Diversity of free-living and adhered bacteria from mangrove swamps

Priya M. D'Costa, Sushanta Kalekar and Saroj Bhosle*

Department of Microbiology, Goa University, Taleigao Plateau, Goa, 403206, India

*Corresponding author; E-mail: saroj@yahoo.co.in

Tel: (91832)2451569

Mangrove samples revealed the presence of large number of adherent bacteria compared to free-living forms. Isolates were able to grow on plant polymers, xylan and cellulose. Most of these bacteria were pigmented, while only 25% of the free-living bacteria were pigmented. Among the pigments produced by *Pseudomonas* and *Serratia* species, prodigiosin and phenazines were detected and characterized. The adherent isolates showed 1.11 - 13.04% adherence to hexadecane with some showing 90% adherence to plant litter. Two isolates showing highest adherence to plant litter were identified as *Staphylococcus.*

Key words: Adherent bacteria, degradation, free-living bacteria, mangroves, pigments, plant litter.

Mangroves represent one of the most productive ecosystems in tropical environments and are characterized by efficient turnover of nutrients[1]. Presence of wide diversity of organisms in mangroves is associated with the degradation of plant litter and this brings about the cycling of carbon and nitrogen sources. Free-living bacteria, fungi and yeasts have been reported to play a significant role in the formation of detritus[2,3]. However, such microorganisms are subjected to varying tidal actions that affect physicochemical parameters such as pH, salinity and nutrients, which in turn affect the rate of mineralization. However, bacteria that can attach to surfaces (adhered bacteria) play an important role in such environments since attachment to plant litter, irrespective of the tidal variation, confers much better decomposing ability[4-6]. Bacterial attachment to particles has been associated with increase in heterotrophic activity, leading to high mineralization rate, biomass production and accumulation of secondary metabolites[7]. We report here the distribution of free-living and adherent bacteria in mangrove ecosystems of Goa and describe their attachment behaviour to solid and liquid substrates.

Materials and Methods

The site chosen for this study was along the Mandovi estuary extending from outside the city of Panaji to Ribandar (15˚ 30’ N; 73˚ 52’ E). The dominant vegetation in this area comprised *Avicennia marina*, *Rhizophora apiculata* and *Rhizophora mucronata.* Surface sediment samples (500 g) along with decomposing plant litter comprising leaves, roots, pneumatophores and bark, were collected and transported to the laboratory in plastic bags for further analysis. Sample (100 g) was suspended in 100 mL distilled water, mixed well and allowed to stand for 1 h for the heavier particles to settle down. The turbid solution was filtered through Whatman filter paper No. 1 and the filtrate used to measure pH, salinity and dissolved oxygen (DO)[8].

For enumeration of bacteria, sediment samples were homogenized in a surface-sterilized mortar and pestle and diluted (upto 10^{-4}) in sterile saline. Free-living bacteria were counted by spreading 0.1 mL aliquots on nutrient agar plates and incubating at room temperature (26-31 ˚C) for 24 h. For estimating adherent bacteria[9], wet sediment (100 g) was washed with 200 mL sterile saline till the washings appeared clear. The washed samples were ground for 5 min in a surface-sterilized mortar and pestle. The extracts were filtered into a sterile screw-capped bottle. The filtrate was diluted (upto 10^{-6}) and plated on nutrient agar prepared in (a) distilled water and (b) seawater, xylan agar[10] and carboxy methylcellulose (CMC) agar[11]. Appropriate dilutions (10^{-1}, 10^{-2}, 10^{-3}) were also spread plated on nutrient agar with 10% glycerol[12], nutrient agar prepared in aged sea water[13], glucose yeast extract agar[14] with 10% skimmed milk, Zobells' marine agar and mineral salts agar with 0.5% glucose[15] to recover pigmented forms. All the plates were incubated at 26 ºC-31ºC for 48 - 72 h. Predominant bacterial morphotypes were purified on respective media and maintained at 4ºC. Isolates were identified based on their morphological, physiological and biochemical characteristics using Bergey's Manual of Systematic Bacteriology[16,17].

Bacterial adherence to hexadecane (BATH assay) and plant litter was determined[18]. The absorption spectra of isolated pigments were determined by inoculating loopfuls of 24 h old bacteria in respective liquid (25 mL in 50 mL flasks)/solid medium and incubating on a shaker (180 rpm)/stationary conditions at room temperature for 72 h. Isolates P22 and P24 did not produce pigment in liquid media; they were thus cultured on respective solid media (Table 1) and then suspended in sterile saline. The broth cultures were centrifuged at 3500 g for 10 min; intracellular pigments were extracted from the cell pellet with 5 mL acetone[19], followed by sonication (pulse of 15 sec for 2 min) in a Vibrionics ultrasonic processor and centrifugation (3500 g for 10 min). The

supernatant was scanned in the visible range i.e., 350 - 700 nm and compared with those reported in the literature.

Results and Discussion

The study site showed significant variation in physicochemical parameters during pre-monsoon and monsoon seasons. The pH varied from 3.7 - 6.6 to 2.69 - 7.66 and salinity fluctuated from 11.045 - 16.730‰ to 8.955 - 13.465‰ during pre-monsoon and monsoon seasons, respectively. However, the level of DO remained nearly constant (9.80 - 20.10 mg L^{-1}, pre-monsoon; 8.48 - 22.10 mg L^{-1}, monsoon). In mangrove ecosystems, DO levels remain constant throughout the year except in stagnant pools that may turn anaerobic on long standing[20].

Viable counts of free-living bacteria were found to be 10.9 x 10^6 cfu g^{-1} and 8.1 x 10^6 cfu g^{-1} and pigmented bacteria ranged from 1.95 x 10^6 cfu g^{-1} (17.97%) to 0.18 x 10^6 cfu g^{-1} (22.22%) during pre-monsoon and monsoon seasons, respectively. During monsoons, detrital organic matter in the water increases due to upwelling caused by torrential rains and flooded rivers, which favours the growth of adherent flora[21]. The average viable counts of adherent organisms on nutrient agar ranged from 2.65 x 10^8 cfu g^{-1} (pre-monsoon) to 3.56 x 10^8 cfu g^{-1} (monsoon).

Majority of the bacteria showed intracellular pigmentation with predominance of yellow-pigmented forms (33.33%) and orange-pigmented bacteria (25.93%). In addition, high counts (1.74 x 10^5 - 1.89 x 10^5 cfu g^{-1} and 45 x 10^3 - 53.8 x 10^3 cfu g^{-1}) were obtained on xylan and CMC agar, respectively, suggesting that such organisms degrade plant material efficiently. These bacteria

appeared to be highly competent and adaptive to changes in pH, salinity and other physico-chemical parameters.

A total of 37 pigmented cultures were isolated from mangrove samples with only 12 isolates showing consistent pigmentation. During sub-culturing, 3 purple pigmented isolates (tentatively identified as *Chromobacterium*) lost viability in 3 days after storage at 4°C. Rapid death of aged *Chromobacterium* cultures has been reported to occur due to accumulation of antibiotics in the medium and autolysis[22].

Among the isolates, Gram-positive rods were predominant, followed by Gram-positive cocci and only few Gram-negative coccobacilli. The adherent isolates belonged to 4 genera namely *Bacillus, Micrococcus, Pseudomonas* and *Staphylococcus;* free-living, pigmented cultures belonged to *Bacillus, Beijerinckia, Erwinia, Microbacterium, Micrococcus, Pseudomonas, Rhodococcus, Serratia*, *Staphylococcus* and *Xanthomonas.* (Table 1). Of these, only *Pseudomonas* isolates produced extracellular pigments.

Pigmentation has been reported to provide protection against uv[23]. Pigments like prodigiosin and phenazines also possess antibiotic properties[24,25]. The production of phenazine pigments has been reported to confer a competitive advantage[26]. Other pigments like carotenoids and melanins alleviate stress. Such photosensitive pigments act as screens, protecting vital macromolecules and cellular elements from the deleterious effects of ultraviolet and visible light[27,28]. Also, as a result of its capacity to absorb Na^+ and K^+ ions, melanin prevents cellular dehydration by sequestering compatible solutes from the environment[29,30].

Of the two *Pseudomonas* cultures which produced extracellular pigment, isolate P31, after 24 h growth, showed presence of phenazines (Table 1). Isolate P36, after 24 h, showed a major peak at 364.5 nm and a minor peak (0.37) at 400.5 nm; after 48 h (brown pigment), the absorption spectrum changed to 400.5 nm (2.066) and 357.5 nm (2.675). The increase in absorbance at 400.5 nm within a span of 24 h is consistent with the development of the brown pigment over the same time period, a characteristic typical of *Ps. solanacearum*[16]. Isolates P22 and P24, identified as *Serratia*, showed presence of prodigiosin-like pigments on agar[19], but pigmentation was not observed in liquid media. Pigments of *Erwinia, Staphylococcus* and *Xanthomonas* could not be extracted successfully. As evidenced from the BATH assay, selected isolates were moderately hydrophobic (maximum adherence 13.04%) (Table 2) with adherence to substrate as high as 90% for two of the isolates. This indicates the capacity of these organisms to attach to the plant material and maintain a strong cell-substrate contact.

The abundance of adherent bacteria in mangrove sediment indicates the significance of the attachment phenomenon in such environments. Pigmentation, widely observed in adherent bacteria, confers an additional advantage and helps them survive stress. Such bacteria possibly exist as stable, adhered communities in tidally influenced environments and appear to play an important role in the decomposition of plant material and formation of detritus.

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Table 1. Characteristics of selected pigment-producing bacteria.

Medium	Isolate no.	Pigmentation	Identification	Peak observed at (nm)	Pigment identification
NAG	P15	Light yellow	*Beijerinckia*	-	-
	P3	Yellow	*Microbacterium*	452	N.I.
	P31	Fluorescent green*	*Pseudomonas*	258	Phenazine
	P22	Pink	*Serratia*	531	Prodigiosin
NA_S	P27	Peach	*Micrococcus*	446	N.I.
	P30	Orange	*Rhodococcus*	463	N.I.
	P28	Yellow	*Staphylococcus*	-	-
G-MSAM	P35	Yellow	*Erwinia*	-	-
	P36	Fluorescent brown*	*Pseudomonas*	400	N.I.
GYEA-SM	P24	Red	*Serratia*	531	Prodigiosin
ZMA	P34	Light orange	*Bacillus*	464	N.I.
	P33	Peach	*Xanthomonas*	-	-

NAG = nutrient agar with 10% glycerol; NA_S = nutrient agar prepared in aged seawater; G-MSAM = mineral salts agar medium with 0.5% glucose; GYEA-SM = glucose yeast extract agar with 10% skimmed milk; ZMA = Zobell's marine agar.
* Extracellular pigmentation; N. I., not identified; - not determined.

Table 2. Adherence of bacteria to hexadecane and plant litter.

Isolate No.	Sampling season	Identification	Adherence (%)	
			Hexadecane	Plant litter
A4	Pre-monsoon	*Bacillus*	2.4	-
A7		*Bacillus*	1.13	12.6
A5		*Micrococcus*	1.72	13.4
A3		*Pseudomonas*	1.42	-
A6		*Pseudomonas*	4.62	22.2
A1		*Staphylococcus*	2.27	99.93
A2		*Staphylococcus*	8.82	15.29
A9	Monsoon	*Bacillus*	8.9	0.84
A10		*Pseudomonas*	1.92	2.8
A8		*Staphylococcus*	1.11	91.73
A11		*Staphylococcus*	5.88	33.05
A12		*Staphylococcus*	13.04	8.4