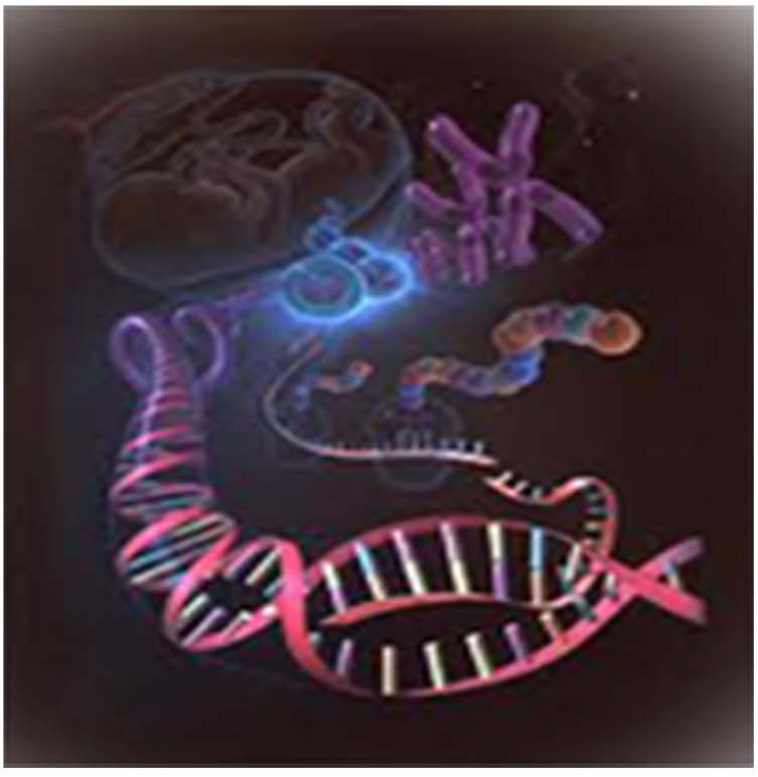
IJLBPR

ISSN 2250 - 3137
Vo1. 2, No. 3, July 2013

International Journal of

Life Sciences Biotechnology and Pharma Research



International Journal of Life Sciences Biotechnology and Pharma Research
Hyderabad, INDIA

www.ijlbpr.com

Email : editorijlbpr@gmail.com or editor@ijlbpr.com

International Journal of Life Sciences Biotechnology and Pharma Research

ISSN 2250-3137 www.ijlbpr.com
Vol. 2, No. 3, July 2013

Research Paper

ISOLATION AND SCREENING OF ACTINOMYCETES FROM MARINE SEDIMENTS FOR THEIR POTENTIAL TO PRODUCE ANTIMICROBIALS

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Actinomycetes are known to produce potential secondary metabolites which are having biological activity. Studies were conducted to isolate and identify the biologically potential marine actinomycetes and also these studies confirm that the isolates have enormous potential for the production of antimicrobial substances. Marine sediments were collected from 28 points in the Bay of Bengal and totally 52 actinomycetes were isolated using different isolation media. All the above isolates were characterized and identified by microscopical and macroscopical observations. Identification of the isolates revealed that all isolates belong to the genus *Streptomyces*. The isolated marine actinomycetes were screened for their antimicrobial activity against the human bacterial pathogens *Salmonella typhi, Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Klebsiella pneumonia* and fungal phytopathogens *Rhizoctonia solani, Macrophomina phaseolina, Fusarium udum* and *Fusarium oxysporum f.* sp. *lycopersici*. Among 52 isolates, 43 showed antibacterial activity and 20 isolates showed antifungal activity. The marine isolate *Streptomyces* sp. LCJ85 was found to be more efficient in the production of secondary metabolites. Also further attention has been paid to study their antimicrobial properties and their other biologically useful properties.

Keywords: Marine actinomycetes, *Streptomyces*, Antibacterial, Antifungal, Starch casein agar

INTRODUCTION

Actinomycetes are Gram-positive bacteria with high G+C content. Actinomycetes play an important role in recycling wastes in the environment and they are also the producers of thousands of metabolic products which exhibit biological activity. After the discovery of the broad spectrum antibiotic Streptomycin by Waksman and Schatz, more attention was paid towards the actinomycetes for isolation of many more antibiotics. Actinomycetes have been exploited successfully for their biologically potential secondary metabolites. They produce diverse group of antimicrobial metabolites notably glycopeptides, beta-lactams, aminoglycosides,

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polyenes, polyketides, macrolides, actinomycins and tetracyclins.

Among the genus of Actinomycetes group, *Streptomyces* is the major and more than 500 species of this genus have been reported by Euzeby (2008). Almost two third of the naturally occurring antibiotics are produced by *Streptomycetes*. The *Streptomyces* species isolated from mangrove environment showed divergent in their phylogenetic analysis and possessed good antibacterial and antifungal activities (Rajesh Kannan and Prakash Vincent, 2011). The Everolimus is a derivative of rapamycin, originally produced by the actinomycete *Streptomyces hygroscopoicus*, is an orally active metabolite which inhibits p70 S6 kinase activity and thus exhibiting immunosuppressive effect (Chapman and Perry, 2004). *Streptomyces rochei* (MTCC 10109) isolated from Visakhapatnam coast showed good antagonistic activities against the human microbial pathogens (Reddy *et al*., 2011). *Streptomyces roseosporus* produces a cyclic lipopeptide, Daptomycin which possess good antibacterial activity and this has been approved for the treatment of complicated skin infections. This metabolite binds to the cell membrane of bacteria and disrupts its membrane potential (Fenton *et al*., 2004).

Marine actinomycetes are a potential source of novel compounds as the environmental conditions of the sea are entirely different from the terrestrial conditions (Meiying and Zhicheng, 1998). Many researchers have isolated novel antibiotics from the marine environment (Sujatha *et al*., 2005; Biabani *et al*., 1997; Maskey *et al*., 2003; Charan *et al*., 2004; and Li *et al*., 2005). The marine actinomycetes produce variety of enzyme inhibitors, antibiotics and anticancer compounds. The marine actinomycetes are the good source of enzyme inhibitors (Imade, 2005). Some of the novel secondary metabolites from marine actinomycetes have been isolated recently include Abyssomicin C, from *Verrucosispora* sp., a secondary metabolite with potent inhibitory action on para aminobenzoic acid synthesis (Riedlinger *et al*., 2004). Salinosporamide A, an anticancer compound from *Salinispora* sp. (Fehling *et al*., 2003) and a novel marinopyrroles from *Streptomyces* sp. (Hughes *et al*., 2008) have been isolated.

Sea water dependent actinomycete *Salinispora* sp. (Maldonado *et al*., 2005) proved that the marine environment is still an untapped source of diverse group of actinomycetes with unique biological functions. However, the reports on biodiversity of actinomycetes are very limited.

The Bay of Bengal an unexploited ecosystem has been recently identified as a source of novel and biologically potent marine derived compounds by several researchers. According to Peela *et al*., (2005) numerous marine actinomycetes have been isolated from the samples collected from Andaman coast in Bay of Bengal and the majority of isolates belong to the genus *Streptomyces* with antibacterial and antifungal activity. In the southern coast of Bangladesh, a novel strain *Nonomuraea* has been isolated by the Japanese researchers (Ara *et al*., 2007). Therefore the present work was focused on the isolation of actinomycetes from the sediment samples of Bay of Bengal and their screening for their ability to produce antimicrobials.

MATERIALS AND METHODS

Sample Collection

Marine sediments were collected from Bay of Bengal, India during one of the Cruise program

with help of National Institute of Ocean Technology, Chennai. The sampling area covers from offshore Covelong to offshore Nagapattinam in Bay of Bengal. The collected sediments samples are kept in sterile polypropylene bags and preserved in the laboratory for further studies.

Isolation of Marine Actinomycetes

Isolation and enumeration of actinomycetes were done by the serial dilution and pour plate technique (Vimal *et al*., 2009). The serial dilution was done by taking one gram of sediment sample and mixed with 10 mL sterile distilled water in a test tube and agitated for 10 min. The suspension was serially diluted by transferring 1 mL aliquots to a series of test tubes; each containing 9 mL sterile distilled water and from respective dilution 1 mL sample was transferred to the sterile petriplates and the molten agar media was poured on to it and allowed to settle. Different media namely Starch Casein Agar (SCA), Starch Nitrate Agar (SNA), Glycerol Asparagine Agar (GAA) and CSPY-ME agar were used for the isolation of actinomycetes from marine samples. The culture media was prepared and sterilized at 121 °C in 15 lbs pressure. The isolation media was supplemented with the antibiotics cycloheximide (25 mg/mL) and nalidixic acid (25 mg/mL) (Kumar and Kannabiran, 2010). The plates were incubated at 28 ± 1 °C for 7 to 21 days. The colonies were identified by their cultural characteristics.

Identification of Marine Actinomycetes

Light microscopical studies were carried out by cover slip culture technique and the characters such as aerial mycelia, spores arrangement are observed under microscope. The cover slip culture technique was done by inserting the sterile coverslip at an angle of 45° in to solidified SCA in Petriplates. A loopful of inoculum of each marine isolate was streaked along the line, where the coverslip meets the agar and the plates were incubated at room temperature. The isolated marine actinomycetes were also cultured on Starch casein agar and their morphological characters were also observed.

Screening of Marine Actinomycetes

The isolated marine actinomycetes were screened for their antimicrobial activity by Cross streak method against the human bacterial pathogens and fungal phytopathogens by antagonistic method. The actinomycetes were streaked on one corner of the Starch Casein Nutrient agar (SCNA) and the bacterial pathogens were streaked perpendicular near to it and incubated at room temperature for 24 h. For the antifungal screening, the actinomycetes cultures were streaked on the sides of the Starch Casein Potato Dextrose Agar (SCPDA) and the mycelia agar block of the phyto-pathogens were kept in the middle of the plates and incubated at room temperature for 2 to 7 days.

The secondary screening of the selected marine isolates was done by agar well diffusion assay. The selected isolates were grown in Soybean Mannitol (SM) medium and incubated on a shaker for 7 days at room temperature. The cultures were filtered and the culture filtrate was used for assay. It was extracted with equal volume of ethyl acetate and condensed. The crude extract was analyzed for the presence of efficient biologically useful metabolites by agar well diffusion assay against the human bacterial pathogens. All the bacterial pathogens were grown in sterile Muller-Hinton broth and incubated at 37 °C ±1 °C for 8 h. After incubation, the cultures were swabbed on sterile nutrient agar plates using a sterile cotton swab. Wells were cut on

the agar plates with a sterile cork borer (9 mm diameter) and the fractions were added into the wells. Wells loaded with Streptomycin served as a positive control whereas wells loaded with ethyl acetate served as negative control. All the plates were incubated at 37 °C and zone of inhibition was measured after 24 h of incubation. The screening was done with five bacterial pathogens *Salmonella typhi, Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Klebsiella pneumonia.* The fungal phyto-pathogens include *Rhizoctonia soloni, Macrophomina phaseolina, Fusarium udum* and *Fusarium oxysporum f.* sp. *lycopersici*. All the cultures were obtained from CAS in Botany, University of Madras.

RESULTS

Isolation of Marine Actinomycetes

A total of 52 marine actinomycetes were isolated from the 28 sediment samples collected from Bay of Bengal (Table 1). In the isolation plates, only the *Streptomycetes* spp. colonies were picked up and subcultured. All 52 marine actinomycetes were subcultured in pure form and maintained as slant culture for further use. Among the different media used only the Starch casein agar proved to be best for the isolation of actinomycetes from marine sediments (Figure 1). Grey colored mycelial cultures were the predominant among the isolates obtained (Figure 2). The cultures were coded with three letter prefix and Arabic numerals- LCJ81 to LCJ132.

IDENTIFICATION OF MARINE ACTINOMYCETES

All the 52 marine actinomycetes were identified up to genus level. The light microscopical studies and the growth characteristics on starch casein agar medium revealed that all the isolates possess characters which are similar to the genus *Streptomyces* and illustrated in Table 2.

Screening of the Isolates for Antimicrobial Properties:

Among the 52 isolates of marine actinomycetes, about 43 marine actinomycetes showed antibacterial activity and the remaining 9 had no activity. Of these, 30 actinomycetes were active against *Escherchia coli*, 35 against *Salmonella typhi*, 12 against *Bacillus cereus* and 8 against *Klebsiella pneumonia* (Figure 3). The activity against fungal phytopathogens showed that a total of 20 marine actinomycetes have antifungal activity out of 52 marine actinomycetes isolated. Among these 2 isolates were active against *Rhizoctonia solani*, 12 isolates against *Fusarium udum*, 8 isolates against *Fusarium oxysporum* f. sp. *lycopersici*, 10 isolates against *Macrophomina phaseolina* and 32 isolates showed no activity against the fungal phytopathogens (Figure 4). From the primary screening, about 10 actinomycetes were selected based on their efficiency. In the secondary screening, all the 10 actinomycete isolates shows activity in well diffusion assay. However the crude extract of the marine actinomycete isolate MMA85 exhibited prominent activity with large area of zone of inhibition against all the pathogens used, when compared to the extracts from other isolates (Table 3).

DISCUSSION

Marine actinomycetes are potential producers of useful secondary metabolites with biological activity. Though many researchers working on actinomycetes in India the studies on marine actinomycetes is very limited. The sediment samples were collected at the Bay of Bengal from

Table 1: Sampling Sites in the Bay of Bengal

Sample No.	Sampling Area	Offshore Distance (km)	Depth(m)	Latitude	Longitude
1	Covelong	20	65	12°.47.09' N	080°.26.78'E
2	Mahabalipuram	20	53.5	12°.35.80'N	080°.22.80'E
3	Palar	20	47	12°.25.94' N	080°.19.12'E
4	Puducheri	20	200	11°.56.06' N	080°.05.96'E
5	Ponnaiyar	20	48	11°.43.50' N	079°.57.80'E
6	Ponnaiyar	10	23.5	11°.45.15' N	079°.82.23'E
7	Ponnaiyar	5	17.4	11°.45.53' N	079°.50.41'E
8	Ponnaiyar	1	11.5	11°.46.08' N	079°.48.76'E
9	Portonovo	1	7	11°.30.80' N	079°.47.20'E
10	Portonovo	5	17.2	11°.31.35' N	079°.49.04'E
11	Uppanar	10	26.4	11°.32.23' N	079°.51.22'E
12	Uppanar	20	73	11°.34.65' N	079°.56.02'E
13	Coleroon river	20	250	11°.36.72' N	079°.56.65'E
14	Pudumaniyar	20	300	11°.24.31' N	079°.59.95'E
15	Pudumaniyar	10	87	11°.11.82' N	080°.01.82'E
16	Pudumaniyar	5	23.3	11°.08.37' N	079°.56.10'E
17	Kaveripoompattinam	20	17.5	11°.08.20' N	079°.54.14'E
18	Kaveripoompattinam	10	6	11°.08.10' N	079°.51.79'E
19	Kaveripoompattinam	5	8	11°.06.77' N	079°.52.11'E
20	Karaikal (Sevenar)	5	8	10°.54.90' N	079°.51.70'E
21	Nagapattinam	20	249	10°.45.80' N	080°.01.54'E
22	Nagapattinam	10	83	11°.04.14' N	080°.20.58'E
23	Nagapattinam	10	68	10°.50.49' N	080°.12.38'E
24	Nagapattinam (water)	20	823	11°.04.52' N	080°.21.06'E
25	Caveri	20	150	11°.11.16' N	080°.02.81'E
26	Caveri	20	250	11°.21.50' N	080°.03.11'E
27	Coleroon River	10	42	11°.21.87' N	079°.54.81'E
28	Coleroon River	5	29	11°.21.95' N	079°.52.89'E

offshore Covelong to offshore Nagapattinam lead to give the picture of biodiversity on marine actinomycetes. The selective screening of marine sediment samples resulted in the isolation of 52 marine actinomycetes. Kumar and Kannabiran (2010) used different media for isolation of marine actinomycetes and among the three different media, the Starch Casein Agar (SCA) was proved

Figure 1: Preference Of Media For Isolation Of Marine Actinomycetes

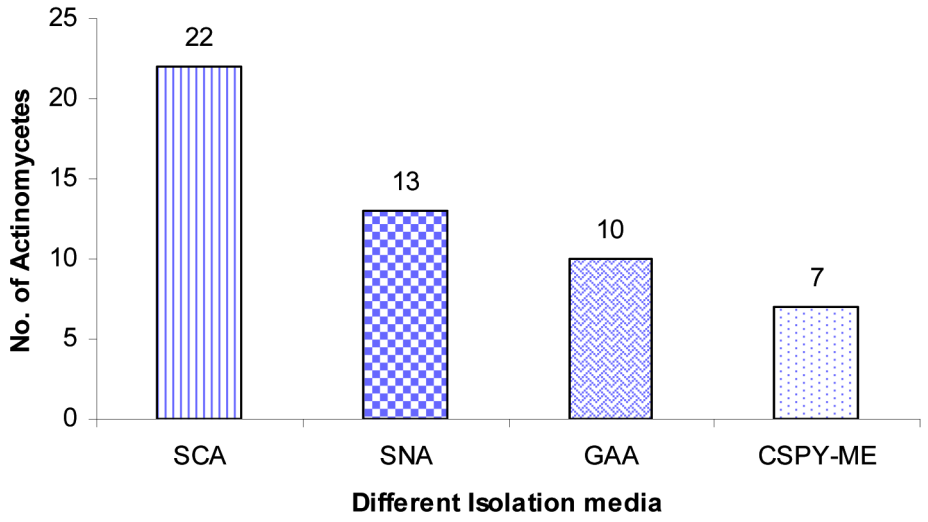
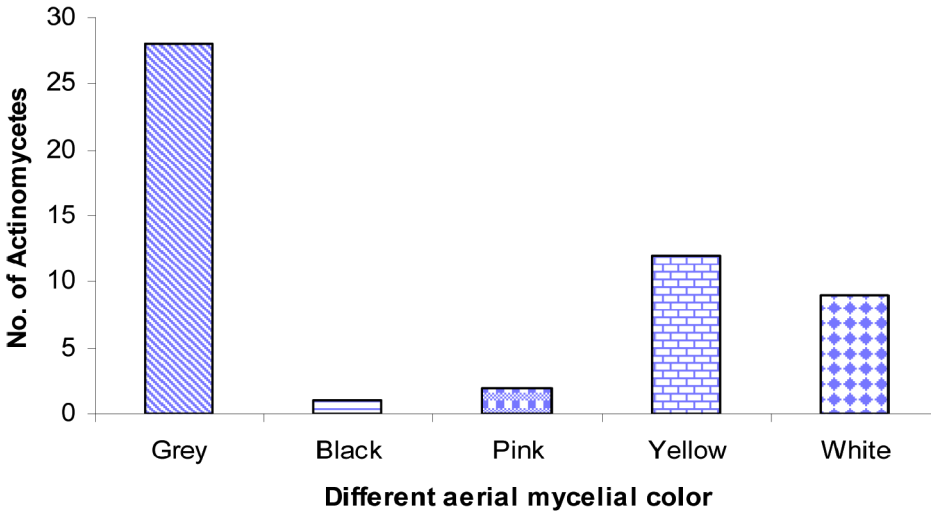


Figure 2: Aerial Mycelial Color Of The Isolates Grown On SCA Medium



to be effective for isolation. Totally 4 different media were used in this study for the isolation of actinomycetes, among them the Starch casein agar proved to be very effective in isolation of marine actinomycetes when compared to other media, *viz*., Starch nitrate agar, Glycerol asparagines agar and CSPY-ME agar. About 22 actinomycetes were isolated using SCA, 13 using SCA, 10 using Glycerol asparagines agar and 7 isolates using CSPY-ME medium. Among various colonies appeared only the *Streptomycetes* type of colonies were picked and purified by repeated subcultures. The light microscopical studies revealed that all the marine isolates have spore bearing hyphae similar to the *Streptomyces* genus. The marine isolates possess simple

Table 2: Characteristics of Marine Actinomycetes Isolated From the Bay of Bengal

Strains	Cultural Characteristics			Micro Morphology		
	AMC	RSP	MP	SM	AM	SCM
LCJ81	Greyish white	-	-	+	+	RF
LCJ82	Grey	-	-	+	+	RF
LCJ83	Grey	-	-	+	+	RF
LCJ84	Black	+	+	+	+	RA
LCJ85	Pink	-	-	+	+	RA
LCJ86	Yellow	+	+	+	+	RA
LCJ87	White	-	-	+	+	S
LCJ88	Yellow	-	-	+	+	S
LCJ89	Yellow	-	-	+	+	S
LCJ90	Yellow	-	-	+	+	RA
LCJ91	Yellow	-	-	+	+	RA
LCJ92	Yellow	-	-	+	+	RA
LCJ93	Grey	+	-	+	+	S
LCJ94	Yellow	-	-	+	+	RA
LCJ95	Yellow	-	-	+	+	RA
LCJ96	Grey	-	-	+	+	RA
LCJ97	Grey	-	-	+	+	RA
LCJ98	White	-	-	+	+	RA
LCJ99	Grey	-	-	+	+	RA
LCJ100	Grey	-	-	+	+	RF
LCJ101	White	-	-	+	+	RA
LCJ102	Grey	+	-	+	+	RA
LCJ103	Grey	-	-	+	+	RF
LCJ104	White	-	-	+	+	RF
LCJ105	Grey	-	-	+	+	RF
LCJ106	White	-	-	+	+	RA
LCJ107	Grey	-	-	+	+	RF
LCJ108	Grey	-	-	+	+	RA
LCJ109	White	-	-	+	+	RF

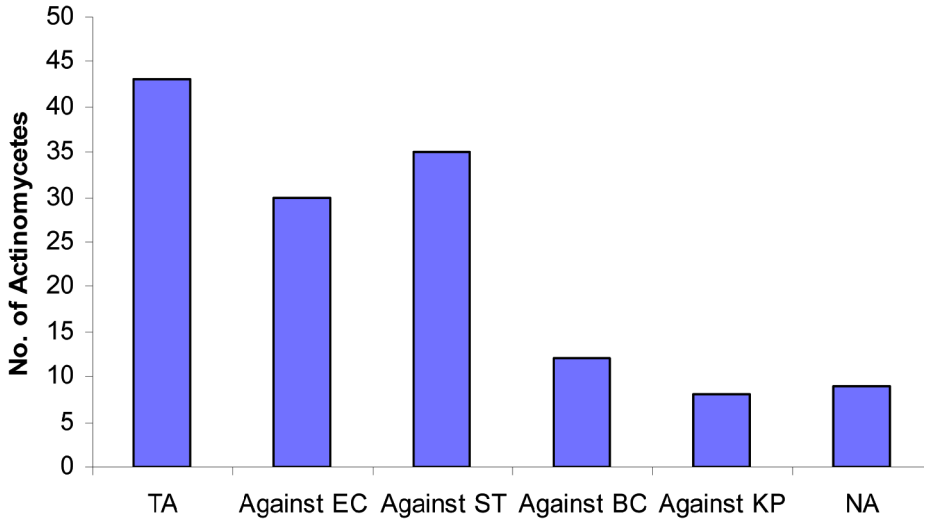
Table 2 (Cont.)

Strains	Cultural Characteristics			Micro Morphology		
	AMC	RSP	MP	SM	AM	SCM
LCJ110	Grey	-	-	+	+	RA
LCJ111	Pink	-	-	+	+	RF
LCJ112	Grey	-	-	+	+	RA
LCJ113	Grey	-	-	+	+	RF
LCJ114	Yellow	-	-	+	+	RF
LCJ115	Grey	-	-	+	+	RA
LCJ116	Grey	+	-	+	+	RF
LCJ117	Grey	-	-	+	+	RF
LCJ118	Yellow	-	-	+	+	S
LCJ119	Yellow	-	-	+	+	RA
LCJ120	Grey	-	-	+	+	RF
LCJ121	Grey	-	-	+	+	RF
LCJ122	White	-	-	+	+	RF
LCJ123	White	-	-	+	+	RA
LCJ124	Yellow	-	-	+	+	RA
LCJ125	Grey	-	-	+	+	RF
LCJ126	Grey	-	-	+	+	RA
LCJ127	Grey	-	-	+	+	RA
LCJ128	Grey	-	-	+	+	RA
LCJ129	White	-	-	+	+	S
LCJ130	Grey	+	-	+	+	RA
LCJ131	Grey	-	-	+	+	RA
LCJ132	Grey	-	-	+	+	RA

Note: AMC – Aerial Mycelial Color, RSP – Reverse side Pigment, SM- Substrate Mycelium, AM- Aerial Mycelium, SCM- Spore chain morphology, RA- retinaculum apertum, RF- Reticulum flexible, S- spiral

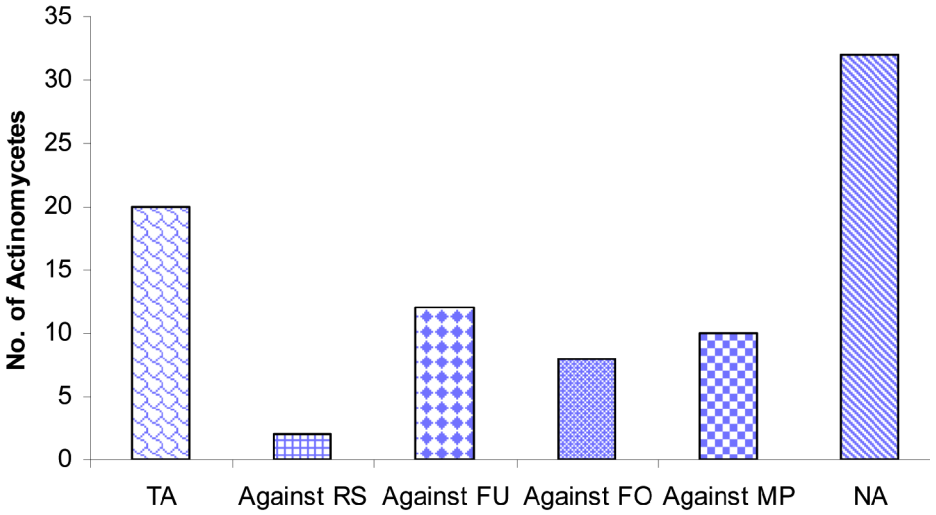
spirals, simple flexible and simple reticulum apertum type of spore chains which were early reported by Shirling and Gottlieb in 1996. The cultures grew well in the starch casein agar and produced the aerial mycelium and substrate mycelium in the medium. In the present study the marine actinomycetes were screened for their antimicrobial potential by cross streak method and antagonistic method. In the cross streak method, among the 52 isolates, 43 isolates possess

Figure 3: Inhibitory Effect Of Culture Filtrates From The Marine Actinomycetes Against Human Pathogenic Bacteria



Note: TA- Total Activity, EC- *Escherichia coli*, ST- *Salmonella typhi*, BC- *Bacillus cereus*, KP- *Klebsiella pneumonia*, NA- No Activity

Figure 4: Inhibitory Effects Of The Marine Actinomycetes Against Various Phytopathogenic Fungi



Note: RS - *Rhizoctonia solani*, FU - *Fusarium udum*, FO - *Fusarium oxysporum* f.sp.lycopersici, MP - *Macrophomina phaseolina.*

antibacterial activity of which 30 isolates shows activity against *Escherichia coli*, 35 isolates against *Salmonella typhi*, 12 isolates against *Bacillus cereus* and 8 isolates against *Klebsiella pneumonia* and 9 isolates showed no activity against any human bacterial pathogens. Similarly Sathiyaseelan and Stella (2011) isolated five marine actinomyctes and among them one isolate active against the human pathogens *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi* and

Table 3. Secondary Screening Of Selected Marine Actinomyctes Against The Human Pathogenic Bacteria

Marine isolates	Human Pathogenic bacteria			
	E.coli	*S.typhi*	*B.cereus*	*S.aureus*
MMA85	30	24	30	31
MMA87	27	15	-	17
MMA90	23	19	-	24
MMA94	14	27	16	29
MMA103	17	29	-	18
MMA108	27	32	13	21
MMA113	19	34	21	27
MMA120	-	-	20	20
MMA124	-	30	13	12
MMA130	24	20	12	18
Streptomycin (Positive control)	34	28	31	34

Vibrio cholera. Shantikumar singh *et al.* (2006) reported 37 actinomycetes from lake sediments, out of them 21 isolates exhibited antimicrobial activity especially 12 active isolates exhibited good antifungal activity. Among 52 isolates performed for antifungal activity test, a total of 20 isolates possess antifungal activity, of which 2, 12, 8 and 10 are active against the *Rhizoctonia solani*, *Fusarium udum*, *Fusarium oxysporum f.* sp. *lycopersici* and *Macrophomina phaseolina* respectively whereas 32 isolates showed no activity. Kathiresan *et al*. (2005) isolated 160 marine actinomycetes and 31% of them are proved to be potential against *Rhizoctonia solani*. The agar well diffusion assay reveal that the marine actinomycete *Streptomyces* sp MMA85 is effective against bacterial pathogens and was found to be a potential strain. Thus the present work reveals that certain marine actinomycetes from the Bay of Bengal may be a potent source of novel antimicrobial compounds.

CONCLUSION

This study can be concluded that the Bay of Bengal may harbor rich source of biologically active actinomycetes which has the capacity to produce novel and effective secondary metabolites with antimicrobial functions.

ACKNOWLEDGMENT

We thank Prof. R. Rengasamy, Director, CAS in Botany, University of Madras for his constant support in the research work. We like to thank National Institute of Ocean Technology, Pallikaranai, Chennai for the Cruise program and sample collections. We also thank Sibi Narayanan and Sugumar paramasivam, CAS in Crystallography and Biophysics for their valuable suggestions.

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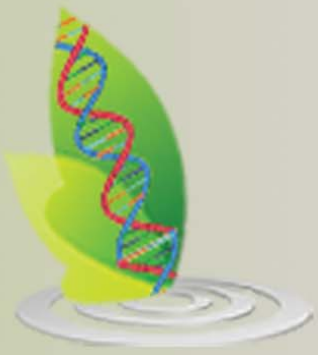

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Hyderabad, INDIA. Ph: +91-09441351700, 09059645577
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ISSN 2250-3137