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Full Length Research Paper

Isolation of potent antibiotic producing Actinomycetes from marine sediments of Andaman and Nicobar Marine Islands

Aparanji Poosarla[1]*, Venkata Ramana L.[1] and R. Murali Krishna[2]

[1]Department of Biochemistry, Andhra University, Visakhapatnam, Andhra Pradesh, India.
[2]Department of Chemistry, Andhra University, Visakhapatnam, Andhra Pradesh, India.

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A total of six isolates of Actinomycetes were collected from marine sediments of Andaman Islands. Each isolate was tested against five pathogenic bacteria and also against some pathogenic fungi. Among the six isolates, one of the isolates showed potent activity against all the bacteria and fungi. These isolates appear to produce high anti-fungal and anti-bacterial compounds on potato dextrose agar and nutrient agar medium respectively, by using the agar diffusion method. The potent Actinomycetes were characterized by morphological methods consist of macroscopic and microscopic methods. The mycelium structure, color and arrangement of conidiophores were observed through the oil immersion (100X). Various biochemical tests performed for the identification of potent isolates are as follows: Melanin reaction, H_2S production, tyrosine reaction, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, milk coagulation and peptonization, nitrate reduction, temperature range of growth, pH tolerance and cell wall type by comparing all these results with the Bergey's manual of Determinative Bacteriology and the organisms were identified. All the isolates were identified to belong to the genus Streptomyces. Further purification of the spent medium may gives more activity than the standard antibiotics and also effective against some multidrug resistant pathogens.

Key words: Actinomycetes, Streptomyces, antimicrobial activity, pathogenic bacteria, pathogenic fungi, spent medium, secondary screening test.

INTRODUCTION

Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites. Among such microorganisms, Actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities (Bredholt et al., 2008). The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Although, considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most versatile source for new antibiotics (Baltz, 2006; Pelaez, 2006). Traditionally, Actinomycetes have been isolated from terrestrial sources although, the first report of mycelium forming Actinomycetes being recovered from marine sediments appeared several decades ago (Weyland, 1969). Recently, the marine derived Actinomycetes have become recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties (Jensen et al., 2005). Actinomycetes represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world and especially significant for their role on the recycling of organic matter (Srinivasan et al., 1991). The literatures suggested that, marine sediment sources are voluble for the isolation of novel Actinomycetes with the potential to yield useful new products (Goodfellow and Haynes, 1984). However, it has been resolved whether Actinomycetes form part of

*Corresponding author. E-mail: apara_p@yahoo.co.in

the autochthonous marine microbial community of sediment samples originated from terrestrial habitats and were simply carried out to sea in the form of resistant spores (Weyland, 1981; Goodfellow and Williams, 1983; Weyland and Helmke, 1988; Takizawa et al., 1993; Ravel et al., 1998). Microorganisms found in marine environments have attracted a great deal of attention, due to the production of various natural compounds and their specialized mechanisms for adaptation to extreme environment (Solingen et al., 2001). The pre-treatment including enrichment, physical and selective media may be used to study the ecology of Actinomycetes in natural habitats such as soil or water samples (Jensen et al., 2005). Since marine sediments represent an environment which is markedly different from that associated with soil samples, it is not clear how effective the pre-treatment of such sediments would be for the recovery of bioactive Actinomycetes. Marine sediment is an inexhaustible resource that has not been properly exploited. Reports from the East Coast of India, suggests that soil is a major source of Actinomycetes (Sivakumar et al., 2005; Vijayakumar et al., 2007; Dhanasekaran et al., 2008; Vijayakumar et al., 2009). Correspondingly, the Andaman and Nicobar Island marine ecosystem is largely unexplored, and may provide a rich source of the microorganisms producing novel and efficient antimicrobial compounds. Hence, the present study was undertaken to isolate the bioactive Actinomycetes from marine sediments of Andaman and Nicobar Islands by various pre-treatment methods using different media and evaluate the antibacterial potentiality of the isolates.

MATERIALS AND METHODS

Collection of sample

The Andaman marine sediment sample was collected for the isolation of Actinomycetes from Pongibalu area at a depth of 10 m using a core sampler. The central portions of the sediments were ascetically transferred to the sterile bottles. The sediment sample was blackish brown color and of a sandy texture.

Isolation of Actinomycetes colonies from the marine

Sediments isolation and enumeration of Actinomycetes were performed by the soil dilution plate technique (Ellaiah et al., 1996) using starch casein agar medium (g/;L: starch 10, casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar 18). 50 ml of starch casein agar media in 250 ml flask were sterilized at 121°C for 20 min by autoclaving. The media was prepared by using 50%(v/v) sea water. 1 g each of the marine sediment sample was taken in 250 ml Erlenmeyer flask containing 50 ml of sterile water. The flasks were shaken on rotary shaker for 30 min for the detachment of spore chains. The flasks were kept aside for 15 min to settle down the particulate matter. The suspension was serially diluted up to 6 fold. 1 ml each of these dilutions were added to each of 50 ml of the aforementioned sterile molten media maintained between 40 to 45°C, thoroughly mixed and poured into Petri plates and incubated at 28°C. The incubated Petri plates were observed from one week onwards for three weeks for colonies of Actinomycetes. The starch casein agar media was supplemented with 2.5 µg/ml of rifampicin and 75 µg/ml of flucanozole to minimize bacterial and fungal contaminations, respectively. Actinomycetes colonies were marked, thus identical colonies were scored out and the selected colonies were sub cultured on SCA slants and incubated at 28°C for one week.

Screening of antibiotic-producing strains

Primary screening test

The anti microbial activities of the isolates were tested by cross-streak plate method employing nutrient agar medium for bacteria and potato dextrose agar medium for fungi. The media were sterilized by autoclaving at 121°C. For 15 min and the molten sterile media were cooled to 40 to 45°C poured in to Petri plates (4 inch diameter) and allowed to solidify. Each plate was streaked with one isolate at the center and incubated at 20°C for 7 days. After 7 days, test organisms were streaked perpendicular to the growth of the isolate; 24 h cultures of bacteria, and 4 day cultures of fungi.

Secondary screening test

The active isolates resulted from primary screening; promising isolates were tested for their extra cellular antibiotic production capabilities under submerged fermentation conditions. The production medium containing Soyabean meal 1%, glucose 1%, NaCl 1% and $CaCO_3$ 0.1% was used for antibiotic production employing isolates obtained from marine sediments of Andaman and Nicobar islands well sporulated, 7 day old slants of the selected isolates were used for the antibiotic production studies. Sterile water was added to each slant and spore suspension was prepared. 5 ml of this spore suspension was added to 45 ml of the respective production media and incubated at 28°C on a rotary shaker (120 rpm) for 4 days. Then samples were collected in to sterile centrifuge tubes and centrifuged at 3000 rpm for 15 min; the clear supernatant was used for antibiotic assay using agar diffusion method (Barry and Thornsberry, 1985), employing nutrient agar for bacteria and potato dextrose agar for fungi. The activity of 50 µl of spent medium of all above isolates was compared with the activity of standard antibiotics like 100 µg/ml of each streptomycin and tetracycline for bacteria, flucanozole and greseofulvin (methanol extract) for fungi. The test organisms mentioned in primary screening were also used in secondary screening test.

Test micro organisms

The bacterial *Streptococcus* (MTCC-2672), *Staphylococcus aureus* (MTCC-96), Bacillus *subtilis* (MTCC-121), *Escherichia coli* (MTCC-118), *Proteusvulgaris* (MTCC-426)} and the fungal samples, *Aspergillus niger* (MTCC-2723), *Candida albicans* (MTCC-227), *Penicilium* (MTCC-161), *Mucor* (MTCC-546), *Rhizopus* (MTCC-262) were taken from Department of Microbiology, Andhra University. By using anti biotic agar diffusion method antibiotic activity of the test samples were measured upon these bacterial and fungal samples. The effectiveness of the extracted broth was measured upon these bacterial and fungal strains.

Biochemical characterization of Actinomycetes

The potent Actinomycetes were characterized by morphological methods consisting of macroscopic and microscopic methods. The mycelium structure, color and arrangement of conidiophores were observed through the oil immersion (100X). The observed structure

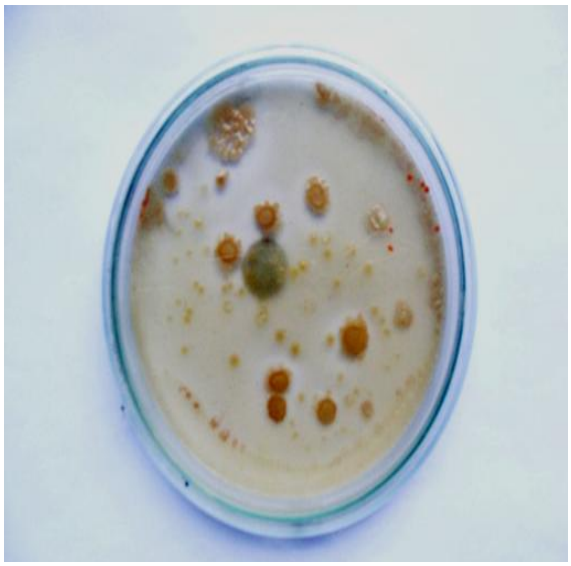

Figure 1. Different Actinomycetes colonies isolated from 10^{-1} dilution on the starch casein agar.

Figure 2. Selective isolates were tested for anti bacterial activity in the primary screening test. Among those, BC-1 isolate showed activity against *B. subtilis.*

was compared with Bergey's manual of Determinative Bacteriology and the organisms were identified. Various biochemical tests performed for the identification of potent isolates are as follows: Melanin reaction, H_2S production, tyrosine reaction, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, milk coagulation and peptonization, nitrate reduction, temperature range of growth, pH tolerance and cell wall type.

RESULTS AND DISCUSSION

A total of six isolates of Actinomycetes from marine

Figure 3. Selective isolates were tested for anti fungal activity in the primary screening test. Among those, BC-1 isolate showed activity against *C. albicans.*

sediments of Andaman Islands collected. As shown in Figure 1, among these, only five isolates exhibited the anti-microbial activity in the primary screening test against bacterial and fungal pathogens (Figures 2 and 3). Where as, in the secondary screening test out of the five active isolates BC-1 isolate showed the potent activity against both bacterial and fungal organisms as shown in the Figures 4 and 5; zone of inhibitions of all active isolates are shown in Figures 6 and 7. The identification of the potent antibiotic producing strains reveal strain belongs to the genus *Streptomyces*. Biochemical tests were performed in positive isolates of Actinomycetes and the results are shown in the Table 1. All the isolated microorganisms were Gram-positive, branching and filamentous bacteria. Spore staining showed BC-1, BC-2 exhibited rectiflexible spore chin and BC-4, BC-5, BC-6 showed spiral shaped spore chain. Except BC-1, all the isolates exhibited grey colored spore mass and BC-1 isolate showed white colored spore mass. BC-1,BC-6 isolates were exhibited grey colored aerial mycelium and BC-2, BC-4, BC-5 isolates showed light grey, brown grey and light brown grey colored aerial mycelium respectively. Except BC-4, all the isolates exhibited yellow brown colored substrate mycelium and BC-4 showed grey brown colored substrate mycelium. BC-1 and BC-2 isolates showed yellow colored soluble pigment and the remaining isolates were colorless. BC-1, BC-4 and BC-5 isolates showed positive results and BC-2 and BC-6 isolates exhibited negative results in melanin reaction. All isolates showed H_2S production negative. BC-1, BC-4 and BC-5 isolates exhibited positive results

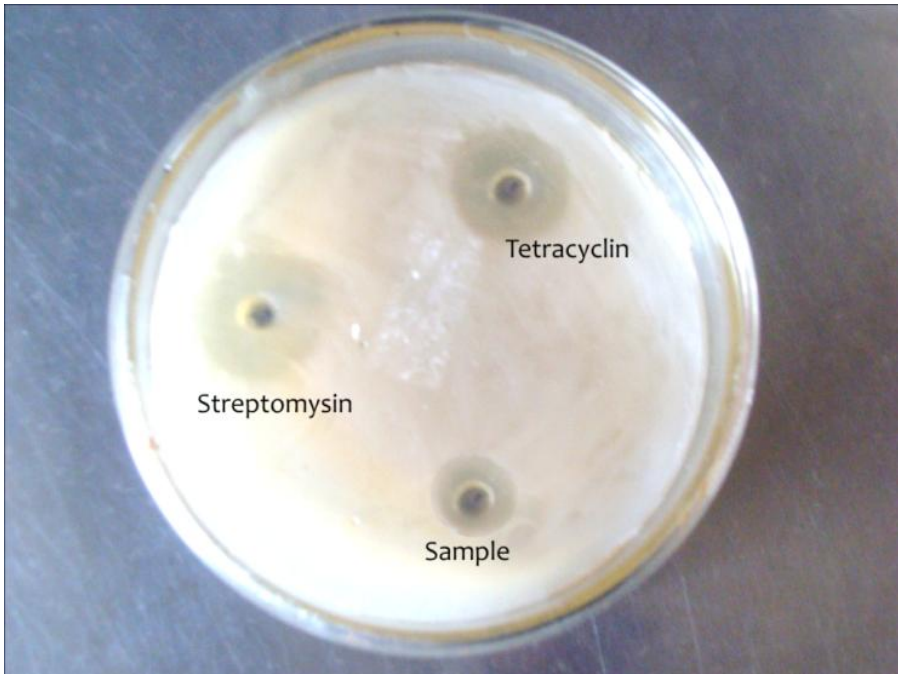

Figure 4. Selective isolates from the primary screening test undergo antibiotic production during the secondary screening test. The spent medium was tested for antimicrobial activity against some bacterial pathogens. Among that BC-1 isolate exhibited potent activity of 16 mm of inhibition zone against *B. suubtilis* compared with standard antibiotics streptomycin and tetracyclin.

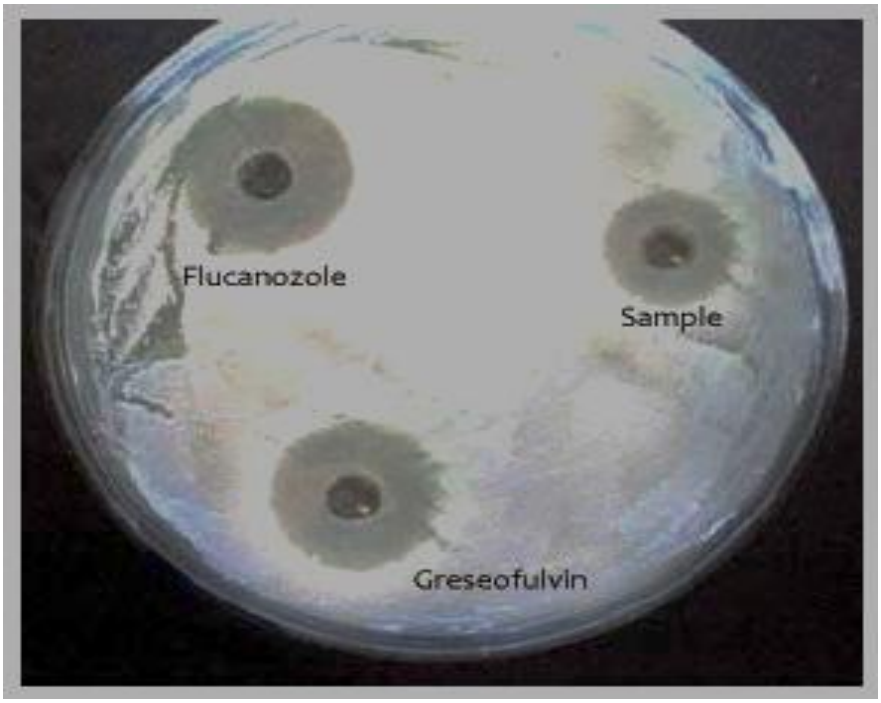

Figure 5. Selective isolates from the primary screening test undergo antibiotic production during the secondary screening test. The spent medium was tested for antimicrobial activity against some bacterial pathogens. Among that, BC-1 isolate exhibited potent activity of 13 mm of inhibition zone against *C. albicans* compared with standard antibiotics, fluconozole and greseofulvin.

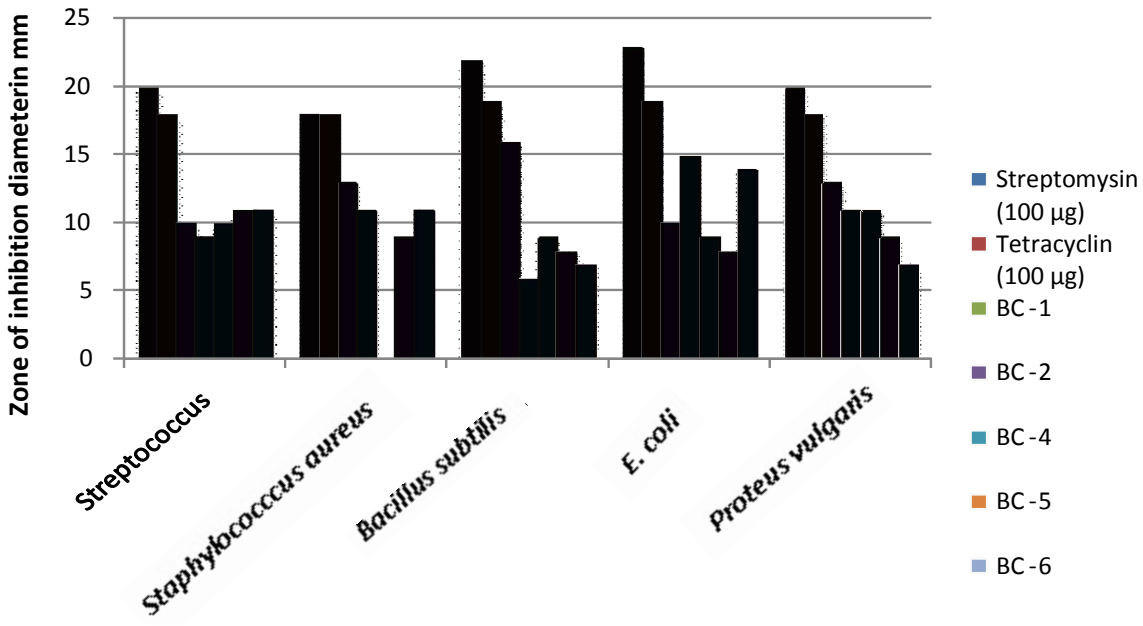

Figure 6. Zone of inhibition of selective isolates against some bacteria by using spent medium produced from the antibiotic production during the secondary screening test.

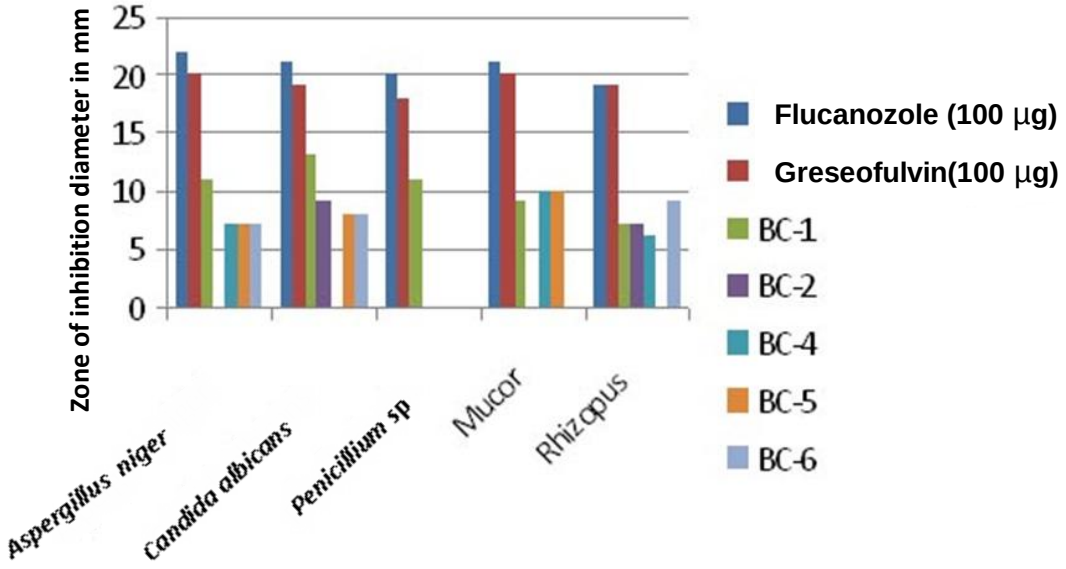

Figure 7. Zone of inhibition of selective isolates against some fungi by using spent medium produced from the antibiotic production during the secondary screening test.

and BC-2 and BC-6 isolates showed negative results in tyrosine reaction. BC-2, BC-4 and BC-6 isolates showed positive results and BC-1 and BC-5 isolates exhibited negative results in starch hydrolysis. BC-4, BC-5 and BC-6 isolates exhibited positive results and BC-1 and BC-2 isolates showed negative results in casein hydrolysis. BC-1, BC-2 and BC-6 isolates showed positive results and BC-4 and BC-5 showed negative results in gelatin hydrolysis. BC-1, BC-2 and BC-6 isolates showed positive results and BC-4 and BC-5 isolates exhibited negative results in milk coagulation and peptonization. Except BC-5, all isolates showed positive results in nitrate reduction test .All the isolates grew well at 28°C and the temperature range of all isolates for growth was from 20 to 37°C temperature. BC-2, BC-4 and BC-6 isolates showed growth in the pH range 6 to 8. While the

Table 1. Biochemical characterization of Actinomycetes strains from sediment sample.

Test	Results				
	BC-1	**BC-2**	**BC-4**	**BC-5**	**BC-6**
Gram staining	**+**	**+**	**+**	**+**	**+**
Spore chain	Rectiflexibles	Rectiflexibles	Spiral	Spiral	Spiral
Spore mass colour	White	Grey	Grey	Grey	Grey
Aerial mycelium	Grey	Light grey	Brown grey	Light brown grey	Grey
Substrate mycelium colour	Yellow brown	Yellow brown	Grey brown	Yellow brown	Yellow brown
Soluble pigment colour	Yellow pigment	Yellow pigment	Nil	Nil	Nil
Melanin reaction	+	−	+	+	−
H_2S production	−	−	−	−	−
Tyrosine reaction	+	−	+	+	−
Starch hydrolysis	−	+	+	−	+
Casein hydrolysis	−	−	+	+	+
Gelatin hydrolysis	+	−	−	+	+
Milk coagulation & peptonization	+	+	−	−	+
Nitrate reduction	+	+	+	−	+
Growth temperature range? (°C)					
10	−	−	−	−	−
20	+	+	+	+	+
28	+	+	+	+	+
37	+	+	+	+	+
pH tolerance	5-8	6-8	6-8	5-9	6-8
Cell wall type	I	I	I	I	I

isolate BC-1 showed growth in pH range 5 to 8 and the isolate BC-5 showed growth in the pH range of 5 to 9. The cell-wall peptidoglycon of all the isolates contained LL-diaminopimilic acid and glycine and the whole cell hydrolysates contained xylose. This indicated that they belong to the cell wall type I which is the characteristic of genus *Streptomyces.*

DISCUSSION

The composition of a starch casein agar (SCA) is suitable for the selective isolation of aerobic Actinomycetes (El-Nakeeb and Lechevalier et al., 1962). In this present study, SCA medium was used for isolation of Actinomycetes from soil sample. The results were observed as filamentous, branching bacteria with a fungal type of morphology. Screening of marine sediment sample near the Pongibalu area of Andaman and Nicobar Marine Islands resulted in the isolation of six isolates of Actinomycetes were shown in Figure 1. Among these, only five isolates exhibited the anti-microbial activity in the primary screening test against bacterial and fungal pathogens; one of those showed in Figures 2 and 3. Where as in the secondary screening out of the five active isolates, BC-1 isolate showed the potent activity against the both bacterial and fungal organisms showed in Figures 4 and 5.

In the earlier study of screening of marine actinobacteria for antimicrobial compounds carried out by Siva et. al. on the marine sediments collected from Bay of Bengal near Pudimadaka coast of Andhra Pradesh, it was observed that out of 78 isolates, 12 isolates have antimicrobial activity, 11 isolates have antifungal activity and 11 isolates have both antimicrobial as well antifungal activity. Isolate BTS 103 showed activity against B. subtilis with the zone of inhibition of 18 mm. Isolate BTS 103 showed activity against C. albicans with a zone of inhibition of 12mm.

In our present study, the zone of inhibition for BC-1 was maximum of 16 mm against *B. subtilis* showed in Figure 2 and BC-1 isolate exhibited potent activity against *C. albicans* and the zone of inhibition was 13 mm as shown in Figure 3.

In this study, various biochemical tests were performed such as melanin reaction, H2S production, tyrosine reaction, starch hydrolysis, casein hydrolysis, gelatin hydrolysis, milk coagulation and peptonization, nitrate reduction, temperature range of growth, pH tolerance and cell wall type to identify the Actinomycetes up to species level.

But due to the lack of other tests, apart from proper identification of genera of Actinomycetes, besides morphological and physiological properties (Kuster, 1972) and by comparing all these results with the Bergey's manual of Determinative Bacteriology , isolates were

identified to belong to the genus Streptomyces.

Conclusion

A total of six isolates of Actinomycetes from marine sediments of Andaman Islands were collected. Each isolate was tested against five pathogenic bacteria and also against some pathogenic fungi. Among the six isolates, one of the isolates showed potent activity against all the bacteria and fungi. After performing some biochemical tests, all isolates were identified to belong to the genus *Streptomyces*. The spent broth which was taken from the antibiotic production media of BC-1 isolate are subjected to antimicrobial activity which showed potent activity against bacterial and fungal pathogens. Further purification of this spent medium may gives the more activity than the standard antibiotics and also effective against some multidrug resistant pathogens.

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