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Bioactive potential of actinobacteria isolated from certain under-studied regions in India

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ABSTRACT

The present study reports the bioactive potential of actinobacteria isolated from certain under-studied regions in India. Soil and sediment samples and mangrove leaves were collected from 16 different under-studied regions in India. Actinobacteria was isolated by adopting selective isolation methods. Totally 158 actinobaterial cultures were selected from the collected terrestrial, marine and plant samples. More number of colonies was isolated from magnesite area, Kolli Hills and forest area in Himachal Pradesh. Majority of the isolates produced powdery (40%) and leathery (25%) colonies with white (38%) or Gray (37%) colour aerial mycelium. Bioactive compound from all the isolates were produced by agar surface fermentation and its activity was tested by agar plug method against *S. aureus, E. coli* and *C. albicans*. About 64 out of 158 cultures showed antibacterial activity in which 62 cultures was active against *S. aureus* whereas 26 were active against *E. coli*. Twenty three actinobacterial cultures were exhibited antifungal activity. About 11 actinobacterial cultures were active against both *S. aureus* and *E. coli*. In antifungal testing whereas fourteen actinobacterial cultures were found to be active against *S. aureus, E. coli* and *C. albicans*. Maximum of 21 antibacterial cultures and antifungal cultures were obtained from Magnesite soil followed by 12 antibacterial and 7 antifungal cultures were obtained from the soil sample collected from Himachal Pradesh. This evidenced that the under-studied ecosystems in India are the promising source for bioactive actinobacteria with broad spectrum antibacterial and antifungal activity. Further studies on the potential actinobacterial strains result in the isolation of broad spectrum antimicrobial metabolites.

INTRODUCTION

The discovery of antibiotics to treat infectious diseases has revolutionized the field of medicine in the mid-twentieth century. However, due to overuse or misuse of antibiotics over a prolonged period, most of the pathogens have become resistant to the antibiotic therapy. Thus, there is a dire need for the discovery and development of new antibiotics to effectively target the life threatening disease causing pathogens (Sharma *et al.*, 2016). Microbial resources are reported as promising source for novel metabolites. Actinobacteria are Gram positive bacteria that constitute one of the largest bacterial phyla and they are ubiquitously distributed in both aquatic and terrestrial ecosystems (Balagurunathan and Radhakrishnan, 2010). Actinobacteria are of great importance in terms of secondary metabolite producers with promising biological activities. They produce two-thirds of all naturally derived antibiotics in current clinical use as well as many anticancer, antifungal and antiviral compounds (Barka *et al.*, 2016).

But, in the past two decades, there has been a decline in the discovery of novel metabolites from actinobacteria including from Streptomyces which yield disappointingly high number of previously described molecules (Berdy, 2012). To solve this issue, bioprospecting of un/less explored ecosystems like marine, desert, forests, caves, and hills has been proved as useful approach for tapping innumerable number of bioactive compounds from novel bioactive actinobacteria (Radhakrishnan *et al.*, 2014).

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In addition, new sources of bioactive metabolites from rare actinobacteria from different ecological niches have promoted recent advances in the discovery of new antibiotic molecules (Nimaichand *et al.,* 2015).With this view the present study has been initiated for bioprospecting actinobacteria from under-studied regions in India to determine their antimicrobial potential.

MATERIALS AND METHODS

Sample collection and pre-treatment

Soil samples were collected from 11 different terrestrial regions and sediment samples were collected from three marine and a fresh water region. Leaves sample from the mangrove plant *Rhizophora apiculata* was also collected from Parangipettai coastal area, Tamil Nadu (Table 1). All the soil and sediment samples were air-dried at room temperature for 3-5 days and the sieved samples were kept at 55°C for 10 minutes in a glass container (Radhakrishnan *et al.,* 2007). Plant leaves were pre-treated by adopting three step process described by Coombs and Franco (2003).

Isolation of actinobacteria

Actinobacteria were isolated by adopting standard spread plate method using different media such as Starch Casein agar, Kuster's agar and Oat meal agar. All the media used in this study was prepared using distilled water whereas 50% sea water was used for the isolation of marine actinobacteria. About 10 gram of pre-treated sediment sample was added into 90 ml of sterile distilled water in 500 ml conical flask. The flask was kept in rotary shaker for 30 minutes for mixing of sample. The particulate matter was allowed to settle down and the suspension was serially diluted up to 10^5 dilutions using sterile distilled water blank. Hundred microliter of aliquot from 10^2, 10^3, 10^4 dilutions was taken and spreaded over agar plate using sterile L-rod. The same procedure was followed for all the remaining samples. All the plates were incubated at 28°C for one month (Radhakrishnan *et al.,* 2007).

Colonies showing suspected actinobacterial morphology were picked up from the isolation agar plates using sterile L-shaped loop and inoculated on yeast extract-malt extract agar (ISP2 medium). The plates were incubated at 28^0C for 7 days. Morphologically different actinobacterial cultures were selected and preserved using ISP2 agar slants as well as in 30% glycerol broth.

Characterization of actinobacteria

Cultural characterization was done by inoculating all the actinobacterial cultures into ISP2 agar medium. All the plates were incubated for 10 days at 28^0C. Cultural characteristics recorded include growth, consistency, aerial mass colour, presence of reverse side pigment and soluble pigment production (Shirling and Gottileb, 1966). Micromorphological characteristics were studied by adopting slide culture method (Balagurunathan *et al.,* 2010). About 2 ml of ISP2 agar medium inoculated with actinobacterial spores were poured as a thin layer over the surface of sterile microscopic slides. The slides were kept in sterile petriplates and incubated at 28 °C for 10 days. Then the slides were observed under bright field microscope at 40X magnification. The recorded microscopic characteristics include presence of aerial mycelium, substrate mycelium, mycelial fragmentation and spore chain morphology.

Based on the results of growth pattern of actinobacteria on ISP2 agar medium and microscopic appearance, similar actinobacterial isolates were discarded and different isolates were selected for further investigations. The selected isolates were grouped into Streptomycetes and non-Streptomycetes/rare actinobacteria.

In vitro screening of actinobacteria for antimicrobial activity

Bioactive compounds from actinobacterial cultures were produced by agar surface fermentation (Radhakrishnan *et al*., 2014). All the actinobacterial cultures were inoculated into ISP2 agar plates and incubated at 28^0C for 10 days for the production of secondary metabolites. During incubation, the extracellular metabolites are secreted into the agar medium. Test pathogens used in this study includes *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739 and *Candida albicans* MTCC227. All the cultures were maintained as slant as well as stab culture using nutrient agar.

Antimicrobial activity of actinobacterial cultures was tested by adopting agar plug method (Radhakrishnan *et al.*, 2014). Actinobacterial cultures grown on IS2 agar plates for bioactive compound production were taken and the mycelial growth was removed from the agar surface using sterile spatula. Test pathogens were inoculated into nutrient agar plates using sterile cotton swab. Agar plug with 5 mm diameter were cut from the ISP agar grown with actinobacterial cultures were placed over nutrient agar seeded with test pathogens. All the plates were incubated at 37 °C for 24 hours. Zone of inhibition was expressed in millimetre in diameter.

RESULTS

Isolation of actinobacteria

Colonies with actinobacterial morphology were observed and recovered from all the samples collected from 16 different regions. More number of actinobacterial colonies was isolated from terrestrial soil and fresh water sediment samples plated on starch casein agar where are Kusters agar yielded more number of actinobacterial colonies from marine sediments and mangrove plant sample.

Totally about 400 actinobacterial colonies were recovered from the samples collected from 16 different places from which 158 morphologically different colonies were selected for further study. More number of colonies was selected from the soil sample collected from Kolli Hills, and Magnesite area, Salem, Tamil Nadu, forest area of Himachal Pradesh and Utharakand (Table 1).

Table 1: Details of samples collected for the isolation of actinobacteria.

Sampling site no	Sampling site	Ecosystem	No. of isolates	Actinobacterial SACC No
1	Borra caves, Andhra Pradesh	Terrestrial	1	1
2	*Rhizophora* sp (mangrove) leaves, Parangipettai, Tamil Nadu	Plant	4	2-6
3	Tuticorin coast, Tamil Nadu	Marine	3	7-9
4	Thottabetta, Western Ghats, Tamil Nadu	Terrestrial	2	10-11
5	Ancient well, Kanchipuram, Tamil Nadu	Freshwater	7	12-18
6	Kolli Hills, Eastern Ghats, Salem, Tamil Nadu	Terrestrial	18	19-36
7	Coorg forest, Kerala	Terrestrial	14	37-50
8	Sabarimalai forest, Kerala	Terrestrial	5	51-55
9	Thalakona forest, Andhra Pradesh	Terrestrial	2	56-57
10	Himachal Pradesh	Terrestrial	28	58-84
11	Pichavaram Mangrove ecosystem, Tamil Nadu	Marine	8	85-92
12	Vellar Estuary, Parangipettai, Tamil Nadu	Marine	7	93-99
13	Magnesite soil, Salem, Tamil Nadu	Terrestrial	27	100-126
14	Forest ecosystem, Utharakand	Terrestrial	19	127-145
15	Oil contaminated area, Salem, Tamil Nadu	Terrestrial	5	146-150
16	Kolli Hills, Eastern Ghats, Salem, Tamil Nadu	Terrestrial	8	151-158

Table 2: Morphological pattern of actinobacteria isolated from different regions in India.

Morphology	Appearance	No. of isolates (%)
Growth	Good	139 (88%)
	Moderate	19 (12%)
Consistency	Powdery	64 (40%)
	Leathery	39 (25%)
	Rough	38 (24%)
	Smooth/spongy/others	17 (11%)
Aerial mass colour	Grey	58 (37%)
	White	60 (38%)
	Brown	7 (4%)
	Orange	9 (5%)
	Others	24 (15%)
Reverse side pigment		59 (37%)
Soluble pigment		31 (20%)
Micromorphology	Aerial and substrate mycelium	136 (86%)
	Substrate mycelium	158 (100%)

Table 3: Actinobacterial cultures showing broad spectrum antibacterial activity against Gram positive and Gram negative bacterial pathogens.

SACC No	Ecosystem	Cultural morphology					Micromorphology		Suspected Genera	Antibacterial activity	
		Growth	Consistency	AMC	RSP	SP	AM	SM		*S. aureus*	*E. coli*
SACC 3	*Rhizophora* sp (mangrove) leaves, Parangipettai	Good	Powdery	Ash	-	-	+	+	Streptomyces	10.33±0.58	10.83±0.29
SACC 4	*Rhizophora* sp (mangrove) leaves, Parangipettai	Good	Powdery	Ash	-	-	+	+	Streptomyces	18.33±0.58	15.33±0.58
SACC 17	Ancient well, >200 years old, Kanchipuram, Tamil Nadu	Good	Powdery	Ash	-	-	+	+	Streptomyces	11.0±1.0	12.0±0.0
SACC 27	Kolli Hills, Eastern Ghats, Salem, Tamil Nadu	Good	Leathery	White	-	-	+	+	Streptomyces	11.33±0.58	9.67±0.58
SACC 28	Kolli Hills, Eastern Ghats, Salem, Tamil Nadu	Good	Powdery	Ash	-	-	+	+	Streptomyces	16.17±1.25	11.5±0.5
SACC 36	Kolli Hills, Eastern Ghats, Salem, Tamil Nadu	Good	Powdery	Ash	-	-	+	+	Streptomyces	14.17±1.25	14.33±0.29
SACC 103	Magnesite area, Salem, Tamil Nadu	Good	Rough	Black	-	-	-	+	RA	15.33±0.58	11.17±0.76
SACC 107	Magnesite area, Salem, Tamil Nadu	Good	Leathery	Brown	+	+	+	+	Streptomyces	14.33±0.58	13.83±0.76
SACC 109	Magnesite area, Salem, Tamil Nadu	Good	Rough	Black	-	-	-	+	RA	22.0±0.0	11.0±0.0
SACC 112	Magnesite area, Salem, Tamil Nadu	Good	Leathery	White	+	-	+	+	Streptomyces	26.83±0.76	10.83±0.76
SACC 122	Magnesite area, Salem, Tamil Nadu	Good	Rough	Black	-	-	-	+	RA	10.83±0.29	11.5±0.5

The results are presented as mean ± SD (n = 3) ; RA – Rare Actinobacteria; + - present; - - absent.

Characterization of actinobacteria

During recovery and preservation, about 88% of the actinobacterial cultures showed good growth on ISP2 agar. Majority of the isolates produced powdery (40%) and leathery (25%) colonies with white (38%) or Gray (37%) colour aerial mycelium.

Under microscopic observation, all the actinobacterial cultures showed the presence of substrate mycelium whereas 86% of the cultures showed the presence of both aerial and substrate mycelium in which majority of them are Streptomyces. The remaining 14 % of the cultures which showed only the presence of substrate mycelium are considered as non-Streptomyces/rare actinobacteria (Table 2).

Screening for antimicrobial activity

In agar plug method, 64 out of 158 cultures showed antibacterial activity in which 62 cultures was active against *S. aureus* whereas 26 were active against *E. coli*. About 11 actinobacterial cultures were active against both *S. aureus* and *E. coli* (Table 3).

Table 4: Actinobacterial cultures showing broad spectrum antibacterial activity against bacterial and fungal pathogens.

SACC No	Ecosystem	Cultural morphology					Micromorphology		Suspected Genera	Antibacterial activity		Antifungal activity
		Growth	Consistency	AMC	RSP	SP	AM	SM		*S. aureus*	*E. coli*	*C. albicans*
SACC 57	Intertidal region, Thiruchendur coastal area, Tamil Nadu	Good	Powdery	Gray	-	-	+	+	Streptomyces	12.17±0.29	12.67±0.58	14.0±0.0
SACC 61	Mahakalimandhir, Himachal Pradesh	Good	Powdery	Gray	+	-	+	+	Streptomyces	18.67±0.58	14.0±0.0	14.67±0.76
SACC 67	Near St. Johns church, Himachal Pradesh	Good	Powdery	Gray	-	-	+	+	Streptomyces	15.67±0.58	12.5±0.5	13.0±0.5
SACC 83	Nadi-Point, Himachal Pradesh	Good	Powdery	Gray	-	-	+	+	Streptomyces	11.5±0.5	11.67±0.29	12.67±0.58
SACC 96	Vellar estuary, Parangipettai, Tamil Nadu	Good	Powdery	Gray	-	-	+	+	Streptomyces	15.0±0.0	15.17±0.29	15.33±0.58
SACC 97	Vellar estuary, Parangipettai, Tamil Nadu	Good	Powdery	Gray	-	-	+	+	Streptomyces	16.17±0.29	13.83±0.76	11.5±0.5
SACC 101	Magnesite area, Salem, Tamil Nadu	Good	Smooth	White	-	-	+	+	RA	23.5±0.5	18.0±0.0	18.33±1.04
SACC 110	Magnesite area, Salem, Tamil Nadu	Good	Smooth	White	-	-	-	+	RA	22.67±0.58	13.0±1.0	12.0±0.0
SACC 111	Magnesite area, Salem, Tamil Nadu	Good	Powdery	Ash	+	-	+	+	Streptomyces	26.0±0.0	19.33±0.58	16.0±0.5
SACC 120	Magnesite area, Salem, Tamil Nadu	Good	Powdery	Gray	+	-	+	+	Streptomyces	21.67±0.58	13.83±0.29	17.67±0.29
SACC 124	Magnesite area, Salem, Tamil Nadu	Good	Rough	Brown	-	-	+	+	RA	28.0±0.0	23.5±0.87	18.0±0.0
SACC 125	Magnesite area, Salem, Tamil Nadu	Good	Powdery	Ash	+	-	+	+	Streptomyces	17.67±0.29	16.83±0.29	18.83±0.76
SACC 126	Magnesite area, Salem, Tamil Nadu	Good	Rough	Black	-	+	-	+	RA	23.83±0.29	22.0±0.0	14.67±0.76
SACC 134	Forest area, Utharakand	Good	Powdery	Gray	+	-	+	+	Streptomyces	14.33±0.58	16.5±0.5	11.83±0.29

The results are presented as mean ± SD (n = 3); RA – Rare Actinobacteria; + - present; - - absent.

In antifungal testing, 23 out of 158 actinobacterial cultures were exhibited antifungal activity against *C. albicans*. Fourteen actinobacterial cultures were found to be active against *S. aureus, E. coli* and *C. Albicans* (Table 4). Maximum of 21 antibacterial cultures and antifungal cultures were obtained from Magnesite soil followed by 12 antibacterial and 7 antifungal cultures were obtained from the soil sample collected from Himachal Pradesh.

DISCUSSION

Based on the hypothesis "poorly researched habitats can offer better prospects for discovering new natural products". This precluded the study of normal terrestrial sources particularly for actinobacteria and has led researchers to explore unique and extreme habitats like deep sea, desert, forest and mountain environment for potentially new biosynthetic diversity. In India, there are many reports on bioprospecting of actinobacteria from various terrestrial (Pazhanimurugan *et al.,* 2010; George *et al.,* 2012) and marine ecosystems (Radhakrishnan *et al.,* 2011; Poosarla *et al.,* 2013) with special reference to antimicrobial and enzymatic activities (Mohanapriya *et al.,* 2011). With this view, the present study is attempted to explore bioactive actinobacteria from certain under-studied aquatic and terrestrial ecosystems in India. In the present study plating of terrestrial soil and aquatic sediments after dry heat treatment at 55^0C for 10 minutes on three different media results in the isolation of more powdery or leathery colonies with substrate and aerial mycelium. Terrestrial samples yielded more colonies on starch casein agar whereas marine sediments yielded more colonies on Kusters agar.

Many authors reported Streptomyces as a major actinobacterial population in both terrestrial (Radhakrishnan *et al.,* 2007; Mohanraj *et al.,* 2014) and marine ecosystems (Sivakumar *et al.,* 2005; Kathiresan *et al.,* 2005). In the present study also majority of the recovered actinobacterial cultures are Streptomyces (86%). Sivakumar *et al.,* (2005) and Mohana and Radhakrishnan (2014) also recommended Kusters agar as a suitable medium for the recovery of actinobacteria from mangrove ecosystem. Further the heat treatment method and antibacterial and antifungal antibiotic supplemented in the isolation medium resulted in the reduction of unwanted bacterial and fungal growth, respectively. Cross streak method using modified nutrient glucose agar (MNGA) is the most common method used for the detection of antagonistic actinobacteria (Radhakrishnan *et al.,* 2007; Balagurunathan *et al.,* 2001).

But this method is not found suitable for actinobacteria isolated from marine ecosystems since the marine actinobacterial cultures require seawater for their growth. The addition of seawater into the MNGA medium to support marine actinobacteria surely affects the growth of test pathogens. Agar plug method using yeast extract malt extract agar (YEME) followed in this study is a simple and suitable method for the detection of actinobacteria especially those which require special conditions / medium supplements for their growth and metabolite production (Radhakrishnnan *et al.,* 2014).

This method also allows to modify the components qualitatively as well as quantitatively which cannot possible in cross streak method. According to Berdy (2012) great number of antibiotic compounds exhibit exclusive activities against gram positive bacteria while only 1.5% are active against gram negative bacteria. Eco-physiological conditions of particular ecosystem greatly influence the biological and metabolic activity and diversity of actinobacteria (Knight *et al.,* 2003). Salamoni *et al.,* (2012) reported that 25 Streptomyces species isolated from compost showed 20 different patterns of activity against 53 bacterial and fungal pathogens.

In the present study also, 39% of the actinobacterial cultures were active against *S. aureus* whereas 16.4% of the cultures were active against E. coli. Interestingly 21% of the antagonistic actinobacterial cultures were showed broad spectrum activity against *S. aureus, E. coli* and *C. albicans*.

CONCLUSION

Findings of the present study evidenced that the under-studied ecosystems in India are the promising source for bioactive actinobacteria with broad spectrum antibacterial and antifungal activity. Further studies on the potential actinobacterial strains result in the isolation of broad spectrum antimicrobial metabolites.

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