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Application of *Bacillus* spp. Isolated from the Intestine of Black Tiger Shrimp (*Penaeus monodon* Fabricius) from Natural Habitat for Control Pathogenic Bacteria in Aquaculture

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ABSTRACT

Bacillus spp. were isolated from the intestine of black tiger shrimp *Penaeus monodon* which were collected from the Gulf of Thailand at Chonburi province area during October 2005 to September 2006. The bacterial antagonist activity were tested with aquacultural pathogenic bacteria. The cross streak method results showed that *Bacillus* W803 and *Bacillus* W120 could inhibit *Aeromonas hydrophila* AQAH after 24 hours. The highest level of antibacterial substances of these *Bacillus* were found in 48 hours. However, *Bacillus* W120 could produce antibacterial substances higher than *Bacillus* W803. *Bacillus* W806 and *Bacillus* W902 could colonize *Streptococcus agalactiae* AQST after 48 hours of incubation. Although cross streak method did not show any effect between *Bacillus* spp. and *Vibrio harveyi* AQVH, but Transmission Electron Microscope (TEM) observations showed the size of *V. harveyi* cell at cross streaking point with *Bacillus* WL01 to be smaller compared to normal cell with width and length reduction of 58.54% and 72.07%, respectively. Application of these selected *Bacillus* strains to use for control the pathogenic bacteria were conducted. The amount of *A. hydrophila* AQAH co-cultured in sterile tap water with *Bacillus* W803 and *Bacillus* W120 were decreased by 22.42 and 27.05%, respectively. The amount of *S. agalactiae* AQST co-cultured in sterile tap water with *Bacillus* W806 and *Bacillus* W902 were decreased by 11.98 and 11.97%, respectively. The amount of *V. harveyi* AQVH co-cultured in sterile artificial sea water (20 ppt) with *Bacillus* WL01 and *Bacillus* W1106 were decreased by 22.75 and 20.23%, respectively. Moreover, *Bacillus* spp. could survive in water more than 5 days and could decrease pathogenic bacteria from 10^6 to 10^5 CFU/ml in 24-48 hours. These results suggest that these *Bacillus* spp. can be applied as effective probiotic to control pathogenic bacteria in aquaculture.

Key words: *Bacillus*, *Aeromonas hydrophila*, *Streptococcus agalectiae*, *Vibrio harveyi*, probiotic

INTRODUCTION

Many bacterial diseases have been reported to cause mortality in cultured shrimp and fish both in the hatchery and grow-out ponds. *Aeromonas hydrophila*, a gram negative, short, motile, rod bacterium, causing motile aeromonads septicemia, is the most common freshwater fish pathogen. *Streptococcus agalactiae*, a gram positive, non motile, non spore-forming coccus that occurs in chains or in pairs of cells, is a cause of streptococcal disease in *Oreochromis niloticus*.

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Vibrio harveyi, a luminous gram negative marine bacterium that causes luminescent bacterial disease, is a serious disease problem in shrimp aquaculture. Disease outbreaks are recognized as important constraints to aquaculture production and trade and since the development of antibiotic resistance has become a matter of growing concern. One of the alternatives to antimicrobials in disease control could be the use of probiotic bacteria as microbial control agents (Verschuere *et al*., 2000).

Probiotics such as the gram positive *Bacillus* offers an alternative to antibiotic therapy for sustainable aquaculture. Although many genera of bacteria were used as probiotic in aquaculture such as *Vibrio alginolyticus*, *Pseudomonas fluorescens* and *Alteromonas* sp. (Douillet and Langdon, 1994; Austin *et al*., 1995; Gram *et al*., 2001), *Bacillus* species offer several advantages over gram negative bacteria, including longer shelf life, because they produce endospores that are tolerant to heat and desiccation, and the broad spectrum activities of their secondary metabolites (Kim *et al*., 2001; Jock *et al*., 2002). *In vitro* production of inhibitory compounds toward known pathogens for the considered species has often been used in the selection of putative probiotics (Verschuere *et al*., 2000).

In this study, the potential probionts which were isolated from intestine of wild *Penaeus monodon* were tested by focusing on competitive and inhibitive capabilities against some common pathogenic bacteria in aquaculture including *V. harveyi*, *A. hydrophila* and *S. agalactiae*.

MATERIALS AND METHODS

Isolation and identification of *Bacillus* spp.

Bacillus spp. were isolated from the intestine of *Penaeus monodon* collected from the Gulf of Thailand at Chonburi province. Every month, during October 2005 to September 2006, 10 samples of shrimp (weight > 80 g) were investigated. Intestines were ground by homogenizer and were dissolved in 5 ml of 1.5% NaCl per animal and the diluted 1.5% NaCl were heat shocked on water bath at 80 °C for 20 min followed by cold shock with normal tap water immediately. Then the intestine solution was spreaded on plates using spread plate technique on Nutrient agar (NA) supplemented with 1.5% NaCl (w/v) and incubated at 30 °C for 24 hours. Isolates were purified by streaking on NA supplemented with 1.5% NaCl (w/v). Catalase test were used for identifying *Bacillus* species. Each of the strain was examined with the basic biochemical tests API 20E (bioMârieux). API CHB Medium and API 50 CH strips were used to study the metabolites of 49 different carbon sources. The API strips were incubated at 37°C. The results were read after 24 and 48 hours and analysed with the APILAB Plus software.

Pathogenic bacteria

V. harveyi AQVH was isolated from diseased *P. monodon*, *A. hydrophila* AQAH was isolated from diseased hybrid catfish and *S. agalactiae* AQST was isolated from diseased *Oreochromis niloticus* obtained from Aquatic Animal Health Management Laboratory, Department of Aquaculture, Faculty of Fisheries, Kasetsart University.

Colonization and inhibition activities of *Bacillus* spp. on the pathogenic bacteria

Bacillus spp., *A. hydrophila* AQAH and *S. agalactiae* AQST were cultured on nutrient agar and incubated at 30°C for 24 hours *V. harveyi* AQVH was cultured on NA supplemented with 1.5% NaCl (w/v). Colonization activities tests were done on NA (*A. hydrophila* AQAH and *S. agalactiae* AQST) and NA supplemented with 1.5% NaCl (w/v) (*V. harveyi* AQVH) by cross streak method (Lemos *et al*., 1985). Pathogenic bacteria was streaked in the first line and then *Bacillus* spp. was streaked perpendicular to it. Each

type of bacterium streaking was done in triplicate and they were incubated at 30°C for 96 hours. Inhibition activities and colonization effect were observed at 24, 48, 72 and 96 hours.

Inhibition activities of selected *Bacillus* spp.

This method was modified from Jock *et al*. (2002). *Bacillus* from single colony was transferred to NA plate as three spots per plate and grown for 1 day, 2 days and 3 days at 30°C. *A. hydrophila* AQAH was grown overnight in NB broth and 0.2 ml of the culture of *A. hydrophila* AQAH in NB was mixed with 20 ml NA agar (40-45°C). This suspension was gently poured on top of the agar with the pregrown *Bacillus* isolates. After incubation for 24-48 hours at 30°C, the plates were inspected for growth inhibition zones on the lawn of *A. hydrophila* AQAH. The comparison between size of clear zone in the *Bacillus* spp. plate which were inoculated 1 day, 2 days and 3 days were determined.

Morphological change of *V. harveyi* AQVH after colonization

V. harveyi was isolated from the colonization area, especially from the cross streaking point as well as from the control. All samples were cultured on TCBS agar and incubated at 30°C for 24 hours. Single colony was used to determine morphological deviation by TEM.

Co-culture of *Bacillus* spp. with pathogenic bacteria in sterile tap water and artificial sea water

Bacillus spp. was tested for antagonistic activity against pathogenic bacteria in a co-culture experiment. *Bacillus* and pathogenic bacteria were separately pre-cultured in 10 ml NB (Nutrient Broth) for 24 hours (110 rpm). Sterile tap water were inoculated with 10^5 CFU/ml pathogenic bacteria together with 10^5 CFU/ml of *Bacillus* spp. *Bacillus* W120 and *Bacillus* W803 were tested with *A. hydrophila* AQAH in sterile tap water. *Bacillus* W806 and *Bacillus* W902 were tested with *S. agalactiae* AQST in sterile tap water. *Bacillus* W1106 and *Bacillus* WL01 were tested with *V. harveyi* AQVH in artificial sea water (20 ppt). Each *Bacillus* and pathogenic bacteria had control group (monoculture) for compare the bacterial concentration. Flasks were incubated at room temperature. All combinations were tested in triplicate. Samples were collected after 0, 12, 24, 48, 72, 96 and 120 hours for enumeration of the number of bacteria.

RESULTS

Isolation and identification of *Bacillus* spp.

From 120 samples of *P. monodon*, 114 strains of genus *Bacillus* were isolated for antagonistic studies.

Colonization and inhibition activities of *Bacillus* spp. on the pathogenic bacteria

After incubated for 48 hours, *Bacillus* W120 and *Bacillus* W803 showed inhibition effect against *A. hydrophila* AQAH. On the test plate, some clear zone areas were existed and more colonization areas were observed after 72 hours (Figure 1.1). *Bacillus* W806 and *Bacillus* W902 showed colonization effect against *S. agalactiae* AQST (Figure 1.2). However, there were no effect between *Bacillus* spp. and *V. harveyi* AQVH (Figure 1.3).

Inhibition activities of selected *Bacillus* spp.

Bacillus W120 and *Bacillus* W803 which showed inhibition effect to *A. hydrophila* AQAH from previous experiment were selected. The results showed that *Bacillus* W120 could produce antibacterial substance higher than *Bacillus* W803 and these *Bacillus* spp. could produce antibacterial substances in highest level in 2 days (Figure 2) but thery were not significantly different at p=0.05 from 3 days as measured by size of clear zone (Table 1).

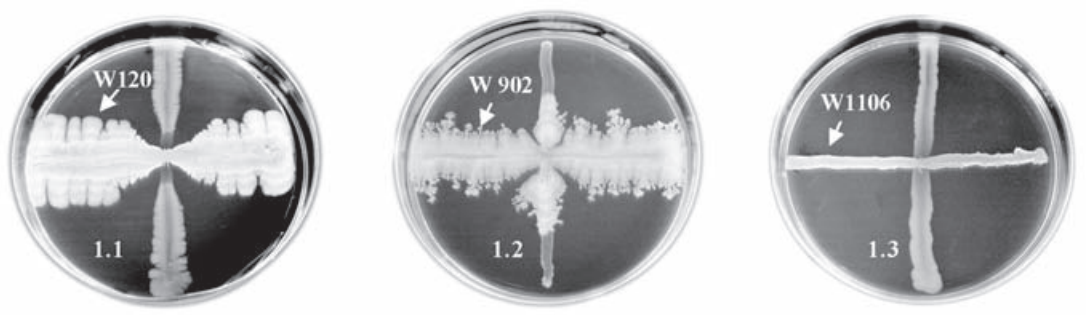

Figure 1 Inhibition and colonization activities of *Bacillus* spp. on pathogenic bacteria *in vitro* at 96 hours.
1.1 inhibition effect of *Bacillus* W120 against *Aeromonas hydrophila* AQAH
1.2 colonization activities of *Bacillus* W902 against *Streptococcus agalectiae* AQST
1.3 no effect between *Bacillus* W1106 and *Vibrio harveyi* AQVH

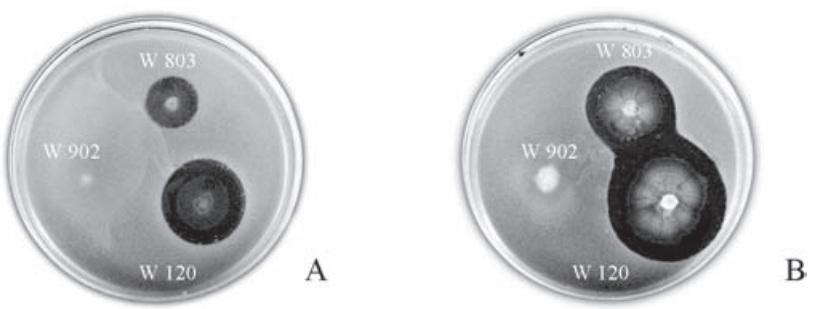

Figure 2 Inhibition zone of *Bacillus* W120 and *Bacillus* W803 against *Aeromonas hydrophila* after incubated *Bacillus* 1 day (A) and after incubated *Bacillus* 2 days (B).

Table 1 Size of clear zone of *Bacillus* W120 and *Bacillus* W803 when pre-cultured *Bacillus* spp. 1, 2 and 3 days showing inhibition effect against *Aeromonas hydrophila*.

	Size of clear zone (mm)		
	1 day	2 days	3 days
Bacillus W120	26.67 ± 1.15^{b}	37.67 ± 0.58^{a}	37.33 ± 0.58^{a}
Bacillus W803	17.00 ± 1.00^{b}	32.00 ± 2.00^{a}	32.67 ± 1.15^{a}

Means values within the same row sharing the same superscript are not significantly different at P = 0.05

Morphological change of *V. harveyi* AQVH after colonization

The cross streaking point between *V. harveyi* and each *Bacillus* spp. on the TCBS agar was isolated for *V. harveyi*. The size of normal *V. harveyi* and those colonized by *Bacillus* WC01, *Bacillus* WBL01, *Bacillus* W1106 and *Bacillus* WL01 were about 0.82 × 1.79 μm, 0.80 × 1.34 μm, 0.67 × 1.21 μm, 0.42 × 0.59 μm and 0.34 × 0.50 μm, respectively as shown in Figure 3. The results indicated that *V. harveyi* colonized by *Bacillus* WL01 showed significant morphological changes.

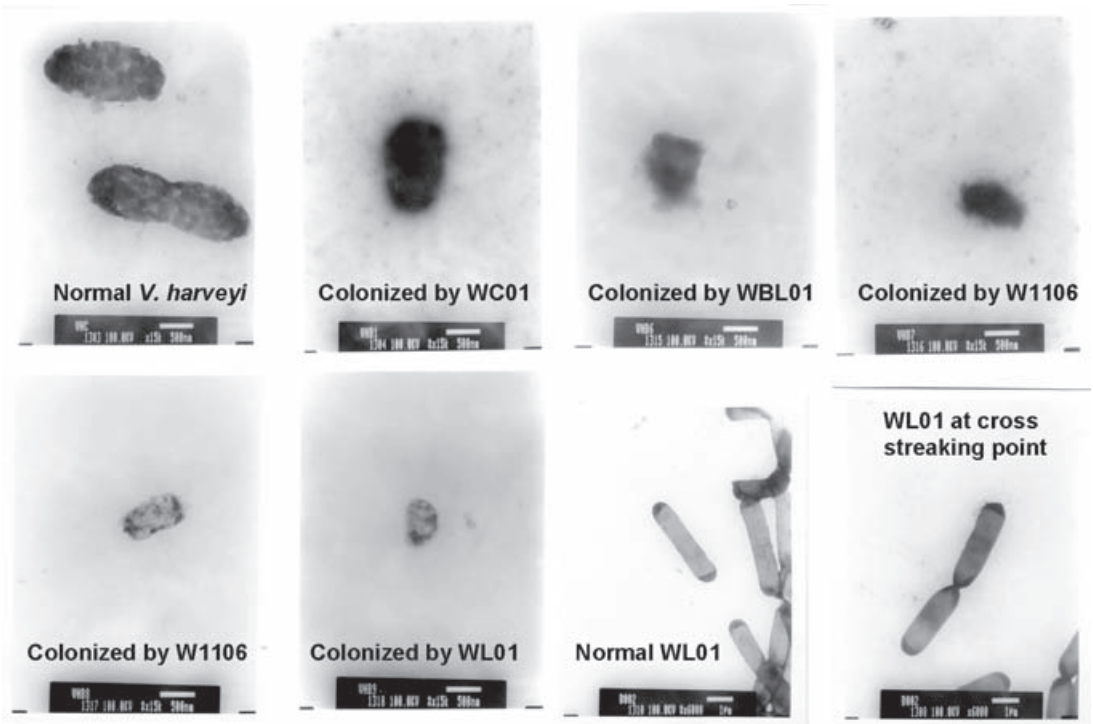

Figure 3 Morphological structure of normal *Vibrio harveyi* and *Bacillus* WL01 compared with those deviated from colonization by TEM.

Co-culture of *Bacillus* spp. with pathogenic bacteria in sterile tap water and artificial sea water

The presence of *Bacillus* W120 and *Bacillus* W803 inhibited growth of *A. hydrophila* AQAH during 48 hours from 4.40×10^6 CFU/ml of control to 9.85×10^5 and 1.99×10^6 CFU/ml, respectively. A further reduction was seen over the following 120 hours, reducing *A. hydrophila* AQAH from 2.77×10^5 CFU/ml to 9.37×10^3 and 1.67×10^4 CFU/ml which counted for 27.05 and 22.42% reduction.

The presence of *Bacillus* W902 and *Bacillus* W806 inhibited growth of *S. agalectiae* AQST during 72 hours from 3.73×10^6 CFU/ml of control to 7.97×10^5 and 2.13×10^6 CFU/ml, respectively. A further reduction was seen over the following 120 hours, reducing *S. agalectiae* AQST from 1.13×10^6 CFU/ml to 2.14×10^5 and 2.13×10^5 CFU/ml which counted for 11.97 and 11.98% reduction.

The presence of *Bacillus* WL01 and *Bacillus* W1106 inhibited growth of *V. harveyi* AQVH during 48 hours from 4.70×10^6 CFU/ml of control to 2.87×10^6 and 2.93×10^6 CFU/ml, respectively. A further reduction was seen over the following 120 hours, reducing *V. harveyi* AQVH from 4.10×10^5 CFU/ml to 2.17×10^4 and 3.00×10^4 CFU/ml which counted for 22.75 and 20.23% reduction. While *Bacillus* spp. concentrations in co-culture treatment did not differ ($P>0.05$) from control treatment (Figure 4).

DISCUSSION

Many researchers are trying to use probiotic bacteria in aquaculture to improve water quality by balancing bacterial population in water and reducing pathogenic bacterial load. Sources of probiotic bacteria may come from animals such

as from intestine (Sugita and Shibuga, 1996) or environments (Nogami and Maeda, 1992). In this study, we focused only genus *Bacillus* sp. which showed antagonistic activity to pathogenic bacteria in aquaculture in many studies (Devaraja *et al*., 2002; Vaseeharan and Ramasamy, 2003). *Bacillus* spp. are commonly found in marine sediments and therefore are naturally ingested by animals such as shrimps that feed in or on the sediments (Moriarty, 1998). So, we isolated *Bacillus* spp. from intestine of *P. monodon* captured from natural habitat and tested for antagonistic activity to pathogenic bacteria in aquaculture.

Bacillus W120 and *Bacillus* W803

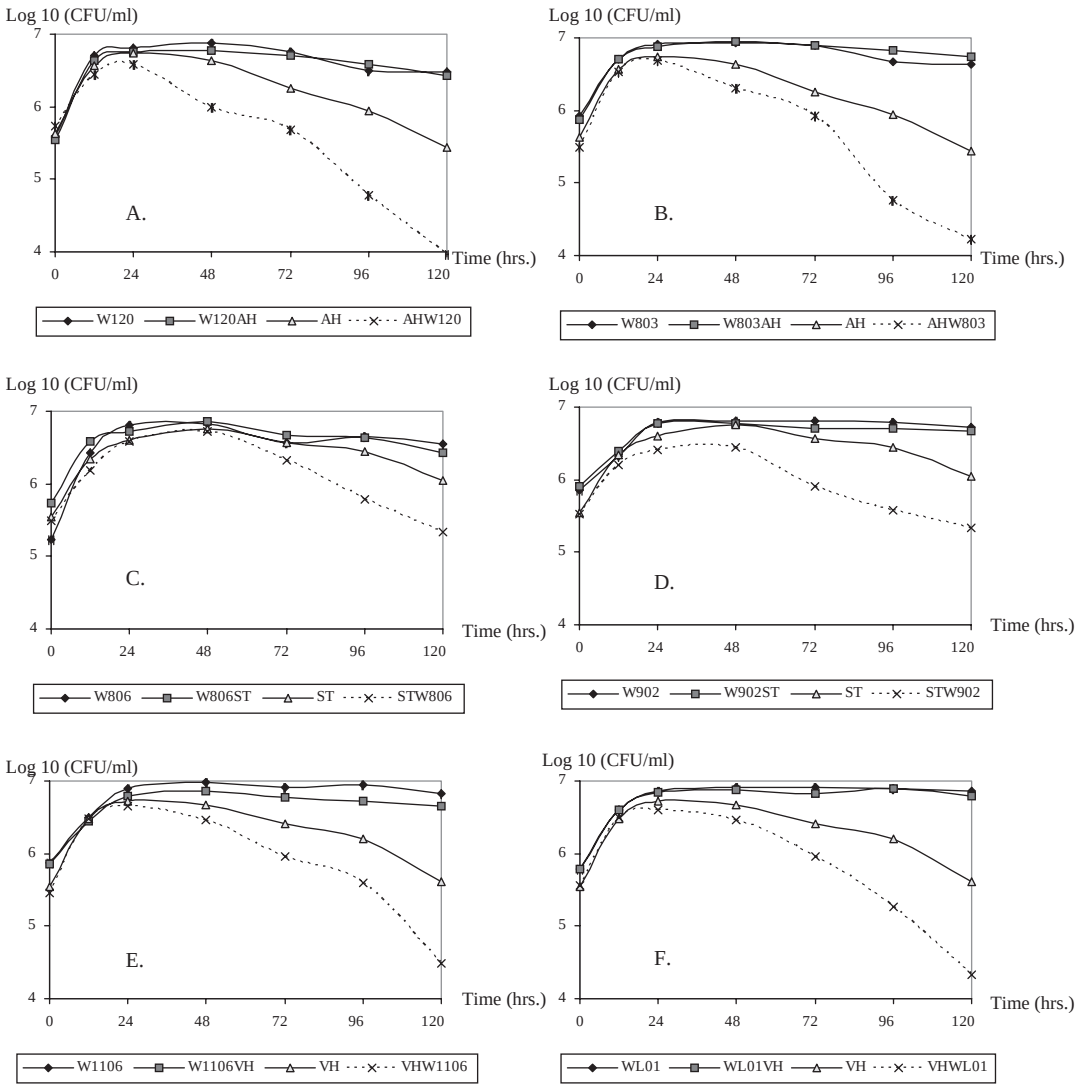

Figure 4 Growth of *Bacillus* spp. and pathogenic bacteria monoculture and co-culture in sterile tap water and artificial sea water.
A. *Bacillus* W120 B. *Bacillus* W803 and *A. hydrophila* AQAH C. *Bacillus* W806
D. *Bacillus* W902 and *S. agalectiae* AQST E. *Bacillus* W1106
F. *Bacillus* WL01 and *V. harveyi* AQVH

showed inhibition effect to *A. hydrophila* AQAH. The antibacterial substance was produced in highest level in 48 hours. This result could explain the reduction of *A. hydrophila* AQAH in co-culture experiment with *Bacillus* W120 and *Bacillus* W803. In 48 hours, *A. hydrophila* AQAH in co-culture was reduced from 10^6 to 10^5 CFU/ml might be caused by high level of antibacterial substance. By using the cross-streaking method, *Bacillus* W806 and *Bacillus* W902 were observed to colonize *S. agalactiae* AQST. But in co-culture experiment, *Bacillus* W806 and *Bacillus* W902 could decrease only 11.97-11.98% of *S. agalactiae* AQST.

Although *Bacillus* WL01 and *Bacillus* W1106 did not show colonization effect but they showed some inhibition effect to *V. harveyi* AQVH, which confirmed by distorted shape of *V. harveyi* AQVH by TEM. The shape of *V. harveyi* AQVH had smaller size and some area of cell wall was destroyed. In this case, *Bacillus* spp. might produce some metabolites, for instance, antibiotic (Williams and Vickers, 1986) or enzymes for inhibition and/or digestion (Bruno and Montville, 1993). However, in co-culture experiment, pathogenic bacteria were used in high level. In general, the number of luminous bacteria in coastal area ranged from 0.7×10^1 to 7.3×10^1 CFU/ml (Sudthongkong, 1996). In freshwater, total bacteria varied from 3.1×10^2 to 1.0×10^3 CFU/ml (de Sousa and Silva-Souza, 2001). In fish pond water, total bacteria ranged from $1.8 \pm 0.9\times10^2$ to $6.0 \pm 1.2\times10^4$ CFU/ml (Al-Harbi, 2003). So, due to the high amount of pathogenic bacteria used with study, the inhibition effect in co-culture experiment might not be that significant.

Many studies supported that *Bacillus* sp. could reduce pathogenic bacteria in aquaculture. Vaseeharan and Ramasamy (2003) reported *P. monodon* immersed in *Bacillus subtilis* BT23 at a density of 10^6-10^8 CFU/ml for 6 days showed 90% reduction in accumulated mortality when challenge with *V. harveyi* at 10^3 - 10^4 CFU/ml for 1 hour. Devaraja *et al*. (2002) used microbial products, *Bacillus* sp., *Saccharomyces* sp., *Nitrosomonas* sp. and *Nitrobacter* sp. in fish and shrimp pond by immersion for 110 days, the results showed that *Bacillus* spp. were dominant in all ponds and the bacterial populations were changed by use this probiotic. In our studies, *Bacillus* sp. in monoculture and co-culture did not decrease during the experiment.

In summary, it has been demonstrated that *Bacillus* W120, *Bacillus* W803 produced substances that could inhibit the growth of the pathogenic bacteria; *Bacillus* W806, *Bacillus* W902, *Bacillus* WL01 and *Bacillus* W1106 showed competitively exclude the pathogenic bacteria. So, the presence of these *Bacillus* spp. could protect the aquatic animals against the infection by pathogenic bacteria and might be applied as good probiotic in aquaculture.

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