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ISOLATION AND CHARACTERIZATION OF MARINE ANTAGONISTIC ACTINOMYCETES FROM WEST COAST OF INDIA

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Summary. *A total of 173 actinomycetes colonies were isolated from near shore marine environment and mangrove ecosystem at 8 different locations of Kerala, West Coast of India. Among them, 64 isolates were morphologically distinct on the basis of spore mass colour, reverse side colour, aerial and substrate mycelia formation and production of diffusible pigment. The majority (47%; n=30) of these isolates were assigned to the genus Streptomyces. Antimicrobial activities of isolates were also tested against various bacterial and fungal pathogens. Of 64 isolates, 21 isolates had antimicrobial activity, with 2 isolates showing broad spectrum of antimicrobial effect.*

Key words: *Soil actinomycetes diversity, West Coast of India, antimicrobial activity, HPLC*

Introduction

Penetration of biotechnology into marine environment has opened up unexpected new horizons for finding novel organisms for trapping their potential resources. However, culturally independent methods have demonstrated that marine sediments contain wide range of unique microorganisms (1, 2). Actinomycetes are the dominant group of soil population together with bacteria and fungi. They are Gram-positive bacteria having high G+C (>55%) content in their DNA and they are originally considered as an intermediate group between bacteria and fungi. They are free living, saprophytic bacteria, and a major source for production of antibiotics. They play a major role in recycling of organic matter (3), production of novel pharmaceuticals, nutritional materials, cosmetics, enzymes, antitumour agents, enzyme inhibitors, immune-modifiers and vitamins. *Streptomyces* are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites (4).

A little is known about the actinomycetes diversity of marine sediments, which is an inexhaustible resource that has not been properly exploited. Many reports describe that in India, the East Coast area is a major source of actinomycetes (5, 6, 7, 8). However, only few reports are available pertaining to Actinomycetes diversity in West Coast of India (9, 10, 11) and mangrove soils of India (11, 12, 13). Hence, the present study made an attempt to estimate the actinomycetes populations in different soil types (sea shore and mangrove) of West Coast of India, as to screen for their antimicrobial properties. Further, the identified antagonistic actinomycetes were characterized based on morphological, biochemical, cultural and physiological characteristics.

Materials and Methods

Collection of soil samples: Marine soil samples were collected from eight different stations namely, seashore soils covering Payambalam beach [Kannur District, Lat. 11^o 52' N and Long. 72^o 25' E], Puthiyangadi beach, Buttroad beach and Calicut beach [Kozhikode District, Lat.11^o 15' N and Long. 75^o 49' E], Muzhipilazhadi beach [Kannur District, Lat.11^o 45' N and Long. 75^o 32' E], Cochin Fort [Eranakulam District, Lat. 9.58^o 52' N and Long. 76^o 17' E] and Beypore beach [Kozhikode District, Lat.11^o 10' N and Long. 75^o 50' E], and Calicut mangrove [Kozhikode District, Lat.11^o 52' N and Long. 72^o 25' E] of West Coast of India, Kerala. Samples were collected from 6-10 cm depth and transported to the laboratory in sterile polythene bags and stored for further study.

Isolation of actinomycetes: Starch casein nitrate (SCN) agar medium (Himedia, Mumbai, India) was used for isolation and enumeration of actinomycetes. The medium was supplemented with 10 µg/ml amphotericin and 25 µg/ml streptomycin (Himedia, Mumbai, India) to inhibit fungal and bacterial contamination respectively (14). In conventional dilution plate technique, 10g of marine soil samples were suspended in 100 ml of sterile sea water and 0.5 ml of suspension from this was spread over 50% sea water starch casein agar medium (15) and incubated for 7-9 days at 28^{o}C. After incubation the actinomycete colonies were purified and sub-cultured on SCN agar plates and stored for further assay.

Screening for antimicrobial activity: Antimicrobial activities of isolates were tested preliminarily by cross streak method (16). Actinomycetes isolates were streaked across diameter on starch casein agar plates. After incubation at 28°C for 6 days, 24 hrs cultures of *Staphylococcus aureus, Escherichia coli* and *Candida albicans* were streaked perpendicular to the central strip of actinomycetes culture. All plates were again incubated at 30°C for 24 hrs and zone of inhibition was measured.

Extraction of antimicrobial compounds: The selected antagonistic actinomycete isolates were inoculated into starch casein broth, and incubated at 28°C in a shaker (200-250 rpm) for seven days. After incubation the broths were filtered through Whatman No.1 filter paper and then through Millipore filter (Millipore Millex-HV Hydrophilic PVDF 0.45 µm). The filtrate was transferred aseptically into a conical flask and stored at 4°C for further assay. To the culture filtrate, equal volume of various solvents (*viz.,* alcohol, chloroform, ethyl acetate and methanol) was added separately and centrifuged at 5000 rpm for 10 min to extract the antimicrobial compound (5). The compound obtained from each solvent was tested for their activity against the test pathogens (*S. aureus*, *Bacillus subtilis*, *E. coli*, *Salmonella typhi*, *Cryptococcus neoformans* and *C. albicans*) by well diffusion method. After incubation the zone of inhibition was measured.

HPLC analysis: The high performance liquid chromatographic (HPLC) separation of antimicrobial compound was carried out on a LC-10 AT vp model HPLC using 250 x 4.60 mm Rheodysne column (C-18). The solvent system methanol and water (HPLC grade) was used in the ratio of 88:12. The operating pressure was 114 kgf, at a flow rate 0.8 ml/min and the temperature was set at 30°C. The UV-Vis (SPD-10 A vp) detector was set at 210 nm. The sample was mixed with the solvent in the ratio of 50:50 and filtered using Millipore filter before injection. About 25 µl of the sample filtrate was injected into the column. The sample was run for 10 min and the retention time was noted. The elution time was compared with the standard and the antimicrobial compound was identified (17, 18).

Characterization of antagonistic isolates: According to the recommendations of International *Streptomyces* Project (ISP) (19) potent antagonistic actinomycete isolates were further characterized based on morphological, biochemical, cultural and physiological features. Microscopic characterization was carried out by cover slip culture method (20) and formation of aerial and substrate mycelium, and arrangement of spores on mycelium were observed under high power objective of light microscope. Cultural characteristics (growth, colouration of aerial and substrate mycelia, formation of soluble pigment) were tested in seven different media including, SCN agar, nutrient agar, yeast extract malt extract agar (ISP- 2), oat meal agar (ISP-3), inorganic salt agar (ISP-4), glycerol - asparagine agar (ISP-5) and tyrosin agar (ISP-7) with the procedures of ISP. Biochemical tests including IMViC, H_2S production, nitrate reduction, urease, catalase, starch gelatine and casein hydrolysis, haemolysis and TSI were also performed as recommended by ISP. Chemotaxonomical properties such as, analysis of cell wall sugars (21) and cell wall amino acid analysis (22) were analyzed. Physiological characterization such as, the effect of pH (5-9), temperature (10°-50°C) and salinity (NaCl concentrations 1-4%) and antibiotic sensitivity against ten different antibiotics (Himedia, Mumbai, India) [cloxacillin (Clo), amikacin (Ak30), ampicillin (A10), bacitracin (B10), chloramphenicol (C30), nalidixic acid (Na30), norfloxicin (Nx10), streptomycin (S10), tetracycline (T30) and trimethoprin (Tr)] were also tested. Utilization of carbon sources such as starch, dextrose, fructose, maltose and mannitol, and nitrogen sources namely D-alanine, L-arginine, potassium nitrate, L-phenylalanine and L-tyrosine were tested on starch casein agar medium.

Results and Discussion

West Coast of India, especially from Ernakulam to Kannur has wide range of salinities and was selected as an ecosystem for studying the diversity of actinomycetes and their antimicrobial properties. Among 173 actinomycete colonies isolated, 64 isolates were morphologically distinct, which included 40 (62%) isolates from sea shore soil and 24 (38%) isolates from mangrove soil, (Calicut mangrove) (Fig.1). All the 64 actinomycetes were identified at a generic level based on the colony morphology and microscopic morphology. Identification of strains by both morphological and cultural characteristics revealed that most (65.6%) of the isolates belonged to white and grey colour series. Out of 64 isolates, 47% (*n*=30) isolates were assigned to the genus *Streptomyces,* and the remaining were identified as *Glycomyces* (*n*=10), *Nocardiopsis* (*n*=7), *Nocardiodes* (*n*=4), *Actinopolyspora* (*n*=3), *Nocardia* (*n*=3), *Kibdelosporangium* (*n*=2), *Actinosynnema* (*n*=1), *Actinomadura* (*n*=1), *Thermoactinomycetes* (*n*=1), *Kineosporia* (*n*=1) and *Saccharopolyspora* (*n*=1). The percentage frequency of the isolates is shown in fig. 2. The present study revealed that among the isolates *Streptomyces* was the dominant genera. Frequency and dominance of *Streptomyces* among actinomycetes in various soil types were reported by several workers (6, 8, 23, 24). Also Alexander (25) reported that about 20-45% of marine actinomycetes exhibited antimicrobial activity; whereas actinomycetes isolated from marine sediments of Visakapatnam, exhibited only 18% of antimicrobial activity as stated by Ellaiah and Reddy (26). Similarly, the present study also determined that out of 64 strains, 21 isolates (32.8%) had antimicrobial activity, of which 12 isolates (18.8%) showed antibacterial activity, 13 isolates (20.3%) showed antifungal activity (against *C. albicans*); 9 isolates (14.1%) showed both antibacterial and antifungal activity. Similarly, 11 isolates showed activity against *E. coli*, 8 isolates showed activity against *S. aureus* and 6 isolates possessed activity against

both *E. coli* and *S. aureus* (Fig. 3). However, only 2 isolates namely *Streptomyces* sp. RM17 and *Streptomyces* sp. RM42 had broad spectral antimicrobial activity, and were selected for further studies, and they represented seashore and mangrove soils respectively.

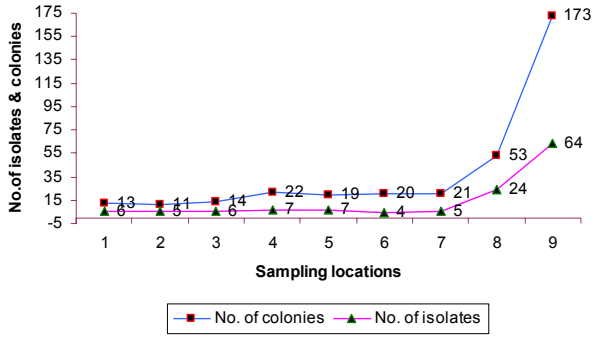


Fig. 1. Actinomycetes population in different soils Seashore isolates: 1-Payambalam Beach (WCRA 1); 2-Puthiyangadi Beach (WCRA 2); 3-Butt road Beach (WCRA 3); 4-Calicut Beach (WCRA 4); 5-Muzhuppilangadi Beach (WCRA 5); 6-Fort Cochin Beach (WCRA 6); 7-Beypore Beach (WCRA 7); Mangrove isolates: 8-Calicut mangrove (WCRA 8); 9-total isolates

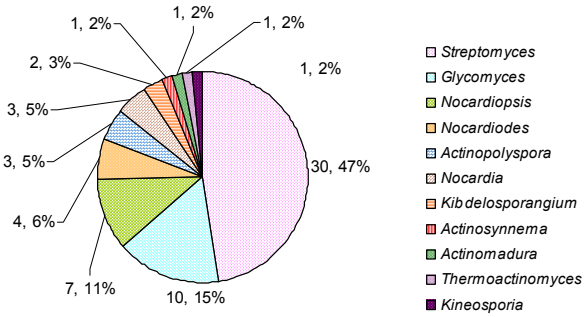


Fig. 2. Percentage frequency of isolated actinomycetes genera

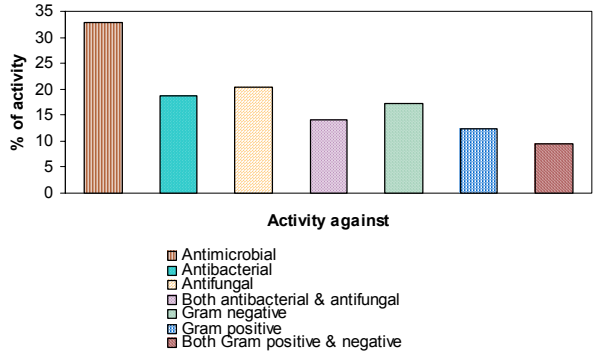


Fig. 3. Antimicrobial activity of actinomycetes isolates

Morphological characterization of both, the broad spectral antagonistic isolates developed dark grey coloured aerial mycelia, and dark grey to white coloured spore mass. However, the strain RM17 developed coffee brown coloured substrate mycelium, and RM42 developed brick red coloured substrate mycelium. Further, the strain RM17 developed spirally nature spore chain in its aerial mycelium, whereas the strain RM42 developed hooked spore chain (Table 1). The details of morphological and biochemical characteristics, utilization of carbon and nitrogen sources, and chemotaxonomical properties of the two test isolates are given in table 1. Sivakumar (13) reported that the characteristics can be used as markers by which an individual strain can be recognized. Particularly, chemotaxonomy plays a major role in identification of actinomycetes to generic level. In this study, both the test isolates contained meso-diaminopimelic acid and there were no sugars found in their cell wall. Both the isolates could utilize all the carbon sources and nitrogen sources (Table 1).

It is also evident that different physiological characteristics are influencing the growth rate of the actinomycetes (27, 28, 29). In the present study, the assessment of physiological characteristics of the strains RM17 and RM42 revealed that they could grow well at 30 and 40°C temperature, pH 7.0 to 9.0 respectively. However, the strains had maximum growth rate at a NaCl concentration of 1g/l (Table 2). The antibiotic sensitivity patterns against the test isolates are shown in table 2. Based on all the above characteristics, seashore isolate (RM17) was identified as *Streptomyces longwoodensis* and mangrove isolate (RM42) was identified to be *Streptomyces viridiviolaceus*. In general, biochemical and physiological characteristics and antimicrobial susceptibility patterns of the actinomycetes vary from isolate to isolate depending on the growth conditions. The present investigation concluded that the physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physical conditions. Upon the growth of both the experimental isolates on various media, SCN agar was observed to be the best medium for maximal growth. Further, the colour of diffusible pigments and the aerial and substrate mycelia produced by the two isolates varied with different media (Table 3). Thus, it was concluded on the basis of the present and previous studies that the nutrient compositions of the medium greatly influence the growth and morphology of organisms (30, 31).

The antimicrobial efficacy of the isolates was tested by using 5 different solvent extracts, and ethyl acetate extract produced maximum inhibitory zone against all the pathogens tested followed by methanol, alcohol and chloroform extracts. Ethyl acetate extract of the strain RM17 showed maximum activity against *B. subtilis* (20 mm) followed by *S. aureus* (18 mm), *S. typhi* (17 mm), *C. neoformans* (16 mm) and *C. albicans* (13 mm). Ethyl acetate extract of the strain RM42 showed maximum activity against *E. coli* (22 mm) followed by *C. neoformans* (20 mm), *S. typhi* (18 mm), *S. aureus* (17 mm), *C. albicans* (16 mm) and *B. subtilis* (15 mm) (Table 4). Similarly, various solvents were used for the extraction of antibiotics from actinomycetes by many workers using ethyl acetate and methanol [Taechowisan *et al.* (32); Ilic *et al.* (33)] and chloroform [Thangadurai *et al.* (34)].

Table 1. Phenotypic characteristics of selected antagonistic actinomycetes

Properties	Streptomyces *sp. RM17*	Streptomyces *sp. RM42*
Morphological characteristics		
Sporophore morphology	Spiral	Hook like
Colour of aerial mycelium	Dark grey	Dark grey
Colour of substrate mycelium	Coffee brown	Brick red
Spore mass	Dark grey	Dark grey
Biochemical characteristics		
Indole production	–	–
Methyl red	–	–
Voges Proskauer	–	–
Citrate utilization	+	+
H_2S production	–	–
Nitrate reduction	–	–
Urease	+	+
Catalase	+	+
Oxidase	–	–
Melanin production	–	–
Starch hydrolysis	+	+
Gelatin hydrolysis	+	+
Lipid hydrolysis	+	+
Casein hydrolysis	+	+
Haemolysis	+	+
Triple sugar iron	alk/alk	alk/alk
Chemotaxonomic characters		
Whole cell sugar analysis	–	–
Cell wall amino acid analysis	L-DAP	L-DAP
Carbon source utilization		
Starch	++++	++++
Dextrose	++	+++
Fructose	+	+
Maltose	+++	+
Mannitol	++++	++++
Nitrogen source utilization		
D-alanine	+++	+++
L-arginine	++	++
Potassium nitrate	++	++
L-phenylalanine	++++	++++
L-tyrosine	++++	++++

+ positive; – negative; alk alkaline; L-DAP L-diaminopimelic acid
Excellent ++++ Good +++ Fair ++ Poor + Nil –

HPLC is being routinely used for the analytical estimation of various antibiotics (35). In the present investigation, HPLC profile of the antimicrobial compounds of *Streptomyces* sp. RM17 and *Streptomyces* sp. RM42 was performed by Rheodysne column (C-18) up to 10 min at 210 nm. The antimicrobial compound of *Streptomyces* sp. RM17 showed absorption peaks at retention time (min) 2.137, 3.263, 3.717, 4.533, 4.843, 6.053 and 6.583. The major peak showed at 3.263 min was the peak representing the antimicrobial activity of *Streptomyces* sp. RM17. Similarly, the antimicrobial compound of *Streptomyces* sp. RM42 showed absorption peaks at retention time 2.123, 3.140, 3.313, 4.573, 4.840, 6.067 and 6.593 min. and the major peaks which had antimicrobial activity were identified to be at 3.140 and 3.313. The retention time 3.263 (min) of antimicrobial compound from *Streptomyces* sp. RM17 on HPLC was similar to oxohexaene (3.23 min) (Fig. 4), a new antifungal antibiotic previously reported by Harindran *et al.* (36) produced by *Streptomyces* sp. CDRIL-312. Whereas, the antimicrobial compound of *Streptomyces* sp. RM 42, showed two retention time of 3.140 and 3.313 on HPLC, the peak at 3.313 (min) was similar to cephalaxine (Fig. 5) a semisynthetic derivative of cephalosporin C previously reported by Aharonowitz and Demain (37) Nakagawa *et al.* (38) produced by *S. clavuligenus*. Further investigation is needed in order to determine the structure of active compound and to scale up the production.

Table 2. Physiological characteristics of antagonistic actinomycetes

Test	*Streptomyces* sp. RM17	*Streptomyces* sp. RM42
pH		
5	++	++
6	+++	+++
7	++++	++++
8	++++	++++
9	++++	++++
Temperature (^oC)		
10	+	+
20	++	++
30	++++	++++
40	++++	++++
50	+++	+++
NaCl concentrations (%)		
Without NaCl	+	+
1	++++	++++
2	+++	+++
3	++	++
4	+	+
Antibiotic sensitivity [zone of inhibition (mm)]		
Cloxacillin (Clo)	R	25
Amikacin (Ak30)	5	5
Ampicillin (A10)	R	R
Bacitracin (B10)	14	15
Chloramphenicol (C30)	1.5	17
Nalidixic acid (Na 30)	R	R
Norfloxicin (Nx10)	15	15
Streptomycin (S10)	34	39
Tetracycline (T30)	18	25
Trimethoprin (Tr)	R	R

Excellent ++++ Good +++ Fair ++ Poor + Nil – R resistant

The search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover an actinomycete population with novel compound of pharmaceutical interest. Because of this, the research will be more promising if diverse and more

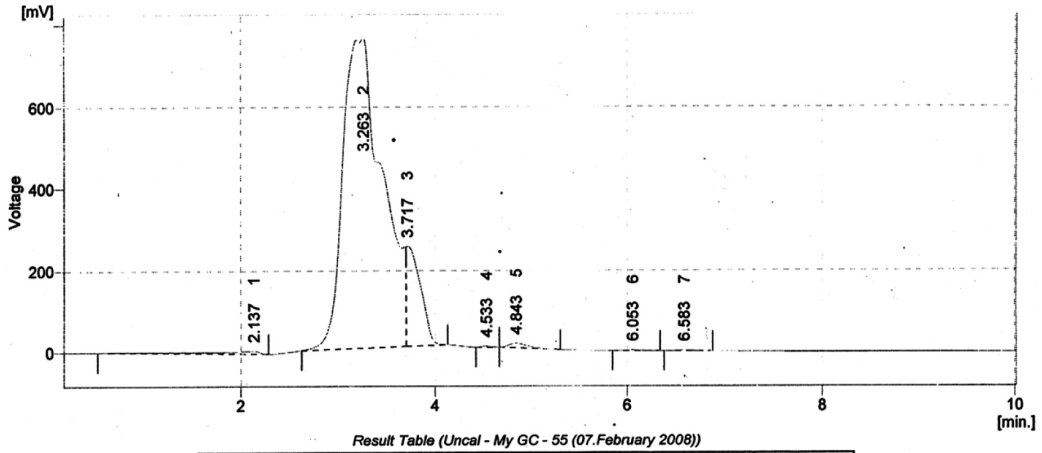
Table 3. Cultural characteristics of antagonistic actinomycetes on different media

S. No.	Name of medium	*Streptomyces* sp. RM17	*Streptomyces* sp. RM42
1.	Malt extract yeast extract agar (ISP-2)	Dull white	Dull white
	Aerial mycelium	Dark yellow	Dark yellow
	Substrate mycelium	Nil	Nil
	Pigmentation		
2.	Oat meal agar (ISP-3)		
	Aerial mycelium	Grey	Medium grey
	Substrate mycelium	Red	Yellow
	Pigmentation	Dark pink	Nil
3.	Inorganic salt starch agar (ISP-4)		
	Aerial mycelium	Grey	Cement grey
	Substrate mycelium	Yellowish green	Light grey
	Pigmentation	Nil	Nil
4.	Glycerol asparagine agar (ISP-5)		
	Aerial mycelium	Grey	Grey
	Substrate mycelium	Light grey	Light grey
	Pigmentation	Nil	Nil
5.	Tyrosin agar (ISP-7)		
	Aerial mycelium	Grey	Light grey
	Substrate mycelium	Yellow	Brown
	Pigmentation	Nil	Nil
6.	Nutrient agar		
	Aerial mycelium	White	Pure white
	Substrate mycelium	Light yellow	Light yellow
	Pigmentation	Nil	Nil
7.	Starch casein nitrate agar		
	Aerial mycelium	Dull grey	Light grey
	Substrate mycelium	Sunset yellow	Dark yellow
	Pigmentation	Nil	Nil

Table 4. Antimicrobial efficacy of actinomycetes

Name of the test organisms	Zone of inhibition (mm)							
	Streptomyces sp. RM17				Streptomyces sp. RM42			
	Ethyl acetate	Methanol	Chloroform	Alcohol	Ethyl acetate	Methanol	Chloroform	Alcohol
Staphylococcus aureus	18	16	13	17	17	9	10	9
Bacillus subtilis	20	17	18	16	15	12	12	15
Escherichia coli	12	10	9	11	22	21	18	19
Salmonella typhi	17	15	13	11	18	15	15	14
Cryptococcus neoformans	16	13	11	10	20	17	16	12
Candida albicans	13	10	8	7	16	9	9	10

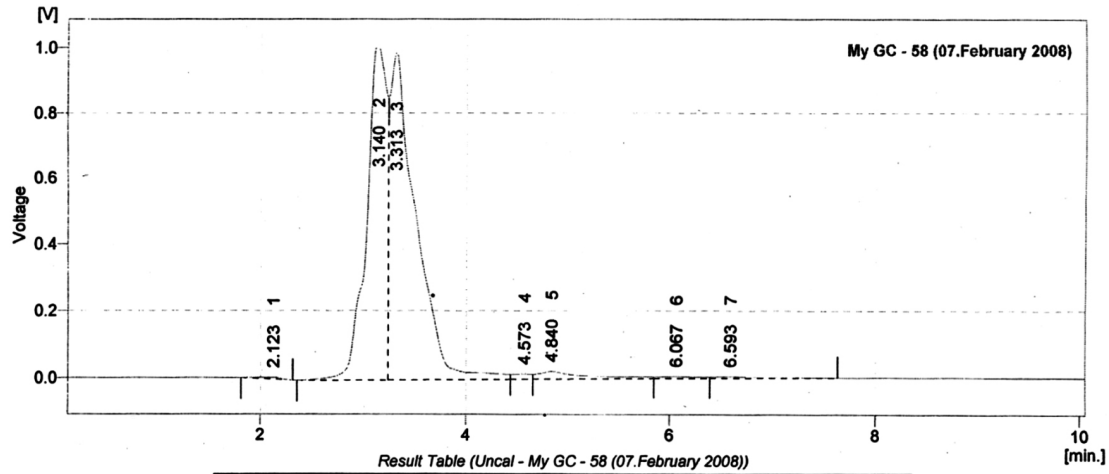
actinomycetes are sampled and screened. In this context, the present study was an attempt to identify and pick-out versatile strains of *Streptomyces* from the regions of the West Coastal area, India that display antimicrobial activity against a variety of microbial pathogens intrinsically. Such attempts need to be continued both in the same area as well as from the adjoining places during various climatic conditions as to screen more isolates for novel therapeutics.



Result Table (Uncal - My GC - 55 (07.February 2008))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.137	283.865	7.450	1.2	0.7	1.69
2	3.263	21579.496	759.120	87.8	73.8	0.48
3	3.717	2485.017	244.988	10.1	23.8	0.17
4	4.533	18.838	2.699	0.1	0.3	0.12
5	4.843	160.788	10.961	0.7	1.1	0.22
6	6.053	17.395	1.651	0.1	0.2	0.15
7	6.583	19.920	1.967	0.1	0.2	0.15
	Total	24565.319	1028.836	100.0	100.0	

Fig. 4. Chromatogram of antimicrobial compound of RM17



Result Table (Uncal - My GC - 58 (07.February 2008))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.123	91.497	6.136	0.3	0.3	0.39
2	3.140	13477.954	1005.855	41.7	49.1	0.20
3	3.313	17465.406	989.749	54.1	48.3	0.27
4	4.573	197.926	15.212	0.6	0.7	0.22
5	4.840	756.535	22.367	2.3	1.1	0.45
6	6.067	154.307	6.003	0.5	0.3	0.55
7	6.593	139.552	4.732	0.4	0.2	0.52
	Total	32283.176	2050.054	100.0	100.0	

Fig. 5. Chromatogram of antimicrobial compound of RM42

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IZOLACIJA I KARAKTERIZACIJA POMORSKIH ANATAGONISTIČKIH AKTINOMICETA SA ZAPADNE OBALE INDIJE

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Kratak sadržaj: *Izolovano je ukupno 173 kolonija aktinomiceta sa obližnje morske obale i ekosistema mangrova na 8 različitih lokcija Kerale, na zapadnoj obali Indije. Medju njima, 64 izolata se morfološki razlikovalo prema boji spora, boji naličja, formaciji vazdušnih i micelija podloge i produkciji difuznog pigmenta. Većina (47%; n=30) izolata je pripisano rodu Streptomyces. Antimikrobna aktivnost izolata je takodje testirana na razne bakterijske i gljivične patogene. Od 64 izolata, 21 je pokazao antimikrobnu aktivnost, od kojih su 2 izolata pokazala širok spektar antimikrobnih efekata.*

Ključne reči: *Raznolikost aktinomiceta iz zemlje, zapadna obala Indije, antimikrobna aktivnost, HPLC*