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Screening of Rhizosphere microorganisms from mangroves forest for saline tolerance and antibiotic resistance

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Abstract

The mangroves ecosystem is considered to be one of the most productive ecosystems. It has some of the richest biodiversity hot spots for both flora and fauna. Better understanding of this ecosystem will help us to exploit the diversified gene pool like saline resistant genes and antibiotic resistant genes. In the present investigation carried out to study the saline resistant and antibiotic resistant properties of soil bacteria of Pichavaram mangroves forest. The rhizosphere soil samples of Rhizophora mucronata and Rhizophora apiculata from reserved forest of Pichavaram were collected and analysed for their physicochemical properties. Total bacterial, fungal and actinomycetes populations of rhizosphere soils of *Rhizophora mucronata* and *Rhizophora apiculata* were enumerated. The resulted in five bacterial isolates (MRB-1 to MRB-5), three fungal isolates (MRF-1 to MRF-5) and one actinomycetes (MRA-1) from rhizosphere of *Rhizophora mucronata* and *Rhizophora apiculata* of Pichavaram forest. The above isolates were subjected to various biochemical tests and tentatively identified bacterial isolates namely, *Serratia marcens* (MRB-1), *Bacillus subtilis* (MRB-2), *Pseudomonas fluorescens* (MRB-3), Alcaligenes sp. (MRB-4) and *Bacillus polymyxa* (MRB-5). The tentatively characterized fungal and actinomycetes isolates are *Aspergillus niger*, *Trichoderma viride*, *Aspergillus flavus*, Actinomycetes and *Nocardia* sp. Respectively. The five bacterial isolates MRB-1 to MRB-5 were tested for their saline tolerance level. The bacterial, fungal and actinomycetes were individually tested for their antibiotic sensitivity spectrum with different antibiotics viz., Kanamycin, Tetracycline, Streptomycin, Chloramphenicol, Cephalosporin and Amphicillin. The five bacterial isolates were screened for presence of antibiotic resistance plasmids by agarose gel electrophoresis technique.

Keywords: mangroves ecosystem, rhizosphere soil, bacterial isolates, fungal and actinomycetes.

Introduction

The coastal zone of India endowed with a very wide range of coastal ecosystems such as estuaries, lagoons, mangroves, back waters, salt marshes, rocky coasts, sandy stretches and coral reefs is characterized by unique biotic and abiotic properties and processes. Mangrove ecosystems command intensive attention among the coastal ecosystem due not only to their peculiar habitat characteristics but also due to their rich biodiversity.

Mangroves situated in tropics and the sub-tropics of the world consist of forest growing under brackish water conditions in the seashore. Mangroves interacting effectively with aquatic, inshore, upstream and terrestrial ecosystems support a diverse flora and fauna of marine, freshwater and terrestrial species. These ecosystems are among the most productive ecosystem of world in terms of both floral and fauna. Many studies have found that mangroves help protect coastlines from erosion, storm damage, and wave action by acting as buffers. They also protect coral reefs and sea grass beds from damaging alteration and pollution.

The total area of the mangrove wetlands of the world is about 181,077 sq.km or 18,1,07,700 ha. According to the Forest Survey of India (1999), the total area of Indian Mangrove Wetland is about 4,87,100 ha of which 565.7% (2,75,800 ha) is on the east coast, 23.5% (1,14,700 ha) on the west coast and the remaining 19.8% (96,600 ha) on the Andaman and Nicobar islands. Tamil Nadu has a coastline of 950 km. Extensive mangrove forests are located in two places in Pichavaram, Cuddalore District and Muthupet in Thiruvarur and Thanjavur Districts. The Pichavaram mangrove forest is located in the Northern extreme of the Cauvery delta, near the mouth of revere Coleroon. Its total area is about 1,350 ha. In the Pichavaram mangrove forests of Cuddalore district, wave energy along the coast is high but a sandy beach, located between the mangroves and the sea, protects the mangrove (Ramasubramanian *et al.*, 2003).

The Pichavaram reserved forest is well known for its biological diversity of plants and microorganisms. The plant species dominated in this area are *Rhizophora* spp. and *Avicenia* spp. *Rhizophora* spp. has roots, which branch from the stem to form stilt-like structures to help prop up the tree in the soft substrate. Several recent studies have documented the presence of antibiotic resistance in human and domestic animal cases. However limited research is available on the presence of antibiotic resistance in marine environment, which is recognized as highly productive ecosystem.

Materials and Methods

The Pichavaram Mangrove forest lies between the Vellar and Coleroon estuaries is well known for its heterogeneous mixture of mangrove elements (lat. 11º 27' N; long. 79 º 47E') and spread over an area of 10 sq. km.

Collection of soil samples

Representative soil samples were collected from rhizosphere of *Rhizophora mucronata* and *Rhizophora apiculata* plant seedlings of mangrove plants of Pichavaram forest reserved area by following standard protocols. The samples were transferred to laboratory and preserved in the refrigerator for microbiological analysis and Physiochemical properties of rhizosphere soil of *Rhizophora* spp of Pichavaram forest, pH, E.C., Macronutrients and micronutrients were analyzed as per standard techniques (Table 1).

Enumeration of total microorganisms from the rhizosphere soil of *Rhizophora mucronata* and *Rhizophora apiculata* collected from mangrove forest, One gram of streptomycin sulphate was dissolved in 100ml of sterile water and from this 0.5ml per liter was added after sterilization to check the growth of bacteria.

Screening of Rhizosphere isolates for saline tolerance

Nutrient broth, Kenknight's broth and Czapexdox's broth were prepared by adjusting the pH from 8.0 to 12.0 and dissolved thoroughly. The respective broths were poured into different test tubes and autoclaved. The isolates MRB_1, MRB_2, MRB_3 and MRB_4 were inoculated into Czapexdox's broths. The isolate MRA_1 was inoculated in Kenknight's broth with varied hydrogen ion concentration from 8.0 to 12.0. The respective broths were observed for the cloudiness. The cloudiness (or) turbidity indicates the growth of the isolates at the different pH.

Screening of isolates for antibiotic resistance

Assay of antibiotic resistance among the bacterial isolates of mangrove forest

The Muller-Hinton agar plates were prepared and allowed to solidify. The Whatman filter paper were made into disc and dipped into the respective antibiotic solutions at 50 ppm concentration overnight. The Muller-Hinton agar plates were spread plated with MRB^{-1}, MRB^{-2}, MRB^{-3} and MFB^{-4} respectively. The antibiotics used for antibiotic resistance studies were Kanamycin, Cephalosporin, Streptomycin, Tetracycline, Amphicillin and Chloramphenicol. The discs were placed at equidistance and incubated for 24 hours at 37ºC. The plates were examined for clearing zone and the diameters of the clear zone were measured.

Study on the plasmid profile of selected microbial isolates from the rhizosphere of Rhizophora mucronata and Rhizophora apiculata of mangrove forest

Plasmids occur widely in nature, and are found in most bacterial species. They vary considerably in size, from a few thousand base pairs up to several hundred kilo bases. The most notable property of plasmids lies in their ability to disseminate antibiotic resistance genes. The present plasmid profile study MRB-1, MRB-3 and MRB-4 showed the presence of plasmid,

the size of the plasmids of all the isolates between 1000-1500 kb with narrow variation among them.

Results and Discussion

The mangrove forest and associated water bodies are together called mangrove forest. Mangroves are defined as woody trees and shrubs that grown in places where river water mixes with seawater. These places are otherwise called estuarine or brackish water environment. An assemblage of such woody trees and shrubs is called a mangrove forest. Since mangrove forest are located in the estuarine environment, they are intersected by a number of small creeks and channels and in many cases large open water bodies are also found associated with them.

Mangrove forests are one of the most productive and bio diverse forest on earth. Yet, these unique coastal tropical forests are among the most threatened habitats in the world. They may be disappearing more quickly than inland tropical rainforests, and so far, with little public notice. Growing in the inter-tidal areas and estuary mouths between land and sea, mangroves provide critical habitat for a diverse marine and terrestrial flora and fauna. Healthy mangrove forests are key to a healthy marine ecology.

Mangroves have two components, mangrove forests and associated water bodies (Selvam *et al.*, 2002). Tidal channels and canals that intersect mangrove forest and divide them into small islands and shallow lagoons and bays found associated with the mangroves constitute the water bodies. Mangrove forests are dominant features of the coastal areas of tropical countries.

Mangroves leaves and wood are made mainly of lingo cellulose components that are degradable by microorganisms (Alongi *et al.*, 1989; Moran and Hodson, 1989). Degradation of fallen mangrove vegetation starts immediately after its colonization by fungi and bacteria residing in the sediment and may last for 2-6 months or more for degradation of the wood (Newell *et al.*, 1984; Steinke *et al.*, 1990). The population of heterotrophic bacteria in Goa's (India) mangroves consists of microorganisms with cellulolytic, pectinolytic, amylolytic and proteolytic activity (Matondkar *et al.*, 1981). The fungus that decomposes mangroves has pectinase, protease and amylase activities and the capacity to degrade lignocellulosic compounds (Findlay *et al.*, 1986). The degradation of mangrove vegetative material produces detritus, which can be defined as organic matter in the active process of decomposition. It is rich in energy and contains a large active microbial population both attached and living free (Odum and Heald 1975a). Microscopic examination of decomposing mangroves leaves reveals a complex community composed of fungi, bacteria, protozoa and microalgae (Odum and Heald, 1975b). The total microbial biomass is never greater than 1.2% of the whole detritus mass and in most cases is substantially less than 1%. It is therefore unlikely that detritivores can rely solely on microorganisms as an energy source (Blum *et al.*, 1988). The nitrogen content in the fallen trunks of *Rhizophora* spp. increased 500 percent during the first 2 month of decomposition (Robertson and Daniel, 1989). In the present study, the bacterial, fungal and actinomycetes populations isolated were found to be in correlation with previous report. The bacteria isolates, *Pseudomonas fluorescens, Alcaligenes* spp., *Bacillus subtilis, Bacillus polymyxa, Serratia marcens* were to and intend a heterotrophic mode of life cycle. They are present in the rhizosphere of *Avicennia officinalis, Avicennia marina* and *Rhizophora* spp. There could be possible explanation to their presence for their ability to adapt to the salinity and possible secretion of pectinas, cellulase, liginase enzyme complex. This will help them to harness the rhizosphere environment. The adaptability of the bacterial isolates is important link in the biogeochemical cycle in the mangroves forest.

The fungal and actinomycetes isolates are *Aspergillus niger, Trichoderma viride, Aspergillus flavus* and *Nocardia spp*. are well established for their degrading ability of lignocellulosic waste materials. The mangroves leaves are rich in lignocellulosic waste. There is evidence of positive correlation for their presence. The plants that are reported in Pichavaram are unique in their biomass building up and been recycle. The rhizosphere isolates that have been recorded in the present study were heterotrophic, lingo cellulose utilizes, capable of producing pectinase, cellulose, ligninase, amylase and proteases. Hence, the results were in accordance with previous results. In addition, the presence of *Pseudomonas fluorescens* (MRB 3) in *Rhizophora* spp. can be correlated for its plant growth promoting abilities, the presence can help in conservation strategies for mangroves. The PGPR microbe *Ps. fluorescens* will produce IAA, Gibberellins and auxins to help the plant to grow faster and establish themselves in hardest environment like salinity and poor eco-based systems.

The influence of hydrogen ion concentration on the growth microbe helps us to understand the type of microorganism and how it can establish them to the

Table-1. Physicochemical properties of rhizosphere soil of Rhizophora spp collected from mangrove forest of Pichavaram

Sl.No	Parameter	Value
1.	PH	8.50
2.	EC (m Mhos)	8.2
3.	Nitrogen	0.78 ppm
4.	Phosphorus	0.77 ppm
5.	Potassium	365 ppm
6.	Sodium	12230 ppm
7.	Chloride	17490 ppm
8.	Calcium	420 ppm
9.	Magnesium	1290 ppm
10.	Sulphate	2500 ppm

PLATE – 2

Bacterial colonies of *Rhizophora* sp. (Pichavaram)

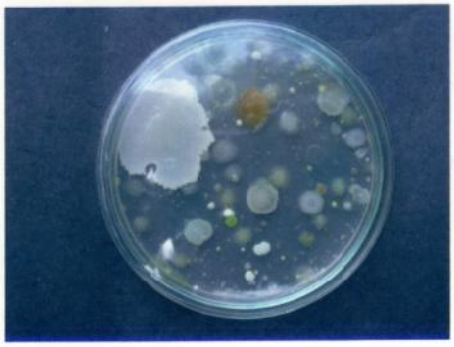

PLATE – 3

Fungal colories of *Rhizophora* spp Pichavaram)

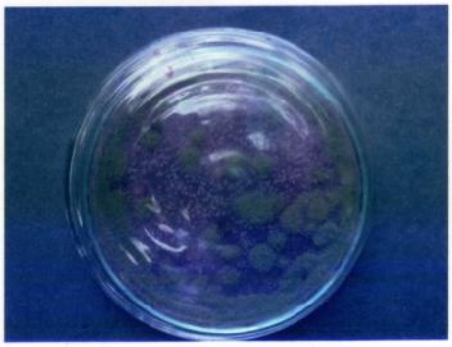

PLATE – 4

ANTIBIOTIC SENSITIVITY SPECTRUM OF PSEUDOMONAS FLUORESCENCE, BACILLUS POLYMYXA

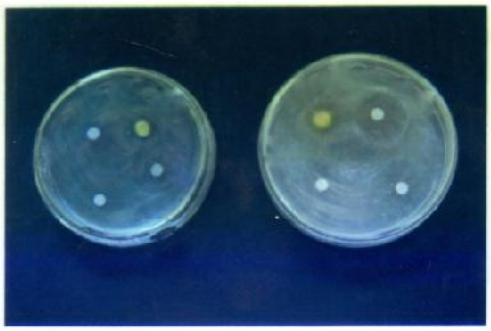

PLATE – 5

ANTIBIOTIC SENSITIVITY SPECTRUM OF ALCALIGENES SPECIES AND PSEUDOMONAS FLUORESCENCE

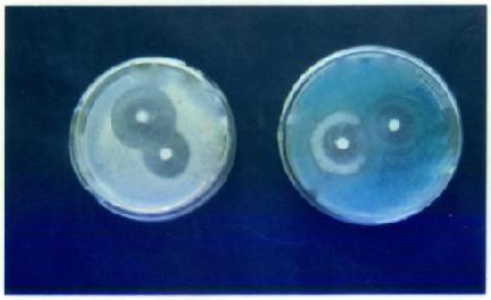

PLATE - 6

ANTIBIOTIC SENSITIVITY SPECTRUM OF BACILLUS SUBTILIS AND BACILLUS POLYMYXA

PLATE – 7

ANTIBIOTIC SENSITIVITY SPECTRUM OF BACILLUS SUBTILIS AND SERATIA MARCENS

particular ecosystem. The ability to adapt to high pH range from pH 8-10 will provide adequate understanding of the isolates ability to control its internal pH. Most of the gram negative organisms control internal pH according external pH by K^+/H^+ and Na^+/H^+ antiporter system (or) Homeostasis (Moat *et al.*, 2002). The important thing in mangroves is that they do not receive external terrestrial nutrients from rivers or other sources. It is vital that the health of the benthic microbial communities be maintained because these microorganisms are responsible for conserving the scarce nutrients with in the ecosystem (Bashan and Holguin, 1997). Further in the present study, the antibiotic resistance along with saline resistance character, it will be vital gene pool for biotechnological exploitation.

Globally, mangrove ecosystems are an important natural resource that should be protected. The detritus generated by the mangrove is the base of an extensive food web that sustains numerous organisms of ecological and commercial importance. Furthermore, mangrove ecosystem provides indispensable shelter and nurturing sites for many marine organisms. The well-being of mangroves is dependent on the diverse and largely unexplored, microbial and faunal activities that transform and recycle nutrients in the ecosystem.

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