Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 8, 2014

Original Article

STUDY ON ANTIMYCOBACTERIAL ACTIVITY OF MARINE ACTINOMYCETES FROM COROMONDAL COASTAL REGION OF SOUTHERN INDIA

P. GAJALAKSHMI[1], A. RAJA[2], R. ARTHUR JAMES[3]

[1]Department of Microbiology, Dhanalakshmi Srinivasan College of Arts and Science for Women, Perambalur 621212, Tamil Nadu, India, [2]Department of Microbiology, Jamal Mohamed college, Tiruchirapalli, Tamilnadu,India, [3]Department of Marine Science, Bharathidasan University, Tiruchirapalli 620024, India.
Email: gaja_black@yahoo.co.in, rajajmcmicro@yahoo.co.in

Received: 19 Jun 2014 Revised and Accepted: 21 Jul 2014

ABSTRACT

Objective: The objective of the present study is to screen the novel actinomycetes from marine sediment active against mycobacterium sp.

Methods: actinomycetes were isolated by crowded plate technique and its antimycobacterial activity was determined by stroke method followed by optimized fermentation. The active fraction was extracted and bio assayed by TLC.

Results: Totally 25 actinomycetes were isolated from 9 Marine sediment samples and frequency of isolates were, 40% of *Streptomyces sp* (ECRST) 32% of *Micromonospora(ECRMM)*, 16% of *Micropolyspora* (ECRMP) and 12% were *Streptoverticillium* (ECRSV). Among the 25 isolates *Microployspora* a rare actinobacteria designated as ECRMP 4 showed potent antimycobacterial activity against *M.tuberculosis.* The zone of inhibition was 20 ± 0.1mm and the percentage of relative inhibitory zone was 62 against *M.tuberculosis*. The bioassay of crude compound prevails that the R_f value of compound is 0.16.

Conclusion: The present study concludes that the isolated marine *Micropolyspora* capable to produce noval antimycobacterial compound effective against *M.tuberculosis.*

Keywords: Micropolyspora, *M.smegmatis,M.tuberculosis,* Relative zone of inhibition.

INTRODUCTION

Multi drug resistant tuberculosis strains are generally considered to be those resistant to at least two drugs, such as INH and Rifampicin most frequently in the countries of the former Soviet Union and in Asia. The current prophylaxis for TB is very long and results in significant toxicity, development of resistant strains and is unable to eliminate the latent bacilli [1]**.** On this view point, attempts have been made to develop new drug against MDR – TB. Marine environment covers almost 70% of the earth surface [2].

Isolation of rare actinomycetes form marine sediment targeted in drug discovery due to its diverse nature of biomolecules. The ocean remains as an unexploited source for many drugs and pharmacologically active substances [3].The role of marine actinomycetes in drug discovery dramatically increased from the past few years due to substantial improvement in screening methods. Actinobacteria are gram positive, filamentous bacteria which are supreme secondary metabolite producers [4] which shows a range of biological activities including antibacterial, antifungal, anticancer, antitumor, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malaria, antiviral, antioxidant, anti-angiogenesis, etc. Of them, many have been obtained from Streptomyces [5] and these natural products have been an extraordinary source for lead structures in the development of new drugs [6]. It is apparent that the marine environment is a potent source for finding new actinobacteria and biologically active substances [7].

The present work was undertaken to isolate potent actinobacteria from marine sample and to assess their antimycobacterial activity.

MATERIALS AND METHODS

Sample collection

Marine sediment samples were collected from east coast region starting from Pondicherry to Mahabalipuram at 10intches depth by hand core sampler in a sterilized container and transferred to the laboratory for further processing.

Isolation & identification of actinobacteria [8]

Isolation and enumeration of actinobacteria was performed on selective media, Actinomycetes Isolation Agar (AIA). The samples were pretreated with 0.1% phenol and after serial dilution one milliliter of 10^7 dilution samples were poured and overlayerd with sterile AIA supplemented with Amphotericin B (10 µg/ml). Plates were incubated at room temperature for 7days. The isolated actinobacteria are characterized by spore and biochemical method and the results were compared with Bergey's manual of Systemic Bacteriology.

Test organism

The test organisms *Mycobacterium smegmatis* MTCC NO-6 and *Mycobacterium tuberculosis* were collected from Bioline laboratory and maintained in Dubos oleic agar at -2^{0}c.

Fermentation process [9]

All the actinobacterial isolates were inoculated into one litre production broth (ISP4 Media) in a bio reactor and fermentation was carried out at 200rpm at 28^{0}c for 7 days. After fermentation, the medium was harvested and centrifuged to remove cell debris. Filtrates were collected and lyophilized at 4^{0}C for further use.

Primary antimicrobial screening [10-11]

Mycobacterium smegmatis was inoculated on dubos plates. In each of these plates, wells were cut out using a sterilized gel borer. 100 µl of lyophilized filtrates were loaded into each well. Plates were incubated at 35+/- 2^{0}c for 3 days. After incubation, all the plates were examined for the presence of zone of inhibition around the Wells.

Extraction of bioactive metabolites [12 - 13]

The bioactive metabolites were collected from the harvested medium by solvent extraction method. The filtrate was mixed with ethyl acetate and shaken vigorously for 1 hour in a solvent extraction funnel. Solvent and filtrate mixture were stabilized for

24-48 hrs. After 48 hrs the solvent phase was separated from aqueous phase. The solvent extracts were concentrated and used for antibacterial activity.

Secondary screening (Disc diffusion method)

Secondary antimicrobial screening of actinobacteria was detected by disc diffusion method on dubos medium. The crude extract was re dissolved in distilled water at 1mg/ml concentration and about 25µg, 50µg and100µg of samples were loaded on the sterile disc and its activity was checked against *Mycobacterium tuberculosis* by stroke method. The percentage of relative zone of inhibition was calculated as follows

$$\text{RIZD} = \frac{\text{Sample zone} - \text{Negative control zone}}{\text{Postive control zone(streptomycin)}} \times 100$$

Thin layer chromatography [14]

10 µl of the ethyl acetate fractions were applied on Silica gel plates and the chromatogram was developed using chloroform: methanol (4:1) as solvent. The spots in the chromatogram were visualized in UV chamber and it was bio assayed. All the tests were conducted in triplicate.

RESULTS AND DISCUSSION

A total 25 actinobacteria colonies (Table-1) was isolated from nine Marine sediment samples and identified based on spore morphology. Of these nine samples, maximum number of isolates was obtained at Auroville site. Among the 25 isolates, *Strptomyces* was the most frequently isolated species.

Based on colony morphology and microscopic appearance the isolated strains were designated as ECRST (*Streptoyces*), ECRMM (*Micromonospora*), ECRMP (*Micropolyspora*), ECRSV (*Strepto verticillium)*. The frequency of isolates were Streptomyces (40%), *Micromonospora* (32%), *Micropolyspora(16%)* and Strptoverticilium (12%). marine sediments were good sources for isolation of actinobacteria and M2 media is the most suitable agent for the purpose [15].

Colonies were 3-4 mm in diameter, initially had a smooth appearance but later developed a weft of aerial mycelium which is floccose, granular, and powdery in nature. The aerial and substrate mycelium are non fragmented (except ECRMP isolate) and showed the production of conidiospores. Monospores and arthrospore on the mycelium.

Table 1: Number of isolated actinomycetes and its sampling site

Sample site	Offshore distance in km	Lattitide	Longitude	Depth (intches)	Number of isolates
Puducherry beach	10	11.9300° N	79.8300° E	10	4
auroville	10	12.0069° N	79.8106° E	10	5
Anichakuppam	10	11.890° N	79.8147° E	10	2
pudukuppam	10	12.053276° N	79.873057	10	2
marakkanam	10	12.187655° N	79.937655 ° E	10	3
vennangupattu	10	12.237715° N	79.96482 ° E	10	3
Ammanur	10	12.3648° N,	80.0122	10	2
Kalpakkam	10	12.5576° N,	80.1754° E	10	2
Mahabalipuram	10	12.357655° N	80.127655 ° E	10	2
Total					25

Table 2: Colony and Spore Morphology of isolated actinomycetes

Site	CFU	Strain Code	Colony Colour	Spore
Puducherry Beach	$14x10^7$	ECRST1	Grey, powdery	rectiflexibiles
		ECRMM1	White, granular	Monospore
		ECRMM2	Ash, granular	Monospore
		ECRST2	Dark Grey, powdery	retinaculiaperti
Anichakuppam	$8 x10^7$	ECRST3	Greyish white powdery	rectiflexibiles
		ECRST4	white,powdery	rectiflexibiles
Auroville	$16 x10^7$	ECRST5	Chalky White, powdery	rectiflexibiles
		ECRST6	Light Ash, fine powdery	retinaculiaperti
		ECRMP1	Dark grey, leathery	Spiral
		ECRSV1	Ash, powdery	Ellipsoidal
		ECRMM3	Chalky white, powdery	Monospore
Vennangupattu	$6 x10^7$	ECRST7	Ash, powdery	retinaculiaperti
		ECRST8	White, powdery	Spiral
		ECRMM4	Ash granular	Monospore
Pudukuppam	$6 x10^7$	ECRST9	Sandal white,powdery	Spiral
		ECRMM5	Grey, granular	Monospore
Ammanur	$5 x10^7$	ECRMM6	Ash, powdery	Monospore
		ECRMP2	Dark grey, granular	Spiral
Marakkanam	$7 x10^7$	ECRMP3	Dark grey, granular	Spiral
		ECRMP4	Dark grey,granular	Spiral
		ECRSV2	Grey,leathery	Ellipsoidal
Kalpakkam	$8 x10^7$	ECRST10	Dull white powdery	Spiral
		ECRMM7	Dull white	Monospore
Mahabalipuram	$6 x10^7$	ECRSV3	Greeish Grey,leathery	Ellipsoidal
		ECRMM8	Ash,granular	Monospore

Among the Strptomyces isolate 70% of them produced spiral chains of spore and less often spirally coiled, retinaculiaperti and refractile features (table 2) on substrate mycelium. Strains of *Micromonospora* sp were gram positive, did not make aerial mycelium, did not produce sporangia, and produced nonmotile mono spores that were borne singly off branched hyphae. *Streptomyces* was described as Streptomycetaceae that formed spores in chains on aerial hyphae [16]. Micropolyspora produced chain of spore are differentiated

from *Streptomyces* by the fragmenting nature of the aerial mycelium [17]. Carbon and nitrogen utilization tests were performed according to standard methods described for actinomycetes [18]. Strains belongs to Strptomyces utilized L asparagines as nitrogen sources. Among the ten Strptomyces (ECRST1 to ECRST10) only two isolates designated as ECRST8 and ECRST9 failed to utilize carbon and nitrogen source. Similarly, isolates belongs to *Streptoverticiliun* (ECRSV) also failed to utilize L asparagine (table 3). In this screening, ISP4 medium was found to be the most significant medium for the growth and antibiotic production. The optimized growth condition was found to be 200 rpm at 28 ° C for 7 days on ISP4 broth (Fig 1).

Table 3: Utilization of carbon and nitrogen source by actinomycetes isolates

Strain Code	*Nitrogen*	Carbon				
		D-glucose	*L rhamnose*	*D-Xylose*	*D-Mannitol*	*L-arabinose*
ECRST1	+	+	+	+	+	+
ECRST2	+	+	+	+	+	+
ECRST3	+	+	+	+	+	+
ECRST4	+	+	+	+	+	+
ECRST5	+	+	+	+	+	+
ECRST6	+	+	+	+	+	+
ECRST7	+	+	+	+	+	+
ECRST8	-	+	+	-	-	-
ECRST9	-	+	+	-	-	-
ECRST10	+	+	+	+	+	+
ECRMM1	+	+	+	+	+	+
ECRMM2	+	+	+	+	+	+
ECRMM3	+	+	+	+	+	+
ECRMM4	+	+	+	+	+	+
ECRMM5	+	+	+	+	+	+
ECRMM6	+	+	+	+	+	-
ECRMM7	+	+	+	+	+	-
ECRMM8	+	+	+	+	+	-
ECRMP1	+	+	+	+	+	-
ECRMP2	+	+	+	+	+	+
ECRMP3	+	+	+	+	+	+
ECRMP4	+	+	+	+	+	+
ECRSV1	-	+	+	+	+	+
ECRSV2	-	+	+	+	+	+
ECRSV3	-	+	+	+	+	+

Table 4: Antimycobacterial Study of Isolated Actinomycetes against Mycobacterium sp

Strain Code	*M.smegmatis*	*Mycobacterium tuberculosis* (disc diffusion)			
		Sample	*PC*	*NC*	*RIZD*
ECRST1	-	-	-	-	-
ECRST2	-	-	-	-	-
ECRST3	-	-	-	-	-
ECRST4	*8 mm*	-	-	-	-
ECRST5	-	-	-	-	
ECRST6	-	-	-	-	-
ECRST7	-	-	-	-	-
ECRST8	-	-	-	-	-
ECRST9	-	-	-	-	-
ECRST10			-	-	-
ECRMM1	-	-	-	-	-
ECRMM2			-	-	-
ECRMM3	-	-	-	-	-
ECRMM4			-	-	-
ECRMM5	-	-	-	-	-
ECRMM6			-	-	-
ECRMM7	-	-	-	-	-
ECRMM8			-	-	-
ECRMP1	-	-	-	-	-
ECRMP2			-	-	-
ECRMP3	-	-	-	-	-
ECRMP4	18±0.68mm	20±0.1mm	16±0.2mm	8±0.24mm	62
ECRSV1	-	-	-	-	-
ECRSV2			-	-	-
ECRSV3	-	-	-	-	-

PC: Positive control Streptomycin, NC: ethyl acetate

In the antimycobacterial study of these 25 isolates, *Microployspora* (ECRMP 4) is a rare actinobacteria showed potent antimycobacterial activity against both Mycobacterium. The maximum inhibition zone was 20 ± 0.1mm against *M. tuberculosis* activity followed by 18 ± 0.68 mm zone of inhibition against *M. smegmatis* (table 4). Similarly the percentage of relative inhibition zone was 66% against

M.tuberculosis at 100µg. However the *Microployspora* (ECRMP 4) grown in ISP1 and ISP2 did not show any antimicrobial activity. Rare actinomycetes are become an increasingly attractive source in the search for new antibiotics tofight drug resistance [19]. From the discovery of streptomycin from *Streptomyces griseus,* numerous anti TB antibiotics such as kanamycin and rifampicin have been reported from actinomycetes isolated only from terrestrial origin but not from marine source [20]. The bioassay of TLC fraction prevails that the R_f value of active compound is 0.16. Consequently further investigations like IR, NMR and MS are needed in order to determine the active metabolite.

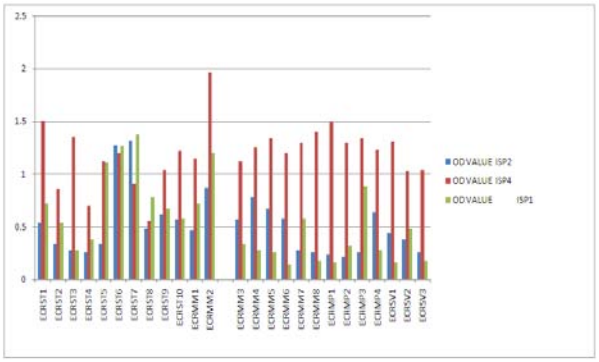

Fig. 1: Growth rate of isolated actinomycetes on different ISP medium at 200 rpm.

REFERENCES

1. Zhang Y. The magic bullets and tuberculosis drug targets. J Annu Rev Pharmacol Toxicol 2005;*45*:529.
2. Valli S, Sugasini S, Aysha OS, Nirmala P, Vinoth Kumar P, Reena A. Antimicrobial potential of Actinomycetes species isolated from marine environment. Asian Pac J Trop Biomed 2012;2:469-73.
3. Sivasubramanian K, Ravichandran S, Vijayapriya M. Antagonistic activity of marine bacteria *Pseudoalteromonas tunicata* against microbial pathogens. Afr J Microbiol Res 2011;*5*:562-67.
4. Olano C, Mendez C, Salas JA. Antitumor Compounds from Marine Actinomycetes. J Mar Drugs 2009;7:210-48.
5. Magee J. Whole-organism fingerprinting. In:Goodfellow M, O'Donnell AG. editors, Handbook of New Bacterial Systematics. J London Academic Press 1993, p. 3–54.
6. Sivakumar K, Sahu MK, Thangaradjou T, Kannan L. Research onmarine Actinobacteria in India. Indian J Microbiol 2007;47:186–96.
7. Gartner A, Ohlendorf B, Schulz D, Zinecker H, Wiese J, Imhoff JF. Levantilides A and B, 20-membered macrolides from a Micromonospora strain isolated from the mediterranean deep sea sediment. J Mar Drugs 2011;9:98-108.
8. Seong CH, Choi JH, Baik KS. An improved selective isolation of rare actinomycetes from forest soil. J Microbiol 2001;17:23-39.
9. Umasankar ME, Gaurav Kumar, Karthik L, Bhaskara Rao K.V Exploration of antagonistic actinobacteria from Amirthi forest. Int J Curr Pharm Res 2010;*2*:16-19.
10. Gaurav Kumar, Karthik L, Bhaskara Rao KV. In vitro anticandidaactivity of Calotropis gigantea against clinical isolates of candida. J Pharm Res 2010;3:539-42.
11. Kumar S, Kannabiran K. Antifungal activity of StreptomycesVITSVK5 sp. against drug resistant Aspergillus clinical isolatesfrom pulmonary tuberculosis patients. J de Mycologie Medicale 2010;20:101-7.
12. Atta HM, Ahmad MS. Antimycin-A A antibiotic biosynthesis produced by Streptomyces Sp. AZ-AR-262:taxonomy, fermentation, purification and biological activities. Aust J Basic Appl Sci 2009;3:126-35.
13. Manivasagan P, Gnanam S, Sivakumar K, Thangaradjou T, Vijayalakshmi S, Balasubramanian T. Antimicrobial and cytotoxicactivities of an actinobacteria (Streptomyces Sp. PM-32) isolated from offshore sediments of the Bay of Bengal in Tamilnadu. Advan Biol Res 2009;*3*:231-6.
14. Usha R, Ananthaselvi P, Venil CK, Palaniswamy M. Antimicrobial and antiangiogenesis activity of *Streptomyces parvulus* KUAP106 from mangrove soil. Eur J of Biol Sci 2010;2:77-83.
15. Karthik L, Gaurav Kumar, Bhaskara Rao KV. Diversity of marine actinomycetes from Nicobar marine sediments and its antifungal activity. Int J Pharm Pharm Sci 2010;1:199-203.
16. Kawato M, Shinobu R. A Simple Technique for the Microscopical Observation. Memoirs of the Osaka University Liberal Arts and Education. 1959;114.
17. Thirumalachmar J, Sukapurre S. Studies on species of the genus Chainia from India. J Hindustan Antibiotics Bull 1964;6:157-66.
18. Shirling EB and Gottlieb D. Methods for characterization of Streptomyces species. Int J Syst Bacteriol 1966;16:313-40.
19. Lazzarini A, Linda C, Giorgio T and Flavia M. Rare genera of actinomycetes as potential producers of new antibiotics. A Van Leeuw J Microb 2000;78:399-405.
20. Kobarfard F. Tuberculosis and traditional medicine:fighting the oldest infectious disease using the oldest source of medicine. Iranian J Phar Res 2004;2:71-72.