

***Streptomyces* sp MA7 isolated from mangrove rhizosphere sediment effective against Gram negative bacterial pathogens**

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Abstract: The present study reports the isolation, characterization and antimicrobial activity of actinobacteria from mangrove rhizosphere sediment against Gram negative bacterial pathogens. Totally 20 morphologically different actinobacterial colonies were isolated from Parangipettai mangrove ecosystem. More number of powdery colonies was observed on Kusters agar plates than the starch casein agar. Antibacterial activity of actinobacterial strains was tested by agar plug method. Twelve out of 20 isolates showed antibacterial activity in which the strain MA7 showed promising activity against 12 pathogenic Gram negative bacterial strains. Results of phenotypic characteristics revealed that the strain MA7 belongs to the genus *Streptomyces*. Antibacterial metabolite from strain MA7 was produced by submerged and agar surface fermentation. Cell free supernatant showed no activity in well diffusion method whereas the methanol extract of fermented agar medium showed promising antibacterial activity in disc diffusion method. Results of this study revealed that the *Streptomyces* sp MA7 isolated from mangrove rhizosphere sediment will be a good source for the isolation of a metabolite effective against Gram negative bacterial pathogens.

Key words: mangrove ecosystem, actinobacteria, *Streptomyces*, antibacterial, agar surface fermentation

Introduction

Gram negative bacterial pathogens pose a serious threat to public health especially in developing countries where hygienic measures are poor. Resistance to multiple antibiotics is increasingly reported in a number of Gram negative bacterial pathogens especially in the enterobacteriaceae, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Acinetobacter* sp. These and other drug resistant bacilli infections impact not only hospitalized patients undergoing surgical and other procedures but also otherwise healthy non-hospitalized individuals¹. Recent discovery and development efforts aimed at MDR gram negative bacterial infections have focused largely on important mechanisms of resistance including beta lactamases and carbapenemases as these are responsible for a large burden of drug resistant infections reported globally. In 2009, IDSA reported no antibacterial agent in development with a purely gram negative spectrum had reached phase 2 of clinical study². Antibacterial agents other than those currently available are in dire need to fight against Gram negative bacterial pathogens.

Actinobacteria are virtually unlimited sources of novel compounds with many therapeutic applications and hold a prominent position due to their diversity, wide range of ecosystems and proven ability to produce

novel bioactive compounds³. Mangroves are salt tolerant plants existing at the interface between land and sea in the tropical and subtropical latitudes. The mangrove forests are one among the world's most productive ecosystems with great ecological and economic significance⁴. This ecosystem supports a rich and diverse group of microorganisms. Especially the mangrove environment is a potent source for the isolation of antibiotic-producing actinomycetes⁵. Many investigators studied the occurrence and antimicrobial and enzymatic activity of actinobacteria from mangrove ecosystems of our country. There is growing interest in investigating actinobacteria from Vellar estuary soil and its associated mangrove region situated at the Parangipettai, Tamil Nadu. Actinobacteria especially *Streptomyces* and *Micromonospora*⁶ isolated from Vellar estuary and its associated mangrove region are reported as a suitable source for production of novel bioactive compounds^{7,8}. The present study was undertaken to investigate the antibacterial activity of actinobacteria from mangrove ecosystem of Vellar estuary against Gram negative bacterial pathogens.

Materials and Methods

Sample collection and actinobacterial isolation

Mangrove rhizosphere sediment samples were collected from the Vellar estuary region at Parangipettai (Lat. 11.4900° N; Long. 79.7600° E), Tamil Nadu. Actinobacteria from the dried sediment samples were isolated by standard spread plate method using Starch casein agar and Kuster's agar medium supplemented with nystatin (50µg/ml) and nalidixic acid (20µg/ml)^{8,9}. Colonies with actinobacterial morphology were recovered using ISP2 agar. All the media used in this study was prepared using 50% sea water, unless otherwise mentioned. Morphologically different colonies were selected and subcultured on ISP2 agar medium.

Preliminary screening for antagonistic activity

All the actinobacterial colonies were grown on ISP2 agar plates for 5 days at 28°C for bioactive metabolite production and tested for antagonistic activity against 17 Gram negative bacterial pathogens (Table 1) by agar plug method¹⁰. All the enteric pathogens were obtained from NICED, Kolkata. Based on the results of antagonistic activity, one potential strain MA7 was selected for further investigation

Characterization of potential strain

Cultural characteristics such as growth rate, colony consistency, aerial mass colour and pigment production were studied using ISP2 agar medium. Micromorphological characters such as presence of aerial and substrate mycelium, spore chain morphology were studied under bright field microscope at 400 magnifications. Growth on different ISP media, carbon utilization characters were studied by adopting the method described by Shirling and Gottlieb¹¹. Tolerance to different pH, temperature and NaCl concentration was also studied using ISP2 agar¹².

Production of bioactive metabolites and testing for activity

Bioactive metabolite from strain MA7 was produced by adopting submerged and agar surface fermentation. For submerged fermentation, about 10% of 48 hours old actinobacterial inoculum was transferred into ISP2 broth and incubated in rotary shaker for 7 days at 28°C. The cell free supernatant (CFS) was collected by centrifugation at 5000 rpm for 10 minutes and tested for antibacterial activity by well diffusion method. The cell free medium was also extracted using ethyl acetate and tested for activity by disc diffusion method at 250µg concentration. In agar surface fermentation, strain MA7 was inoculated into YEME agar plates and incubated for 7 days at 28°C. After scrapping the mycelial growth, bioactive metabolite from the agar medium was extracted solid-liquid extraction method using methanol, chloroform and n-hexane. The crude extracts were concentrated and tested for activity by disc diffusion method at 250µg concentration⁹.

Results and Discussion

Isolation and enumeration of actinobacteria

Among the two different medium used for the actinobacterial isolation Kuster's agar plates are showed morphologically different colonies than in starch casein agar plates. The number of colonies formed was also higher in Kuster's agar plates (1.4×10^{-4} cfu/gm) than in starch casein agar plates (0.8×10^{-4} cfu/gm). In the previous studies also authors reported Kusters agar is a suitable medium for the isolation of actinobacteria from

mangrove and estuarine ecosystems^{13,14,15}. Totally 20 different actinobacterial colonies were selected based on their visible morphological differences. Fifteen out of 20 actinobacterial cultures showed good growth with varying aerial mass color on ISP2 agar medium supplemented with 50% sea water. All the isolates are showed the presence of aerial and substrate mycelium. Many authors reported *Streptomyces* as a major actinobacterial population from mangrove ecosystem^{8,16,17}. In the present study also, based on the cultural and micro morphology all the actinobacterial isolates are suspected as species of the genus *Streptomyces*.

Antagonistic activity of marine actinobacteria

There is a dire need to isolate antibacterial agents to fight against Gram negative bacterial pathogens². Berdy¹⁸ reported that a great part of antibiotic compounds exhibit exclusive activity against gram-positive bacteria (app. 30% of all) and only 1.5% of the compounds (some 250 metabolites) showed activity only against gram-negative bacteria. In the present study 12 out of 20 isolates showed antagonistic activity against atleast any one of the 14 bacterial pathogens tested. Especially strain MA7 which inhibited maximum of 12 bacterial pathogens. So the actinobacterial strain MA7 was selected as the best strain for further investigations.

Table 1. Antagonistic activity of marine actinobacteria MA7 against Gram negative bacterial pathogens

Bacterial pathogens	Zone of inhibition*
<i>Vibrio cholerae O1</i>	18
<i>Vibrio cholerae O139</i>	12
<i>Vibrio parahaemolyticus</i>	17
<i>Vibrio mimicus</i>	15
<i>Enterotoxigenic E. coli</i>	14
<i>Enteropathogenic E. coli</i>	13
<i>Enteraggregative E. coli</i>	11
<i>Serotoxigenic E. coli</i>	11
<i>Salmonella typhi</i>	13
<i>Salmonella typhimurium</i>	10
<i>Shigella flexneri</i>	10
<i>Shigella boydii</i>	-
<i>Shigella sonnei</i>	-
<i>Aeromonas hydrophila</i>	10

*millimeter in diameter

Characterization of actinobacterial strain MA7

Under microscopic observation, strain MA7 showed the presence of both aerial and substrate mycelium with rectus flexible arrangement. No mycelial fragmentation was observed. About 30-40 spores are present in spore chain. Strain MA7 showed good growth on ISP1, ISP2 (Figure 1), ISP3 and ISP5 medium. Moderate and poor growth was observed on ISP4 and ISP6 medium, respectively. Strain MA7 showed growth at 30-40°C and 7-9 pH range and also utilized wide range of sugars as carbon source (Table 2). Based on the studied phenotypic characteristics strain MA7 was identified as *Streptomyces* sp.

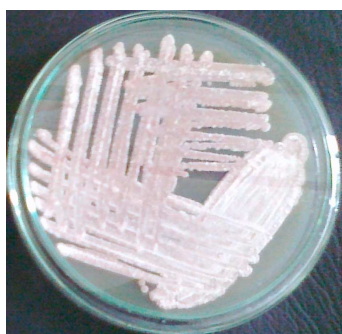


Figure 1. Cultural morphology of actinobacterial strain MA7 on ISP2 agar medium

Table 2. Characteristics of actinobacterial strain MA7

Characteristics	Result
Micromorphology	
Aerial mycelium	Present
Substrate mycelium	Present
Spore chain morphology	Rectus flexibile (RF)
Spore surface	Smooth
Cultural characteristics	
Colony consistency	Powdery
Aerial mass colour	Pinkish White
Reverseside pigment	Absent
Soluble pigment	Present
Growth on different ISP media	
ISP1	Good
ISP2	Good
ISP3	Good
ISP4	Moderate
ISP5	Good
ISP6	Moderate
ISP7	Good
Growth at pH	7-9
Growth at temperature	30-40 ⁰ C
Utilized carbon sources	Glucose, fructose, sucrose, rhamnose mannitol, xylose, inositol
NaCl tolerance	
0-2.5%	Good
5%	Moderate
7.5-10%	Poor

Production of bioactive compound and antibacterial activity

Strain MA7 showed good growth on ISP2 broth during submerged fermentation by shake flask method. In well diffusion test, the cell free supernatant showed no activity against the test organisms. The ethyl acetate extract of cell free supernatant also showed no activity in disc diffusion method. In agar surface fermentation, strain MA7 showed good growth and the methanol extract obtained from the fermented medium showed promising activity against the Gram negative enteric pathogens (Table 3). Production of a majority of industrially important secondary metabolites from actinomycetes is carried out using submerged fermentation processes where they exhibit diverse morphological forms^{19,20}. Morphology is influenced by environmental conditions such as medium composition and shear stress^{21,22}. Further, morphology and product formation have been observed to be closely related²³. In a study by Shomura et al.²⁴ the antibiotic production of *Streptomyces halstedii*, which showed activity against gram negative bacteria only in agar dishes, was well correlated with its mycelial morphology. In our early study²⁵, we also reported that *Streptomyces* sp D25 isolated from Thar desert soil produced an antitubercular pigment only in solid culture but not in liquid culture even we used the diluted nutrient medium as suggested by Shomura et al.²⁴. Further optimization of medium component and concentration may be needed to produce the compound from strain MA7 by submerged fermentation.

Table 3. Antimicrobial activity of cell free supernatant and crude extracts of strain MA7

Pathogens	Submerged fermentation		Agar surface fermentation		
	Agar well diffusion	Disc diffusion	ME	ChE	n-HE
<i>Vibrio cholerae O1</i>	-	-	20	-	-
<i>Vibrio cholera O139</i>	-	-	15	-	-
<i>Vibrio parahaemolyticus</i>	-	-	21	-	-
<i>Vibrio mimicus</i>	-	-	18	-	-
<i>Enterotoxigenic E. coli</i>	-	-	18	-	-
<i>Enteropathogenic E. coli</i>	-	-	16	-	9
<i>Enteraggregative E. coli</i>	-	-	12	-	-
<i>Serotoxigenic E. coli</i>	-	-	11	-	-
<i>Salmonella typhi</i>	-	-	15	-	13
<i>Salmonella typhimurium</i>	-	-	12	-	15
<i>Shigella flexneri</i>	-	-	10	-	-
<i>Shigella boydii</i>	-	-	-	-	-
<i>Shigella sonnei</i>	-	-	-	-	-
<i>Aeromonas hydrophila</i>	-	-	15	-	-

Acknowledgement

The authors thank the Principal and the management of Sri Sankara Arts & Science College, Kanchipuram for the research facilities provided. Authors also thank the NICED, Kolkata for the pathogenic strains provided.

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