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ORIGINAL ARTICLE

Diversity and exploration of bioactive marine actinomycetes in the Bay of Bengal of the Puducherry coast of India

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Abstract The present study was designed to investigate the Puducherry coast of the Bay of Bengal, India for the diversity of bioactive actinomycetes. A total of 50 actinomycete strains were isolated from the marine sediments and most of the strains were belongs to *Streptomyces.* These strains were identified by means of morphological physiological, biochemical and cultural characteristics. The isolates were subjected to shake flask fermentation and the secondary metabolites were extracted with ethyl acetate and screened for cytotoxicity, hemolytic activity and antimicrobial activity against selected bacterial and fungal pathogens. The cytotoxic activity was evaluated using HeLa cell lines by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay, hemolytic activity on mouse erythrocytes and the antifungal activity was evaluated by MTT cytotoxic assay against *Aspergillus niger, Aspergillus fumigatus* and *Candida albicans.* The antibacterial activity was studied against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Klebsiella pneumoniae.* The cytotoxicity and antimicrobial activity of secondary metabolite was found to be concentration dependent and nearly 24% of isolates showed significant antimicrobial, hemolytic and cytotoxic activity. The results of our study indicate the diversity and bioactive potential of marine actinomycetes isolated in the Puducherry coast.

Keywords Actinomycetes · Streptomyces · Secondary metabolite · Antagonism · Cytotoxicity

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Introduction

The rate of discovery of novel drugs from terrestrial actinomycetes has been decreased which make researchers to evaluate new sources for the isolation of actinomycetes and to discover new chemical compounds. There are approximately 32,500 natural products reported from microbial sources including about 1,000 derived from marine microbe [1]. Wide variety of secondary metabolites produced by the marine microbes is used for drug development and remains to be fundamental of modern pharmaceutical companies [2]. The marine source is rich in both biological and chemical diversity and there are several reports that the marine actinomycetes yielded several metabolites such as anticancer compounds, antimicrobials as well as industrially important enzymes [3–5].

A number of terrestrial actinomycetes, especially those belongs to the genus *Streptomyces*, are being extensively used for commercial production of different medically important compounds [6]. *Streptomyces* has yielded many therapeutic agents, which includes antibacterials such as tetracyclines, antifungal agents such as amphotericin, anticancer drugs exemplified by adriamycin and the immunosuppressant tacrolimus [7]. *Streptomyces* has been reported to contribute nearly 70% of metabolites described under actinobacteria [8].

In Indian Peninsula only 41 species of actinobacteria belonging to eight genera have been isolated and studied for its potential role [9]. Most of the studies conducted in Indian Peninsula have been restricted to isolation, identification and maintenance of actinobacteria in different culture media and only few studies alone conducted to evaluate the antagonistic potential of marine actinomycetes. *Streptomyces* were the major isolates in most of marine samples collected in the south east coastal region of India.

Marine *Streptomyces* isolated from the Vellar estuary, Tamil Nadu have been reported to possess antagonistic activity on human bacterial pathogens [10]. Antagonistic activity of *Streptomyces* isolated from the Pichavaram (Tamil Nadu) mangrove environment has been reported [11].

Isolation of 39 *Streptomyces* strains from Palk Strait region, India has been reported [12]. Presence of number of extracellular enzymes such as lipase, caseinase, gelatinase, amylase and cellulase in marine actinomycetes have been reported in the samples collected in the Tamil Nadu coast of Bay of Bengal [13]. A *Streptomyces* strain isolated from salt pans of Cuddalore, Tamil Nadu have been shown to possess good antimicrobial activity on eight tested pathogens [14]. In this study a systematic screening of bioactive marine actinomycetes was carried out in the sediment samples collected at the Puducherry coast of the Bay of Bengal, India.

Materials and methods

Sampling

A total number of 43 marine sediments were collected from the Pondicherry coast (Latitude (N) – 11°56′; Longitude (E) - 79°53′) of India at the depth of 50–300 cm. The sediments collected were maintained at ambient temperature with sea water and transported to laboratory.

Isolation of actinomycetes

Soil samples were subjected to10-fold serial dilution and aliquots (0.1 ml) were plated on starch casein agar (SCA), ISP No. 1 medium and actinomycetes isolation agar, supplemented with 25% sea water, 25% soil extracts and antibiotics (cycloheximide, 25 µg/ml and nalidixic acid, 25 µg/ml). Plates were incubated for 7–10 days at optimal conditions. Colony forming units (CFU) were counted and the average number of bacteria referred to the swabbed area (cm^2). The isolated actinomycetes strains were kept in slant cultures at 4°C.

Primary screening for antagonistic activity

The isolated actinomycetes were screened for the antagonistic activity using conventional well diffusion assay against selected bacteria and fungi and the lead candidates were selected and studied further.

Characterization of actinomycetes cultures

The selected potential actinomycetes strains were studied for morphological, cultural, physiological and biochemical characteristics according to previously described methods [15]. The morphology of spore chain and spore bearing hyphae were identified using high power optical microscope (Olympus) at 1,000 × magnification [16]. The color of spore mass was examined under light microscope and estimated by color chart [17].

Optimization of cultural conditions and medium composition

The effect of different culture media (SCA, ISP No. 1 and actinomycetes isolation agar), pH (5, 7.4 and 9), temperature (15, 27, 37 and 50°C) and NaCl (5, 7, 9 and 12% w/v) concentration on the growth of isolates were studied. The suitable carbon and nitrogen sources were studied by inoculating the isolates into the SCA with different carbon and nitrogen sources. The concentration of carbon sources required and carbon utilization tests were carried out as described earlier [15, 16].

Fermentation

Well grown slant cultures of the isolates in SCA were inoculated into 50 ml medium in 250 ml Erlenmeyer flasks containing the production medium and incubated for 2 days in rotary shaker (200 rpm) at 28°C. Ten percent of these inoculums were transferred into 200 ml production medium in 1 liter Erlenmeyer flasks. The inoculated cultures in the production medium were incubated for 72 h on a rotary shaker (200 rpm) at 28°C.

Extraction of crude secondary metabolite

After fermentation the fermented broth (2 l) was centrifuged at 4,000 rpm for 10 min at 10°C and the filtrate was separated. The supernatant was extracted twice with ethyl acetate (400 ml) and washed with 500 ml water. The mycelium cell extract was extracted with acetone (500 ml) and centrifuged at 4,000 rpm at 10°C for 10 min. Both the ethyl acetate and acetone extracts were concentrated in rotary vacuum and lyophilized to obtain a crude secondary metabolite. A stock was prepared by dissolving in dimethyl sulfoxide (DMSO) (1 mg/ml) and stored at 4°C. The appropriate concentration of the secondary metabolite was prepared by serial dilution with culture medium.

Bacterial and fungal pathogens

The following bacterial strains *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 10273) and fungal strains *Aspergillus niger* (ATCC 16404), *Aspergillus fumigatus* (ATCC 46645), *Candida albicans* (ATCC 10231) were used to evaluate the antifungal activity.

Antifungal assay

The antifungal activity of the secondary metabolite was tested by MTT (3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, a tetrazole) assay [18]. In brief, 1 ml sabouraud dextrose agar (SDA) media containing 1×10^8 cells of fungal strain in eppendorf tubes was mixed with different concentrations of the crude secondary metabolite in each tube and incubated for 90 min at 22°C. MTT (5 mg/ml) 50 µl was added to each tube and incubated for 2 h at 30°C. The 500 µl of propan-2-ol containing 0.04 mole HCl was added to each tube and the mixture was vigorously vortexes to remove MTT-formazon complex from the cells. Then the tubes were centrifuged at 12,200 rpm for 2 min; the supernatant was collected in a different set of tubes and the absorbance was measured at 570 nm against the cell-free medium as control. The drug amphotericin was used as positive control and the average was calculated from the triplicate values.

***In vitro* antibacterial assay**

The antibacterial activity of secondary metabolites (25 µg/ml) was tested by agar diffusion assay. The plates were incubated at 37°C for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite when compared to the controls. Chloramphenicol was used as positive control.

***In vitro* hemolytic assay**

The hemolytic activity of the secondary metabolite was tested by hemolytic assay [19] under *in vitro* conditions in 96-well plates. Each well received 100 µl of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well served as negative control contained only water, and in the second well, 100 µl of secondary metabolite of various concentrations (5–500 µg/ml) were added. The last well served as positive control containing 20 µl of 0.1% Triton X-100 in 0.85% saline. Then, each well received 100 µl of a 2% suspension of mouse erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After 30 min incubation at room temperature and centrifugation, the supernatant was removed and the absorbance of the liberated hemoglobin was measured at 540 nm. The average value was calculated from triplicate assay.

Cell culture

HeLa cell lines were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) (v/v) and 100 mg/l streptomycin and 100 IU/ml penicillin at 37°C in a CO_2 incubator with 5% carbon dioxide.

MTT cell proliferation assay

The cytotoxic activity of the secondary metabolite (diluted in DMSO 0–100 µg/ml) on HeLa cells (1×10^5 cells/well) were tested by using the CellQuanti-MTT cell viability assay kit (Bioassay Systems). The wells with only culture medium or cells treated with 0.1% of DMSO served as control. The graph was plotted with cell viability against the time period in hours at increasing concentrations of secondary metabolite. The mean and the IC_{50} value were calculated by non-linear regression analysis using the data analysis software (Prism) from three independent experiments.

Results and discussion

The sampling site is a narrow sloppy coastal belt of Bay of Bengal of Indian cost with salt range of 40–70% (Fig. 1). It has a wide diversity of marine life including flora, fauna and microbes. The sediment samples are collected during the tidal wave ranging from 4 to 6 m. The systemic screening process resulted in isolation of 50 actinomycetes from 18 sediment samples. The numbers of actinomycetes present in various sediments varied from 1.5×10^6 CFU and with a minimum of 1.1×10^3/g. Twelve colonies were isolated using SCA, 17 from ISP No. 1 and 21 from actinomycetes isolation agar. The actinomycetes identified are subcultured and maintained in SCA slants at 4°C. The diversity of the collected actinomycetes is given in Fig. 2. Most of the actinomycete strains isolated from sediment samples are belongs to the genera *Streptomyces* (48%), *Micromonospora* 18%, *Actinopolyspora* 14%, *Saccharopolyspora* 8%, *Microbispora* 6%, *Actinomyces* 4% and *Actinoplanes* 2%. It was already been reported that *Streptomyces* are

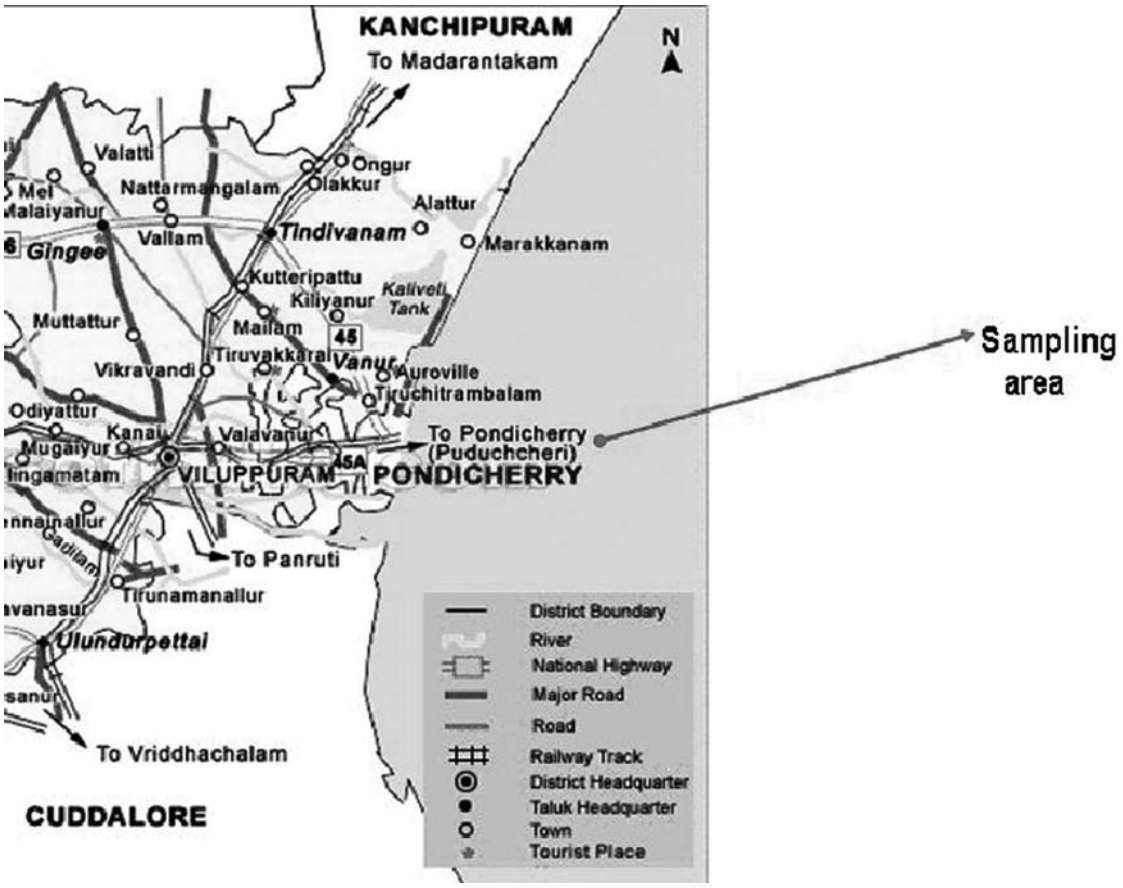

Fig. 1 Marine sediments sampling site.

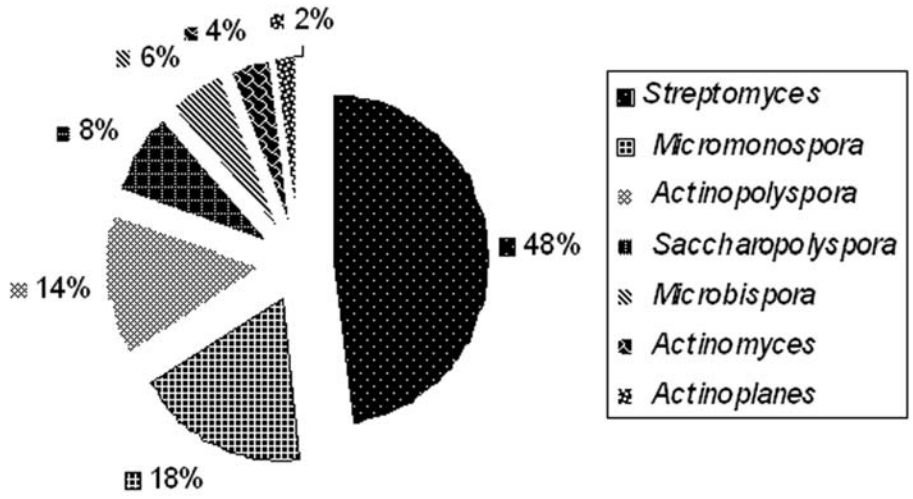

Fig. 2 Diversity and percentage distribution of actinomycetes isolated from marine sediments collected at the Puducherry coast.

predominant species in the marine environment among all actinomycetes studied so far and followed by *Micromonospora* [20]. Screening of marine actinomycetes for bioactivity, out of 50 strains 12 (24%) strains have broad-spectrum of activity and those are selected for further studies. The potential actinomycetes strains belong to four genera namely, *Streptomycetes, Micromonospora, Actinopolyspora* and *Saccharopolyspora*. The diversity of actinomycetes in the Palk Strait region of the Bay of Bengal was reported by Vijayakumar et al. [12]. Peela et al. [21] have shown the antagonistic activity of marine actinomycetes isolated from the Bay of Bengal. Similarly, isolation of antagonistic halophilic *Actinopolyspora* species AH1 was reported from a marine sediment sample collected at the Alibag coast of Maharashtra, India which showed good antibacterial and antifungal activities [22].

Only moderate growth is seen in the commercial media used for most of the potential isolates but the designed production media helped for better growth and antibiotic production. The production media (PM) consists of glucose 0.5%, soluble starch 2%, beef extract 0.4%, yeast extract 0.5%, tryptone 0.5%, calcium carbonate 0.4%, sodium sulfate 0.1%, potassium chloride 0.05%, magnesium chloride 0.2%, dipotassium phosphate 0.05%, sodium chloride 9%, soil extract 25%, sea water 25%, distilled water 50% and pH is 7.4. The growth of isolates is maximum when supplemented the media with 25% sea water and 25% soil extract, prepared from the marine sediments collected and moreover the growth and antibiotic production are depends on sea water and soil extract supplementation. The maximal growth was seen at 27°C with optimal temperature range of 27–30°C. The optimal pH was found to be 7.4. The isolates capable of growing well at NaCl concentrations ranging from 9 to 12% and the growth was almost inhibited at 3% salt concentration. It is clear that the growth and antibiotic production depends on salt concentration.

Among the 50 isolates screened for antifungal activity, six strains showed good cytotoxic activity against fungal strains tested. The *Actinopolyspora* species VITSDK43 and *Streptomyces* species VITSDK37 exhibited a marked antagonistic activity against all the three fungal pathogens with the MIC value ranging from 0.48 to 1.18 µg/ml. The antagonistic activity of the secondary metabolite extracted from VITSDK43 and 37 is equivalent to amphotericin activity (Table 1). The minimal inhibitory concentration (MIC) values were calculated only for those extracts which showed more than 90% inhibition over cell population as compared with control. The MTT assay was carried out with different concentration (0.1–5 µg/ml) of the secondary metabolite and the inhibitory action (cytotoxicity) of the extracts was found to be dose-dependent. Though most of the available antibiotics are derived from actinomycetes, the marine actinobacteria of Indian peninsula is poorly explored for diversity and antagonistic activity. The actinomycetes genera is considered as a potential producer of antibiotics and the

Table 1 *In vitro* antibacterial and antifungal activity of secondary metabolites extracted from actinomycetes against selected pathogens by agar diffusion assay for bacteria and MTT assay for fungi

Actinomycete isolates	Average zone of inhibition (mm)*				MIC (µg/ml) #		
	SA	BS	EC	KP	AN	AF	CA
Streptomyces sp. VITSDK36	–	–	18.0	13.8	1.14	1.32	–
Streptomyces sp. VITSDK37	–	13.4	24.9	31.0	0.56	0.74	0.87
Streptomyces sp. VITSDK38	20.1	18.5	9.2	18.0	1.49	1.64	–
Streptomyces sp. VITSDK39	25.0	19.6	31.0	33.6	1.55	1.78	0.96
Streptomyces sp. VITSDK40	8.3	21.8	14.7	17.5	–	–	0.54
Streptomyces sp. VITSDK41	4.0	7.4	–	–	2.44	2.68	1.12
Streptomyces sp. VITSDK42	27.3	22.1	26.8	32.4	1.47	1.58	2.23
Actinopolyspora sp. VITSDK43	26.0	30.5	25.5	29.7	1.47	1.58	2.23
Actinopolyspora sp. VITSDK44	11.8	15.2	–	11.4	–	2.54	0.77
Micromonospora sp.VITSDK45	13.5	–	–	–	–	–	0.64
Micromonospora sp. VITSDK46	8.5	11.0	20.3	18.5	1.25	1.40	1.96
Sachharopolyspora sp. VITSDK47	5.4	7.5	–	4.6	0.84	0.90	–
Chloramphenicol (10 µg/disc)	16.0	14.0	23.0	21.0	–	–	–
Amphotericin B (25 µg/disc)	–	–	–	–	0.41	0.37	0.32

*Values of zone of inhibition excluding diameter of bore (6 mm).

#MIC values are calculated only for those extract showed ≥90% inhibition when compared to control.

SA: *S. aureus,* BS: *B. subtilis,* EC: *E. coli,* KP: *K. pneumoniae,* AN: *A. niger,* AF: *A. fumigatus,* CA: *C. albicans.*

Table 2 *In vitro* hemolytic activity of secondary metabolites extracted from actinomycetes on mouse erythrocytes (25 µg/ml) and cytotoxicity on HeLa cells (100 µg/ml)

Actinomycete isolates	Hemolytic activity EC_{50} (µg/ml) CI 95%	Cytotoxicity on HeLa cells IC_{50} (µg/ml) CI 95%
Streptomyces sp. VITSDK36	>500	>100
Streptomyces sp. VITSDK37	>500	87.8
Streptomyces sp. VITSDK38	>500	22.0
Streptomyces sp. VITSDK39	294.6	67.2
Streptomyces sp. VITSDK40	>500	>100
Streptomyces sp. VITSDK41	211.7	6.8
Streptomyces sp. VITSDK42	>500	26.9
Actinopolyspora sp. VITSDK43	>500	11.4
Actinopolyspora sp. VITSDK44	>500	>100
Micromonospora sp. VITSDK45	423.9	>100
Micromonospora sp. VITSDK46	318.8	>100
Sachharopolyspora sp. VITSDK47	195	62.8

The total hemolysis was obtained with 20 µl of Triton X-100 (0.1%) and 1 h incubation. The EC_{50}, IC_{50} and 95% confidence interval (CI 95%) was obtained by non-linear regression analyses. EC_{50} value lower than 250 µg/ml was considered as active for hemolytic activity. IC_{50} value lower than 40 µg/ml was considered as active for cytotoxicity on HeLa cells.

marine actinobacteria are capable of producing different bioactive compounds, some could be novel, since the marine environmental conditions are different from the terrestrial conditions.

The actinomycetes isolates have shown good anti-bacterial activity against selected bacterial pathogens (Table 1). The *Streptomyces* species VITSDK39 has shown the maximum inhibition (33.6 mm) against *K. pneumoniae*. The genus *Streptomyces* has more antagonistic activity followed by *Actinopolyspora*. The *Streptomyces* species VITSDK37, 39 and 42 have shown broad-spectrum activity on both gram-positive and gram-negative bacterial pathogens followed by *Actinopolyspora* species VITSDK43. The *Sachharopolyspora* species VITSDK47 and *Streptomyces* species VITSDK41 are effective in inducing hemolysis with the EC_{50} value of 195 µg/ml and 211.7 µg/ml, respectively (Table 2). Among the 12 isolates, six showed significant cytotoxic activity on HeLa cells. The *Streptomyces* species VITSDK38 is effective in inhibiting the proliferation of HeLa cells with the IC_{50} value of 22 µg/ml followed by VITSDK41 and VITSDK42 with the IC_{50} value of 26.8 and 39.1, respectively (Table 2).

The isolated actinomycetes strains have shown broad-spectrum of activity against tested bacterial and fungal pathogens with the antagonistic potential equivalent to that of standard antibiotics tested. The strains also possess moderate cytotoxic property on cancer cellline and hemolytic activity on mouse erythrocytes. The outcome of this study indicates that the marine actinomycetes are potential source for isolation of lead antagonistic compounds.

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