

Inhibitory Activity of Antibiotics and Anti-Vibrio Probiotics Against *Vibrio harveyi* Isolated from Penaeid Shrimp Hatcheries

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Abstract

In the present study, water samples were collected from different hatcheries during vibriosis outbreak. Overall, ten species of *Vibrio* were isolated on the basis of biochemical tests in which three were identified as *Vibrio harveyi* (C3, C5 and C9). Antibigram of bacterial isolates were ascertained using the disk diffusion method and the result revealed that all three isolates were susceptible to ceftriaxone, chloramphenicol, ciprofloxacin, gentamycin, nalidixic acid, norfloxacin, oxolinic acid, oxytetracycline, polymyxin B, rifampicin and tetracycline whereas it rendered resistance to three antibiotics viz., ampicillin, erythromycin and kanamycin. A total of 12 probiotic bacteria were isolated from the gut of black tiger shrimp (*Penaeus monodon*) in which four exhibited antagonistic activity against *V. harveyi* and they were identified belonging to *Bacillus* spp. (AVP03, AVP04, AVP07 and AVP11). Anti-vibrio activity of the probiotic cultures were tested using agar well diffusion method (zone of inhibition ranging from 13.4±0.6 to 19.0±0.1 mm) and the isolate, AVP07 was identified as most efficient probiont. Production of anti-vibrio (extracellular) compounds of AVP07 started from the late log phase (10.6 mm inhibition zone in 12 hr) and the highest activity was found in early and late stationary phase (17.1 mm in 18 hr and 16.9 mm in 24 hr). The culture filtrate (0.45 µm) of probiont AVP07 also showed the cell lytic activity on heat killed (4 to 10 hr below 50% and 18 to 22 hr below 75%) and live cells (at 4 hr below 50% and 6 to 22 hr below 75%) of *V. harveyi* C5.

Introduction

Aquaculture is the fastest growing food production in the world among other sectors over the past 30 years (Josupief et al. 2000). Though the shrimp hatchery technology has advanced over the decades, the hatchery production is more often hampered by severe mortalities caused by luminous vibriosis, a major problem for shrimp cultivation in Asian countries (Musa et al. 2008). Among the vibrios, *Vibrio harveyi* is the main cause of shrimp death, infecting larva in the hatchery as well as in the cultivation pond (Vinod et al. 2005; Chari and Dubey, 2006; Won and Park, 2008). The virulence of *V. harveyi* causes 100% losses at a time in shrimp production (Chythanya et al. 2002; Musa et al. 2008). Antibiotics are commonly used to control the disease in shrimp hatcheries. But, their extensive use creates many problems such as resistant strains (Musa et al. 2008) and the residual drug in animals are hazardous for human consumption. Therefore, it is necessary to carry out sensitivity studies on pathogens like *V. harveyi* to different antibiotics. Antibiotic resistant *V.*

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harveyi causes mass mortality in black tiger shrimp (*Penaeus monodon*) larvae in hatcheries (Karunasagar et al. 1994). In order to limit the use of antibiotics, many workers have been exploring the use of new bioactive compounds to control the diseases, particularly that caused by *V. harveyi*. For the last few years, probiotics have been used to control pathogens in aquaculture following the development of disease resistance strains in aquatic pathogens. Probiotics in contrast to antibiotics can be a safer ecological alternative tool for sustainable aquaculture. Hence, the present study was aimed to examine the efficacy of anti-vibrio probiotics against the *V. harveyi* isolated from commercial hatcheries along with its sensitivity towards different antibiotics.

Materials and Methods

Sample collection

Random samplings from the infected tanks were obtained from the four commercial shrimp hatcheries in Tamil Nadu, located along the Southeast coast of India during the disease outbreak. The samples were brought to the Microbial Biotechnology Laboratory of Manonmaniam Sundaranar University, South India.

Isolation and Identification of Vibrio harveyi

After plating the samples on nutrient agar to enumerate the total heterotrophic bacteria, isolation of luminous *Vibrio* and other *Vibrio* spp. was performed by spreading on TCBS (Thiosulphate Citrate Bile Salt) agar (Hi-media, Mumbai) and VHA (*Vibrio harveyi* Agar) medium (Harris et al. 1996). Following the incubation at 30 °C, plates were observed for colony morphology and luminescence. After 24 hr, only the luminous colonies were selected and stored in deep tube tryptic soy agar (TSA) with 2% NaCl as stock and identified by using various biochemical test (Wiik et al. 1989; Alsina and Blanch, 1994).

Antibiogram assay

The sensitivity pattern of the isolated vibrios was done by growing the cells on Mueller-Hinton agar (Hi-media, Mumbai) impregnated with commercial antibiotic discs (Bauer et al. 1966) and the results were interpreted as mentioned by NCCLS (National Committee for Clinical Laboratory Standards), thereby the antibiogram was constructed.

Isolation and characterization of probiotics

Live shrimps were collected from a hatchery located in Tuticorin, Tamil Nadu and stored in frozen condition in the laboratory. The gut portion was dissected and homogenized using aseptic condition in sterile saline and then serially diluted up to 10^{-5} . Spread plating was performed on Zobell Marine Agar and incubated at 37 °C for 24 hr. The anti-vibrio activity of the isolates from the agar plate was screened by cross streak (Lemos et al. 1985) and agar spot method (Dopazo et al.

1988). Bacteria selected for its anti-vibrio activity were identified up to genus level using macroscopic, microscopic and physiological tests (Claus and Berkeley, 1986). The following four bacteria from shrimp gut (AVP03, AVP04, AVP07 and AVP11) and one reference strain of *Bacillus subtilis* (BS6710) obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India were selected as anti-vibrio probiotics.

Anti-vibrio probiotics assay

Stock cultures of *V. harveyi* were subcultured at least twice (18 hr, 30 °C) on fresh tryptic soy broth (TSB) with 1.5% NaCl to ensure their active growth. The pathogens in TSB were individually adjusted to the 0.5 McFarland standards (1.5×10^8 CFU·ml⁻¹) and then swabbed over the surface of TSA (1.5% NaCl) plates. Swabbing was repeated to ensure an even distribution of 188 inoculums. Wells (diameter 7 mm) were punched in the TSA plate by a Pasteur pipette. Seventy µL of an 18 hr culture (Chythanya et al. 2002) of the isolated (AVP03, AVP04, AVP07 and AVP11) and reference (BS6710) probiotics were transferred into each well and incubated at 30 °C for 24 hr. The antibiotic discs used as positive controls were: oxonilic acid (2 µg), norfloxacin (10 µg) and tetracycline (30 µg) and the inhibition zones were measured using a vernier caliper. The experiment was carried out in six replicates and the results were interpreted as means ± their standard deviations.

Effect of culture age on anti-vibrio activity

From the above assay one effective anti-vibrio probiotic was selected and cultured in TSB medium supplemented with 2% NaCl under the optimal conditions. The culture broth was sampled at 0, 4, 8, 12, 16, 18, 24 and 36 hr to measure the growth based on the cell turbidity at 660 nm (OD 660) using a spectrophotometer. Simultaneously each sample was filtered using a 0.45 µm cellulose acetate filter and tested for their inhibitory activity against *V. harveyi* by well diffusion method.

Assay of bacteriolytic activity

The culture filtrate (0.45 µm) obtained from 24 hr culture of selected probiotic was used to test its bacteriolytic activity (Than et al. 2004). A 48 hr culture of *V. harveyi* (C5) was centrifuged at 6,000 rpm for 25 min to collect the cell pellet and dispersed in 3 mL sterile artificial sea water (ASW), incubated in a water bath at 50 °C ± 0.5 °C for 30 min to obtain heat killed cells. A mixture consisting of 7 mL of the 0.45 µm culture filtrate and 3 mL of the heat-killed *V. harveyi* cell suspension was adjusted to an initial absorbance of approximately 0.5 (OD 540 nm) and then incubated at 30 °C for investigating cell lytic activity. The cell lytic activity was assayed by monitoring the absorbance at 540 nm at different time intervals viz., 0, 2, 4, 6, 8, 10, 18 and 22 hr and the ASW was used as a blank. A 24 hr culture filtrate was also used to test its bacteriolytic activity against live cells of *V. harveyi* by using the same method as for heat-killed cells except that dead cells were replaced by live cells. The lytic activity was determined according to the percentage decrease in absorbance at 540 nm in comparison with the control (Niwa et al. 2005).

Results

Isolation and identification of Vibrio harveyi

After overnight incubation, different colonies such as green, yellow, bluish and colorless colonies were found on TCBS agar. Among the ten isolated colonies on VHA, three colonies (C3, C5 and C9) appeared to produce typical *V. harveyi* colonies, while one colony (C6) produced blue and the rest were green on VHA media. Twenty eight biochemical tests were performed to identify the isolated colonies. All the isolates were found to belong to the genus *Vibrio* but 40% of the cultures could not be assigned to any particular species by these biochemical characters. Further, some isolates died during storage. Overall identified *Vibrio* spp. is tabulated in Table 1. Further studies are needed in order to include other atypical vibrios in the classification scheme.

Antibiogram assay

Antibiotic susceptibility testing of the pathogenic *V. harveyi* was performed by disc diffusion method. All the three strains tested were susceptible to ceftriaxone, chloramphenicol, ciprofloxacin, gentamycin, nalidixic acid, norfloxacin, oxolinic acid, oxytetracycline, polymyxin B, rifampicin and tetracycline but resistant to ampicillin, erythromycin and kanamycin. Furazolidone and sulphamethoxazole gave an intermediate zone of inhibition to strains C3, C5 and C5, C9 but susceptible to C9 and C3 respectively. C3 and C9 were also found to be susceptible to streptomycin whereas C5 showed intermediate to this antibiotic. The antibiogram results are presented in Table 2.

Characteristics of anti-vibrio probiotics

Around 12 bacteria were obtained from the gut homogenates based on colony morphology on Zobell marine agar and labeled as AVP01 to AVP12. All the isolates were checked for its anti-vibrio activity using cross streak and agar spot method. Among the 12 isolates, four (AVP03, AVP04, AVP06 and AVP11) had antagonistic activity and they were partially identified as the genus *Bacillus* spp. based on the microscopic and physiological tests (Table 3).

Table 1. Biochemical properties of the isolated colonies from shrimp hatchery water.

Biochemical Test	Culture No									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Arabinose utilization	ND	-	-	-	-	-	ND	ND	-	ND
Arginine dihydrolase	ND	+	+	+	+	+	ND	ND	+	ND
Catalase	+	+	+	+	+	+	-	-	+	+
Citrate utilization	-	-	-	-	-	+	-	-	-	-
Gelatinase	+	-	+	+	+	-	ND	ND	+	+
Glucose utilization	+	-	-	-	-	+	-	-	-	-
Gram stain	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S	-/S
Growth at 0% NaCl	ND	+	-	+	-	-	ND	ND	-	ND

Table 1 (contd.)

Growth at 1% NaCl	+	+	+	+	+	+	+	+	+	+
Growth at 3% NaCl	+	+	+	+	+	+	+	+	+	+
Growth at 6% NaCl	+	+	+	+	+	+	-	-	+	+
Growth at 8% NaCl	+	+	+	+	+	+	-	-	+	-
Growth at 10% NaCl	-	+	-	+	-	-	-	-	-	-
Growth at 30 °C	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	-	+	-	+	ND	-	-	-
H ₂ S	-	-	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-
Luminescence	+/-	-	+	-	+	-	-	-	+	-
Lysine decarboxylase	-	-	+	-	+	-	-	-	+	-
Mannitol utilization	+	-	+	-	+	+	-	-	+	-
Motility	+	+	+	+	+	+	+	+	+	+
MR test	-	+	+	+	+	-	-	-	+	-
Ornithine decarboxylase	+	+	-	-	-	-	-	+	-	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Sucrose utilization	+	-	-	+	-	+	-	-	-	-
Swarming	+	-	-	-	-	-	-	-	-	-
VP test	-	-	-	-	-	-	-	-	-	-
Suspected organisms	Va	V	Vh	Vm	Vh	Vn	V	V	Vh	V

+ = positive, -= negative, ND = not determined, S = Short rod

Va= *V. alginolyticus*, V= *Vibrio* sp., Vh= *V. harveyi*, Vm= *V. metschnikovii*, Vn= *V. nereis***Table 2.** Susceptibility of *Vibrio harveyi* strains to commercial antibiotics (mm of inhibition zone).

Antimicrobial agent	Disk content µg/disk	<i>Vibrio harveyi</i>		
		C3	C5	C9
Ampicillin	10	R(0)	R(0)	R(0)
Ceftriaxone	30	S(23)	S(23)	S(22)
Chloramphenicol	30	S(18)	S(19)	S(18)
Ciprofloxacin	5	S(22)	S(21)	S(23)
Erythromycin	15	R(0)	R(0)	R(0)
Furazolidone	15	I(17)	I(17)	S(18)
Gentamycin	10	S(18)	S(18)	S(19)
Kanamycin	30	R(11)	R(13)	R(12)
Nalidixic acid	30	S(23)	S(23)	S(23)
Norfloxacin	10	S(21)	S(22)	S(21)
Oxolinic acid	2	S(20)	S(19)	S(20)
Oxytetracycline	30	S(20)	S(20)	S(20)
Polymyxin B	300	S(13)	S(12)	S(13)
Rifampicin	5	S(22)	S(22)	S(22)
Streptomycin	10	S(16)	I(13)	S(16)
Sulphamethoxazole	25	S(20)	I(13)	I(14)
Tetracycline	30	S(24)	S(24)	S(24)

Table 3. Characteristics of isolated antivibrial probiotics from shrimp gut.

Culture Code	Colony on marine agar	Spore type	Antivibrial activity	Suspected organisms
AVP01	Pale yellow/round	-	-	ND
AVP02	White/flat surface	-	-	ND
AVP03	Creamy white/thin round	Terminal/Ellipsoidal	+	<i>Bacillus</i> spp.
AVP04	Grey-white/irregular	Central/cylindrical	+	<i>Bacillus</i> spp.
AVP05	White/mucoid	-	-	ND
AVP06	White/flat surface	-	-	ND
AVP07	Yellow/opaque	Central/round	+	<i>Bacillus</i> spp.
AVP08	Pale yellow/round	-	-	ND
AVP09	White/mucoid	-	-	ND
AVP10	Yellowish/round	-	-	ND
AVP11	White/opaque	Subterminal/Ellipsoidal	+	<i>Bacillus</i> spp.
AVP12	White/mucoid	-	-	ND
BS6710	Creamy white/round	Terminal/Ellipsoidal	+	<i>B. subtilis</i>

+ = present, - = absent, ND = not determined

Inhibitory activity of anti-vibrio probiotics

Table 4 indicates that a 70 μ L of the culture from anti-vibrio probiotics inhibited the growth of the following shrimp pathogens *V. harveyi* (C3), *V. harveyi* (C5) and *V. harveyi* (C9) with clear zones in a size range of 13.43 ± 0.69 to 19.02 ± 0.13 mm. The most efficient probiotic was identified as AVP07 while AVP04 was less effective against all the three pathogens. The reference strain rendered a highest inhibition on C3 followed by C9 and C5. Among the three antibiotics tested, tetracycline had high inhibitory zone (24.26 ± 0.80) to all the pathogens followed by oxonilic acid and norfloxacin.

Table 4. Inhibitory effect of anti-vibrio probiotics against *V. harveyi*.

	Zone of inhibition (mm)							
	AVP03	AVP04	AVP07	AVP11	BS6710	Nx	T	Oa
C3	17.1 ± 0.4	13.7 ± 0.8	17.2 ± 0.8	15.1 ± 0.4	16.7 ± 0.4	21.9 ± 0.2	24.0 ± 0.8	20.9 ± 1.3
C5	16.2 ± 0.6	13.4 ± 0.6	19.0 ± 0.1	14.3 ± 0.8	16.9 ± 0.6	22.3 ± 0.7	24.1 ± 0.7	19.9 ± 0.9
C9	16.6 ± 0.9	14.0 ± 0.1	18.6 ± 0.9	14.2 ± 0.4	16.4 ± 0.3	21.9 ± 0.2	24.2 ± 0.8	20.7 ± 1.4

Nx = norfloxacin (10 μ g), T = tetracycline (30 μ g), Oa = oxolinic acid (2 μ g)

AVP03, AVP04, AVP07 & AVP11 = Anti-vibrio probiotics (70 μ L)

BS6710 = Reference strain of *B. subtilis* (70 μ L)

C3, C5, C9 = *V. harveyi*

Effect of culture age on anti-vibrio activity

The culture filtrate (0.45 μ m) from the probiotic bacteria AVP07 grown in TSB medium incorporated with 2% NaCl under the optimal conditions indicated that the cells started to produce bioactive compounds (extracellular compounds) that inhibited *V. harveyi* (C5) reaching the late log phase of growth at 12 hr whose inhibition zone was 10.6 mm (Fig. 1). Highest activity (17.1 mm) was observed in the early stationary phase (18 hr) against the target organism which significantly decreased to 16.9 mm at the late stationary phase (24 hr).

Assay of bacteriolytic activity

The bacteriolytic activity of 0.45 μ m culture filtrate from 24 hr of probiotic (AVP07) culture against heat killed and live cells of *V. harveyi* (C5) is presented in Fig. 2. The filtrate rendered a low lytic activity against dead cells which was first observed at 2 hr until the end of 10 hr (10-50%) and further increased to higher level (50-75%) at 18 hr to 22 hr. The lytic activity of the culture filtrate against live cells was also low (below 25%) until 4 hr of incubation followed by a higher lytic activity (50-75%).

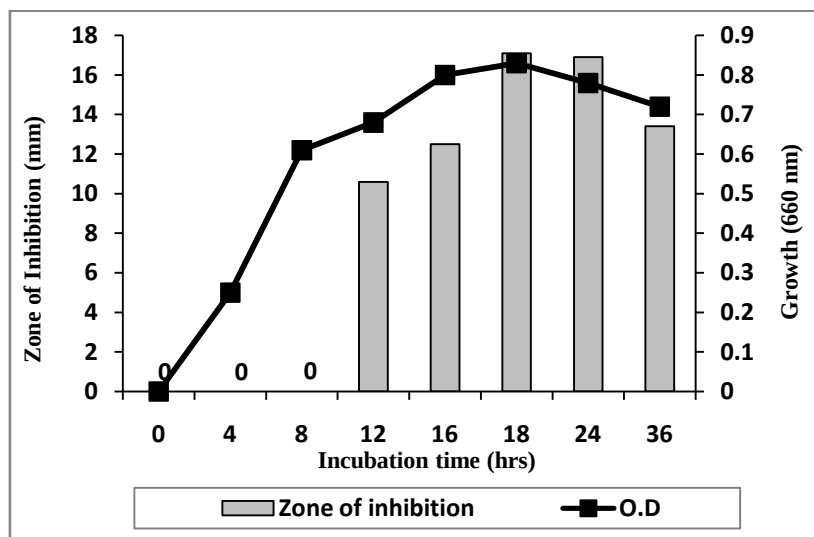


Fig. 1. Inhibition of *V. harveyi* (C5) by culture filtrate from a probiotic bacterium AVP07 at different stages of growth.

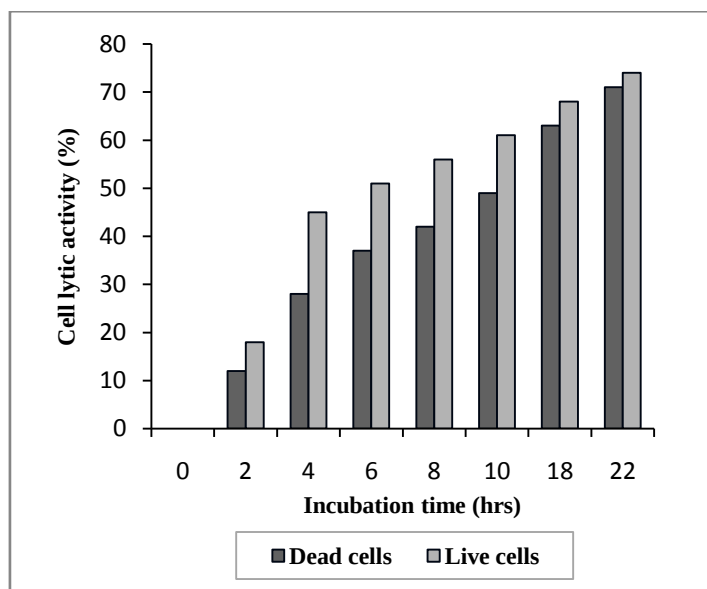


Fig. 2. Lytic activity of culture filtrate from AVP07 on live and killed cells of *V. harveyi* at different time intervals of incubation.

Discussion

On TCBS, the yellow colonies appeared as a result of sucrose utilization whereas the green colonies were unable to utilize sucrose. The VHA medium was used for the isolation of *V. harveyi* and *V. alginolyticus* (Harris et al. 1996). *V. harveyi* produced typical light green colonies with dark centered or yellow halo on VHA because of the utilization of both cellobiose and ornithine as their carbon source. In this study, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase (A/L/O) based dendrogram was followed for the primary identification of *Vibrio* spp. (Alsina and Blanch, 1994). The result of the dendrogram placed C3, C5, and C9 as *V. harveyi* likewise C1, C4 and C6 as *V. alginolyticus*, *V. metschnikovii* and *V. nereis* respectively. The remaining were found to belong to the genus *Vibrio* but could not be assigned to any particular species by these biochemical characters.

Most of the β -lactam antibiotics were no longer able to prevent luminous vibriosis (Teo et al. 2002). In addition, many *V. harveyi* strains showed resistance to multiple antibiotics such as tetracycline, chloramphenicol, streptomycin and spectinomycin (Shariff et al. 1996). This fact prompted us to evaluate the sensitivity pattern of the isolated *Vibrios* to different antibiotics. From the antibiogram it was observed that all the three strains were sensitive to most of the antibiotics tested but resistant to ampicillin, erythromycin and kanamycin. The resistance of ampicillin was similar to the findings of Otta et al. (2001) where 92% of isolated *V. harveyi* from *P. monodon* hatcheries in India were resistant to ampicillin. Ampicillin is categorized as broad spectrum β -lactam antibiotic and it functions as inhibitors of bacteria cell wall biosynthesis (Backhaus and Grimme, 1999). Thus, the finding of the present study indicated that the isolated *V. harveyi* possessed β -lactamase. On the other hand all three isolates were found to be highly sensitive to

tetracycline based on the observation of largest inhibition zone. Similar result was also obtained by Otta et al. (2001) where 97% isolates demonstrate high sensitivity against tetracycline.

Many studies have shown that compounds produced by bacteria from various sources could be used to inhibit bacterial pathogens in aquaculture (Alavandi et al. 2004; Vijayan et al. 2006). In this study the *Bacillus* spp. (AVP03, AVP04, AVP07 and AVP11) isolated from the shrimp gut inhibited the growth of *V. harveyi*. Vaseeharan and Ramasamy (2003) found that growth of pathogenic *V. harveyi* in tiger shrimp was controlled by the probiotic effect of *B. subtilis* BT23 in vitro and in vivo. Disease resistance was improved with reduced accumulated mortality by 90% when juvenile *P. monodon* were exposed to *B. subtilis* BT23 before a challenge with *V. harveyi*. Similarly Rengpipat et al. (1998) reported that inoculation with *Bacillus* S11 which had previously demonstrated its inhibitory effect in vitro against *V. parahaemolyticus* and *V. harveyi* resulted in greater survival of *P. monodon* challenged with pathogenic luminescent bacteria. The present study demonstrated that the identified four anti-vibrio probiotics belonging to genus *Bacillus* spp. from which one isolate (AVP07) showed highest activity against all the three *V. harveyi*.

Late log phase cultures of AVP07 inhibited the growth of shrimp pathogen (*V. harveyi* C3) tested in vitro and a maximum activity was present during their early stationary phase (Fig. 1). This indicated that the anti-vibrio compounds could be “secondary metabolites”. The unfiltered supernatant culture produced the best inhibition of *V. harveyi* than the 0.45 µm filtrate. Some of the growth inhibitory compounds produced by AVP07 may be bacteriolytic against dead and live cells of *V. harveyi* C3. This might be due to an attack on the cell envelope structure of *V. harveyi*. In contrast, a *Pseudomonas* spp. isolated from the surface seawater of Japan produced an enzyme that completely lysed the dead cells of *V. parahaemolyticus* VPHK-46 (Than et al. 2004). In conclusion, the results from this study indicate that the anti-vibrio probiotic isolated from shrimp gut produced antibacterial substances responsible for inhibiting the growth of shrimp pathogen and caused cell lysis in vitro.

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