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Screening of Marine Actinobacteria for Antimicrobial Compounds

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ABSTRACT

Actinobacteria producing bioactive compounds were isolated by serial dilution method from marine sediments collected from Bay of Bengal at a depth of 10-40 m near pudimadaka coast of Andhra Pradesh. A total of 78 isolates were obtained and *Streptomyces* is predominant among all the isolates. Out of 78 isolates, 22 isolates exhibited antibacterial activity alone and 12 isolates exhibited both antibacterial and antifungal activity, respectively. Among the active isolates BTS-112, BTS-314 and BTS-401 isolates showed promising activities. The isolates were further characterized and identified to be belonging to the genus *Rhodococcus* and *Streptomyces*.

Key words: Marine sediments, actinobacteria, *Streptomyces*, *Nocardia*, *Micromonospora*, antimicrobial activity

INTRODUCTION

Actinobacteria, the filamentous bacteria have provided many important bioactive compounds of high commercial value and are being routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics have been isolated from actinobacteria (Solanki *et al*., 2008). Most of the bioactive microbial metabolites were isolated from actinobacteria especially from streptomycetes and also from some rare actinomycetes. During the last 20-30 years, the interest in the marine microflora increased due to the investigation of novel bioactive compounds especially antibiotics and enzymes. As the frequency of novel bioactive compounds obtained from terrestrial actinobacteria decreases with time, actinobacteria from diverse environments have been increasingly screened for their ability to produce new secondary metabolites. It has been emphasized that actinobacteria from marine sediments may be valuable for the isolation of novel strains which could potentially yield a broad spectrum of secondary metabolites (Ismet *et al.*, 2004; Jensen *et al.*, 1991; Maskey *et al.*, 2003). However, it has been resolved whether actinobacteria form part of the marine microbial community of sediment samples originated from terrestrial habitats and were simply carried out to sea in the form of resistant spores (Goodfellow and Haynes, 1984). Many commercially important bioactive compounds and antitumor agents in addition to enzymes of industrial interest have been produced from actinobacteria (Imasda, 2005). It has been estimated that approximately 203 of the naturally occurring antibiotics have been isolated from these organisms (Takizawa *et al*., 1993).

When conventional isolation techniques were applied, most of the isolates recovered on agar plates have been identified as genus *Streptomyces* which are the dominant actinobacteria in soil (Iwai and Takahashi, 1992; Bascom-Slack *et al*., 2009). For the purpose of screening novel bioactive

molecules, several factors must be considered: choice of screening source, pretreatment, selective medium, culture condition and recognition of candidate colonies on a primary isolation plate. An alternative approach was to make the isolation procedure more selective by adding chemicals such as phenol to the soil suspension (Nonomura, 1988; Hayakawa *et al.*, 1991). Many actinobacteria have shown multiple resistances to wide ranges of antibiotics. Several antibiotic molecules were used in selective medium to inhibit the competing bacteria including fast-growing actinobacteria (Jiang *et al.*, 2008). Specialized growth media were developed to isolate specific actinobacteria genera macromolecules such as starch, glycerol, chitin, casein, humic acid and amino acids were chosen as the beat carbon and nitrogen sources of rare actinobacteria (Cho *et al.*, 1994; Hayakawa and Nonomura, 1987).

So far the terrestrial soil was the most predominant and widely exploited source. A very little is known about the microbial diversity of marine sediments which is an inexhaustible resource that has not been properly exploited. However, the full potential of this domain as the basis for biotechnology, particularly in India, remains largely unexplored. One of the most successful approaches to obtain new types of useful microbial metabolites is to investigate marine sediments. The strategies for the investigation of marine organisms have been described by Jensen and Fenical (1994) and Okami *et al.* (1976).

In the present investigation an effort was made to screen different marine sediments of the pudimadaka coast of the Bay of Bengal, India, which is large, diverse and unscreened ecosystem for the isolation of potent antibiotic-producing actinobacteria and deals with their distribution pattern and taxonomy.

MATERIALS AND METHODS

Collection of sediment samples: A total of ten Marine sediment samples were collected in June 2008 from Bay of Bengal near pudimadaka coast of Andhra Pradesh at a depth of 10-40 m using a core sampler. The sediment samples were brown to black in colour and of sandy texture. The location and depths of these sampling stations are summarized in Table 1.

Isolation of actinomycetes from sediment samples: Starch casein agar media, Glycerol asparagine agar media, Chitin agar media (Kampfer, 2006) and glucose yeast extract malt extract agar media (Shirling and Gottlieb, 1966) were used for the isolation of actinobacteria. Each media containing 50% filter (0.42 μm) sterilized sea water supplemented with rifampcin 5 μg mL^{-1} and nystatin (Himedia Mumbai) 25 μg mL^{-1} to inhibit bacterial and fungal contamination, respectively (Porter, 1971). The sediment samples were appropriately diluted with sterilized seawater and an aliquot of 0.1 mL was spread on the media. After incubation for 1-3 weeks at 28°C, the actinomycetes colonies that developed on the plates were picked out and purified before being stored in starch casein agar slants.

Screening of actinobacteria for antibiotics: Preliminary screening for antibiotic production was done by cross streak method. The isolates having the activity were cultured in about 50 mL of production media having the composition glucose 1%, soybean meal 1%, NaCl 1% and $CaCO_3$ 0.1% in 250 mL Erlenmeyer flask under submerged fermentation conditions at 28°C for 96 h at 180 rpm and the clear supernatant broth samples were tested for their antimicrobial activities. The antimicrobial activity was determined by disk diffusion method (Audrey, 2007). The assay plates were seeded with *S. aureus* (MTCC 3160), *B. subtilis* (MTCC 441), *B. cereus* (MTCC 430),

Table 1: Samples collected near pudimadaka coast of Andhra Pradesh

	Location	
Depth (m)	Latitude	Longitude
10	+17.30500	+83.02300
20	+17.30500	+83.04400
30	+17.30500	+83.08000
30	+17.29000	+83.00816
20	+17.29000	+83.02205
30	+17.29000	+83.05086
40	+17.29015	+83.07480
10	+17.27000	+82.59398
20	+17.27000	+82.59295
30	+17.27000	+83.01314

P. aeruginosa (MTCC 424), *E. coli* (MTCC 443), *P. vulgaris* (MTCC 426) using Muller Hinton agar and *S. cerevisiae* (MTCC 170), *C. albicans* (MTCC 227), *A. niger* (MTCC 961) and *A. flavus* (MTCC 3396) using yeast extract -malt extract agar were used for the antimicrobial assay, respectively. The antimicrobial activity was observed after 24 h of incubation at 37°C for bacteria and 48 h of incubation at 25°C for fungi and the zone of inhibitions were expressed as diameter (mm).

Characterization of the isolates: The isolates were characterized up to genus level by observing the spore bearing hyphae, structure of spore chain, colour of the spore, aerial mass colour and colour of substrate mycelia as described by Bergey (1989) and International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966; Pridham, 1965).

RESULTS AND DISCUSSION

Distribution of actinobacteria: The occurrence and distribution of different actinobacteria genera in different marine sediment samples is shown in Fig. 1. Distribution and diversity of actinobacteria have been reported from marine habitats such as marine sediments by Jensen *et al.* (1991) and Takizawa *et al.* (1993).

A total of 78 actinobacteria strains were isolated from ten marine sediment samples collected from Bay of Bengal near pudimadaka coast of Andhra Pradesh. Out of 78 isolates, 70 isolates were identified as genus *Streptomyces* (spore chain with coiling, spiral and looped), 3 as *Micromonospora*, (clusters of single conidia on substrate mycelium), 2 as *Nocardia* (conidia on powdery appearance aerial hyphae, carotenoid like pigments), 2 as *Streptoverticilium* (whorls of straight chain of conidia formed) and 1 as *Rhodococccus*. The actinomycetes obtained from the sediment samples varied in number from one location to another and especially *Streptomyces* is predominant in all the locations and whereas, *Streptoverticilium* and *Nocardia* are least dominant.

Screening of actinobacteria for antibiotics: Biological activity testing of fermentation products from the marine-derived actinobacteria revealed to have activities against pathogenic bacteria and fungi (Magarney *et al.*, 2004; Peela *et al.*, 2005). Out of 78 isolates, 22 isolates exhibited antibacterial activity, 12 isolates exhibited antifungal activity and 11 isolates exhibited both antibacterial and antifungal activities. The antimicrobial activities against the test organisms are indicated in Table 2 and 3. Out of the 22 active isolates (7 isolates) BTS-103, BTS-112, BTS-301,BTS-314, BTS-401, BTS-706 and BTS-707 show the best antibacterial activities and the

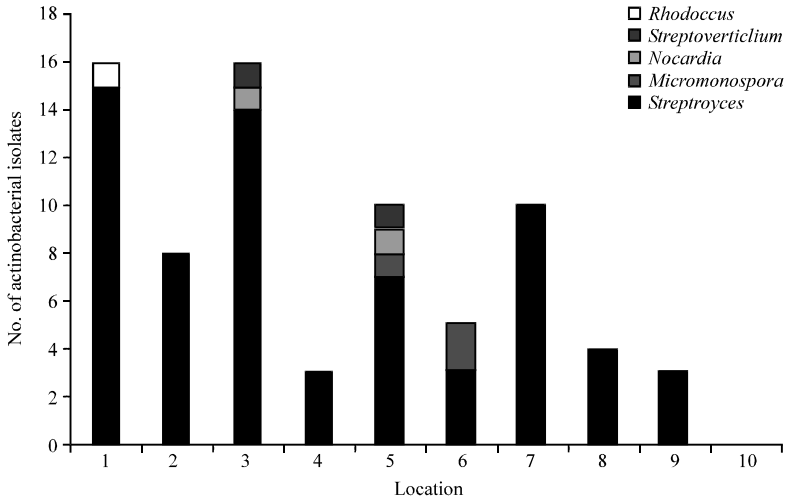

Fig. 1: Distribution of actinobacteria in different marine sediment samples

Table 2: Antibacterial activity of the active isolates

	Name of the test organism (Inhibition zone diameter in mm)					
Isolate No.	*E.coli* (MTCC-443)	*Proteus vulgaris* (MTCC-426)	*Pseudomonas aeruginosa* (MTCC-424)	*Bacillus subtilis* (MTCC-441)	*Bacillus cereus* (MTCC-430)	*Staphylococcus aureus* (MTCC-3160)
BTS-103	16	12	-	18	-	12
BTS-104	10	11	-	10	12	12
BTS-106	12	10	10	-	-	10
BTS-112	18	13	18	15	16	17
BTS-204	12	10	12	-	-	10
BTS-206	10	-	10	12	12	12
BTS-301	14	12	10	12	10	14
BTS-306	10	10	-	-	-	-
BTS-310	10	12	-	-	-	-
BTS-312	12	-	-	10	10	-
BTS-314	12	12	14	12	12	10
BTS-315	12	10	10	12	10	-
BTS-316	10	10	12	12	10	-
BTS-401	14	12	12	14	14	10
BTS-505	10	12	-	12	10	-
BTS-506	12	-	-	11	10	-
BTS-509	10	-	-	-	-	-
BTS-706	14	12	12	14	14	12
BTS-707	12	14	10	12	10	10
BTS-801	10	10	-	12	10	-
BTS-802	12	-	-	-	12	-
BTS-903	12	12	-	-	-	-

- = No activity

remaining isolates exhibited intermediate activity against the test organisms. Among these isolates BTS-112, BTS-314 and BTS-401 show the highest activities against *E. coli, P. aeruginosa, B. cereus* and *S. aureus*. Whereas, BTS-103 has no activity against the test organisms *P. aeruginosa* and *B. cereus* but it show the highest activity against *B.subtilis*. In case of antifungal studies, out of 12

active isolates-3 isolates (BTS-112, BTS-314 and BTS-401) showed best activities against the fungi and the remaining isolates exhibited the lowest activities. The isolate BTS-401 exhibited the highest activity against *A. niger* and it showed less activity towards the other fungi. The remaining two isolates BTS-314 and BTS-112 were intermediate in their activities against the pathogenic fungi used in the study. The present findings highlight the importance for further investigation towards the goal of obtaining antimicrobial agents. In a similar study by Devi *et al.* (2006) and Singh *et al.* (2006) showed parallel results towards the antagonistic activity of marine actinomycetes against the pathogenic bacteria and fungi.

Characterization of isolates: Among all the active isolates, only three isolates BTS-112, BTS-314 and BTS-401 exhibited promising activities against the test organisms used in the study. The biochemical characteristics of these three isolates were further studied. The morphological and physiological characteristics of the promising isolates were represented in Table 4. The isolate BTS-112 (Fig. 2) was identified as *Rhodococcus* species belongs to Nocardiaceae and the remaining two isolates BTS-314 (Fig. 3) and BTS-401 (Fig. 4) were identified as *Streptomyces* species. Among all the members of the actinobacteria the *Streptomyces* species were known as the producers; of the secondary metabolites such as antimicrobial compounds (Krishna *et al.*, 2006; Rizk *et al.*, 2007) and cytotoxic compounds (Yoo *et al.*, 2002; Thangapandian *et al.*, 2007) that have the potential to control wide range of pathogens.

Table 3: Antifungal activity of the active isolates

	Name of the test organism (Inhibition zone diameter in mm)			
Isolate No.	*A. niger* (MTCC-961)	*A. flavus* (MTCC-3396)	*C. albicans* (MTCC-227)	*S. cerevisiae* (MTCC-170)
BTS-103	10	10	12	-
BTS-106	-	-	10	-
BTS-112	10	12	12	10
BTS-206	-	-	12	-
BTS-301	-	-	10	-
BTS-312	-	10	12	-
BTS-314	12	10	12	10
BTS-316	-	-	10	-
BTS-401	14	10	10	12
BTS-506	-	10	-	10
BTS-706	-	-	-	12
BTS-803	-	12	10	10

- = No activity

Table 4: Morphological and physiological characteristics of potent isolates

	Isolate No.		
Characteristics	BTS-112	BTS- 314	BTS- 401
Substrate mycelia	Red	Green	White
Aerial mycelia	Red	Green	Brown
Soluble pigment	Purple	Blackish green	Orange red
Spore chain	ND	Spiral	*Retinaculum apertum*
Spore mass colour	ND	Green	Brown
Melanin formation	-	+	+

Table 4: Continued

Characteristics	Isolate No. BTS-112	BTS- 314	BTS- 401
H_2S Production	-	+	+
Tyrosine reaction	+	+	+
Starch hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Nitrate reductase	+	-	+
Catalase	+	-	-
Growth temperature (°C)			
10	-	-	-
20	+	+	+
28	+	+	+
37	+	+	-
pH tolerance	5-8	6-8	6-8
Cell wall type	IV	I	I
Carbon source			
D-Glucose	Good	Good	Good
D-Fructose	Moderate	Good	Moderate
D-Galactose	Good	Moderate	Moderate
D-Xylose	Moderate	Moderate	Moderate
L-Arabinose	No growth	No growth	No growth
Glycerol	Good	Good	Good
Nitrogen source			
Asparagine	+	+	+
Arginine	+	+	+
Histidine	±	+	±
KNO_3	+	±	±
NaCl tolerance (%)			
1	Good	Good	Good
4	Good	Good	Good
7	Good	Good	Good
10	Moderate	Moderate	Moderate

ND = Not determined; + = Positive reaction; - = Negative reaction; ± = Weakly positive reaction

Fig. 2: Isolate No. BTS-112 identified as *Rhodococcus* sp. on starch casein agar media

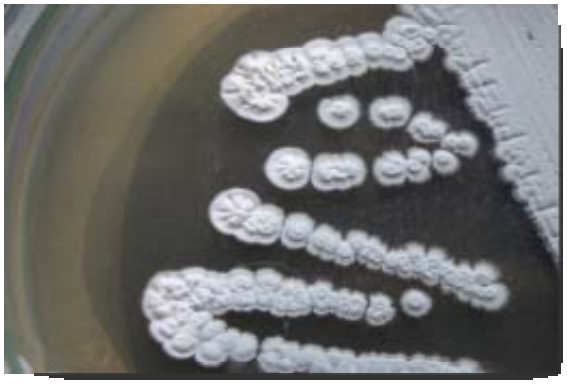

Fig. 3: Isolate No. BTS-314 identified as *Streptomyces* sp. on starch casein agar media

Fig. 4: Isolate No. BTS-401 identified as *Streptomyces* sp. on glycerol asparagine agar media

CONCLUSION

By combining pretreatment with suitable media supplemented with specific antibiotics, diverse rare actinobacteria genera that previously were only recovered incidentally by conventional dilution-plating techniques can now be successfully isolated. Further studies on the molecular characterization of the isolates, purification of the antibiotic substance and elucidation of its production pathways are underway.

It is expected that the current attempt for the isolation, characterization and the study on marine actinobacteria of Pudimadaka coast of Bay of Bengal will be useful for the identification of new antibiotics effective against challenging pathogens.

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