Indian J. Fish., 66(1): 124-130, 2019
DOI: 10.21077/ijf.2019.66.1.79877-17

Note

Isolation and characterisation of antagonistic bacteria against *Vibrio harveyi* from milkfish *Chanos chanos*

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ABSTRACT

Vibrio species are the most dominant multidrug-resistant opportunistic bacterial pathogens in coastal aquaculture environments. There is an urgent need for biocontrol strategy to enhance commercially viable shrimp production. In the present study, eight strains of *Bacillus* were isolated and characterised from surface mucus of milk fish maintained in greenwater system and their antagonistic effects were evaluated against shrimp pathogenic bacteria *Vibrio harveyi,* by agar well diffusion and co-culture methods. Promising organisms were immobilised onto a matrix for preparation of bioaugmentor and again tested for antagonistic activity against *V. harveyi*.

Keywords: Bioagumentation, Brackishwater aquaculture, Probiotics, Shrimp, *Vibrio harveyi*

Emerging major constraints in coastal shrimp farming around the world are problems linked to deterioration of pond water quality and stress associated disease incidence. *Vibrio* species are the predominant bacterial pathogens in the marine environment, causing vibriosis especially in farmed shrimp (Kang *et al.,* 2014). Among them, particularly, *Vibrio harveyi* is identified as a major pathogen, which is a luminous Gram negative bacterium associated with vibriosis in penaeid shrimp (Moriarty, 1999; Moreno *et al.*, 2017). In addition, there have been several reports indicating the antibiotic resistance of *V. harveyi*, causing mortalities among larvae, post-larvae and cultured shrimp leading to economic losses (Stalin and Srinivasan, 2016; Moreno *et al.,* 2017).

Vibrio spp. are difficult to be controlled in aquaculture and related aquatic environment when the stocking density is high. Antagonistic activity of probiotic bacteria against pathogenic bacteria have been evaluated (Pan *et al.,* 2008; Fjellheim *et al.,* 2010). Reports on isolation, purification and structural elucidation of active compounds from pharmacologically promising marine organisms associated bacteria from Indian marine waters are scanty (Anand *et al.,* 2006; Krishnani, 2010). There is a huge potential for venturing into the micro niche to explore the potential of the bacterial diversity associated with marine organisms as a source of novel biomolecules. Recent developments in molecular biology based techniques have led to rapid and accurate strategies for monitoring, discovery and identification of novel bacteria (Anand *et al.,* 2006; Krishnani *et al.,* 2009; 2010; Krishnani and Kathiravan, 2010), which can be helpful to understand the interaction of marine bacteria. The research findings of Stalin and Srinivasan (2016) are helpful in understanding the multiple antibiotics resistance of *V. harveyi*, which indicated the urgent need for targeted alternative biocontrol strategies to enhance the prospects of commercially viable shrimp cultivation. In the present study, promising antagonistic bacteria have been isolated from surface mucus of fish maintained in greenwater system for controlling pathogenic *V. harveyi*.

Bacterial strains were isolated from the surface mucus of milk fish maintained in greenwater system in coastal aquaculture pond. In brief, about 0.1 g milkfish slime (mucus from fish surface) was incubated in 10 ml of sterile seawater. After serial dilution, the mixture was inoculated on tryptic soy agar (TSA) by spread plate method and incubated at 28°C until visible colonies were formed. Bacterial colonies were further purified and the isolates were initially characterised using standard biochemical tests and later employing molecular techniques.

Couple of isolates were cultured on tryptic soy agar medium (TSA) at 28°C for 48 to 72 h. The cells were harvested from the plates by scraping with a sterile loop and used for fatty acid methyl ester (FAME) analysis. Saponification, methylation and extraction were performed using the procedure described in the MIDI

manual (Microbial Identification, Inc.) (Sasser, 1990). Cluster analysis was performed using an in-house cluster program and the MIDI software. Sherlock MIS uses fatty acids 9-20 carbons in length. The peaks are automatically named and quantitated by the system.

Genomic DNA was extracted from pure cultures using a DNA isolation kit (Himedia, India) according to the manufacturer's instructions. The bacterial 16S rRNA gene was amplified using universal primers. The PCR thermal program was followed as previously described (Kathiravan and Krishnani, 2013). The sequences were analysed using ABI3100 Genetic Analyser. The sequences obtained were analysed for bacterial species identification using BLASTN programme of NCBI (Altschul *et al.,* 1990). Phylogenetic tree was constructed using the neighbor-joining method from MEGA 4 program package (Tamura *et al.,* 2007).

The antibacterial activity of isolated strains was assessed against two pathogenic bacterial strains *viz*., *V. harveyi* strain A1 and *V. harveyi* strain B5, which were isolated from coastal hatchery using special *V. harveyi* medium (Lachlan *et al.,* 1996). These bacteria were selected after confirming pathogenicity based on the PCR amplification of hemolysin *vhh* gene. Originally designed primers (Conejero and Hedreyda, 2004) (forward: 5-CTTCACGCTTGATGGCTACTG-3 and reverse: 5-GTCACCCAATGCTACGACCT-3) were used for amplification of 235 bp fragment of hemolysin *vhh* gene. Pure cultures of the pathogenic strains were inoculated in trypticase soy broth (TSB) and incubated at 28ºC. After 24 h of incubation, bacterial suspension (inoculum) was diluted with sterile physiological saline, to get a cell density of 10^8 cfu $ml^{-1.}$

Bacterial strains isolated from the fish slime were evaluated for their antagonistic activity against the two pathogenic strains of *V. harveyi* using agar well diffusion method. The isolated strains were inoculated into 5 ml of TSB and then incubated at 28ºC. Bacterial suspensions of the two pathogenic strains of *V. harveyi* strains were uniformly spread on TSA plates using sterile cotton swab. The plates having wells of 6 mm dia were filled with 50 µl inoculum of the test strains. The plates were then incubated for 24 h under aerobic conditions. Inhibition zones of the bacterial growth around the wells were measured in mm. All tests were performed in duplicates.

Co-culture of the test strains along with the pathogenic bacteria was also employed to determine the antagonistic activity of the isolated bacterial strains. Promising antagonistic strains were immobilised onto a matrix (sterile tapioca flour) for preparation of antagonistic bioaugmentor. One hundred microliter each of the bacterial strains was mixed with 500 ml of TS broth containing 10 g sterilised tapioca flour and incubated for 48 h at 28ºC on a rotary shaker (100 rpm). The mixture was centrifuged for 15 min at 10,000 rpm, then washed with sterile phosphate buffered saline (PBS) (>5 ml) to remove the loosely attached cells. The mixture was dried at 40°C for 3 h and stored at 4°C, until use.

Viability of the antagosistic bacteria in the bioaugmentor was checked for a period of 8 months. For this, known quantity of matrix was taken soon after the immobilisation process and thereafter at monthly intervals into sterile centrifuge tubes containing known volume of sterile PBS. The biofilm cells were dislodged using vortex and then serially diluted and plated onto the suitable agar media (TSA) for determining the total viable count.

Antagonistic effect of bioaugmentor was tested and revalidated against *V. harveyi* using co-culture method. Each of the immobilised strain was added in 25 ml of TSB in a conical flask which was inoculated with the pathogenic strain of *V. harveyi* (10^6 cells). Control broth inoculated with *V. harveyi*, without antagonistic bacterial isolate was also prepared. The co-cultures, made in duplicates were incubated at 28ºC and samples were taken at an interval of 24 h for 72 hours for enumeration of *Vibrio* count on *V. harveyi* medium (Lachlan *et al.,* 1996). Based on the dilution used in the plating, the concentration of the residual amount of viable cells per milliliter at different time intervals were calculated. The reduction percentage of the bacteria was estimated using the following equation:

Reduction percentage (R%) = Bacterial cell density in control - Bacterial cell density in in co-culture / Bacterial cell density in control × 100

Eight bacterial isolates designated as DBTGSB1, DBTS12B2, DBTS18B3, DBTS34B4, DBTS21B5, DBTS29B6, DBTS3B7 and DBTS11B8 were isolated from the surface mucus of milk fish maintained in greenwater system in coastal aquaculture ponds. DBTGSB1, DBTS21B5 and DBTS29B6 were positive for oxidase, catalase, voges-proskauer (VP) and negative for gelatinase, indole production and citrate utilisation. DBTS12B2 and DBTS11B8 were positive for oxidase, catalase, indole, VP, gelatin test and negative for methyl red (MR) test. DBTS18B3 and DBTS34B4 were negative for MR test and positive for oxidase, catalase, indole, VP and gelatin hydrolysis tests. DBTS3B7 was positive for catalase and gelatinase; while negative for oxidase, MR-VP and indole (Table 1).

The fatty acid profiles of two promising antagonistic bacterial strains *viz*., DBTS29B6 and DBT S12B2

obtained from FAME analysis, confirmed the identity of the strains as *Bacillus pumilus* and *Bacillus subtilis* respectively (Fig. 1).

The eight bacterial strains were also identified based on 16S rRNA gene sequence analysis, using BLASTN, based on maximum sequence similarity to species in the NCBI database**.** 16S rRNA sequences of the eight *Bacillus* strains were submitted in NCBI-GenBank with accession nos. as given in Table 1. Phylogenetic tree was constructed based on 16S rRNA gene sequence using MEGA 4 (Fig. 2).

V. harveyi strain A1 (Accession No. JF264472) and *V. harveyi* strain B5 (Accession No. JF264473) isolated from coastal hatchery using *V. harveyi* medium were used as test bacteria. The pathogenicity of both the strains were confirmed by amplification and sequencing of the hemolysin (*vhh*) gene (Fig. 3). The functional gene sequences of *vhh* gene of *V. harveyi* strain B5 and A1

Table 1. GenBank accession numbers of 16S rRNA gene and percentage of similarity

Isolate and GenBank Acc No.	Biochemical test	16S rRNA matching % & Accession No.	Bacteria
DBTGSB1 & KF860867	Indole -ve and +ve for oxidase, catalase, MR, VP, gelatin hydrolysis	96 & KC250164	*Bacillus pumilus*
DBTS12B2 & KF860868	+ve for oxidase, catalase, indole, VP, gelatin and -ve for MR.	97 & JX102497	*Bacillus subtilis*
DBTS18B3 & KF860869	-ve for MR and +ve for oxidase, catalase, indole, VP, gelatin hydrolysis	99 & JX901378	*Bacillus licheniformis*
DBTS34B4 & KF860870	-ve for MR and +ve for oxidase, catalase, indole, VP, gelatin hydrolysis	99 & JX901378	*Bacillus licheniformis*
DBTS21B5 & KF860871	-ve for indole and +ve for oxidase, catalase, MR, VP, gelatin hydrolysis	99 & JX841107	*Bacillus pumilus*
DBTS29B6 & KF860872	-ve for indole and +ve for oxidase, catalase, MR, VP, gelatin hydrolysis	99 & AB741462	*Bacillus pumilus*
DBTS3B7 & KF860873	Catalase, gelatin +ve; Oxidase, MR-VP, indole –ve	99 & KC329822	*Bacillus megaterium*
DBTS11B8 & KF860874	+ve for oxidase, catalase, indole, VP, gelatin and -ve for MR test.	98 & GU193980	*Bacillus subtilis*

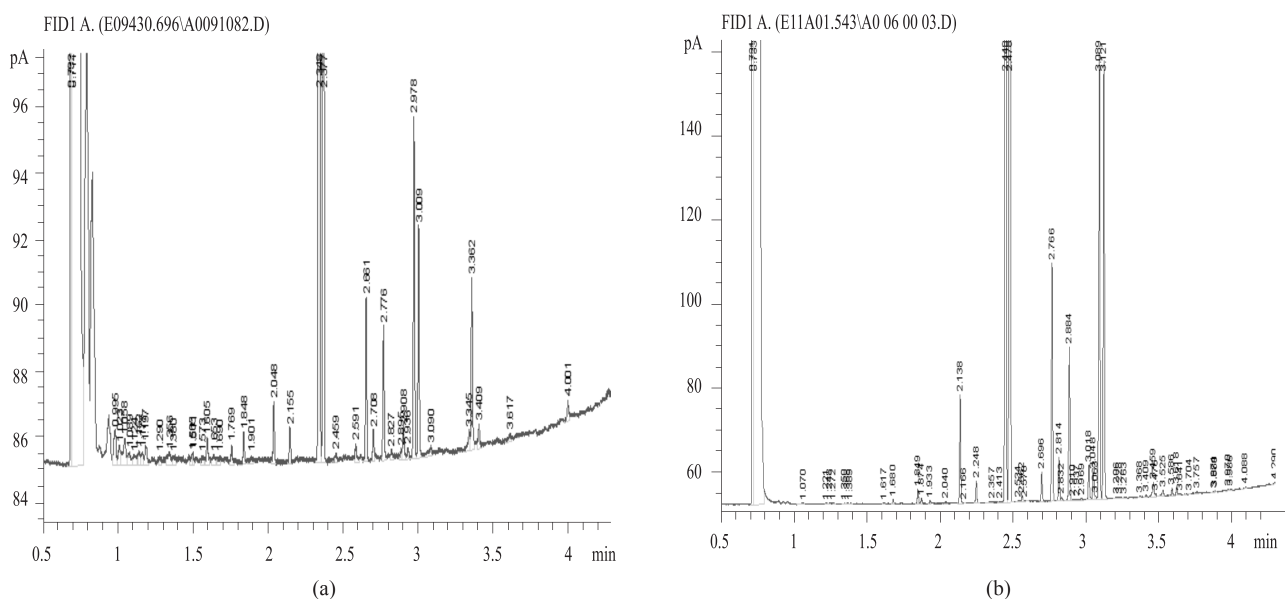

Fig. 1. FAME analysis of a couple of antagonistic bacterial isolates (a) *Bacillus pumilus* DBTS29B6 and (b) *Bacillus subtilis* DBTS12B2

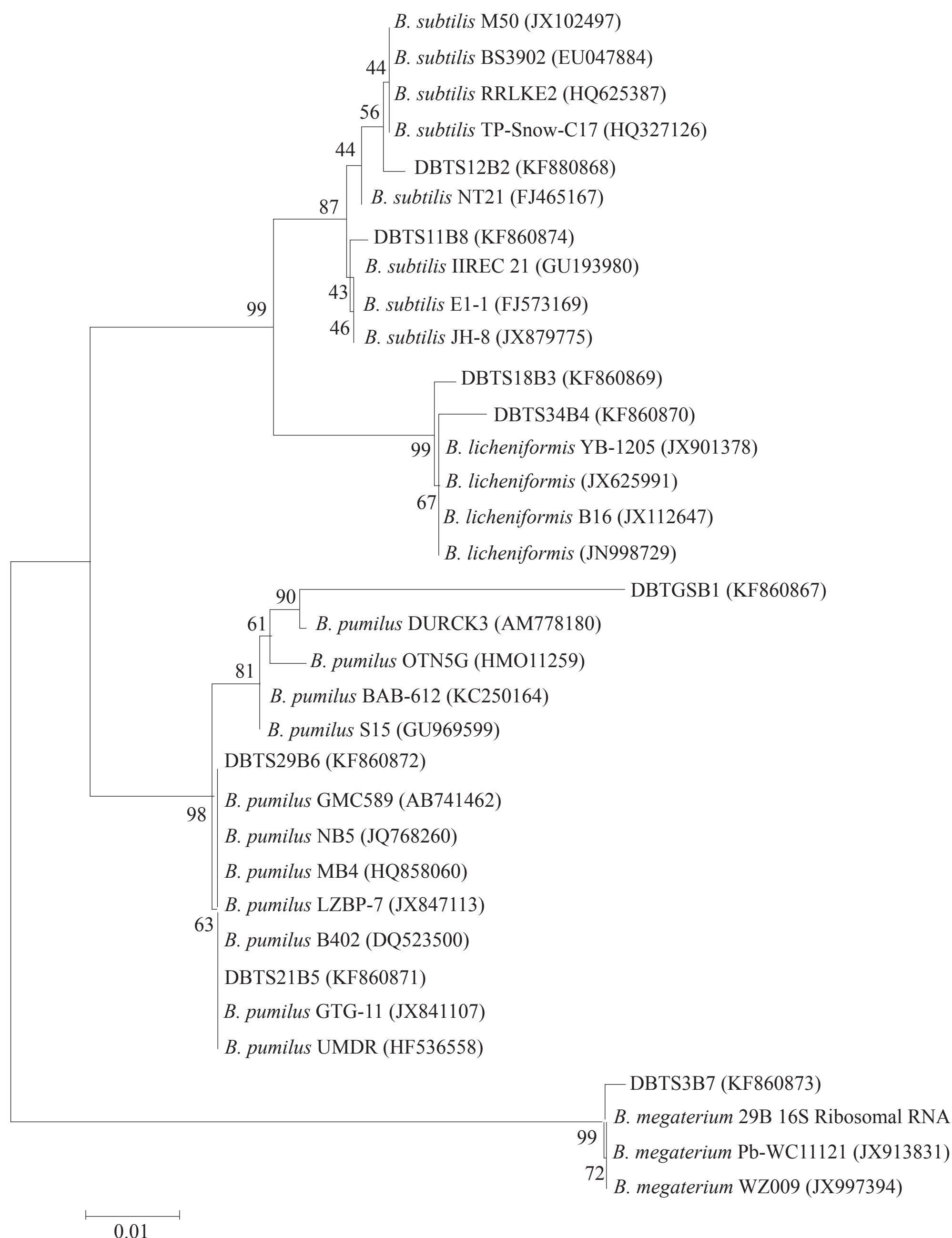

Fig. 2. Phylogenetic tree based on 16S rRNA gene as determined by maximum likelihood method

were also submitted in the GenBank and their accession numbers are KJ000877 and KJ000878 respectively. The pathogenic bacteria were inhibited by antagonistic strains as evidenced by the production of clear zones of inhibition against the growth of the target pathogens. All *Bacillus* isolates were found most effective against *V. harveyi* strain A1 as compared to *V. harveyi* strain B5 as they yielded 3-16 mm zones of inhibition. Strong inhibition zone of 16 mm against *V. harveyi* strain A1 was obtained for *B. subtilis* strain DBTS12B2 and *B. pumilus* strain DBTS29B6. *B. licheniformis* strains DBTS18B3 and DBTS34B4 showed moderate activity against target pathogens.

Cell viability during storage of the bioaugmentor matrix showed that initial viable bacterial counts in the immobilised matrix was 10^8-10^9 cells per g. It was also found that the matrix can retain 10^8-10^9 viable cells per g for the first three months of storage at room temperature and thereafter 10^7-10^8 viable cells per g during 4-6 months

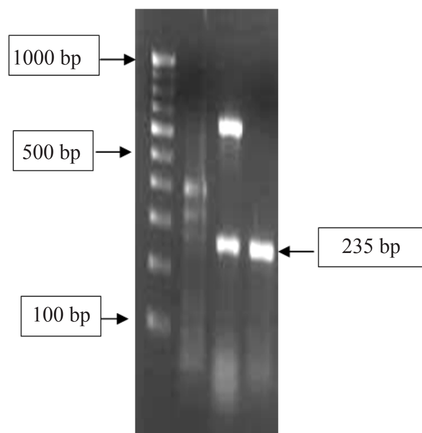

Fig. 3. PCR amplification of *Vhh* gene in *V. harveyi* strain

and 10^6-10^7 cells per g during 6-8 months of the storage at room temperature. The potential advantages of tapioca powder as immobilising matrix are: longer shelf life at room temperature, low cost, easy availability, adequate substrate concentration, excellent viable cell loading capacity/storage, high surface area for maintenance of a high microbial cell density and optimisation of microbial growth, easily biodegradable and no residue problem and longer biomass retention time. In the co-culture experiment, immobilised *Bacillus* strains effectively controlled *V. harveyi* as evidenced from the reduction of 40.54 to 88.65%, 60 to 98.86% and 73.33 to 99.99% in 24, 48 and 72 h respectively (Fig. 4).

There was no growth on *V. harveyi* agar after 72 h from *B. subtilis* strain DBTS12B2, *B. pumilus* strain DBTS29B6 and *B. licheniformis* strain DBTS4B4 flasks (Fig. 4). Almost 99% reduction of *V. harveyi* strain A1 was noticed. *B. megaterium* strain DBTS3B7 and *B. subtilis* strain DBTS11B8 showed moderate reduction (73-82%) as compared to other isolates.

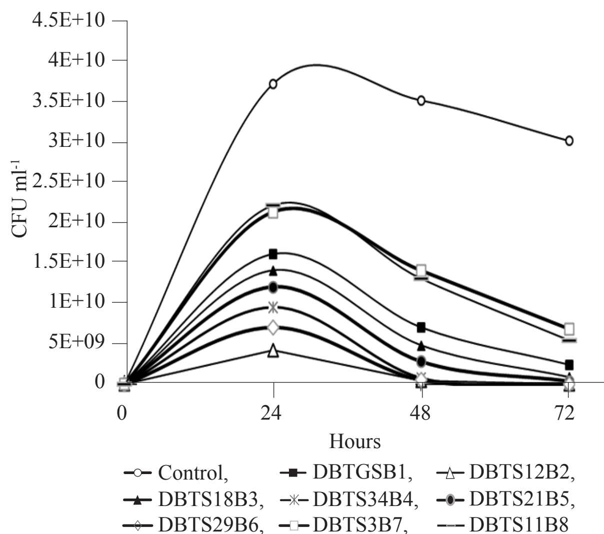

Fig. 4. Changes in residual amount of *V. harveyi* strain A1 with eight strains of *Bacillus* spp. using co-culture method

Fig. 5 shows the inhibitory activity of crude extracts from isolated bacterial strains. *B. pumilus* strain DBTS29B6 and *B. subtilis* strain DBTS12B2 showed highest activity against both pathogenic *Vibrio* species. They have shown considerably higher zone of inhibition against *V. harveyi* strain A1 (15-16 mm) and *V. harveyi* strain B5 (4-6 mm). *B. licheniformis* strains DBTS18B3 and DBTS34B4 showed moderate activity against the pathogens.

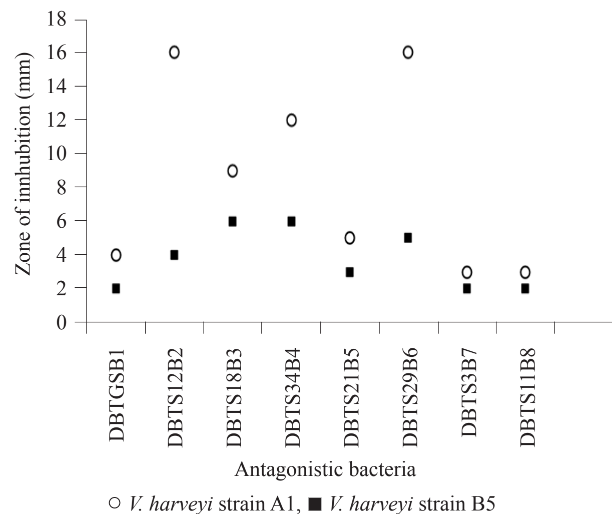

Fig. 5. Results of antagonism assay from well-diffusion method that produced clear zones of inhibition against *V. harveyi* strains A1 and B5

Beneficial bacteria associated with fish slime helps condition the water by inhibiting the growth of *Vibrio* spp.. *Bacillus* spp. especially *Bacillus subtilis* and *B. licheniformis* are widely present in the aquatic environment and are commonly used as probiotic strains against disease causing organisms due to their ability to grow in different conditions (Moriarty, 1999; Decamp, 2004). Control of pathogenic bacteria is effected through a variety of mechanisms such as competitive exclusion, improvement of water quality (Garriques and Arevalo, 1995), enhancement of immune response of host species (Andlid *et al.,* 1995) as well as enhancement of nutrition of host species (Garriques and Arevalo 1995).

Pathogenic *Vibrio* spp. especially the luminous *V. harveyi* often adversely affects aquaculture production. In the present investigation, eight strains of beneficial *Bacillus* spp. isolated from fish slime were found

effective in inhibiting the growth of pathogenic *Vibrio harveyi* Antagonistic bacterial formulation producing biomolecules with antibacterial activity could be effectively used against pathogenic bacteria in aquaculture and related aquatic environment.

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Date of Receipt : 17.05.2018
Date of Acceptance : 22.12.2018