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Immobilized Nitrifying Bacterial Consortium for Improving Water Quality, Survival and Growth of *Penaeus monodon* Fabricius 1798 Postlarvae in Hatchery System

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Abstract

The exposure of shrimp postlarvae (PL) to toxic compounds such as ammonia leads to stress, diseases and mortality. In this study, a consortium of immobilized nitrifying bacteria M1 was tested for its efficacy in reducing total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2-N) so as to improve the survival and growth of *Penaeus monodon* Fabricius 1798 PL in hatchery system. The immobilized nitrifying bacterial consortium consisted of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Nocardiodes albus*. There were three treatments, i.e., 1) tanks with beads containing immobilized nitrifying bacteria (10^6 cfu·mL^{-1}), 2) tanks with beads without nitrifying bacteria, 3) tanks with 50% water exchange on alternate days (no nitrifying bacteria and beads), and 4) control tanks (no water exchange and without treatment). Results showed that tanks treated with immobilized bacteria were able to maintain TAN and NO_2-N below 0.5 mg·L^{-1}. In addition, shrimp PLs in tanks treated with immobilized bacteria had significantly (P<0.05) higher survival rate (72.44%) and specific growth rate (12.86%) compared to other treatments. This technology of using immobilized nitrifying bacteria should be further explored for use on a commercial scale for improving water quality, survival and growth of cultured shrimp PLs in hatcheries.

Introduction

The shrimp industry in recent years has grown rapidly due to its popularity. The intensive rearing of *Penaeus monodon* Fabricius 1798 larvae is certainly of great interest because of its high demand for commercial production. One of the bottlenecks in this industry is the large loss of shrimp during the post-larval stages. In intensive hatchery system, high concentration of total ammonia nitrogen (TAN) and other organic matter are common as a result from overloading of feed input and animal wastes (Audelo Naranjo et al. 2012). Previous studies have shown that in most shrimp hatchery systems 40-90% of nitrogenous compounds are excreted from crustacean's metabolic processes which also comprises of ammonia (Parry 1960; Hartenstein 1970; Chen et al. 1991). Both ammonia and nitrite (NH_3 and NO_2^-) can be toxic to shrimp (Chen and Lei 1990;

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Alcaraz et al. 1999).The "safe level" of TAN and nitrite nitrogen (NO_2-N) for larval rearing of *P. monodon* are 1.15 $mg{\cdot}L^{-1}$ and 1.36 $mg{\cdot}L^{-1}$, respectively (Chin and Chen 1987; Chen and Chin 1988).

Water quality has been considered to be the most important factor that influences the growth and survival of postlarvae (PLs) in a hatchery system. In culture systems, sequestration of ammonia has been achieved using biological (Malone and Pfeiffer 2006) and chemical (Gräslund and Bengtsson 2001) filters as well as application of microbes (Rombaut et al. 2003). The development of systems such as rotary biological contractor (Shankha 1980) or fluidized bed bioreactor (Miller and Libey 1985) prevents the accumulation of ammonia and nitrite (Chen et al. 1991). However, due to the high cost and technical intricacies, use of chemical and biological filtration system is not practical and economically viable for small-scale shrimp hatchery system.

Among several techniques applied for nitrogen removal from aquaculture systems biofiltration using nitrification process is accepted as the most feasible nitrogen treatment process. Nitrification takes place due to ammonia oxidizing bacteria and nitrite oxidizing bacteria which comprise slow-growing species and are sensitive to the changing environment (Lertsutthiwong et al. 2013). In addition, due to the loss of bacteria being washed out from the system, the effectiveness of the nitrification process in the system is minimized.

Therefore, the use of immobilized bacteria in culture system is a better eco-friendly practice. The procedure of immobilization protects the bacterial cells against harsh external conditions such as pH and organic solvents. In addition, immobilization has been proven to circumvent the inherently slow rate of biological nitrogen removal due to the slow growth of the microorganisms (Khin and Annachhatre 2004). Hence, the objective of this study was to maintain low concentration of TAN and NO_2-N and improve growth and survival of *P. monodon* postlarvae using immobilized nitrifying bacterial consortium in a shrimp hatchery system without water exchange.

Materials and Methods

Selection of potential ammonia oxidizing bacteria

Based on our previous studies (Maya et al. 2013), the ammonia oxidizing bacteria consortium M1 consisting of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Nocardioides albus* was selected in this present study for improving water quality, survival and growth of *P. monodon* PLs.

Immobilization of nitrifying bacterial consortium

Alginate solution was used for preparation of beads as described by Smidsrød and Skjåk-Bræk (1990). Alginate beads were formed by dissolving 3% (w/v) of alginate (Protanal LF 10/60, FMC Biopolymer, Norway) in autoclaved seawater for 2 h. Bacterial culture (1–2 x

10^6cfu·mL^{-1}) was centrifuged at 10,000 rpm for 1 min before the bacteria suspension was mixed with the 3% (w/v) of alginate solution. The mixture of bacteria and alginate was dropped using a syringe (0.2 mm internal diameter) into a cation solution of 0.1M strontium chloride ($SrCl_2$, Merck, Germany) from a height of approximately 15-20 cm and at a rate of approximately 1 drop·sec^{-1}. Beads were immediately formed in the $SrCl_2$ with gentle stirring and left in the cation solution for 1 h to allow complete hardening of the alginate beads. Beads were then washed several times using distilled water followed by seawater and then stored in Skinner and Walker (1961) medium at 4 °C prior to use. The same step was used for preparation of beads without bacteria, except bacteria were not immobilized in the beads.

Experimental design

There were three treatments, i.e., 1) tanks with 1,250 beads (bead count based on Maya et al. 2013) containing immobilized nitrifying bacteria (10^6cfu·mL^{-1}), 2) tanks with 1,250 beads without nitrifying bacteria, 3) tanks with 50% water exchange on alternate days (no nitrifying bacteria and beads), and, 4) control tanks (no water exchange and without treatment). All treatments were performed in triplicates. Twelve 4 L aquarium tanks were used in this experiment. Each tank was filled with 2.5 L of filtered dechlorinated seawater before the introduction of PLs. *Penaeus monodon* postlarvae stage 3 (PL3) were purchased from a local hatchery and were stocked at a density of 30 PL·L^{-1}. Postlarvae were fed with a commercial diet (over 52% crude protein and 9% crude fat; Higashimaru, Japan) and *Artemia* (Golden Dolphin, Malaysia) three times a day according to manufacturers' instructions. The tanks were aerated to provide oxygen for the PLs. Parameters such as temperature, pH, salinity and dissolved oxygen were measured every morning at 10:00 a.m. using the YSI 556 MPS (YSI, USA). Total ammonia nitrogen concentration of water in tanks with shrimp PLs were allowed to increase until 2.0 mg·L^{-1} before the beads were introduced. Subsequently, water samples for analysis of TAN and NO_2-N were collected on alternate days for 14 days.

Total ammonia nitrogen was determined according to Parsons et al. (1984). Standard stock solution was prepared using $(NH_4)_2SO_4$. From the stock solution, a series of standard solutions were prepared. Samples and standard solutions (10 mL) were placed in test tube and 0.4 mL of phenol solution and 0.4 mL of sodium nitroprusside was added in sequence. Finally, 1 mL of oxidizing solution was added and allowed to cool at room temperature (20-27 °C) for 1 h. The extinction was measured at 640 nm (UV-1601, Shimadzu, Japan).

Nitrite nitrogen was determined according to Parsons et al. (1984). Standard stock solution was prepared by using $NaNO_2$. From the stock solution, a series of standard solutions were prepared by mixing with deionized water. Samples and standard solutions (10 mL) were placed in test tubes. Then 0.2 mL of sulfanilamide solution was added.

After more than 2 min but less than 10 min, 1 mL of *N*-(1-naphthyl)ethylenediamine (NED) reagent was added and mixed immediately. After 10 min and within 2 h the extinction was measured at a wavelength of 543 nm (UV-1601, Shimadzu, Japan).

Biological analysis

The specific growth rate (SGR) of PL was calculated from the body weight of PL based on the formula of Ricker (1990);

$SGR = [\ln(\text{final weight}) - \ln(\text{initial weight})] / \Delta t \times 100.$

Where; initial weight is the natural logarithm of body weight at the beginning of the experiment, final weight is the natural logarithm of body weight at the end of the experiment and Δt the culture time. The survival of PL was determined at the end of the experiment. Then, reverse salinity stress test (commonly practised by commercial hatcheries to distinguish between healthy and weak PLs) was carried out to distinguish between healthy and weak PLs. For the stress test, 10 PLs from each tank were added into different respective beakers containing distilled water (1L; 0 ppt) and PL mortality was observed every 30 min for 90 min.

Data analysis

Data collected were analysed using the one-way analysis of variance (ANOVA). Significant differences amongst treatments were determined using Duncan's multiple range test at 0.05 level. Statistical analyses were done using the SPSS computer package.

Results

Water quality parameters

Dissolved oxygen, pH, salinity and water temperature did not differ significantly ($P>0.05$) during the experimental period (Table 1). However, there was significant difference in TAN concentration ($P<0.05$) between different treatments until day 4. From day 6 until day 14, no significant difference ($P>0.05$) was found in tanks treated with immobilized bacteria and 50% water exchange (Fig. 1). Throughout the 14 days experiment, TAN concentration in tanks treated with immobilized bacteria was below 0.5 $mg{\cdot}L^{-1}$. With regards to NO_2-N concentration, tanks treated with immobilized bacteria and 50% water exchange had significantly ($P<0.05$) lower concentration compared to the other treatment and control throughout the experimental period (Fig. 2). Therefore, reduction of TAN and NO_2-N were significantly higher ($P<0.05$) by immobilized nitrifying bacterial consortium (76%, 43%) compared to 50% water exchange (72%, 8%), beads without bacteria (0.3%, 0%) and the control (0%, 0%).

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Table 1. Water quality parameters in control and treated tanks.

Treatment tanks	Salinity (ppt)	Temperature (°C)	pH	Dissolved oxygen ($mg \cdot L^{-1}$)
Immobilized bacteria	$^{a}31.9 \pm 2.7$	$^{a}26.3 \pm 0.7$	$^{a}7.7 \pm 0.3$	$^{a}5.9 \pm 0.3$
Beads with no bacteria	$^{a}32.1 \pm 3.7$	$^{a}26.2 \pm 0.7$	$^{a}7.7 \pm 0.2$	$^{a}5.8 \pm 0.5$
50% water exchange	$^{a}32.0 \pm 3.2$	$^{a}26.0 \pm 0.7$	$^{a}7.6 \pm 0.2$	$^{a}5.9 \pm 0.4$
Control	$^{a}32.1 \pm 3.7$	$^{a}26.1 \pm 0.6$	$^{a}7.7 \pm 0.3$	$^{a}5.9 \pm 0.2$

Note: Mean values ± standard error with different superscripts are significantly different in the column ($P<0.05$).

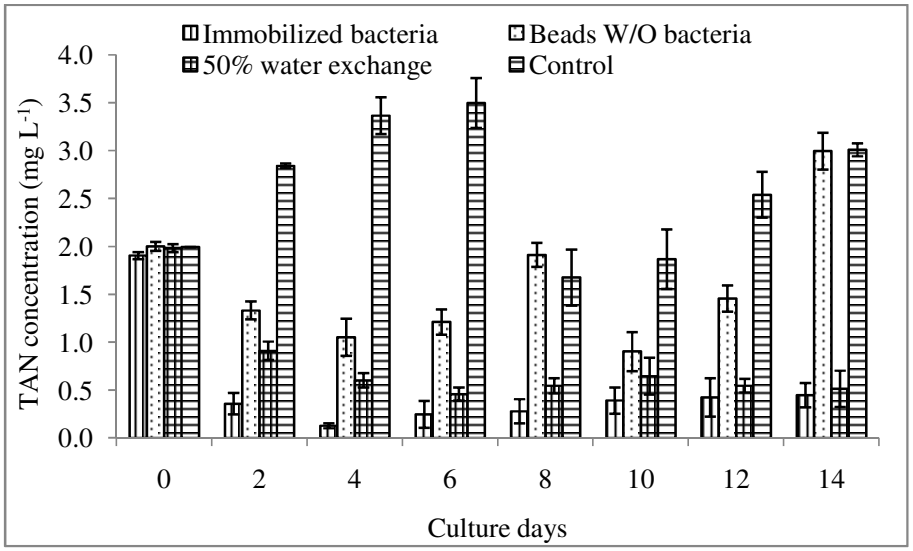

Fig. 1. Total ammonia nitrogen concentration in experimental and control tanks. Ammonia was allowed to increase to 2 $mg \cdot L^{-1}$ before the introduction of immobilized bacteria. Vertical bars indicate standard error of the means.

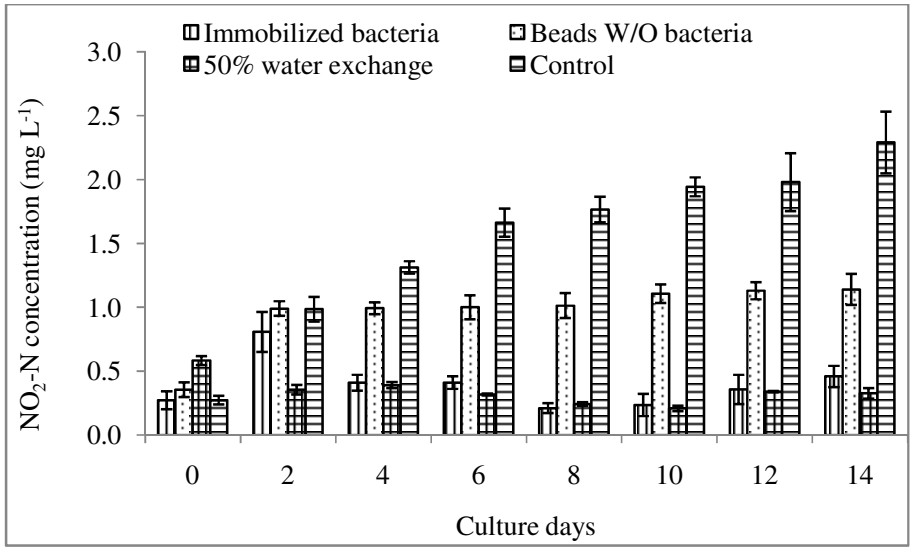

Fig. 2. Nitrite-nitrogen concentration in experimental and control tanks. Vertical bars indicate standard error of the means.

Survival and growth of postlarvae

The highest survival rate of PLs was found in tanks containing immobilized bacteria (72.44%) which was significantly different ($P<0.05$) from tanks treated with beads without bacteria (65.33%) and the control tanks (49.33%) but not significantly different ($P>0.05$) between tanks with 50% water exchange (70.22%) (Fig. 3). With regards to SGR, the pattern followed a similar trend as survival of PLs reared in tanks with immobilized bacteria that showed the highest SGR (12.86%) followed by tanks with 50% water exchange (12.72%), tanks with beads without bacteria (11.86%) and the control tanks (11.5%) (Fig. 4).

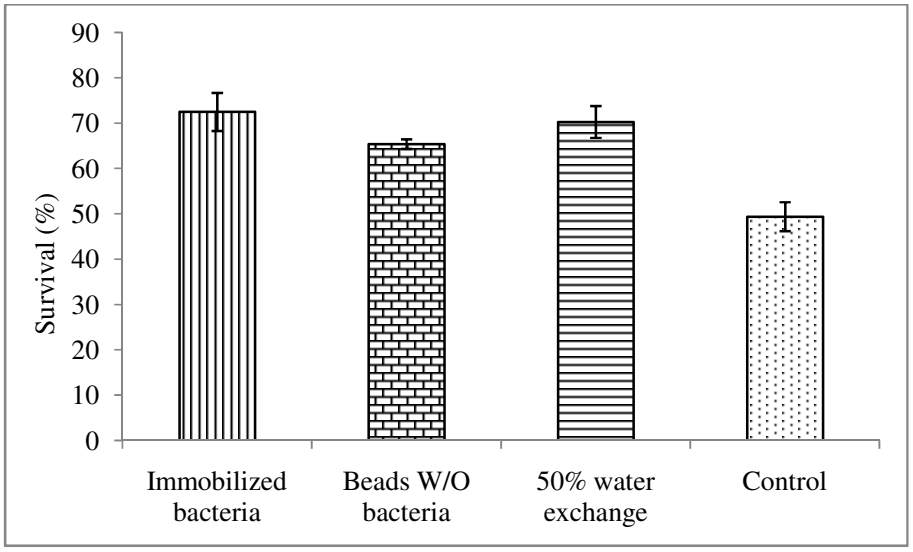

Fig. 3. Survival rate of shrimp postlarvae in experimental and control tanks after 14 days. Vertical bars indicate standard error of the means.

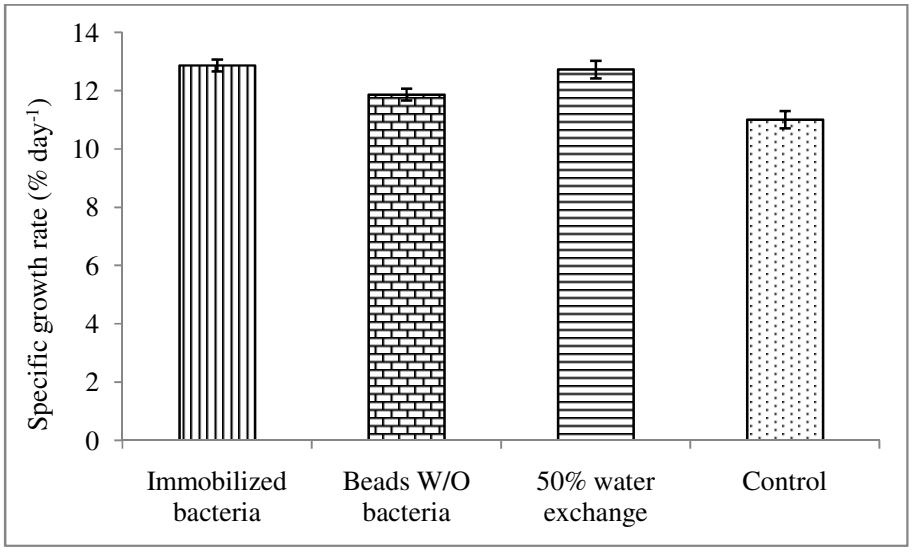

Fig. 4. Specific growth rate of shrimp postlarvae after the end of experiment in treatment and control tanks. Vertical bars indicate standard error of the means.

Based on the salinity stress test, survival of PLs in tanks treated with immobilized bacteria had significantly (P<0.05) higher survival rate (73.33%) compared to tanks with 50% water exchange which had an overall survival of 66.67%, followed by tanks with beads without bacteria (60%) and control tanks (50%) (Fig. 5).

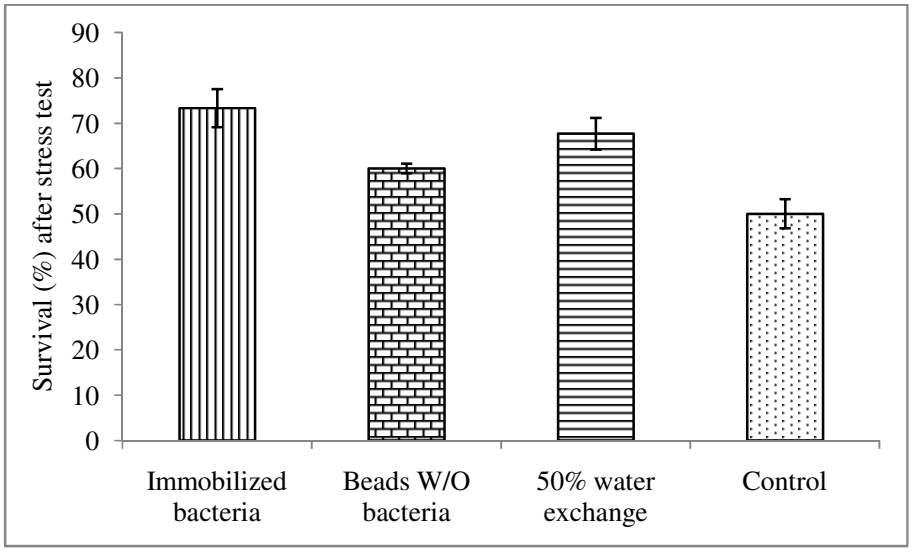

Fig. 5. Survival rate after reverse salinity stress test. Vertical bars indicate standard error of the means.

Discussion

Accumulation of high concentrations of ammonia is harmful in aquaculture systems leading to retarded growth, poor survival, reduced feed intake and susceptibility to diseases of the cultured organisms (Manju et al. 2009). Chin and Chen (1987) and Chen and Chin (1988) reported that the "safe level" of TAN and nitrite nitrogen (NO_2-N) for larval rearing of *P. monodon* are 1.15 $mg{\cdot}L^{-1}$ and 1.36 $mg{\cdot}L^{-1}$, respectively. As TAN is produced continuously during the culture period, an intensive method for the nitrification of ammonia is required in managing the TAN concentrations of culture systems. Shan and Obbard (2003) reported that reduction of TAN in aquaculture system can be facilitated by providing and maintaining an optimum environment condition for nitrifying bacteria. Therefore, Buttner et al. (1993) suggested that by maintaining an optimal environment condition such as water pH between 7 to 9 and temperature of about 24 to 30 °C in the hatchery will increase the utilization of TAN by nitrifying bacteria. In the present experiment, TAN and NO_2-N removals of 76% and 43% were achieved by immobilized nitrifying bacterial consortium, which were significantly higher compared to other treatments and the control. This may be due to the optimum pH (7.7) and temperature (26.3 °C) maintained in the culture tank and also due to the fact that the nitrifying bacteria were immobilized. The entrapment of cells in alginate gels provided more stability of its primary metabolic activity that allowed the immobilized bacteria to consume more nitrogenous compounds (Garbayo et al. 2000).

Ammonia and nitrite are toxic to fish and crustaceans and can have detrimental effects on larvae in a closed culture system (Chen and Chin 1988) and therefore their removal is important. According to Chen et al. (1986), ammonia can increase to more than 0.8 $mg{\cdot}L^{-1}$ ammonia-N during shrimp larval development in a hatchery, even with frequent water exchange. They found that when ammonia-N and nitrite-N increased to 808.4 and 118.1 $\mu g{\cdot}L^{-1}$ respectively, survival of PL was 4% inspite of water exchange. In another instance, no PLs survived when ammonia-N increased from 25.3 to 269.1 $\mu g{\cdot}L^{-1}$and NO_2-N increased from 0.8 to 78.3 $\mu g{\cdot}L^{-1}$although one-third of water was changed. In the present experiment, the survival of PL was significantly higher in tanks containing the immobilized nitrifying bacterial consortium compared to the other treatments and the control probably due to the low concentration of TAN and NO_2-N. In the tanks with 50% water exchange, the TAN concentration did not vary significantly when compared to tanks treated with immobilized bacteria. However, it was interesting to observe that PL survival rate in tanks with 50% water exchange was relatively lower than PLs in tanks treated with immobilized bacteria. This difference might be attributed to the sudden environmental changes caused by the frequent water exchange which subsequently stressed the shrimp PLs. According to Chien (1992), sudden environmental change resulting from water replacement in small ponds or tanks can cause stress to the shrimp. It was also interesting to note that high PL mortality also occurred in control tanks associated with high TAN concentration. Thus, the application of immobilized bacteria for bioremediation can reduce stress thereby avoiding mortalities.

In most hatcheries, reverse salinity stress test is used to assess the quality of PLs before the PLs are introduced into the growout ponds (Samocha et al. 1998; Palacios et al. 2004). The optimum qualities of PLs are important in order to get the maximum yield during the stocking of PLs into the growout ponds. In the present study, reverse salinity stress test showed that there was a significant effect in the survival rate of PLs undergoing stress. PLs reared in tanks treated with immobilized bacteria and those in tanks with 50% water exchange showed significantly higher resistance to reverse salinity stress tests compared to those reared with beads without bacteria and control tanks. This proves that when PLs are grown in an environment with low ammonia and nitrite concentration, they have higher survival rate. Similar study by Peirong and Wei (2013) also showed that immobilized bacteria for ammonia reduction can improve water quality and maintained the health of cultured animals. In the present study, SGR of PLs was significantly higher in tanks treated with immobilized bacteria as compared to all other treatments. Millamena (1990) also reported that by maintaining low TAN concentration during experimental period can increase the SGR of PLs.

Conclusion

This study demonstrated that the use of immobilized nitrifying bacterial consortium consisting of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri* and *Nocardiodes albus* in shrimp PL hatchery system represents a viable alternative for maintaining low concentration of TAN and NO_2-N in water while supporting better PL survival and growth.

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