Isolation and Screening of Actinomycetes for Antimicrobial Activities from Marine Soil Sediments and Sponges

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ABSTRACT

Objective: The aim of this study was to isolate and screen for the antimicrobial properties of actinomycetes from marine soil sediments and sponges.

Methods: The samples of sponges and sediments were collected from various locations from the coast of Andhra Pradesh. Isolation of actinomycetes was done by plating them on starch casein agar, kuster's agar, Actinomycete isolation agar and potassium tellurite agar medium using dilution technique. Various pathogenic bacterial strains, fungal strains and yeasts were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), Pune, India.

Results: A total of 6 different soil sediments were collected from the coast of Andhra Pradesh at depths ranging from 1 to 20 m. From the 9 different samples collected, 22 colonies of actinomycetes were isolated. Well separated and pure colonies of 12 actinomycetes were selected. Of these, 9 actinomycetes were active against Gram positive bacteria, 3 against Gram negative bacteria, 4 against filamentous fungi, and 1 against yeast. The crude extracts prepared from 3 potential isolates showed exhibited both antibacterial and antifungal activities. On the basis of morphological, physiological and biochemical characteristics, the most effective isolate identified (AP 13) as belonging to the genus *Streptomyces*.

Conclusion: Careful evaluation using previously published species of the genus *Streptomyces*, the isolate was identified as the strain *Streptomyces fradiae*.

Keywords: Actinomyces, antimicrobial property, soil sediments, sponges

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INTRODUCTION

Despite the success of the discovery of antibiotics, and advances in the techniques of their production, infectious diseases still remain the second leading cause of death worldwide, among which bacterial infections cause approximately 17 million deaths annually, affecting mainly children and the elderly (1). This is mainly due to the rapid development of resistance in most pathogenic microorganisms to new antibiotic. Thus the demand of novel antibiotics to treat various diseases is increasing day by day.

Actinomycetes play a significant role in the production of various other antimicrobial agents and industrially important substances such as enzymes used as therapeutic agents in human cancer, mostly in acute lymphoblastic leukemia. *Actinomycetes* are useful in cancer treatment and bioremediation. *Actinomycetes* are also used as plant growth promoting agents, biocontrol tools, biopesticide agents, antifungal compounds, and bio-corrosion and as a source of agro-active compounds (2).

Marine environmental conditions are extremely different from the terrestrial environment, it turned out that marine microorganisms have different characteristics from those of their terrestrial counterparts therefore, might produce different types of compounds (3). In recent years, marine *actinomycetes* have emerged as a rich source of novel compounds (4). The *Streptomyces* species isolated from mangrove environment showed divergent in their phylogenetic analysis and possessed good antibacterial and antifungal activities (5). *Streptomyces rochei* (MTCC 10109) isolated from Visakhapatnam coast showed good antagonistic activities against the human microbial pathogens (6). *Actinomycetes* are important sources of new bioactive compounds such as antibiotics and enzymes (7-8) which have diverse clinical effects and are active against many pathogenic organisms. *Actinomycetes* and their

bioactive compound show antibacterial and anti-microbial against various pathogens and multidrug resistant pathogens e.g. Vancomycin-Resistant *Enterococci*, Methicillin-Resistant *Staphylococcus aureus* (*S. aureus*), *Shigella dysenteriae* (*S. dysenteriae*), *Klebsiella sp.* and *Pseudomonas aeruginosa* (*P. aeruginosa*) etc. (9-11).

This study therefore isolate and screen for the antimicrobial properties of actinomycetes from marine soil sediments and sponges in an attempt for a new, safe and effective antimicrobial agent.

METHODS

Sampling area

The samples of sponges and sediments were collected at various locations from the coast of Andhra Pradesh.

Sample collection

Sponge collection

Three sponge samples were collected in shallow water within the depth of 2 to 5 m. Sponge samples were cut from the sponge using a knife and individual pieces were collected in separate plastic collection bags. Samples were kept on ice in fresh seawater and later transported to the laboratory where they were stored at -20ºC.

Sediment collection

The sediments were collected from the coast of Andhra Pradesh. A total of six marine sediment samples were collected at a depth of 1 to 20 meters into the soil with the help of a sterile spatula. The sample was transferred to a sterile polythene bag and transported to the laboratory. The

sediments were aseptically transferred into sterile bottles, with a sterile spatula and stored under refrigeration conditions. The samples were black-brown-grey in color and of clay texture.

Isolation of actinomycetes

The isolation of *actinomycetes* from marine sediment samples was done by plating them on suitable agar media using dilution technique. One gram of the sample was 10 fold serially diluted sterile saline water and plated on the following media: Starch casein agar, Kuster's Agar, Potassium tellurite agar medium, Actinomycete Isolation agar.

Sample processing

Sponge processing

To remove transient and loosely attached bacteria, each sponge sample was thoroughly washed at least 5 times with sterile artificial seawater (ASW). The specimen was placed on a sterile cutting surface, and a $1cm^3$ section was cut from the sponge with a sterile scalpel blade. The $1cm^3$ sponge sample was placed in a sterile mortar with 9ml of sterile ASW and thoroughly homogenized for 2-3 min. The homogenates were heated in a water bath at 55ºC for 6 min. The supernatant was diluted in ten-fold series and subsequently plated out on agar plates.

Sediment processing

The soil samples were air dried for one week at room temperature, crushed in mortar and pestle to make fine particles. The samples were then subjected to physical pretreatment method in order to facilitate the isolation of *actinomycetes.* Heat treatment was performed by holding sediment samples in a water bath at 50ºC for 60 min. *Actinomycetes* were isolated by serial dilution method from sediments. Stock solution was prepared by diluting 1g of sediment in 9ml of sterile

saline water and shaken well by vortex mixer. From the stock solution, 1ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} by serial dilution. Sample was then inoculated on the agar plates.

All media were prepared using 50% (v/v) sea water. Each medium was supplemented with 50µg/ml cyclohexamide to minimize contamination with fungi and 5µg/ml rifampacin to minimize bacterial contamination. Plates were incubated for 3 to 20 days at 28°C. The plates were observed periodically for the growth of actinomycetes. The well separated colonies were selected, isolated and sub-cultured onto starch casein agar media and incubated at 28^0C. The pure colonies were then selected and maintained in starch casein agar slants at 4ºC for further antimicrobial screening.

Test organisms

The Pathogenic Bacterial strains include *Bacillus subtilis* MTCC 8141, *Escherichia coli* MTCC 6365, *Proteus vulgaris* MTCC 2813 and *Staphylococcus aureus* MTCC 7443. Fungal organisms include *Aspergillus niger* MTCC 6484, *Aspergillus awamori* MTCC 7711, *Candida albicans* MTCC 1346 and the yeast *Saccharomyces cerevisiae* were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India and National Collection of Industrial Microorganisms (NCIM), Pune, India.

McFarland standard was prepared by adding 0.5 ml of 0.048M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) into 99.5 ml of 0.18M H_2SO_4 (1% w/v) with constant stirring and stored at room before use.

Screening for antimicrobial activity

Procedures for inoculum preparation and inoculation

Pure 24 hr old bacterial culture of test bacteria was taken by using a sterile wire loop and transferred into test tubes having a sterile nutrient broth. Pure 3-5 days culture of fungi and 2 days old culture of yeast on potato dextrose agar was taken and scrapped to form a suspension in sterile water on a vortex mixer. The homogenous suspension was adjusted to visible turbidity equal to that of 0.5 McFarland standards.

After adjusting the turbidity, sterile cotton swabs was dipped into the suspensions and streaked over the entire surface of the plate medium using four quadrant streaking technique.

Primary screening of isolates

Antibacterial activity of isolated actinobacteria against *Bacillus subtilis* MTCC 8141, *Escherichia coli* MTCC 6365, *Staphylococcus aureus* MTCC 7443, *Proteus vulgaris* MTCC 2813, *Aspergillus niger* MTCC 6484, *Aspergillus awamori* MTCC 7711, *Candida albicans* MTCC 1346 and the yeast *Saccharomyces cerevisiae* was studied. The tested organisms used for the study were 24hrs old cultures of bacteria, 4 days old culture for fungi and 2 days old culture of yeast.

Cross- streak method

The antimicrobial activity for the isolates was tested, using the cross streak method employing nutrient agar medium for bacteria and potato dextrose agar medium for fungi and yeast. Single streak of the isolate was made at the center of the plate and incubated at 20^0C for 7 days. After observing a good ribbon-like growth of the Actinobacteria on the petri plates, the pathogen was streaked at right angles to the original streak of the Actinobacteria. After 24 to 48hrs of incubating at 20^0C for bacterial cells and 96 to 120hrs of incubation at 28ºC for fungal cells, the inhibition zone was measured. A control plate was maintained without inoculating the

Actinobacteria, to assess the normal growth of bacteria. From this screening, strains of potential antagonistic Actinobacteria were selected.

Secondary screening

Production of crude extracts

The isolates showing potential antibacterial activities from the primary screening were subjected to submerged state fermentation methods to produce crude extracts.

Agar well diffusion method

Petri plates containing nutrient agar medium were seeded with 24hr culture of bacterial strains and potato dextrose agar medium was inoculated with 5 d culture of fungi and 2 d culture of yeast. The well was prepared in the plate by using sterile cork borer (6 mm in diameter). A volume of 100 µl of 10 mg/ml of crude extracts was carefully dispensed into each well and allowed to diffuse for 2 h and incubated at 37°C for 24 h. The sterilized methanol was filtered and used as negative control. After 24 h of incubation, zone of inhibition around each well was recorded and the experiment was repeated for three times.

Characterization of Actinomycetes

The potent actinomycetes were further characterized based on taxonomic study. Characterization study includes morphological studies, cultural studies, and various biochemical reactions like-melanin formation, H_2S production, tyrosine reaction, gelatin hydrolysis, casein hydrolysis, starch hydrolysis, coagulation and peptonization of milk, carbon source utilization, nitrogen source utilization, sodium chloride tolerance, growth temperature range and pH tolerance.

Media for Taxonomic studies

The media recommended by ISP were: Yeast extract malt extract agar (ISP-2), Oatmeal agar (ISP-3), Inorganic salts starch agar medium (ISP-4), Glycerol asparagine agar medium (ISP-5). Inoculum was prepared for morphological cultural characteristics. Identifications were done by macro morphology and micro morphology (Inclined cover slip method -Direct method), biochemical characteristics (Tyrosine reaction, melanin formation, Casein hydrolysis, Starch hydrolysis, gelatin hydrolysis, coagulation and peptonization of milk, H_2S production, carbon utilization test, nitrate reduction, nitrogen source utilization, Physiological characteristics (pH, temperature, NaCl tolerance).

RESULTS

Sample collection

A total of 6 different soil sediments were collected from the coast of Andhra Pradesh at depths ranging from 1 to 20 m and kept in sterile plastic bags. The characteristic appearance of the marine sediments ranges from grey, black and brown. The description, distribution and antagonistic activities of the sediment samples are given in Table 1. Three sponge samples were collected comprising of greenish and/or brownish body parts (each sample being about 5 to 10 cm long and 2 cm in diameter) were served from replicate individual sponges collected in shallow waters (2 to 5 m depth) along the coast of Andhra Pradesh (Table 2).

Screening and isolation of actinomycetes

Primary screening

From the 9 different samples collected, 22 colonies of actinomycetes were isolated. Well separated and pure colonies of 12 actinomycetes were selected. Of these, 9 actinomycetes were active against Gram positive bacteria, 3 against Gram negative bacteria, 4 against filamentous fungi, and 1 against yeast. The results are displayed in Figure 1. From the primary screening, 3 actinomycetes were selected based on their efficiencies. The 3 isolates showed antibacterial and antifungal activities against at least one of the tested organism Table 3.

Secondary screening

The crude extracts prepared from 3 potential isolates by using submerged fermentation methods was subjected to secondary screening by agar well diffusion methods. Among the 3 isolates, it was observed that 1 isolates, AP 13 exhibited both antibacterial and antifungal activities (Figure 2); zone of inhibition of the active isolate is shown in Figure 3 and Figure 4.

Characterizations of Actinomycetes

Morphological characteristics

All the 12 isolates isolated along the coast of Andhra Pradesh were identified as belonging to the genus *Streptomyces* family *Streptomycetaceae* (spore chain coiled and branched). The morphological and cultural characteristics of different *Streptomyces* isolates are shown in Table 4. The morphology of spore bearing hyphae indicate that most of the isolates showed spiral sporophores (41.6%) followed by straight (25%), flexuous sporophores (16.6%), and retinaculum apertum (16.6%), reverse color (8.3%), soluble color (25%), pigmentation (33.3%) was seen.

The cultural characteristics of the most promising antagonistic isolate AP 13 on ISP media and different other media are shown in Table 5. The isolate showed moderate to good growth in different media. Vegetative mycelium showed yellow-brown color and aerial mycelium showed grey color. No soluble pigment was produced in any of the media.

Physiological and Biochemical characterization

The morphological, physiological and biochemical characteristics of isolate AP 13 is shown in Table 6. The isolate AP 13 is characterized by positive production of melanoid on medium ISP-1, ISP-6, ISP-7; positive hydrolysis of starch, gelatin and casein; negative for H_2S production; no reduction of tyrosine nor nitrate; positive for coagulation and peptonization of milk; grows at 28^0C and has a pH tolerance of 6-8.

Good growth was observed on nitrogen source such as L-asparagine, L-cysteine, and L-histidine. However poor or no growth was observed on Potassium nitrate, Sodium nitrate, Ammonium nitrate, L-Arginine, and L-Valine. Isolate AP 13 showed good growth in the presence of low salt concentration but no growth was observed at a concentration about 7% (Table 7).

On the basis of morphological, physiological and biochemical characteristics, the isolate AP 13 was identified as belonging to the genus *Streptomyces*. The characteristic of the isolate was compared with the characteristics of previously published species of the genus *Streptomyces*. Based on the above observation, the isolate was identified as the strain *Streptomyces fradiae* (Fig 5)

DISCUSSION

The isolation of antibacterial compounds from the marine environment is of interest to isolate novel bioactive *actinomycetes.* Recent investigations indicate the tremendous potential of marine *actinomycetes*, particularly *Streptomyces* species as a useful and sustainable source of new bioactive natural products (12). This study has identified the *actinomycetes* by the presence of powdered colonies on the surface of agar plate. Kokare *et al* (2004) have stated the filamentous nature of *actinomycetes* which are gram positive (13).

During the screening of the novel secondary metabolites, isolated *actinomycetes* showed more activity against gram positive bacteria than gram negative bacteria and fungi were often encountered. This was similar to the findings of most studies (13-15). According to a study done in 2013, a marine isolated strain, *Streptomyces fradiae* BDMS1, was found to have aminoglycoside biomolecules. Several compounds were identified of which two were fradimycin C and urdamycin. A showing potent antibacterial and anticancer activity (16).

CONCLUSION

The results of this investigation revealed that the marine *actinomycetes* collected from the coast of Andhra Pradesh might be a potent source of novel antibiotics. It is anticipated that isolation, characterization and study of actinomycetes can be useful for the discovery of novel species of bacteria producing bioactive compounds.

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Table 1: Description, distribution and antagonistic activities of sediments samples collected on the coast of Andhra Pradesh

Sample no.	Depth of sample collection (m)	Characteristic appearance of the sample	No. of actinos/ gms	No. actinos observed	No. of actinos isolated	Active isolates	Active isolates (%)
01	3	Grey to black	1600	16	5	4	33.3
02	18	Grey to back	4000	40	3	1	8.3
03	5	Grey	1700	17	6	2	16.6
04	16	Grey to brown	3200	32	2	3	25
05	14	Grey to black	1100	11	1	1	8.3
06	7	Grey to brown	600	06	1	1	8.3

Table 2: Antagonistic active of *Actinomycetes* isolated from sponges

Sample no.	No. of actinos observed	No. of actinos isolated	Active isolates	Active isolates (%)
1	43	2	1	8.3
2	21	3	-	-
3	19	1	-	-

Table 3: Zone of inhibition of selected isolates against some bacteria and fungi by agar well diffusion method

Selected isolate	Inhibition zone (mm)							
	Bacteria				Filamentous fungi & yeast			
	S.a.	B.s.	E.c.	P.v	C.a.	S.c.	A.n.	A.a
AP 6	13	13	10	11	-	-	-	-
AP 13	15	18	13	12	17	16	14	14
AP 19	15	16	13	13	-	-	-	-

B.s. = *Bacillus subtilis* (MTCC 8141); E.c. = *Escherichia coli* (MTCC 6365);
S.a. = *Staphylococcus aureus* (MTCC 7443); A.n. = *Aspergillus niger* (MTCC 6484);
A.a. = *Aspergillus awamori* (MTCC 7711); C.a. = *Candida albicans* (MTCC 1346);
S.c. = *Saccharomyces cerevisiae* (MTCC 463); P.v. = *Proteus vulgaris* (MTCC 2813);

Table 4: Sporophore Morphology and Pigment Production of *Streptomyces* Isolates along coast of Andhra Pradesh

Character	No. of isolates
Sporophore Morphology	
Spiral	5 (41.6%)
Flexuous	2 (16.6%)
Straight	3 (25%)
Retinaculum apertum	2 (16.6%)
Pigment production	
Melanin	4 (33.3%)
Reverse color	1 (8.3%)
Soluble color	3 (25%)
Isolates showing pigmentation	4 (33.3%)
Total isolates	12 (100%)

Table 5: Cultural characteristics of the isolate AP 13

Medium	**Characteristics**				
	Growth	**Vegetative mycelia**	**Aerial mycelia**	**Spore mass**	**Soluble pigment**
Nutrient agar	Good	Moderate, pale yellow-brown	Moderate, grey	Poor, grey	Nil
Yeast extract malt extract agar (ISP-2)	Abundant	Moderate, yellow-brown	Abundant, grey	Moderate, grey	Nil
Oatmeal agar (ISP-3)	Abundant	Good, pale yellow-brown	Abundant, grey	Moderate, grey	Nil
Inorganic salts starch agar (ISP-4)	Good	Moderate, yellow-brown	Good, grey	Poor, grey	Nil
Glycerol asparagine agar (ISP-5)	Good	Moderate, yellow-brown	Good, grey	Moderate, grey	Nil
Tyrosine agar (ISP-7)	Moderate	Moderate, pale yellow-brown	Good, grey	Poor, grey	Nil
Peptone agar	Moderate - good	Good, pale yellow-brown	Good, grey	Moderate, grey	Nil
Tryptone yeast glucose agar	Moderate	Moderate, pale yellow-brown	Moderate, grey	Poor, grey	Nil

Table 6: A comparative study of the morphological, physiological and biochemical property of the isolate AP 13, in relation to the reference strains *Streptomyces fradiae*

Morphological characteristics	**Isolate AP 13**	***Streptomyces fradiae***
Motility	-	-
Spore mass white	-	-
Spore mass gray	+	±
Spore chain Spirales	+	±
Spore chain Rectiflexibiles	-	-
Melanoid prodiction on		
Medium ISP-1	+	+
Medium ISP-6	+	+
Medium ISP-7	+	+
Hydrolysis of starch	+	+
Medium ISP-4		
Gelatin	+	+
Casein	+	+
H_2S production	-	-
Tyrosine reduction (ISP-7)	-	-
Nitrate reduction	-	-
Milk coagulation and Peptonization	+	+
Growth temperature		
10^0C	-	-
20^0C	+	+
28^0C	+	+
37^0C	+	+
45^0C	-	-
pH tolerance	**6-8**	**6-8**

Table 7: Carbon and nitrogen source utilization pattern along with sodium chloride tolerance of AP 13

Characteristics	**AP 13**
Carbon source	
D-glucose	Good
Sucrose	No growth
Fructose	Good
Iso-inositol	No growth
D-mannitol	No growth
Rhamnose	Poor
Raffinose	Poor
D-xylose	Moderate
Nitrogen source	
L-Asparagine	Good
Potassium nitrate	Poor
Sodium nitrate	Poor
Ammonium nitrate	No growth
L-Arginine	Poor
L-Cysteine	Good
L-Valine	Poor
L-Histidine	Good
Growth in NaCl	
1%	Good
3%	Good
6%	Good
9%	No growth
12%	No growth

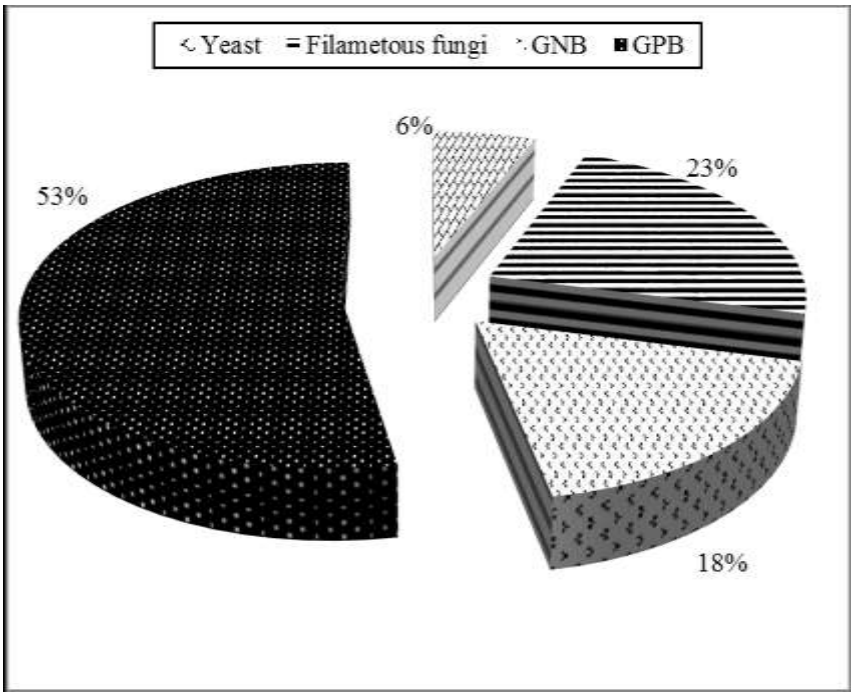

Fig. 1: Pie chart showing antimicrobial activity of the isolated *Actinomycetes*.

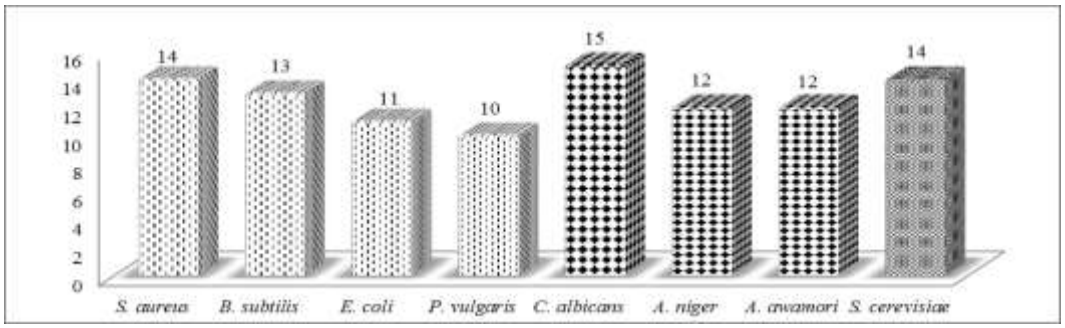

Fig. 2: Bar graph showing antimicrobial activity of the isolate AP 13.

Fig 3: The isolate, AP 13, exhibited the widest zone of inhibition (18mm) against *B. subtilis*.

Fig. 4: Isolate AP 13 exhibited potent activity of 17 mm of inhibition zone against C. *albicans*.

Fig. 5: Pure culture of *Streptomycetes fradiae* on oatmeal agar (ISP-3)