The Efficiency of *Bacillus* spp. to Remove Ammonia in Shrimp Aquaculture

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Abstract

Salt-tolerant *Bacillus* was used to eliminate ammonia in shrimp aquaculture wastewater. *Bacillus* strains were isolated from sediment and water collected from shrimp farms and domestic wastewater. Ammonium oxidizing ability was screened by Griess-Ilosvay method. Five isolates were identified as *Bacillus* spp. with salt requirement within the range of 0-40 g/L NaCl and an optimal pH of 7. *Bacillus* strains TS41, TW31, HS12, HW34 and ES33 exhibited preliminary ammonium removal efficiency on HNM medium for 84.21%-94.86%. Improved ability of synthesized shrimp wastewaters was determined by applying 1% and 5% cell suspension for 7 days. SF experiment with 1% cell suspension of ES33 showed highest ammonium removal of 66.38%, while the results of other treatments showed no significance ($p > 0.05$). SNF experiment, all five *Bacillus* strains showed ammonium removal of 78-96% at day 7. NSF experiment, ES33 provided the highest ammonium removal efficiency of 1% cell suspension at day 7 for 93.14%. The amounts of nitrite and nitrate were presented in all experiments and removed by *Bacillus* species. The results demonstrated the process of nitrification-denitrification reaction. Consequently, our *Bacillus* strains may propose as heterotrophic nitrification-aerobic denitrification species.

Keywords: Salt-tolerant *Bacillus*, Shrimp aquaculture wastewater, Ammonium removal efficiency, Cell suspension, Nitrification-denitrification

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Introduction

Ammonia is a natural component of the nitrogen cycle in the ecosystem because of the substantial amounts of uneaten feedstuff and faeces generated in the process (Lin and Wu, 1996; Read and Fernandes, 2003), which causes rapid accumulation of ammonia in the water. Thus, ammonia induces a large variety of physiological disturbances and immunosuppression on aquatic animals. It is toxic to aquatic animals.

Nitrogen-containing compounds released into the environment can create serious problems, such as eutrophication in river and coastal areas (Fennesy and Cronk, 1997). Furthermore, nitrite accumulation was a critical issue inherent in the aquaculture industry, because coastal aquaculture such as marine shrimp and sea bass are important to the economy of Thailand (Paungfoo *et al*., 2007). However, a more serious risk is that the toxicity of ammonia could cause outbreak of shrimp diseases and ultimately result in huge economic losses (Cheng and Chen, 1999). Hence, treatment of culture water to decrease the rapid accumulation of ammonia for such an activity has become increasingly crucial.

There are three mechanisms to remove ammonia, nitrite, and nitrate in aquaculture, including physical, chemical and biological methods (Wang *et al*., 2008), but other physical and chemical methods are not able to remove nitrite completely. Typically, adding of chemical substances is totally accumulative in shrimp culture pond and environment (Charoendat *et al*., 2016). The best way to accomplish is through the natural biological mechanism which occurs without pollution and residues (Li *et al*., 2005). Usawakesmanee (2016) suggested that the treatment of wastewater by biological method helps to reduce treating cost.

The biological process that has the ability to transform ammonia to nitrite and further transform nitrite to nitrate is called the nitrification process which is performed by nitrifying bacteria. In the past, nitrifying bacteria was well known as autotrophic bacteria such as *Nitrobacter* and *Nitrosomonas* (Zhang *et al*., 2012; Chankaew *et al*., 2018). Despite nitrogen's transformative ability to nitrify bacteria, they have slow growth and less competition when compared with other heterotrophic bacteria (Sirianuntapiboon *et al*., 2015; Chankaew *et al*., 2018). Nitrogen content in wastewater has large decrease by activities of heterotrophic bacteria, nitrifying bacteria and denitrifying bacteria. These organisms have ability to transform nitrogen compounds as energy source and cell precursor (Sirianuntapiboon *et al*., 2015). Recently, heterotrophic bacteria capable of removing ammonia, nitrite and nitrate have been reported. Some of them can reduce nitrate

to free nitrogen under aerobic conditions via aerobic-denitrification process. Heterotrophic nitrification-aerobic denitrification bacteria such as *Bacillus* sp., *Pseudomonas* sp. and *Alcaligenes faecalis* were isolated and their nitrogen removal efficiency determined by several research studies (Joo *et al*., 2005; Wan *et al*., 2011; Yang *et al*., 2011). One of the promising bacterium used for ammonium removal or wastewater treatment is *Bacillus*. *Bacillus* is widely used in the aquaculture industry to improve water quality, promote growth and prevent diseases of aquatic animals (Vaseeharan and Ramasamy, 2003; Chankaew *et al*., 2018). In case of nitrification-denitrification process, *Bacillus* could perform not only heterotrophic nitrification, but also aerobic denitrification. All *Bacilli* are aerobic heterotrophs, implying that they can simultaneously work on nitrification and denitrification (Gupta and Gupta, 2001). Moreover, *Bacillus* species are capable of using nitrate and nitrite as alternative electron acceptors when oxygen is absent (Seenivasagan *et al*., 2017). Other unique characteristics of *Bacillus* are rapid growth, good competition, variety of carbon and nitrogen utilization, salt tolerance, and endo-spore formation (Rosovitz *et al*., 1998; Suharti and Vries, 2004; Manzo *et al*., 2013; Meeboon and Saimmai, 2019). Isolation and understanding of characteristics of *Bacillus* in the nitrification-denitrification process is very important in order to develop and apply them for wastewater treatment. One desirable property for brackish and marine aquaculture treatment is salt-tolerant ability. The bacteria must able to survive and provide good performance in saline conditions. Therefore, the aims of this study are to isolate salt-tolerant *Bacillus* and to determine the efficiency of ammonium removal in wastewater from marine shrimp aquaculture.

Materials and Methods

1. Sample collection

Sediment and water samples were collected from marine shrimp farms and domestic wastewater in Pak Meng Beach ($7^{\circ}30'$ N and $99^{\circ}19'$ E), Trang Province, Thailand. Samples were kept in sterile polyethylene bags and stored at 4 $^{\circ}$C for further study.

2. Isolation and screening of *Bacillus*

Each 1 g or 1 ml of samples was enriched into 100 ml of nutrient broth supplemented with 2% NaCl and an enrichment medium (peptone 5 g, beef extract 3 g, sea salt 2 g, shrimp feed 1g and H_2O 1000 ml) at 35 $^{\circ}$C and shaken at 170 rpm for 24-48 hours. One milliliter of the suspended liquid was serially diluted from 10^{-1} to 10^{-8} in a tube containing 9 ml of distilled water. Suspension tubes were treated in

an 80 ℃ water bath for 10 min. Then 0.1 ml of diluted solution was taken from each tube and spread on nutrient agar supplemented with 2% NaCl (Zhao *et al*., 2017). Gram positive, rod shape and endo-spore forming colony was selected and transferred to fresh medium. Purified isolates of *Bacillus* were obtained by repeated streaking on fresh agar plates. Biochemical characteristics, catalase and oxidase test of *Bacillus* were examined.

3. Preliminary testing for ammonium oxidizing ability

One milliliter of *Bacillus* inoculum was transferred to the nutrient broth (NB) medium (peptone 5 g, beef extract 3 g, ammonium sulfate 15 g and H_2O 1000 ml) and incubated at 35 $^{\circ}$C for 14 days. Nitrite-oxidizing reaction was tested by Griess-Ilosvay method (Lu *et al*., 2012). Positively tested suspensions with red color were further isolated by spreading on nutrient agar medium.

4. Preliminary testing for heterotrophic nitrifying-denitrifying characteristic

Bacillus spp. were incubated in a 250 ml serum bottle containing 100 ml of heterotrophic nitrification medium (HNM) ($(NH_4)_2SO_4$ 0.66 g, sodium succinate 4.72 g, KH_2PO_4 0.50 g, Na_2HPO_4 0.50 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, NaCl 20.00 g and H_2O 1000 ml) (Zhang *et al*., 2012). The incubation was performed at 30 ℃ on a rotary shaker at 160 rpm for 7 days. The concentration of ammonium nitrogen (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) were determined on day 7.

5. Salt requirement and optimal pH on growth

One milliliter of cell suspension was cultured in a tube with 10 ml of NB medium. The pH of NB was varied at 3, 5, 7, 9 and 11. Salt requirement trial was set at 0, 10, 20, 30 and 40 g/L (ppt). Cultivation tubes were shaken at 170 rpm for 24 hrs. Growth profile of *Bacillus* was determined by optical density (OD) at 600 nm (Song *et al*., 2011; Seenivasagan *et al*., 2017).

6. Shrimp wastewater preparing

Four experiments of different shrimp wastewaters were designed. Experimental wastewaters were devised as sterilized and non-sterilized treatments. Each of them was exactly separated as fermented and non-fermented with shrimp feed. For fermented trials, shrimp wastewaters were synthesized by fermentation of 1% commercial shrimp feed for 3 days. The synthesized shrimp wastewaters are shown in Table 1.

7. Efficiency of ammonium removal

Bacillus strains grown in Heterotrophic nitrification medium and preliminary showed high efficiency of ammonium removal and were further studied for inorganic nitrogen removal of four synthesized shrimp wastewaters (Table 1). Cell

suspensions of *Bacillus* spp. were prepared in NB until 10^7 CFU/ml. In each experimental study, cell suspension used both of 1% (50 ml) and 5% (250 ml) in 5 L of prepared wastewaters. All designed experiments and control treatment (CTRL) were performed for 7 days in an aerated system. Each CTRL treatment was same prepared as that experiments but no added bacterial cell suspension. Every day 4 and day 7, wastewaters were sampled and examined for the concentration of ammonia, nitrite and nitrate followed by the standard colorimetric method (Strickland and Parsons, 1972).

Table 1 Synthesized shrimp wastewaters for ammonium removal study

Experiment	Sterilization (121 °C, 15 mins)	Fermentation (1% shrimp feed, 3 days)
1-SF	sterilized	fermented
2-SNF	sterilized	non-fermented
3-NSF	non-sterilized	fermented
4-NSNF	non-sterilized	non-fermented

8. Statistics analysis

Results were shown as the average of at least three independent experiments and were presented as means±SD (standard deviation of means). All statistical analyses were carried out by one-way ANOVA. That performed using the software package SPSS and minimum significant differences were calculated by the Duncan multiple range test ($p < 0.05$).

Results

1. Isolation and characterization of *Bacillus*

Thirty-three isolates were proposed as *Bacillus*. Then, 24 out of 33 isolates exhibited positive results with Griess-Ilosvay testing. All 24 isolates were preliminarily determined for nitrogen removal (NH_3, NO_2^- and NO_3^-) on HMN medium. Only 5 strains, including TS41, TW31, HS12, HW34 and ES33, showed high ammonium removal efficiency of more than 90% (94.86%, 93.94%, 93.17%, 90.65% and 84.21%, respectively) (Figure 1). These 5 strains were able to transform ammonia to nitrite, but only minimally continue to nitrate. Figure 2 shows ability of strains TS41, TW31, HS12, HW34 and ES33 on nitrite production of 11.55, 18.76, 6.43,

0.98 and 16.15 mg-N/L, respectively and nitrate production of 2.09, 2.11, 1.31, 1.73 and 1.65 mg-N/L, respectively.

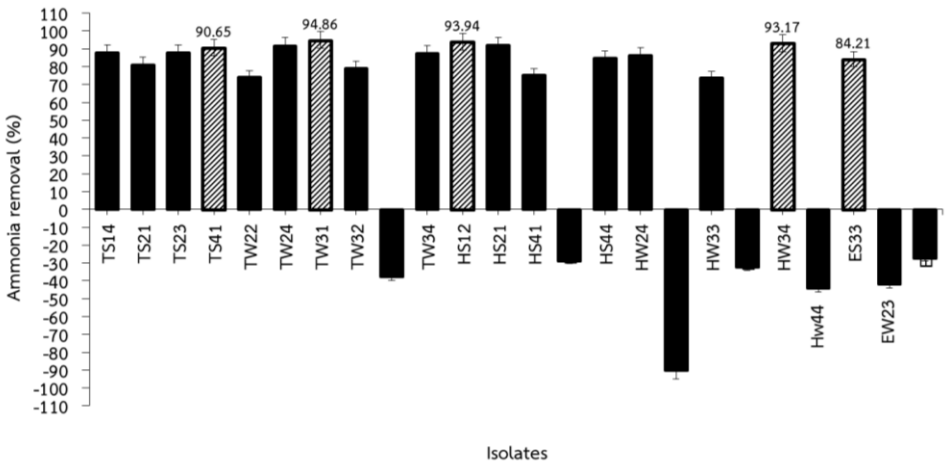

Figure 1 Ammonium oxidizing ability of *Bacillus* spp. on HNM medium. Values are means±SD (error bars) for three replicates.

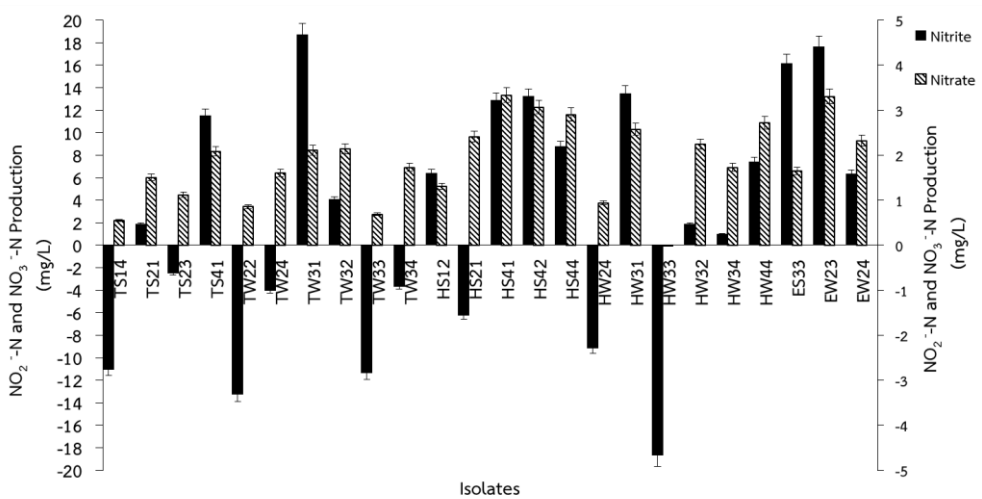

Figure 2 Nitrite and nitrate production abilities of *Bacillus* spp. on HNM medium. Values are means±SD (error bars) for three replicates.

2. Salt requirement and optimal pH on growth of *Bacillus*

The results showed that five isolated *Bacillus* strains have slight growth at pH 3-5. They have optimal growth at pH 7 and then growth slightly decreases to a pH of 9-11 (Figure 3). Our *Bacillus* isolates can grow in wide spectra

of salt concentration of 0-40 g/L NaCl. Whereas strain, HW34, had slight growth at 0-10 g/L NaCl, but it displayed maximum growth when the salt concentration rose to 20-40 g/L NaCl (Figure 3).

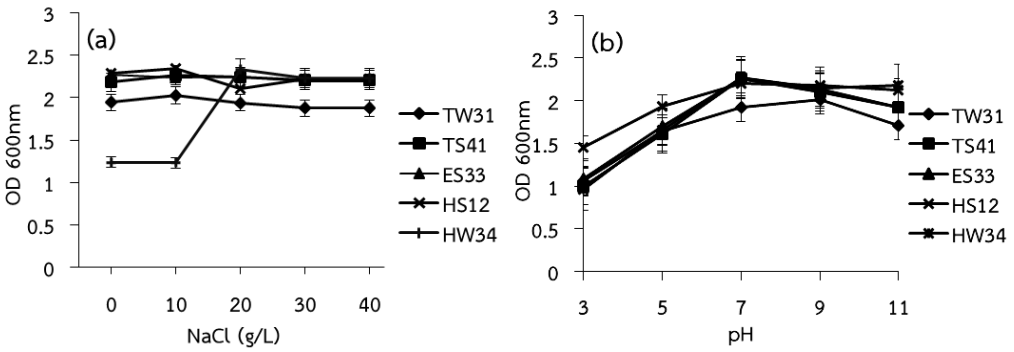

Figure 3 Growth profiles of *Bacillus* spp. under different salinity (a) and pH (b)
Values are means±SD (error bars) for three replicates.

3. Efficiency of *Bacillus* on ammonium removal of synthesized shrimp wastewater

3.1 Ammonium removal efficiency

Which all five *Bacillus* strains were studied for treatment in four different experiments of synthesized shrimp wastewaters as shown in Table 1. Each *Bacillus* cell suspension of 1% and 5% was applied to synthesized wastewaters and was aerated for 7 days. The result of experiment 1 (SF) showed that 1% and 5% cell suspension adding all five *Bacillus* provided slight ammonium removal at day 4 (initial ammonia was 3.61±0.04 mg-N/L). The amount of ammonium removed was about 53-66% until day 7, whereas removal showed no significance ($p > 0.05$) of all strains and control treatments (Figure 4a). Experiment 2 (SNF), 1% cell suspension of all five *Bacillus* strains showed ammonium removal ability of 78-96% at day 7 displaying significant ammonium removal ability 5% higher than cell suspension and control treatments. Ammonium removal capabilities of strains TW31, ES33 and HW34 (1% cell suspension) were fast and high after 4 days of operation (initial ammonia was 2.15±0.05 mg-N/L). In contrast, 5% cell suspension of strains TW31, ES33, HW34 and HS12 at day 4 exhibited an increasing amount of ammonium. High volume addition of these bacteria may firstly produce ammonia during cell adjustment into new conditions. Then cells will be harmonized to wastewater and ammonium removal capability (Figure 4b). Experiment 3 (NSF), 1% cell suspension of strains ES33, HW34 and TS41 exhibited rapid ammonium removal at day 4 (initial

ammonia was 1.18±0.01 mg-N/L). Strain ES33 provided the highest ammonium removal efficiency of 1% cell suspension at day 4 and 7 for 90.01% and 93.14%, respectively. Ammonium removal of strains TW31 and TS41 showed effectiveness at 5% cell suspension at day 4 for 81.04% and 87.22%, respectively (Figure 4c). Strains TS41 and HS12 with 5% cell suspension have provided ammonium removal ability of 83.67% and 85.27%, respectively. Non-sterilized and non-fermented shrimp wastewater treatments (NSNF; experiment 4) showed overall ammonium removal efficiency with a range of 60-96% (initial ammonia was 1.18±0.01 mg-N/L). However, the results have no significance ($p > 0.05$) between 1% and 5% of cell suspension as well as dates of treatments (Figure 4d).

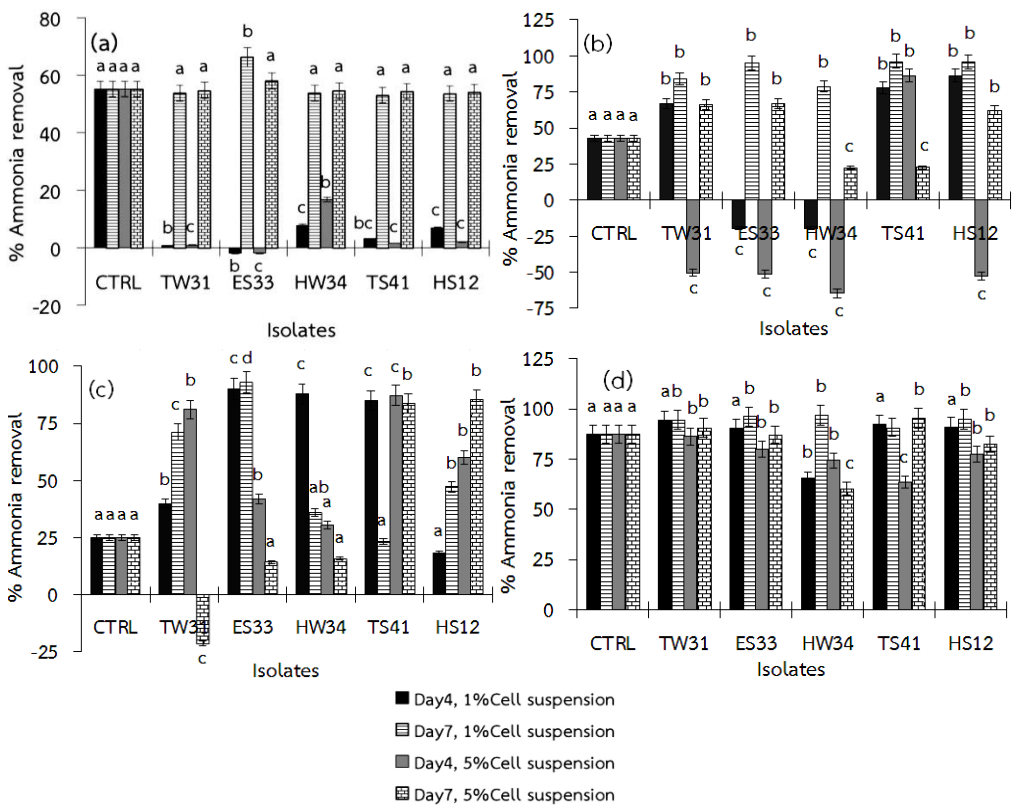

Figure 4 Ammonium removal efficiencies of *Bacillus* spp. (a), 1-SF; (b), 2–SNF; (c), 3-NSF; (d), 4-NSNF. Values represent the mean±SD (n=3). Values with different letters indicate significant differences ($p < 0.05$) among them.

3.2 Nitrite removal efficiency and nitrate production

The initial nitrite concentration of synthesized wastewater was maintained at the low level (<1 mg-N/L). However, NO_2^- volumes of experiments

were produced and eliminated in the incubation process. In the SNF experiment, *Bacillus* strain TW31 with 5% cell suspension had removed nitrite volume at day 4 and 7, about 94.69% and 95.30%, respectively and showed higher than 1% cell suspension. Moreover, strains ES33, HW34, TS41 and HS12 demonstrated highest nitrite removal with 1% cell suspension at day 4 for 96.66%, 88.18%, 86.51 and 91.06%, respectively (Figure 5a). The control trial of SNF, in contrast, showed very low nitrite production and removal due to death of microorganisms in raw wastewater by autoclaving. Hence, the control trial of NSF showed nitrite removal of approximately 29%. This confirmed our assumption that live microorganism cells in raw wastewater played a role in nitrogen removal proficiency. *Bacillus* strains TW31, ES33, HW34, TS41 and HS12 with 1% cell suspension exhibited remarkably high nitrite removal for 87.40%, 93.70%, 92.12%, 68.50 and 96.06%, respectively (Figure 5b). Nitrate removal in the SF experiment showed highest ability for nitrate removal with 98.80% at day 4 (5% cell suspension) by strain HS12. Followed by 95.11% removal at day 4 (5% cell suspension) for strain TS41. While, other 3 isolates showed no significance ($p > 0.05$) for nitrate removal, except strain TW31 with 5% cell suspension (day 7), but had no nitrite removal ability (Figure 5c). The NSF experiment of nitrate result displayed 1% cell suspension of strains TW31, ES33, HW34 and HS12 and provided high nitrate removal at day 4 of 45.54%, 84.81%, 89.49% and 86.62%, respectively (Figure 5d). Whereas, strain TS41 exhibited the highest nitrate removal ability with 5% cell suspension after incubating for 7 days.

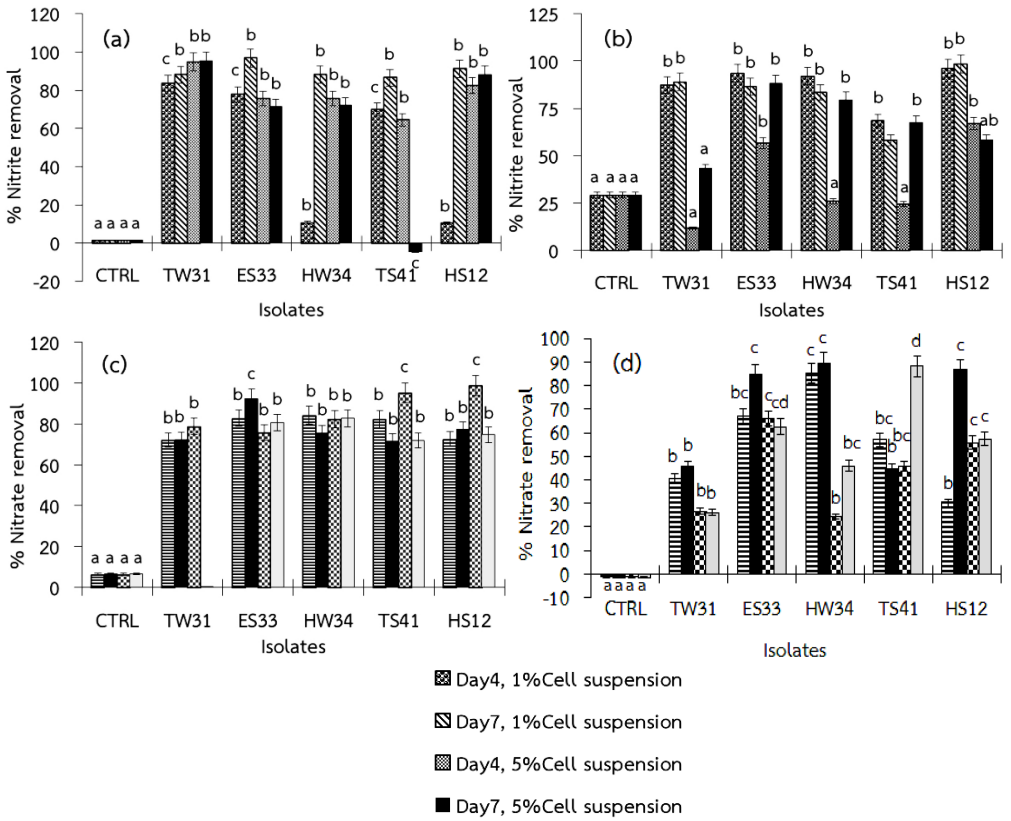

Figure 5 Nitrite (a-b) and nitrate (c-d) removal efficiencies of *Bacillus* spp. (a), SNF; (b), NSF; (c), SF; (d), NSF. Values represent the mean±SD (n=3). Values with different letters indicate significant differences ($p < 0.05$) among them.

Discussion

Zhang *et al.* (2012) reported a similar study of growth and nitrification efficiency of other *Bacillus* spp. which displayed high growth at an initial pH of 7-8. Consistent with those reported in this present study, acidic (pH 5-6) or alkaline (pH 9-10) conditions were hazardous to the growth of *Bacillus*. The slightly alkaline environment was conducive to heterotrophic nitrification (Mevel and Prieur, 2000). Mevel and Prieur (2000) reported that *Bacillus* MS 30 had an optimal growth salinity of 16 g/L NaCl, and it could not grow when the salinity was increased to 28.50 g/L NaCl. Therefore, our five *Bacillus* strains should be proposed as salt–tolerant species with a wide range of salinity of at least 0-40 g/L NaCl. That salinity range is for optimal growth of coastal species (Sutin, 2010). This characteristic expands their application scope, regardless of the coastal and mariculture wastewaters containing high salinity.

The ammonium removal result of experiment 3 (NSF) of all *Bacillus* strains provided higher efficiency than other experiments. NSF experiment was non-sterilized; hence, it still consisted of indigenous bacteria (Ongsara *et al*., 2012). Moreover, fermented shrimp feed released organic compounds as nutrients serving bacteria. A combination of microorganisms between our *Bacillus* isolates and indigenous microorganisms showed co-working and synergy on ammonium elimination ability. While sterilized wastewater in experiment 2 (SNF), displayed no contaminated microorganisms in the trial. The result of this treatment showed lower ammonium removal efficiency than NSF treatment. This suggested that the ability of only *Bacillus* strains for ammonium removal was less, in contrast with the cooperation of microorganisms. Shrimp feed consists mainly of protein and phosphorus (Hmadhloo *et al*., 2013). It will release nitrogen and phosphorus compounds to serve *Bacillus* spp. and other microorganisms (Mclntosh *et al*., 2001; Dechmahitkul *et al*., 2007). Non-sterilized shrimp wastewater decomposed organic and inorganic matters by wild microorganisms (O-Thong *et al*., 2003). *Bacillus* strains were heterotrophic nitrification bacteria. They have efficiency for ammonium removal and a less complex nitrification process than autotrophic bacteria (Gupta and Gupta, 2001). Therefore, heterotrophic *Bacillus* may have high potential to be applied in aquaculture wastewater treatment.

In this study, our *Bacillus* isolates were found to be leading to aerobic nitrification. Yang *et al*. (2011) described that NO_2^- is converted to NO_3^- by the nitrification process and nitrate can be further converted to free nitrogen in aerobic denitrification reactions by heterotrophic nitrification bacterium. In our investigation, five *Bacillus* strains showed ammonium removal characteristics as well as the ability to transform ammonium to nitrite. Furthermore, they can eliminate and transform nitrite to nitrate as well as remove nitrate. However, free nitrogen gas that is reduced from nitrate in the operation system should be further analyzed.

Conclusions

Five *Bacillus* strains, including TW31, HS12, HW34, TS41 and ES33, were isolated based on the characteristic high efficiency ammonium removal ability. These *Bacillus* strains have the property of salt tolerance. When treating the synthesized shrimp wastewaters with 5 isolated *Bacillus*, 1% and 5% of cell starters were not effectively significant for ammonium removal ability. Indeed, 1% cell starter was suggested for use in order to reduce costs. Nitrite amounts were presented in all experiments and further highly removed reaching 96% by *Bacillus* species. In addition, nitrate volumes were produced in all experiments and then

removed almost 100% by our *Bacillus* isolates. Consequently, our five *Bacillus* isolates can be proposed as heterotrophic nitrification–aerobic denitrification species. Moreover, these five *Bacillus* strains can be used for removal of ammonia, nitrite and nitrate in shrimp aquaculture with saline conditions.

Acknowledgments

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