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A holistic approach for selection of *Bacillus* spp. as a bioremediator for shrimp postlarvae culture

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Abstract: Indigenous *Bacillus pumilus*, *B. licheniformis*, and *B. subtilis* were isolated from marine water and soil samples and investigated for potential bioremediation ability in *Penaeus monodon* culture. *Bacillus* spp. were selected based on their wide range of growth conditions, ease of mass culture, tolerance to total ammonia nitrogen (TAN), inhibition of pathogenic vibrios, nonpathogenicity, and ability to reduce TAN. Results showed that optimum growth of the selected *Bacillus* spp. occurred at 30 °C, pH 7.5, and 1.5% NaCl, and they secreted protease, amylase, and lipase. *Vibrio* spp. were also inhibited by 3 *Bacillus* spp. In addition, the selected *Bacillus* spp. had no pathogenic effect on shrimp postlarvae (PL) and were able to reduce TAN. They promoted better growth and survival in shrimp PL without water exchange. This study was a systematic approach undertaken for the selection of suitable *Bacillus* spp. as bioremediators for a *Penaeus monodon* culture system.

Key words: *Bacillus*, bioremediation, *Vibrio* spp., *Penaeus monodon*, shrimp postlarvae

1. Introduction

The rapid expansion of shrimp culture has resulted in huge demand for shrimp postlarvae (PL). This has led to the intensification of PL production in hatcheries. Water quality in hatcheries plays an important role in health, survival, and growth in PL production. The water quality rapidly deteriorates due to nitrogenous compounds such as ammonia and nitrite, which result from excess feed and excretory products (1). Water exchange is used to maintain good water quality during the larval rearing period. Studies in hatcheries by Soundarapandian and Babu (2) have shown that survival of *Penaeus monodon* (PL15) in control tanks was 30% with water exchange beginning from mysis III stage. Randrianarivelo et al. (3) reported a survival rate of 15% for *P. monodon* (PL8) with a water exchange average of 50%. However, frequent water exchange to overcome poor water quality problems in tanks is laborious and expensive (4), and it may increase the risk of introducing disease-causing agents. Environmental problems often lead to shrimp becoming more vulnerable to ubiquitous pathogens (5). The resulting disease outbreaks have caused severe economic losses and other socioeconomic fallout in several Asian countries (6,7). Indiscriminate use of antibiotics for disease prevention and treatment has led to the emergence of resistant strains of bacteria, which negatively impact the health of PL and the environment. Thus, for the sustainability of the shrimp industry, it is essential that basic problems associated with hatchery practices be managed using environmentally friendly approaches (8,9).

There is increasing evidence that the use of microorganisms such as *Bacillus* spp. can play an important role in improving water quality (8,10) and increasing the growth and survival of *Penaeus monodon* (11). Rapid degradation of accumulated organic wastes and overall improvement in the health and yield of cultured organisms have been recorded upon introducing the active cells of selected microorganisms into ponds (12–14). This external seeding of useful microorganisms, popularly known as bioremediation, is gaining impetus, especially in environmental management. Bioremediation is a process of reducing hazardous organic wastes to environmentally safe levels through the use of micro- or macroorganisms (15). However, Wang et al. (16) believed

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that *B. subtilis* was the cause of nonfatal bacterial white spots in adult shrimp, which may have resulted from regular use of microbial consortia containing nonnative *Bacillus* species. Disadvantages associated with the use of an introduced microbial consortium are the inability of seeded microorganisms to adapt to the local environment, possible introduction of new pathogens, negative impact on the local ecosystem, and higher cost (17).

In Malaysia, there are several imported microbial consortia used with the intention of improving water quality and fish or shellfish production (5,9,18,19). However, only a few well-documented studies concerning the culture of fish and shrimp using an imported microbial consortium appear in the literature, and there have been conflicting reports on its efficacy (13). Therefore, the usefulness of microbial consortia has always been contentious (5,14,18). Hence, it is necessary to scientifically develop a microbial consortium comprising indigenous microorganisms with proven efficacy in aquaculture. The aim of the present study was to investigate indigenous isolates of *Bacillus* spp. for use as bioremediators in shrimp culture.

2. Materials and methods

2.1. Isolation and identification of *Bacillus* spp.

Brackish water sediment samples were collected in sterile polythene bags along the west coast of peninsular Malaysia (3°17′N, 101°17′E) and transported on ice to the Aquatic Animal Health Unit, Universiti Putra, Malaysia, for further analysis. Samples were subjected to bacterial isolation using physiological saline (0.85% sodium chloride, NaCl; Merck, Germany) on tryptic soy agar (TSA; Merck) plates supplemented with 1.5% NaCl (Merck). Inoculated plates were incubated at 30 °C for 24–48 h, and randomly selected colonies were purified on TSA plates and subjected to Gram staining. Cultures of gram-positive rod-shaped cells were incubated on TSA plates for 5 days, allowing for the formation of endospores (20). Endospore-forming isolates were grown in tryptic soy broth (TSB; Merck) supplemented with 1.5% NaCl and identified to the species level using biochemical tests (21) and an API CH kit (BioMerieux, France). In addition, random amplification of polymorphic DNA (RAPD) was carried out using the Ready-To-Go RAPD analysis kit (Amersham Pharmacia Biotech Inc., USA) to check for species variation among *Bacillus* isolates (22).

2.2. Temperature, pH, and NaCl concentration

Bacillus spp. were grown separately in TSB at varying temperatures (0–65 °C at 5 °C intervals; 1.5% NaCl and pH 7.5), pH levels (2–10 at 0.5 unit pH intervals; 1.5% NaCl and 30 °C), and NaCl concentrations (0%–10% at 1% intervals; pH 7.5 and 30 °C). The optimum growth conditions were determined by measuring cell numbers (cfu mL^{-1}) by spread plate technique on TSA plates (23,24) and optical density (600 nm) using a spectrophotometer (Shimadzu UV-VIS 1601, Japan) (25).

2.3. Extracellular enzyme secretion

Bacillus spp. were examined for secretion of protease, amylase, and lipase, and the diameter of the clear zone (mm) formed on respective substrate plates was measured (26).

2.4. Tolerance towards total ammonia nitrogen

Isolates were tested for their ability to tolerate different levels of total ammonia nitrogen (TAN) (0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 15.0, 20.0, and 25.0 mg L^{-1}). A stock solution of ammonia was prepared by dissolving 9.43 g of dehydrated ammonium sulfate, $(NH_4)_2SO_4$, in 1000 mL of deionized water (27). Each dilution was prepared in 200-mL volumes into which 3.0% TSB and 1.5% NaCl were added. The mixture was distributed into 10-mL test tubes with 5 replicates each. To serve as the control (zero concentration of TAN), 5 test tubes containing 10 mL of deionized water with 3.0% TSB and 1.5% NaCl were prepared. A loopful of overnight culture of each *Bacillus* isolate grown on TSA was inoculated into each sterilized test tube separately. All inoculated test tubes were incubated at 30 °C for 24–48 h. Growth was measured in the form of cell density by spread plate technique on TSA plates (23).

2.5. Inhibition of pathogenic vibrios

Vibrio spp. are of particular concern due to their ubiquitous nature and frequent occurrence in marine aquaculture (28). Therefore, the competitive inhibition/exclusion ability of *Bacillus* spp. in the presence of pathogenic vibrios was tested by disk diffusion method (29), perpendicular streak plate method (30), and viable count of vibrios in mixed culture. Isolates showing an inhibition zone of at least 5.0 mm were considered inhibitory to *Vibrio* spp. (31). The disk diffusion method was also tried using various combinations of 3 isolates in different combinations with a 0.5:1:1.5 volume ratio to test for inhibition properties against vibrios.

Inhibition or exclusion properties of the bacterial isolates were assessed by grading the inhibition as minimal (+), moderate (++), or heavy (+++) at the point of intersection.

2.6. Confirmation of nonpathogenicity to shrimp postlarvae

P. monodon PL (PL17) were bought from a local hatchery and acclimatized for 12 days. Three treatments, *B. pumilus*, *B. subtilis*, and *B. licheniformis*, and a control (with no bacterial inoculum) were evaluated for pathogenicity to PL in triplicates for a period of 14 days. Rectangular glass aquaria (10 L) containing 6 L of filtered (0.5 μm), chlorinated, and autoclaved seawater were stocked with 50 PL L^{-1}. An air compressor was used to provide constant aeration to each aquarium. Shrimp were fed with

micropelleted commercial feed (Grobest, Thailand) ad libitum. *Bacillus* spp. (10^6 cfu mL^{-1}) (32) were separately added to the respective treatment tanks. Temperature, salinity, pH, and total plate count were measured on days 1, 7, and 14. On day 8, the treatment tanks were supplemented with *Bacillus* spp. to restore the initial load of bacteria. Mortality of shrimp PL was recorded up to day 14. PL were observed for any abnormal behavior or external morphological changes during the experiment. From each tank, 5 PL were observed on day 14 under a light microscope (Nikon Eclipse E600, Japan) for any external changes. External body surfaces such as antennae, antennal scales, pleopods, pereopods, uropods, rostrums, carapaces, and muscles were observed for abnormalities as these are susceptible to bacterial infections (33). PL in control tanks were compared with the treated PL. The experiment was carried out without water exchange.

2.7. Reduction of TAN by indigenous *Bacillus* spp.

To evaluate their ability to reduce TAN, 3 microbial consortia purchased from the local supplier were compared with 3 indigenous *Bacillus* spp. For this experiment, 21 glass aquaria (50 L) were used in triplicate. To simulate ammonia production, sediment and water were brought from a 90-day-old shrimp pond located at Kuala Selangor, Malaysia (3°17′N, 101°17′E). All tanks were laid with 8 kg $tank^{-1}$ of brownish black sediment. The tanks were then filled with 20 L of shrimp pond water and left undisturbed for 3 days. The treatment tanks received their respective bacterial (*B. pumilus*, *B. subtilis*, and *B. licheniformis*) inoculum (10^6 cfu mL^{-1}) and 3 microbial consortia (P1, P2, and P3) separately, and control tanks received an equal volume of seawater. The experiment was conducted for 20 days, during which booster doses (10^6 cfu mL^{-1}) of *Bacillus* spp. were added on days 7 and 14 and commercial microbial products were added on a weekly basis into each of the treatment tanks at a concentration of 10 mg L^{-1} as recommended by the manufacturer. Temperature, salinity, pH, and dissolved oxygen in all tanks were measured once every 3 days using a multiparameter system (YSI 556 MPS, YSI Life Sciences, USA). Total ammonia nitrogen was analyzed once every 3 days following the method of Parsons et al. (27). For bacteriological analysis, water samples were collected aseptically from all aquaria. Bacteria were enumerated by plating suitably diluted samples on TSA (Merck) plates with 2% NaCl (Merck) by spread plate technique.

2.8. Use of *Bacillus pumilus* in rearing *Penaeus monodon* postlarvae

Based on the preliminary optimum performance of ammonia reduction, enzyme secretion, and ability to inhibit vibrios, *B. pumilus* was selected for the experiment. The experiment consisted of *B. pumilus* alone and a control with water containing no bacteria. All treatments were performed in triplicate. For this experiment, 6 rectangular glass aquaria (200 L), each containing 170 L of filtered (mesh size: 0.5 µm) and chlorinated seawater (28 ppt), were used. *B. pumilus* (10^6 cfu mL^{-1} in culture tanks) was introduced into the experimental tanks 1 day before the tanks were stocked with PL (28). *P. monodon* PL at stage 1 (PL1) were bought from a commercial hatchery and stocked in glass aquaria at a density of 50 PL1 L^{-1}. The concentration of *Bacillus* sp. was measured in the form of cell density by spread plate technique on TSA plates; based on the result, fresh culture was introduced into the treatment tanks every 3 days to maintain a sufficient concentration of *B. pumilus*. An air compressor was used to provide constant aeration to each aquarium. The same amount of commercial shrimp feed (Feed No. 1-3; Higashimaru, Japan) and *Artemia* (Golden Dolphin, Malaysia) was fed to the PL in all tanks 6 times a day. The experiment was carried out in a static system without water exchange and terminated after 18 days (PL18 stage). Temperature, salinity, pH, and dissolved oxygen levels in the culture tanks were measured daily using the YSI 556 MPS. TAN was analyzed on alternate days following the method of Parsons et al. (27).

2.9. Statistical analysis

Means of *Bacillus* cell numbers, diameters in millimeters of the clear zones, and viable cell numbers of vibrios were statistically analyzed using 2-way ANOVA ($P < 0.05$). To find significant differences in survival between the control and treated PL groups, 1-way ANOVA was performed, and 2-way ANOVA and Duncan's multiple range critical tests were used to determine the significant differences ($P < 0.05$) in TAN concentrations among the treatment and control tanks (34).

3. Results

3.1. Identification of *Bacillus* spp.

From 358 colonies randomly selected for Gram staining, 67 were gram-positive. Out of these 67 isolates, 29 were endospore-forming, short to long rods. Species identification using API kits revealed the following isolates: *B. pumilus* (99.2% test efficiency), *B. subtilis* (96.2% test efficiency), and *B. licheniformis* (97.3% test efficiency). RAPD reaction clearly distinguished the isolates as different species. Based on optimum growth parameters, 3 species were selected for further study.

3.2. Temperature, pH, NaCl concentration, and extracellular enzyme secretion

The optimum growth of selected *Bacillus* spp. occurred at 30 °C, pH 7.5, and 1.5% NaCl based on a mean cell density of $(4.9 \pm 0.6) \times 10^9$ cells mL^{-1}. As indicated by different inhibition zones, 3 *Bacillus* spp. were found to secrete varying quantities of extracellular enzymes (Table 1).

Table 1. Mean ± standard error of clear zone (mm) formed by extracellular enzymes secreted from *Bacillus* spp.

Species	Protease	Amylase	Lipase
B. pumilus AB58	[a]19.0 ± 2.0	[b]21.0 ± 3.0	[a]20.0 ± 3.0
B. subtilis AB65	[a]23.0 ± 4.0	[a]31.0 ± 5.0	[a]21.0 ± 4.0
B. licheniformis AB69	[b]14.0 ± 3.0	[b]22.0 ± 5.0	[a]22.0 ± 4.0

Means with different superscripts in columns are significantly different ($P < 0.05$); n = 6.

3.3. Tolerance towards total ammonia nitrogen

The selected *Bacillus* spp. showed growth at all levels of TAN except the 25 mg L^{-1} concentration. Cell numbers of *B. pumilus* at 15 mg L^{-1} TAN were significantly higher than in the other 2 species ($P < 0.05$) (Table 2).

3.4. Inhibition of pathogenic *Vibrio* spp.

In general, *B. pumilus* and *B. licheniformis* showed higher inhibition ability than *B. subtilis* against the pathogenic vibrios (Table 3). All combinations of the 3 selected isolates showed significantly higher ($P < 0.05$) inhibition against pathogenic vibrios than the isolates singly, indicating the advantage of using them in consortium (Table 4).

3.5. Nonpathogenicity test of *Bacillus* spp. to shrimp postlarvae

Temperature (26.0–28.0 °C), salinity (29.0–29.5 ppt), and pH (7.0–7.5) in all tanks were in the optimum range for shrimp PL growth and there was no significant difference ($P > 0.05$) among treatments. All treated and control PL observed under the microscope did not show any morphological changes or abnormalities on their external surfaces. Muscles, body appendages, and other body parts of the shrimp PL from the treatment tanks were normal. Shrimp PL exhibited normal swimming and feeding patterns during the experiment. PL survival (%) in tanks treated with *B. pumilus* was the highest (84.0 ± 7.0), followed by *B. licheniformis* (81.0 ± 6.0) and *B. subtilis* (78.0 ± 4.0), and then the control (76.0 ± 4.0). There was no significant difference among treatments ($P > 0.05$).

3.6. Reduction of total ammonia nitrogen by indigenous *Bacillus* spp. in comparison to commercial microbial consortia

No significant differences ($P > 0.05$) were detected in the water with regards to salinity (ppt), dissolved oxygen (mg L^{-1}), and temperature (°C) between the treatments (28.0 ±

Table 2. Mean ± SE of cell density (cfu mL^{-1}) of *Bacillus* spp. after 18 h of incubation in different concentrations of total ammonia nitrogen.

Total ammonia nitrogen (mg L^{-1})	*B. pumilus* AB58	*B. subtilis* AB65	*B. licheniformis* AB69
*0.0	$5.4 \times 10^9 \pm 0.7$	$3.9 \times 10^9 \pm 0.5$	$5.2 \times 10^9 \pm 0.5$
*0.05	$5.5 \times 10^9 \pm 0.7$	$4.3 \times 10^9 \pm 1.1$	$5.1 \times 10^9 \pm 0.9$
*0.1	$5.9 \times 10^9 \pm 0.5$	$3.6 \times 10^9 \pm 0.5$	$5.3 \times 10^9 \pm 0.7$
*0.5	$5.7 \times 10^9 \pm 0.8$	$3.7 \times 10^9 \pm 0.6$	$4.4 \times 10^9 \pm 1.1$
*1.0	$4.9 \times 10^9 \pm 0.9$	$3.8 \times 10^9 \pm 0.9$	$5.2 \times 10^9 \pm 1.1$
*5.0	$5.5 \times 10^9 \pm 0.8$	$3.3 \times 10^9 \pm 0.7$	$4.9 \times 10^9 \pm 0.7$
**10.0	[##]$5.5 \times 10^8 \pm 0.5$	$4.4 \times 10^8 \pm 0.4$	$3.9 \times 10^8 \pm 1.1$
**15.0	[a,##]$5.5 \times 10^8 \pm 1.0$	[#]$3.5 \times 10^7 \pm 0.9$	[#]$5.6 \times 10^7 \pm 0.9$
***20.0	[#]$4.3 \times 10^7 \pm 0.8$	[#]$4.8 \times 10^7 \pm 0.6$	[#]$5.7 \times 10^7 \pm 0.6$
25.0	NG	NG	NG

Means of cell numbers with superscript [a] are significantly different from others in the same row ($P < 0.05$). Means of cell numbers with similar asterisks are not significantly different from the others in the same columns ($P > 0.05$). Means of cell numbers with # and ## are significantly different from others in the same columns ($P < 0.05$). NG = no growth.

Table 3. Mean ± SE of inhibition zones (mm) formed by disk diffusion method using selected *Bacillus* spp. against pathogenic *Vibrio* spp.

Vibrio isolates	*Bacillus* isolates		
	B. pumilus AB58	*B. subtilis* AB65	*B. licheniformis* AB69
V. alginolyticus M11	*20.0 ± 1.0	a,*6.0 ± 1.0	*20.0 ± 2.0
V. alginolyticus M12	*20.0 ± 1.0	a,*7.0 ± 1.0	*16.0 ± 1.0
V. parahaemolyticus M1	**5.0 ± 1.0	*8.0 ± 1.0	**10.0 ± 2.0
V. parahaemolyticus M3	**6.0 ± 1.0	*9.0 ± 2.0	**10.0 ± 1.0
V. parahaemolyticus M6	**9.0 ± 1.0	*10.0 ± 1.0	**10.0 ± 2.0
V. alginolyticus Th	*18.0 ± 2.0	a,*9.0 ± 1.0	*17.0 ± 2.0
V. parahaemolyticus Th	**11.0 ± 1.0	*12.0 ± 1.0	**13.0 ± 2.0
V. harveyi Id	a,*24.0 ± 2.0	**20.0 ± 3.0	*19.0 ± 2.0
V. parahaemolyticus Id	**13.0 ± 1.0	*8.0 ± 1.0	**11.0 ± 1.0

Mean values with superscript [a] are significantly different from others in row ($P < 0.05$) and mean values with * and ** are significantly different from others in column ($P < 0.05$).

0.3; 5.1 ± 0.0; 28.7 ± 0.5, respectively) and the control (28.2 ± 0.1; 5.5 ± 0.0; 29.0 ± 0.5, respectively). All treated tanks containing *Bacillus* spp. showed a significantly higher ($P < 0.05$) percent reduction of TAN compared to the imported commercial microbial consortium and the control tanks (Figure 1).

3.7. Use of *Bacillus pumilus* in rearing *Penaeus monodon* postlarvae

There was no significant difference in dissolved oxygen, pH, salinity, and water temperature among the treatment and the control tanks. TAN was significantly lower ($P < 0.05$) in the tank containing *B. pumilus* compared to the control (Figure 2). In addition, significantly higher ($P < 0.05$) survival and specific growth rate (SGR) were observed among PL reared in tanks containing *B. pumilus* (Figure 3).

4. Discussion

The application of bioremediation agents is one of the current approaches to improving water quality in aquaculture. There are definite selection steps as bioremediation organisms need to adapt to different host species and environments. In the present study, the criteria for selecting high-performing bioremediators were based on range of growth conditions, ease of mass culture, tolerance to TAN, inhibition of pathogenic vibrios, nonpathogenicity, ability to reduce TAN and promote

Table 4. Inhibition zones (diameter in mm) formed in disk diffusion method by combination of *Bacillus* isolates against the pathogenic *Vibrio* spp.

Vibrio isolates	*Bacillus* consortium ®					
	Bp:Bs:Bl	Bp:Bl:Bs	Bs:Bp:Bl	Bs:Bl:Bp	Bl:Bp:Bs	Bl:Bs:Bp
V. alginolyticus	*19.0 ± 2.0	*19.0 ± 2.0	*20.0 ± 1.0	a,*24.0 ± 1.0	a,*25.0 ± 1.0	a,*25.0 ± 2.0
V. alginolyticus	*20.0 ± 1.0	*19.0 ± 1.0	*22.0 ± 3.0	a,*25.0 ± 1.0	a,*24.0 ± 2.0	a,*24.0 ± 1.0
V. parahaemolyticus	**15.0 ± 1.0	**12.0 ± 1.0	**14.0 ± 2.0	**13.0 ± 2.0	**12.0 ± 2.0	**15.0 ± 1.0
V. parahaemolyticus	**14.0 ± 2.0	**13.0 ± 1.0	**14.0 ± 2.0	**11.0 ± 1.0	**16.0 ± 2.0	**16.0 ± 2.0

All values represent mean ± standard error; mean values with superscript [a] are significantly different from others in row ($P < 0.05$), and mean values with * and ** are significantly different from others in column ($P < 0.05$). Bp: *Bacillus pumilus,* Bl: *Bacillus licheniformis,* Bs: *Bacillus subtilis*

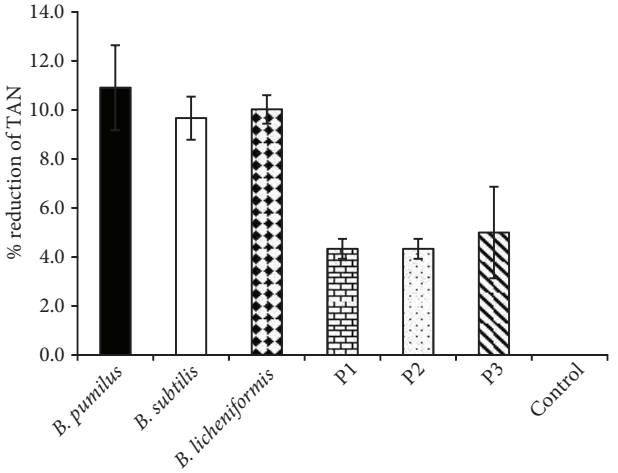

Figure 1. Percentage difference of TAN reduction among the 3 indigenous *Bacillus* spp., introduced microbial consortia, and control tanks during the experiment period.

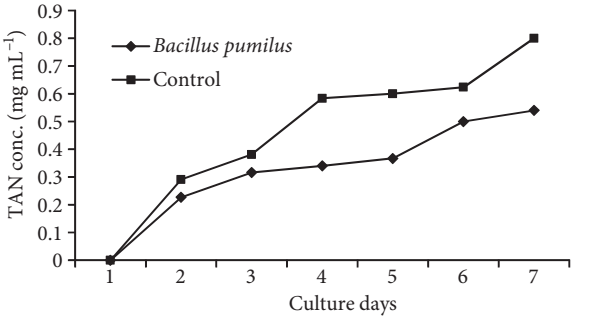

Figure 2. TAN concentration in *Bacillus pumilus* and control tanks during the experimental period. Error bars show standard error; n = 3 tanks.

better growth, and PL survival; all these criteria were tested in a holistic approach.

Salinity in Malaysian shrimp aquaculture normally ranges from 24 to 30 ppt, but sometimes increases up to 36 ppt (5,19). *B. pumilus*, *B. subtilis*, and *B. licheniformis* from marine environments had an inherent versatility as they are able to grow at a wide range of temperatures and pH, salinity, and ammonia levels. These characteristics make them useful bioremediating agents in shrimp culture systems. In addition, all *Bacillus* spp. used in this study secreted major extracellular enzymes such as protease, amylase, and lipase (35), which initiate the process of organic matter degradation in aquatic ecosystems (36).

Use of microorganisms to control pathogens is gaining greater attention in aquaculture as an alternative to antibiotics (12,37). The results of the present experiment suggest that the action of the marine *Bacillus* spp. appears to be significant in protecting the host shrimp against pathogenic vibrios. According to Fabregas et al. (38), bacteria prevailing in aquatic ecosystems have the ability to inhibit the growth of other microorganisms by producing antimicrobial substances. Sugita et al. (39) and Gibson et al. (40) recorded the production of antibacterial compounds by marine bacteria. Studies by Dharmaraj (41) showed that marine actinobacterial isolates from sponges have positive inhibition against fish and shellfish pathogens such as *Aeromonas hydrophila*, *Serratia* sp., and *Vibrio* spp. On the other hand, Türker et al. (42) reported antibacterial activities on common fish pathogens in extracts from some Turkish endemic plants. The inhibition ability of *Bacillus* spp. against the pathogenic vibrios is useful in the biocontrol process as *Bacillus* spp. show competitive inhibition and exclusion of the tested pathogens.

For desired results, isolates used for bioremediation in aquaculture are applied in high numbers in the form of commercial microbial products (43), and these should not be pathogenic to the host organism; they should support its health and improve water quality (44). In the present study, *Bacillus* isolates tested for pathogenicity on *P. monodon* PL did not appear to cause infection or disease when tested at a concentration of 10^6 cfu mL^{-1}. This concentration was selected based on reports that most diseases in aquatic animals are caused by bacteria at and above a level of 10^4 cfu mL^{-1} (32). Studies by Moriarty (43,45), Sugita et al. (39), and Rengpipat et al. (46) found that *Bacillus* species (*Bacillus* S11 and *Bacillus* NM12) supported the survival and growth of hosts and did not affect shrimp or fish during the culture cycle. According to Untergasser (47), exposed body parts are highly susceptible to bacterial actions, and any damage by bacteria is easily seen under the microscope. In this study, we observed the telson, cuticular spines, and transparent muscle (32,48) and did not find any indication of infection, thus emphasizing the fact that the selected *Bacillus* isolates were nonpathogenic to shrimp PL.

High concentrations of TAN can be detrimental to PL, especially in the tropics, where water temperature and pH are usually high, resulting in high concentrations of toxic unionized ammonia (9). In the present study, the indigenous *Bacillus* spp. were able to reduce TAN levels significantly in all treated tanks, indicating the beneficial effects of local isolates in comparison to the introduced microbial consortium imported by suppliers. Banerjee et al. (8) reported that the use of indigenous *B. pumilus* in shrimp PL rearing tanks lowered the concentration of TAN (0.80 mg L^{-1}) and nitrite-nitrogen (NO_2-N) (0.68 mg L^{-1}) compared to the control tanks (TAN, 1.11; NO_2-N, 1.12 mg L^{-1}). However, there are reports of nonperforming bioremediators. Timmermans and Gerard (49) tested 2 commercial bacterial suspensions, ABA-S for decomposition of organic matter and ABA-N

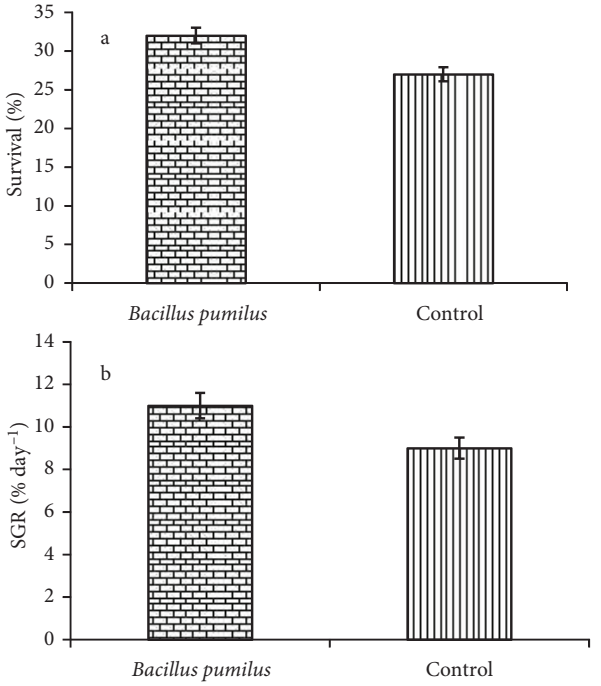

Figure 3. Survival (a) and specific growth rate (SGR; n = 100 PL) (b) in *Bacillus pumilus* and control tanks after a culture period of 18 days. Error bars show standard error.

for nitrification processes, under laboratory conditions and did not find a significant difference in water quality between the treated and untreated tanks. Thus, it is important to use indigenous species that are adapted to the local environment and tested for high performance. The present study also showed the importance of estimating the frequency of application required to achieve the desired results. A booster dose of *Bacillus* spp. was necessary to restore the initial cell concentration in the treatments.

Bacillus spp., including their spores, have been suggested as probiotics and biocontrol agents in fish and shellfish culture systems (12,44,50,51). Lalloo et al. (10) demonstrated that the use of *Bacillus* spp. was safe as there was a reduction in pathogen load and concentrations of waste ions, in vitro and in vivo, in ornamental fish aquaculture. In the present experiment, the use of *B. pumilus* in *P. monodon* PL rearing produced higher survival and SGR and low concentrations of TAN, implying the usefulness of *Bacillus* sp. Studies by Rengpipat et al. (11) reported that *Bacillus* S11, when supplemented with feed, produced significantly higher growth and survival of pond-reared *P. monodon* PL. Along similar lines, Lalloo et al. (10) demonstrated that *Bacillus* isolates used in the culture of ornamental fish exhibited a synergistic positive effect on pathogen inhibition and water quality, in vitro and in vivo.

The most important factor in shrimp larviculture is the maintenance of good water quality. Studies by researchers on the effects of microbial products on water quality have shown inconsistent results. Perhaps improper dosage and frequency of application, lack of suitable microorganisms, inability of microorganisms to adapt to new environments, and varying management practices might be the reasons for ineffectiveness of some imported microbial products in improving water quality. This study takes a systematic approach to identifying suitable isolates that can secrete extracellular enzymes and that display ease of mass culture under laboratory conditions, tolerance to toxic substances like ammonia, and competitive inhibition or exclusion of pathogenic vibrios. In addition, the current study attempts to identify isolates that act as bioremediation agents, utilize toxic ammonia as an energy source for metabolism, and promote survival and growth of the cultured organism while remaining nonpathogenic to shrimp PL. In light of these parameters, *Bacillus* spp. qualify as bioremediators for use in aquaculture.

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