

ANTAGONISTIC ACTINOBACTERIAL DIVERSITY OF MARINE SEDIMENTS COLLECTED FROM COASTAL TAMIL NADU

MANIKANDAN MADHESLU¹, SWAPNA MANI², DURAIMURUGAN KASIVISWANATHAN³,
SOLAI RAMATCHANDIRANE PRABAGARAN^{4*}

¹Department of Biotechnology, Sri Krishna Arts and Science College, Coimbatore, Tamil Nadu, India. ²Department of Biotechnology, Mercy College, Palakkad, Kerala, India. ³Department of Food Processing and Nutrition Science, School of Community Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur, West Bengal, India. ⁴Department of Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India. Email: prabagaran@buc.edu.in

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ABSTRACT

Objective: The main objective of the current investigation is to identify the antagonistic potential of marine sediment actinobacterial population collected from coastal regions of Tamil Nadu and Kerala.

Methods: A total of 60 marine sediment samples were collected from 27 locations of coastal Tamil Nadu and Kerala. A total of 85 morphologically distinct actinobacterial strains were isolated and taxonomically characterized. The strains were screened for its antagonistic potential against selected bacteria strains, and the obtained results were statistically analyzed. Further, the strains excelled in producing antimicrobial substances were subjected to electron microscopic and chemotaxonomic studies to get more taxonomic details.

Results: The isolates were identified as different species of *Streptomyces*, *Micromonospora*, *Saccharopolyspora*, *Actinopolyspora*, *Nocardia*, *Nocardioides*, *Kitasatospora*, and the rest of them were categorized as "unidentified." All the isolates were subjected to antimicrobial assay through cross-streak and well diffusion methods that endorsed 34 (40%) of them to be active against clinical and reference bacterial strains such as *Staphylococcus aureus* MTCC 3160, *S. aureus* (methicillin-resistant), *Bacillus pumilus* NCIM 2327, *Escherichia coli* MTCC 1698, *E. coli*, *Shigella flexneri* MTCC 1457, and *Proteus vulgaris*. The predominance of *Streptomyces* (18) was ascertained and followed by *Saccharopolyspora* (7), *Micromonospora* (5), *Nocardia* (2), and *Kitasatospora* (2) with considerable antimicrobial activities. Statistical analysis revealed that the isolates BDK01, BMT01, BPY03, and BRS07 showed significant antimicrobial activities. Phenotypic and chemotaxonomic analysis revealed that all four isolates are belonging under *Streptomyces* genus.

Conclusion: The output of the present investigation revealed that the *Streptomyces* species are predominant in marine sediments and gain more attention for their antagonistic potential. Therefore, assessment and evaluation of marine microbial diversity were done, which could serve as a potential source for novel antibiotics.

Keywords: Antagonistic potential, Intertidal and sub-littoral zone, Marine actinobacteria, Marine sediment, Tamil Nadu coast.

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INTRODUCTION

Marine ecosystem occupies a larger part of the Earth containing different physiochemical and biological features, particularly, the salinity, which makes it different from the terrestrial condition. Discovery of new microbial taxa from the marine environment is a novel approach to discover new metabolites from such regimes. The marine environment facilitates actinobacteria to adapt extreme conditions such as high atmospheric pressure, temperature (0°C–100°C), and high acidic conditions. It is often reflected in the genetic and metabolic diversity of marine actinobacteria [1], which makes the marine-derived compounds special for medical investigation and industrial applications.

Out of 53 known bacterial phyla, only five were reported for antimicrobial activities [2]. Among them, five bacteria are belonging to the order *Actinomycetales* account for approximately 7000 bioactive compounds, as reported in the Dictionary of Natural Products. However, only 140 genera of actinobacteria are well described for synthesizing a vast variety of natural products. It is, therefore, vital to isolate novel strains for synthesizing new compounds [3]. Actinobacteria, in the marine environment, are largely unexplored, indigenous population that remains elusive in marine water and sand. This may be due to the lack of concrete effort and a high-profile investigation for the complete understanding. Sometimes terrestrial waterbodies carry actinobacterial spores into the marine habitats, leading to later identification [4].

The Indian subcontinent is covered by two major seas and an ocean with a coastal region of about 7500 km with an area of 2.02 million sq. km in our exclusive economic zone, besides an additional stretch of 2000 km from Andaman-Nicobar and Lakshadweep islands chains. It consists of sandy beaches, rocky shores, cliffs, and mudflats or marshy shores. Tamil Nadu coastal region is almost occupied by the Bay of Bengal for the entire length, and a bit is covered by the Indian Ocean. This region comprises diverse marine habitats such as sea-shore, hypersaline lakes, estuaries, salt pans, and a variety of soil habitats. Microbial diversity and its applications along coastal regions of India were well studied by various investigators in the past decade [5-9]. In the present study, an attempt was made to explore antagonistically potential actinobacterial diversity from the marine sediment samples collected from the coastal Tamil Nadu.

METHODS

Study area

Sampling was conducted at the Bay of Bengal with fair coverage along the east coast of Tamil Nadu, India (Fig. 1). The sampling area included various ecological niches such as estuaries, brackish water lagoons, and mangrove forest, which varied in physiochemical and biological constituents. In addition to these, tsunami, in 2004, moved some deep sea organism to the coastal regions, which adds merit to our study [10]. Most of the samples were collected in the east coast and two (Calicut and Muttom) were collected from the west coast.

Collection of marine sediment samples

During the months of April-May in 2012 and 2013, 60 sediment samples were collected from 27 different locations (Table 1). At cruise program, five marine sediment soil samples were collected using the facilities available at Center for Advanced Studies (CAS), Marine Biology, Annamalai University (Parangipettai, Tamil Nadu, India). Other samples were accumulated from intertidal and sub-littoral zones using corer in depth from 10 to 30 cm and transferred to sterile polythene bag. Samples were transferred to the laboratory as early as possible and stored in a cold room at 4°C for future use.

Sample processing and isolation of actinobacteria

To facilitate actinobacterial isolation, the samples were air-dried and then kept in the hot air oven under 55°C for 30 min to eliminate other vegetative bacterial flora. 1g of dried marine sediment sample was serially diluted up to 10^{-6} in 0.5% saline before plating on

media [11]. The starch casein agar (HiMedia) and Actinomycete isolation agar (HiMedia) were prepared in filtered marine water and autoclaved at 121°C in 15 lbs for 15 min. The filter sterilized antibiotics cycloheximide (HiMedia) (50 µg/ml) and nalidixic acid (HiMedia) (50 µg/ml) were incorporated into the sterile media at approximately 55°C [12]. The serially diluted samples were seeded on appropriate plates and incubated at room temperature for 10–12 days. At the end of the incubation period, all plates were observed for the complete growth of actinobacterial colonies and were selected for further studies. The fully grown actinobacteria are characterized by tough leathery colonies having well-developed, branched vegetative, and aerial mycelia with spores [13]. They were selected based on the location, colony size, morphology, and color of the aerial and substrate mycelia. A total of 85 isolates were sub-cultured on yeast extract malt extract agar medium (ISP2), and pure cultures were prepared as 20% glycerol stock and stored under -20°C [14].

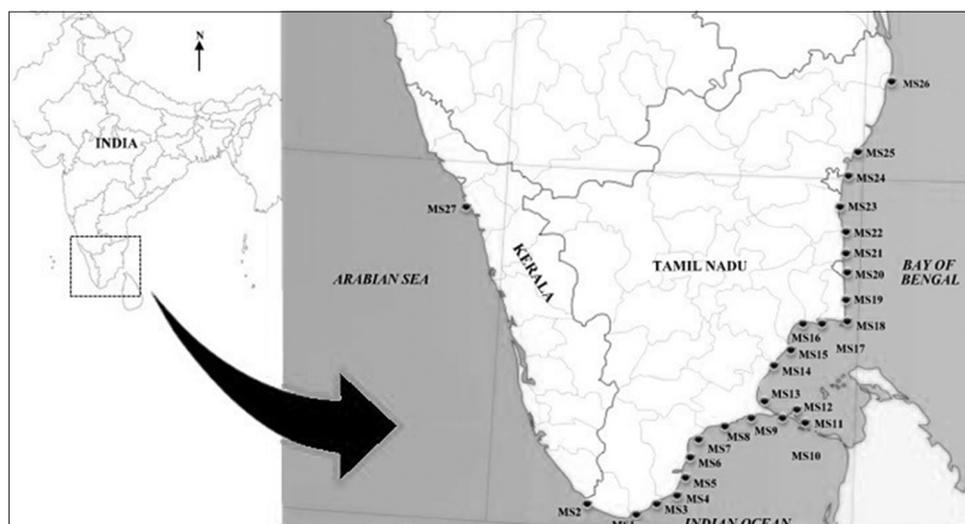


Fig. 1: Schematic representation of map showing sampling area in the Coastal Tamil Nadu and Kerala

Table 1: Details of marine sediment samples collected from different geographical regions of coastal Tamil Nadu and Kerala

Sample No.	Locations	Latitude (N)	Longitude (E)	Number of samples collected	Characteristics of soil sample
MS1	Kanyakumari	08°04'44.56"	77°33'05.52"	2	SWS*
MS2	Muttom	08°07'25.26"	77°19'08.63"	1	DSS**
MS3	Kootapuli	08°08'43.73"	77°36'02.05"	2	SWS*
MS4	Kudankulam	08°10'22.23"	77°43'46.18"	2	SWS*
MS5	Kodavilai	08°15'10.14"	77°50'32.69"	2	SWS*
MS6	Nadar uvari	08°17'05.35"	77°54'01.74"	2	SWS*
MS7	Thiruchendur	08°29'40.87"	78°07'44.67"	2	SWS*
MS8	Tuticorin	08°48'28.45"	78°09'46.63"	3	SWS*
MS9	Vembara	09°04'41.22"	78°22'01.36"	2	SWS*
MS10	Keelakarai	09°13'38.06"	78°47'08.55"	2	SWS*
MS11	Dhanuskodi	09°09'07.29"	79°26'35.82"	4	SWS*
MS12	Rameshwaram	09°16'34.58"	79°18'59.69"	3	SWS*
MS13	Devipattinam	09°28'25.06"	78°53'57.96"	2	SWS*
MS14	Uppur	09°36'30.15"	78°56'05.68"	2	SWS*
MS15	Thondi	09°44'34.32"	79°01'17.79"	2	SWS*
MS16	Mimesal	09°55'29.07"	79°09'09.84"	2	SWS*
MS17	Vedaranyam	10°23'34.79"	79°52'08.88"	2	SWS*
MS18	Velankanni	10°41'01.00"	79°51'13.74"	2	MS†
MS19	Nagapattinam	10°46'04.33"	79°51'06.37"	2	SWS*
MS20	Nagoor	10°48'52.37"	79°51'04.61"	2	SWS*
MS21	Karaikal	10°56'27.22"	79°51'12.26"	2	SWS*
MS22	Tharangambadi	11°01'41.26"	79°51'22.29"	2	SWS*
MS23	Pichavaram	11°25'53.31"	79°46'51.94"	3	MS†
MS24	Parangipettai	11°31'45.36"	79°45'54.54"	4	DSS**
MS25	Pondicherry	11°55'29.08"	79°50'04.16"	2	SWS*
MS26	Chennai	12°36'32.60"	80°11'47.64"	2	SWS*
MS27	Calicut	11°15'34.42"	75°46'04.04"	2	SWS*

SWS*=Shallow water sediment, DSS**=Deep sea sediment, MS†=Mangrove sediment

Preliminary identification of isolated actinobacteria

All the isolates were identified based on colony morphology, spore chain arrangement, and biochemical characterization according to Shirling and Gottlieb [15] and Bergey's Manual of Systematic Bacteriology [16]. The color of the mycelia was visually perceived, and all the isolates were made as a thin film on glass slide, heat fixed, and subjected to Gram staining and acid-fast staining. Microscopic coverslip was placed at an angle of the growing actinobacterial colonies in an agar plate. After observing good mycelial propagation on the coverslip, it was removed and placed on a glass slide for detailed observation of spore morphology, spore-bearing hyphae, structure and arrangement of the spore chain on the substrate, and aerial mycelium by light microscopy at $\times 1500$ magnification (Olympus, India).

Screening for antimicrobial compound producing actinobacteria

To identify the antimicrobial effectiveness of the isolates, reference and clinically derived bacterial cultures were collected from Institute of Microbial Technology, Chandigarh, India, and Kovai Medical Center and Hospital, Coimbatore, India. The bacterial cultures such as *Streptomyces griseus* subsp. *Griseus* MTCC 4734, *Staphylococcus aureus* MTCC 3160, *Bacillus pumilus* NCIM 2327, *S. aureus* (methicillin-resistant), *Escherichia coli* MTCC 1698, *E. coli* (ESBL), *Shigella flexneri* MTCC 1457, *Proteus vulgaris*, and *Enterobacter cloacae* were availed from the above said sources. *S. griseus* subsp. *griseus* MTCC 4734 was used as positive control for the entire study.

The agar plug method was performed for all the isolates to identify the antimicrobial compound-producing actinobacteria [17]. The purified actinobacteria were streaked on ISP2 media and incubated for 7 days at $28\pm 2^\circ\text{C}$. After observing good growth, the listed test bacterial strains were swabbed on the nutrient agar and a small well sized about 5 mm in diameter were made with a sterile corer. Later, the empty nutrient agar wells were replaced with similar sized agar plug from the ISP2 and incubated at 37°C . The inhibition zone was measured after 24 and 48 h. Based on the presence of inhibition zone, the antimicrobial compounds-producing actinobacteria were selected. The isolates excelled in agar plug assay were inoculated into 250 mL conical flask containing starch-casein broth (75 mL/flask) and incubated in an orbital shaker at 180 rpm under 26°C – 28°C for 7–8 days. After growth, the fermented broth was harvested by centrifugation at 6000 rpm for 10 min, and the collected supernatant was challenged against the previously mentioned pathogenic bacteria as suggested by Parthasarathi et al. [18]. Concurrently, a positive control *S. griseus* MTCC 4734 was subjected for all the above-mentioned analysis.

Statistical analysis of antagonistic efficacy of isolates

The statistical analysis (SPSS 16.0) was performed to compare the antagonistic efficacy of the isolates. The well diffusion results of all 34 isolates against test bacteria were taken into account, and the difference among the antimicrobial efficacy of every individual isolates was analyzed by *post hoc* (Turkey's test).

Characterization of antagonistic significant isolates

The selected antagonistically significant isolates were subjected to electron microscopic and chemotaxonomic characterization. Coverslip culture technique was adopted for light microscopic studies. Actinobacterial culture plate was prepared, and sterile coverslips were placed at an angle of 45° . After the appropriate growth, the coverslips were used for scanning electron microscopic observation. For cell wall sugar, samples were prepared and investigated by the prescribed methods of Suput et al. [19]. Cell wall amino acids were extracted using the standard protocol [20] and analyzed using reverse phase high-performance liquid chromatography (HPLC) [21] to validate the presence of LL-2,6-Diaminopimelic acid. For cellular fatty acid analysis, the samples were prepared by the method of Sasser [22], and methylated fatty acids were analyzed through GC-MS (Model - THERMO GC - TRACE ULTRA VER: 5.0, MS DSQ).

RESULTS

In this study, 60 marine sediment samples were collected from 27 locations of coastal Tamil Nadu and selected parts of Kerala (Fig. 1). All the

samples were collected during the months of April–May 2012 and 2013 to avoid the influence of the rainy season. Color of the sediment samples varied from black to brown in nature. Sediment textures were found to be clayey, sandy, and with small pebble-like structures. Besides, the samples from mangrove sediment were light-brown to dark brown and sticky like fine mud. The pH of the collected sediment samples varied from 7.2 to 8.4.

The actinobacterial populations were investigated from sediment samples through spread plate using two media, namely starch casein agar and actinomycetes isolation agar prepared in the sea water. On both the media, colonies with well-developed, tough leathery, mycelia, sometime pigmented, and sporulating, often observed to differ in shape, color, and size [13]. Likewise, 85 morphologically distinct actinobacterial strains were isolated from both the media. Isolation of similar colonies from same samples was avoided to evade the re-isolation of the same strain. Among them, 57 colonies were isolated on starch casein agar, whereas the rest 28 colonies were isolated from actinomycete isolation agar. Nearly 88.23% of the actinobacterial strains were isolated from marine shallow-water sediment, 9.41% from mangrove sediment, and 2.35% from deep-sea sediment. Reason for such results could be excessive number of samples, of which 50 samples are from shallow water sediment. The mangrove sediment yielded a high proportion of the actinobacterial population, whereas deep-sea sediment yielded significantly less. The samples collected from Rameshwaram (MS12) (7 colonies), Pichavaram (MS23) (7 colonies), and Dhanushkodi (MS11) yielded considerable number of diverse colonies in terms of morphology. While low population density was recorded in four places such as Nadavavurai (MS06), Vembara (MS09), Velankanni (MS18), and Tharangambadi (MS22), only one kind of morphologically alike actinobacterial population was seen in both Uppur (MS14) and Karaikal (MS21).

Majority of the isolates showed powdery morphology, whereas others showed mucoidal and shrunken skin-like appearance. Colony morphology differed in the aspects of form, elevation, and margin of the colony. Most of the colonies were circular, whereas a few were filamentous and irregular. The colonies were uniquely elevated as convex, raised, pulvinate, and umbonate. Margin of the colonies was different such as entire, undulate, erose, and filamentous. Moreover, a moderate number of colonies had multiple layers of the concentric circle. Predominant colonies were white and related colors, though others include pink, red, yellow, brown, blue, and black.

All the isolates were subjected to staining and microscopic examination. Gram staining revealed that all the 85 isolates were Gram-positive. Acid-fast staining results showed that many of them (84.7%) were non-acid fast and the remaining were weakly acid fast (15.3%). Considering the results of colony morphology, pigmentation, color of aerial and substrate mycelia, staining results, spore chain ornamentation, biochemical characterization, and chemotaxonomic characterization, of 85 isolates, 40% were identified as *Streptomyces*, 18% as *Micromonospora*, 14% as *Saccharopolyspora*, 7% as *Actinopolyspora*, 8% as *Nocardia*, 2% as *Nocardioidea*, 6% as *Kitasatospora*, and 5% remained as unidentified (Fig. 2).

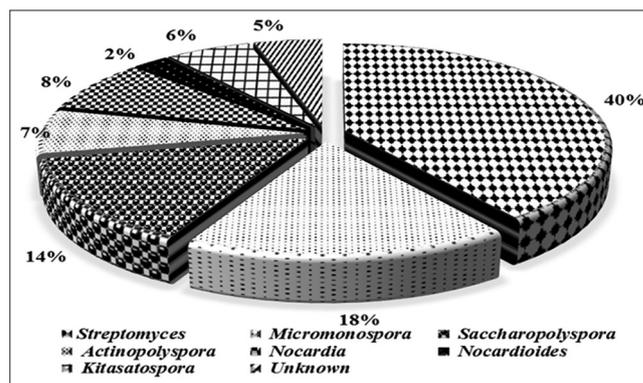


Fig. 2: Distribution of actinobacterial populations in marine sediments

In the present investigation, the actinobacteria made of entire samples have both antagonistic and non-antagonistic abilities. Among them, 85 actinobacterial isolates challenged against various clinical and reference bacterial strains by agar plug assay, and 34 (40%) isolates showed considerable antagonistic activity. Pichavaram, Dhanushkodi, and Mimesal excelled in antagonistic activity with >3 isolates in each sample. Rest of the samples had between one to two potent actinobacterial colonies, whereas the samples from Kanyakumari, Nadar uvari, Vembara, Velankanni, and Karaikal did not yield any single potent isolate (Fig. 3). Of 34 potential isolates, 18 (53%) were *Streptomyces*. Moreover, the other genera such as seven of *Saccharopolyspora* (20%), five of *Micromonospora* (15%), two of *Nocardia* (6%), and two of *Kitasatospora* (6%) are reported in the current study for their antagonistic ability.

Among the potential isolates, 35% of the isolates were active against Gram-positive clinical and reference bacterial strains, and 18% of the isolates had activity against Gram-negative, whereas 47% had broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Overall, 58.8% of the potential isolates had good antagonistic activity against *B. pumilus* NCIM 2327. In addition, 50% of the potential antagonistic isolates were very active against *S. aureus* MTCC 3160 and *E. coli* MTCC 1698. Similarly, 29.4% of the isolates were active against *S. aureus* (methicillin-resistant) and *E. coli* (ESBL). Moreover, 26.4% and 20.5% of the isolates were capable of producing antimicrobial compounds against *S. flexneri* MTCC 1457 and *P. vulgaris*, respectively. The obtained results of well diffusion assay were qualitatively almost similar as agar plug assay but quantitatively differed (Table 2). On the basis of mean average, all the isolates were categorized into five components through the statistical analysis method. It is identified that the fifth component, which includes BDK01, BMT01, BPY03, and BRS07, exhibited high antimicrobial ability. Furthermore, the isolate BDK01 excelled in antimicrobial ability as indicated by its mean performance.

The cultural morphology and biochemical characterization of all the four selected isolates were tested on different media, and the results were tabulated (Table 3).

In electron microscopic study, BDK01 and BPY03 showed rectiflexibles sporophore morphology, and BMT01 belonged to retinaculiaperti. Isolate BRS07 exhibited spiral spore chain morphology (Fig. 4).

Chemotaxonomic characterization of BDK01, BMT01, BPY03, and BRS07 revealed that no sugar was present in the cell wall, whereas glycine and LL-2,6 diaminopimelic acid were present. The chromatogram obtained for standard amino acid revealed that compound will get eluted at 3.44 min from the column.

Similarly, the cell wall amino acid isolated from isolates exhibited a similar chromatogram (Fig. 5) at the specific time with minor deviations. The result of the cell wall fatty acid analysis is given in Table 4.

DISCUSSION

Actinobacteria are potential sources for many new antibiotics, and specifically, *Streptomyces* is an important genus among them with a considerable contribution to pharmaceutical industries. Its diverse population and metabolites are promising factors to explore next-generation antibiotics. Some of the recent studies [23,24] highlight the concerns on the inappropriate usage of antibiotics and the development of multiple drug-resistant bacteria. Despite

Table 2: Antibacterial activity of fermented broth by isolates by well diffusion method

Isolate code	1	2	3	4	5	6	7	8
(Zone of inhibition in mm)								
Control	14.3	9.6	-	14.0	11.3	8.3	-	-
BMT1	21.6	16.3	14.0	14.6	12.3	9.60	22.6	17.6
BKT1	-	7.6	8.3	-	-	-	-	-
BKD1	14.3	-	-	-	-	-	-	-
BKD2	-	-	-	8.6	-	-	-	-
BKV2	14.6	11.6	-	15.6	-	-	-	-
BTC2	-	-	-	16.0	-	15.6	13.6	-
BTT1	-	-	16.6	14.0	-	8.3	-	-
BKL1	-	11.3	-	-	-	-	-	-
BDK1	20.0	18.3	24.3	28.0	33.6	28.0	22.0	12.3
BDK3	25.6	18.0	-	20.3	-	-	-	-
BDK6	19.6	-	16.3	-	-	-	-	-
BRS4	10.6	8.3	8.6	6.6	-	-	-	-
BRS7	14.3	12.3	13.6	13.6	20.0	11.3	14.0	16.0
BDV1	9.3	7.6	-	-	7.3	-	-	-
BUP1	-	20.6	16.6	14.6	15.6	-	8.6	-
BTD1	-	-	-	-	8.3	-	-	-
BMM1	-	-	-	-	-	15.6	15.3	-
BMM2	-	9.6	-	-	-	-	-	-
BMM3	-	8.6	-	11.3	-	-	-	-
BVR1	10.3	-	-	-	-	-	-	-
BVR3	-	-	-	-	-	9.6	-	-
BNG4	-	15.3	-	-	-	-	-	-
BNG6	10.3	-	-	-	-	-	-	-
BNO1	-	8.0	-	-	10.6	-	-	-
BTR1	-	17.6	-	-	-	-	-	-
BPH 1	18.6	-	-	28.3	24.3	-	-	-
BPH 2	11.6	10.3	-	19.3	20.3	-	-	-
BPH 5	-	-	-	14.3	-	-	-	-
BPH 6	18.6	-	-	16.6	-	22.6	-	-
BPP1	17.6	16.6	-	13.6	-	-	-	-
BPY1	-	18.6	-	-	-	-	-	-
BPY3	12.6	21.6	20.0	30.3	32.3	13.6	14.6	10.6
BCH1	14.3	-	14.6	-	-	-	-	-
BCC3	-	9.6	-	-	-	-	-	-

Control *Streptomyces griseus* subsp. *griseus* MTCC 4734; 1. *Staphylococcus aureus* MTCC 3160; 2. *Bacillus pumilis* NCIM 2327; 3. *Staphylococcus aureus* (methicillin resistant); 4. *Escherichia coli* MTCC 1698; 5. *Escherichia coli* (ESBL); 6. *Shigella flexneri* MTCC 1457; 7. *Proteus vulgaris*; 8. *Enterobacter cloacae*, mm, millimeter; - No activity

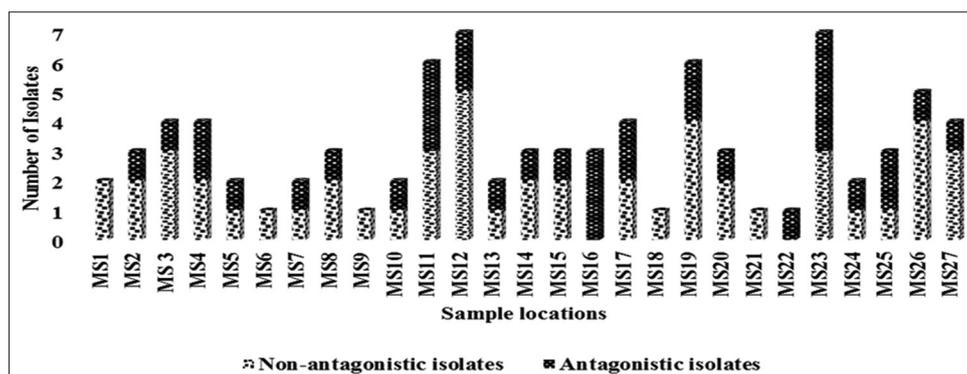


Fig. 3: Comparison of non-antagonistic and antagonistic isolates from different locations

Table 3: Phenotypic characterization of the isolates BDK01, BPY03, BMT01, and BRS07 on starch casein agar

Characteristics	BDK01	BPY03	BMT01	BRS07
Colony morphology	Circular, umbonate, entire	Circular, umbonate, entire	Circular, flat, curled	Circular, crateriform, curled
Sporophore morphology	Straight or rectiflexibiles	Straight or rectiflexibiles	Retinaculiaperti	Spiral
Spore surface	Smooth	Smooth	Spiny	Warty
Spore chain length	>30 spores	>30 spores	>10 spores	>30 spores
Spore size	0.840×0.508 µm	1.036×0.508 µm	1.123×0.5 µm	1.075 µm×459.4 nm
Colour of aerial mycelium	Pale orange	Pale orange	Gray in middle and white concentric circle around the colonies	White with white concentric circle
Color of substrate mycelium	Brown	Brown	Dull brown	Dull brown
Spore mass	Pale orange	Pale orange	Whitish-gray	Chalky white
Pigment	Brown color	Brown color	No pigment	No pigment

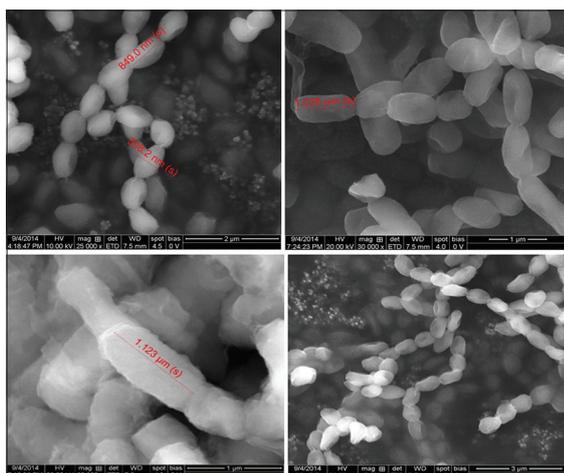


Fig. 4: Electron micrographs of (a) BDK01, (b) BPY03, (c) BMT01, and (d) BRS07

the continuous investigation on marine natural products, there is a large lacuna in the field of medicine for alternate therapeutic agents against most pathogenic microbes and clinical disorders. This prompts researchers to investigate for novel antimicrobials, particularly from actinobacteria. In 2009, Ramesh and Mathivanan also emphasized the importance of discovery of the new antibiotics to challenge plant and animal pathogens. The marine sediment was pondered as a potential source for diverse actinobacterial populations and its biologically active metabolite by many investigators [7,25,26].

Previous studies conducted on microbial population in the South-east coast of India after the 2004 tsunami [27-29] had opened further arena to the quest. When giant tidal waves like tsunami occurred in the sea, it was possible that the deeper nutrient-rich water came up to the surface [30]. This could have influenced the marine environment to a great extent due to nutrient and mineral flux resulting in variation in marine biodiversity in pre- and post-tsunami sediment. Based on these evidences, the marine sediment samples were collected from the East coast with the goal of isolation, identification of potential marine antagonistic actinobacterial population, and characterization of their products. In the present study, the sediment samples of coastal Tamil Nadu and Kerala were collected during pre-monsoon period of 2012 and 2013. Consequently, if samples collected after monsoon, inward freshwater might have affected the isolation of indigenous marine actinobacteria.

The places with good number of actinobacterial populations were identified, and the reason for this population density could be the influence of soil factor. The sediment sample of Pichavaram mangrove was black, sticky fine mud probably with high organic content and

elevated levels of carbon and nitrogen sources. Other places such as Rameshwaram and Dhanushkodi also yielded a high number of actinobacteria. Previously, while studying Palk Strait region of Bay of Bengal, Vijayakumar *et al.* [31] recorded a high number of actinobacteria, particularly, 39 *Streptomyces* strains. Rest of the sampling sites yielded moderate or less number of actinobacteria, which were taken from the littoral zone, probably with poor organic contents. Culturing the samples in more than one media always increase the probability for isolation of diverse actinobacterial populations. Hence, the recovered bacteria were also influenced by the type of media and the growth conditions employed, which reflects the nutritional contents [32,33]. About 81.4% of the collected marine sediment samples had the genus *Streptomyces*. Such results highly comply with the previous studies of Mitra *et al.* [26] and Suthindhiran and Kannabiran [34]. The genus *Streptomyces* is more predominant in marine environment compared with all other actinobacterial population [8,35]. Phenotypically the streptomycetes colonies are elevated, convex, and powdery in nature [36]. The spore morphology of *Streptomyces* and *Micromonospora* was compared with the results obtained by Vijayakumar *et al.* [31]. Since 1970, >3000 species of *Streptomyces* were reported in various literature and patents [37]. However, only 600 *Streptomyces* species alone were well defined by most investigators [38].

Micromonospora is the second largest population in the marine environment [39]. Similarly, in this study, >44.4% of the marine sediment samples had *Micromonospora*. Out of 85 total isolates, 18% of them belonged to the genus *Micromonospora* followed by *Saccharopolyspora* (14%). Small proportions of other rare actinobacterial populations such as *Actinopolyspora*, *Nocardia*, *Nocardioideis*, and *Kitasatospora* were reported [7,26,40]. The sporophore morphology of *Saccharopolyspora*, *Actinopolyspora*, *Nocardia*, and *Nocardioideis* was highly similar [41,42]. The morphology of *Kitasatospora* of the current study is in high agreement with the previous investigation of Gill *et al.* (2014) [52]. Almost 5% of the colonies were reported as unidentified due to unstable growth and irregular spore chain arrangement. Numerous isolated genera of actinobacteria as reported in marine water of Bay of Bengal, in his study includes *Streptomyces*, *Actinopolyspora*, *Kitasatospora*, and *Micromonospora*. Other potential species that have been reported are *Faenia*, *Ampullariella*, *Intrasporangium*, *Sporichthya*, *Nocardia*, and *Nocardioideis* [43].

After preliminary screening by agar plug method, of 85 isolates, 34 (40%) were identified as potential producers of antimicrobial compounds against clinical and reference bacterial strains. Around 82% of the sediment samples had antagonistic potential, whereas 18% showed no significant performance. Among seven colonies of Pichavaram mangrove, four were capable of producing antimicrobials. Mangrove sediment is always highly influenced by rich organic content of the soil that can promote the diversity of the microbial population, leading to nutritional competition for survival of every organism to combat a chemical war. Hence, the microbial population which exists in this zone may contain high antagonistic activity [44].

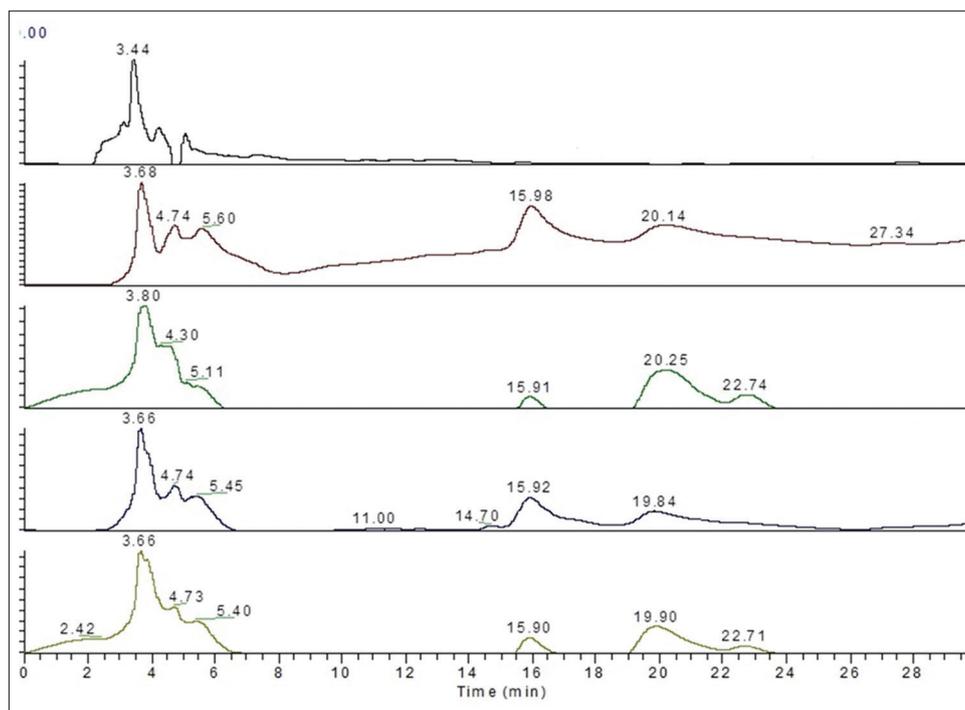


Fig. 5: Cell wall amino acid profile using reverse-phase high-performance liquid chromatography; (a) standard LL-2,6-diaminopimelic acid, (b) BDK01, (c) BPY03, (d) BMT01, and (e) BRS07

Table 4: Taxonomically significant fatty acid methyl esters present in cell wall extract of isolates BDK01, BPY03, BMT01, and BRS07 (fatty acid composition in percentage)

Fatty acid	BDK01	BPY03	BMT01	BRS07
C _{15:0} anteiso (12-Methyl)	46.03	56.60	39.57	23.42
C _{15:0}	-	7.10	-	-
C _{16:0}	12.56	21.30	42.11	48.48
C _{17:0}	-	14.99	-	-
C _{17:0} anteiso (14-Methyl)	-	-	18.30	-
C _{18:0}	-	-	-	20.08
C _{18:2} w6c (9Z,12Z)	41.39	-	-	-

Similarly, other regions such as Dhanushkodi and Mimesal (Part of Palk Strait of Bay of Bengal) yielded more than 3 antagonistic potential actinobacteria. Earlier, Vijayakumar *et al.* [8] isolated 66 actinobacterial strains from the Palk Strait region of Bay of Bengal between India and Sri Lanka. About 37% (25) of them had significant antimicrobial activity against various bacterial and fungal pathogens. Rest of the marine sediment samples screened had at least one antagonistically potential actinobacteria. Overall, 50% of the isolates had outstanding antagonistic potential against *B. pumilus* NCIM 2327, *S. aureus* MTCC 3160, and *E. coli* MTCC 1698. Therefore, Bay of Bengal is proven to be a pivot of biologically valuable compound producing actinobacterial population. Ramesh and Mathivanan [7] reported the potential actinobacterial populations of Bay of Bengal, which is active against human and plant pathogens. Out of 288 isolates, 53% (153) of them showed antagonistic activity against human pathogens such as *E. coli*, *Streptococcus epidermis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Candida albicans*. A similar study carried by Nithyanand *et al.* [45] indicated the antimicrobial ability of actinobacteria isolated from Gulf of Mannar.

In the current study, of 34 potential isolates, >53% (18) belong to the genus *Streptomyces*. Among them, isolates BDK01, BPY03, BRS07, and BMT01 were excelled in antimicrobial efficacy against the test bacterial strains. According to Miyadoh [46], *Streptomyces*, a potent genus in this group, produce as much as 75% of the commercially and medically

useful antibiotics. It does not mean that non-streptomycetes species are not producing any secondary metabolite. The reason may be lack of proper isolation media and techniques for the isolation of these strains from the environment [47]. Different genera such as *Saccharopolyspora*, *Micromonospora*, *Nocardia*, and *Kitasatospora* reported in this study still had good antimicrobial activity. Earlier, *Saccharopolyspora erythraea* has reported for the production of erythromycin-A by Wu *et al.* [48]. Carlson *et al.* [49] reported a chemopreventive macrolide antibiotic from a marine-derived *Micromonospora* sp. Tranvalencin-Z, an antimicrobial compound produced by *Nocardia transvalensis* IFM 10065, was reported by Mukai *et al.* [50]. In addition, in the previous study of Tsuda *et al.* [51], *Nocardia brasiliensis* was reported for the production of a cytotoxic compound Brasilibactin A. *Kitasatospora cystarginea* was identified as a potential source for cystargolides (A) 1 and (B) 2, by Gill *et al.* [52].

The shake flask method is the common tool used by many investigators for checking the antagonistic ability of their isolates [18]. To check the prolonged production ability of the organism, shake flask fermentation was performed for all the isolates, and the results were recorded. The outputs of well diffusion assay were qualitatively similar to the agar plug assay but differed quantitatively against each test pathogen (Table 2). The statistical analysis of the results obtained through one-way ANOVA grouped the isolates into five categories based on the mean average (Table 5). Particularly, the isolates BDK01, BPY03, BRS07, and BMT01 (which were identified as *Streptomyces* genus) were categorized into separate group with the elevated mean average of the zone of inhibition.

In chemotaxonomic characterization, no sugar was found in all the four selected isolates: BDK01, BPY03, BRS07, and BMT01. Similarly, in cell wall amino acid analysis, glycine and LL 2,6 diaminopimelic acid were found in all the four isolates. Reverse phase HPLC chromatogram shows that the retention of the standard LL-2,6-Diaminopimelic acid is at 3.44 min and correspondingly the elution of LL-2,6-Diaminopimelic acid from selected isolates exhibited similar retention time with minor variations. This clearly shows that isolates contain LL-2,6-Diaminopimelic acid in their cell wall. These results were high agreement with the study of Phongsopitanun *et al.* (2014) [53]. Hence, it is confirmed that the cell wall of all the isolates belonged to Type I, according to Suput *et al.* Fatty

Table 5: Statistical analysis of antimicrobial ability of actinobacterial populations by post hoc (Turkey's test)

Tukey test		Subset for alpha=0.05				
Group	n	1	2	3	4	5
BTD1	8	1.0375				
BKD2	8	1.0750				
BMM2	8	1.2000				
BVR3	8	1.2000				
BCC3	8	1.2000				
BVR1	8	1.2875				
BNG6	8	1.2875				
BKL1	8	1.4125				
BKD1	8	1.7875	1.7875			
BPH 5	8	1.7875	1.7875			
BNG4	8	1.9125	1.9125			
BKT1	8	1.9875	1.9875			
BTR1	8	2.2000	2.2000			
BNO1	8	2.3250	2.3250			
BPY1	8	2.3250	2.3250			
BMM3	8	2.4875	2.4875			
BDV1	8	3.0250	3.0250			
BCH2	8	3.6125	3.6125	3.6125		
BMM1	8	3.8625	3.8625	3.8625		
BDK6	8	4.4875	4.4875	4.4875		
BT1	8	4.8625	4.8625	4.8625		
BKV2	8	5.2250	5.2250	5.2250		
BRS4	8	5.5125	5.5125	5.5125		
BTC2	8	5.6500	5.6500	5.6500		
BPP1	8	5.9750	5.9750	5.9750		
BPH 6	8	7.2250	7.2250	7.2250	7.2250	
BPH 2	8	7.6875	7.6875	7.6875	7.6875	
BDK3	8	7.9875	7.9875	7.9875	7.9875	
BPH 1	8	8.9000	8.9000	8.9000	8.9000	
BUP1	8	9.5000	9.5000	9.5000	9.5000	
BRS7	8		14.3875	14.3875	14.3875	14.3875
BMT1	8			16.0750	16.0750	16.0750
BPY3	8				19.4500	19.4500
BDK1	8					23.3125
Sig.		0.768	0.050	0.058	0.072	0.663

*Means for groups in homogeneous subsets are displayed

acid methyl ester analysis revealed the presence of several straight and branched chain fatty acids including C_{15:0} anteiso (12-Methyl), C_{15:0}, C_{16:0}, C_{17:0}, and C_{17:0} anteiso (14-Methyl), C_{18:0} and C_{18:2} w6c (9Z,12Z) in the potential isolates. All these findings suggested that the selected isolates could belong to the genus *Streptomyces*. To identify the species, all the four strains should be subjected to 16S rRNA gene sequencing.

According to Bérdy [54], 75% of the commercially important antibiotics produced are by *Streptomyces*, and 25% of them were produced by rare actinobacteria. The rare actinobacterial species produces chemically diverse, unique, unprecedented, and biologically potent compound with low toxicity [54,55]. Particularly, *Micromonospora*, *Nocardia*, and *Saccharopolyspora* play a very important role in the production of antimicrobial compounds and antibiotics [1,54,56].

More than 99% of the known valuable antimicrobial compounds in the market are derived from the terrestrial actinobacterial compounds. Recently, the efforts decreased gradually due to the distrust that this resource has been exhaustively studied. Hence, the present investigation was done on the marine environment for novel compounds to combat the developing pathogens. Marine actinobacteria were well known for its bioactivities, such as antimicrobial and cytotoxic activities. Particularly, marine *Streptomyces* are widely distributed in biological sources such as fishes, molluscs, sponges, seaweeds, algae, and mangroves beside seawater sediments [57]. It is gaining importance not only for its taxonomic and ecological perspectives but also for their production

of novel bioactive compounds such as antibiotics, enzymes, enzyme inhibitors, pigments, and for its biotechnological applications such as the production of probiotics and single cell proteins. In proving the above statement, research has explored a total of 205 actinomycetes species notably producing industrially important enzymes such as amylase, protease, lipase, esterase, and gelatinase [58]. Furthermore, a recent study at coromandel coastal region of southern India has explored effective marine actinomycete belonging to the species *Micropolyspora* producing antimycobacterial compounds with potent activity against *Mycobacterium tuberculosis* [59].

Vested with an enormous wealth of biodiversity in the Bay of Bengal, this study on bioprospecting gains more importance. Marine sediment is an excellent source for the isolation of novel actinobacterial diversity that produces novel metabolites. Particularly, the coastal regions of Tamil Nadu and Kerala are reported to be the target site by several authors for its diverse microbial population and rich metabolic profile. Through this study, we confirm that the coastal marine sediment contains abundant morphologically distinct actinobacterial populations, and notably, *Streptomyces* is predominant. The sediments from mangrove forests with rich organic content yielded high number of antagonistic actinobacteria than any other samples. The antimicrobial compound production efficacy of the isolated strains of actinobacteria was remarkable against various bacterial pathogens. The statistical analysis revealed that the four strains BDK01, BPY03, BRS07, and BMT01, which are identified as *Streptomyces* genus, were highly competitive compared to all other isolates investigated. In addition, the results throw light on the importance of several techniques to harness actinobacterial diversity from such regions for comprehensive understanding and exploring the application potential.

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AUTHORS' CONTRIBUTIONS

Dr. Manikandan Madheslu - Data collection, Data analysis, and interpretation. Ms. Swapna Mani - Drafting the article. Mr. Duraimurugan Kasiviswanathan - Marine sediment samples collection. Dr. Solai Ramachandirane Prabakaran - Design of the work, Supervision of the work, and Final approval of the version to be published.

CONFLICTS OF INTEREST

All authors have no conflicts of interest.

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