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In vitro Antimicrobial Activity of *Bacillus* spp. Against Pathogenic *Vibrio* spp. in Black Tiger Shrimp (*Penaeus monodon*)

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ABSTRACT

Twenty-five isolates of *Bacillus* spp. from hepatopancreas of black tiger shrimp were screened for antimicrobial activity against four shrimp pathogenic *Vibrio* spp. (*V. harveyi* VHY02, *V. harveyi* VHG03, *V. alginolyticus* VA01, *V. parahaemolyticus* VP02) by agar well diffusion assay. Four isolates of *Bacillus* spp. (B17, B19, B21 and B25) were found active against all strains of *Vibrio* spp. and 4 isolates of *Bacillus* spp. (B06, B10, B13 and B22) showed some degrees of antimicrobial activities against at least one strain of *Vibrio* spp. The minimum inhibitory concentration (MIC) of 4 isolates of *Bacillus* spp. against all strains of *Vibrio* spp.(10^4 cfu/ml) were determined. The MIC, at 5.0×10^5 cfu/ml, belonged to *Bacillus* B17, *Bacillus* B21 and *Bacillus* 25 and at 5.0×10^6 cfu/ml to *Bacillus* B19. Broth studies were also determined by culturing *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 in broth containing *Vibrio harveyi* VHG03 (10^4 cfu/ml). The challenging experiment showed that *Bacillus* B17 and *Bacillus* B21 with 5.0×10^7 cfu/ml were enough to completely suppress *V. harveyi* VHG03 within 12 hours and *Bacillus* B25 was only able to reduce *Vibrio harveyi* VHG03 lower than control. The isolated strains *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 could have potential against *Vibrio harveyi* under *in vitro* condition and might be useful as biological control agents in the culture of black tiger shrimp.

Key words: *Penaeus monodon*, shrimp, *Vibrio harveyi*, *Bacillus* spp., antimicrobial activity

INTRODUCTION

Over the last few decades, the aquaculture industry has been growing tremendously, especially the shrimp industry. Concomitant with the growth of the shrimp culture industry has been the recognition of the ever increasing importance of diseases, especially those caused by infectious agents. The most important diseases of cultured penaeid shrimp are virus and bacteria. For instance, White spot syndrome virus (WSSV), the causative virus of the white spot syndrome disease, is found in most shrimp farming in Asia and has now spread to America (Sudha, 1998 and Corsin *et al*., 2002). Luminous *Vibrio*, the causative bacteria of the luminous vibriosis, is a major bacteria pathogen in shrimp culture such as *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. damsela*. However, *V. harveyi* has emerged as important because it causes large economic losses to the shrimp farming industry. In addition, a few important diseases have fungal and protozoan agents as their causes (Lightner and Redman,

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1998, Leano *et al*., 1998 and Sung *et al*., 2001). Shrimp farms can be broadly classified into three types based on major economic and technological differences: intensive, semi-intensive and extensive systems. The dominant intensive shrimp species is *Penaeus monodon* (Ling *et al*., 1999). The high density of shrimps in ponds is conductive to the spread of pathogens. Use of commercial antibiotics for disease treatment produces undesirable side effects, which may result in virulence of pathogens and, furthermore, are causes for concern in promoting transfer of antibiotic resistance to human pathogens. Some chemicals used in shrimp farming, such as organotin compounds, copper compounds, and other compounds with a high affinity to sediments, leave persistent, toxic residues, and are likely to have a negative impact on the environment (Graslund and Bengtsson, 2001, Tendencia and de la Pena, 2001 and Graslund *et al*., 2003). One of the techniques reported is the use of beneficial bacterial to displace pathogens in shrimp culture by competitive processes. The application of probiotics, *Bacillus* spp. *Lactobacillus* spp. and *Pseudomonas* spp. lessens pathogenic *Vibrio* spp. and enhances beneficial bacilli in the culture leading to improve water quality and promote growth of shrimps. The *Bacillus* species have a wide range of antimicrobial activity. It was found that some *Bacillus* spp. were effective against Gram-positive and Gram-negative bacteria (Yilamz *et al*., 2006). Rengpipat *et al*. (1998) reported the isolation of a bacteria probiont, *Bacillus* S11, from healthy *Penaeus monodon*, which reduced *P. monodon* mortality when challenged with *V. harveyi*. Chythanya *et al*. (2002) reported that *Pseudomonas* I-2 strain displayed antimicrobial activity against shrimp pathogens (*V. harveyi*, *V. fluvialis*, *V. damsela*, *V. parahaemolyticus* and *V. vulnificus*). Vaseeharan and Ramasamy (2003) noted *Bacillus subtilis* BT23 increased the survival of black tiger shrimp and suppressed the pathogen *V. harveyi*. Gullian *et al*. (2004) found that the probiont *Bacillus* P64 applied to shrimps could reduce diseases caused by *V. harveyi*. The aim of this study was to screen for effective *Bacillus* spp. against luminous *Vibrio harveyi* in an attempt to use it as a feed additive for black tiger shrimp.

MATERIALS AND METHODS

Bacteria strain

The strains used in this work were *Bacillus* spp. and *Vibrio* spp. obtained from the Department of Aquaculture and Department of Biotechnology. The 25 *Bacillus* spp. were isolated from hepatopancreas of *Penaeus monodon*. The 4 pathogenic strains were isolated from moribund shrimp on thiosulphate citrate bile salt sucrose (TCBS) agar (Table 1).

Bacterial culture preparation

Bacillus spp. and *Vibrio* spp. were grown in 250 ml erlenmeyer flasks containing 100 ml of nutrient broth containing 1.5 % sodium chloride at 37°C and shaked at 250 rpm for 24 h prior to being used in experiments.

Determination of antimicrobial activity

The antimicrobial activity was first determined by agar diffusion method (Baydar *et al.*, 2004 and Dobner *et al.*, 2003). Further study was made by broth assay where *Bacillus* spp. and *Vibrio* spp. were mixed and survival determined by plate counting at various time intervals from 0 to 48 h (Chythanya *et al.*, 2002).

Table 1 *Vibrio* spp. pathogenic strains.

Vibrio spp.	Code
Vibrio harveyi (yellow colony)	VHY02
Vibrio harveyi (green colony)	VHG03
Vibrio alginolyticus	VA01
Vibrio parahaemolyticus	VP02

Agar diffusion assay

Antimicrobial activity of 25 isolates of *Bacillus* spp. was carried out against 4 target strains. The active cultures of *Vibrio* spp. (250 µl), adjusted to 10^6 cfu/ml final cell concentration, were added to flasks containing 25 ml sterile nutrient agar containing 1.5 % sodium chloride at 45°C, and poured into petri plates (9 cm diameter). The agar was allowed to solidity for 1 hour. Wells (8 mm-diameter) were punched out of the solid agar using sterile cork borer, and 50 µl of the culture broth of *Bacillus* spp. (adjusted to 5×10^8 cfu/ml in 0.85% sodium chloride) or the control (nutrient broth containing 1.5 % sodium chloride) were added into each well and incubated at 37°C. After 24 h, the diameter of inhibition zones were measured.

Determination of MIC values was performed using the agar dilution method (Voravuthikunchai *et al*., 2004). This assay was performed with the same method as above but before testing, suspensions of *Bacillus* spp. were serially diluted with 0.85% sodium chloride giving concentrations of 5×10^7, 5×10^6, 5×10^5, 5×10^4 and 5×10^3 cfu/ml, respectively, and nutrient broth containing 1.5 % sodium chloride was used as the control. Then the minimum inhibitory concentration (MIC) defined as the lowest concentration at which no visible inhibition zone could be detected, was determined. Each experiment was repeated two times.

Effect of *Bacillus* spp. on growth of *Vibrio* spp. in sterile nutrient broth

The cultures of *Bacillus* spp. (B17, B19, B21 and B25) and *V. harveyi* VHG03 were prepared as described above. Four 250 ml flasks containing 100 ml of nutrient broth containing 1.5 % sodium chloride were sterilised at 121°C for 15 min and designated as FI, FII, FIII, FIV. Cell suspension of *V. harveyi* VHG03 was then added to all flasks to get a cell density of approximately 5×10^4 cfu/ml. Cell suspensions of *Bacillus* spp. adjusted to 5×10^8, 5×10^7 and 5×10^6 cfu/ml final cell concentration were added to flasks FI, FII and FIII, respectively, while flask FIV without *Bacillus* spp. added served as the control. The cultures were incubated at 37°C for 48 h with shaking at 250 rpm. *Bacillus* spp. and *V. harveyi* VHG03 were enumerated at 0, 12, 24, 36 and 48 h on nutrient agar and TCBS agar, respectively, by standard spread plate method.

RESULTS AND DISCUSSION

Screening of *Bacillus* spp for antimicrobial activity

Antimicrobial activity of 25 *Bacillus* spp. isolates was screened against 4 target strains, *V. harveyi* VHY02, *V. harveyi* VHG03, *V. alginolyticus* VA01 and *V. parahaemolyticus* VP02, using agar well diffusion assay. The 8 *Bacillus* spp. isolates had antibacterial activity against at least one of the *Vibrio* spp. Four isolates of *Bacillus* spp. (B17, B19, B21 and B25) were found active against all strains of *Vibrio* spp. (Table 2 and Figure 1). The *Bacillus* spp. B17, B21 and B25 presented the highest inhibition zones (1.68±0.04, 1.80±0.07 and 1.95±0.14 cm, respectively) on *V. parahaemolyticus* VP02 and *Bacillus* B19 presented the highest inhibition zone (1.63±0.04 cm) on *V. harveyi* VHG03.

Various strains of *Bacillus* spp. and *Pseudomonas* spp. have been reported as effective against *V. harveyi* and other *Vibrio* species determined by using agar well diffusion technique. Sugita *et al*. (1998) isolated *Bacillus* NM12 from coastal fish and showed high activity against *V. vulnificus* RIMD 219009, where the diameter of zone of inhibition was 1.9 cm. Chythanya *et al*. (2002) reported that *Pseudomonas* I-2 strain displayed antimicrobial activity against shrimp pathogen, *V. harveyi* (diameter about 1.7 cm).

Bacillus spp. B17, B19, B21 and B25 were identified as *B. amyloliquefaciens* BA01, *B. pumilus*, *B. amyloliquefaciens* BA02 and *B. megaterium*, respectively, by Thailand Institute of

Table 2 Antibacterial activity of *Vibrio* spp. by various *Bacillus* spp.

Bacillus spp.	Inhibition zone (cm) ± S.D.			
	V. harveyi	*V. harveyi*	*V. alginolyticus*	*V. parahaemolyticus*
B01	-	-	-	-
B02	-	-	-	-
B03	-	-	-	-
B04	-	-	-	-
B05	-	-	-	-
B06	1.20 ± 0.00	-	-	-
B07	-	-	-	-
B08	-	-	-	-
B09	-	-	-	-
B10	1.65 ± 0.07	-	-	-
B11	-	-	-	-
B12	-	-	-	-
B13	1.20 ± 0.07	-	-	-
B14	-	-	-	-
B15	-	-	-	-
B16	-	-	-	-
B17	1.48 ± 0.04	1.50 ± 0.14	1.58 ± 0.11	1.68 ± 0.04
B18	-	-	-	1.88 ± 0.04
B19	1.40 ± 0.00	1.63 ± 0.04	1.55 ± 0.28	1.58 ± 0.04
B20	-	-	-	1.78 ± 0.04
B21	1.48 ± 0.04	1.35 ± 0.07	1.63 ± 0.11	1.80 ± 0.07
B22	1.35 ± 0.00	-	-	-
B23	-	-	-	1.20 ± 0.00
B24	-	-	-	1.25 ± 0.00
B25	1.63 ± 0.04	1.45 ± 0.14	1.78 ± 0.25	1.95 ± 0.14

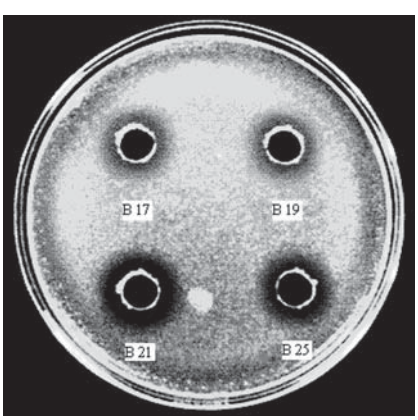

Figure 1 Inhibition zones of *Bacillus* B17, *Bacillus* B19, *Bacillus* B21 and *Bacillus* B25 against *Vibrio harveyi* (VHG 03).

Scientific and Technological Research. Those bacteria produced inhibition zones higher than 0.8 cm and against all strains of *Vibrio* spp. *Bacillus* B17, B19, B21 and B25 were selected for further study on minimum inhibition concentration (MIC) using agar diffusion assay.

Minimum inhibition concentrations (MICs)

Agar diffusion assay

The lowest concentrations of *Bacillus* spp., which did not show any growth of *Vibrio* spp. were determined as MIC. The results of MIC to control *Vibrio* spp. are shown in Table 2. *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 had

Table 3 Minimum inhibitory concentrations (MIC) of *Bacillus* spp. against *Vibrio* spp.

Concentration of	*Bacillus*	Inhibition zone (cm)			
		V. harveyi	*V. harveyi*	*V. alginolyticus*	*V. parahaemolyticus*
5×10^7 cfu/ml	B 17	1.40	1.53	1.53	1.65
	B 19	1.55	1.58	1.65	1.63
	B 21	1.55	1.30	1.63	1.68
	B 25	1.60	1.45	1.78	1.95
5×10^6 cfu/ml	B 17	1.48	1.38	1.48	1.58
	B 19	1.43	1.45	1.40	1.55
	B 21	1.43	1.30	1.58	1.58
	B 25	1.55	1.60	1.60	1.85
5×10^5 cfu/ml	B 17	1.33	1.25	1.43	1.55
	B 19	1.33	-	1.10	1.18
	B 21	1.28	1.23	1.65	1.25
	B 25	1.55	1.38	1.45	1.95
5×10^4 cfu/ml	B 17	-	-	1.43	1.40
	B 19	-	-	-	-
	B 21	-	-	1.68	-
	B 25	1.38	1.28	1.33	-
5×10^3 cfu/ml	B 17	-	-	-	-
	B 19	-	-	-	-
	B 21	-	-	-	-
	B 25	-	-	-	-

the same MIC value of 5.0×10^5 cfu/ml. *Bacillus* B19 showed a higher MIC value (5.0×10^6 cfu/ml) than the other species. The lower MIC of *Bacillus* spp. to control *Vibrio* spp. indicated greater antimicrobial activity than the other species. Therefore, only *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 were chosen for futher study.

Effect of *Bacillus* spp. on growth of *Vibrio* spp. in nutrient broth

The inhibition of *V. harveyi* VHG03 (10^4 cfu/ml) by various concentrations of *Bacillus* spp. (adjusted to 5×10^8, 5×10^7 and 5×10^6 cfu/ml final cell concentration) in nutrient broth containing 1.5% sodium chloride are shown in Figure 2 to Figure 4.

Figure 2 shows the time course of bacterial concentration in the culture of *Bacillus* B17 (*B. amyloliquefaciens* BA01) and *V. harveyi* VHG03 after incubation in nutrient broth containing 1.5 % sodium chloride. The *Bacillus* B17 at the concentration of 5×10^6 cfu/ml had no effect on the growth of *Vibrio harveyi* VHG03. *Bacillus* B17 (5×10^7 cfu/ml) could inhibit *V. harveyi* VHG03 growth within 12 hours. It was found that the concentration of *Vibrio harveyi* VHG03 was constant (about 10^2 cfu/ml) until 48 hours. Similar results were obtained when *Bacillus* B17 concentration increased (5×10^8 cfu/ml). For the control, an increase of *Vibrio harveyi* VHG03 was observed from about 10^4 to 10^8 cfu/ml. From the results, the MIC value of *Bacillus* B17 to control the growth of *Vibrio harveyi* VHG03 was 5×10^7 cfu/ml.

Figure 3 shows that *Bacillus* B21 (*B. amyloliquefaciens* BA01) concentration at 5×10^6 cfu/ml had no effect on the growth of *Vibrio*

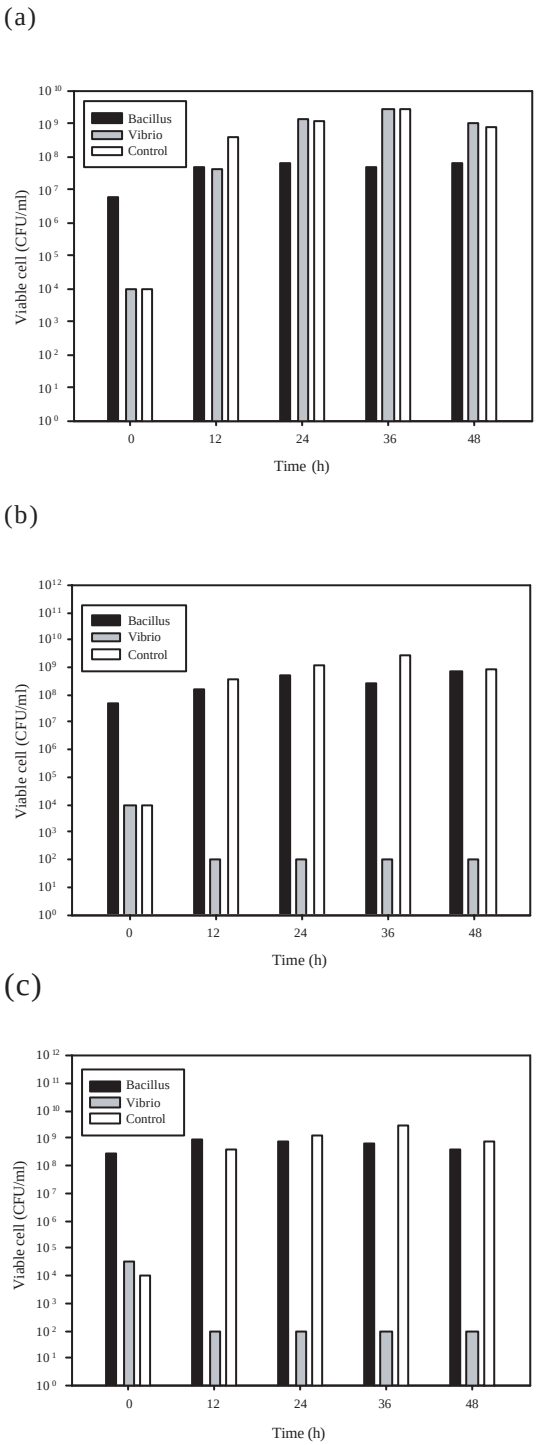

Figure 2 Effect of different concentrations of *Bacillus* B17 at 5×10^6 cfu/ml:(a), 5×10^7 cfu/ml:(b), 5×10^8 cfu/ml: (c) on growth of *V. harveyi* VHG03 in nutrient broth containing 1.5 % sodium chloride.

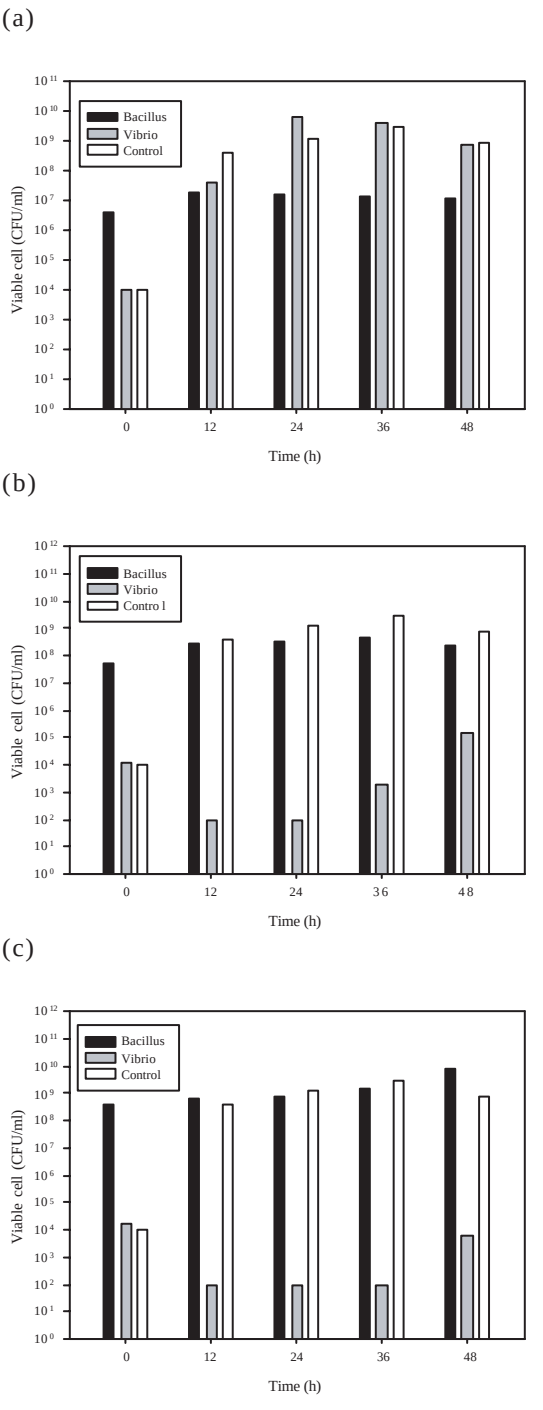

Figure 3 Effect of different concentrations of *Bacillus* B21 at 5×10^6 cfu/ml (a), 5×10^7 cfu/ml (b), 5×10^8 cfu/ml (c) on growth of *V. harveyi* VHG03 in nutrient broth containing 1.5 % sodium chloride.

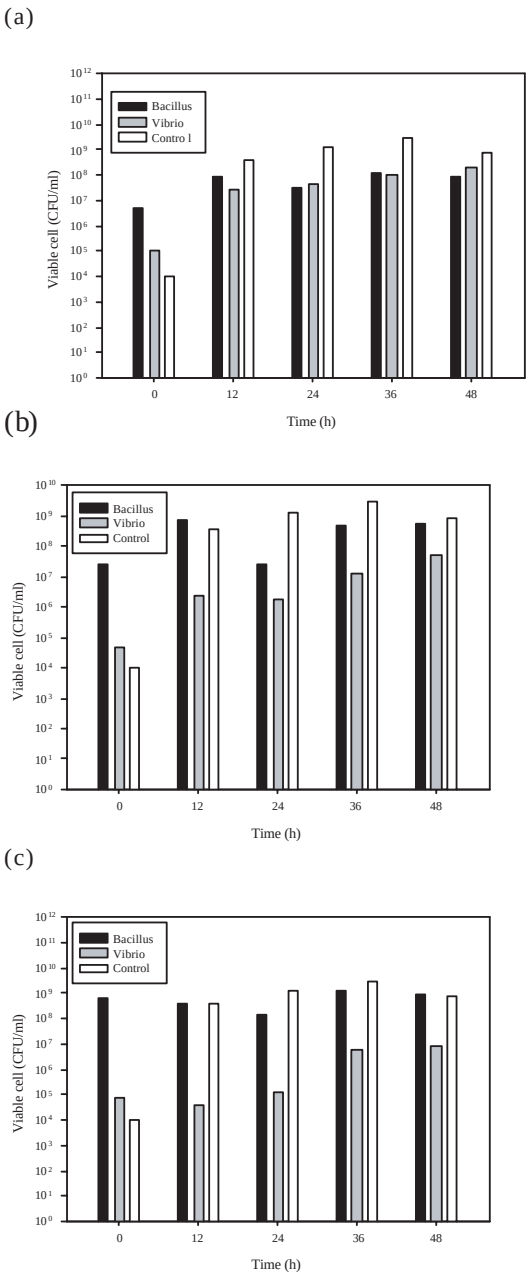

Figure 4 Effect of different concentrations of *Bacillus* B25 at 5×10^6 cfu/ml (a), 5×10^7 cfu/ml (b), 5×10^8 cfu/ml (c) on growth of *V. harveyi* VHG03 in nutrient broth containing 1.5 % sodium chloride.

harveyi VHG03. At concentration of 5×10^7 cfu/ml, *Bacillus* B21 showed the inhibition effect on *V. harveyi* VHG03 within 12 to 24 h as the *V. harveyi* VHG03 concentration decreased to about 10^2 cfu/ml. After 36 h incubation, the pathogenic strain increased to about 10^3 cfu/ml. Then, cell concentration of the pathogenic strain increased to about 10^3 to 10^5 cfu/ml at 36 to 48 h. At concentration of 5×10^8 cfu/ml, *Bacillus* B21 showed the inhibition effect on *V. harveyi* VHG03 within 12 to 36 h (*V. harveyi* VHG03 decreased to about about 10^2 cfu/ml). After incubation for 48 h the *V. harveyi* VHG03 increased to about 10^4 cfu/ml. For the control, an increase of *Vibrio harveyi* VHG03 was observed from about 10^4 to 10^8 cfu/ml. Although the results showed that the MIC value of *Bacillus* B21 to control the growth of *Vibrio harveyi* VHG03 was 5×10^7 cfu/ml, it required boosting again at 36 h for complete control of *Vibrio harveyi* VHG03.

There are several reasons why the growth of *Vibrio* spp. is inhibited by *Bacillus* species because *Bacillus* spp. can secrete many exoenzymes and antimicrobial compounds. (Moriarty, 1998). *B. amyloliquefaciens* (*Bacillus* B17 and *Bacillus* B21) secretes a variety of enzymes, such as amylase, galactanase, isoamylase, mannanase, xylanase, metal protease, serine protease, alkaline phosphatase and deoxyribonuclease (Priest, 1977). *B. amyloliquefaciens* was applied as an antifungal agent as reported by Kim and Chung (2004) that antifungal protein produced by *B. amyloliquefaciens* MET 0908 (isolated from soil) showed strong activity against the plant pathogen, *Colletotrichum lagenarium*, that caused watermelon anthracnose.

Figure 4 shows that all concentrations of *Bacillus* B25 (*B. megaterium*) are not able to reduce *V. harveyi* VHG03. However, the high concentrations of *Bacillus* B25 (5×10^7 and 5×10^8 cfu/ml) increased the inhibition effect on the growth of *V. harveyi* VHG03. Many reports have

shown that several strains of *B. megaterium* produce many exoenzymes and antimicrobial compounds, such as amylase, dextranase, protease, lactamase and nucleotidase (Priest, 1977). Some strains of *B. megaterium* produce bacteriocins. Brusilow and Nelson (1981) reported that *B. megaterium* strain 337 produced such a protein, megacin Cx, which killed sensitive bacterial cells by specifically blocking protein synthesis.

This experiment showed that the inhibitory effect of *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 on *Vibrio harveyi* VHG03 in sterile nutrient broth increased with increasing density of the *Bacillus* spp. Low density of *Bacillus* spp. (5×10^6 cfu/ml) had no inhibitory effect. This result corresponded to the co-culture experiment of *Bacillus subtilis* BT23 and *V. harveyi* reported by Vaseeharan and Ramasamy (2003). The growth of pathogenic *V. harveyi* was inhibited by *B. subtilis* BT23 culture inoculated at an initial level of 10^5 to 10^9 cfu/ml. Co-culture experiments showed that the inhibitory activity of *B. subtilis* BT23 increased with increasing density of the antagonist. A high concentration of *B. subtilis* BT23 (antagonist) was required to inhibit *V. harveyi* in the co-culture experiments. The study showed that the antagonist must be present at significantly higher levels than the pathogen and the degree of inhibition increased with the level of antagonist. During the co-culture, 10^7 to 10^9 cfu/ml were required to inhibit the growth of the pathogen *V. harveyi* (approx 10^2 cfu/ml).

CONCLUSION

Bacillus B17, *Bacillus* B21 and *Bacillus* B25 isolated from hepatopancreas of *Penaeus monodon* produced a wide zone of inhibition against *Vibrio spp*. Co-culture in sterile nutrient broth experimental results showed that *Bacillus* B17 and *Bacillus* B21 with 5.0×10^7 cfu/ml were enough to completely suppress *V. harveyi* VHG03 (5×10^4 cfu/ml) within 12 hours and *Bacillus* B25 was able to reduce *Vibrio harveyi* VHG03 as compared to the control. The isolated strains *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 had the properties of a biocontrol agent for use in control of *Vibrio harveyi* and might be useful for replacing the commercial antibiotic. Further study is needed for formulation of *Bacillus* B17, *Bacillus* B21 and *Bacillus* B25 by spray drying. It is used for preservation and concentration of these *bacillus* spp. Moreover, the powder is easy to use in the culture of black tiger shrimp.

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