113 isolates, 37 showed some degree of activity against all pure enzymes, of which 8 showed strong positive (++) activity. Total of 17, 7 and 9 isolates showed PI activity exclusively against chymotrypsin, trypsin and subtilisin respectively (Fig. 2). Majority of isolates which showed strong positive activity against enzyme chymotrypsin, also showed strong positive activity against subtilisin and trypsin. Interestingly, even though 20% of the *Streptomyces* members did not show any inhibition against any of the proteases, all the isolates showing strong positive activity against all enzymes belonged entirely to *Streptomyces* sp. Very few isolates of *Nocardiopsis* sp. showed PI activity against subtilisin. Regarding the source, highest number of strong positive isolates (7 out of 8) were obtained from the sediments and only 1 was obtained from the sponge. (Fig. 1).

Out of 113 PI producers, strong positive (++) activity was shown by 10, 54, and 27 isolates against chymotrypsin, trypsin and subtilisin respectively (Table 2). A subset of 90 isolates belonging to most common genera viz. *Streptomyces*, *Nocardiopsis* and *Micromonospora* were chosen to check the activity against another extracellular bacterial protease which was obtained from cell free supernatant of *P.*

Table 1 Number of isolates belonging to different families obtained from various sources

Families	Sediment	Water	Sponge	Total
Actinomycetaceae	47	9	32	88
Brevibacteriaceae	–	1	–	1
Intrasporangiaceae	1	–	–	1
Microbacteriaceae	–	1	–	1
Micrococcaceae	1	1	2	4
Micromonosporaceae	3	–	2	5
Nocardiaceae	1	1	2	4
Nocardiopsaceae	18	3	11	32
Pseudonocardiaceae	–	3	–	3
Jonesiaceae	–	–	1	1
Grand total	71	19	50	140

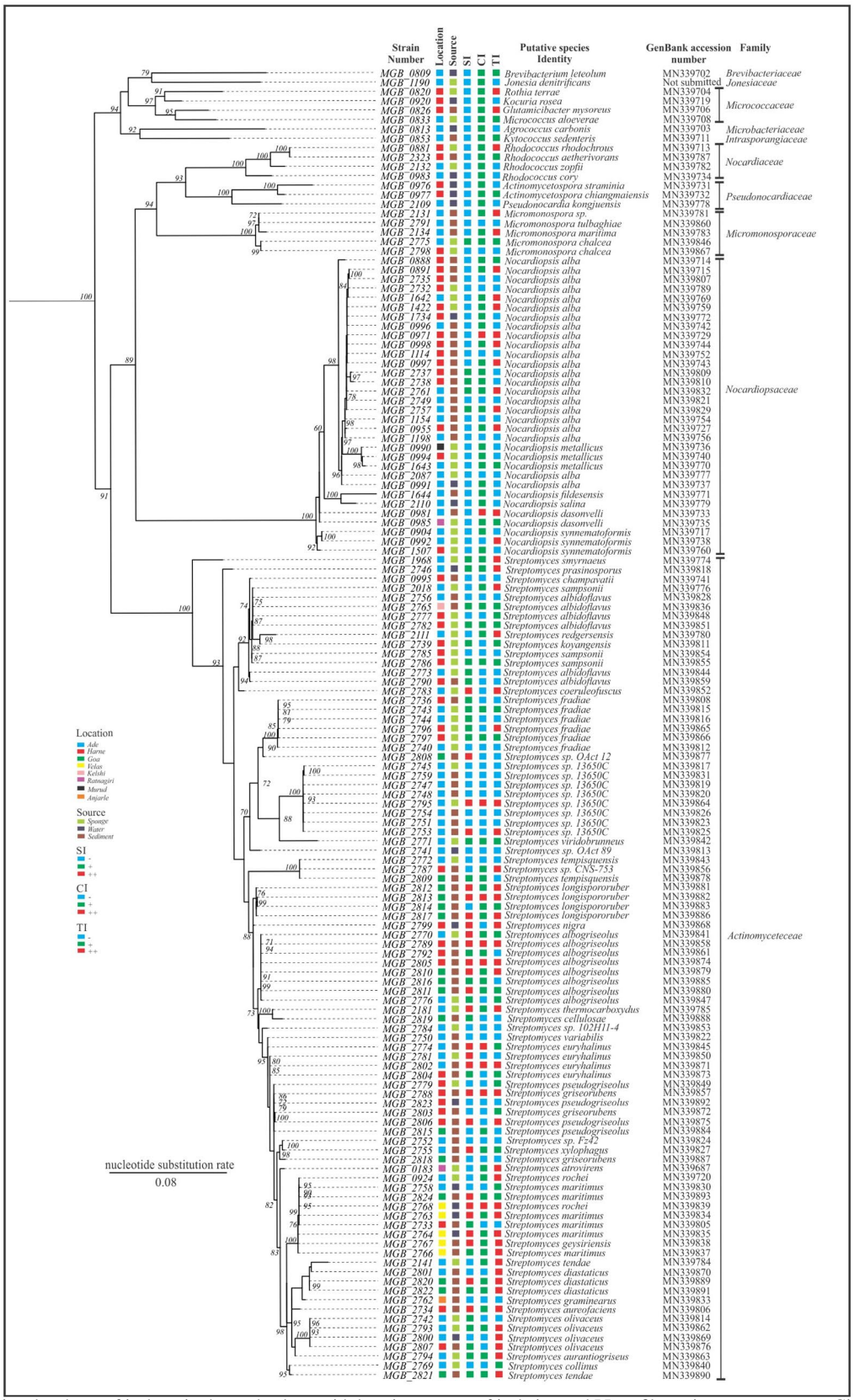

Fig. 1 Phylogenetic relatedness of isolates in the study along with location, source of isolation and PI profile against pure enzymes. Given is a maximum likelihood tree of actinobacteria used for screening of PIs. Out group taxa (*Bacillus* sp.) not shown. Percent bootstrap values of 1000 iterations are provided along the nodes. Also shown are location and source of isolation and their ability of production of PIs against subtilisin (SI), chymotrypsin (CI) and trypsin (TI) where (−: negative, +: positive and ++: strong positive)

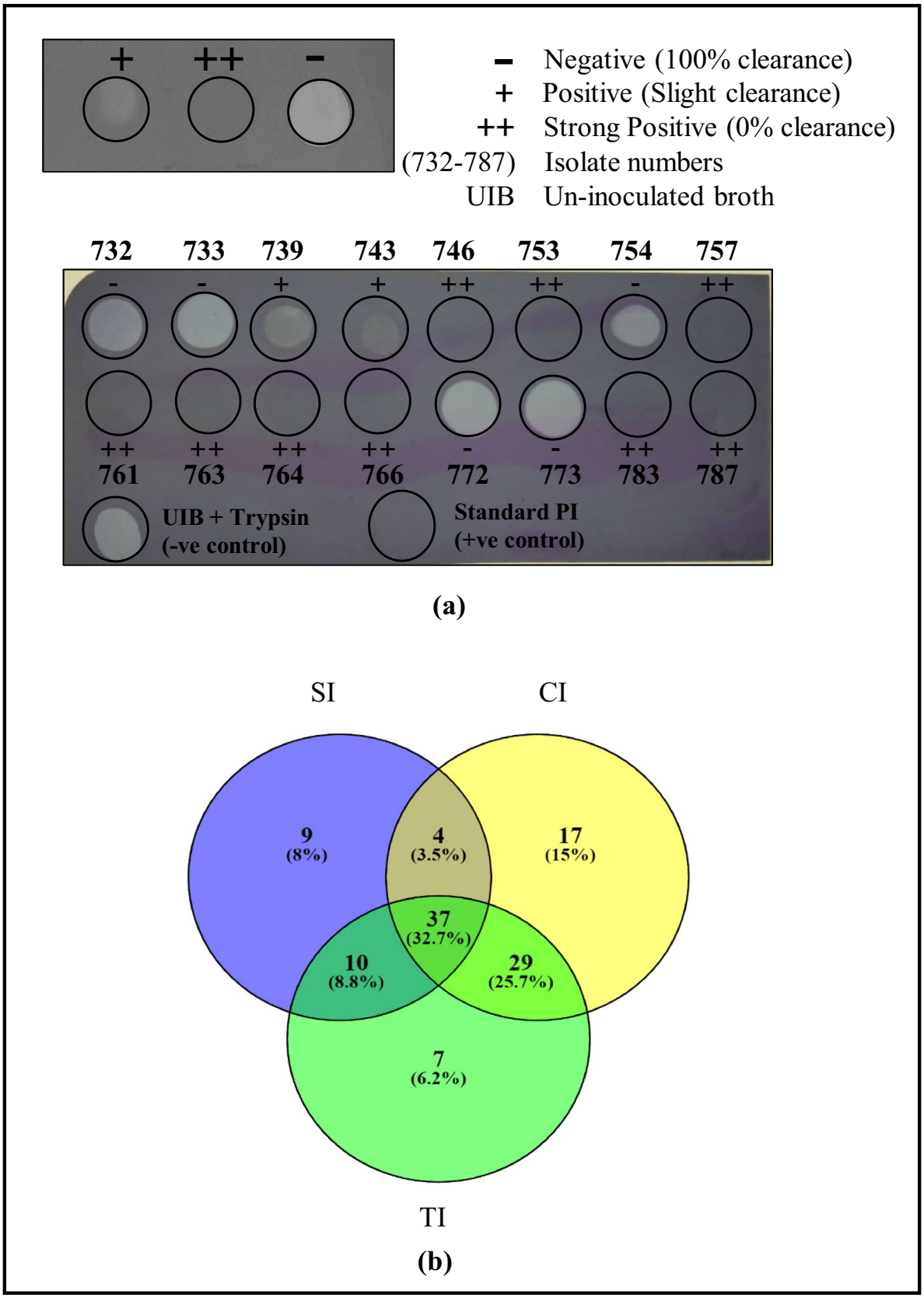

Fig. 2 Spot assay representing PI activity against trypsin (**a**); and Venn diagram showing overlap of number of isolates producing subtilisin, chymotrypsin and trypsin inhibitors. Venn diagram is not to scale and prepared using Venny (version 2.1) [50] (**b**)

Table 2 Genus-wise counts of PI producers active against pure proteases

Genus	Total no. of isolates	Chymotrypsin			Trypsin			Subtilisin		
		−	+	++	−	+	++	−	+	++
Actinomycetospora	2	0	2	0	1	1	0	2	0	0
Agrococcus	1	0	1	0	1	0	0	1	0	0
Brevibacterium	1	0	1	0	0	1	0	1	0	0
Glutamicibacter	1	0	1	0	0	0	1	1	0	0
Jonesia	1	0	1	0	1	0	0	1	0	0
Kocuria	1	0	1	0	1	0	0	1	0	0
Kytococcus	1	0	1	0	0	1	0	1	0	0
Micrococcus	1	0	1	0	0	1	0	1	0	0
Micromonospora	5	2	3	0	2	1	2	4	1	0
Nocardiopsis	32	7	23	2	18	3	11	28	4	0
Pseudonocardia	1	0	1	0	1	0	0	1	0	0
Rhodococcus	4	0	4	0	2	1	1	4	0	0
Rothia	1	0	1	0	0	0	1	1	0	0
Streptomyces	88	43	37	8	31	19	38	33	28	27
Grand Total	140	52	78	10	58	28	54	80	33	27

PI activity is represented as '−': negative, '+': positive and '++': strong positive

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

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

PI activity is represented as '−': negative, '+': positive and '++': strong positive

aeruginosa, 36 isolates gave some degree of activity against this crude bacterial protease (Table 3).

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 (Fig. 3a). From Ade and Harnai, over all, occurrence of inhibitors against chymotrypsin and trypsin was more as compared to that against subtilisin (Fig. 3b). 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 8.5%) (Fig. 3c). 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.

Discussion

Actinobacteria are widespread in terrestrial and aquatic environments. Many of them also produce external spores which are resistant to dehydration [19]. Various reports say that marine actinobacteria have a high potential for production of biomolecules [34, 35] however, the number of actinobacterial compounds discovered so far are limited not by the number of compounds produced by them but by the amount of screening efforts put in [36]. Many reports have highlighted the need for studying marine actinomycete diversity and their potential use for obtaining novel metabolites [37–40]. Researchers from India have also isolated actinobacteria from coastal regions of West Bengal [41, 42], Gujarat [34], Tamil Nadu [43] and Andaman and Nicobar [11] to name a few for bioprospecting studies. However, there are very few reports of isolation of actinobacteria and still fewer reports on PI producing actinobacteria from the coast of Maharashtra. Thus considering these facts, current study focused on isolation of actinobacteria from coast of Maharashtra and their potential as producers of PIs.

Phylogenetics of Actinobacteria from Intertidal Regions of Maharashtra

From seven different sites including Ade and Harnai used in the study, high diversity of actinobacterial species was

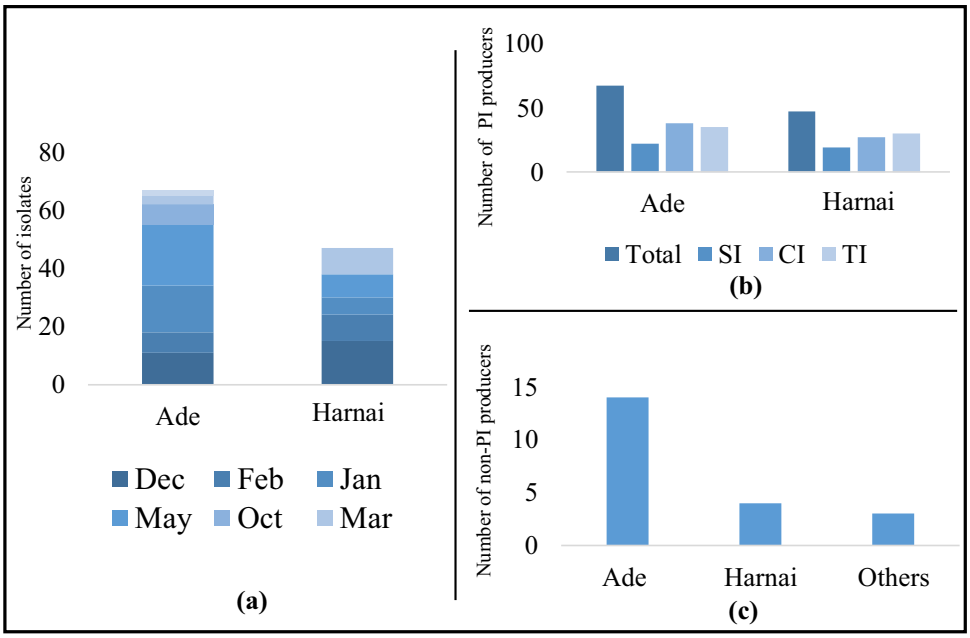

Fig. 3 Graphs showing number of actinobacterial isolates obtained from Ade and Harnai in different seasons (**a**), number of isolates showing inhibitory activity against subtilisin (SI), chymotrypsin (CI) and trypsin (TI) is shown in **b** and number of isolates negative for PI production are shown in **c**

obtained. Highest number of isolates were obtained from sediment samples followed by sponge and water samples. Majority of isolates belonged to *Streptomyces sp.* followed by *Nocardiopsis sp.* and *Micromonospora sp.* The observations coincided with observations of a study from Gujarat which is situated north of Maharashtra [34]. These findings might suggest that members of above three genera have adapted well to live in marine environments and show a widespread distribution along the western coast of India. Few earlier reports strongly support the existence of sponge-specific microbes [44, 45]. In the current study, higher Shannon index values for sponge associated actinobacteria likely suggest their sponge specific nature.

PI Production by Marine Actinobacteria

Marine actinobacteria are known to produce large number of bioactive molecules like antimicrobial and anticancer compounds intra- as well as extracellularly [40], however, it is difficult to understand the strategy of producing extracellular molecules in the marine ecosystems, since, the molecules secreted outside can be easily diluted in the surroundings. Endopeptidases like chymotrypsin and trypsin are also secreted extracellularly by bacteria and invertebrates in the marine environments [46, 47]. Moreover, reports have shown that coastal waters contain significant amounts of trypsin-type and chymotrypsin-type endopeptidases [48]. Interestingly, results of current study demonstrated that high number of actinobacteria produced PIs against chymotrypsin and trypsin. Further, more than 80% of cultured actinobacteria produced extracellular inhibitors against at least one of the four enzymes and almost 20% isolates showed inhibition against all the enzymes used in the study.

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

Micro-ecological Dynamics in PI Producing Actinobacteria

In this study, PI producing actinobacteria were obtained from all locations and from samples collected in all seasons. However, there was a remarkable difference in proportions of PI producing actinobacteria obtained from sites with varying human disturbance. As seen from Shannon's diversity index, comparatively less disturbed site Ade showed more species diversity and majority of non-PI producing isolates were reported from this site. On the contrary, almost 80% isolates obtained from Harnai (which has more human interference and thus chances of containing higher organic load) were producing PIs. Therefore, it would be interesting to see whether organic load and presence of extracellular proteases in the surroundings affect the production of extracellular PIs. These results can be used in the same regard as a lead for carrying out studies on a larger expanse to check correlation of organic load and production of PIs by the marine actinobacteria.

Actinobacteria are involved in bacterial predation at oligophilic conditions [49], they might need to produce proteases for killing prey species. Hence, one of the possibilities of finding high number of PI producers amongst

actinobacterial isolates might be to achieve self-protection so that their own proteases are rendered ineffective against their own selves. Hence, it would be interesting to check if there is any significant correlation between production of PI and bacterial predation, [28].

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

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Author Contributions ND, VT, NS and RP conceived and designed the study. NS, UB, and AP performed the study. NS and VT analysed the data. NS, VT, and ND wrote the manuscript with inputs from other authors. All authors contributed to the proofreading of the manuscript.

Data Availability Sequences of 16S rRNA gene of studied isolates are submitted to GenBank NCBI under the accession numbers MN339687–MN339893. Data used for analysis are provided in Supplementary Table 1.

Compliance with Ethical Standards

Competing interests The authors declare no competing interests.

References

1. von Groll U, Berger D, Altmann T (2002) The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during Arabidopsis stomatal development. Plant Cell 14(7):1527–1539
2. Koblinski JE, Ahram M, Sloane BF (2000) Unraveling the role of proteases in cancer. Clin Chim Acta 291(2):113–135
3. Overall CM, Blobel CP (2007) In search of partners: linking extracellular proteases to substrates. Nat Rev Mol Cell Biol 3:245–257
4. Roberts IN, Caputo C, Criado MV, Funk C (2012) Senescence-associated proteases in plants. Physiol Plant 145(1):130–139
5. Ingmer H, Brøndsted L (2009) Proteases in bacterial pathogenesis. Res Microbiol 160(9):704–710
6. Maeda H (1996) Role of microbial proteases in pathogenesis. Microbiol Immunol 40(10):685–699
7. Martin MO (2002) Predatory prokaryotes: an emerging research opportunity. J Mol Microbiol Biotechnol 5:467–477
8. Habib H, Fazili KM (2007) Plant protease inhibitors: a defense strategy in plants. https://academicjournals.org/journal/BMBR/article
9. Hartl M, Giri AP, Kaur H, Baldwin IT (2011) The multiple functions of plant serine protease inhibitors: defense against herbivores and beyond. Plant Signal Behav 7:1009–1011
10. Mourão CBF, Schwartz EF (2013) Protease inhibitors from marine venomous animals and their counterparts in terrestrial venomous animals. Mar Drugs 11(6):2069–2112
11. Karthik L, Kumar G, Keswani T, Bhattacharyya A, Chandar SS, Rao KVB (2014) Protease inhibitors from marine actinobacteria as a potential source for antimalarial compound. PLoS ONE 9(3):e90972
12. Liu X, Gan M, Dong B, Zhang T, Li Y, Zhang Y et al (4862F) 4862F, a new inhibitor of HIV-1 protease, from the culture of Streptomyces I03A–04862. Molecules 18(1):236–243
13. Sreedharan V, Bhaskara Rao KV (2017) Efficacy of protease inhibitor from marine Streptomyces sp. VITBVK2 against *Leishmania donovani*—an in vitro study. Exp Parasitol 174:45–51
14. Engel S, Jensen PR, Fenical W (2002) Chemical ecology of marine microbial defense. J Chem Ecol 28(10):1971–1985
15. Pawlik JR (1993) Marine invertebrate chemical defenses. Chem Rev 93(5):1911–1922
16. Hay ME (2009) Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. Annu Rev Mar Sci 1:193–212
17. Covaleda G, del Rivero MA, Chávez MA, Avilés FX, Reverter D (2012) Crystal structure of novel metallocarboxypeptidase inhibitor from marine mollusk *Nerita versicolor* in complex with human carboxypeptidase A4. J Biol Chem 287(12):9250–9258
18. Xie C-L, Xia J-M, Wang J-S, Lin D-H, Yang X-W (2018) Metabolomic investigations on *Nesterenkonia flava* revealed significant differences between marine and terrestrial actinomycetes. Mar Drugs 16(10):356
19. Trujillo ME (2016) Actinobacteria. In: eLS. American Cancer Society, pp 1–16. https://onlinelibrary.wiley.com/doi/abs/10.1002/9780470015902.a0020366.pub2
20. Maldonado LA, Stach JEM, Pathom-aree W, Ward AC, Bull AT, Goodfellow M (2005) Diversity of cultivable actinobacteria in geographically widespread marine sediments. Antonie Van Leeuwenhoek 87(1):11–18
21. Ward AC, Bora N (2006) Diversity and biogeography of marine actinobacteria. Curr Opin Microbiol 9(3):279–286
22. Ruocco N, Costantini S, Palumbo F, Costantini M (2017) Marine sponges and bacteria as challenging sources of enzyme inhibitors for pharmacological applications. Mar Drugs 15(6):173
23. Haygood MG, Schmidt EW, Davidson SK, Faulkner DJ (1999) Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology. J Mol Microbiol Biotechnol 1(1):33–43
24. Mehbub MF, Lei J, Franco C, Zhang W (2014) Marine sponge derived natural products between 2001 and 2010: trends and opportunities for discovery of bioactives. Mar Drugs 12(8):4539–4577
25. Watve M, Shejval V, Sonawane C, Rahalkar M, Matapurkar A, Shouche Y et al (2000) The "K" selected oligophilic bacteria: a key to uncultured diversity? Curr Sci 78:1535–1542
26. Zobell CE (1941) Studies on marine bacteria, I : the cultural requirements of heterotrophic aerobes. J Mar Res 4(1):42–75
27. Magarvey NA, Keller JM, Bernan V, Dworkin M, Sherman DH (2004) Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. Appl Environ Microbiol 70(12):7520–7529
28. Baig U, Dahanukar N, Shintre N, Holkar K, Pund A, Lele U et al (2020) Phylogenetic diversity and activity screening of cultivable actinobacteria isolated from marine sponges and associated environments from the western coast of India. bioRxiv.
29. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ (2015) IQ-Tree: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32(1):268–274
30. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS (2017) ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods 14(6):587–589

31. Shintre N, Baig U, Pund A, Patwardhan R, Tamhane V, Deshpande N (2020) Diversity of culturable actinobacteria producing protease inhibitors isolated from the intertidal zones of Maharashtra, India. bioRxiv
32. Tripathi VR, Kumar S, Garg SK (2011) A study on trypsin, Aspergillus flavus and Bacillus sp protease inhibitory activity in Cassia tora (L.) syn Senna tora (L.) Roxb. seed extract. BMC Complement Altern Med 11:56
33. Cheung AL, Ying P, Fischetti VA (1991) A method to detect proteinase activity using unprocessed X-ray films. Anal Biochem 193(1):20–23
34. Jose PA, Jha B (2017) Intertidal marine sediment harbours Actinobacteria with promising bioactive and biosynthetic potential. Sci Rep 7(1):1–15
35. Solanki R, Khanna M, Lal R (2008) Bioactive compounds from marine actinomycetes. Indian J Microbiol 48(4):410–431
36. Watve M, Tickoo R, Jog M, Bhole B (2001) How many antibiotics are produced by the genus *Streptomyces* ? Arch Microbiol 176(5):386–390
37. Dharmaraj S (2010) Marine Streptomyces as a novel source of bioactive substances. World J Microbiol Biotechnol 26(12):2123–2139
38. Jensen PR, Mincer TJ, Williams PG, Fenical W (2005) Marine actinomycete diversity and natural product discovery. Antonie Van Leeuwenhoek 87(1):43–48
39. Lam KS (2006) Discovery of novel metabolites from marine actinomycetes. Curr Opin Microbiol 9(3):245–251
40. Subramani R, Aalbersberg W (2012) Marine actinomycetes: an ongoing source of novel bioactive metabolites. Microbiol Res 167(10):571–580
41. Peela S, Kurada VB, Terli R (2005) Studies on antagonistic marine actinomycetes from the Bay of Bengal. World J Microbiol Biotechnol 21(4):583–585
42. Ramesh S, Mathivanan N (2009) Screening of marine actinomycetes isolated from the Bay of Bengal, India for antimicrobial activity and industrial enzymes. World J Microbiol Biotechnol 25(12):2103–2111
43. Raja A, Prabakaran P (2011) Actinomycetes and drug-an overview. Am J Drug Discov Dev 1(2):75–84
44. Simister RL, Deines P, Botté ES, Webster NS, Taylor MW (2012) Sponge-specific clusters revisited: a comprehensive phylogeny of sponge-associated microorganisms. Environ Microbiol 14(2):517–524
45. Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71(2):295–347
46. Holmström C, Kjelleberg S (1999) Marine Pseudoalteromonas species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiol Ecol 30(4):285–293
47. Thao NV, Nozawa A, Obayashi Y, Kitamura S-I, Yokokawa T, Suzuki S (2015) Extracellular proteases are released by ciliates in defined seawater microcosms. Mar Environ Res 109:95–102
48. Obayashi Y, Suzuki S (2005) Proteolytic enzymes in coastal surface seawater: Significant activity of endopeptidases and exopeptidases. Limnol Oceanogr 50(2):722–726
49. Kumbhar C, Mudliar P, Bhatia L, Kshirsagar A, Watve M (2014) Widespread predatory abilities in the genus Streptomyces. Arch Microbiol 196(4):235–248
50. Oliveros, JC (2007) Venny. An interactive tool for comparing lists with Venn diagrams. https://bioinfogp.cnb.csic.es/tools/venny/index.html.