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ORIGINAL ARTICLE

Isolation and Screening of Marine Actinobacteria for their Antimicrobial Compounds

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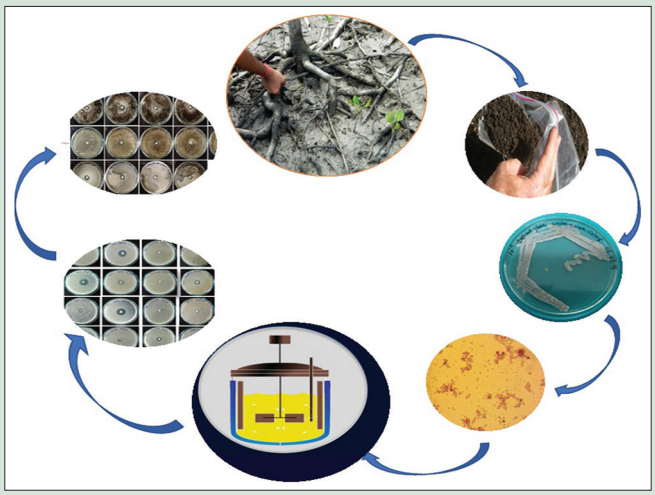
ABSTRACT

Objective: The objective of this study is to assess the antimicrobial activity of marine actinobacteria isolated from coastal regions of Andhra Pradesh, India. **Materials and Methods:** Using sterile techniques, soil samples were collected from Machilipatnam (MCP), Krishna district, Andhra Pradesh, India. Biochemical tests were performed to identify isolated actinobacteria. Antimicrobial activity of the isolated organisms was performed by the disk plate technique. **Results:** In this study, six mangrove soil samples were collected and performed culture-dependent methods to obtain marine actinobacteria. A total of five actinobacteria were isolated in which two exhibited antibacterial as well as antifungal activity. Among the five, MCP-2 isolate had shown promising antibacterial and antifungal activity. **Conclusion:** The findings of this investigation revealed that the coastal marine actinobacteria are an important source of novel antibiotics

Key words: Actinobacterial screening, antibacterial, antifungal activities, Machilipatnam, mangrove soil, novel isolates

SUMMARY

- Marine habitat has been shown to be an excellent and fascinating resource for the invention of new and potent bioactive-producing micro-organism. In this study, 6 mangrove soil samples were collected and performed culture-dependent method to obtain marine actinobacteria. A total of five actinobacteria were isolated in which 2 exhibited antibacterial as well as antifungal activity.



Abbreviations Used: MCP: Machilipatnam, RT: Room Temperature, SCA: Starch Casein Agar, ISP-2: International *Streptomyces* Project-2, NBS: National Bureau of Standards, ISCC: Inter-Society Color Council, ATCC: American Type Culture Collection, NSS: Normal saline solution, MHB: Mueller-Hinton broth, SVIMS: Sri Venkateswara Institute of Medical Sciences, UGC: University grant commission, BSR: Basic scientific research.

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INTRODUCTION

Microbial secondary metabolites have received considerable attention due to their novel and potent biological activities, particularly as antimicrobial agents against human pathogens.[1] Actinobacteria is one of the largest bacterial phyla with diverse morphological, physiological, and chemotaxonomic properties occupying equally diverse aquatic and terrestrial habitats.[2,3] Marine Actinobacteria's secondary metabolites are unique and exhibit interesting biological activities (antibacterials-abyssomicins; antifungal compounds-phenazines; antiviral compounds-benzastatin C).[4-6] The mangrove Marine invertebrates are known to possess physiological and biochemical mechanisms, which produce special metabolic pathways strikingly unique as seen in terrestrial organisms.[5,6] These novel chemical compounds or marine bioactive compounds can serve as precursors for the development of next-generation bioactive compounds employable in the biomedical sector.[7] Hundreds of bioactive compounds have been isolated and characterized, many of which are finding applications in human health, animal care, and agricultural sectors.[6,8,9] Accordingly, actinobacteria are considered the most diverse and potent source

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of secondary metabolites and other bioactive compounds.[6,10-14] Many reports in recent and contemporary literature indicate that actinobacteria are potent antibiotic producers, accounting for 75% of all known antibiotics used and Streptomyces occupies a special place in the production of antibiotics.[15] Screening, isolation, and characterization of promising actinobacteria strains that produce potential antibiotics and other therapeutics is, therefore, an important part of the research.[16] According to recent statistics, the number of novel compounds isolated from marine actinobacteria in the 21st century has doubled.[17] Hence, the current study was undertaken to isolate actinobacteria from the Coastal soils of Machilipatnam (MCP), Andhra Pradesh, India, and screen for antimicrobial compounds.

MATERIALS AND METHODS

Collection mangrove soil samples

Mangrove soil sediments were collected from regions of MCP coastal area, Andhra Pradesh, India [Figure 1]. Each sample was collected at a depth of 5–10 cm and placed in sterile plastic bags. All samples were transported to the laboratory, at the Department of virology, Sri Venkateswara University for further study.

Isolation of actinobacteria

Sediment samples were air-dried at room temperature (RT) for 3–5 days and grounded aseptically with a pestle and mortar. The soil sample then was dried at 50°C for 1 h in a dry oven to reduce the bacterial and mold populations.[18] To isolate actinobacteria, 1 g soil samples were serially diluted in sterile 50% seawater. Then, 0.1 ml from each dilution was spread plated onto different isolation and selective media, starch casein agar media, actinobacteria isolation agar (AIA), and modified soil extract agar.[19,20] The plates were incubated at RT for 4 weeks. Colonies of actinobacteria were sub-cultured onto modified soil extract agar and incubated at RT for 7–14 days to obtain pure isolates. The isolated actinobacteria strains were stored in International *Streptomyces* Project-2 (ISP-2) agar slants.

Cultural characteristics

Morphological features of the selected actinobacteria strains were established on the ISP2 agar medium after incubation at 28°C for 7–14 days. The cultural (pigmentation and colony morphology) and morphological (Gram's nature, spore formation, and motility) characteristics were established using standard procedures. The color of metabolized substrate and aerial mycelium and soluble pigment production were determined using the National Bureau of Standards/Inter-Society Color Council color system.[21] For taxonomic identification traits like the utilization of carbon and nitrogen sources of *Actinobacterial* sp., Nonomura's key and Bergey's Manual were utilized.[22]

Figure 1: Coastal region from Machilipatnam, Krishna District, Andhra Pradesh, India

Test micro-organisms

The test micro-organisms used in this study, *Escherichia coli* (American Type Culture Collection [ATCC]-9837) *Staphylococcus aureus* (ATCC-6538), *Bacillus subtilis* (ATCC-9856), and *Pseudomonas aeruginosa* (ATCC-9027) were procured from Sri Venkateswara Institute of Medical Sciences, Tirupati. Bacterial suspensions of respective test organisms were prepared by overnight culture in Nutrient Broth under continuous shaking (120 rpm and 37°C). The bacterial suspensions were adjusted to B equal the turbidity of 0.5 McFarland standards (approx. 10^6 CFU ml^{-1}) with sterile saline. Similarly, for antifungal activity, *Aspergillus niger* and *Aspergillus fumigatus* spore suspension in sterile water were used (approx. 10^4 CFU ml^{-1}).

Antibacterial activity screening

The primary antibacterial screening was performed by spread culture technique.[23] In this, each actinobacteria isolate spread uniformly on AIA medium prepared with 50% normal distilled water and 50% of seawater and incubated at 28°C for 7–14 days. One loop of each test strain (10^8 CFU ml^{-1}) was streaked perpendicularly at the edge of the actinobacteria. Plates were incubated at 37°C for 24–48 h and the inhibition zones were recorded. The control plate was maintained without inoculating actinobacteria. The experiment was performed in triplicates and the results are presented as average with standard deviation.

Antifungal activity screening

The antifungal activity of actinobacteria was assessed by using the standard agar disc-diffusion method.[24] Briefly, the spore suspension was prepared by inoculating the fungal test organisms in sterile Potato Dextrose Broth (PDB) and incubated at 28°C for 3–7 days. After incubation, the fungal mass was vigorously shaken and the spores were filtered and used for the assay. The test fungal spores were uniformly spread on PDB agar medium and the actinobacteria-containing agar piece is placed in the center of the PDB agar plate after inoculating the fungal spores. The plates were incubated for 3–5 days and the zone of inhibition was measured.

Actinobacterial fermentation and active compound extraction

The Actinobacteria possessing antibacterial activity were further assessed to isolate potential active compounds. Hence, the potent isolates (as established from the initial antibacterial and antifungal screening assays) inoculated in 100 ml ISP2 broth and incubated at 28°C for 7–14 days for isolating secondary metabolites. A total of 3 L culture filtrate was produced using a lab-scale fermenter (Bio-Age fermenter, BIO-AGE instruments). The fermentation broth was treated with equal volumes of ethyl acetate and the EtOAc phase collected and the process was repeated thrice to maximize the extraction. Further, the EtOAc phase collected was evaporated completely under reduced pressure using a rotary evaporator to obtain a broth ethyl acetate extract. Similarly, the aqueous phase was dried to give aqueous extract. Both the EtOAc and aqueous extracts were further combined and converted to single amorphous powder through Lyophilization using a Lyophilizer (LYODEL Freeze dryer), this increases the chances of obtaining potential active compounds in future fractionation and identification studies.

Antibacterial activity of active extracts-disk diffusion method

Each isolates extract (both aqueous and EtOAc) was separately assessed against both gram-positive and Gram-negative isolates.[25] Disk diffusion assay was performed as standard protocol, briefly, the test bacteria were spread plated on to nutrient agar and sterile Whatman No. 1 filter paper disks were placed in the center loaded with actinobacteria extract (25 µl). The plates were then incubated for 48 h at 37°C and the zone of inhibition was observed and recorded. The experiment was performed in triplicates and the results are presented as average with standard deviation.

RESULTS AND DISCUSSION

Morphological and biochemical characteristics

Different isolates were selected based on the growth characteristics as observed on the AIA and other selective media as described earlier. The isolates with unique growth features were selected for the study and labeled as MCP-1, MCP-2, MCP-3, MCP-4, and MCP-5. The growth traits along with the morphological and biochemical characteristics are presented in Table 1 (only the potent isolates are presented excluding the rest, based on antibacterial assays performed later).

Antibacterial activity from Machilipatnam isolates

The actinobacteria isolated from the coastal soils of MCP were assessed for the antibacterial activity initially via growth inhibition assay using dual culture method. The isolates showing inhibition of growth around the colony, creating a clear zone are selected as potent isolates and the inhibition potential is presented in Table 2. The results exhibited indicate the different actinobacteria have varied degrees of inhibition activity toward Gram-positive and negative bacteria. Isolates MCP-5 showed significantly higher activity against both the Gram-positive test isolates (*Bacillus* and *Staphylococcus*), but the rest showed no such pattern. Furthermore, isolate MCP-1 showed the highest antibacterial activity of □12.2 mm against bacillus but only 1.3 mm against *Staphylococcus* despite both being Gram-positive. While MCP-2 showed a similar pattern (highest against *E. coli* and lowest against *Pseudomonas*). Isolate MCP-3 showed exceptional inhibition in the case of *B. subtilis* but was only mildly effective against the rest. As an odd member from the group, MCP-4 showed significant activity against all the test isolates but Gram-negative species were much susceptible. Streptomycin served as control.[26] Antibacterial activity of fermentative extracts from the potent isolates was determined by disk diffusion assay as presented later.

Table 1: Morphological and biochemical characteristics of Machilipatnam isolates

Characters	MCP-1	MCP-2	MCP-3	MCP-4	MCP-5
Colony appearance	Mycelial	Cotton mycelial	Cotton mycelial	Cotton mycelial	Mycelial
Sporulation of aerial mycelia	Long chain	Long chain	Long chain	Long chain	Long chain
Motility	Non-motile	Non-motile	Non-motile	Non-motile	Non-motile
Colony color	Ash	Cream	White	Light gray	Ash
Gram staining	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+
Gelatine hydrolysis	+	+	+	+	+
Casein hydrolysis	+	+	+	+	+
Carbon utilization					
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	+

MCP: Machilipatnam isolate

Table 2: Antibacterial activity of Machilipatnam isolates

Name of the isolates	Zone of inhibition (mm)			
	Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
MCP-1	8.0±0.30	12.2±0.41	1.3±0.04	2.5±0.38
MCP-2	12.1±0.26	6.5±0.55	7.5±0.24	1.6±0.20
MCP-3	5.1±0.21	13.2±0.53	2.0±0.24	0.5±0.04
MCP-4	11.1±0.78	4.9±0.47	7.5±0.16	8.1±0.24
MCP-5	9.1±0.85	12.2±0.85	10.1±0.70	2.7±0.53
Control (streptomycin)	7.5±0.42	13.4±0.44	10.3±0.33	14.1±0.57

MCP: Machilipatnam

Table 3: Zone of inhibition of antifungal activity from Machilipatnam isolates

Name of isolates	Zone of inhibition of *Aspergillus niger* (mm)	Zone of inhibition of *Aspergillus fumigatus* (mm)
MCP-1	6.2±0.33	2.5±0.38
MCP-2	11.5±0.54	10.1±0.69
MCP-3	9.1±0.41	11.3±0.38
MCP-4	8.9±0.69	14.1±0.55
MCP-5	8.6±0.65	18.2±0.49
Control nystatin	7.1±0.65	9.1±0.41
Cycloheximide	10.3±0.98	11.4±0.85

MCP: Machilipatnam

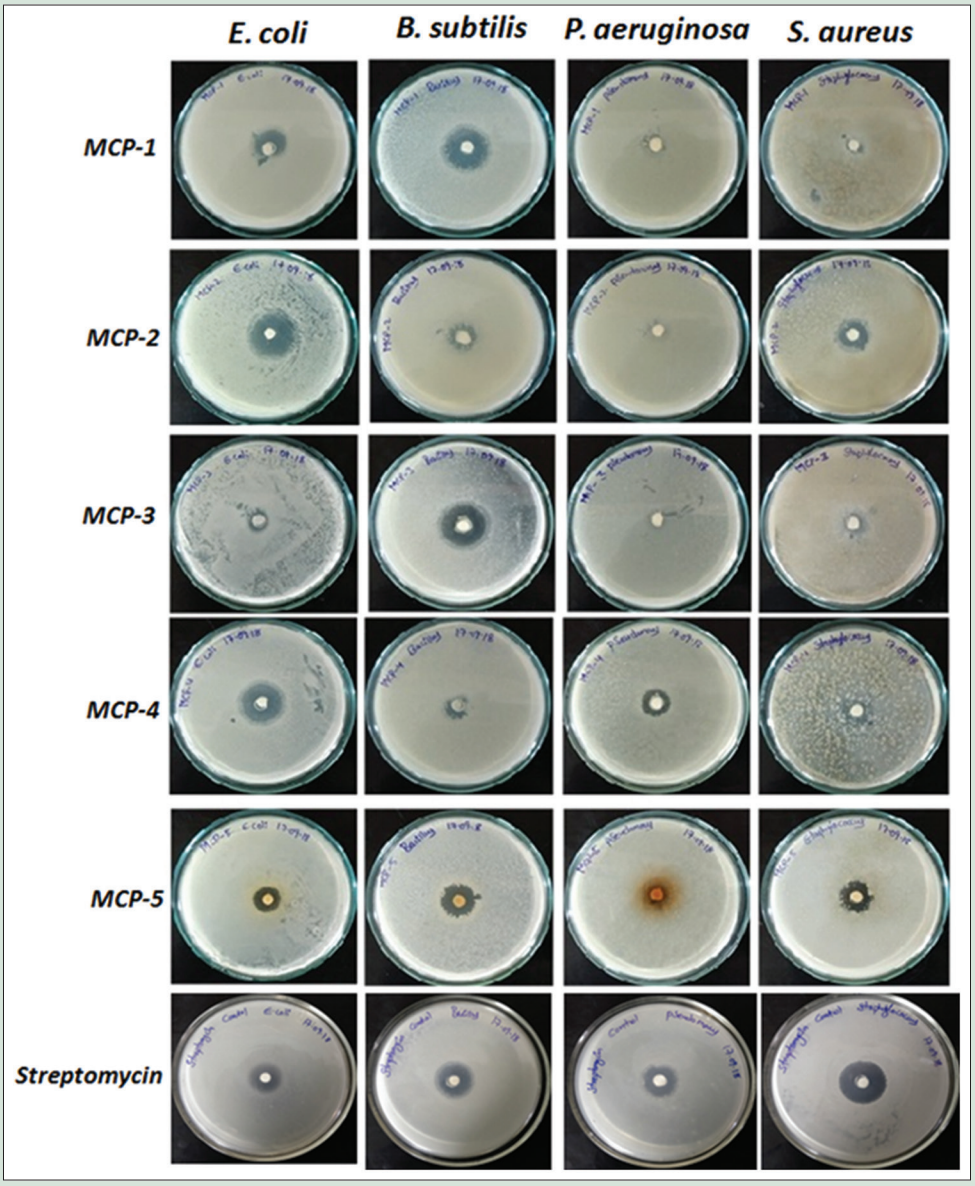

Figure 2: Antibacterial activity of extracts from Machilipatnam isolates

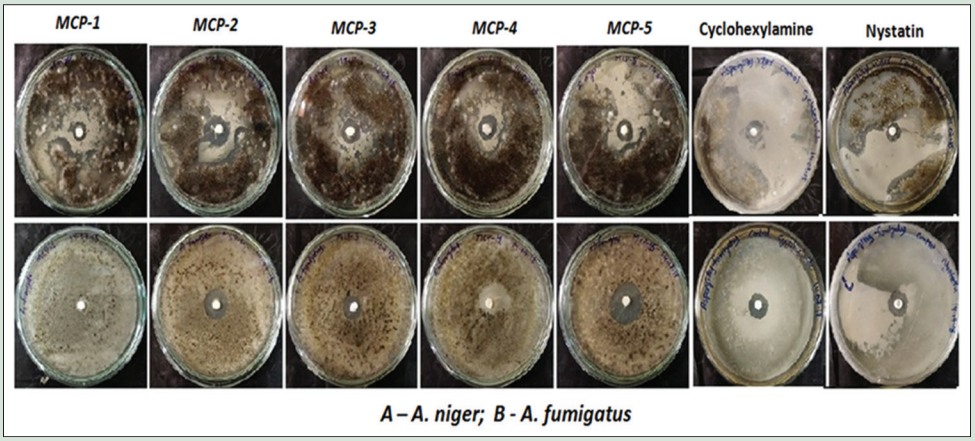

Figure 3: Antifungal activity of the extracts from Machilipatnam isolates

Antifungal activity from Machilipatnam isolates

The antifungal activity of marine actinobacteria against phytopathogenic fungal species in primary screening is represented by Figure 2. *A. niger and A. fumigatus* both were significantly inhibited by the actinobacterial isolates. As seen with antibacterial activity, the antifungal activity as well, was varied. All the isolates showed maximum inhibition of *A. fumigatus* when compared with *A. niger* except MCP-1. Isolate MCP-5 showed the highest inhibition (□18.2 mm) toward *A. fumigatus* even higher than the controls used. Isolate MCP-2 showed the highest inhibition of *A. niger* reaching □11.5 mm more than that of the controls used [Table 3]. The results observed agreed with some previous reports.[27,28]

Antibacterial and antifungal activity of the actinobacterial extracts of the selected isolates

The extract prepared from the fermented broth of the selected isolates was assessed for the antibacterial and antifungal activity by the disk diffusion method as described earlier. The extracts showed similar activity against both bacteria and fungi tested herein. When compared to the antibacterial activity alone the antagonistic activity of the MCP-5 was effective against all the bacteria tested. In the case of antifungal activity, the isolate MCP-1, MCP-2, and MCP-5 showed significant antagonistic activity against both the tested fungi but lower than the controls. Furthermore, the control cycloheximide was more effective antifungal agent than Nystatin as observed in this study. Both the antibacterial and antifungal activities of the extracts are presented in Figures 2 and 3, respectively.

CONCLUSION

In the current endeavor, we succeeded in isolating potent actinobacteria with significant antibacterial as well as antifungal activity from MCP mangrove sediment. Initially, the growth, morphological and biochemical characteristics were established based on which unique isolates were selected for the study. The isolates, as well as their metabolic extracts, showed promising antimicrobial properties, warranting their further characterization to identify the specific active compounds conferring such properties to their producers

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Conflicts of interest

There are no conflicts of interest.

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