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Isolation and Characterization of Actinomycetes from Mangrove Samples

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Abstract

Mangroves are a group of plants that occur in the coastal intertidal zones of tropics and the sub-tropics. It is a unique ecosystem that is considered as a huge treasure-house of mangrove actinomycetes resources. Microorganisms of this unique system are also unique in several aspects. Among these, actinomycetes have gained special attention as they are producers of almost half of the discovered bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. Mangroves are nutritionally versatile which affect the genetic diversity of microbes over there and therefore produce novel metabolites. It is difficult to isolate novel actinomycetes from this ecosystem because of abundance of identified organisms. The present study was conducted with the aim of isolating biologically important Actinomycetes from mangrove ecosystem for the production of bioactive products. For this initially water samples were collected from a mangrove ecosystem at Veli lake, Thiruvananthapuram. A total of 4 actinomycetes strains were isolated from the selected sample. They were partially characterized morphologically and biochemically.

Keywords: Mangroves, Actinomycetes, biochemical characterization

Introduction

Actinomycetes are an important group of microorganisms which are reported to have a wide range of activities including production of industrial enzymes and bioactive metabolites. The population on earth is ever increasing. The industrial sector in India is developing fast for meeting the needs of food processing, pharmacy and textile industries. Mangrove organisms as a whole and microorganisms in specific have unique properties since they have to adapt to extreme mangrove environment conditions such as high temperature, pH and hardness which are characteristic of mangrove ecosystems. These distinctive characteristics have attracted many researchers to explore the mangrove systems in depth since there is the potential of mangrove microorganisms to be used in industry. Thus, it is very crucial for the isolation of new groups of actinomycetes from unexplored or under exploited habitats be pursued as sources of novel bioactive secondary metabolites.

Actinomycetes are a group of microorganisms which morphologically resemble fungi and physiologically resemble bacteria, (Gayatri Devi *et al*., 2013). They are Gram-positive, free living and saprophytic bacteria having high guanine-cytosine content (> 55%) in their DNA. They are the most economical and biotechnologically important class of prokaryotes producing secondary metabolites notably antibiotics, anti-tumor agents, immunosuppressive agents, enzymes and enzyme inhibitors, (Valli *et al*., 2012).

Actinomycetes are of ubiquitous occurrence. They are distributed widely in almost every habitat. Jensen *et al*., (1995) reported that actinomycetes isolated from marine environment are metabolically active and have adapted to life in the sea. So many actinomycetes have been reported from mangrove ecosystem as well. Mangroves, a unique ecosystem is an intertidal zone located in the changeover between land and sea. This ecosystem is characterized by periodic tidal flooding which makes environmental factors such as salinity and nutrient availability extremely variable, resulting in unique and precise characteristic, (Holguin *et al.,* 2001).

Hong *et al*., (2009) opined that discovery of novel bioactive compounds from any environment is a wasteful process because there are compounds already discovered and an attempt to isolate novel bioactives will lead to a repeated isolation of the known compounds. Thus it is emphasized

that undiscovered bacteria should be first isolated and then search for novel bioactives from them. Therefore, researchers are now looking into underexplored habitats such as the oceans (Bian *et al.*, 2009), deserts (Hozzein *et al.*, 2004) and mangrove forests (Xu *et al*., 2014; Prabhahar *et al*., 2014) for new resources of actinomycetes. Since actinomycetes are now known to be potent microbes producing so many bioactive metabolites, it is stipulated that new resources of actinomycetes will lead to the discovery of new bioactive compounds which can be converted to a myriad of chemical analogues with a range of biological activities.

With a view of the significance of actinomycetes in mangroves, which is a rich source of novel bio active compounds and the increased industrial applications of the enzymes secreted by them, the present study is aimed to isolate actinomycetes from a selected mangrove ecosystem.

Materials and Methods

Sample collection

Water samples for the isolation of actinomycetes were collected from mangrove ecosystem at Veli, Thiruvananthapuram. The samples were collected in sterile bottles as per standard microbiological procedures and were brought to laboratory maintaining a cold chain and refrigerated.

Enrichment and Isolation of Actinomycetes

For enrichment 1 mL of sample was transferred to 100mL of starch casein broth supplemented with 25 mg/mL cycloheximide and 25 mg/mL nalidixic acid for preventing the growth of bacteria (Kumar and Kannabiran, 2010) and incubated at 30^{o}C for 7 days in shaker at 200rpm. Isolation of actinomycetes was done by the serial dilution and pour plate technique. A loopful of inoculum from the starch casein broth was streaked onto the starch casein agar (SCA) supplemented with 50 µg/mL fluconazole and incubated at 30°C for 7 days (Savitri and Azmi, 2003). Single separated colonies were selected and the subcultures were maintained on starch casein slants at 4°C until further use.

Identification of marine actinomycetes by cover slip method

The isolated strains were confirmed as actinomycetes by observing their morphology under microscope. Starch casein agar was poured on sterile slide and allowed to solidify. Then the organisms were streaked on it and incubated at 37°C for 48hrs. After that, 2 drops of methylene blue dye was added and allowed it to stand for a minute. Then the slide was covered with cover slip and observed their morphology under microscope (Prazeres *et al.*, 2006).

Biochemical characterization

Biochemical characterization of the actinomycete cultures was carried out based on Bergeys Manual of Determinative Bacteriology (1994) and Cappucino and Sherman, (1992).

Methyl red and vogues' proskaeur test (MR-VP)

(A) Glucose phosphate medium:

Peptone	- 1.4 g
Dextrose	- 1.0g
Di potassium phosphate	- 1.0g
Distilled water	- 200mL

(B) Methyl red indicator

Methyl red	- 10g
Ethanol	- 25mL
Distilled water	- 25mL

(C) **Barrits reagents**

(a) Solution A - Dissolve 3gm of α- naphthol in 50mL of 95% ethanol

(b) Solution B - dissolve 8gm of KOH in 50mL of distilled water

Glucose phosphate medium was taken in two set of test tube. Hundred micro liters of actinomycete cultures were inoculated to each of test tube at 37^0C for 48 hours. After incubation a few drops of methyl red was added, to one set of tubes and noticed the colour change. Appearance of a red colour indicated positive reactions. The glucose phosphate medium having

neutral pH was converted to acidic due to the fermentation of glucose by the introduced microorganisms. Methyl red indicator shows red colour at pH 6.9.

Barrits reagent solution A (0.5 mL) was added to the second set of tubes, and shaken vigorously till pink/ red colour appeared.

Voges-Proskeaur test differentiates the actinomycetes producing large amounts of acid. These organisms grow anaerobically on glucose via respiratory mechanism and shift to butenediol fermentation via EMP pathway (Embden-Meyerhof-Parnas Pathway). Acetion formed in the culture is oxidized into diacetyl in the presence of oxygen and potassium hydroxide. Diacetyl reacts with VP reagent gives a crimson red colour complex (Navami *et al.*, 2015).

Oxidase test

Reagent is prepared by dissolving 100 mg (1 mg in 10 mL) of N, N, N, N, tetramethyl- P-Phenyl- enediamine dihydrochloride in 100 mL distilled water. Filter paper soaked with oxidase reagent was placed on glass slides. Actinomycete cultures were streaked on the filter paper. Positive reaction was indicated by the appearance of purple colour. This test was used to detect the production of oxidase enzyme by microorganisms.

Urea hydrolysis

Actinomycete cultures were grown in media containing (gL^{-1}) yeast extract (0.1), monopotassium phosphate (9.1), dipotassium phosphate (9.5), urea (20), and phenol red (0.01). pH was adjusted to 6.8. The medium was filter sterilized as 5mL volume in tubes. The tubes were inoculated with the culture and incubated for 48-72 hours. Positive reaction was indicated by cherry red colour.

Catalase test

A drop of 3% hydrogen peroxide was placed on a clean glass slide. Actinomycete colonies transferred into it. Formation of effervescence was considered as a positive catalase test. Biochemical characterization of the isolated actinomycete strains is given in the table.

Gram's staining

The isolated cultures were subjected to Gram's stain to make sure they are actinomycetes. Reagents used for this were crystal violet, gram's iodine, 95% ethyl alcohol and safranin. The actinomycete cultures were stained with crystal violet for one minute and excess stain was washed off with tap water. Gram's iodine application was carried out for one minute and then washed off with tap water. 95% ethyl alcohol was added drop by drop and washed off with tap water. The slide was counter stained with safranin for 45 seconds. Then it was washed off with tap water, and examined under microscope. Development of purple color represented positive and pink color represented negative test.

Results and Discussion

Mangrove samples when inoculated in the enrichment media showed heavy growth of microorganisms after 7 days of incubation. Standard Plate Count was made to find the actinomycete load and to isolate actinomycetes from enrichment. A total of 4 actinomycetes strains were isolated from the selected marine samples (M1, M2, M3 and M4). The morphological characters of the strains were identified using cover slip method. The results are shown in Table 1. Previous studies have shown that, actinomycetes are ubiquitous in nature including water and sediments of marine and estuarine environments.

Biochemical characterizations of the isolated actinomycetes strains were done using standard procedures and the results are shown in Table 2. The ecological features of the habitat in which marine organisms bloom will have an effect on their metabolic functions, subsequently enabling their bio molecular machinery. Further studies on the molecular characterization of the isolates are in progress.

Table: 1. Colony morphology of isolated actinomycetes

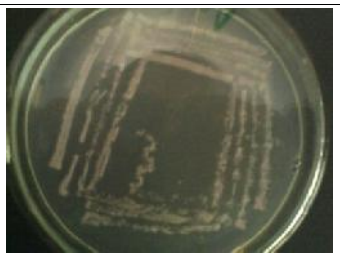
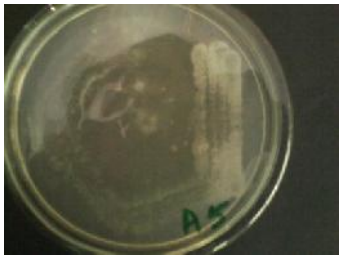
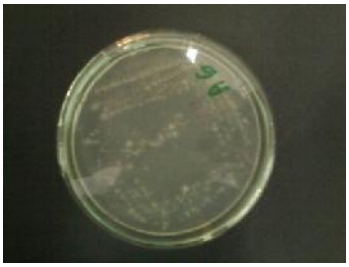
Name of the Colony isolated	Morphology	Figure
M1	Pink, Branching, Irregular, Dry	
M2	Grey, Powdery, Irregular, Dry, Early stage-off white	
M3	Grey, Powdery, Irregular, Branching, Dry	
M4	Slight grey, Powdery, Branching, Irregular	

Table: 2. Biochemical characterization of the isolated actinomycetes strains

Strains	M1	M2	M3	M4
Gram's staining	+	+	+	+
Methyl red	_	_	_	_
Voges proskauer	+	_	+	_
Oxidase test	+	+	_	+
Urea hydrolysis	_	_	_	_
Catalase test	_	+	+	+

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