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ORIGINAL RESEARCH ARTICLE

Isolation, Identification and Antagonistic Activity of Marine Actinomycetes Isolated From the Muthupet Mangrove Environment

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ABSTRACT

Actinomycetes are the representative of terrestrial microorganisms and usually are isolated from soils. When compared to terrestrial actinomycetes, however, a small portion of work has been conducted on marine actinomycetes. The marine Actinomycetes were well known for its antagonistic activity. In the present study, the sediment samples were collected from different stations of the Muthupet mangrove ecosystem (10°15'-10°35'N and 79°20'–79°55'E), situated along the Southeast coast of India, for isolation of actinomycetes, using Kuster agar medium. The isolated strains were identified using physiological and biochemical methods. Seven Actinomycetes sp were isolated and tested for their antagonistic activity against human pathogens like, *Escherichia coli, Pseudomonas* sp., *Klebsiella* sp. and *Bacillus* sp.

Key words: Actinomycetes, Mangrove ecosystem, Antagonistic activity and Human pathogens.

1. INTRODUCTION

Actinomycetes in marine and estuarine sediments have not been investigated extensively although their ubiquitous presence in marine sediments has been well documented [1].They were believed to be of terrestrial origin, transported to rivers by rain or irrigation water, and finally to the marine environment where they are exposed to water with salt concentrations and temperatures that differ from those of the terrestrial environment. As a result, some metabolic changes may occur in the organisms, and it was observed that 50% of Streptomyces were isolated from the marine environment showed antimicrobial activity, and the percentage was found to be increased when tests were conducted in the presence of seawater. As mentioned above, the marine environment is considerably different from the terrestrial environment, and therefore, it needs to be explored and exploited for new biological products. So far, microorganisms producing various bioactive compounds have been isolated largely from neritic environments [2].

Marine environments are considered to be largely untapped source for the isolation of new microorganisms which had the potentiality to produce active secondary metabolites. Among such microorganisms, actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities. The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Nowadays, considerable progress is being continuing within the fields of chemical synthesis and in the field of engineered biosynthesis of antibacterial compounds. So, the nature still remains the richest and the most versatile source for new antibiotics [3].

A search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research. Natural products having novel structures have been observed to possess useful biological activities. Soil is a natural reservoir for microorganisms and their antimicrobial products [4].

Marine actinomycetes reflected in the genetic and metabolic diversity, which remains largely unknown. Indeed, the marine environment is a virtually untapped source of novel *Actinomycetes* diversity and, therefore, of new metabolites. *Micromonospora* are common inhabitants of aquatic habitats and have proved to be a continuing source of novel bio active compound including antibacterial and antitumor agents. *Streptomycetes* possess maximum bio active

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secondary metabolites having great structural and functional diversity including antibiotics, antifungal, antiviral, anticancer, immunosuppressant agents and insecticides

In the recent years, marine microorganisms have known for antimicrobial, antiviral, antitumour, anticoagulant, anti-diabetic and cardioactive properties. Antibiotic effect of actinomycetes has been used in many fields including agriculture, veterinary and pharmaceutical industries. Most of the bioactive microbial metabolites were isolated from actinobacteria especially from Streptomycetes and also from some rare actinomycetes. During the last 20-30 years, the interest in the marine microflora increased due to the investigation of novel bioactive compounds especially antibiotics and enzymes. As the frequency of novel bioactive compounds obtained from terrestrial actinobacteria decreases with time, actinobacteria from diverse environments have been increasingly screened for their ability to produce new secondary metabolites. It has been emphasized that actinobacteria from marine sediments may be valuable for the isolation of novel strains which could potentially yield a broad spectrum of secondary metabolites [1].

The microbes keep on producing novel metabolites as they move into the diverse ecological units. From the biologically active compounds that have been obtained so far from microbes, 45 percent are produced by Actinomycetes, 38 percent by fungi and 17 percent by unicellular bacteria. Microbes have always been a better resource for getting lead molecule with novel scaffold to overcome any such limitation of existing drugs. Actinomycetes are the representative of terrestrial microorganisms and usually are isolated from soils. Compared to terrestrial actinomycetes, however, very little work has been conducted on marine actinomycetes. As marine environmental conditions are extremely different from terrestrial ones, it was observed that marine actinomycetes have characteristics different from those of terrestrial actinomycetes and therefore may produce different types of bioactive compounds [5].

2. MATERIALS AND METHODS

2.1. Sampling:

A study was done by the collection of samples from two stations in Muthupet Mangroves.

2.2. Sample collection:

The samples were collected in the month of February, from top 4 cm soil profile, where most of the microbial activity takes place and thus where most of the bacterial population was concentrated. Soil samples (approximately 500g) were collected using clean, dry and sterile polythene bags along with sterile spatula and other accessories. The site selection was done by taking care of the point where widely varying characteristics as possible with regard to the organic matter, moisture content, and particle size and colour of soil and to avoid contamination as far as possible. Samples were stored in ice boxes and transported to the laboratory where they were kept in refrigerator at 4^0C until analysis.

2.3. Isolation and enumeration of Actinomycetes population from collected soil samples

The soil samples collected from the station mentioned above were pooled and one ml soil sample was transferred to 100ml of sterile distilled water in a 250ml Erlenmeyer flask and incubated on a rotator shaker (100rpm) for 30 minutes at 28°C. The well mixed suspension was then diluted appropriately up to 10^{-6} dilution. One ml of suspension from 10^{-4} dilution was aseptically transferred to sterile petriplates and then 10-20ml of selective Starch Casein agar medium was added and incubated at 28ºC for 7 days. Three replications were maintained for each dilution and the colonies were counted using Qubec colony counter. The total number of colonies in the original samples was expressed as cfu g^{-1}. The isolated Actinomycetes strains were identified on the basis of their phenotypic characterization, physiological characteristics and a biochemical characteristic which includes some basic tests, aerial mass colour, reverse side pigment, melanoid pigments and spore chain morphology.

2.3.1. Aerial mass colour:

For the grouping and identification of Actinomycetes, the Chromogenicity of the aerial mycelium was considered to be an important character. The colours of the mature sporulating aerial mycelium are white, gray, red, green, blue and violet [7]. When the aerial mass colour falls between two colours series, both the colors were recorded. Whereas, in the cases where aerial mass colour of a strain showed intermediate tints, then in that place both the colour series should be noted.

2.3.2. Reverse side pigments*:*

The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and not distinctive (-). A colour

with low chroma such as pale yellow, olive or yellowish brown occurs, it should included in the latter group (-).

2.3.3. Melanoid Pigments*:*

The grouping is made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colours) on the medium. The strains are grouped as melanoid pigment produced (+) and not produced (-) [6]. For the melanoid pigment observation, the inoculated plates where kept under incubator for 4 to 5 days. The strains which shows cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color are recorded as positive (+), total absence of diffusible pigment, are recorded as negative (-) for melanoid pigment production.

2.3.4. Spore chain morphology:

The species with spore bearing hyphae are grouped into three types *viz*., Flexible- Rectiflexibiles (RF), Open loops- Retinaculiaperti (RA) and Spira- Spirales (S). A characteristic of the spore bearing hyphae and spore chains were determined by the direct microscopic examination of the culture area. Adequate magnification used to establish the presence or absence of spore chains and to observe the nature of spore chains. By the standard protocol of Cover slip culture technique, the plates were prepared and after the incubation of 7 to 10 days it was observed. During this method of spore morphological study, plates have to be prepared containing ISP2 medium. After solidification, using a sharp scalpel from the central portion of the plate, medium should be scooped out making a rectangular area. Then three sterile cover slips have to be placed on the hollow rectangular space. Slowly actinomycetes spores have to be inoculated at the edge of the cover slips touching the medium. The plates must be incubated at 28±2°C for 5 days and examined periodically taking out the cover slips.

2.3.5. Biochemical characteristics:

Assimilation of carbon sources*:*

The ability of different actinomycetes strains in utilizing various carbon compounds as source of energy was studied by the following method recommended in International Streptomyces Project. The Carbon utilization medium was used in this Carbon assimilation test. Stock solution of 10 sugars i.e; D-Glucose, L-Arabinose, D-Sucrose , D-Fructose, D – Xylose, D-Raffinose, D-Mannitol , Cellulose, Rhamnose and Inositol having concentration of 10x was prepared in sterilized water and sterilized by filtering through 0.22µm pore size membrane filters and stored at 4°C. Growth of actinomycetes strain was checked by taking 1% carbon source in ISP2 media. Plates were streaked by inoculation loop by Flame sterilization technique and Incubated at 28^0C for 7 to 10 days. Growth of the isolates was observed by comparing them with positive and negative control.

2.4. Screening for antimicrobial activity

Five clinically important human pathogenic bacteria *Pseudomonas* sp., *Escherichia coli*, *Klebsiella* sp., *Bacillus* sp., and *Proteus* sp. were inoculated in Nutrient broth. The test organisms were spread over the surface of the medium. After solidification, a well was made on the agar medium using sterile cork borer and cell filtrates were placed in the well and incubated at 28º C for 48 - 72 hours. After incubation, the diameter of the zone of inhibition around the well was measured to evaluate the antibacterial activity of actinomycetes isolates. The inhibitory activity of the Actinomycetes isolates against the test pathogens was determined by measuring the diameter of the zone of inhibition and expressed in mm.

3. RESULTS AND DISCUSSION

3.1. Isolation and enumeration of Actinomycetes sp:

Distribution and diversity of actinobacteria have been reported from marine habitats such marine sediments by Jensen *et al.*, (1991) (7). The Actinomycetes sp. was isolated from the soil samples of two different sites of Mangroves in Muthupet, East costal region of Tamil Nadu. The sediment soil sample was analyzed by Serial dilution techniques and seven pure actinomycetes isolates were obtained by Spread plates technique and maximum population was noted in dense mangroves (18.23 cfu g^{-4}) (**Table 1**). The strains were identified on the basis of their physiological and biochemical characteristics.

The cultural and microscopic characterizations of actinomycetes were showed in (**Table 2**). Aerial mass colour of the substrate mycelium was determined by observing the plates after 7 to 10 days. It was done only after observing the heavy spore mass surface. The common colours found in the strains were White (W), Yellow (Y), Grey (G) and dark ash. The aerial mass colour of the isolates AM1and AM4 showed white colour. The isolate AM3 exhibited yellow coloured aerial mass and the isolates AM2, AM6 and AM7 were

Greyish white in colour. This isolate AM5 showed grey aerial mass colour.

The strains were divided into two groups according to their ability to produce pigments on the reverse side of the colony, namely distinctive (+) and not distinctive or none (-). Reverse side pigments and melanoid pigmentation was observed by the formation of greenish brown, brownish black or distinct brown pigment. The colours observed for not distinctive isolates were pale yellow, olive or yellowish brown colour marked as (-). Spore chain morphology was done by the Coverslip culture technique. The slides were examined under microscope of 40x. Spira was shown by maximum strains namely AM1, AM2, AM3, AM4, AM5 and AM6.

The ability of different actinomycetes strains for utilizing various carbon compounds as source of energy (**Table 3**) was done by following the method recommended in International Streptomyces Project. After compairing growth with negative and positive control, it was observed that mannitol was the most assimilated carbon source by all strains of Actinomycetes and the arabinose was least assimilated carbon source.

After obtaining all the results from the experiment done were matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project). The match was done on the basis of maximum percentage of resemblance of characteristics. The seven isolates were *S.neyagawaensis, A.aureocirculatus, A.aureocirculatus, S.spheroides, S.albulus, S.antibioticus, S.mirabilis* and *S.umbrosus.*

The antimicrobial activities of the Actinomycetes isolates against test pathogens were showed in (**Table 4**). These studies indicates that the AM3 strains have shown good antimicrobial activity against *Klebsiella* sp. and *Proteus* sp. Growth of *Proteus* sp. was inhibited by most of the strains. AM4 has shown activity against *Proteus* sp. only. *Pseudomonas* sp. was the least inhibited pathogenic strain, AM1 and AM3 has shown moderate inhibition against it. *Escherichia coli* have also shown very least resistance against any strains. Earlier studies carried out by [8] actinomycetes exhibited antimicrobial activity against human and plant pathogens, respectively. Among eight isolates from the pH range 7–7.5, 3 (37.5%) and 8 (100%) marine actinomycetes showed antimicrobial activity against human and plant pathogens.

Table 1:Enumeration of actinomycetes

S.No	Total actinomycetes Population ($x10^4$CFU/g) in Muthupet
1	14.23
2	12.65
3	16.94
4	14.28
5	10.23
6	18.23
7	15.23

Table 2: Physiological characteristics of the Actinomycetes isolates

Isolates	Aerial mass colour	Melanoid pigmentation	Reverse side pigments	Spore chain morphology
AM1	W	-	-	S
AM2	Gy (W)	-	-	S
AM3	Y	-	-	S
AM4	W	-	-	S
AM5	Gy	+	-	S
AM6	Gy (W)	+	+	S
AM7	Gy (W)	+	-	RA

Table 3: Assimilation of carbon sources

Isolates	Xylose	Inositol	Sucrose	Raffinose	Fructose	Rhamnose	Mannitol
AM1	+	+	+	+	+	+	+
AM2	+	+	+	-	+	+	+
AM3	+	-	+	±	+	+	+
AM4	-	+	-	-	-	++	+
AM5	-	+	-	-	-	+	+
AM6	-	+	-	-	-	+	+
AM7	+	+	-	-	-	+	+

Table 4: Antimicrobial activity against human pathogens

S.No	Strain	*Pseudomonas* sp	*Escherichia coli*	*Klebsiella* sp.	*Bacillus* sp.
1	AM1	±	+	+	+
2	AM2	- -	-	+	-
3	AM3	±	-	++	++
4	AM4	-	-	-	+
5	AM5	-	-	+	+
6	AM6	-	-	+	+
7	AM7	-	±	-	-

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