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ORIGINAL RESEARCH ARTICLE

Isolation and Characterization of Total Heterotrophic Bacteria and Exopolysaccharide Produced From Mangrove Ecosystem

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ABSTRACT

Heterotrophic bacteria and their processes in the mangrove environment into focus, an understanding on their abundance, distribution, production and, their involvement in nutrient cycling and how they are at the base of microbial food web is essential. Total Heterotrophic Bacteria (THB) was screened from Pitchavaram mangrove sediment. Eight isolates were selected based on the colony morphology and of colonies were identified by phenotypic and biochemical character such as *Bacillus subtilis, Streptococcus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Photobacterium* sp., *Enterobacteriaceae* sp., *Escherichia coli* and *Azotobacter* sp. All the isolates were screened for producing Exopolysaccharide (EPS), of which *Azotobacter* sp. and *Pseudomonas* sp. produced high appreciable amount of EPS.

Key words: Mangrove, Biochemical characterization, Heterotrophic bacteria and Exopolysaccharide

1. INTRODUCTION

Mangrove forests occupy several million hectares of coastal areas worldwide and distributed in over 112 countries and territories comprising a total area of about 1,81,000 km^2 in over one fourth of the world's coastline[1,2]. According to Forest Survey of India (FSI) (State of Forest Report, 1999), out of 4,87,100 ha of mangrove wetlands in India, nearly 56.7% (2,75,800 ha) is present along the east coast, and 23.5% (1,14,700 ha) along the west coast and the remaining 19.8% (96,600 ha) is found in the Andaman and Nicobar islands. The largest single area of mangroves in the world lies in the Bangladesh part of the Sunderbans, covering an area of almost 6,00,000 ha including waterways. There are about 6.9 million ha in the Indo-Pacific region, 3.5 million ha in Africa, 4.1 million ha in Americas including the Carribbean. Mangroves also survive in some temperate zones but there is a rapid decrease in the number of species including latitude [3,4,5].

Mangrove ecosystems are rich in bacterial flora. Fertility of the mangrove waters results from the microbial decomposition of organic matter and recycling of nutrients. Among the microbes, the bacterial population in mangroves is many-fold greater than the fungi. In tropical mangroves, bacteria and fungi constitute 91% of the total microbial biomass, whereas algae and protozoa represent only 7% and 2% respectively [6].

Most of the sea bacteria belong to Gram-negative [7]. Gram-positive bacteria are less than 10% of the total bacterial population and higher percentage in sediments. *Arthrobacter* and endospore producing forms *Bacillus* and *Clostridium* (Family: Bacillaceae) have also been isolated. Especially *Bacillus* species readily grow in medium containing nutrients. Majority of bacteria belong to the families Pseudomonadaceae and Vibrionaceae. Most marine bacteria are aerobic or facultatively anaerobic because large parts of the ocean are well oxygenated. Nitrogen-fixing bacteria such as members of the genera *Azospirillum, Azotobacter, Rhizobium, Clostridium* and *Klebsiella* were isolated from the sediments, rhizosphere and root surfaces of various mangrove species. Several strains of diazotrophic bacteria such as *Vibrio campbelli, Listonella anguillarum, V. aestuarianus* and *Phyllobacterium* sp. were isolated from the rhizosphere of the mangroves in Mexico [8].

Mangroves provide a unique ecological environment for diverse bacterial communities. The bacteria fill a number of niches and are fundamental to the functioning of these habitats. For example, sulfate-reducing bacteria (e.g., *Desulfovibrio, Desulfotomaculu, Desulfosarcina*, and *Desulfococcus* [9,10]) are the primary

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decomposers in anoxic mangrove sediments. These bacteria largely control iron, phosphorus, and sulfur dynamics and contribute to soil and vegetation patterns [11]. Methanogenic bacteria are seasonally abundant in sediments where *Avicennia* species dominate [12,13]. Subsurface bacterial communities (along with epibenthic microalgae) may sequester nutrients and hold them within nutrient-limited mangrove muds [14].

Alginates are exopolysaccharides synthesized by marine algae [15,16], as well as by bacteria such as *Pseudomonas aeruginosa* and *Azotobacter vinelandii* [17]. *A vinelandii* synthesizes a polymer that is similar to that of the algae [17], [18]. The present study was aimed to isolate the total heterotrophic bacteria present in the mangrove sediments and rhizospheric soil and screening of exopolysaccharide producing bacteria.

2. MATERIALS AND METHODS

Sample collection

20 samples were collected from different sites in Pitchavaram. *Rhizophora apiculata* and *Rhizophora mucronata* plant rhizosphere and sediment (non rhizosphere) samples were collected in the clean polyethylene bags and transported to the laboratory and processed within 3 hours and microbial analysis were carried within 4 hours. Mangroves sediment are acidic and clay in nature. Sediment becomes loose because of the presence of the decaying organic matter and sediment is black in colour.

Enumeration of microorganisms from rhizosphere and non- rhizosphere soil of mangrove ecosystem

The soil samples collected from various sources were serially diluted up to 10^{-6} dilution to determine the population of bacteria, fungi and Actinomycetes [19]. The 10^{-6} dilutions were plated on sterile Petri plates containing nutrient agar (NA) medium and incubated at 28 ± 2°C for two days for enumerating the bacterial population, 10^{-4} dilutions were plated on sterile Petri plates containing Rose Bengal Agar medium (RBA) and incubated at 28 ± 2°C for 3 days for enumerating fungal colonies and 10^{-5} dilutions were plated on sterile Petri plates containing Kenknight's agar medium (KKA) and incubated at 30 ± 2°C for 5 to 7 days for enumerating actinomycete colonies. After incubation the number of bacterial, fungal and actinomycetes colonies in the respective plates were counted and the population was expressed in terms of cfu g^{-1} soil on oven dry basis. The R: S ratio was calculated by using the formula proposed by Aneja[20].

Screening and Identification of Total Heterotrophic Bacteria (THB)

The sediment sample were diluted and plated in Nutrient agar medium prepared with 50% aged seawater [21]. Triplicate plates from each dilution were incubated at 28°C. After incubation the colonies were counted by colony forming unit (CFU) and subculture by colony morphology. The different morphological and biochemical characterization [22] of the isolates were investigated according to the Bergey's Manual of determinative bacteriology [23].

Isolation of heterotrophic bacteria for EPS production

Culture conditions

The standard seed and production media contained per liter of distilled water: 6 g yeast extract; 0.6 g $(NH_4)_2SO_4$, 2 g Na_2HPO_4; 0.3 g $MgSO_4.7H_2O$; pH was adjusted to 7. To assess the effect of cultural conditions on bacterial exopolysaccharide production, standard medium was supplemented with different amounts of glucose, $(NH_4)_2SO_4$ Na_2HPO_4 and sodium acetate. Then, to limit the variation of pH within the range 7.2 - 6.7, this medium was buffered by adding 50 mM 3(N-morpholino) propane- sulfonic acid (MOPS) and then adjusting the pH to 7.2 with NaOH [24]**.** All media used were sterilized at 121°C for 15 min.

The maintenance culture was transferred to 100ml Erlenmeyer flasks containing 25 ml of sterile medium each. The seed culture was incubated on a rotary shaker at 300 min^{-1} and 35°C for 24 hrs. A fraction (1.25 ml) of the vegetative seed culture was then used to inoculate a 100 ml Erlenmeyer flask containing 25 ml of the same medium which was incubated at different shaking speeds (25 and 450 min^{-1}) and temperatures (23 - 42°C) for 48 to 120 hrs.

3. RESULTS AND DISCUSSION

Enumeration of microbial biodiversity in rhizosphere and non rhizosphere of mangrove soil

The enumeration of microorganisms in the rhizosphere and non- rhizosphere soil samples revealed that the rhizosphere soil sample contained higher microbial populations compared to non- rhizosphere soil (**Table 1**). The rhizosphere population was recorded as 2.84×10^9 for bacteria, 2.50×10^7 for fungi and 1.12×10^8 for actinomycetes and in non- rhizosphere 1.86×10^9, 1.65×10^7 and 1.00×10^8 for bacteria, fungus and actinomycetes respectively was observed.

Table 1: Enumeration of microorganisms from rhizosphere and non- rhizosphere soil samples

S. No	Source	Bacteria 1×10^9 g^{-1}			Fungi 1×10^7 g^{-1}			Actinomycetes 1×10^8 g^{-1}		
		Rhizosphere soil	Non-rhizosphere soil	R:S	Rhizosphere soil	Non-rhizosphere soil	R:S	Rhizosphere soil	Non-rhizosphere soil	R:S
1	Mangrove field soil	2.84	1.86	1.52	2.50	1.65	1.51	1.12	1.00	1.12

Phenotypic Characterization

Bacteria inhabiting mangroves environment are dominant microorganisms fairly well adapted to the extreme condition of mangrove ecosystem. Recent bacteriological studies of mangrove environment concerned mainly their sanitary, pollution and bacterial number [25]. Eight different isolates were identified and sub cultured in the Nutrient agar (**Table 2**). The following isolates 2, 3 and 8 observed as round colonies, isolate 5 is transparent in nature. Rhizoid structure was observed in the isolate 4, isolate 1 and 6 was mucoid and isolate 7 was filamentous in nature. Colony size of the isolates also varied from the range of 0.1- 1.5 mm. the colour was also varied as orange, yellow white and pale white.

Table 2: Showing the Morphological Characters of different bacterial isolates

Isolates	Colony Colour	Colony Size (mm)	Morphology and Nature of the Colony
1	White	0.1- 0.3	Round, mucoid
2	Orange	0.1-0.5	Round
3	Yellow	0.5- 1.5	Round, Convex
4	White	0.5- 1.0	Rhizoid, sticky
5	White	0.5- 1.2	Transparent
6	Pale white	0.3- 0.7	Mucoid, circular
7	White	0.7- 1.5	Filamentous, irregular
8	White	0.1-0.6	Round, convex

Biochemical Characterization

There are many methods for identifying bacteria. Traditionally, an observational and biochemical approach has been used. Simply looking at (and even smelling) a bacterial colony growing on an agar plate can give an experienced researcher clues to a bacterium's identity. Bacteria are categorized as "Gram Positive" or "Gram Negative" according to whether or not they are stained by a chemical dye, a common biochemical technique [22]. The screened isolates were subjected in to the biochemical characterization, test results of Indole, Methyl red, Voges Proskauer, citrate utilization, carbohydrate fermentation (Glucose, Sucrose and Lactose), Catalase and Oxidase (**Table 3**).

The results obtained in this study portray the bacterial community associated to the mangrove rhizosphere as a dynamic one, experiencing important changes in abundance of both total and active bacteria. In contrast to the vertical exponential decline in the bacterial abundance often observed in muddy terrigenous sediments [26,27] the community analyzed here was, in terms of abundance, rather homogenous through the depth. The results from our study indicate a higher proportion of Gram negative bacteria than Gram positive bacteria among the species of heterotrophic bacteria. The results were in agreement with the general rules that the proportion of Gram negative bacteria is much higher than the proportion of Gram positive bacteria in the ocean [28].

Table 3: Phenotypic and Biochemical characteristics of isolates

Tests	Isolates							
	1	2	3	4	5	6	7	8
Gram staining	-Ve Rod	-Ve Cocci	+Ve Rod	-Ve Rod	-Ve Rod	-Ve Cocci	+Ve Cocci	-Ve Rod
Indole	-	+	+	+	+	+	+	+
Methyl red	-	+	+	+	+	+	+	+
Voges proskauer	-	-	-	+	-	+	-	+
Citrate utilization	+	-	-	-	+	+	-	-
Glucose	+	+	+	+	+	+	+	-
Sucrose	+	-	-	+		+	-	+
Lactose	-	-	-	+		+	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	-	-	-	+		-	-	-
Starch hydrolysis	-	-	-	+	+	+	-	-

In our study, it indicated that *Pseudomonas* sp., *Bacillus* sp., *Enterobacteriaceae* sp., *Escherichia coli*, *Streptococcus* sp., *Azotobacter* sp., *Staphylococcus* sp. and *Photobacterium* sp. were abundant in the mangrove rhizosphere sediment samples. The result of *Streptococcus* and *Escherichia coli* were comparable to the earlier reports of Thompson[31][29][30] recorded 9 genera and Paramasivam (2002) recorded 10 genera of THB from Pitchavaram and Muthupettai mangrove environment respectively.

The genus *Bacillus* comprises a phylogenetically and phenotypically heterogeneous group of species. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats [32]. Several *Bacillus* strains from soils and mangrove sediments have already been reported as hydrocarbon degraders

and emulsifier producers [33]. Macrae [34] found bacilli as dominant rhizosphere organisms in mangroves and suggested that they should be targeted to provide microbial solutions which ameliorate polluted environments. All the isolates were further characterized based on EPS production. *Pseudomonas* sp. (Isolate 1) and *Azotobacter* sp. (Isolate 6) were found more efficient in producing EPS and were further analyzed.

Exopolysaccharide Production

Of all the EPS producing isolates, Isolate 1 showed the maximum (6.8 mg/ml) followed by isolate 6 (5.3 mg/ml) and isolate 7 showed the minimum (3.0 mg/ml) level of EPS production and the reference strain produced 5.2 mg/ml of EPS (**Table 4**).

In the present study all the isolates produced EPS in appreciable quantities. Among the isolates *Pseudomonas* sp. (Isolate 1) secreted highest amount of extracellular polymeric substances followed by *Azotobacter* sp. (Isolate 6) and other isolates.

Extracellular polymeric substances (EPS) are biosynthetic polymers produced by both prokaryotic and eukaryotic microorganisms growing in natural as well as artificial environments either as single species, in binary association or in heterogeneous communities. Burd [35] found that by decreasing heavy metal toxicity, PGPR increased plant growth. Bacterial EPS play an important role in cell adhesion, formation of microbial aggregates such as biofilms, flocs, sludges and biogranules and protect cells from hostile environments [36].

Table 4: Production of Exopolysaccharide (EPS) by different mangrove isolates

S. No	Name of the Isolate	Production of EPS(mg/ml)
1	Isolate 1	6.8
2	Isolate 2	4.9
3	Isolate 3	4.8
4	Isolate 4	4.7
5	Isolate 5	5.1
6	Isolate 6	5.3
7	Isolate 7	3.0
8	Isolate 8	3.6
9	*ATCC 14579	5.2

4. CONCLUSION

The present study concluded that the phenotypic and biochemical analysis is a suitable tool for characterize the Total Heterotrophic Bacterial (THB) community in Pitchavaram mangrove sediment and better understand the functioning of their related ecosystems. In addition to their distribution pattern and involved in nutrient cycling how they are act as a tool for biodegrading the nutrients in food web. *Pseudomonas* sp. (Isolate 1) and *Azotobacter* sp. (Isolate 6) were found more efficient in producing EPS and were further characterized for alginate production.

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