ORIGINAL ARTICLE

Isolation and Screening of *Actinomycetes* from Marine Soil Sediments and Sponges for Anti-microbial Activities

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ABSTRACT

Objective: *To isolate and screen for the anti-microbial properties of actinomycetes from marine soil sediments and sponges.*

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

Results: *A total of six 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, nine actinomycetes were active against Gram-positive bacteria, three against Gram-negative bacteria, four against filamentous fungi, and one against yeast. The crude extracts prepared from three potential isolates exhibited both anti-bacterial and anti-fungal activities. Based on morphological, physiological, and biochemical characteristics, the most effective isolate was identified (AP 13) as belonging to the genus* Streptomyces*.*

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

Keywords: *Actinomycetes*, anti-microbial property, soil sediments, sponges

INTRODUCTION

Despite the success of the discovery of antibiotics and advances in the techniques of their production, infectious diseases 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 to new antibiotics in most pathogenic microorganisms. Thus, the demand for novel antibiotics to treat various diseases is increasing day by day.

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

Marine environmental conditions are significantly different from the terrestrial environment and marine microorganisms have different characteristics from those of their terrestrial counterparts; therefore, they 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

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from the mangrove environment, showed divergence in their phylogenetic analysis and possessed good anti-bacterial and anti-fungal activities (5). *Streptomyces rochei* (Microbial Type Culture Collection [MTCC] 10109), isolated from the Visakhapatnam coast, showed good antagonistic activities against 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 compounds show anti-bacterial and anti-microbial activities against various pathogens and multidrug-resistant pathogens, for example, vancomycin-resistant *Enterococci*, methicillin-resistant *Staphylococcus aureus*, *Shigella dysenteriae*, *Klebsiella* sp. and *Pseudomonas aeruginosa*, etc (9–11).

This study isolates and screens for the anti-microbial properties of actinomycetes from marine soil sediments and sponges in an attempt to find a new, safe, and effective anti-microbial agent.

MATERIALS AND METHODS

Sampling area

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

Sample collection

Sponge collection

Three sponge samples were collected in shallow water within the depth of 2–5 m. Sponge samples were cut from the sponge using a knife, and individual pieces were collected in separate plastic collection bags. The samples were immersed in fresh seawater, covered with ice 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 1–20 m into the soil (drilling) and the samples collected 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 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 the dilution technique. One gram of the sample was 10-fold, serially diluted with sterile physiological saline and plated on the following media: starch casein agar, Kuster's agar, potassium tellurite agar medium, and actinomycetes isolation agar.

Sample processing

Sponge processing

To remove transient and loosely attached bacteria, each sponge sample was thoroughly washed at least five times with sterile artificial seawater (ASW). The specimen was placed on a sterile cutting surface, and a 1 cm^3 section was cut from the sponge with a sterile scalpel blade. The 1 cm^3 sponge sample was placed in a sterile mortar with 9 ml of sterile ASW and thoroughly homogenized for 2–3 minutes. The homogenates were heated in a water bath at 55ºC for 6 minutes. The supernatant was diluted in 10-fold series and subsequently plated out on agar plates.

Sediment processing

The soil samples were air dried for 1 week at room temperature and crushed in mortar and pestle to make fine particles. The samples were then subjected to the physical pretreatment method to facilitate the isolation of actinomycetes. Heat treatment was performed by holding sediment samples in a water bath at 50ºC for 60 minutes. Actinomycetes were isolated by the serial dilution method from sediments. The stock solution was prepared by diluting 1 g of sediment in 9 ml of sterile, saline water and shaken well by a vortex mixer. From the stock solution, 1 ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} by serial dilution. The sample was then inoculated on the agar plates.

All media were prepared using 50% (v/v) seawater. Each medium was supplemented with 50 µg/ml cycloheximide to minimize contamination with fungi and 5 µg/ml rifampicin to minimize bacterial contamination. Plates were incubated for 3–20 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°C. The pure colonies were then selected and maintained in starch casein agar slants at 4ºC for further anti-microbial screening.

Test organisms

The pathogenic bacterial strains include *Bacillus subtilis* MTCC 8141, *Escherichia coli* MTCC 6365, *Proteus vulgaris* MTCC 2813, and *S. 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 $BaCl_2 \cdot 2H_2O$) into 99.5 ml of 0.18M H_2SO_4 (1% w/v) with constant stirring and was stored at room temperature before use.

Screening for anti-microbial activity

Procedures for inoculum preparation and inoculation

Pure 24-hour-old bacterial culture of test bacteria was taken by using a sterile wire loop and transferred into test tubes with a sterile nutrient broth. Pure 3–5 day-old culture of fungi and 2 day-old culture of yeast on potato dextrose agar were 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 the 0.5 McFarland standard.

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

Primary screening of isolates

Anti-bacterial activity of isolated actinobacteria against *Bacillus subtilis* MTCC 8141, *E. coli* MTCC 6365, *S. aureus* MTCC 7443, *P. vulgaris* MTCC 2813, *Aspergillus niger* MTCC 6484, *Aspergillus awamori* MTCC 7711, *Candida albicans* MTCC 1346 and the yeast *S. cerevisiae* was studied. The tested organisms used for the study were 24-hour old cultures of bacteria, 4-day-old cultures for fungi, and 2-day-old cultures of yeast.

Cross-streak method

The anti-microbial activity of the isolates was tested, using the cross-streak method, employing the nutrient agar medium for bacteria and the potato dextrose agar medium for fungi and yeast. A single streak of the isolate was made at the centre of the plate and incubated at 20ºC 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 24–48 hours of incubation at 20ºC for bacterial cells and 96–120 hours 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 anti-bacterial activities from the primary screening were subjected to submerged state fermentation methods to produce crude extracts.

Agar well diffusion method

Petri plates containing the nutrient agar medium were seeded with a 24-hour culture of bacterial strains, and the potato dextrose agar medium was inoculated with a 5-day culture of fungi and a 2-day culture of yeast. The well was prepared in the plate by using a 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 hours and incubated at 37°C for 24 hours. The sterilized methanol was filtered and used as a negative control. After 24 hours of incubation, the zone of inhibition around each well was recorded and the experiment was repeated three times.

Characterization of actinomycetes

The potent actinomycetes were further characterized based on a taxonomic study. The characterization study included morphological studies, cultural studies, and various biochemical reactions such as 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 the Information Society Project (ISP), Yale Law School, were: Yeast extract malt extract agar (ISP-2), oatmeal agar (ISP-3), inorganic salts starch agar medium (ISP-4), and glycerol asparagine agar medium (ISP-5).

The inoculum was prepared for morphological cultural characteristics. Identifications were done by macromorphology and micromorphology (inclined coverslip 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 and physiological characteristics (pH, temperature and NaCl tolerance).

RESULTS

Sample collection

A total of six 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 ranged from grey to black and brown. The description, distribution, and antagonistic activities of the sediment samples are shown in Table 1. Three sponge samples were collected comprising greenish and/or brownish body parts (each sample being about 5–10 cm long and 2 cm in diameter) and were served from replicate individual sponges collected in shallow waters (2–5 m in 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, nine actinomycetes were active against Gram-positive bacteria, three against Gram-negative bacteria, four against filamentous fungi, and one against yeast. The results are displayed in Fig. 1. From the primary screening, three actinomycetes were selected based on their efficiencies. The three isolates showed anti-bacterial and anti-fungal activities against at least one of the tested organisms (Table 3).

Secondary screening

The crude extracts prepared from three potential isolates by using submerged fermentation methods were subjected to secondary screening by agar well diffusion methods. Among the three isolates, it was observed that one isolate, AP 13, exhibited both anti-bacterial and anti-fungal activities (Fig. 2); the zone of inhibition of the active isolate is shown in Figs. 3 and 4.

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

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

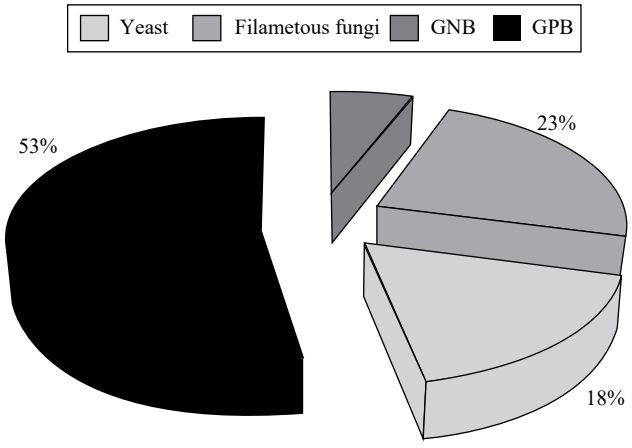

Figure 1: Pie chart showing anti-microbial activity of the isolated actinomycetes.

Table 2: Antagonistic activities 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 the agar well diffusion method

	Inhibition zone (mm)							
Selected isolate	Bacteria				Filamentous fungi and 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).

Characterization 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 indicates that most of the isolates showed spiral sporophores (41.6%), followed by straight (25%), flexuous sporophores (16.6%), and retinaculum apertum (16.6%), reverse colour (8.3%), soluble colour (25%) and pigmentation (33.3%) were seen.

The cultural characteristics of the most promising antagonistic isolate AP 13 on ISP media and other different media are shown in Table 5. The isolate showed

Table 4: Sporophore morphology and pigment production of Streptomyces isolates along the coast of Andhra Pradesh

Characteristics	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 colour	1 (8.3%)
Soluble colour	3 (25%)
Isolates showing pigmentation	4 (33.3%)
Total isolates	12 (100%)

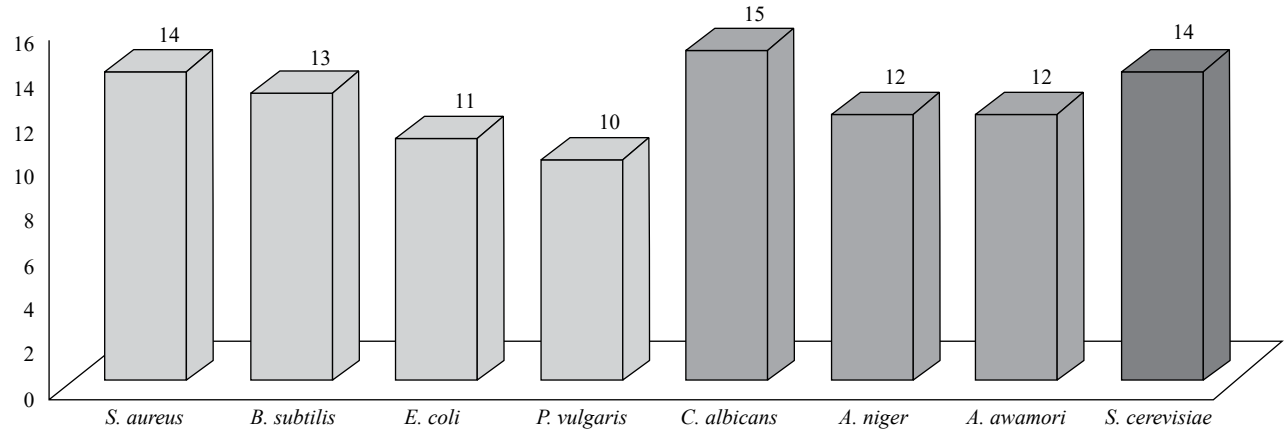

Figure 2: Bar graph showing anti-microbial activity of the isolate AP 13.

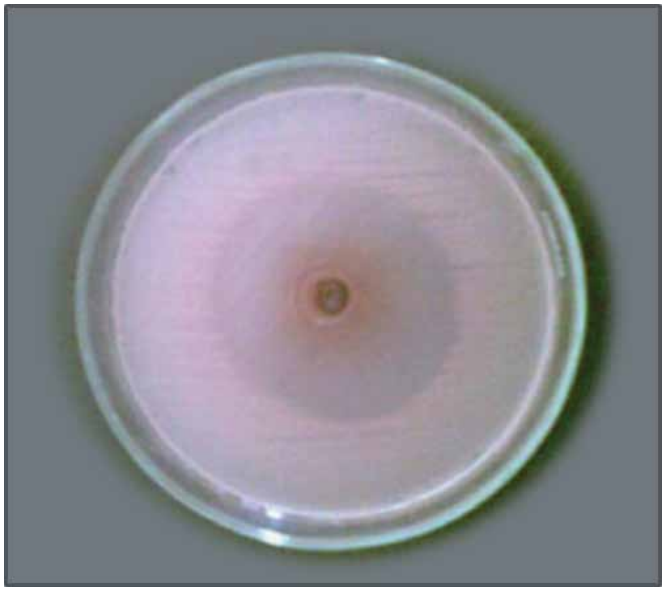

Figure 3: The isolate, AP 13, exhibited the widest zone of inhibition (18 mm) against *Bacillus subtilis*.

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

moderate to good growth in different media. The vegetative mycelium showed yellow-brown colour and the aerial mycelium showed grey colour. No soluble pigment was produced in any of the media.

Physiological and biochemical characterization

The morphological, physiological, and biochemical characteristics of isolate AP 13 are shown in Table 6. The isolate AP 13 is characterized by the positive production of the melanoid on the media ISP-1, ISP-6, and 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; growth at 28°C and pH tolerance of 6–8.

Good growth was observed in the 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 of about 7% (Table 7).

Table 5: Cultural characteristics of the isolate AP 13

	Characteristics				
Medium	**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 properties of the isolate AP 13, with the reference strains *Streptomyces fradiae*.

Morphological characteristics	**Isolate AP 13**	***Streptomyces fradiae***
Motility	–	–
Spore mass, white	–	–
Spore mass, grey	+	±
Spore chain, spirales	+	±
Spore chain, rectiflexibiles	–	–
Melanoid production 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°C	–	–
20°C	+	+
28°C	+	+
37°C	+	+
45°C	–	–
pH tolerance	6–8	6–8

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

During the screening of the novel secondary metabolites, isolated actinomycetes showed more activity against Gram-positive bacteria than Gram-negative bacteria. This was similar to the findings of most studies (12–14). According to a study done in 2013, a marine-isolated strain, *Streptomyces fradiae* BDMS1, was found to have aminoglycoside biomolecules. Several compounds

Table 7: Carbon and nitrogen source utilization pattern, along with sodium chloride tolerance of AP 13 (16)

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

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

were identified of which two were Fradimycin C and Urdamycin A that were showing potent, anti-bacterial, and anti-cancer activities (15).

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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