Nitrite oxidizing bacteria for water treatment in coastal aquaculture system

S Noorak1, S Rakkhiaw1, K Limjirakhajornt1, A Uppabullung1,2, T Keawtawee1,2 and Y Sangnoi1,2*

1Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla 90110 Thailand
2Discipline of Excellence for Sustainable Aquaculture, Prince of Songkla University, Hat Yai, Songkhla 90110 Thailand

E-mail: yutthapong.s@psu.ac.th

Abstract. This research aimed to isolate and characterize nitrite oxidizing bacteria and to study their capability for water quality improvement. Fourteen strains of bacteria with nitrite-oxidizing character were isolated after 21 days of enrichment in Pep-Beef-NOB medium contained NaNO2. Two strains, SF-1 and SF-5, showed highest nitrite removal rate for 42.42% and 37.2%, respectively. These strains were determined an efficiency of open-system wastewater treatment for 14 days. The results showed that control, SF-1 and SF-5 had remove ammonia from day 1 to day 6. At the end of the study, ammonia was removed by the control, SF-1 and SF-5 for 81.27%, 70.1% and 69.82%, respectively. Nitrite concentration was lowest at day 8 with removal rate of 98.73%, 98.3% and 97.24% from control, SF-1 and SF-5, respectively. However, nitrite concentration in control experiment was increased again at day 11 whereas in SF-1 and SF-5 were increased at day 13. Chemical Oxygen Demand (COD) was decreased by 77.78%, 73.50% and 78.63% in the control, SF-1 and SF-5, respectively. Biological Oxygen Demand (BOD) in the control, SF-1 and SF-5 were reduced by 85.92%, 79.53% and 82.09%, respectively. Based on 16S rRNA gene, SF-1 and SF-5 were identified as *Bacillus vietnemensis* and *B. firmus*, respectively.

1. Introduction
Coastal aquacultures have an important economy of Thailand [1]. These are including of marine shrimp and seabass. However, the accumulation of inorganic nitrogen compounds such as ammonia, nitrite and nitrate in coastal aquaculture system is serious toxic to aquatic animals. Biological process of nitrification including ammonium oxidation and nitrite oxidation has transformed ammonia and then nitrite to nitrate. These two process are involved with nitrifying bacteria; ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Our previous study suggested that heterotrophic nitrifying bacteria have ability to oxidize ammonia to nitrite but they cannot oxidize nitrite to nitrate. Therefore, this study aimed to isolate heterotrophic nitrite oxidizing bacteria and determine the water treatment efficiency.
2. Methodology

2.1 Isolation and screening of NOB
Water and sediment samples were collected from seabass farms located in Koh Yor Island, Songkhla province. Each 1 g or 1 ml of sample was enriched into 100 ml of Pep-Beef-NOB medium (peptone 5 g, beef extract 3 g, NaNO₂ 0.2 g, Na₂CO₃ 1 g, K₂HPO₄ 0.5 g, MgSO₄ 0.5 g, FeSO₄ 0.4 g, sea salt 7 g, H₂O 1000 ml) at 28 °C and shaken at 150 rpm for 21 days [2]. At days 7 and 14, ten milliliters of suspension were transferred to fresh Pep-Beef-NOB medium with 0.3 g and then 0.4 g of NaNO₂, respectively. Nitrite oxidizing reaction was tested by Griess-Ilosvay method [3, 4, 5]. Positive tested suspensions were further isolated by using serial dilution technique spreading on Pep-Beef-NOB agar medium.

2.2 Morphological characteristic and optimal salinity of NOB
The NOB isolates were Gram stained and observed morphology under a light microscope. Salinity tolerance ranging of 0, 5, 10, 15, 20, 25, 30, 35 and 40 ppt were evaluated.

2.3 16S rRNA gene sequencing and phylogenetic analysis
DNA of NOB isolates were extracted by using Phenol/Chloroform method. The 16S rRNA genes were amplified by PCR using 27F and 1492R primers. The DNA sequences were analyzed and compared within the GenBank/EMBL/DDBJ database. Phylogenetic tree was constructed by using MEGA6 program [6].

2.4 Nitrogen removal efficiency of NOB in waste water treatment
Seabass-cultured water was mixed and fermented with fish feed (200 g/40 L) for 3 days before used. NOB was prepared to 10⁷ CFU/ml and then 50 ml (1%) were inoculated into open container of 5 L fermented wastewater. The experiment was studied for 14 days. Every day, wastewater samples were collected in order to examine the quantities of ammonia, nitrite and nitrate. While, the amounts of BOD and COD were determined at days 7 and 14.

3. Results and Discussion

3.1 Isolation and characterization of NOB
Fourteen bacterial strains including 10 of Gram negative and 4 of Gram positive were isolated. However, 2 from 4 Gram positive strains, SF-1 and SF-5, showed a highest nitrite removal rate in preliminary study. Strains SF-1 and SF-5 were rod shape (figure 1) and isolated from sediment samples. Optimal salinity for growth of strains SF-1 and SF-5 were 20-40 ppt and 20-30 ppt, respectively.

Figure 1. Vegetative cells of isolated SF-1 (left) and SF-5 (right). Bar = 5 μm.
3.2. 16S rRNA gene sequence and phylogenetic analyses
Comparison result of partial 16S rRNA gene sequence showed that strain SF-1 has relatedness with Bacillus vietnamensis for 92% similarity. Whereas, strain SF-5 has relatedness with Bacillus firmus for 99% similarity. These result were relevance with phylogenetic tree analysis. It demonstrated strains SF-1 and SF-5 were located into the group of Bacillus and showed evolutionary linear distance from relatedness neighbor species (figure 2).

Figure 2. Phylogenetic tree of 16S rRNA gene of Bacillus strains SF-1, SF-5 and neighbor species. Bar = 0.01.

3.3. Efficiency of waste water treatment
The results showed that experiments of control (no bacteria added), SF-1 and SF-5 have successively remove ammonia from day 1 to day 6. Then ammonia concentration were continuously decreased until day 14. At the end of the study (day 14), ammonia was totally removed by the control, SF-1 and SF-5 for 81.27%, 70.1% and 69.82%, respectively (figure 3).

Figure 3. Ammonium removal efficiency of strains SF-1 and SF-5.
The initial nitrite concentration of fermented wastewater was very low (<1 mg-N/L) when compared with Pep-Beef-NOB medium. This resulted to no more nitrite for assimilation of *Bacillus* strains. However, the nitrite concentration was lowest at day 8 with removal rate 98.73%, 98.3% and 97.24% from control, SF-1 and SF-5, respectively. Nitrite concentration in control experiment was increased again at day 11 and has very high (3.09 mg-N/L) at day 14. Whereas the experiments of SF-1 and SF-5, nitrite was increased at day 13. This suggested that these two NOB isolates could remove and control nitrite better than the control experiment (figure 4).

![Figure 4. Nitrite removal efficiency of strains SF-1 and SF-5.](image)

In case of biological oxygen demand (BOD), the control, SF-1 and SF-5 experiments showed the capable of decreasing for 85.92%, 79.53% and 82.09%, respectively. Likewise, chemical oxygen demand (COD) was reduced by 77.78%, 73.50% and 78.63% in the control, SF-1 and SF-5 experiments, respectively (figure 5-6). Although overall efficiency of wastewater treatment by NOB *Bacillus* SF-1 and SF-5 was no significance with the control experiment. Also, they have eliminated nitrite better than control experiment. This study, we added only 1 time and 1% of NOB inoculum which it may not enough quantity of cells. This resulted to reduce an efficiency of NOB. Therefore, the increasing of initial amount inoculum and frequency of inoculum adding will significant increase an efficiency of wastewater treatment of NOB *Bacillus* isolates.
4. Conclusion
Two NOB *Bacillus* SF-1 and SF-5 were isolated and identified as *B. vietnamensis* and *B. firmus*, respectively. Efficiency of these isolates for wastewater treatment including ammonia, BOD and COD
removal was no significance when compared with control experiment. However, these NOB isolates have ability to remove nitrite better than the control experiment.

**Acknowledgements**
The authors are grateful Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University and Discipline of Excellence for Sustainable Aquaculture, Prince of Songkla University.

**5. References**

[1] Paungfoo, C, Prasertsan, P, Burrell, P C, Intrasungkha, N and Blackall, L L 2007 Nitrifying bacterial communities in an aquaculture wastewater treatment system using fluorescence in situ hybridization (FISH), 16S rRNA gene cloning, and phylogenetic analysis *Biotechnology and Bioengineering* **97**: 985–990

[2] Ma S, Zhang D, Zhang W and Wang, Y 2014 Ammonia stimulates growth and nitrite oxidizing activity of *Nitrobacter winogradskyi* *Biotechnology and Biotechnological Equipment* **28**: 27–32

[3] Lu Y, Wang X, Liu B, Liu Y and Yang X 2012 Isolation and characterization of heterotrophic nitrifying strain W1 *Chinese Journal of Chemical Engineering* **20**: 995–1002

[4] Sangnoi Y, Chankaew S and O-Thong S 2017 Indigenous *Halomonas* spp., the potential nitrifying bacteria for saline ammonium waste water treatment *Pakistan J. Bio. Sci.* **20**: 52–58

[5] Yang X P, Wang S M, Zhang D W and Zhou L X 2011 Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying–denitrifying bacterium, *Bacillus subtilis* A1 *Bioresource Technology* **102**: 854–862

[6] Tamura K, Stecher G, Peterson D, Filipski A and Kumar S 2013 MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729