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Halomonas sp. SKNB4, a Proficient Ammonium Oxidizing Bacterium

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Abstract : Ammonium removal in aquaculture ponds is significant for preventing aquatic animals from ammonium toxicity. Nitrification is biological process of nitrogen transformation performed by two groups of nitrifying bacteria. This process, ammonia is oxidized to nitrite; and nitrite is oxidized to nitrate by ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), respectively. This study, AOB strain SKNB4 was isolated from sediment collected in a Pacific white shrimp farm. A sample was enriched in modified Pep-Beef-AOM medium for 28 days and ammonium removal ability was screened by Griess-Ilosvay method. Morphological, physiological and biochemical characteristics of the strain SKNB 4 included gram negative, rod shape, optimum growth at salinity 2-4% NaCl and pH 6-9, positives for oxidase and catalase tests. The 16S rRNA gene sequencing analysis showed similarity relatedness to *Halomonas aquamarina* of 99.7%. The ammonium removal efficiency of the isolate was determined in modified Pep-Beef-AOM medium (adjusted $(NH_4)_2SO_4$ to 4 g) for 5 days. SKNB4 showed abilities of ammonium removing for 23.32%, nitrite producing for 0.0106 mg-N/L and nitrate producing for 0.0086 mg-N/L. Then, the strain SKNB4 was examined for treating of saline ammonium waste water. Seed culture of *Halomonas* sp. SKNB4 (1% v/v) was inoculated into 4 L of waste water collected from a shrimp farm. After 7 days of experiment, ammonia was removed for 99.96% (decreased from 78.03±0.72 to0.00±7.71 mg-N/L), while nitrite and nitrate were produced for 19.77% and for 42.31%. This suggests that *Halomonas* sp. SKNB4 is an effective ammonium oxidizing bacterium which could be developed for saline ammonium waste water treatment.

Keywords : Nitrification, nitrifying bacteria, ammonium oxidizing bacteria, *Halomonas aquamarina*

Introduction

Shrimp culture has a major sector in the growth of Thailand's economy (Paungfoo *et al*., 2007). To get more productivity, the super-intensive aquaculture system with exceed of feeds and high shrimp density are performed. However, the nutrient discharges from these activities particular nitrogenous compounds (ammonia and nitrite) can be toxic to shrimps (Achuthan *et al*., 2006, Ziembinska *et al*., 2009). Nitrification process by nitrifying bacteria is biological process occurred in the shrimp farm ecosystem. The nitrification process can be converted ammonia to nitrite by ammonium oxidizing bacteria (AOB) and converted nitrite to nitrate by nitrite oxidizing bacteria (NOB) which result to less toxicity (Ahn, 2006). This study aimed to isolate heterotrophic ammonium oxidizing bacteria and determine the nitrogen removal efficiency.

Methodology

1. Isolation and screening of ammonium oxidizing bacterium (AOB)

AOB was isolated from sediment collected from a Pacific white shrimp (*Litopenaeus vannamei*) farm located in Hat Yai, Songkhla province, South of Thailand (GPS location 7.13 N 100.5034 E). One gram of sediment was inoculated into 100 ml of modified Pep-Beef-AOM medium (peptone 5 g, beef extract 2 g, $(NH_4)_2SO_4$ 2.0 g,

K_2HPO_4 0.75 g, NaH_2PO_4 0.25 g, $MgSO_4$ 0.03 g, $MnSO_4$ 0.01 g, sodium citrate 17.8054 g, sea salt 20 g, H_2O 1000 ml, pH 7.0) in 250 ml Erlenmeyer flask and shaken on a rotary shaker at 150 rpm, 28°C for 28 days. Nitrogen oxidizing activity was tested every 3 days by Griess-Ilosvay method. In brief, 5–7 drops of nitrite reagent were dropped into 1 ml of suspension medium and left for 1 minute. Occurrence of red color indicated that a positive for nitrogen oxidation. Then the positive suspension was diluted and spread on fresh modified Pep-Beef-AOM agar medium. Culture strain of AOB was obtained by repeated streaking on the isolation medium (Lu *et al.,* 2012; Yang *et al*., 2011).

2. Morphological and biochemical analysis of AOB

Gram staining and cell morphology of the isolate were observed under a light microscope (Olympus BX50). Catalase activity was tested by bubble formation in 3% H_2O_2 solution. Oxidase activity was tested on test strip (Merck) to examine the oxidation of *N,N*-dimethyl-1, 4-phenylene diammonium dichloride. Optimal salt requirement ranging of 0-9 % NaCl(w/v) and optimal pH ranging of 6-9 of the isolate were evaluated.

3. 16S rRNA gene sequencing and phylogenetic analysis

The genomic DNA of AOB was extracted by using Genomic DNA minikit (Geneaid). The 16S rRNA gene was amplified by PCR using the 16S rRNA gene universal primers of 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′). Amplified PCR product was purified by GF-1 AmbiClean Kit (PCR/Gel) (Vivantis). The DNA sequence was compared with other related sequences by using the BLAST program within the GenBank/EMBL/DDBJ database. Phylogenetic tree was constructed by using MEGA6 program (Tamura *et al*., 2013). A bootstrap value was performed with 1,000 replicatesand phylogenetic tree was determined by using neighbor-joining, maximum-parsimony, and maximum-likelihood.

4. Ammonium removal efficiency of AOB in flask scale

The efficiency of nitrogen removal of AOB isolate was tested for flask scale into synthetic medium. Cell suspension of AOB (1.5 ml) was inoculated into 150 ml of modified Pep-Beef-AOM medium ($(NH_4)_2SO_4$ was adjusted to 4 g) in 250 ml Erlenmeyer flask shaken at 160 rpm, 28°C. After 5 days of cultivation, broth medium was centrifuged at 3,500 rpm, 40 minutes for removing bacterial cells. Supernatant was collected, and then the concentrations of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were measured following by the standard method (Strickland and Parsons, 1972).

5. Ammonium removal efficiency of AOB in large scale

Non-autoclaved saline waste water from a shrimp farm was used to examine an efficiency of nitrogen removal of AOB isolate. Ammonia concentration of waste water was increased by fermenting with shrimp feed for 3 days before used. Cell suspension (1%, v/v) was inoculated into 4 L of prepared waste water and aeration was given throughout the trial period for 7 days. Every day, 200 ml of waste water were collected and evaluated the amounts of ammonium, nitrite, and nitrate.

Results and Discussion

1. Isolation and characteristics of AOB

After re-streaking on isolation medium, the AOB strain SKNB4 was obtained. Then, the morphology and some characteristics of SKNB4 were observed. The strain SKNB4 was Gram negative. The colony on modified Pep-Beef-AOM medium was creamy white (Figure. 1).

Vegetative cells of the strain were rod shape with the size in length of 0.5 x 3 µm. Both catalase and oxidase tests were positives. Strain SKNB4 grew well in optimal salt of 2 – 4 % NaCl (w/v) and optimal pH of 6 – 9 (Table 1).

2. 16S rRNA gene sequence and phylogenetic analysis

The GenBank/EMBL/DDBJ accession number of partial 16S rRNA gene sequence of AOB strain SKNB4 was LC027952. A blast search result of its partial 16S rRNA gene sequence showed a species relatedness with *Halomonas aquamarina* for 99.7 % similarity. Phylogenetic tree analysis demonstrated that the isolate located in *Halomonas* group. The strain formed a linage slightly separated from *H. aquamarina.* (Figure. 2).

3. Efficiency of ammonium removal

After 5 days of flask scale testing, the result provided an evidence of ammonium removal ability of AOB strain SKNB4. The strain showed ammonium removal efficiency of 23.32%, and nitrite and nitrate productions of 0.0106 mg-N/L and 0.0086 mg-N/L, respectively (Table 2). Although an ammonium removal efficiency of the isolate was not high, it performed for high ammonium loaded condition (initial ammonia is 815.88 mg-N/L). Other nitrifying bacteria (AOB) have a good function in low ammonium concentration. For example, *Bacillus subtilis* had reported its ammonium removal efficiency of 58% with an initial ammonium concentration about 104 mg-N/L (Yang *et al*., 2011). While *Alcaligenes* sp. provided maximum ammonium removal of 95% with an initial ammonium concentration about 400 mg-N/L (Lu *et al*., 2012). The initial ammonium concentration is a critical point for ammonium removal efficiency because high ammonium concentration inhibits and toxics to AOB cells (Kim *et al*., 2006). The production of nitrite and particular nitrate in this study was very low. This suggests that most ammonia may be consumed by bacterial cells and less was converted to nitrite.

4. Efficiency of waste water treatment

AOB strain SKNB4 was determined an efficiency of waste water treatment under microcosm condition. After 7 days of treatment, the amount of ammonia was almost absent. Efficiency of ammonium removal was more than 99 % (Table 3.). The experiment could produce nitrite and nitrate for approximate of 19 mg-N/L and 42 mg-N/L, respectively. Occurrence of nitrate indicated that nitrite was oxidized to nitrate by native nitrite oxidizing bacteria (NOB) inhabited in waste water. Another report of tilapia waste water treatment belongs to *Pseudomonas* sp. HBf01 and *Acinetobacter baumannii* HHf01 which have the ammonium removal efficiency after 48 h for 67.9% and 76.7%, respectively (Fan *et al*., 2015). This information suggests that the strain SKNB4 has good proficient for ammonium treatment.

High salinity of waste water from shrimp farm decreased the efficiency of microorganisms for ammonium removing (Guo *et al*., 2013). However, the unique characteristic of genus *Halomonas* is salt loving. This characteristic supported *Halomonas*to use for saline waste water treatment in shrimp farm. Moreover, *H. aquamarina* have proposed as beneficial probiotic for shrimp and used to enhance growth of shrimp larva (Suantika *et al*., 2013; Zhang *et al*., 2009). Therefore, the *Halomonas* sp. SKNB4 might develop to treat ammonium saline waste water and to promote growth of shrimp.

Conclusion

Ammonium oxidizing bacterium strain SKNB4 was isolated from white shrimp farm and was identified as *Halomonas aquamarina*. The *Halomonas* sp. SKNB4 showed ammonium removal efficiencies of 23.32% in flask scale and of 99% in large scale of shrimp waste water treatment for 7 days. This suggests that *Halomonas* sp. SKNB4 is an effective bacterium which might use for saline ammonium waste water treatment.

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Figure 1. Morphology of AOB strain SKNB4

Table 1. Characteristics of AOB strain SKNB4

Strain	Characteristics							
	Color	Shape	Size (μm)	Gram	Oxidase	Catalase	Optimal pH	Optimal salt (%, w/v)
SKNB4	Creamy-white	Rod	0.5 x 3	Negative	Positive	Positive	6-9	2-4

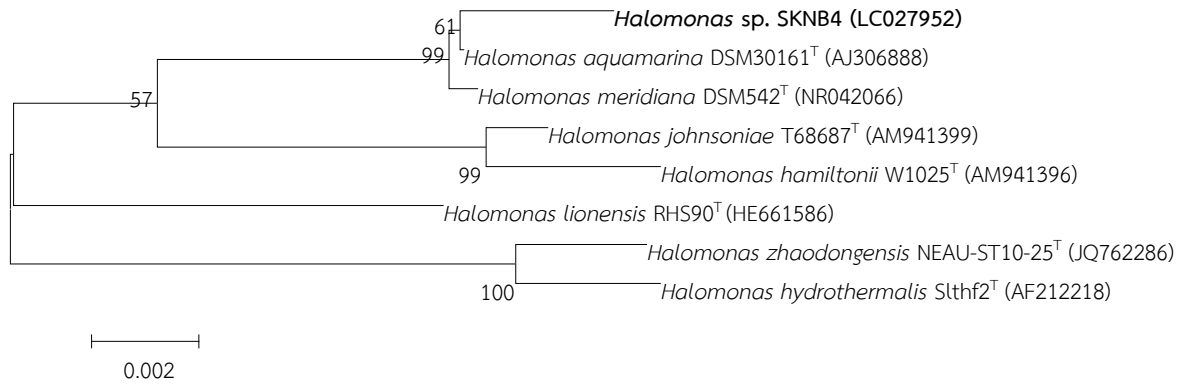

Figure 2. Phylogenetic tree of partial 16S rRNA gene sequence of *Halomonas* sp. SKNB4 and its related species (Bar = 0.002)

Table 2. Ammonium removal efficiency of *Halomonas* sp. SKNB4

Strain	Ammonia				Nitrite (mg-N/L)	Nitrate (mg-N/L)
	Initial (mg-N/L)	Final (mg-N/L)	Removal (mg-N/L)	Removal (%)		
SKNB4	815.88±0.00	625.63±1.66	190.25±1.66	23.32±0.20	0.0106±0.00	0.0086±0.00

Table 3. Efficiency of waste water treatment of *Halomonas* sp. SKNB4

SKNB4	Ammonia				Nitrite (mg-N/L)	Nitrate (mg-N/L)
	Initial (mg-N/L)	Final (mg-N/L)	removal (mg-N/L)	removal (percent)		
Day 0	78.03±0.72	-	-	-	0.01±0.00	0.00±0.00
Day 7	-	0.00±7.71	77.98±0.76	99.96±0.06	19.78±1.28	42.31±6.05