Malaysian Journal of Microbiology, Vol 7(1) 2011, pp. 54-56
http://dx.doi.org/10.21161/mjm.23510

## SHORT COMMUNICATION

# Antagonistic activities of purple non-sulfur bacterial extracts against antibiotic resistant *Vibrio* sp.

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Received 12 January 2010; received in revised form 22 March 2010; accepted 31 March 2010

**ABSTRACT**

Solvent extracts of native purple non-sulfur bacterial (PNSB) isolates from the effluents of brackish shrimp culture ponds, near Nagapattinam coast (South India) were evaluated for antibacterial activity by the disc diffusion method. Best results were shown by the chloroform extracts against oxytetracycline resistant *Vibrio harveyi* and *Vibrio fischerii*. Among the purple non-sulfur bacterial isolates, *Rhodobacter sphaeroides*, showed maximum antagonistic activity. The findings suggest that the antagonistic extracts from *Rba. sphaeroides* could be used as an effective antibiotic in controlling *Vibrio* spp., in aquaculture systems.

*Keywords:* antibacterial activity, *Rhodobacter sphaeroides*, oxytetracycline resistant *Vibrio* sp.

## INTRODUCTION

The occurrence of diseases in shrimp culture systems pose a great threat as the shrimp farmers suffer from heavy losses. Indiscriminate use of antibacterial compounds in aquaculture, has led to the development of antibacterial resistant *Vibrio* sp. (Eleonor and Leobert, 2001) and controlling them has become a challenge in the aquaculture systems. According to the FAO report (2005), conventional antibiotics like Cloramphenicol have been banned to be used in aquaculture. Due to this legislation, shrimp farmers who are exporting their produce are facing huge financial loses. Hence, it is high time that novel antibacterial compounds with better therapeutic potential are developed, for which luminous *Vibrio* sp., may not confer resistance. The earlier works of Kaspari and Klemme (1977) and Burgess *et al.* (1991) reported the occurrence of the antibiotic substances from anoxygenic photosynthetic bacteria and they were effective in inhibiting only the Gram positive bacterial members. Likewise, the cis-vaccenic acid extracted from the PNSB member, *Rhodopseudomonas capsulata* posses antiviral properties, which are active against T5 phages (Hirotani *et al.*, 1991). Present study was undertaken with the aim of isolating PNSB from marine environment and to assess their antagonistic potential against *Vibrio* sp., isolated from infected, *Penaeus monodon.*

## MATERIALS AND METHODS

Water samples of brackish shrimp pond were collected from Papakovil (10°44'39.75"N and 79°49'39.61"E) Nagapattinam district, Tamilnadu, India using sterile polycarbonate sampling bottles. The water samples were enriched using Phototrophic bacterial medium (PTBM), (Rabinson, 2006) in screw capped bottles, with the following composition (g/L), 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: 1 mL. Trace metal solution: [(mg/L): $ZnCl_2$: 70; $MnCl_2 \cdot 4H_2O$: 100; $H_3BO_3$: 60; $CoCl_2 \cdot 6H_2O$: 200; CuCl2·H2O: 20; $NiCl_2 \cdot 6H_2O$: 20; $Na_2MoO_4 \cdot 2H_2O$: 40] at the pH = 8 ± 2. The medium and samples were added in the ratio 9:1, the medium was poured up to the brim and screwcapped tightly, 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 color.

Enrichment cultures were purified by repeatedly streaking on PTBM agar slants prepared in 25 x 150 mm rim-less test tubes, sealed with polybutyrate rubber stoppers (suba seals). The gas phase in the test tube was replaced by flushing with argon for 2 min and incubated at 2400 lux at 32 °C. The resulting colonies were subjected to microscopic, biochemical and physiological studies based on Imhoff (2005; 2006). Amplification of 16S rRNA was performed by using primers, Eub27F

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(5′GAGTTTGATCCTGGCTCAG-3′) and Univ1492R (5′-GGTTACCTTGTTACGACT T-3′). The obtained sequences of length approximately 1350 to 1450 bp was submitted to the NCBI-BLAST. Based on the blast search results, type strain sequences of the closely related members were obtained. The type strain numbers were either obtained from Bergey's Manual of Systematic Bacteriology (Imhoff, 2005) and List of prokaryotic names with standing in nomenclature (LPSN – http://www.bacterio.cict.fr/index.html). EzTaxon server ver. 2.1 was also used for comparison 16S rRNA gene sequences with type strain sequences.

The characterized PNSB isolates, were cultured en-masse and kept under constant illumination at 2400 lux at 30 ± 2 °C, for 7 days until the color changed 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 min, 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), was obtained based on (Burgess *et al.*,1991), acetone methanol (7:2) extracts (Yuzo and Tsutomu,1984) and toluene:methanol (3:1) extracts were obtained by vortexing the cell pellets with pre-sterilized 0.5 mm glass beads and filtration using ultrafine nylon filters. The organic phase of all the extracts was concentrated under rotary evaporator at 35 °C and the resulting dry residues were resuspended individually in ethanol to a final concentration of 5 mg/mL.

The activity of solvent extracts was assayed by the paper disc method, using 7 mm diameter paper discs. The cultures of *Vibrio* species namely *Vibrio harveyi* (vpk L4c), V. *fisheri* (vpk L1a) and *V. alginolyticus* (Ppk L4b) which were previously isolated, from infected shrimp, *Penaeus monodon*, and were maintained as pure cultures in our laboratory.

*Vibrio* sp., (Vpk L4c ,Vpk L1a and Ppk L4b) were diluted to approximately $10^8$ CFU/mL (0.5 McFarland standard), spreaded and incubated for 24 h at 37 °C on Mueller-Hinton agar (Hi-media) incorporated with 1 % NaCl. The disc-diffusion method (Branislav *et al.*, 2009) was used to study antimicrobial activity. After the appropriate inoculum was seeded in Mueller-Hinton agar (1% NaCl) plates, then paper discs were laid on the inoculated substrate after being soaked with 50 µL of PNSB extract at a concentration of 5 mg/mL.

Antimicrobial activity was determined by measuring the diameter of the zone of inhibition around the disc. As a positive control of growth inhibition, chloramphenicol 30 µg (Hi-media) was used and oxytetracycline 30 µg (Hi-media) was used as negative control.

## RESULTS AND DISCUSSION

Screening the shrimp ponds for purple non-sulphur bacteria, in order to study their Vibriostatic potential yielded a total of 17 PNSB isolates. Based on microscopic (data not shown), biochemical (data not shown) and phylogenitic identification based on partial 16S rRNA gene sequencing, of which ,nine species were identified as *Rhodovulum sulfidophilum* (BRP5) on the basis of similarity levels of more than 99% relative to the type strain of the species *R. sulfidophilum* DSM 1374$^T$, strain, two isolates were identified as *Rhodobium orienties* (BRP7) affiliated to *R. orienties*, showing 99.7% sequence similarity with that of *Rbi.orienties* DSM 11290, six isolates were identified as *Rhodobacter sphaeroides* (BRP9) with an 99.5 % similarity with that of *Rba. sphaeroides* ATCC 17023.

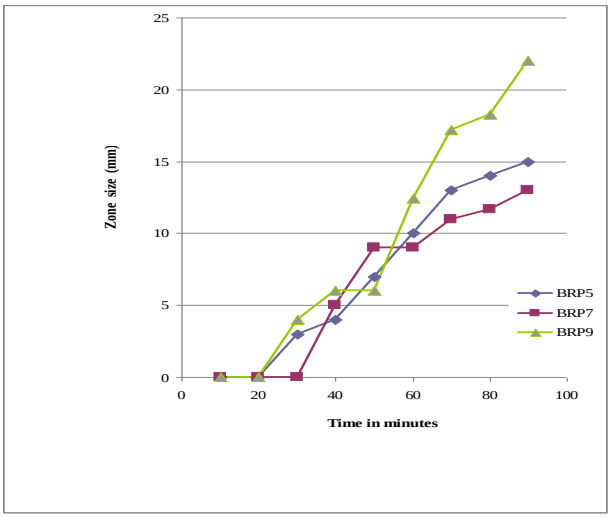


**Figure 1:** Influence of boiling time on the antibiotic properties of PNSB against *Vibrio harveyi*

**Table 1:** Antibacterial activities of PNSB extracts against *Vibrio* sp., tested based on Disc-diffusion[a]

| PNSB strains | *V. harveyi* | | | *V. fischeri* | | | *V.alginolyticus* | | |
|---|---|---|---|---|---|---|---|---|---|
| | E1 | E2 | E3 | E1 | E2 | E3 | E1 | E2 | E3 |
| *Rhodovulum sulfidophilum* (BRP5) | 12 | – | 11 | 11 | – | 12 | – | – | – |
| *Rhodobium orienties* (BRP7) | 9 | 11 | – | 11 | – | – | – | – | – |
| *Rhodobacter sphaeroides* (BRP9) | 27 | 22 | 14 | 24 | 22 | 12 | 12 | – | 23 |

[a] Diameter of inhibition zone (mm) including disc diameter of 7 mm.
–: Negative; E1: Chloroform:methanol:water extract; E2: Acetone methanol extracts; E3:Toluene:methanol extracts; Chloramphenicol (30 µg): Positive control; Oxytetracycline (30 µg): negative control.

Inhibition of *Vibrio* spp.*,* by the extracts prepared from the three PNSB strains is summarized in Table 1. The inhibitory values obtained from *Rba. sphaeroides* were greater, than any other PNSB. Vibriostatic activity has been observed from the recent works in *Bacillus* spp.(Watchariya and Nontawith, 2007), *Flavobacterium* spp.(Zizhong *et al*., 2009), *Lactobacillus* spp.( Ajitha *et al*., 2004; Vieira *et al*., 2007), *Pseudomonas* spp*.* (Bushra *et al.*, 2009), *Phaeobacter* spp. (Prado *et al.*, 2009). The effect of antibiotic activity by the hot water extracts prepared from PNSB, was proved to be time dependent (Figure 1), as the supernatants of PNSB inhibited the *V. harveyi* strains only after more than 25 min of boiling. This indicates that the antibacterial compounds are intracellular as observed by (Burgess *et al.*, 1991) and the bioactive compounds are thermostable even at 100 °C, by eliciting and antibacterial response against test organisms. In this study the chloroform:methanol:water extracts showed a higher zone of clearance when compared to other extracts. As no previous reports are available on the utilization of PNSB against marine *Vibrio* sp., or any other shrimp pathogen the effectiveness of utilizing these Anoxygenic purple non-sulphur bacteria depends on the outcome of extensive research in this perspective in the coming future. 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.

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