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# RESEARCH ARTICLE

# ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FROM MARINE SEDIMENTS AND EVALUATION OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF ACTINOMYCETES AGAINST PATHOGENS

***Sari Dharmaveera, Sandhya Deepika, D. and Yamuna, M.M.**

Department of Botany, Andhra University, Visakhapatnam, India, Pin- 530 003

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## ABSTRACT

The main objective of the present study was isolation, purification, and characterization of actinomycetes from soil samples, having antimicrobial activity against 10 selected pathogenic strains. Marine soil samples were collected from the Bay of Bengal, off the coast of Andhra Pradesh, India. Potential colonies were screened, purified, and stored in glycerol stock. Isolates were morphologically and biochemically characterized. Totally 31 actinomycete isolates were tested for antagonistic activity against 10 pathogenic microorganisms. Isolates DHB-201, DHB-308, DHB-603 and DHB-702 were highly active, while other showed less activity against the pathogenic microorganisms. Isolate DHB-603 exhibited the highest antagonistic activity against all bacterial organisms and DHB-505 showed the highest activity against fungal organisms. All actinomycetes isolates showed antibacterial activity against *B. subtilis*, while they showed less activity against *P. aeruginosa*. These isolates had antibacterial activity and could be used in the development of new antibiotics for pharmaceutical or agricultural purposes.

***Key words:*** Actinomycetes, Isolations, Antibiotics, Antimicrobial.



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## INTRODUCTION

The demand for new antibiotics continues to increase due to the rapid emergence of multiple antibiotic resistant pathogens causing life threatening diseases (Pelaez, 2006). Thus, it is highly essential and important to search for novel antibiotics particularly from microorganisms to combat the threat of increasing population of antibiotic-resistant bacteria. Based on the literature survey, marine environments of India particularly off the coast of Andhra Pradesh, soil habitats have not been properly surveyed or exploited for the diversity of actinobacteria and their novel secondary metabolites. Hence, the present investigation has been designed to facilitate the isolation and screening of potent actinobacteria with high antimicrobial compound production from the marine soil samples collected from the Bay of Bengal, off the coast of Andhra Pradesh, India. The study also included the evaluation of the antimicrobial efficacy of these actinobacterial isolates against some pathogenic microorganisms followed by the characterization of the antimicrobial compounds and its producers. Detection and isolation of high-yielding species from the natural source material containing a heterogeneous microbial population called screening (Patel et al, 1995).

***Corresponding author:*** **Sari Dharmaveera,**
Department of Botany, Andhra University, Visakhapatnam, India, Pin- 530 003.

Usually screening programs include primary screening and secondary screening. Primary screening consists of some elementary tests required to detect and to isolate microorganisms possessing the desired property from along a large microbial population. The simplest primary screening technique for antibiotic producers is the crowded plate technique (Patel et al.,1995). This technique has a limited application, since it merely provides information regarding the inhibitory activity of a colony against certain microbes that may be present by chance on the plate. Therefore, the technique has been enhanced upon by introducing the use of a test organism. The isolates were further screened for their antimicrobial activities using cross steak method. Some of the best antibiotic producers isolated in this way are further investigated by secondary screening. Secondary screening helps in the detection of useful microorganisms in a fermentation process and is strictly necessary in any systematic screening programme (Patel et al, 1995). Primary screening purely allows the detection and isolation of microorganisms possessing potentially interesting industrial applications while secondary screening is projected to isolate industrially useful microorganisms. Secondary screening is carried out employed liquid media in flasks. The culture filtrate was tested for antimicrobial activities using cup plate method and paper disc diffusion assay method. Several methods were suggested for the screening of bioactive actinomycetes.

The marine sediment as a source of bioactive actinomycetes was less exploited. One of the successful approaches to obtain new types of useful microbial metabolites is to carry out investigations on marine sediments where the different ecological conditions and requirements may produce different types of metabolites in contrast to organisms occurring in terrestrial environment. The strategies for the investigation of marine organisms have been described by Fenical and Jensen 1994. Since the first report on antagonistic marine actinomycetes, only few efforts were made by various researchers (Okami et al., 1986; Pisano et al., 1989). The list of novel actinomycetes and products derived from poorly explored areas of the world, stresses the importance of investigating new habitats (Nolan and Cross 1988). The Bay of Bengal near South East coast of Andhra Pradesh, India is an unexploited marine source for marine actinomycetes and evaluation of their antimicrobial activities.

## MATERIALS AND METHODS

### Collection of Marine Sediment Samples

A total of 8 marine sediment samples were collected from 4 different locations of the Bay of Bengal along the South East coast of India. These samples were collected using a grab sampler. These sediment samples were collected in sterile zipped polythene bags and are carried to the lab for further analysis. The sediment samples were brown to black in colour and of muddy texture.

### Isolation of Actinomycetes from Marine Samples

Actinobacteria were isolated from marine sediment samples by plating them on suitable agar media with different dilutions. Actinobacteria colonies can easily be distinguished on the plate from those of fungi and non-filamentous bacteria. They are filamentous, compact, chalky, firm and often leathery giving a conical appearance and have a dry surface. The following media were used for the isolation of actinobacteria: 1. Chitin agar media (Hsu and Lockwood, 1975); 2. Glucose yeast extract malt extract agar media (Shirling and Gottlieb, 1966); 3. Starch casein agar media (Küster and Williams, 1964); 4. Glycerol asparagine agar media (GA agar) (Shirling and Gottlieb, 1966)

### Screening of Actinomycetes for Antimicrobial Activity

### Primary Screening for Antimicrobial Activity

The antimicrobial activities of the isolates were tested by Cross-Streak method employing Nutrient agar 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 40-45°C, poured into Petri plates (4 inch diameter) and allowed to solidify. Each plate was streaked with one isolate at the centre and incubated at 28°C for 7 days. After 7 days, test organisms were streaked perpendicular to the growth of the isolate; 4 day cultures of bacteria and fungi were used as the test organisms. All the test organisms employed in the present investigation were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. The test organisms used for the determination of antifungal activities are *S.aureus (MTCC 3160), B.Subtilis (MTCC 441), P. aeruginosa (MTCC 424), B. cereus (MTCC 430), P. vulgaris (MTCC 426), E. coli (MTCC 443), S. cerevisiae (MTCC 170), A. niger (MTCC 961), A. flavus (MTCC 3396), C. albicans (MTCC 227)*

### Secondary Screening for Antimicrobial Activity

The active isolates resulting from the primary screening were tested for their extra cellular antifungal compound production capabilities under submerged fermentation conditions. The production medium containing glucose 1%, soyabean meal 1%, NaCl 1% and $CaCO_3$ 0.1% with pH 7.6.

### Cup-plate Method

Cups of 6 mm diameter were made using sterile cork borer in media plate. Fifty ml of clear supernatant from fermentation broth was added to each cup. The plates were kept in refrigerator for about 2 hours to allow the diffusion of antifungal and anti bacterial compound and later incubated at 37°C and 28°C for bacterial and fungal growth respectively. The inhibition zones were measured using an antibiotic zone reader after 48 h of incubation.

### Characterization of Actinomycetes

### Morphological Characteristics

The macro, micro morphological features of the colonies, colour characteristics of the aerial mycelium, substrate mycelium, soluble pigments and biochemical characters were evaluated after 14 days of incubation at 28°C.

## RESULTS AND DISCUSSION

### Isolation of Actinomycetes

Soil sediment samples were obtained from 8 locations at different depths of the Bay of Bengal area. 36 isolates of actinomycetes were obtained from marine soil sediments from the Bay of Bengal in our investigation. 12 isolates were showed activity against the bacterial test organisms. 8 isolates were showed activity against the bacterial and fungal test organisms. The 8 organisms which showed potent antimicrobial activity against the test organisms were selected for further studies. Alexopoulous et al., (1941) were the first to show that as many as 56% of all cultures of actinomycetes isolated from the soil possessed some antifungal properties. Nearly 17.5% of the cultures were strong inhibitors of fungal growth. This wide distribution of antifungal agents among the actinomycetes was confirmed by Cooper and Chilton (1949). As many as half of the freshly isolated cultures from soil were found to have some effect upon fungi and 20% or more had a market effect. The antibiotics produced by these organisms have a wide spectrum, although some are highly active against yeast like fungi and others against filamentous fungi (Waksman et al., 1952; Mustafa Oskay et al., 2009).

### Primary Screening for Antimicrobial Activity

During the course of survey, a total of 36 actinobacteria were isolated from the 8 marine sediment samples, by using Chitin agar, Starch Casein Agar, Glycerol Asparagine Agar, and Glucose Yeast extract Malt extract Agar media. The culture of the isolation plates are shown in Figure 1 and the pure cultures of some of the isolates were shown in Figure 2. The isolates were analyzed for antimicrobial activity preliminarily by cross streak method.

**Table 1. Isolates showing antibacterial activity**

| Isolate No. | Name of the Test Organism (Inhibition zone diameter in mm) | | | | | |
|---|---|---|---|---|---|---|
| | *E.coli* | *Proteus vulgaris* | *Pseudomonas aeruginosa* | *Bacillus subtilis* | *Bacillus cereus* | *Staphylococcus aureus* |
| DHB – 101 | 12 | 10 | 12 | 12 | -- | 10 |
| DHB – 201 | 16 | 13 | 12 | 10 | 13 | 12 |
| DHB – 308 | 20 | 18 | 16 | 16 | 20 | 28 |
| DHB – 311 | 14 | -- | -- | 12 | -- | -- |
| DHB – 505 | 14 | 10 | -- | 14 | -- | -- |
| DHB – 603 | 31 | 32 | 28 | 28 | 34 | 28 |
| DHB – 404 | 12 | 10 | -- | 10 | 12 | -- |
| DHB – 806 | 16 | -- | 12 | 10 | -- | 12 |
| DHB – 702 | 18 | 13 | 12 | 10 | 12 | 14 |

'—' Inhibition zone not observed.

**Table 2. Isolates showing antifungal activity**

| Isolate No. | Name of the Test Organism (Inhibition zone diameter in mm) | | | |
|---|---|---|---|---|
| | *A.niger* | *A.flavus* | *C.albicans* | *S.cerevisiae* |
| DHB – 101 | -- | 12 | 14 | 12 |
| DHB – 201 | 28 | 32 | 30 | 28 |
| DHB – 308 | 10 | -- | 14 | 10 |
| DHB – 311 | 14 | 20 | 14 | 21 |
| DHB – 505 | 18 | 20 | 14 | 12 |
| DHB – 603 | -- | 16 | -- | 18 |
| DHB – 404 | 16 | -- | 14 | 12 |
| DHB – 806 | 18 | 20 | -- | 14 |
| DHB – 702 | 16 | 18 | 14 | -- |

**Table 3. Morphological and Cultural Properties of Potent Isolates**

| Morphological and Cultural characteristics of the active isolates | | | | | | |
|---|---|---|---|---|---|---|
| Isolate No. | Morphological characteristics | | | Cultural characteristics | | |
| | Spore bearing hyphae | Spore mass colour | Growth | Vegetative mycelia colour | Aerial mycelia colour | Soluble pigment |
| DHB–101 | Retinaculum apertum | Brown | Abundant | Light brown | Brown | Reddish Brown |
| DHB–201 | Flexous | Light yellow | Good | Yellow | Brown | Brown |
| DHB–308 | Retinaculum apertum | Dark brown | Abundant | Dark yellow | Brown | Reddish brown |
| DHB–311 | Monoverticillus | Black | Abundant | Light brown | Brown | Nil |
| DHB–505 | Retinaculum apertum | Green | Abundant | White | Green | Green |
| DHB–603 | spirals | Grey | Good | Light brown | Grey | Brown |
| DHB–404 | Flexous | Light yellow | Good | Yellow | Brown | Nil |
| DHB–806 | Retinaculum apertum | Black | Abundant | Light brown | Grey | Brown |
| DHB–702 | Spirals | Green | Good | White | Brown | Green |

'—' Inhibition zone not observed.

Among these isolates, 12 isolates exhibited antibacterial activity, out of which 8 isolates showed potent antifungal activity. All the isolates which exhibited antifungal activity also showed effective antibacterial activity. These active isolates were later subjected to submerged fermentation studies.

## Secondary Screening for Antimicrobial Activity

Secondary screening is useful in sorting out microorganisms that have real commercial value from many isolates obtained during fermentation process. 8 isolates showed potent antibacterial and antifungal activity (Table 1& 2). Among them, the isolate no. DHB-201 exhibited highest antimicrobial activity when compared to the other isolates. The isolate DHB-603 showed good antibacterial activity against E. coli, B. subtilis and other test organisms. But it showing less antifungal activity on fungal test organisms *Aspergillus niger, Geotrichum candidum, Saccharomyces cerevisiae,* and *C. albicans*, whereas, it showed mild activity against *Saccharomyces cerevisiae.* Similarly the isolate DHB-311 showed good antifungal activity against *A.niger and A.flavus*. Some of the isolates such as DHB-101, DHB -308 and DHB - 505 exhibited good antimicrobial activity against a specific organism and showed mild activity against the other test organisms.

Lim *et al*., (2000) reported that among 32 thermophilic actinomycetes which showed antifungal activity against 6 plant pathogenic fungi on V8 agar, 12 actinomycetes were selected for further *in vitro* bioassay for antifungal activity of culture filtrates. Among the tested actinomycetes, the butanol and methanol extracts of the actinomycetes strain S5-55 showed a broad spectrum of antifungal activity. Antagonistic actinobacterial strains were isolated from different marine sediment samples collected from Tuticorin coast, South India, exhibited inhibitory activity against fish pathogens namely *Aeromonas hydrophila*, *Aeromonas sobria* and *Edwardsiella tarda*. Three *Streptomyces* strains designated as DKDVIT 1, 2 and 3, isolated from the marine soil samples of Ennore coastal region of Tamil Nadu, India showed antidermatophytic activity (Lakshmipathy and Krishnan 2009). Actinobacteria from the surface microlayer in the Trondheim fjord, Norway, have been isolated and characterized. The strain exhibited antagonistic activity against non-filamentous fungi and Gram-negative and Gram positive bacteria (Hakvag *et al.,* 2008). Praveen Kumar Jain *et al.,* (2007) reported the antifungal activity of *Streptomyces* strain GS 1322, isolated from garden soil sediment, which inhibited the growth of fungi (*Aspergillus niger, Candida albicans, Microsporium gypseum and Trichophyton sp.*) suggesting the broad spectrum of the bioactive compounds.

**Table 4. Physiological and Biochemical Characteristics of Promising Isolates**

| | Isolates | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Reaction | DHB–101 | DHB–201 | DHB–308 | DHB–311 | DHB–505 | DHB–603 | DHB–404 | DHB–806 | DHB–702 |
| Melanin reaction | - | + | + | + | - | - | - | - | - |
| • ISP-1 | + | + | + | + | - | + | + | + | - |
| • ISP-6 | + | + | + | + | - | + | + | + | - |
| • ISP-7 | | | | | | | | | |
| H2S production | + | + | + | + | - | + | + | + | - |
| • ISP-6 | | | | | | | | | |
| Tyrosine reaction | + | + | + | + | + | - | + | - | + |
| • ISP-7 | | | | | | | | | |
| Starch hydrolysis | + | - | + | + | + | + | + | + | + |
| Casein hydrolysis | + | - | + | - | + | + | + | + | + |
| Gelatin hydrolysis | + | + | + | + | + | + | + | + | + |
| Milk coagulation & peptonization | + | + | - | - | + | + | + | + | + |
| Nitrate reduction | + | + | + | + | - | + | + | + | - |
| Methyl red | - | + | - | + | + | + | - | + | + |
| Voges-Proskauer | - | - | - | - | - | - | - | - | - |
| Citrate | + | + | + | + | + | + | + | + | + |
| Oxidase | - | + | - | - | - | - | - | - | - |
| Urease | + | + | + | + | + | + | + | + | + |
| Catalase | - | + | - | - | + | - | - | - | + |
| Growth temperature | | | | | | | | | |
| 10°C | - | - | - | - | - | - | - | - | - |
| 20°C | + | + | + | + | + | + | + | + | + |
| 28°C | + | + | + | + | + | + | + | + | + |
| 37°C | + | + | + | + | + | + | + | + | + |
| $P^H$ tolerance | 6-9 | 5-9 | 5-9 | 5-9 | 6-9 | 6-9 | 6-9 | 6-9 | 6-9 |

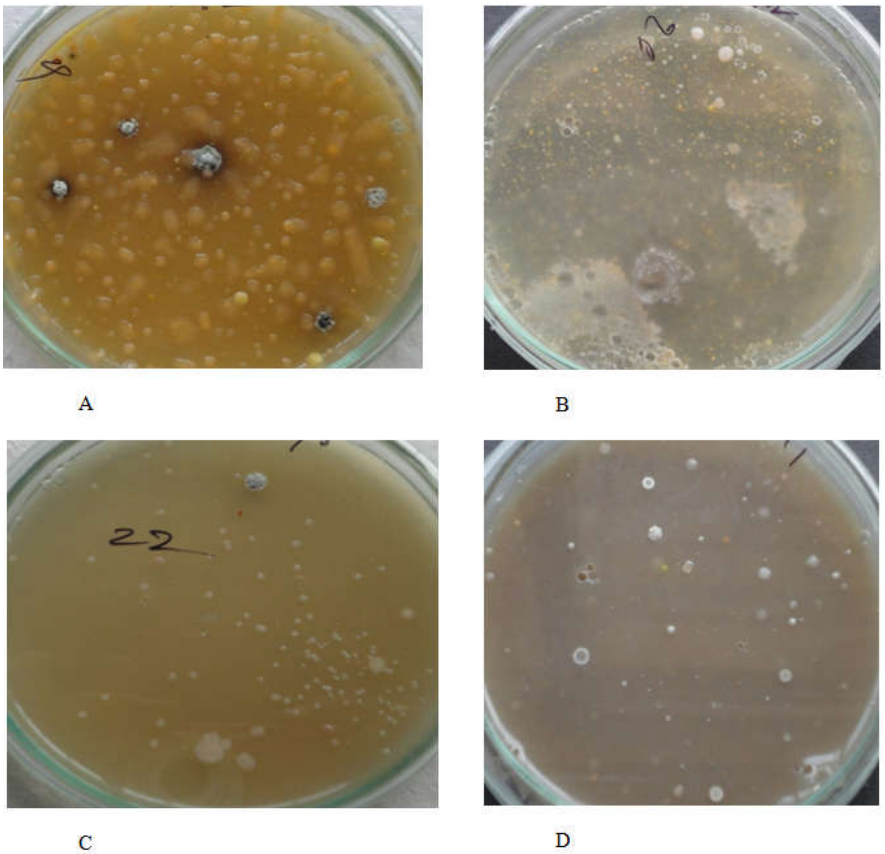


**Figure 1: Isolation of Actinobacteria. A) GYM agar plate showing grey colony Actinomycetes Isolate; B) Different colonies of Aerobic Actinobacteria on Starch casein agar media plate; C) Folded colonies of Actinomycete Isolate; D) Actinomycetes isolation agar plate showing different Actinomycete Isolate.**

Research on the biodiversity of marine actinobacteria is not only important for the basic studies but also necessary for their exploitation. There are only few comprehensive reports on the marine antagonistic actinobacteria from India. The existence of these bacteria in the marine sediments collected at different depths, along the south east coast of Bay of Bengal, India specifies that it is an underexploited highly diversified ecosystem suitable for screening actinobacteria capable of producing bioactive compounds.

## Characterization of Actinomycetes

**Morphological Characteristics:** All the six isolates which showed promising antimicrobial activities were identified as belonging to the genus *Streptomyces,* family

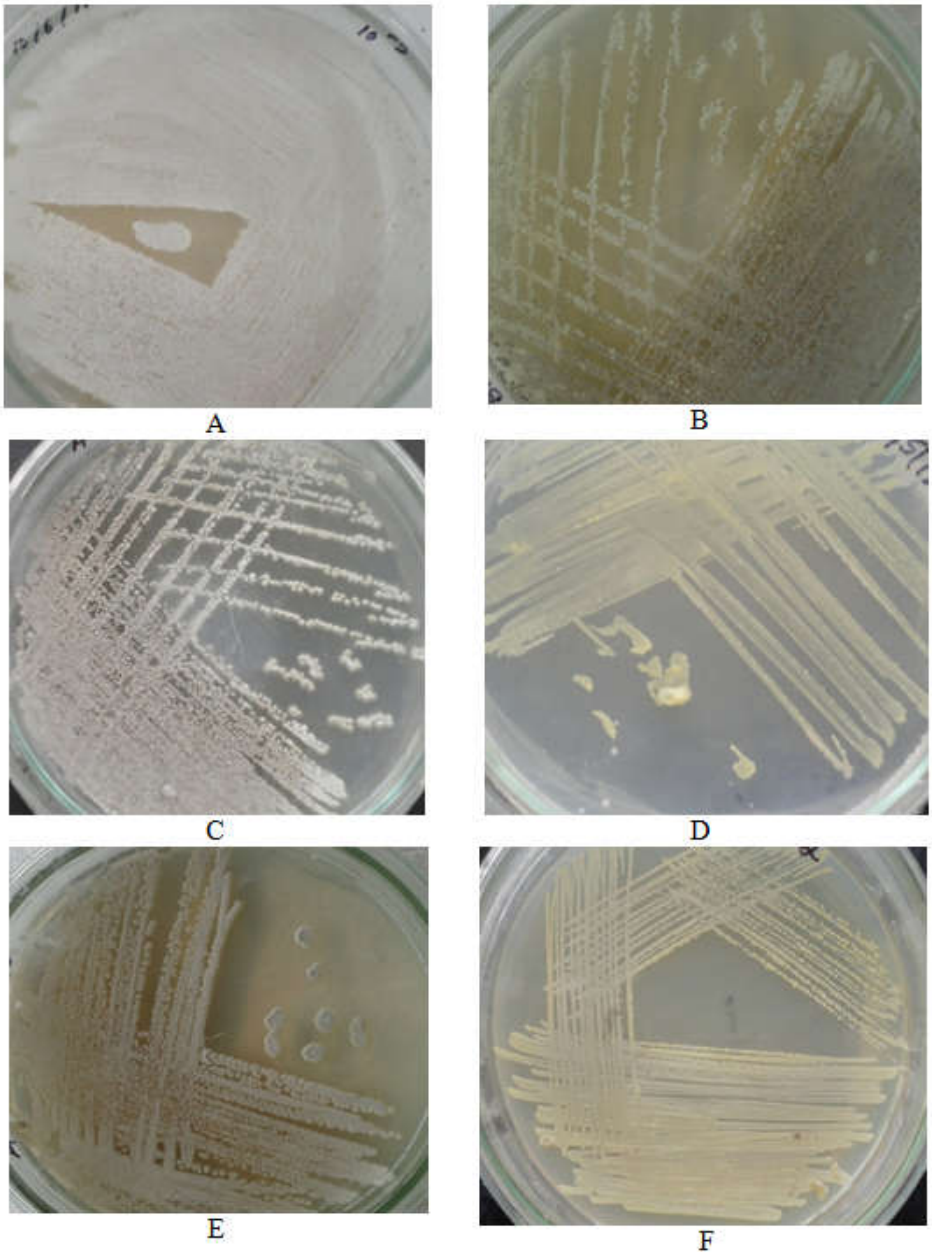


**Figure 2. Pure Culture of Actinobacteria. A) White Coloured Actinobacterial Colonies with Chalky Appearance over the Surface; B) Cream Coloured with Folded Colonies; C) Grey Coloured Folded Colonies; D) Culture with yellow Coloured colonies; E) Light brown Coloured Colonies; F) Cream Coloured Colonies**

**Table 5. Sodium chloride Tolerance of the Promising Isolates**

| | Sodium chloride Tolerance | | | | |
|---|---|---|---|---|---|
| Isolates | 1% | 4% | 7% | 10% | 13% |
| DHB – 101 | Good | Good | Good | Moderate | No growth |
| DHB – 201 | Good | Good | Good | Moderate | No growth |
| DHB – 308 | Good | Good | Good | Moderate | No growth |
| DHB – 311 | Good | Good | Good | No growth | No growth |
| DHB – 505 | Good | Good | Good | Moderate | No growth |
| DHB – 603 | Good | Good | Good | No growth | No growth |
| DHB – 404 | Good | Good | Good | No growth | No growth |
| DHB – 806 | Good | Good | Good | Moderate | No growth |
| DHB – 702 | Good | Good | Good | No growth | No growth |

Streptomycetaceae (spore chain with coiling and branching). The predominance of *Streptomyces* in any actinomycete population is a well-known fact. Jensen *et al.,* (1991) reported the number of marine *Streptomyces* in the actinomycete population in relation to the depth, where the distribution accounted for by a rapid decrease in *Streptomycetes* and an increase in actinoplanetes and *Micromonospora* with increasing depth.

This is also reflected in our present investigation on marine sediments of south east coast of Bay of Bengal. In a similar study, Maldonado *et al.,* 2004 demonstrated that the genus *Streptomyces* is a widely distributed actinomycete in diverse marine habitats. The morphological and cultural properties of promising isolates are shown in Table-3. The results indicated that among 6 isolates, 1 was characterized as spiral type, 1 were as flexous type and 3 were as retinaculum apertum type

of spore bearing hyphae. Morphological observation of 14 day old culture of isolates grown on different ISP media revealed that the vegetative mycelia and the aerial hyphae were abundant, well developed and varied from grey – white, creamy – yellow, and yellow – light brown colour on different test media. Most of the isolates grew well on the ISP -2, ISP-4 and ISP -5 media. Whereas, on ISP -3 medium the vegetative mycelia and aerial hyphae were poorly developed.

**Physiological and Biochemical Characteristics**

The physiological and biochemical properties of 6 promising isolates are shown in Table 4, Melanin pigmentation was shown by DHB-201, DHB-308 and DHB-311 on all the three test media. While the isolates DHB-101 and DHB-603 produced melanin in only two test media. Whereas, the isolates DHB-505 did not show melanin pigmentation in any of the media used in the study. All the isolates were $H_2S$ positive except DHB-505. Tyrosine reaction was positive for all the isolates except DHB-603.

Starch hydrolysis was positive for all isolates expect DHB-201. Casein hydrolysis was positive for isolates (DHB-201 and DHB-311) and negative for the remaining isolates. Gelatin hydrolysis was positive for all the isolates. Milk coagulation and peptonization test was negative for 2 isolates (DHB-308 and DHB-311), while the remaining isolates showed positive test. Nitrate reduction test was negative for 1 isolates (DHB-505), while the other isolates exhibited positive test. All the promising isolates showed negative reaction to Voges-Proskauer and oxidase tests. The isolates DHB-201, DHB-311, DHB-505 and DHB-603 showed positive test for methyl red. The citrate and urease tests were positive for all the isolates. Out of 6 isolates, only 2 isolate (DHB-201 and DHB-505) were positive for catalase test. The isolates grew well at all the temperatures ranging from 20 to 37˚C. All the isolates were studied for their ability to tolerate different pH values. Out of 6 isolates, 3 isolates (DHB-201, DHB-308 and DHB-311) showed growth between pH 5.0 and 9.0 and 3 isolates (DHB-101, DHB-505 and DHB-603) between pH 6.0 and 9.0. This indicates that these isolates can withstand pH range from acidic to alkaline.

The cell wall peptidoglycan of all the 6 isolates contained LL-diaminopimelic acid and glycine; the presence of LL-diaminopimelic acid was indicated by a characteristic olive green ninhydrin reaction fading to yellow during paper chromatography. It is a rapid method of differentiation between the *Streptomycetes* and most other actinomycetes by paper chromatography of whole cell hydrolysates (Becker *et al.,* 1964). The whole cell hydrolysates do not contain characteristic sugar. This indicated that they belong to cell wall type I, which is a characteristic of the genus *Streptomyces* (Lechevalier and Lechevalier 1970a, b). By performing the morphological, biochemical and chemotaxonomic studies, it was revealed that these promising isolates belong to the widely distributed genus *Streptomyces*. The sodium chloride tolerance of the isolates is shown in Table 5. All the isolates exhibited good growth up to 7% (w/v) NaCl concentration. 4 isolates (DHB – 101, DHB – 201, DHB – 308 and DHB – 505) showed moderate growth at 10% (w/v) NaCl concentration and the remaining isolates exhibited no growth. At 13% (w/v) NaCl concentration growth was not observed for any isolate. On the basis of morphological and chemotaxonomic characteristics and by the comparison of the characteristics of previously published species with that of the isolates, they were identified as belonging to the genus *Streptomyces.* In order to characterize an isolate up to species level, an in-depth study of molecular analysis and a polyphasic approach is essential.

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