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Research Article

Bacteriostatic potential of Purple sulfur bacteria against *Pseudomonas aeruginosa.*

[1] Dr. G.V. Ashokkumar, [2] Dr. P. Kavitha and [3] K . Vijaya

[1& 2]Asst. prof., Dept. of Microbiology, Srimad Andavan college of Arts and Science (Autonomous), Trichy -5.
[3]Research scholar, Dept.of Microbiology, Srimad Andavan college of Arts and Science (Autonomous), Trichy – 5.
[1]ashokmicro@yahoo.com [2] balakavi2009@gmail.com

ABSTRACT

Mangroves are important for marine productivity and are suitable niches for the growth and development of purple sulfur bacteria (PSB). Hence the present study focuses to harness the benefits of native PSB isolated from the mangroves of Thondi, situated in the east coast of TamilNadu and use them as biocontrol agents against UTI producing *Pseudomonas aeruginosa*. The Thondi mangrove sediment harbors PSB members and there exists diversity among the *Chromatium spp.*, populations, and there might be an unreported species with unusual physiological characteristics, this proves the coexistence of more species of single genera in small pocket of micro-niches. This signals much more intensive explorations and polyphasic taxonomical approaches have to be undertaken to bring out various PSB species that may lay hidden in these natural environments. The studies on the potential application of PSB strains against UTI producing *Pseudomonas aeruginosa* give us an insight into their antagonistic potentialities as well.

Keywords

Mangrove soil; Purple sulfur bacteria; Chromatium sp.; Urinary tract infections; Pseudomonas aeruginosa

1.INTRODUCTION

Mangroves are present in the tropical and subtropical intertidal estuarine region and river deltas of the world. They contain biologically diversified habitats where various life forms including plants, animals and microorganisms thrive (Holguin *et.al.*,, 2001, Gomes *et.al.*,2010). Along sea shorelines, purple sulfur bacteria can be found in anoxic sediments and in shallow water bodies, such as coastal lagoons. These conditions can be observed in estuaries and mangrooves where microbial mats develop exhibiting very high population densities in purple coloured thin layers (Van Gemerden and Mas, 1995).

PSB belongs to the order *Chromatiaceae* and *Ectothiorhodospiraceae* families, which can be easily distinguished from other purple photographs by their capacity to accumulate elemental sulfur inside or outside the cells as an intermediary byproduct during photosynthesis. (Pfennig and Truper

1992). Marine Purple sulfur bacteria are useful to mankind as they are wonderful probiotics, they produce antibacterial substances, they are a source of single cell protein, and they are involved in biohydrogen production (Burgess *et al.*, 1994, Jensen *et al.*, 1995).

The people who reside in the coastal areas of Tamil Nadu are prone to *Pseudomonas spp.*, borne urinary tract infections. Lot of cases have been reported and documented in the coastal areas of Thondi in Ramanathapuram District, Tamilnadu. (Ravikumar *et.al.*, 2010 and Ravikumar *et.al.*, 2012)

The most important factor in the pathogenicity of *Pseudomonas aeruginosa* is the elaboration of a group of protein exotoxins leading to leukopenia, acidosis, circulatory collapse, necrosis of liver, pulmonary edema, hemorrhage, and tubular necrosis of kidneys (Pinghui, 1974). Commercial chemotherapeutic agents like gentamicin, carbenicillin, ticarcillin, ureidopenicillins and cephalosporins are infective in dealing with these pathogens (Leigh and Emmanuel, 1984 and Schaeffer, 1990). Various marine biota have been previously researched upon to control these pathogens, but in vain, most of the UTI causing pathogens have gained resistance in due course of time (Sivaperumal *et.al.*, 2010., Ravikumar *et.al.*, 2010 and Ravikumar *et.al.*, 2012). The availability of scientific information on the pathogenic feature of UTIs causing Pseudomonas *aeruginosa* impedes the research on the development of suitable chemotherapeutic agents and other preventive approaches. (Rahul Mittal *et.al., 2009*)

Hence the present work was carried out, to harness the antibiotic potentials of native purple sulfur bacteria prevalent in the Thondi coast against pathogenic *Pseudomonas aeruginosa* causing urinary tract infections in humans.

2. MATERIALS AND METHODS

2.1 Area of Study and Sample Collection

Mangrove sediment soil from Thondi mangroves (Ramanathapuram Dist.) east coast of Tamil Nadu, were collected in fresh zip-lock polythene covers and transported to the laboratory and refrigerated at 18°C.

2.1.1 Selective Enrichment of Mangrove Sediment Soil Samples for PSB (Imhoff, and Truper 1989)

The soil samples were enriched separately in 100 ml screw capped bottles, using modified Biebl and Pfennig's medium (1981) and the tubes were kept under constant illumination (2,400 lux) at 30 ± 2ºC for 7 to 12 days and observed for brown / brownish-red / purple colour, which indicates the presence of PSB.

2.1.2 Purification of PSB (Archana *et al.*, 2003 modified)

Enrichment cultures were purified by repeatedly streaking on Biebl and Pfennig's agar slants (1981) and incubated under constant illumination (2400 lux) at 32°C.The purification was performed till the colonies appearing on two successive slants were all identical.

2.1.3 Characterization and Identification of Purple Sulfur Bacterial Strains

The purified PSB strains were characterized and identified based on the Bergey's manual of systematic bacteriology (2005).

2.2 Isolation of *Pseudomonas aeruginosa*

Pure cultures of *Pseudomonas aeruginosa*, previously isolated from patients suffering from urethritis has been maintained in our laboratory as stabs. The stab cultures were aseptically plated on the plates containing *Pseudomonas* isolation agar and incubated at 37°C for 24 hours and observed for growth and purity.

2.3 Biocontrol of *Pseudomonas aeruginosa* using PSB strains

The purified PSB strains were cultured en-mass, using modified Biebl and Pfennig's (1981) broth filled in 1000ml glass bottles. Extraction of intracellular extracts from PSB strains using chemical solvents were done by the modified method of Chandrasekaran and Kumar, (2011), where PSB strains (50ml) were centrifuged at 4000 rpm for 6 minutes, the supernatant was discarded and the cell pellet was washed twice by centrifugation, with double deionized distilled water. The cell pellets was subjected to solvent extraction with the following solvents *viz.*, chloroform: methanol 1:2, Acetone: methanol 7:2, Toulene : methanol 3:1,

The solvent extracts were collected and then concentrated in vacuum at 40°C using a rotary evaporator. The dry residues (0.5grams) were further dissolved in 10 ml of 2% dimethyl sulfoxide (DMSO) for studying the antibacterial activity by disk diffusion method.

2.3.1 Bioassay of Crude Extracts By Disc Diffusion (BAUER *et al.*, 1966)

The broth containing pure culture of *Pseudomonas aeruginosa* diluted to approximately 10^8 CFU/mL, according to the 0.5 McFarland standard. 0.1ml of the inoculum were spreaded on Mueller-Hinton agar and sterile paper disks (6 mm in diameter) were laid on the inoculated substrate after being soaked with 15 µL of PSB extract at a concentration of 50 mg/ml. The plates were incubated for 24 hours at 37°C. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the disk.

3. **RESULTS**

All the selective soil sediment enrichments for PSB isolates were brownish red to brown in colour, Fig. 1. Two distinctive PSB strains namely PSB1 and PSB2 could be isolated based on phenotypic and their physiological properties, Fig. 2 &3, their cultural and morphological characters are summarized in the Table I and II. Both PSB isolates have NaCl growth range above 8.0, and optimal Nacl range for both strain was 9.0% and 8.5% respectively,(Table-II). The pH ranges for PSB-1 and PSB-2 were 6.5-8.5% and 7.5-9.5% respectively and the optimal pH for PSB-1 and PSB -2 were 8.5% and 8.0%. The temperature ranges for PSB-1and PSB-2 were 20- 35°C and 30-30.5°C. The temperature optimal range for PSB -1 and PSB-2 was 35°C and 30 °C. The PSB isolates showed

photoorganoheterotrophic growth. The PSB isolate 2 alone showed chemoorganoheterotropic growth. Both the PSB strains were positive for Indole, Voges proskauer and utilized Citrate, Nitrate and produced urease. The PSB strain 2 was Catalase positive and the strain 1 was negative. Both the strains could not liquefy Gelatin and were methylred and oxidase negative (Table II).

3.1 Organic carbon/e⁻donor utilization by Purple sulfur bacterial strains

Both the PSB strains used Formate, Pyruvate, Fumarate , Acetate, Succinate, Malate. Both the PSB strains could not use Thiosulfate, Sulfite, Propanol, Mannitol, Glycolate, Tartrate, Citrate, Benzoate, Glutamate, butyrate, lactate sugars as Fructose and Glucose (Table III). The PSB strain1 were capable of utilizing Propionate, where as PSB 2 strain could not use. On the contrary, Na2S.9H2O, Ethanol, Glycerol was used by PSB 2 but not the strain PSB -1 (Table III).

3.1.1 Identification of PSB strains

Based on the phenotypic, physiological and biochemical characterization of the purified PSB strains the strains 1 and 2 which showed phenotypic similarity with *Chromatium* sp., at the genera level, but could not be tentatively identified up to their species based on Bergey's manual of systematic bacteriology 2005.

3.2 Biochemical characters of test *Pseudomonas aeruginosa*

The test urinary tract pathogen *Pseudomonas aeruginosa* exhibited positive results for citrate, arginine, catalase, Nitrate, Glucose, Mannose ,motility but indole, methyl red, ONPG, voges proskauer, H_2S, phenylalanine, ornithine, lysine, esculin, lactose, xylose, trehalose, sucrose negative.

3.3 Antagonistic activity of solvent extract of PSB strains against *Pseudomonas aeruginosa*

The Acetone: Methanol 7:2 extracts from *Chromatium spp.*1alone to produce zone of clearance against the test *Pseudomonas aeruginosa*, where as the Chloroform: Methanol 1:2 showed the zone of clearance, Toluene: Methanol 3:1 extracts slightly produce zone of clearance. All the extracts of *Chromatium spp.,* showed zones of inhibition but the Acetone: Methanol 1:2 extracts showed a better zone of inhibition than the other extracts, Fig. 4 to Fig. 9.

4. **DISCUSSION**

Marine environment is plentiful of purple photosynthetic bacteria and *Chromatium spp.,* are the major isolates inhabiting the marine environments of tropical countries (Hiraishi and Ueda, 1995, Srinivas et al., 2006, Srinivas *et al.,* 2007a,Srinivas *et al.,* 2007 b).

It is a well documented fact that purple photosynthetic bacteria inhibits many marine pathogens like *Vibrio* spp., (Chandrasekaran and Kumar, 2011), this gave us the impetus to continue further to explore their potentialities to act as bacteriostatic agents against urinary tract pathogen like *Pseudomonas aeruginosa* .Besides India being a tropical country UTI is quite prevalent irrespective of age (Abrutyn *et al*.,1988), the emergence of antibiotic resistance in many strains of *Pseudomonas*

aerugnosa, is a matter of concern, so alternative natural therapeutic agents are the need of the hour. Marine microbes are promising agents which have quite phenomenal capabilities to act as panacea for microbial diseases and it has been scientifically proved. Many marine bacterial members have been employed to assess their bacteriostatic efficacy against *Pseudomonas aerugnosa* (Rajesh Singh., *et al.*, 2013, Raj Kumar, *et al.*, 2012)

Keeping this in mind, the application of native mangrove PSB extracts on *Pseudomonas aeruginosa* was considered to find out if these humble PSB had any such bacteriostatic activity. Among *Cromatium sp.,* isolated the intracellular extracts exhibited a considerable zones of inhibition against *Pseudomonas aeruginosa*, whereas the chloroform: methanol and the acetone: methanol extracts of *Chromatium* showed zone of inhibition while the other toluene: methanol extracts slightly showed zone. The above results proves the fact that purple sulfur bacteria have the capability to exhibit bacteriostatic activity against many pathogens as stated earlier by Burgess *et al.*,1991.

This clearly proves that marine purple sulfur bacteria holds the key for treating urinary tract infection in the coming future, where extensive research in this perspective will definitely bear fruit and help mankind to overcome microbial diseases.

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TABLE– I Purified Purple Sulfur Bacterial Isolates From Mangroves Their Cultural And Morphological Characters.

PSB isolate	Colour of the culture	Colony morphology	Microscopic observations
1	Brownish red	Round, convex, smooth, and pale reddish brown	Rod shaped, 0.8-1.5-2.0×2.0-5.0 µm,deposited sulfur granules inside the cells and multiplied by binary fission and motile.
2	Pale brown	Round, convex, smooth and sandy brown coloured. Size of the colony has reached to 1-2 mm in diameter after 6 days of incubation under fluorescent light (2,400lux) at 28^0C.	Ovoid to rod shaped in chains, 2.0-2.5×3.0-5.0µm,deposited sulfur granules inside the cells multiplied by binary fission, motile,some non motile cells could be observed

TABLE-II Physiological and biochemical characters of PSB isolates

Characteristic features	**PSB Isolate 1**	**PSB Isolate 2**
NaCl range (%)	7-9.5	6-8.5
NaCl optimum (%)	9	8.5
pH range	6.5-8.5	7.5-9.5
pH optimum	8.5	8.0º
Temperature range (°C)	20-35^{0}C	30-30.5 °C
Temperature optimum (°C)	35	30
Photoorganoheterotrophy	+	+
Chemoorganoheterotrophy	-	+
Gelatin liquefaction	-	-
Indole production from L-tryptophan	+	+
Methylred	-	-
Voges proskauer	+	+
Citrate utilization	+	+
Catalase	-	+
Oxidase	-	-
Nitrate utilization	+	+
Urease	+	+

TABLE- III Organic carbon/e$^-$donor utilization by Purple sulfur bacterial strains

S. No.	Carbon source $^{\#}$/e$^-$ donor	PSB Isolate 1		PSB Isolate 2	
		Growth yield (O.D. $_{660\ nm}$)	Growth (+ / -)	Growth yield (O.D. $_{660\ nm}$)	Growth (+ / -)
1	Control(without carbon source)	0.00	NA	0.00	NA
2	Thiosulfate0.3%	0.03	-	0.32	-
3	$Na_2S.9H_2O$ 0.4%	0.01	-	0.22	+
4	Sulfite 0.3%	0.02	-	0.01	-
5	Formate	0.45	+	0.42	+
6	Pyruvate	0.65	+	0.68	+
7	Fumarate	0.48	+	0.62	+
8	Acetate	0.52	+	0.55	+
9	Propionate	0.44	+	0.02	-
10	Butyrate	0.00	-	0.01	-
11	Lactate	0.02	-	0.02	-
12	Succinate	0.58	+	0.50	+
13	Malate	0.60	+	0.55	+
14	Glutamate	0.02	-	0.01	-
15	Fructose	0.01	-	0.00	-
16	Glucose	0.02	-	0.01	-
17	Ethanol	0.01	-	0.25	+
18	Propanol	0.00	-	0.01	-
19	Mannitol	0.01	-	0.00	-
20	Glycerol	0.00	-	0.45	+
21	Glycolate	0.00	-	0.01	-
22	Tartrate	0.00	-	0.01	-
23	Citrate	0.01	-	0.00	-
24	Benzoate	0.02	-	0.00	-

Results expressed are average values of an experiment done in triplicates after 72h of light (2,400 lux) anaerobic incubation at 30± 2°C. Symbols: + = Growth present (OD660,>0.2); - = No growth, PG= poor growth (OD660, 0.1 to 0.2), NA: Not applicable.

TABLE-IV Antagonistic Activity of Solvent Extract of PSB Strains against *Pseudomonas aeruginosa:*

S.No	PSB strain	Solvent extracts	Zone in diameter (mm)
1	PSB-I *Chromatium sp.,*	Chloroform:methanol 1:2	0
		Acetone:methanol 7:2	0
		Toluene: methanol 3:1	0
2	PSB –II *Chromatium sp.,*	Chloroform:methanol 1:2	10.7
		Acetone:methanol 7:2	11.5
		Toluene: methanol 3:1	Slightly positive

Fig.1Enrichments of mangrove sediment soil sample for PSB

Fig.2 Phase contrast micrographs of PSB isolate 1

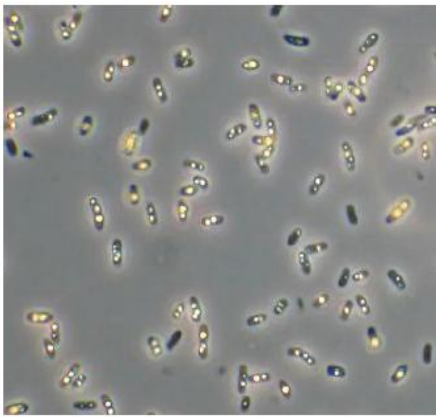


Fig.3. Phase contrast micrographs of PSB isolate 2

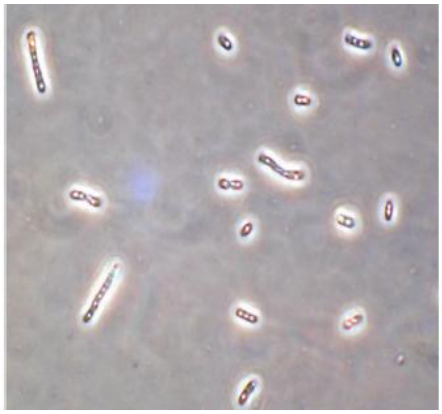
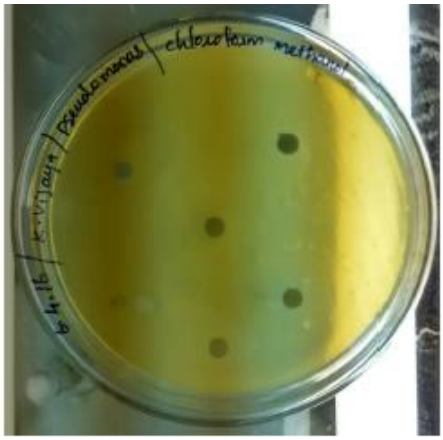


Fig.4. PSB 1

Chloroform: methanol extracts

Fig.5. PSB 2

Chloroform: methanol extracts



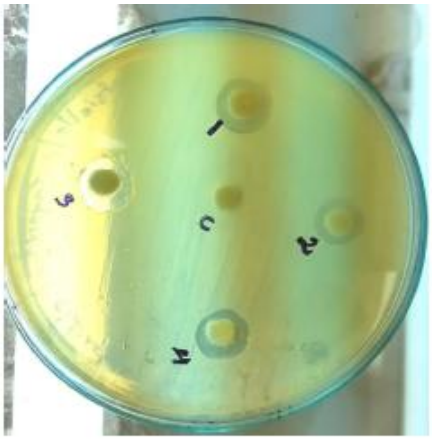


Fig.6. PSB 1

Acetone: methanol extracts

Fig.7. PSB 2

Acetone: methanol extracts

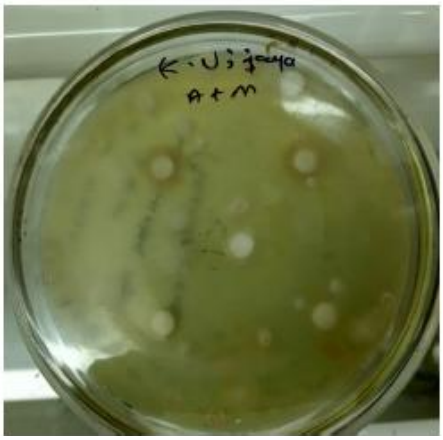


Fig.8. PSB 1

Toluene: methanol extracts

Fig.9. PSB 2

Toluene: methanol extracts