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Efficacies of some Beneficial Bacteria on the Colonization and Inhibition of *Vibrio harveyi* in Black Tiger Shrimp (*Penaeus monodon* Fabricius) Larvae

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ABSTRACT

Eight beneficial bacteria from nature were tested for their colonization and inhibition efficacies on *Vibrio harveyi*, the causative agent of luminous disease in black tiger shrimp by cross streaking method *in vitro*. Three types of bacteria showed the best effects against *V. harveyi* especially in terms of causing its mophological deviation. *Nitrosomonas* sp. AM-11 showed the fastest colonization activity followed by *Bacillus licheniformis* AM-04 and *B. subtilis* AM-01, respectively. However, scanning electron microscope (SEM) revealed that *B. subtilis* AM-01 caused size and shape of *V. harveyi* deviated more than others. The virulency of the normal *V. harveyi* was compared with the *V. harveyi* colonized by both types of *Bacillus in vivo*. There were 5 treatments with 3 replications: the first treatment was controlled without *V. harveyi* ; the second treatment was controlled with APS broth but without *V. harveyi* ; the third treatment was the normal *V. harveyi* grown in APS broth ; the fourth treatment was the *V. harveyi* colonized by *B. subtilis* AM-01 grown in APS broth ; the fifth treatment was *V. harveyi* colonized by *B. licheniformis* AM-04 grown in APS broth. Each replication used 15 larvae aged PL15. Results after 48 hours showed that the mortality of black tiger shrimp from normal *V. harveyi* was 60% while the mortality of black tiger shrimp from the abnormal *V. harveyi* colonized by both types of the *Bacillus* equalled 40%. They were significantly different at 95% confidence from the normal *V. harveyi* and the control. Therefore, both types of *Bacillus* could inhibit *V. harveyi* virulency to some extent. These deviated *V. harveyi* were still capable of initiating the disease. Nevertheless, in good aquacultural practice, these colonizing bacteria should be used more than once during rearing period.

Key words: beneficial bacteria, *Vibrio harveyi*, *Penaeus monodon*

INTRODUCTION

Black tiger shrimp *(Peneaus monodon* Fabricius*)* is one of Thailand important export commodity. Recent practice for rearing based on intensive farming which gives high yield but severe risk from several diseases. Among these, bacterial infection is a primary case which should be studied in more detail because there are several reports confirmed that it is quite prevalent with black tiger shrimp. Luminous bacteria causing disease, particularly *Vibrio harveyi*, has been reported as a typical problem obstructing shrimp raising in the ponds (Austin and Austin, 1987; Chanvatchakul, 1999). During 1997-1998 this disease caused heavy loss in shrimp production (Ruangpan, 1998). *V. harveyi* grows well in salt water with the salinity higher than 15 part-per-thousand by feeding on

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organic waste from feed and shrimp excreta accumulated at the bottom of the pond. Black tiger shrimps usually feed at the bottom of the pond, so it is very likely that they may be infected by *V. harveyi* when exposed to the accumulated waste. Therefore, prevention of the disease should be emphasized on good management of water quality and the cleanliness of the bottom of the pond for sustainable aquaculture.

The use of bacteria for waste digestion in the pond is a very popular method in water-quality management. Most common are those of the Bacillus group, and *Bacillus subtilis* is highly efficient in decomposing organic matter and waste with enzymes for extra-cell decomposition (Thanaviriyakul, 1992), followed by nitrifying bacteria such as *Nitrosomonas* sp., *Nitrosococcus* sp. which change ammonia to nitrite, and then changed to nitrate by *Nitrobactor* sp. or *Nitrococcus* sp., a group of phytoplankton feed on nitrate ion (Maketon, 1997). There are also bacteria belonging to the *Thiobacillus* group which turn hydrogen sulphide to sulphate. These beneficial bacteria, apart from digesting wastes, may also assist in controlling the amount of *V. harveyi* by colonization and/or inhibition its growth opportunity (Anantasilpa, 2002).

This research was conducted in two parts, the first was performed *in vitro* in order to see the colonization activities of several known bacteria and also the possibility of morphological deviation of *Vibrio harveyi* after colonization took place. The second part was continued by employing the best two effective colonization bacteria to study their colonization activities *in vivo* using post larvae 15 (PL 15) as a studying object. The mortality rate of the PL 15 after infected with normal *V. harveyi* and two colonized *V. harveyi* were compared with the control group to see whether those well-performed colonization bacteria could increase the shrimp's survival rate.

MATERIALS AND METHODS

1. The colonization activities of eight bacteria on *Vibrio harveyi in vitro*

1.1 The colonization potential

V. harveyi was isolated from black tiger shrimp and streaked on nutrient agar (NA) + 1.5 % NaCl as a straight line as shown in Figure 1. Eight different types of beneficial bacteria, including *Bacillus subtilis* AM-01, *Bacillus licheniformis* AM-04, *Bacillus* sp. AM-14, *Bacillus* sp. AM-3065, *Nitrosomonas* sp. AM-11, *Nitrobactor* sp. AM-12, *Thiobacillus* sp. FW-01 and *Thiobacillus* sp. SW-01 isolated from water samples were obtained from shrimp ponds in Chantaburi and Surattani provinces. Each bacteria was streaked on the same medium by crossing the *V. harveyi.* Each type of the bacteria was done in triplicate and kept at room temperature for 72 hours. Also, only *V. harveyi* streaked on NA+1.5% NaCl was used as a control (Buchanan *et al.*, 1974).

1.2 The morphological change of *V. harveyi* after colonization

Each *V. harveyi* was isolated from the colonization area, especially from the cross-streaking point and *V. harveyi* from the control was also isolated. All samples were cultured in Thiosulfate Citrate Bile Salt Sucrose agar (TCBS agar). After 72 hours, each *V. Harveyi* was inspected for the morphological deviation using Scanning Electron Microscope (SEM) and compared with the control.

1.3 The possibility of *V. harveyi* to return to its normal shape

All *V. harveyi* samples isolated from the cross streaking point were cultured on TCBS agar and then subcultured on TCBS agar every 24 hours for three consecutive times and then each sample was inspected for the morphological change using SEM in order to observe whether it could return to normal shape.

2. The inhibition efficacies of some selected bacteria on *V. harveyi in vivo*

2.1 Quantification of *V. harveyi* colonized by *B. subtilis* AM-01, *B. licheniformis* AM-04, and the control

Each *V. haryeyi* colonized by *B. subtilis* AM-01, *B. licheniformis* AM-04, and the control was cultured in the APS broth, shaked at 250 rpm for 48 hours at 25°C and diluted to the concentration of about 5×10^7 cfu/ml.

2.2 Experimental design using black tiger shrimp post larvae 15 (PL 15)

Three hundred PL 15 were used. These larvae were reared in the brine with salinity of 25 part-per-thousand (ppt) and fed 4 meals per day and the brine was also changed every day, this was performed for three days prior to start the experiment. Completely randomized designed (CRD) was employed by dividing those larvae into five treatments with three replications each as shown in Table 1.

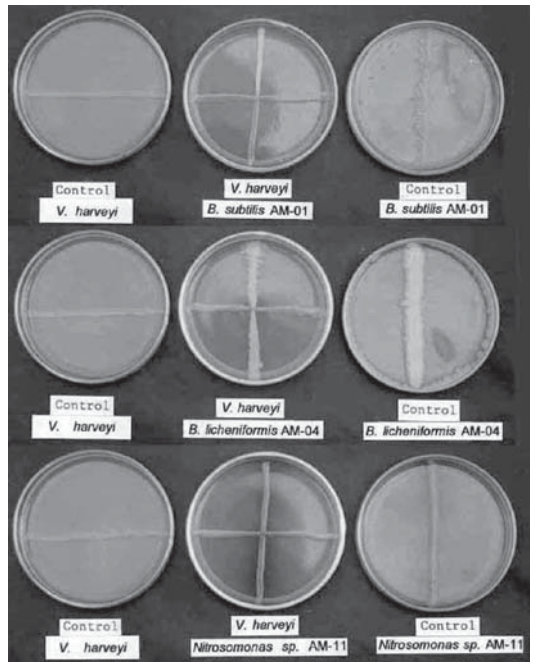
Then those survived larvae were randomly divided and transfered into 15 jars with 15 larvae each, and set with an air hose connected from the pump. The larvae survival rates were recorded every 6 hours till 48 hours. Dead larvae were rinsed with chlorox® 0.1% and followed by distilled water twice then *V. harveyi* was isolated from their digestive tracts and streaked on TCBS agar. The death of larvae was confirmed by the existence of *V. harveyi* in the digestive tract.

RESULTS

1. The colonization activities of eight bacteria on *V. harveyi in vitro*

1.1 The colonization potential

Among eight bacteria, *Nitrosomonas* sp. AM-11 showed superior growth and colonization on *V. harveyi* (Figure 1). It also changed the color of NA from light-brown to greenish-blue which



Figrue 1 Colonization activities of three most effective bacteria on *V. harveyi in vitro.*

Table 1 Treatments of black tiger shrimp post larvae 15 (PL 15).

Treatment	Ingredient	Volume
1	Control (brine)	1,000 ml
2	Brine + APS broth	950+50 ml
3	Brine + suspension of normal *V. harveyi* in APS broth	950+50 ml
4	Brine + suspension of *V. harveyi* colonized by *B. subtilis* AM-01 in APS broth	950+50 ml
5	Brine + suspension of *V. harveyi* colonized by *B.licheniformis* AM-04 in APS broth	950+50 ml

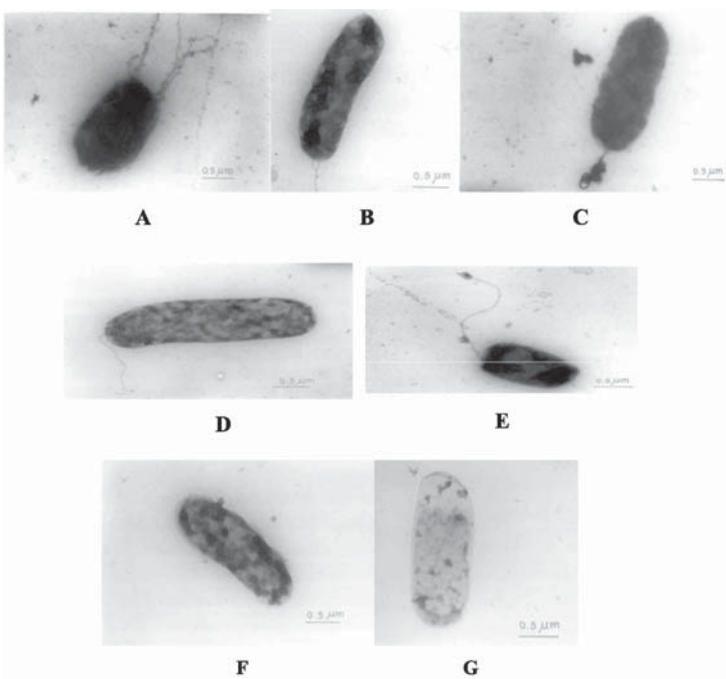
might occur either from some antagonistic metabolites excreted from itself or the metabolites from the *V. harveyi*. While the growth rate and colonization efficiencies of *B. licheniformes* AM-04 and *B. subtilis* AM-01 were slightly lower than *Nitrosomonas* sp. AM-11 as shown in Figure 1, the others five bacteria did not show any good sign of colonization capabilitys.

1.2 The morphological change of *V. harveyi* after colonization

At the cross streaking point between *V. harveyi* and each colonizing bacterium on the TCBS agar was isolated for *V. harveyi* and studied under SEM. Figures 2A, 2B, and 2C show the size of *V. harveyi* colonized by *B. subtilis* AM-01, *Nitrosomonas* sp. AM-11, and *B. licheniformis* AM-04 to be about 0.84×1.63 μm, 0.68×2.19 μm, and 0.90×2.27 μm, respectively. While Figure 2D shows the size of normal *V. harveyi* to be about 0.82×2.94 μm.

1.3 The possibility of *V. harveyi* to return to its normal shape

After three consecutive subcultures those deviated *V. harveyi* colonized from each bacterium on the TCBS agar at every 24 hours confirmed that their morphologies permanently changed compared with the control. Thus, it might not be possible for the colonized *V. harveyi* to return to its regular size and shape again as shown in Figure 2E, 2F, and 2G, respectively.



Figrue 2 Morphological structure of *Vibrio harveyi* by scanning electron microscope.
A. *V. harveyi* colonized by *B. subtilis* AM-01.
B. *V. harveyi* colonized by *Nitrosomonas* sp. AM-11.
C. *V. harveyi* colonized by *B. licheniformis* AM-04.
D. Normal *V. harveyi*.
E. *V. harveyi* colonized by *B. subtilis* AM-01 after three consecutive subcultures.
F. V. harveyi colonized by *Nitrosomonas* sp. AM-11 after three consecutive subcultures.
G. V. harveyi colonized by *B. licheniformis* AM-04 after three consecutive subcultures.

2. The inhibition efficacies of some selected bacteria on *V. harveyi in vivo*

Even if the *Nitrosomonas* sp. AM-11 showed the fastest growth and colonization activity on *V. harveyi*, its limitation in either large scale production or utilization in shrimp rearing was not practical due to the fact that it was a non-spore forming bacteria thus it had a very short shelf-life. Therefore, only two types of spore forming bacteria, *B. subtilis* AM-01 and *B. licheniformis* AM-04 were further studied.

Table 2 shows the percent mortalities of shrimp larvae PL 15 in each treatment after treated with *V. harveyi* as compared with the control. Results showed that in treatment No. 3 which was treated with normal *V. harveyi*, the percent mortality of the PL 15 after 6, 12, 18, 24, 30, 36, 42, and 48 hours were 22.2, 31.1, 35.6, 37.7, 48.9, 48.9, 51.1, and 60.0, respectively. In treatment No. 4 which was treated with *V. harveyi* colonized by *B. subtilis* AM-01 the percent mortality of PL 15 were 11.1, 11.1, 17.8, 20.0, 28.9, 28.9, 31.1, and 40.0 respectively. And treatment No. 5 which was treated with *V. harveyi* colonized by *B. licheniformis* AM-04 the percent mortality of the PL 15 were 13.3, 13.3, 17.7, 26.6, 31.1, 31.1, 31.1, and 40.0 respectively, while, there was neither mortality in treatment No.1 nor No.2. The percent mortality of PL 15 in treatment No.3 was significantly different from treatments No.1, No.2, No.4 and No.5, while the percent mortalities of treatments No.4 and No.5 were the same but significantly different from No.1, No.2 and No.3 at $P \leq 0.05$ confidence interval.

Figure 3 shows the increasing of percent mortality through time. After twelve hours, the mortality rate of PL 15 in treatment No.3 increased rapidly because of the infection from normal *V. harveyi* while the mortality rates of PL 15 in treatment No.4 and No.5. were still in the similar trend as in the previous six hours. However, after eighteen hours the percent mortality of each treatment increased tremendously especially in treatment No.3 while the increasing rates were slower in treatment No.4 and No.5.

DISCUSSION

After studying for the capabilities of eight types of bacteria, namely *Bacillus subtilis* AM-01, *Bacillus licheniformis* AM-04, *Bacillus* sp. AM-14, *Bacillus* sp. AM-3065, *Nitrosomonas* sp. AM-11, *Nitrobactor* sp. AM-12, *Thiobacillus* sp. FW-01 and *Thiobacillus* sp. SW-01, in colonizing *Vibrio harveyi*, it was found that after 72 hours of competition and colonization activities on the nutrient agar media, the colony of *Nitrosomonas* sp. AM-11 showed the best growth rate and clearly dominated the colony of *V. harveyi*. It also changed the color of NA media to greenish blue, while *B. licheniformis* AM-04 and *B. subtilis* AM-01 showed slower effects. However, in terms of

Table 2 The percentmortalities of PL 15 after treated with *V. harveyi.*

Treatment	Hours							
	6	12	18	24	30	36	42	48
T1	0.0 c[1/]	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c
T2	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c
T3	22.2 a	31.1 a	35.6 a	37.7 a	48.9 a	48.9 a	51.1 a	60.0 a
T4	11.1 b	11.1 b	17.8 b	20.0 b	28.9 b	28.9 b	31.1 b	40.0 b
T5	13.3 b	13.3 b	17.7 b	26.6 b	31.1 b	31.1 b	31.1 b	40.0 b

Remark : There is no significant difference of mean followed by the same letter at $p \leq 0.05$ confidence interval.

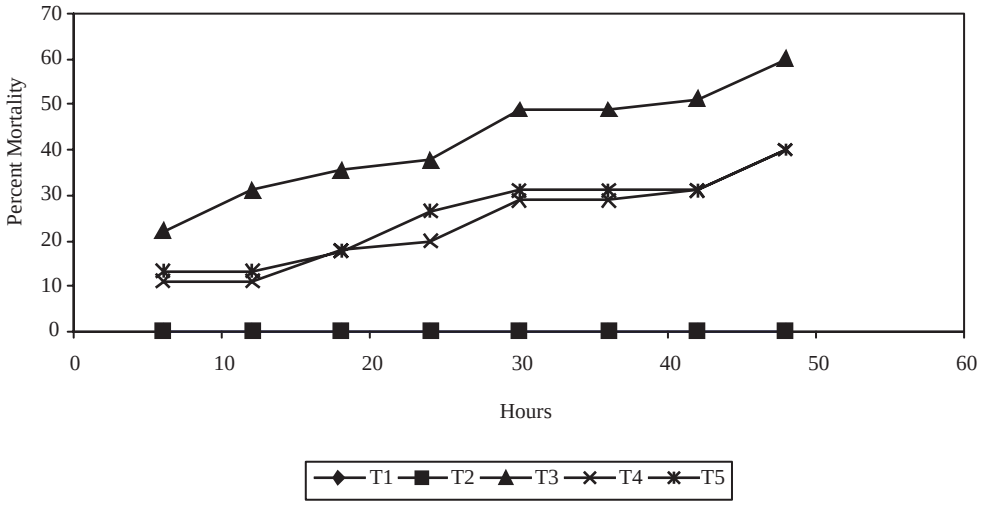


Figure 3 Percent mortalities of larvae PL 15 in five treatments after treated with *Vibryo harveyi* through time.

morphological change of *V. harveyi* from SEM studies showed that *B. subtilis* AM-01 was the most effective, followed by *Nitrosomonas* sp. AM-11 and *B. licheniformis* AM-04, by turning it into a shorter and rounder shape. The mechanism of this shrinking process is not known yet. There might be several reasons, one reason might be from some metabolites produced by those bacilli (Colin, 1989) which should be investigated in more detail in the future.

With regard to the capability of returning to its normal shape, the morphology of *V. harveyi* colonized by these three bacterium changed permanently, with no likelihood of returning to its normal condition despite three successive subcultures.

However, due to the limitation of *Nitrosomonas* sp. AM-11 for future scale up study, it was omitted from *in vivo* tested as mentioned earier, only two promissing bacteria which were *B. subtilis* AM-01 and *B. licheniformis* AM-04 were tested in the second part.

In the second part, the black tiger shrimp larvae PL 15 were tested for their mortality rate from those *V. harveyi* colonized by *B. subtilis* AM-01, *B. licheniformis* AM-04, and the normal *V. harveyi* compared with controls in order to confirm the results of the *Bacillus* inhibition efficacies against *V. harveyi in vitro*. The results revealed that both *B. subtilis* AM-01 and *B. licheniformis* AM-04 showed some promising efficacies, which were similar to the report of Rungsri (2002). However, the percent mortality of shrimp might be lower if these *Bacillus* were applied more than once or they could be studied as probiotic by blending with shrimp feed in order to colonize in shrimp's digestive tract. Therefore, this hypothesis should be further investigated in more detail because it will benefit shrimp industry in terms of avoiding the over uses of antibiotic which causes environmental contamination.

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