Research Article

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Potential bioactive compound from novel marine actinobacteria associated with antagonistic activity against human pathogens

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ABSTRACT

A total of fifty six marine actinobacteria were isolated from the soil sediment of South Indian coastal region. The isolates were screened for invitro anti-microbial activity study. The anti-microbial screening showed that, three isolates (1, 2 and 18) exhibited potential activity against human pathogens such as *Borrelia bargdosferi, Bartonella henselae, Listeria monocytogens, Helicobacter pylori and Camphylobacter jejuni* by extracting bioactive compounds using ethyl acetate, acetone and dimethyl sulfoxide (DMSO). Among them, ethyl acetate extracted bioactive compounds from the isolate 1 exhibited virulent activity (18±0.67, 15±0.39, 11±0.75, 16±0.32 and 10±0.10) against all human pathogens than the other solvents and isolates. The partial characterization of the ethyl acetate extract (isolate - 1) by IR (Infra Red) spectra analysis revealed the possible presence of functional groups such as hydroxyl groups, unsaturated fatty acids and amine derivatives compounds in the extract. A unique 16S rRNA gene sequences and phylogenetic analysis provided strong evidence for the three novel actinobacterial strains (1, 2 and 18) namely *Streptomyces acrimycini* NGP (JX843532), *Streptomyces albogriseolus* NGP (JX843531), *Streptomyces variabilis* NGP (JX843530).

Key words: Actinobacteria, 16s rRNA, anti-microbial activity, phylogenetic analysis.

INTRODUCTION

Oceans are highly complex environments and harbor a diverse assemblage of microbes that occur in environments with extreme variations in pressure, salinity, temperature and low nutrition, besides it is estimated that only 1% of marine microorganisms have been cultured and identified [1]. Marine microorganisms have attracted great attention since they have developed unique metabolic and physiological capabilities that not only ensure survival in an extreme habitats but also the potential to produce compounds with unique metabolic pathways and structures that may differ from terrestrial microorganisms [2].

The marine ecosystem has the unique group of actinobacteria which produce diverse nature of secondary metabolites. The researches on actinobacteria have provided many vital bioactive compounds with high commercial valuable antibiotics like salinosporamide and abyssomycin [3, 4]. Sum of 10,000 of bioactive compounds were isolated from actinobacteria, especially the genus Steptomyces, which possesses the wide range of secondary metabolites [5]. Earlier studies revealed that, due to the particular living conditions, salinity, nutrition, higher pressure, temperature variations, competition with bacteria, viruses and fungi, the secondary metabolites producing marine actinobacteria have distinct chemical structure, which may form the synthesis of new drugs [6].

Bioactive compounds occurring in an actinobacteria consists of multicomponent mixtures, their separation and functional groups determination still creates problem. FTIR has proven to be a valuable tool for the determination of functional groups (chemical bonds) present in an unknown mixture [7]. Besides, FTIR spectra of the compounds are identified by comparison to a library of known compounds. IR scanning yields the vibrational peaks of the functional groups present in the compound, which are efficient and identifications of the disease specific drugs [8, 9].

In addition to, the identification of actinobacteria using microscopic techniques and biochemical tests were not enough to ensure certainty. 16S rRNA sequencing method is being considered as the advancement of technology to identify the isolated actinobacteria at molecular level [10]. Previous study stated that, sequence based approaches can now be addressed to find the relationship between the groups of related strains and the tree was constructed using Molecular Evolutionary Genetic Analysis (MEGA) version. So, the identification of actinobacteria at genetic level was made possible in a fast and accurate manner [11].

The aim of the present study elucidates, an interesting chemical compounds extracted from the novel isolates *S.acrimycini* NGP (JX843532) *S.albogriseolus* NGP (JX843531) and *S.variabilis* NGP (JX843530) as a bioactive agent tested against the human pathogens.

MATERIALS AND METHODS

Collection and isolation of Actinobacteria

The actinobacterial isolates were isolated from marine sediment sample of South Indian coastal region, Tamilnadu, India. One gram each of the sediment samples were treated with 100 ml of sterile sea water complex broth and incubated at an ambient temperature for about a

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week at 200 rpm. The pretreated samples were diluted 1:10 v/v with sterile distilled water and serial dilution prepared down to 10^{-6}. One hundred microlitres of the 10^{-1} to 10^{-6} suspensions were spread onto Starch Casein Agar (SCA - ISP 4) medium. The medium component included fuconazole (20 mg/l) to prevent other non-actinomycetes bacteria and fungal growth. After incubation of 7 to 10 days at 28°C, the actinobacterial colonies that developed on the plates were enumerated [12]. The isolates were identified by various parameters such as colony morphology, physiological, biochemical and microscopic characterization [13].

Antimicrobial Screening

The pathogens used for the antimicrobial screening were clinical isolates includes the following; *B.burgdorferi, B.henselae, L.monocytogenes, H.pylori and C.jejuni.* All pathogens were streaked on nutrient agar and incubated at 37°C for 24 h. After obtaining superior growth, the slants were refrigerated at 4°C and they were frequently checked for viability and re-prepared when appropriate.

Antibiotic sensitivity test

Antibiotic sensitivity test of the human pathogens were performed. The pathogens were streaked on Muller Hinton Agar and the susceptibility resistances to various types of antibiotics were tested by observing and measuring the zone of incubation [14]. The following standard antibiotic discs were tested for resistance activity studies against the human pathogens were amoxicillin, tetracycline, erythromycin, rifampicin, ticarcillin and vancomycin.

Primary screening

The antagonistic activity was tested by conventional cross streak method [15]. Single streak of the 56 actinobacterial isolates were made on nutrient agar medium and incubated at 28°C for 7 days. After observing a good growth, the human pathogens were streaked at right angles to the original streak of actinobacteria and incubated at 37°C for 48 hrs. Actinobacterial isolates which showed prominent and broad spectrum activity were used for secondary screening.

Extraction of Bioactive compound

Promising antagonistic stains were subjected for the mass cultivation for the extraction of bioactive compound with the solvents, ethyl acetate, acetone and DMSO by the modified method of earlier research [16]. The sterile disc impregnated with ethyl acetate, acetone and DMSO were used as a control and all the plates were incubated at 37°C for 72 hrs. After incubation, the zone of inhibition appearing around the discs were measured and recorded as millimeter diameter.

Characterization of crude extract

Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown extracts [7]. The IR spectra of the crude extracts were measured (as KBr discs) between 800-4000 cm^{-1} using FTIR (Shimatzu). The important IR bands, such as -OH, C-H, =CH, C=C, CH_2 and C-O stretching frequencies were studied to determine the functional group in the ethyl acetate crude extract with the reference chart [17].

16s rRNA amplification and sequencing

The genetic level identification of potential actinobacteria was carried out. Genomic DNA was extracted according to the standard procedure [18]. The 16s rRNA of the strains were amplified using the 16s rRNA primers, forward primer (5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-ACGGCTACCTTGTTACGACTT-3'). PCR products were purified using QIAquick PCR purification kit. The purified DNA fragments were sequenced using sequencer model ABI 3100 sequencer according to the protocol provided by the manufactures (ABI PRISM 3100 Genetic Analyzer User's Manual). The conservative part (16s rRNA) of three actinobacteria were aligned manually with available nucleotide sequences retrieved from the GenBank using CLUSTRAL W [19].

Phylogenetic analysis

Nucleotide sequences were compared to those in the Gene Bank database with the Basic Local Alignment Search Tool (BLAST) algorithm to identify known closely related sequences. 16S rRNA gene sequence analysis was performed with the aid of MEGA. 4 software package by using neighbour-joining method [20]. The number of species in each clone library was determined by comparing closely related sequences using bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast.cgi) [19].

RESULTS

Isolation of Actinobacteria

The present investigation has carried out for the isolation of actinobacteria in the marine sediment samples from south Indian coastal region which was not explored so far. 56 isolates were selected based up on the morphological characteristics (Data not shown) irrespective of the collection site and seasons. Those were enriched with sea water complex broth for seven days for the progressive growth and investigated for antagonistic activity study against human pathogens.

Antagonistic activity of Actinobacteria

The results of multi drug resistant activity of human pathogens are shown in table 1. Of the six standard antibiotic discs screened, all the pathogens showed only a narrow spectrum activity against the discs. Simultaneously, all the pathogens exhibited absence of an inhibition zone against any one of the antibiotic discs. The primary screening method was used to select the actino bacterial isolates and determine the range of pathogens that were sensitive to the antibiotics. The isolates 1, 2, 3, 4 and 18 showed increased antibacterial activity against all the human pathogens. The formations of inhibitery zone around the pathogenic strains were due to the production of secondary metabolites by actinobacterial isolates. The metabolites were extracted from culture filtrates of the organisms (1, 2, 3, 4 and 18) by partitioning with organic solvents viz, ethyl acetate, acetone and DMSO. The three solvents were being tried for extraction of secondary metabolites and studied their bio efficiency of each solvent against human pathogens. All these data have been mentioned in table 2. Among the metabolites tested against human pathogens, the metabolites extracted from isolate 1 with ethyl acetate was efficiently inhibited all the pathogens tested. The maximum zones of inhibition were noticed as 18±0.67, 15±0.39mm against *B.burgdorferi and B.henselae.* Similarly, the broad spectrum activity with big zone of inhibition was observed against *L.monocytogens, H.pylori and C.jejuni* which were notified as 11±0.75, 16±0.32 and 10±0.10 respectively. On the other hand the extracts with acetone and DMSO were in effective against any one of the pathogens. As in the case of control study, ethyl

Table 1: Multi drug resistant activity of human pathogens

Antibiotics	*B.burgdorferi*	*B.henselae*	*L.monocytogenes*	*H.pylori*	*C.jejuni*
		Zone of inhibition (mm)			
Amoxycillin	1.0±0.2	2.0±0.63	1.5±0.33	-	1.0±0.15
Tetracyclin	-	3.5±0.39	1.0±0.69	1.0±0.50	-
Erythromycin	1.5±0.2	-	3.0±0.99	3.5±0.66	2.5±0.39
Rifampicin	-	2.0±0.66	-	4.0±0.71	2.0±0.94
Ticarcillin	1.0±0.36	1.0±0.54	2.0±0.50	2.0±0.11	-
Vancomycin	-	-	1.5±0.75	-	3.0±0.55

'-': No activity; values are the average of three replicates

Table 2: Antimicrobial susceptibility of antagonistic actinobacteria metabolites against human pathogen

Isolates (Is)	Solvents	*B.burgdorferi*	*B.henselae*	*L.monocytogenes*	*H. pylori*	*C.jejuni*
			Zone of inhibition (mm)			
Control	Ethyl acetate	3±0.25	2±0.18	2±0.33	2±0.69	2.5±0.12
	Acetone	2±0.11	1.5±0.10	2±0.11	1.5±0.22	1±0.10
	DMSO	-	-	1±0.05	-	0.5±0.22
Is - 1	Ethyl acetate	18±0.67	15±0.39	11±0.75	16±0.32	10±0.10
	Acetone	16±0.85	14±0.96	10±0.89	15±0.96	9±0.55
	DMSO	17±0.79	14±0.22	11±0.66	13±0.14	10±0.05
Is - 2	Ethyl acetate	13±0.66	12±0.55	9±0.45	15±0.33	7±0.55
	Acetone	12±0.96	11±0.11	7±0.99	11±0.84	-
	DMSO	9±0.12	11±0.69	8±0.22	12±0.71	-
Is - 3	Ethyl acetate	5±0.25	5±0.63	3±0.78	-	-
	Acetone	-	-	-	-	-
	DMSO	-	-	-	-	-
Is - 4	Ethyl acetate	3±0.36	-	8±0.50	7±0.56	6±0.33
	Acetone	-	-	-	-	3±0.11
	DMSO	-	5±0.47	-	-	-
Is -18	Ethyl acetate	12±0.47	17±0.66	15±0.55	10±0.67	8±0.55
	Acetone	11±0.98	9±0.22	-	10±0.14	5±0.22
	DMSO	11±0.79	-	6±0.41	8±0.66	-

'-': No activity; values are the average of three replicates

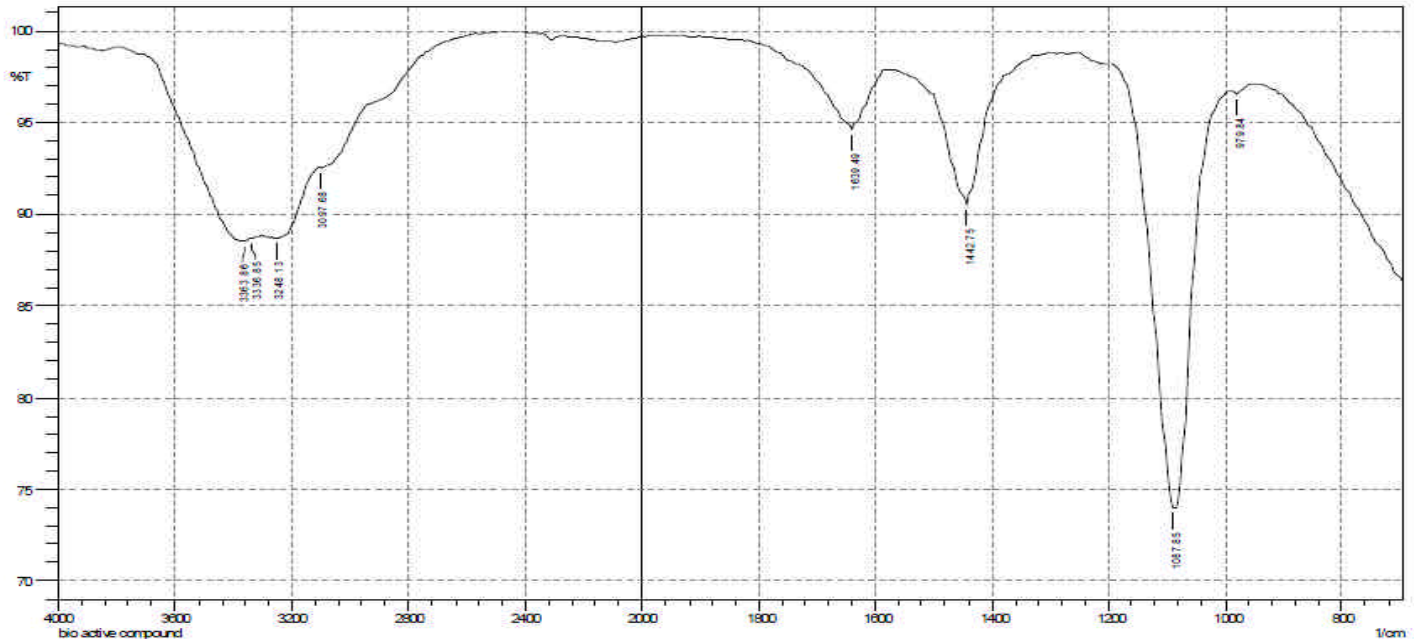
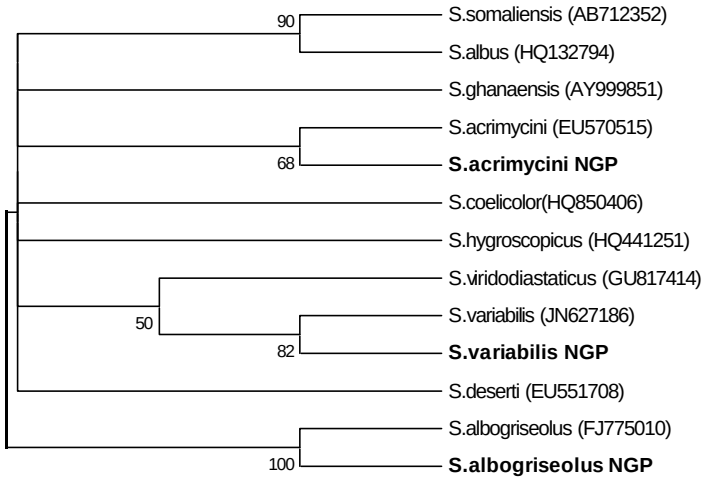


Fig. 1: FTIR analysis of bio active compound from ethyl acetate extract of isolate 1

Table 3: FTIR spectra showed the absorbency bands of different chemical functional groups

Functional groups	Absorbency peaks (800-4000 cm^{-1})
-OH (stretch)	3363.86 and 3336.85
C-H (stretch)	3248.13
=CH (stretch)	3097.68
C=C (conjugated)	1639.49
CH_2 (bend)	1442.75
C-O (stretch)	1087.85
C-H (stretch)	979.84

Fig. 2: Neighbour joining phylogenetic tree based 16S rRNA gene sequences



acetate extracts had being exhibited an increased zone of inhibition against the pathogens among the solvents.

Characterization of bioactive compound

The ethyl acetate extracts obtained from self free culture of isolate one was subjected to IR for the identification of mebolites present in the extracts. The presence of some functional group is shown in table 3 and figure 1. The FTIR spectrum showed hydroxyl functional group between 3300 and 3370 cm^{-1} and the unsaturated fatty acids group were characterized between the vibrational peaks of 2800 and 3200 cm^{-1}. The vibrational peaks between 970 and 1700 cm^{-1} were distinctiveness of amine functional groups based on the characteristic features of IR vibrational peaks in the spectra, hydroxyl groups (-OH), unsaturated fatty acids (C=C) and amine derivates were the possible compounds in the extract.

Phylogenetic tree analysis

The analysis of the 16S rRNA gene is most important tool for correct identification of microbial species; the isolates 1, 2 and 18 were sequenced and the phylogenetic trees were constructed by neighbor joining method. A BLAST analysis carried out by blastn search through GenBank revealed that all the three isolated are the members of the genus *Streptomyces.* The levels of similarity between the 16S rRNA gene of the *Streptomyces* species are shown in figure 2. The NCBI (National Centre for Biotechnological Information) and EMBL (European Molecular Biological Laboratory) sequences data showed that isolate 1 - *Streptomyces acrimycini* NGP (JX843532), isolate 2 - *Streptomyces albogriseolus* NGP(JX843531) and isolate 18 - *Streptomyces variabilis* NGP (JX843530) having 68, 100 and 82 percent similarity with EU570515, FJ775010 and JN627186.

DISCUSSION

Selection of potential actinobacteria depends upon the huge diversity of strains and sample size. Actinobacteria are indeed well adopted and are functional members of the aquatic microbial community [21]. The result of this study corroborates the report of earlier study explained that, the significant attention is currently being paid to the isolation and characterization of *Streptomyces* from poorly researched habitats that had given the foundation to discover new natural products for developing the ideal resource for biotechnology [7]. A total of 106 strains were isolated from the yellow sea, China and in that, 78 actinobacteria strains were belonged to *Streptomyces sp*. Further sums of 34 actinomycete strains were isolated from marine sediment samples of Parangipettai for the extraction of secondary metabolites [22, 23].

It has been reported that, ethyl acetate extract of bioactive compound from novel actinomycete *Streptomyces radiopugnans* MS 1 showed activity against multidrug resistant bacteria and malignant cells [24]**.** Although, a total of 40 actinobacteria isolated from Antarctica were tested for antagonistic activity against 7 gram-positive and negative bacteria, yeasts and 16 phytopathogenic fungi. Similarly, eighty actinobacteria were selected as antagonists against *Pestalotiopsis magniferae*, a causative agent of brown rot fungi. The antimicrobial nature of the extracts from the actinobacteria are depends on the presence of biochemical constituents in the extract especially rich in chlorine and bromine elements [25, 26]. It is also interesting to corroborate the research report , 31 strains out of 63 isolates showed sensitivity against *Candida albicans*. However, actinobacterial strains isolated from the sediment of Parangipettai coast did not show sensitivity againt *Candida albicans*. Further, the human eye pathogens such as *Aspergillus flavus, A.niger* and *Rhodococcus* were inhibited by the marine actinobacteria from Arabian Sea coast [27, 16].

The IR spectrum exhibited for the ethyl acetate extracts of the isolate 1 highlight their potentials and suggests that, they could be a vital candidate for antibiotics. The vibrational peaks of these functional groups depicts that the extract has diverse activity against the test organisms [17]. The IR spectrum of an antifungal compound: 4' phenyl -1-napthyl -phenyl acetamide from *Streptomyces sp.* DPTB16 indicates that the compound had NH_2 and –OH group. Bioactive compounds from a Polypore fungus *Ganoderma applanatum* (Per s.ex Wallr.) Pat was analysed with FTIR spectrum [28, 29]. The similar technique implemented for the isolation of antibacterial compound from marine soil actinomycetes, which were analysed by vibrational peaks of FTIR spectrum [30]. The 16SrRNA sequence data supported that assignment of this isolate 1, 2 and 18 to the *Streptomyces acrimicini, S.albogriseolus and S.variabilis* respectively. The phylogenetic analysis of the 16SrRNA gene sequence was aligned using the CLUSTRAL W programme from the MEGA 4. Version [31, 32]. The topologies of the constructed tree were evaluated by bootstrap analysis with 1000 resamplings. Molecular tools have a great potential to assist in isolating yet - uncultured bacteria with known rRNA sequences for further investigations .One of these molecular tools is the PCR amplification of variable regions of the genes encoding 16S rRNA, by use of primers homologous to the conserved region of gene [33].

REFERENCES

1. Sivakumar K, Sahu MK, Thangaradjan T, Kannan L , Research on marine actinobacteria in India. Indian journal of Microbiology, 2007 47, 186-196.
2. Baltz RH, Renaissaiance in antimicrobial discovery from actinomycetes. *Current openion in pharmacology*, 2008, 8, 557-563.
3. Solanki R, Khanna M, Rup Lal R, Bioactive compounds from marine actinomycetes. Indian J. Microbiol, 2008, 48, 410-431.
4. Bhavya M, Mohanapriya R, pazhanimurugan and Balagurunathan R , Potential bioactive compound from marine actinomycetes against antifouling bacteria. Indian journal of geo-marine sciences, 2011, 40, 578-582.
5. Dharmaraj S, Ashok kumar B and Dhevendaran K, Fermentative production of carotenoids from marine actinomycetes. Iranian journal of Microbiology, 2009, 1, 36-41.
6. Aneja KR, Experiments in Microbiology plant pathology and Biotechnology. Fourth edition, New age international (P) Limited Publishers, 2003, 245-275, 390. ISBN 81-224-1494-X.
7. Eccteston GP, Brooks PR, Kurtboke DI , The occurance of bioactive micromonosporae in afautic habitats of the sunshine, coast in Ausrtralia mar Dauys, 2008, 6, 243-261.
8. Bharathidasan R and Paneerselvam A, Anti bacterial activity and bio active compounds analysis of mangrove endophytic fungi *Penicillium janthinellum, Aspergillum conicus* and *Phomosis sp.*, International journal of pharmaceutical research and development, 2012, 4, 159-168.
9. Najam-ul-Haq M, Majeed A, Saeed S, Huck CW, Huck-pezzei VA, Pallua J, Bittner L, Schonbichler S, Pezzei C, Athar M, Mahmood N and Bonn GK , Role of infrared spectroscopy in medical plant research in Pakisthan. Current bioactive compounds.2012, 7, 85-92.
10. Wellington EMH, Stackebrandt E, Sanders D, Wolstrup J, Jorgensen NOG, Taxononic studies of kitasatosporia and proposed unification with streptomyces on the basis of phenotypic 16SrpNA analysis and emendation of streptomyces. AL.Inl.I.Systs Bacteriol. 1994, 42,156-160
11. Mangamuri UK, Vijalakshmi Muvva, Sudhakar poda, Sreenivasalu kamma ,Isolation, Identification and molecular characterization of rare actinomycetes from mangrore ecosystem of Nizapatanam, Malasian Journal of Microbilogy,2012, 8:83-91
12. Oskay M, Usame A and azeri C, Anti bacterial activity of some actinomycetes isolated from farming soils of Turkey. AJB, 2004, 3, 441-446.
13. Lachevalier HA ,The actinomycetes III, A practical guide to generic identification on actinomycetes. Bergey's manual of systematric bacteriology. Williams and wilkins, Baltimore, 1989, **4**, 2344 - 2347.
14. Bauer AW, Kirby WM and Sherris JC, Antibiotic susceptibility testing by a standard single disc method. Am. J. Clin. Pathol, 1996, **451,**493-496.
15. Vijayakumar R, Muthukumar C, Thajuddin N, Panneerselnam A, Saravanamuthu R, Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. *Actinomycetologica*, 2007, 21, 59-65.
16. Ravikumar S, Samikan Krishnakumar, Samuel Jacob Inbaneson and Murugesan Gnanadesigan , Antagonistic activity of marine actinomycetes from Arabian Sea coast. Archieves of applied science research, 2010, 29: 273-280.
17. Ogunmwonyi IH, Mazomba N, Mabinya L, Ngwenya E, Green E, Akinpelu DA, Olaniran AO, Bernard K and Okoh AI, Studies on the culturable marine actinomycetes isolated from the Nafoon beach in the Eastern Cape Province of South Africa. African Journal of Microbiology Research, 2010, 4, 2223-2230.
18. Marmur J, A procedure for the isolation of deoxyriobonucleic acid from microorganisms. J.Mol.Biol. 1961, 3, 208-218.
19. Maidak BL, Coloe JR, Lilburn TG, Parker Jr CT, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen GJ, Pramanik S, Schmidt TM, Tiedje JM , The RDP (Ribosomal Database Project) continues. Nucl.Acids Res, 2000, 28, 173-174.
20. Saitou N aand Nei M, The neighbour joining method: a new method for reconstructing phylogenetic trees. Mol.Biol.Evol. **B**, 1987, 406-425.
21. Jensen PR, Dwight R, Fenical W, Distribution of actinimycetes in near – shore tropical marine sediments. Application of environment microbial. 1991, 52,1102-1108
22. Zhang L, An R, Wang J, Sun N, Zhang S, Hu J and Kuai J, Exploring novel bioactive compounds from marine microbes. Curr.opin, Microbiol, 2005, 8, 276-281.
23. Poorani E, Saseetharan MK, Dhevagi P, L-asparaginase production and molecular identification of marine streptomyces sp strain EPD 27. International journal of integrative biology, 2009, 7, 150-155.
24. Senthil kumar M and Selvam K, Prospective efficacy of bioactive extract of novel actinomycetes against multidrug cell and multidrug resistant bacteria. Pharmacology online, 2011, 1, 1262-1290.
25. Nathan A, Magarvey, Keller JM, Bernan V, Dworkin M, Sherman DH, Isolation and characterization of novel marine-derived actinomycete taxa rich in bioactive metabolites. Appl Environ Microbiol, 2004, 70, 7520-7529.
26. Arifuzzaman M, Khatun MR, Rahman H, Isolation and screening of actinomycetes from sundarbans soil for antimicrobial activity. African journal of Biotechnology, 2010, 9, 4615-4619.
27. Balagurunathan R and Subramanian A, Antagonistic actinomycetes from marine sediments. Ad. Bios, 2001, 20, 71 - 76.
28. Dhanasekaran D, Thajuddin N and Paneerselvam A, Antifungal compound: 4' phenyl -1-napthyl -phenyl acetamide from *Streptomyces* sp. DPTB16. Facta universitatis Series: Med. Biol, 2008, 15, 7-12.
29. Muhsin TM, Hafiz AA, Duboon A and Khalaf KT, Bioactive compounds from a Polypore Fungus *Genoderma applanatum* (Perr s.ex Wallr.)Pat. Jordan Journal of Biological Sciences, 2011, 4, 205-212.
30. Bais YG, Nimbekar TP, Wanjari BE and Timande SP, Isolation of antibacterial compound from marine soil actinomycetes. International Journal of Biomedical and Advance Research, 2012, 3, 193-196.
31. Teske A, Sigalevich P, Cohen Y, Muyzer G, Molecular identification of bacteria from coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. Appl. Environ. Microbiol, 1996, 62, 4210-4215.
32. Tamura K, Dudley J, Nei M and Kumar S, MEGA 4: Molecular Evolutionary Genetics Analysis software version 4.0. Molecular Biology Evalution, 2007. 24, 1596-1599.
33. Whitman WB, Coleman DC, Wiebe WJ, Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. USA, 1998, 95, 6578-6583.

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