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Phylogenetic identification of antimicrobial active marine bacteria from sediments off the coast of South East India

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The antagonistic action of the marine sediment associated bacteria from three different area of Gulf of Mannar Coast was tested against five bacterial fish/shrimp pathogens viz. *Aeromonas hydrophila*, *A.sobria*, *Vibrio vulnificus*, *Vibrio harveyi* and *V. fischeri* using the double agar overlay method. The total bacterial load of sediment ranged from 10^5 -10^6 cfu/g. The bacterial composition of marine sediments predominantly consisted of *Bacillus* sp. (40%), followed by *Vibrio* sp., *Flavobacterium* sp., *Alteromonas* sp., *Micrococcus* sp., *Staphylococcus* sp. and *Pseudomonas* sp. Phylogenetic analysis of the sediments associated producer strains showed that 12 strains are clustered within the Firmicutes group belonging to several *Bacillus* sp. and *Halobacillus* sp. with 94 - 98% similarity between them. Of the 35 isolates tested, 42% showed antagonistic action against fish/shrimp pathogens. The marine sediment associated bacteria shall therefore be evolved as putative probiotic bacteria to counteract fish/shrimp disease problems.

Marine biology/Molecular Biology

Marine microorganisms are essential for nutrient turnover in the oceans, and the growth and metabolism of marine microorganisms has been studied to understand their ecology and role in biochemical processes (Falkowski *et al.* 1998; Field et al. 1998; Azam and Malfatti 2007). Marine microorganisms are also the focus of attention due to their production of secondary metabolites that may have a range of pharmaceutical and biotechnological applications (Jensen and Fenical, 1996); Burgess et al. 1999; Fenical and Jensen, (2006); Bull and Stach, (2007); Debnath et al. 2007; Egan *et al.* 2008; Blunt et al. 2008). In the marine environment banterioplankton are one of the most frequently studied communities by Giovannoni and Stingl (2005). Though bacterial communities have been frequently studied, recent studies show that lots of bacterial communities need to be still characterized in marine environment (Gontang *et al.* 2007). Some marine bacteria are inhibitory to other bacteria (Burkhold *et al.* 1966; Gauthier and Flatau, (1976); Barja *et al.* (1989), suggesting that bacterial interactions could play an important role in marine ecology (Long and Azam ,2001; Strom 2008). Control of unwanted microorganisms is essential in all aspects of life, and microbial diseases must be treated in humans, animals, and plants. The oceans cover more than 70% of the earth's surface, and little is known about the microbial diversity of marine sediments (Magarvey et al. 2004).

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Bacteria within coastal and shelf sediments play an important role in global biogeochemical cycles, as they are the ultimate sink of most terrestrially derived compounds and a high proportion of marine flux. Despite a growing understanding of the global biogeochemical importance of these sediment habitats (Codispoti et al. 2001), little is known of the bacterial communities inhabiting them (Kim *et al*., 2004) nor the factors influencing their distribution (Polymenakou *et al.* 2005). Marine microbial diversity in the ocean has been the subject of intense recent study (Giovannoni and Rappe 2000; Venter et al. 2004). Only in the past two decades have molecular tools for addressing microbial ecology become available, which circumvent culture biases associated with an estimated 95% of bacterial taxa (Giovannoni et al. 1990; Fuhrman et al.1992).

The wide-spread occurrence of antibiotic resistance is threatening both fishes and agriculture production. Therefore, the discovery and development of new antibiotics is essential to combat drug resistant pathogens. The bacterial diversity in marine sediments is little known, which is an inexhaustible resource that has not been properly exploited. The full potential of this domain remains largely unexplored as the basis for biotechnology, particularly in India. Hence, we in the present study sampled from sediments associated marine organisms with antimicrobial actives and to identify potent bacterial strains using a 16S rRNA phylogenetic analysis.

Material and Methods

Collection of samples

Sediments samples were collected from Hare Island and Rameswaram area of Gulf of Mannar Coast. Sediments were collected from near shore, 1.5m and 2.5m depth using sediment sampler. During the sample collection the temperature was 30^{o} C to 32^{o} C and the pH was 8.5 to 9.1. The samples were collected in sterile plastic bags and kept cool until transported to laboratory for further processing.

Isolation of marine bacteria

Sediment sample (1g) was transferred to sterile test tube with 1mL autoclaved-sterilized artificial seawater (ASW) in a sterile hood. The bacteria were suspended in ASW by vigorous vortexing for 5 min. Representatives of each colony morphotype were isolated using standard serial dilution and plating techniques in triplicate on Zobell Marine Agar 2216 (Himedia Laboratories, India), a medium for isolation and enumeration of marine of marine hetrotrophic bacteria, and the pH was adjusted to 8.5 in accordance with the sediment pH. All plates were incubated at 25^{o} C - 28^{o}C, for 3- 7 days. Pure cultures were isolated and subcultured in the same medium at 25^{o} C- 28^{o} C. Glycerol stocks were prepared with 30% glycerol in Zobell Marine medium and stored at -80^{o}C for future work. Bacterial counts were represented as CFU/g for each sediment samples.

Biochemical identification of sediment associated bacteria

All the isolated bacteria were identified by performing various biochemical tests according to Bergey's manual and Lampert et al. (2006). The sediments bacterial isolates were subjected to various morphological and biochemical tests. Carbohydrate tests were performed using the Hicarbohydrate kit (Himedia Laboratories; Cat. No. KB009).

Antimicrobial activity of sediment associated bacteria

The bactericidal effects of sediments associated bacterial strain were tested against fish pathogens like (*Aeromonas sobria* (MTCC 1608), *Aeromonas hydrophila* (ATCC 7966), *Vibrio fischeri* (MTCC 1738), *V. harveyi* (MTCC3438) and *Vibrio vulnificus* (ATCC 29307) by double layer method described by (Riquelme et al. 1997). Briefly, MZA plates were spot inoculated with 5µl of overnight cultures of each bacterial strain to be tested. Following incubation for 24h at room temperature, the developed colonies were killed with chloroform vapour over a period of 45 min. These plates were overlaid using 6 ml of TSB supplemented with 1% NaCl (salt used depending on growing strain) and 0.9% agar, containing 100µl of a 1/10 dilution of 12h culture of fish pathogens. After 24 h of incubation, antagonistic effects of bacterial strains tested were observed by measuring the zone of inhibition, which appeared as clearance zone around these bacterial colonies. A minimum six plates was used for each assay. The results of minimum detectable

concentration obtained were based on the means of triplicate.

Genomic DNA extraction from sediments associated bacteria

The bacterial cultures grown on Zobell Marine broth overnight at 27ºC was centrifuged at 4600 g for 3 minutes. Bacterial genomic DNA was isolated according to Babu et al. (2009). The pellet was resuspended in 400 µl of Sucrose TE. Lysozyme was added to a final concentration of 8mg/mL^{-1} and incubated for 1 h at 37 ºC. To the tube, 100 µl of 0.5M EDTA (pH 8.0), 60 µl of 10% SDS and 3µl of proteinase K from 20mgmL^{-1} were added and incubated at 55 ºC overnight. The supernatant was extracted twice with phenol:chloroform (1:1) and once with chloroform : isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water.

Amplification of 16S rRNA gene from bacteria and actinomycetes

Bacterial 16S rRNA gene was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA gene primers: forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' (*E. coli* positions 8–27) and reverse primer 5'-ACGGCTACCTTGTTACGACTT-3' (*E. coli* positions 1492–1513). PCR was performed in a 50 µl reaction mixture containing 2µl (10ng) of DNA as the template, each primer at a concentration of 0.5 µM, 1.5mM MgCl2 and each deoxynucleoside triphosphate at a concentration of 50 µM, as well as 1U of Taq polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 3 min at 95 ºC, 40 cycles consisting of denaturation at 95 ºC for 1min, annealing at 55 ºC for 1min and extension at 72 ºC for 2min, and a final extension step of 5min at 72 ºC were carried out (Mastercycler Personal, Eppendorf, Germany). The amplification of 16S rDNA gene was confirmed by running the amplification product in 1% agarose gel in 1 x Tris-acetate-EDTA.

Amplified ribosomal DNA restriction analysis (ARDRA)

With the objective of determining bacterial diversity, all the 16S rRNA gene amplicons representing various isolates were subjected to ARDRA. To examine the ARDRA profile, 10µl of the PCR product was digested with *Hinf1* at 37^{o} C for 3 h. Digested DNA samples were analysed in 2% agarose gel.

Cloning and sequencing of 16S rRNA gene sequence analysis

The amplified products were purified using GFXTM PCR DNA and Gel Band Purification Kit (Geni, Bangalore) according to the manufacturer's instructions. The 16S rRNA gene amplicon was cloned in pTZ57R/T vector according to the manufacturer's instructions (InsT/A clone^{TM} PCR Product Cloning Kit #K1214, MBI Fermentas). Full-length sequencing of the rRNA gene for all the sediments associated antagonistic bacterial isolates were carried out in Geni (Bangalore, India).

Nucleotide sequence analysis

The full-length sequences obtained were matched with sequences available in NCBI using BLAST (Altschul et al. 1997). Multiple sequence analysis was carried out using CLUSTALW (Thompson et al.1997) and a further neighbor-joining plot by Perriere and Gouy,1996) and PHYLODRAW (Choi et al. 2000) were used to construct the phylogenetic tree. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

Results

Isolation of bacteria from marine sediments

In the present study, a total of thirty five bacterial strains were isolated from three different places sediments samples. Total count was high in sample SSS at 1.3 x 10^6 CFU/g and low in CSD sample at 3.7 x 10^5. The total bacterial counts of three different sediments and their representative bacteria (n=35) were given in Table 1. The strains were characterized using biochemical and molecular methods.

Table - 1: Total heterotrophic bacteria from marine sediments

Sl. No.	Samples	Total count CFU/g	No. of representative bacteria
1.	Sediment - SSS	1.3 x 10^6	14
2.	Sediment - SWS	4.2 x 10^5	9
3.	Sediment - CSD	3.7 x 10^5	12

Taxonomic classification of sediments associated bacteria

Sediments associated bacteria were identified to generic level along with their percentage incidence are given in Fig 1. Of the 35 isolates from the three sediment samples, *Bacillus* sp. (40%) was dominant flora followed by *Vibrio* sp. (31.42%), *Flavobacterium* sp. (8.57%), *Alteromonas* sp. (5.71%), *Staphylococcus* sp. (5.71%), Micrococcus sp. (5.71%) and *Pseudomonas* sp. (2.85%).

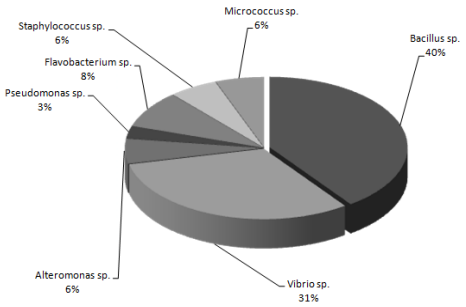

Fig. 1: Pie chart illustrating the diversity of bacterial groups associated with marine sediments

ARDRA analysis

ARDRA showed the presence of different polymorphic groups of bacteria in the sediments. ARDRA anslysis revealed 15 polymorphic groups. Among the 15 ARDRA groups of the sediment found, 15 polymorphic patterns for *Hinf1* were observed (Fig 2).

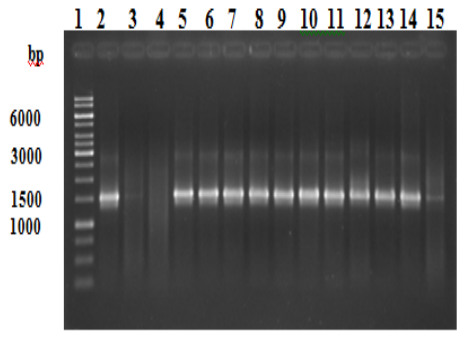

Fig. - 2: ARDRA profile of sediments associated bacteria with *Hinf*1 (Lane 1: 1kb ladder (Fermentas), Lane 3,4 & 15 share a similar ARDRA profile, Lanes 2, 5, 6, 7,8,9, 10 &14 show similar ARDRA profiles Lane 11&12 share similar profile).

Antibacterial activity of marine sediments associated bacteria

Double agar assays showed that 15 isolates of sediments (42.85%) released antimicrobial compounds against challenged pathogens (Fig 3). The antibiotic activity differed from strain to strain and both broad-specific and species-specific activities were noted. Five antimicrobial isolates (SSS01, SSS02, CSD02, CSD09 and CSD12) displayed inhibitory activity against *V. fischeri*, *V. vulnificus*, *V. harveyi*, *A. hydrophila* and *A. sorbia*. The strongest antibacterial activities were obtained by SWS01 with inhibition zones of 16.83 mm against *V. vulnificus*. The higher activity noted SWS01 against *V. vulnificus* follwed by *A. sobria*, *V. harveyi*, *A. hydrophila* and *V. fischeri*. Considerable antimicrobial activities against *V. fischeri* and *A. sobria* were presented also by SSS02. The two susceptible organisms were SSS06 and SWS09, which were inhibited by *V. fischeri*, *V. vulnificus*, *A. hydrophila* and *A. sobria*. An influence on the growth of *V. harveyi* was detected for 10 species of sediments associated bacteria. One of the isolates, SWS01 had most susceptible activity against *V. vulnificus*. The same inhibition zone was obtained from the isolate CSD02 against *A. sobria*.

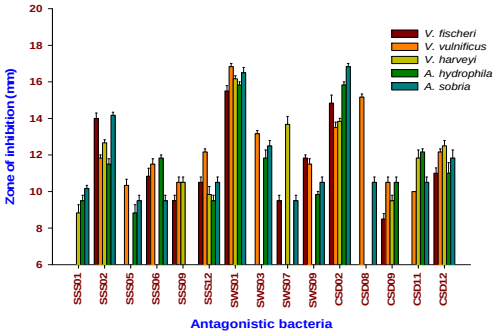

Fig.3: Antagonistic activity of sediments associated bacteria against different fish pathogens

Phylogenetic analysis

Full-length of 16S rRNA gene sequence from 15 of the 35 characterized marine sediment isolates. The 15 bacterial isolates from three different sediments could be assigned to 14 phylogenetically different clusters (Fig.4). Among the tested isolates majority of them are grouped with genus *Bacillus* sp., *Vibrio* sp., *Staphylococcus* sp., *Streptococcus* sp. and

Halobacillus sp. with 95 - 98% similarity. In the *Bacillus* cluster three of the isolates SSS02, SWS07 and CSD11 show close relationship with *Bacillus cereus*. However, the strain CSD12 showed a very high homology with *Streptococcus* sp. The cluster of the strains SSS01 and SWS09 were closely related to *Bacillus indicus* and *Bacillus pumilus* with >90% sequence similarity. The other producer strains viz. SSS09, SWS03, CSD05, CSD08 and CSD12 were found to be significantly close to *Staphylococus flocculus, V. parahaemolyticus, B. arsenicus, B. licheniformis* and *Streptococcus albus,* respectively. All the sequences were submitted in GenBank and the accession numbers for all the sequenced producer strains are given in Table 2 (www.ncbi.nlm.nih.gov/Genbank/).

Table - 2: Identification by 16S rRNA gene sequencing analysis of antagonistic active marine sediments associated bacteria based on BLAST analysis

Isolates No	Genus	Accession No.	Bacterial groups	Base pairs
SSS01	*Bacillus indicus*	HM100209	Firmicutes	1430
SSS02	*Bacillus cereus*	HM100210	Firmicutes	1488
SSS05	*Halobacillus trueperi*	HM100211	Firmicutes	1501
SSS06	*Bacillus pumilus*	HM100212	Firmicutes	1477
SSS09	*Streptomyces flocculus*	HM100213	Actinobacteria	719
SSS12	*Bacillus firmus*	HM100214	Firmicutes	1471
SWS01	*Bacillus subtilis*	HM100215	Firmicutes	1455
SWS03	*Vibrio parahaemolyticus*	HM100216	Proteobacteria	1461
SWS07	*Bacillus cereus*	HM100217	Firmicutes	1474
SWS09	*Bacillus pumilus*	HM100218	Firmicutes	1475
CSD02	*Bacillus subtilis*	HM100219	Firmicutes	1475
CSD05	*Bacillus arsenicus*	HM100220	Firmicutes	1434
CSD08	*Bacillus lichniformis*	HM100221	Firmicutes	1461
CSD11	*Bacillus cereus*	HM100222	Firmicutes	1473
CSD12	*Streptomyces albus*	HM100223	Actinobacteria	828

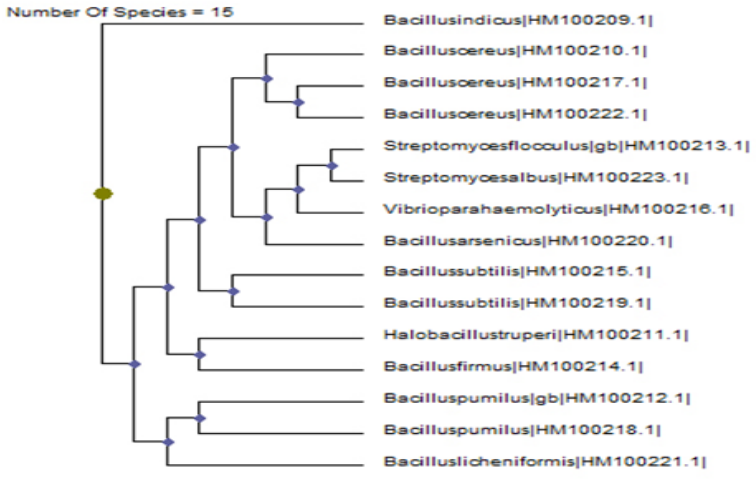

Fig 4: Neighbour-joining phylogenetic tree from analysis of 16S rRNA gene sequence of Sediments. The numbers are the percentages indicating the levels of boot strap support, based on a neighbour joining analysis of 1000 resampled data sets.

Fig.5: The DNA alignment showing the homology of isolate SWS01 and *Bacillus subtilis* based on ClustalW Multiple sequence alignment

Phylogenetic analysis of the sediments associated strains showed that 12 strains are clustered within the Firmicutes group belonging to several *Bacillus* sp. and *Halobacillus* sp. with 94 - 98% similarity between them. The higher antagonistic active bacteria SWS01 (*Bacillus subtilis*) had 96% similarity to the Palk bay sediment isolates (EU620412) (Fig. 5). BLAST analysis of the 15 strains showed SSS02 was close relatives of *Bacillus cereus* (EU624434) had 98% similarity. The strain SWS07 had 96% similarity with *Bacillus* cereus (EU620410), isolated from palk bay sediments of India. Two strains of *Bacillus pumilus* (SSS06 and SWS09) from the Firmicutes group with 97% similarity to EU620417 of palk bay sediment of India. Two strains (SSS09 and CSD12) from the Actinobacteria group fall under the family *Streptomyces* sp. with 96% similarity to *Streptomyces flocculus*. (FJ662867) from marine sediments, India and *Streptomyces albus* (FJ662866) isolated from corals *Acropora digitifera*, Gulf of Mannar coast, India. The bacterial strain SWS03 which has found to be a member of the Proteobacteria was a close relative, with 97% similarity, of the strain *Vibrio parahaemolyticus* was isolated

from Palk bay sediments of India. Only one strain, CSD08, among the sediments isolates; BLAST analysis revealed that this strain is a close relative with 96% similarity to the strain *Bacillus licheniformis* (EF635428) isolated from China. Strain CSD05 is a close relative, with 96% similarity to *Bacillus arsenicus* (EU620414) isolated from marine sediments of palk bay, India.

Discussion

The diversity of marine sediments associated bacteria has been extraordinary significance in sever areas of science and medicine. The marine represent an underexplored environment for microbial discovery, and although new methods are under development, relatively few have been applied to reveal the microbial diversity of the marine environment (Kaeberclein et al. 2002). Marine sediments, in particular, have been largely overlooked. The current study is the first endeavor to assess culturable heterotrophic bacterial diversity using standard biochemical test and 16S rRNA gene sequencing analysis from the sediments of Gulf of Mannar Coast. Bacteria (N=35) isolated from the sediment samples were diverse and represented three bacterial phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*). In this cultivation, *Firmicutes* and *Actinobacteria* were the predominant phyla and the findings go well with the previous studies of marine sediment (Gray and Herwing, 1996; Urakawa et al. 1999). A significant proportion of the bacterial community from 12 belonging to the Gram positive, namely *Firmicutes* (low G+C content bacteria accounting for 40%) and *Actinobacteria* (high G+C content bacteria, 4%) correlates very well with the findings of the earlier studies (Jensen et al. 2005; Stach and Bull (2005); Gontang et al. 2007). This study yet again establishes the abundance and diversity of Gram positive bacteria from marine sediment. Most of the isolated strains were closely related to other cultured bacteria, while 15 strains (43% of the isolates) had more than 90% to the nearest match present in GeneBank database.

The commonly used criterion for proposing novel species of bacteria was more than 90% identity of the sequences to previously published 16S rRNA gene sequences in the GenBank (Rohwer et al. 2002), so we envisage that these strains may belong to novel species. This study provides the first evidence for the existence of bacterial strains such as *Bacillus arsenicus*, *Bacillus indicus, Bacillus pumilus, Bacillus flocculus* and *Bacillus subtilis* in marine sediments representing *Firmicutes*. Among these five strains show more than 90% similarity with arsenic-resistant bacterial strains (Suresh et al. 2004).

In the present study *Alteromonas* sp (13%) and *Pseudomonas* sp (16%) were high in incidence of sediments samples. Smith and Davey, (1993) reported that *Pseudomonas fluorescences* reduced disease caused by *Salmonicida*. Sugita et al. (1997) have reported that a strain of *Vibrio* isolated from fish farm exhibited a wide antibacterial spectrum against *V. vulnificus*. The results were reported at first time on the studies of antibacterial agents from bacteria isolated from Gulf of Mannar region. With the ever increasing emergence of resistant strains (developed against existing antibiotics), there is a need to discover novel antibacterial compounds. Majority of the strains were showed the antibacterial activity against both Gram positive and Gram negative bacteria. The isolates had different strengths and spectra of activity against the various human and aquaculture pathogens suggesting that the metabolites from these microbes are diverse. When the antibiotic producers that were isolated from marine sediments were tested against each other as per previous report by Shnit-Orland and Kushmaro, (2009), no inhibition occurred, suggesting that the strains isolated could be resistant to these antagonistic mechanisms.

Zheng et al. (2005) reported that only 5% of the sediments associated bacteria had antimicrobial activity against the human pathogens. In the present study, 42% of sediments associated bacteria from Gulf of Mannar coast had antagonism activity against the fish pathogens. Normally, the most of the antagonistic active bacteria against fish pathogens as *Bacillus* sp. (Shakila et al. 2006). Moriarty, (1998) also reported that *Bacillus* strains showed antibiotic activity against luminescent *Vibrio* sp. In our study, *Bacillus subtilis* (SWS01 and CSD02), *Bacillus cereus* (SSS02), *Bacilus firmus* (SSS12) and *Streptomyces albus* (CSD12) were shown to have antibiotic activity against most of the Gram positive and Gram negative bacteria. Patil et al. (2001) isolated

Streptomyces sp. from marine sediments were inhibitory to various human pathogens. It has also reported that the *Streptomyces* from marine sediments inhibited many bacterial pathogens (Okazaki and Okami, (1972). Since some of the isolates in our study belongs to novel species category, the active compounds possessed by these marine isolates may 15 provide new hope for fighting against the drug-resistant pathogens. Among these isolates, *Bacillus subtilis* (SWS01) alone high inhibitory activity against fish pathogens. Actinomycetes, *Streptomyces albus* (CSD12) also exhibited inhibitory activity against challenged various pathogens. The *Bacillus pumilus* (SWS09) isolated from 1.5m depth sediment had the broadest range of activity and inhibited a range of aquaculture pathogens. The existence of antibacterial metabolites in the natural habitat may lead to resistance of bacterial species against such compounds (Shnit-Orland and Kushmaro,2009).

These results not only describe bacterial diversity of the sediments but also indicate the 15 biotechnological prospects of the cultures. In addition, cultured strains have some advantages that they can be subjected to taxonomic characterization and their physiological, ecological and biotechnological potential can be explored. It was clear from this survey that considerable new examples of eubacterial diversity can be readily cultured from marine sediments.

Conclusion

Marine organisms collected from the Southeast coast of India have been shown to possess a number of biological activities against fish pathogens. In our studies, the most interesting species are the *Bacillus subtilis* and *Bacillus cereus*. To the best of our knowledge, this is the first report demonstrating the antimicrobial activity of most of the marine organisms taken up in this study, with few exceptions. These organisms are currently undergoing detailed investigations with the objective of isolating biologically active molecules along with the search for novel compounds using HPLC. The high antibiotic substance produced by the isolates show the real potential of the hitherto unexplored this coastal region. So the continued use of cultivation-dependent techniques will undoubtedly lead to the discovery of additional bacterial diversity and provide a direct means to learn more about their ecophysiology and applications in biotechnology from the unexplored Gulf of Mannar Coastal area. Further, the encouraging biological activities seen in this study show that the Indian coastline is a potential source of variety of marine organism worthy of further investigation.

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