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Isolation, Screening and Characterization of Marine Actinomycetes for Novel Bioactive Compounds

Mantripragada Visalakshi^{1*}, Veerendra Kumar Bapatla¹

*1. Department of Biotechnology, GITAM Institute of Science, GITAM University,
Visakhapatnam, India.*

ABSTRACT

The present study deals with the isolation, screening and characterization of the actinomycetes from the marine sediments, which were collected from different locations of Bay of Bengal. Selective media and the pre-treatment strategies enhanced the isolation and screening of novel marine actinomycetes. A total of 8 marine samples were collected. The pre-heat treatment method and a combination of 4 enrichment media were found to be effective in selectively isolating marine actinomycetes. The top six potent isolates were subjected to detailed morphological, cultural, biochemical and physiological characterization. A total of 63 isolates were isolated. The antimicrobial activity was studied for all the 63 isolates. The preliminary study of 63 isolates for antimicrobial activity by cross streak method indicated that 42 isolates had antagonistic properties. All these 42 isolates were subjected to submerged fermentation studies. It was observed that 12 isolates exhibited antibacterial activity, 6 isolates showed antifungal activity whereas 6 isolates exhibited both antibacterial and antifungal activities. The present study was an attempt to use different methods to screen, select and isolate marine actinomycetes from the sediments of Bay of Bengal with intrinsic antimicrobial activity against a variety of microbial pathogens.

Keywords: Marine actinomycetes, Antimicrobial activity, Bioactive compounds.

*Corresponding Author Email: pragada86@gmail.com

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INTRODUCTION

Marine environment contains a wide range of distinct microorganisms that are not present in the terrestrial environment. Though some reports are available on antibiotic and enzyme production by marine actinomycetes, the marine environment is still a potential source for new actinomycetes, which can yield novel bioactive compounds and industrially important enzymes¹. Recently the rate of discovery of new compounds from terrestrial actinomycetes has decreased whereas the rate of re-isolation of known compounds has increased. Thus, it is excited that new groups of actinomycetes from unexplored or under exploited habitats be pursued as sources of novel bioactive secondary metabolites². Although the diversity of life in the terrestrial environment is extraordinary, the greatest biodiversity is in the oceans³. As marine environmental conditions are extremely different from terrestrial ones, it is surmised that marine actinomycetes have different characteristics from those of terrestrial counter parts, and therefore might produce different types of bioactive compounds^{4,5}. Around 23000 bioactive secondary metabolites produced by microorganisms have been reported and among them 10000 compounds were produced by actinomycetes⁶. Vast numbers of these antimicrobial agents are discovered from actinomycetes by screening natural habitat such as soil and water bodies⁷. Since late 1980's the number of novel compounds isolated from terrestrial microorganisms has steadily decreased. To cope up with demand for new pharmaceutical compounds and to combat the antibiotic resistant pathogens, researches have been forced to look for novel microorganisms in unusual environment. Relatively, the present study deals with screening for the isolation of actinomycetes from the marine sediments in and around Visakhapatnam coastal regions, which produce antibiotic compounds, in turn we also aimed for the evaluation of their antimicrobial activities.

MATERIALS AND METHOD

Sampling

A total of 8 marine sediments were collected along the South East coast of the Bay of Bengal at various depths of 2-5m using grab sampler. They were maintained at ambient temperature with sea water and brought to the laboratory in sterile zipped polypropylene bags for further analysis.

Isolation of actinomycetes

Serial dilution method

Isolation and enumeration of marine actinomycetes were performed by the serial dilute plate technique⁸. 1 g each of the marine sediment sample was added to 50ml of sterilised sea water taken in a 250ml Erlenmeyer flask. The flasks were agitated for about one hour, to ensure the separation

of the filamented actinobacteria from the sediment and are released into the medium. The marine sediment was filtered and the filtrate was used for the serial dilution. The filtrate was serially diluted in a series of test tubes to obtain 10^{-1} to 10^{-7} dilution using the sterilized sea water. 1 ml each of these dilution were added to 50 ml of sterile molten starch casein agar medium thoroughly mixed and poured into petriplates and incubated at 28°C for 3 days to 3 weeks. Different media like starch casein agar media, chitin agar media, glycerol asparagine agar media, glucose yeast extract malt extract agar media are used for isolation technique.

Heat treatment

The samples were heated by incubating at 55°C for 15 min in a water bath⁹. 10 fold serial dilutions of these dement samples were made using sterile 50 % sea water¹⁰. About 0.1ml of the serially diluted samples was spread over Starch case in agar medium¹¹ and Actinomycetes isolation agar medium. Both the media were supplemented with 5µg/ml rifampicin and 25 µg/ml of Nystatin (Himedia, Mumbai) to minimize the other bacterial and fungal growth¹². All the plates were incubated at 28°C for 21 days. The appearance and growth of marine actinomycetes colonies were recognized by their characteristic chalky to leathery appearance. All the morphologically different actinomycetes colonies were sub-cultured on yeast extract malt extract agar medium.

Pre-enrichment method

One gram of sediment was transferred to conical flasks containing 100ml of sterile sea water, starch casein broth and glucose asparagine broth prepared with natural seawater separately for the pre-enrichment of samples. The flasks were incubated at 30°C for 14days in a shaker incubator. A loop-full of in oculum from the pre-enriched starch case in broth and glucose as paragine broth was streaked on starch casein agar (SCA) and glucose as paragon agar separately and the plates were incubated at 30°C for 7 days. Single discrete colonies were isolated and identified. All the morphologically different act in omycete colonies were sub cultured on yeast extract malt extract agar medium (ISP No.2)¹³ by streak plate technique. After growth appeared, the actinomycetes colonies were maintained in ISPNo.2 agar slants.

Primary Screening for Antimicrobial Activity

The antimicrobial activities of the isolates were tested by Cross-Streak method employing Mueller Hinton agar medium for bacteria and Sabouraud dextrose agar medium for fungi. The media was sterilized by autoclaving at 121°C and 15lbs pressure for 15 min and the molten sterile media was cooled to 40-45°C, poured into petriplates (4 inch diameter) and allowed to solidify. Each plate was streaked with one isolate at the center and incubated at 28°C for 7 days. After 7 days, test organisms were streaked perpendicular to the growth of the isolate; 24 hours old cultures of

bacteria, 4 days old cultures of fungi and 2 days old cultures of yeast were used to test the 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 antimicrobial activity are *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 430), *Pseudomonas aeruginosa* (MTCC424), *Escherichia coli*(MTCC443), *Proteus vulgaris* (MTCC 426), *Saccharomyces cerevisiae* (MTCC170), *Candida albicans* (MTCC 227), *Aspergillus niger* (MTCC961), and *Aspergillus flavus*(MTCC3396).

Secondary Screening for Antimicrobial Activity

Based on the results of primary screening 6 potent *Streptomyces* isolates namely GIBT-101, GIBT-201, GIBT-308, GIBT-311, GIBT-505, GIBT-603 were used for the antibiotic production by fermentation method.

Secondary Screening for Antibiotic Production Agar Well Diffusion Method

Secondary screening of promising isolates was done by submerged fermentation. Slant cultures of mature actinomycete strains were inoculated in the medium contain in gsoyabeanmeal 20g, glucose 20g, NaC 14g, K₂HPO₄ 0.05g, MgSO₄ 0.50g and CaCO₃ 5g for 1000 ml and maintained at pH 7.2. The cultures were incubated in arotary shaker (180rev/min) at 27°C for 7 days and the fermented broth was centrifuged at 10000 rpm at4°C for20 min. The supernatant was filtered using 0.45µm pore size membrane filter (Millipore)¹⁴. The clear supernatant samples were tested for their antimicrobial activity by agar well diffusion method. To determine the antibacterial spectrum, pathogenic bacteria cultured on nutrient broth at 37°C for 24 hours; the cultures were swapped on nutrient agar media. The relative activities of metabolites were determined based on the diameter of zones of inhibition formed. Secondary screening of potent actinomycetes confirmed the results of primary screening.

Characterization of Actinomycetes Cultures

The top six potent actinomycetes isolates selected for screening were characterized, based on morphological, cultural, biochemical and physiological features. Morphological and cultural characteristics such as type of aerial hyphae, growth of vegetative hyphae, diffusible pigment and spore formation were observed. Biochemical tests including melanin pigmentation, H₂S production, tyrosinere action, starch, casein, gelatin hydrolysis, Milk coagulation & peptonization, Methyl red, Voges-Proskauer, citrate, Oxidase, Urease and Catalase tests were also performed using starch casein agar medium. Physical parameters such as the effect of p^H(5-9) and temperature (10°C-50°C)were also tested.

Utilization of carbon sources such as Glucose, Fructose, Mannitol, Rhamnose, Raffinose, Maltose, Lactose, Sucrose, Glycerol, Starch and Nitrogen sources namely L-Arginine, L-Tyrosine, L-Asparagine, L-Leucine, L-Cysteine, L-Histidine, L-Valine, and L-Glycine were tested on starch case in agar medium (Table 5 and 6). Sodium chloride tolerance: Sodium chloride tolerance level¹⁵ of the isolates were estimated on starch case in agar medium supplemented with graded doses of NaCl (1,4,7,10 and 13%) maximum NaCl tolerance concentration in the medium inhibiting growth was recorded (Table 7).

Table 5: Carbon Source Utilization Patterns for Promising Isolates

Carbon source (1% w/v)	Isolates					
	GIBT – 101	GIBT – 201	GIBT – 308	GIBT – 311	GIBT – 505	GIBT – 603
D-Glucose	Good	Good	Good	Good	Good	Good
L-Arabinose	Moderate	No growth	No growth	Moderate	Moderate	No growth
D-Galactose	Good	Moderate	Moderate	Good	Moderate	Moderate
D-Fructose	Good	Good	Good	Good	Good	Good
D-Xylose	Good	Moderate	Good	Moderate	Moderate	No growth
Meso-inositol	Good	Good	No growth	Good	Good	Good
D-mannitol	Good	Good	Good	Good	Good	Good
L-rhamnose	Moderate	Moderate	Moderate	No growth	Moderate	Moderate
Raffinose	Moderate	No growth				
Maltose	Good	Good	Good	No growth	No growth	Moderate
Lactose	Moderate	Moderate	Moderate	No growth	Moderate	Moderate
Sucrose	Good	Good	Good	Moderate	Moderate	Moderate
Glycerol	Good	Good	Good	Moderate	Moderate	Moderate
Starch	Good	Good	Good	Moderate	Moderate	Moderate

Table 6: Nitrogen source utilization patterns for promising isolates

Isolates	Nitrogen source (0.2% w/v)								
	KNO ₃	L- Arginine	L- Tyrosine	L- Asparagine	L- Leucine	L-Cysteine HCl	L- Histidine	L- Valine	Glycine
GIBT – 101	+	+	+	+	+	+	+	-	-
GIBT – 201	+	+	+	+	+	-	+	-	+
GIBT – 308	+	+	-	+	-	+	+	+	-
GIBT – 311	+	+	-	+	-	-	+	+	-
GIBT – 505	+	+	-	+	-	+	+	-	-
GIBT – 603	+	+	+	+	+	-	+	+	+

Table 7: Sodium chloride tolerance for promising isolates

Isolates	Sodium chloride tolerance				
	1%	4%	7%	10%	13%
GIBT – 101	Good	Good	Good	Moderate	No growth
GIBT – 201	Good	Good	Good	Moderate	No growth
GIBT – 308	Good	Good	Good	Moderate	No growth
GIBT – 311	Good	Good	Good	No growth	No growth

GIBT – 505	Good	Good	Good	Moderate	No growth
GIBT – 603	Good	Good	Good	No growth	No growth

RESULTS AND DISCUSSION

Marine sediments from South East coast of Bay of Bengal were selected as a potential source of marine actinomycetes and possible bioactivity. Various pre-treatment procedures and selective media were applied to assess the optimal conditions for the isolation of marine actinomycetes from sediments. Three different pre-treatment methods were employed for maximum isolation of actinomycetes. Serial dilution technique allowed the growth of actinomycetes, bacterial and fungal colonies (figure1). But the next two pre-treatment methods (figure2 and 3) inhibited growth of bacterial and fungal colonies. Hence it has been inferred that when these sediments were cultured without pretreatment, large number of bacterial and fungal colonies were grown, the dominance of other bacterial and fungal contamination was found to inhibit the colonization of actinomycetes. Whereas when the soil was pretreated, their numbers decreased on culture plates. Previously, several researchers suggested pre-treatment methods for isolation of actinomycetes^{10,16,17}. The pretreatment of wet-heating at 55°C for 15 min. Starch casein agar and glucose as paragine agar media were the most effective for the isolation of actinomycetes. The bacterial and fungal contamination was diminished by pre-heat treatment and allowed selective isolation of actinomycetes. Antibacterial and antifungal agents namely rifampicin 5µg/ml and nystatin 25 µg/ml were supplemented into the isolation medium, the number of bacteria and fungi were further decreased. The growth of marine actinomycetes colonies was recognized by their characteristic chalky to leathery appearance. All the morphologically different actinomycete colonies were sub-cultured on yeast extract malt extract agars lants (ISPNo.2). Amongst 8 marine sediments screened, 63 actinomycete colonies were isolated and 42 (66.7%) isolates exhibited antimicrobial activities. 12 (19.1%) isolates showed antibacterial, 6 (9.5%) showed antifungal activities and 6 (9.5%) confirmed both antibacterial and antifungal activities (Table 1 and 2). All the 63 isolates were identified at generic level based on the colony morphology and microscopic morphology. Frequency and dominance of *Streptomyces* among actinomycetes in various soil types was reported by several workers^{17,18}. The present study correlates with earlier reports, among that the isolates of *Streptomyces* were the dominant genera.

Table1: Antibacterial Inhibitory Zones for Isolates

Isolate No.	Name of the test organism (Inhibition zone diameter in mm)					
	<i>E.coli</i>	<i>Proteus vulgaris</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Staphylococcus Aureus</i>
GIBT – 101	12±2	12±1.5	12±3	10±2	--	12±3
GIBT – 201	14±2.5	12±2	11±2.5	12±2	12±3	10±3.5
GIBT – 308	16±2	14±2.5	14±2	14±2.5	18±3	24±2
GIBT – 311	12±2	--	--	10±2	--	--
GIBT – 505	10±3	12±1	--	12±2	--	--
GIBT – 603	32±2.5	31±1.5	29±3	31±2	33±2.5	29±3

* Each value represents the mean ± SD of three replicates

Table2: Antifungal inhibitory zones for isolates

Isolate No.	Name of the test organism (Inhibition zone diameter in mm)			
	<i>A.niger</i>	<i>A.flavus</i>	<i>C.albicans</i>	<i>S.cerevisiae</i>
GIBT – 101	--	10±2	10±2	12±3
GIBT – 201	28±1	30±2	27±2.5	29±3
GIBT – 308	10±1	10±3	--	10±2
GIBT – 311	16±2	22±2.5	12±2	18±3
GIBT – 505	20±1.5	16±2	12±2.5	--
GIBT – 603	--	16±2	20±2	18±3

**Figure 1: Growth of actinomycetes colonies on agar plate**

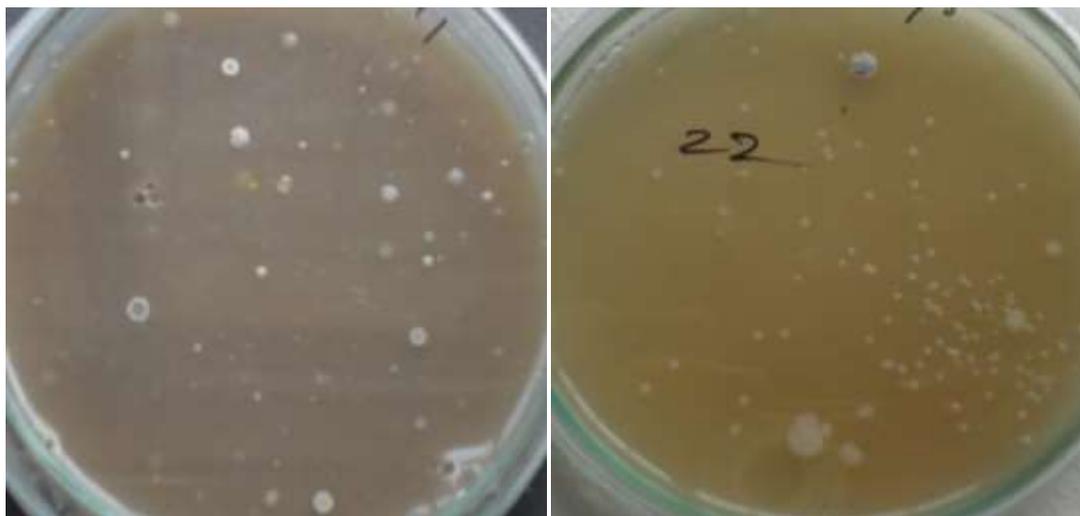


Figure 2 and 3: Selective growth of actinomycetes colonies on Starch casein and Glycerol Asparagine agar plates respectively

On both nutrient agar and PDA media, remarkably the strains GIBT- 101, GIBT- 201, GIBT- 308, GIBT-311, GIBT-505 and GIBT-603 have supreme activity against the organisms tested (Table 1 and 2). On Mueller Hinton agar medium and Sabouraud dextrose agar medium, the strain GIBT-603 exhibited maximum zone of inhibition against bacteria *S.aureus* (29mm) and *B.subtilis* (31mm) and fungus namely *C. albicans* (20mm) (figure6) and *S. Cereviseae* (18mm). Consequently, GIBT-603 was selected as a promising isolate and used for further identification. Morphological characterization of the broad spectral antagonistic isolates revealed dark grey coloured aerial mycelia, and dark grey to white coloured spore mass. However, the strain GIBT-101 developed light brown vegetative mycelium and brown aerial mycelium, GIBT-201 developed yellow vegetative mycelium and brown aerial mycelium, GIBT-308 developed dark yellow vegetative mycelium and brown aerial mycelium. The strain GIBT-311 developed monoverticillus spores and black spore mass, GIBT-505 developed green spore mass and retina culum apertum spores. Further, the strain GIBT-603 developed spiral spores, grey spore mass light brown vegetative mycelium and grey aerial mycelium (Table3). The details of biochemical and physiological characteristics, utilization of carbon and nitrogen sources of the isolates were displayed in (Table4, 5 and 6). It is also evident that different physiological characteristics are influencing the growth rate of the actinomycetes¹⁹. In general, biochemical and physiological characteristics and antimicrobial susceptibility patterns of the actinomycetes vary from isolate to isolate depending on the growth conditions. The present exploration concludes that the physiological characteristics of actinomycetes varied depending on the available nutrients in the medium and the physical conditions. Thus, it was concluded on the basis of the present and previous studies that the nutrient

compositions of the medium greatly influence the growth and morphology of organisms²⁰. The cultural characteristics and spore morphology isolates were placed under the family *Streptomyces* and genus *Streptomyces*. Further study is in progress to evaluate the potential of the organism for production of anti-microbial compounds.

Table3: Morphological and Cultural Characteristics of Selective actinomycete 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
GIBT – 101	Retinaculum apertum	Brown	Abundant	Light brown	Brown	Reddish Brown
GIBT – 201	Flexous	Light yellow	Good	Yellow	Brown	Brown
GIBT – 308	Retinaculum apertum	Dark brown	Abundant	Dark yellow	Brown	Reddish brown
GIBT – 311	Monoverticillus	Black	Abundant	Light brown	Brown	Nil
GIBT – 505	Retinaculum apertum	Green	Abundant	White	Green	Green
GIBT – 603	spirals	Grey	Good	Light brown	Grey	Brown

Table4: Physiological and biochemical Characteristics of promising isolates

Isolates	GIBT – 101	GIBT – 201	GIBT – 308	GIBT – 311	GIBT – 505	GIBT – 603
Reaction						
Melanin reaction						
a. ISP-1	-	+	+	+	-	-
b. ISP-6	+	+	+	+	-	+
c. ISP-7	+	+	+	+	-	+
H ₂ S production	+	+	+	+	-	+
a. ISP-6						
Tyrosine reaction	+	+	+	+	+	-
a. 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



Figure 4: Morphologically different actinomycetes sub cultured on yeast extract malt extract agar medium (ISP No. 2).

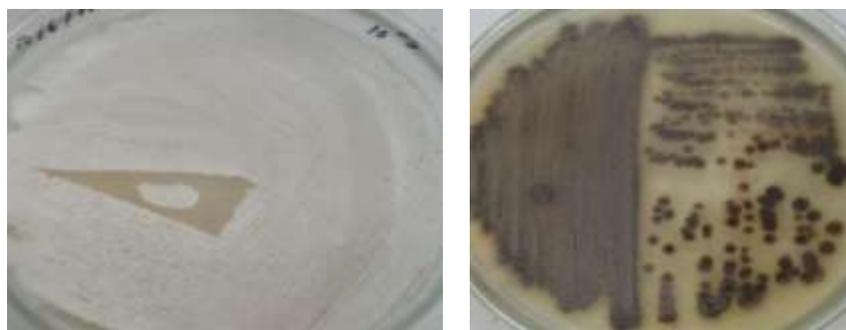


Figure 5: Morphologically different actinomycetes sub cultured on starch casein agar medium.



Figure 6: Inhibitory Zones for the Bacterial and Fungal Test Organisms

CONCLUSION

The search for novel metabolites especially from actinomycetes requires screening large number of isolates (over thousands) in order to discover actinomycete population with novel compound of pharmaceutical interest. The present study was an attempt to use pretreatment methods to screen,

select and isolate marine actinomycetes, within trinsicanti microbial activity against a variety of microbial pathogens, from these dements of Bay of Bengal.

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