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Antibacterial Activity of Purple non Sulphur Bacteria against *Vibrio* spp., Associated with Diseased Shrimp

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The antibacterial activity of organic solvent extracts from purple non sulphur bacteria (PNSB), isolated from shrimp culture ponds, showed inhibitory activity against the growth of, *Vibrio spp.*, isolated from infected Tiger prawn (*Penaeus monodon*).The antibiotic activity was studied by Disk diffusion method and Broth tube dilution method. Amongst the purple non sulphur bacterial isolates *Rhodobacter sphaeroides* showed antagonism against *Vibrio harveyi, Vibrio fischeri* ,and *Vibrio alginolyticus,* with an minimum inhibitory concentration (MIC) range of 0.39- 6.25mg/ml. The chloroform extracts showed highest inhibitory zones than other solvents. These results reveal that extracts from the PNSB strains may hold novel bioactive compounds with effective antimicrobial properties.

Key words: Purple non sulphur bacteria, Antimicrobial activity, Shrimp pond, *Vibrio harveyi.*

Shrimp cultivation in India is plagued by diseases like vibriosis and they are responsible for higher incidence of shrimp mortality[1]. Six species of *Vibrio* namely, *V. harveyi, V.parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus* and *V. splendidus* are associated with the diseased shrimp[2-4]. Antibiotics are applied to shrimp pond to reduce the severity of vibriosis. The massive misuse of antibiotics to control infections in aquaculture has resulted in the development of resistant strains, which have rendered antibiotic treatments ineffective[5]. Moreover, the horizontal transfer of resistance determinants to human pathogens and the presence of antibiotic residues in aquaculture products for human consumption constitute important threats to public health. Therefore, to make the aquaculture industry more sustainable, new strategies to control infections are urgently needed[6]. Probiotic bacterial strains are commonly employed to prevent vibriosis in shrimp farms. Nowadays photosynthetic bacterial members are used as probiotics in aquaculture and they enhance the water quality and prevent diseases[7]. The Anoxygenic photosynthetic bacterial members do exhibit antibacterial properties against both gram negative and gram positive bacteria[8,9].

Likewise, the cis-vaccenic acid extracted from the purple non sulphur bacterial (PNSB) member, *Rhodopseudomonas capsulata* exhibited antiviral properties, which are active against T5 phages[10].

The PNSB are widely distributed in marine and hypersaline habitats as microbial mats in brackish water ponds, salt marshes, marine waters, surface/tidal waters, tidal seawater pools, anoxic sediments from sea and sediments of intertidal mud flats[11].

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The present study was undertaken with the aim of isolating PNSB from shrimp ponds and to assess their antagonistic potential against *Vibrio* sp., isolated from infected *Penaeus monodon.*

MATERIALS AND METHODS

The cultures of V*ibrio* species namely *Vibrio harveyi* (vpk L4c) ,V*ibrio fisheri* (vpk L1 a) and *Vibrio alginolyticus* (Ppk L4b) which were isolated, from infected Tiger prawn(*Penaeus monodon*) and were maintained as pure cultures in our laboratory . These *Vibrio* strains were cultured in Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS Agar) plates, and used as indicator strains against PNSB extracts in this study.

Screening, shrimp ponds for PNSB

The shrimp culture pond effluents were collected in sterile polysterine containers from Papakovil, Nagapattinam district, and direct sea water shrimp farm effluents from Karankadu , Ramanathapuram district, Tamilnadu. India. The shrimp pond water samples were screened for PNSB and enriched using Phototrophic bacterial medium (PTBM), in screw capped bottles, at the pH = 8±2 , with the following composition (g/l^{-1}) Ammonium chloride: 1, Magnesium sulphate: 0.3, Calcium chloride: 0.2 Potassium hydrogen phosphate : 0.5, Sodium chloride : 0.5, Sodium succinate: 2, Yeast extract : 1.5 , Disodium hydrogen phosphate: 0.3, Ferric-Citrate (0.1% w/ v) : 5 ml; Trace metal solution: 1ml.

Trace metal solution

[(mg/l^{-1}): $ZnCl_2$:70; $MnCl_2$. $4H_2O$:100; H_3BO_3 :60; $CoCl_2$. $6H_2O$:200; $CuCl_2$ d H_2O :20; $NiCl_2.6H_2O$: 20; $Na_2MoO_4.2H_2O$: 40]

The medium and samples were added in the ratio 9:1, the medium was poured up to the brim and screwcapped tightly and sealed with parafilm, and kept in incandescent illumination at 2400 lux at 32°C for 7 to 12 days and observed for brown / brownish-red colour.

Characterization of PNSB

Enrichment cultures were purified by repeatedly streaking on PTBM agar slants prepared in 25 x 150 mm. rim-less test tubes, containing 15 ml media and sealed with Polybutyrate rubber stoppers (suba seals). The gas phase in the test tube was replaced by flushing with argon for 2 min by using a pair of hypodermic needles and incubated at 2400 lux at 32°C. The purification was performed till the colonies appearing on two successive slants were all identical. Purity of the culture was checked by streaking on Nutrient agar. Contamination from other phototrophic bacteria was checked by monitoring the cultural characters like color of the culture, colony morphology and by microscopic observation.

The resulting pure cultures of PNSB were biochemically characterized[11] and cell material for 16S rDNA sequencing was taken from 1-2 ml of well grown liquid cultures. DNA was extracted and purified according to the method of Marmur[12]. Amplification of 16S rRNA gene was performed based on the methods of Sivaji *et al*.,[13] .The obtained sequences were submitted in the, National Center for Biotechnology Information, NCBI-BLAST search in order to know the nearest phylogenetic relative. Based on the blast search results, type strains of the closely related members were determined. Based on the blast search results, type strain sequences of the closely related members were obtained in fasta format from (NCBI). EzTaxon server ver. 2.1 was also used for comparison 16S rRNA gene sequences with type strain sequences.

Preparation of extracts from PNSB

The characterized PNSB isolates, were cultured en-masse, using PTBM broth filled in 6 litre high grade glass jars and sealed with non-corrosive stainless steel lid –with scilicone rubber lining . The medium was poured up to the brim, leaving no head space, bubbled with argon for 5 minutes and kept under constant illumination at 2400lux at 30 ± 2°C, for 7 days until the colour changes to red - brick red/ brown.

The mass cultured photosynthetic bacterial isolates were harvested by centrifugation and re-suspended in deionized water and placed in a boiling water bath for up to 90 minutes, cleared supernatant was recovered at various time intervals and designated as, Hot water aqueous extract and used for the bioassay against *Vibrio* spp., by the agar well diffusion method.

Solvent extracts like Chloroform: methanol: water (1:2:0.8), Acetone methanol (7:2) and Toluene: methanol (3:1) extracts were

obtained by vortexing the cell pellets with pre-sterilized 0.5mm glass beads and filtered using ultrafine nylon filter.

The solvent extracts were collected and then concentrated in vacuum at 40°C using a Rotary evaporator (Photonix,) and the dry extracts obtained, were further dissolved in dimethyl sulfoxide (DMSO) for studying the antibacterial activity by disk diffusion and Broth tube dilution method.

Bioassay of extracted antibiotic

The sensitivity of *Vibrio* sp., to solvent extracts of PNSB was tested by measuring the zone of inhibition of a given concentration of the extract by the disk-diffusion method and by determining the minimal inhibitory concentration (MIC) by Broth tube dilution method. *Vibrio harveyi* (vpk L4c) ,V*ibrio fisheri* (vpk L1 a) and *Vibrio alginolyticus* (Ppk L4b) were diluted to approximately 10^8 CFU/mL, according to the 0.5 McFarland standard, spreaded and incubated for 24 h at 37°C on Mueller-Hinton agar (Hi-media) incorporated with (1 % Nacl). The standard disk-diffusion method was used to study antimicrobial activity. After the appropriate inoculum was seeded in Mueller-Hinton agar plates (1% NaCl) and then paper disks (7 mm in diameter) were laid on the inoculated substrate after being soaked with 15 μL of PNSB extract at a concentration of 50 mg/mL. Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the disk. As a positive control of growth inhibition, Chloramphenicol 30 µg (Hi-media) antibiotic disc was prepared and used for the test *Vibrio* sp. The minimal inhibitory concentration (MIC) was determined by the broth tube dilution method. A series of dilutions with concentrations ranging from 50 to 0.15 mg/mL was used in the experiment with each extracts for the test organisms. The starting solutions of extracts with a concentration of 50 mg/mL were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Twofold dilutions of extracts were prepared in Mueller-Hinton broth with 1% NaCl. The boundary dilutions without any visible growth was defined as the Minimal inhibitory concentration (MIC) for the tested microorganism at the given PNSB extract concentration. As a positive control of growth inhibition, chloramphenicol was used and DMSO solution was used as a negative control of the influence of solvents. All experiments were performed in duplicate.

Table 1. Antibacterial activities of solvent extracts of PNSB against *Vibrio spp.,* by disk diffusion method

PNSB strains	Solvent extracts	*V. harveyi* DD *	*V. fischeri* DD *	*V.alginolyticus* DD *
Rhodovulum sulfidophilum (BRP5)	E1	12	11	-
	E2	-	-	-
	E3	11	12	-
Rhodobium orienties (BRP7)	E1	12	11	-
	E2	11	-	-
	E3	-	-	-
Rhodobium marinum(BRP8)	E1	21	-	-
	E2	-	-	11
	E3	-	10	-
Rhodobacter sphaeroides (BRP9)	E1	27	24	12
	E2	22	22	-
	E3	14	12	23
Rhodobacter aestuari (BRP12)	E1	21	18	-
	E2	-	-	9
	E3	-	-	-

DD: disk diffusion, (-): Negative

*: Diameter of inhibition zone (mm)including disc diameter of 7 mm.,

E1: Chloroform: methanol: water (1:2:0.8); E2: Acetone methanol(7:2) and E3: Toluene: methanol (3:1)

RESULTS AND DISCUSSION

A total of 38 PNSB strains were isolated from shrimp culture ponds, based on microscopic (data not shown), biochemical(data not shown) and phylogenitic identification based on partial 16s rRNA gene sequencing .Nine were identified as *Rhodovulum sulfidophilum (BRP5)* with similarity levels of more than 99% relative to the type strain of the species *Rhodovulum sulfidophilum* DSM 1374^T, strain., Three isolates were identified as *Rhodobium orienties* (BRP7) affiliated to *Rhodobium orienties*, showing 99.7% sequence similarity with that of *Rbi.orienties* DSM 11290, seven isolates identified as *Rhodobium marinum*(BRP8) with an 99 % sequence similarity to *Rbi.marinum* CCUG 55129^T, six isolates were identified as *Rhodobacter aestuarii* (BRP12) with a 99.5% similarity with *Rba. aestuarii* JCM 14887^T, 14 isolates were identified as *Rhodobacter sphaeroides* (BRP9) with an 99.5 % similarity with that of *Rba. sphaeroides* ATCC 17023. Even though *Rba.sphaeroides* was designated as fresh water bacteria, they tolerated upto 3% Nacl. Same as observed by (9), which makes them versatile to be present even in a brackish environment.

This study using PNSB members, was done primarily to assess their antagonistic potential against *Vibrio sp.,* Antibacterial activity of PNSB extracts (summarized in Table 1&2) of which, the minimal inhibitory concentrations were in the range from 0.39- 6.25mg/ml of extract in relation to the bacteria tested.

The effect of antibiotic activity by the hot water extracts prepared from PNSB, was proved to be time dependent (Fig-1), as the supernatants of PNSB inhibited the *Vibrio harveyi* strains after more than 25 minutes of boiling, this indicates that the antibacterial compounds are intracellular [9] and the bioactive compounds are thermostable even at 100º C, by eliciting an antibacterial response against the test organism, *V.harveyi* .

In this study the Chloroform: methanol: water extracts showed a higher zone of clearnance when compared to other extracts, which indicates that the major antagonistic substance may be a lipid containing compound.

Even though the inhibitory values obtained in the study against *Vibrio sp.,* might be fluctuating , it is evident that these PNSB members do possess antimicrobial substances and

Table 2. Antibacterial activities of solvent extracts of PNSB against *Vibrio spp.*, broth tube dilution method

PNSB strains	Solvent extracts	*V. harveyi* DD *	*V. fischeri* DD *	*V.alginolyticus* DD *
Rhodovulum sulfidophilum (BRP5)	E1	3.12	3.12	-
	E2	-	-	-
	E3	6.25		-
Rhodobium orienties (BRP7)	E1	3.12	6.25	-
	E2	12.5	-	-
	E3	-	-	-
Rhodobium marinum(BRP8)	E1	1.56	-	-
	E2	-	-	12.5
	E3	-	-	-
Rhodobacter sphaeroides (BRP9)	E1	0.39	1.56	3.12
	E2	0.78	1.56	-
	E3	6.25	3.12	0.78
Rhodobacter aestuari (BRP12)	E1	1.56	3.12	-
	E2	-	-	25
	E3	-	-	-

MIC: Minimum inhibitory concentration, (-): Negative,
**: values given as mg/mL for PNSB extract
E1: Chloroform: methanol: water (1:2:0.8)
E2: Acetone methanol(7:2) and E3: Toluene: methanol (3:1)

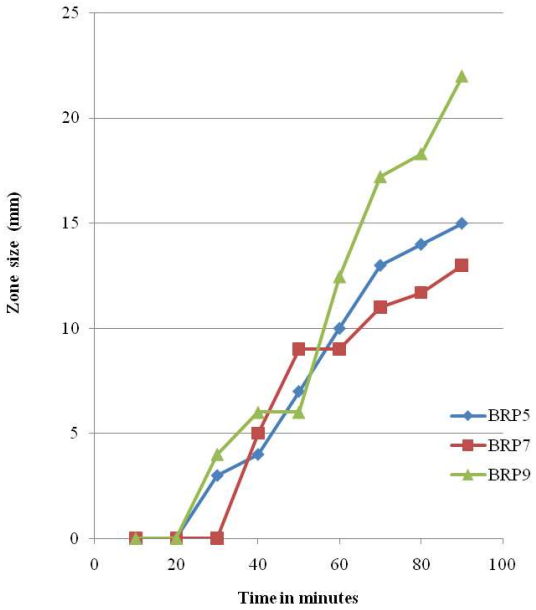

Fig. 1. Influence of boiling time on the Antibiotic properties of PNSB against *Vibrio harveyi*

active components ,which might be more closely related to the classical antibiotics .

Results obtained from this study revealed that *Rba. sphaeroides*(BRP9) displayed, better antibiotic activity against *Vibrio spp*., than other PNSB strains. Now with the outcome of this study it is possible to consider using PNSB as candidates to control *Vibriosis* in Penaid shrimps and the scope is wide open to harness the bio-pharmaceutical potential of *Rba.sphaeroides* as well.

As, the marine photosynthetic bacteria are untapped resources for novel bioactive products, the same approach can be assayed for finding out new antagonistic PNSB against various other types of aquaculture pathogens.

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