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Contributed Paper

Nitrogen Removal Efficiency of Salt-tolerant Heterotrophic Nitrifying Bacteria

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ABSTRACT

Salt-tolerant heterotrophic nitrifying bacteria including strains SKNB1, SKNB2, SKNB4, and SKNB7 were collected and isolated from Pacific white shrimp farm. Strain SKNB1 was rod shape, gram positive, and endospore formation. SKNB2 and SKNB4 were rod shape. SKNB7 was coccus shape. Strains SKNB2, SKNB4, and SKNB7 were gram negative and did not form endospore. The partial 16S rRNA gene sequencing analysis with similarity range of 92-97% indicated that four strains were identified as *Bacillus* sp. (SKNB1), *Halomonas* sp. (SKNB2 and SKNB4) and *Psychrobacter* sp. (SKNB7). These isolates were halophilic heterotrophic bacteria and showed nitrification characteristic. They could eliminated high strength of initial ammonia concentration (815.86 mg-N/L) under saline condition. Ammonium removal efficiency of *Halomonas* sp. SKNB2, *Psychrobacter* sp. SKNB7, *Bacillus* sp. SKNB1 and *Halomonas* sp. SKNB4 were 56.18%, 42.35%, 42.28%, and 23.32%, respectively. Also, nitrite producing ability of SKNB2, SKNB1, SKNB7, and SKNB4 were 0.0751, 0.0172, 0.0169, and 0.0106 mg-N/L, respectively. While very low nitrate presentation by SKNB4 and SKNB1 (0.0086 and 0.0035 mg-N/L, respectively) and no nitrate observation was done for SKNB2 and SKNB7. Based on most ammonium removal ability, these nitrifying bacteria could be suggest as ammonium oxidizing bacteria (AOB) and they might proficient for high ammonium saline wastewater treatment.

Keywords: salt-tolerant heterotrophic nitrifying bacteria, halophilic heterotrophic bacteria, initial ammonia concentration, ammonium removal efficiency, ammonium oxidizing bacteria (AOB)

1. INTRODUCTION

Coastal aquacultures, especially marine shrimp culture has been practiced in Thailand for about three decades [1]. Shrimp culture has been moved to intensive aquaculture system with higher of shrimp density and feed supplementary. Closed or semi-closed aquaculture systems are conducted together with intensive aquaculture in order to avoid risks of pathogenic infections. These closed and semi-closed systems, water in shrimp

ponds will have no or less contacted to outside environments. However, closed aquaculture system makes an accumulation of wastes produced from shrimp excretion and exceeded feed residues. Serious toxic of nitrogen compounds in shrimp pond are ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-). High concentration of nitrogen compounds will cause low growth rate, inhibits molting, makes high stress, decreases immunity, and risks to disease infections of aquatic animals [2]. Moreover, nitrogen compounds also causes pollution, eutrophication, and biotoxin production by phytoplankton in aquatic ecosystems [3]. In order to solve the problem of nitrogen compounds, nitrification is the biological solution and process of nitrogen transformation. During the process, ammonia will be oxidized to nitrite, and then nitrite will be oxidized to nitrate. These processes are involved two different groups of nitrifying bacteria. First, ammonium oxidizing bacteria (AOB) such as *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosovibrio*, and *Nitrosolobus* which further oxidize ammonia to nitrite. Another group of nitrite oxidizing bacteria (NOB) such as *Nitrobacter, Nitrospira, Nitrospina, Nitrococcus,* and *Nitrocystis* will oxidize nitrite to nitrate. Nitrate has lower toxicity than ammonia [4]. Generally, nitrification is occurred in most aquatic ecosystems both fresh, brackish, and marine environments [1, 5]. However, coastal aquacultures have very large scales in the world both space and economic areas. Therefore, attempting for effective salt-tolerant nitrifying bacteria isolation is very important. Recently, several isolations of salt-tolerant or halophilic AOB and NOB from saline environments were reported. For example, *Nitrosomonas* spp. and *Nitrospira* spp. were isolated and obtained from marine aquacultures and samples [5-7]. Moreover, there were reports of finding some other halophilic nitrifying groups such as *Halomonas* and *Alcaligenes* [8-11]. These literatures gave evidences of nitrifying bacteria diversity which authors might be able to isolate some of these untouchable before. This study aimed to isolate salt-tolerant heterotrophic nitrifying bacteria and to determine their efficiency of nitrogen removal.

2. MATERIALS AND METHODS

2.1 Isolation and Screening of Nitrifying Bacteria

Salt-tolerant heterotrophic nitrifying bacteria were isolated from sediment and water samples collected from Pacific white shrimp (*Litopenaeus vannamei*) farm located in Hat Yai, Songkhla province, South of Thailand (GPS location 7.13 N 100.5034 E). The water salinity in shrimp farm was 20 ppt (part per thousand). The samples of water (1 ml) and sediment (1 g) were separately 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. Both samples were individually shaken on a rotary shaker at 160 rpm, 28°C in order to enrich salt-tolerant nitrifying bacteria. Nitrogen oxidizing (nitrite/nitrate producing) was tested every 3 days by Griess-Ilosvay method [12]. Nitrite reagent (5-7 drops) was dropped into 1 ml of suspension medium and left for 1 minute. Red color produced from the observation will indicate the positive test (nitrite/nitrate producing). No color changed will indicate the negative test (no nitrite/nitrate producing). Then the suspension of positive sample was diluted and transferred to modified Pep-Beef-AOM agar medium. Purified nitrifying bacteria were obtained after 3-4 times of re-streaking.

2.2 Efficiency of Nitrogen Removal of Isolated Nitrifying Bacteria

Heterotrophic nitrifying bacteria were tested for the efficiency of nitrogen removal in flask scale. Bacterial suspension (1.5 ml of 10^9 CFU/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 for 40 minutes in order to remove 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 colorimetric method [13]. Ammonium concentration was measured by mixing together of supernatant (10 ml), phenol solution (0.5 ml), sodium nitroprusside solution (0.5 ml), oxidizing solution (1 ml) and sodium hypochlorite solution (5 ml) in culture tube. After allowing the tube for 1 hour, sample solution was measured by spectrophotometer at 640 nm. Nitrite concentration was measured by mixing of supernatant (50 ml) and sulphanilamide solution in 125 ml Erlenmeyer flask for 2-8 minutes. Then, naphthylethylenediamine solution (1 ml) was added and immediately mixed between 10 minutes to 2 hours before measuring by spectrophotometer at 540 nm. For nitrate concentration, ammonium chloride (2 ml) was added into supernatant (100 ml) in 250 Erlenmeyer flask. Sample solution was loaded into cadmium column and 40 ml of solution which passed through the column were drained. Later 50 ml of solution were collected in the collection tube. Then, sample was added by sulphanilamide solution (1 ml) and after 2-8 minutes, sample was mixed by naphthylethylenediamine solution. After sample standing between 2 minutes and 2 hours, nitrate concentration was measure by spectrophotometer at 543 nm.

2.3 Morphological and Biochemical Analysis

Gram staining and cell morphology of the isolates 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 observe the oxidation of *N,N*-dimethyl-1, 4-phenylene diammonium dichloride. Optimal salt requirement of 0-40 ppt was examined.

2.4 16S rRNA Gene Sequence and Phylogenetic Analysis

The genomic DNA of the nitrifying bacteria was extracted by using Genomic DNA minikit (Geneaid). The 16S rRNA genes were amplified by PCR by using the 16S rRNA gene universal primers of 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (52-TACCTTGTTACGACTT). The amplified PCR products were purified by GF-1 AmbiClean Kit (PCR/Gel) (Vivantis). Sequencing reactions were performed with the same of that universal primers by First BASE Laboratories Sdn Bhd's Manufacture. Partial DNA sequences were compared with related sequences by using the BLAST program within the GenBank/EMBL/DDBJ database. Multiple alignments of the 16S rRNA gene sequences were carried out by using the CLUSTAL_X program version 1.83 [14]. Nucleotide substitution rates (K_{nuc} values) were determined, and phylogenetic tree was constructed by using MEGA6 program [15]. A bootstrap value was performed with 1,000 replicates, and phylogenetic tree was determined by using neighbor-joining, maximum-parsimony, and maximum-likelihood.

2.5 Nucleotide Sequence Accession Numbers

The 16S rRNA gene sequence data of all strains were submitted at DNA Data Bank of Japan (DDBJ). The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences of the strains SKNB1, SKNB2, SKNB4 (=TBRC 4995^T), and SKNB7 are LC027949, LC027950, LC027952, and LC027953, respectively.

3. RESULTS AND DISCUSSION

3.1 Isolation of Salt-tolerant Heterotrophic Nitrifying Bacteria

Four salt-tolerant heterotrophic nitrifying bacteria were isolated from 7 specimens including waters (4 samples) and sediments (3 samples) collected from Pacific white shrimp farm. All isolates showed positive results for nitrogen oxidizing with red color reaction of nitrite reagent. After re-streaking on modified Pep-Beef-AOM agar medium, four purified nitrifying bacteria including SKNB1, SKNB2, SKNB4, and SKNB7 were obtained. The modified Pep-Beef-AOM medium which contained of peptone, beef extract, ammonium sulfate ($(NH_4)_2SO_4$), and sea salt was used to isolate salt-tolerant heterotrophic nitrifying bacteria for this study. Peptone and beef extract were rich nutrient sources of heterotrophic bacteria while ammonium sulfate would help to get rid those unwanted and to receive the nitrifying bacteria desired. Normally, ammonia is an inhibitant to limit cells growth of general bacteria. Therefore, adding of ammonium sulfate into isolation medium will then inhibit unwanted non-nitrifying bacteria and will enhance desired nitrifying bacteria. Then an ammonium sulfate and supplementation of sea salt were proper to isolate salt-tolerant nitrifying bacteria. During isolation process, it was found that some colony showed negative test for nitrite/nitrate reagent could grow in Pep-Beef-AOM medium. This indicated that those bacteria could not transform ammonia to nitrite and further nitrate. They might only tolerate to ammonium sulfate and utilize peptone and beef extract as carbon and nitrogen sources. Another reason of negative nitrite/nitrate test, the bacteria may completely converted nitrate to gaseous nitrogen. During a process of nitrogen gas converting, however, remained nitrite and nitrate should be detected. There is a report indicated that if cultivation was left longer days, the unwanted heterotrophic bacteria could be eliminated because of organic nutrient depleting [11]. Moreover, using of continuous or fed-batch culture techniques which adding of ammonium sources during cultivation would help to promote the growth of slow-growing nitrifying bacteria quite well.

3.2 Identification and Characterization of Nitrifying Bacteria

The morphology and some characteristics of nitrifying bacterial isolates were determined. Strains SKNB1 and SKNB4 were shape rods with the sizes in length of 0.5 × 2 μm and 0.5 × 3 μm, respectively, whereas SKNB2 was short rod with the size in length of 0.5 × 2 μm. Another strain SKNB7 was coccus with the size of 0.5 μm. The colony colors after 48 hr. incubation on modified Pep-Beef-AOM agar medium of strains SKNB2, SKNB4, and SKNB7 were white, creamy white, and opaque white, respectively, while strain SKNB1 was opaque yellow. Only SKNB1 was gram positive with endospore formation whereas other isolates were gram negative without endospore formation (Figure 1). Catalase tests of all strains were positive. Oxidase tests were positive for SKNB2 and SKNB4, and negative for

SKNB1 and SKNB7. Table 1 showed that strains SKNB4 and SKNB7 have grown well with optimal salt requirement of 30-40 ppt so that these strains should be considered to meso-halophilic nitrifying bacteria. This meso-halophilic isolation revealed the same investigations with other studies such as *Nitrosomonas* spp. and *Nitrobacter alkalicus* [5, 16]. A blast search result of partial 16S rRNA gene sequences of four nitrifying bacteria showed 3 genera relatedness including *Bacillus*, *Halomonas*, and *Psychrobacter*. Strains SKNB1 had revealed a similarity of 97% to *Bacillus aryabhattai* B8W22^T (EF114313), SKNB2 and SKNB4 revealed similarity of 93-94% to *Halomonas aquamarina* 2PR52-11^T (EU440965), and SKNB7 revealed similarity of 92% to *Psychrobacter marincola* aa-33^T (EU652050). Identification result of partial 16S rRNA gene sequences was relevant to the phylogenetic tree analysis (Figure 2). This also demonstrated that the isolates were located in different three clusters of heterotrophic bacteria including *Bacillus*, *Halomonas*, and *Psychrobacter*. Surprisingly, the most famous nitrifying bacteria such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus*, *Nitrospira*, *Nitrococcus*, and *Nitrobacter* were not found in this study. While other heterotrophic nitrifiers (*Bacillus*, *Halomonas*, and *Psychrobacter*) were found instead. These because modified Pep-Beef-AOM agar medium was more suitable for fast-growing heterotrophic nitrifying bacteria than slow-growing autotrophic nitrifying bacteria. Although our 3 genera were quite different from famous nitrifiers, these 3 genera have joined characteristics and ability of nitrogen removal. Previously, *Bacillus* spp. were reported for their nitrogen removal characteristics. However, the isolating sources of these *Bacillu*s were come from other places such as the municipal waste water, bioreactor and soil which were not from the saline environments [12, 17-18]. In 1980, the first *Halomonas* (*H. elongata*) was isolated and described as salt-tolerant bacteria [19]. *H. elongata* could reduce nitrate to nitrite which was described as denitrifying bacteria. Those denitrifying bacteria or denitrifiers played important roles in denitrification which was the biological process that continuous by reduced nitrate product from nitrification process to free nitrogen (N_2). In addition, other halophilic *Halomonas* including *H. fontilapidosi*, *H. cerina*, *H. denitrificans*, *H. korlensis*, and *H. shengliensis*, were also reported as denitrifying bacteria [8-10, 20-21] while *H. campisalis* showed both nitrification and denitrification characteristics [22]. Genus *Psychrobacter* was proposed as psychrophilic bacteria which grew at the temperature lower than 4 °C and also at that higher than 20 °C. *Psychrobacter* could be found in various marine environments [23-24]. However, there is rare publication that described *Psychrobacter* as denitrifying bacteria [25] when nitrifying *Psychrobacter* has not been reported.

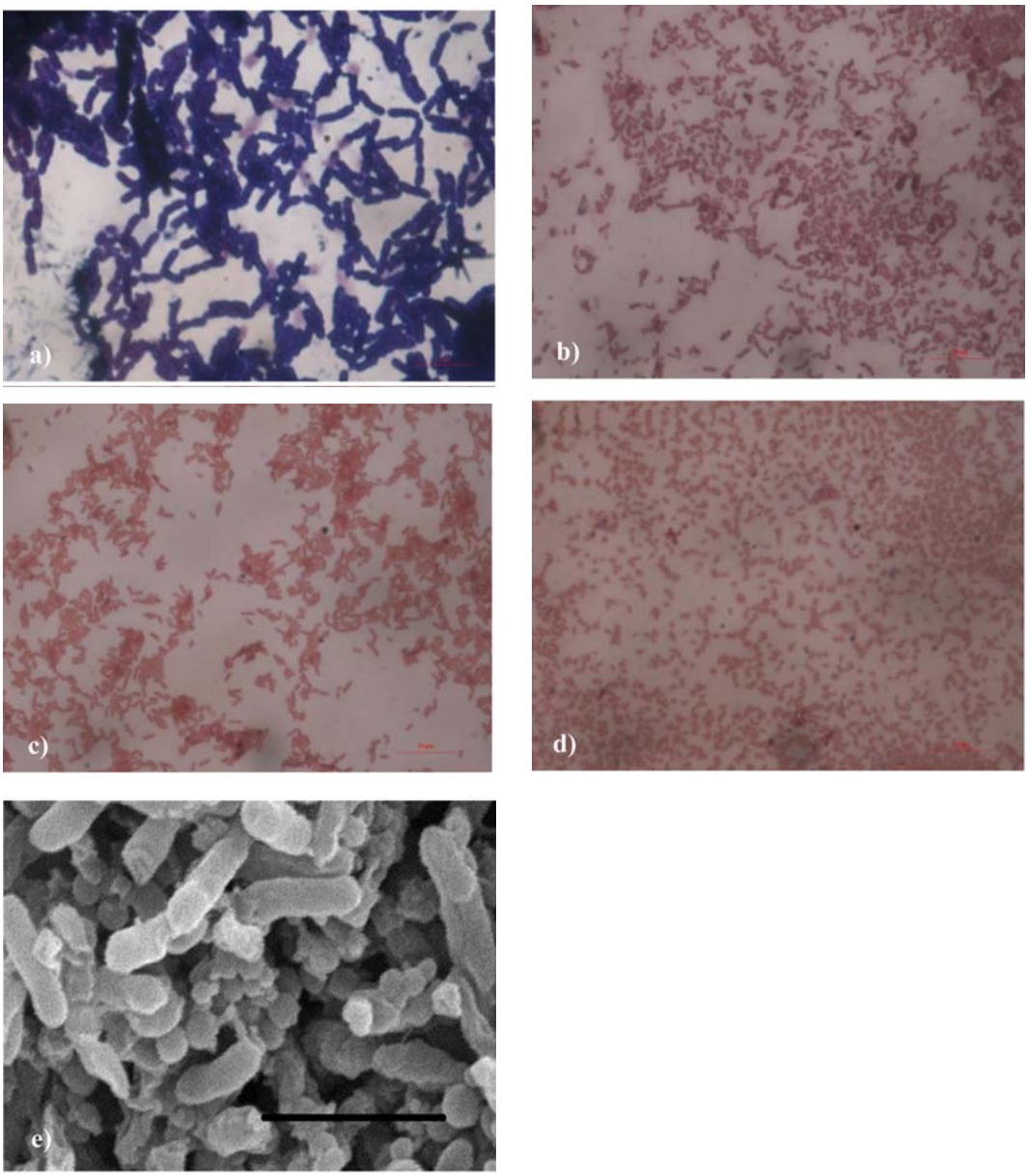

Figure 1. Vegetative cell morphology of nitrifying bacteria; a) SKNB1; b) SKNB2; c) SKNB4; d) SKNB7 and e) scanning electron micrograph of SKNB4. (a, b, c, d; bars = 10 µm, e; bar = 2 µm).

Table 1. Phenotypic characteristics of the nitrifying bacteria isolates.

Characteristic	SKNB1	SKNB2	SKNB4	SKNB7
Cell shape	Rod	Short rod	Rod	Coccus
Size in length (μm)	0.5 × 2	0.5 × 2	0.5 × 3	0.5
Colony color	Opaque yellow	White	Creamy white	Opaque white
Endospore forming	Yes	No	No	No
Gram's stain	Positive	Negative	Negative	Negative
Oxidase	Negative	Positive	Positive	Negative
Catalase	Positive	Positive	Positive	Positive
Salt requirement (ppt)	10-25	10-20	25-30	35-40

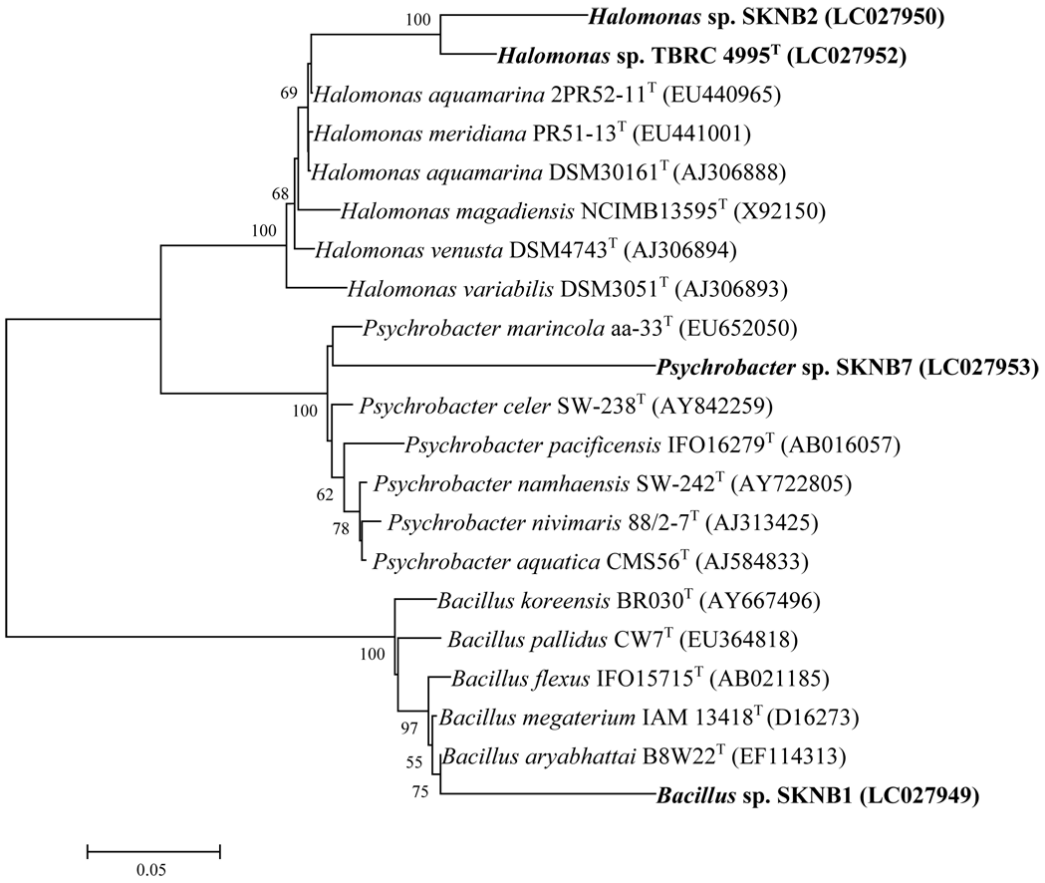

Figure 2. Phylogenetic tree of partial 16S rRNA gene sequences of salt-tolerant heterotrophic nitrifying bacteria and related species (Bar = 0.05).

3.3 Nitrogen Removal Efficiency

In the course of nitrogen removal efficiency study, the concentration of ammonia, nitrite, and nitrate were examined. The results showed that *Halomonas* sp. SKNB2 had the highest ammonium removal efficiency with 56.18% followed by *Psychrobacter* sp. SKNB7, *Bacillus* sp. SKNB1, and *Halomonas* sp. SKNB4 for the ammonium removal efficiency of 42.35%, 42.28%, and 23.32%, respectively (Figure 3). Noteworthy, the initial ammonium concentration of this study was very high (815.86 mg-N/L) when compared with other studies. For examples, *Bacillus subtilis* from another study was reported of 36.3-2.3% ammonium removal efficiency which initial ammonium concentration was 105.58-536.21 mg/L [12]. Ammonium removal efficiency of *Alcaligenes* sp. was around 80% with 437.47 mg/L initial ammonium concentration [11]. These evidences suggested that initial ammonium concentration was very important for ammonium removal ability of microorganisms because initial ammonia was toxic to microbial cells [26]. Generally, nitrifying bacteria had high ability of ammonium removal efficiency at low level of initial ammonium concentration, the ability would be decreased or limited when increasing of initial ammonium concentration [11]. This suggested that the isolates of this study could benefit for treatment of high ammonium loaded saline wastewater. In case of *Halomonas* spp., most literatures reported that *Halomonas* spp. was denitrifier [8-10, 20] while *H. campisalis* exhibited higher denitrification than nitrification abilities [22]. Also, most published *Psychrobacter* spp. had no involvement into nitrogen removal process excepted for *Psychrobacter* sp. TSBY-70 described as denitrifier [25]. According to these evidences, four nitrifying bacterial isolates in this study might have potential to develop for group of effective salt-tolerant heterotrophic nitrifying bacteria. Figure 3 indicated that SKNB2 had the highest efficiency to transform ammonium to nitrite (0.0751 mg-N/L) followed by strains SKNB1, SKNB7, and SKNB4 for which the nitrite concentration of 0.0172, 0.0169, and 0.0106 mg-N/L, respectively. Even most of nitrate concentrations were very low detected; only strains SKNB4 and SKNB1 had produced nitrates for 0.0086 and 0.0035 mg-N/L, respectively. However, nitrates from SKNB2 and SKNB7 were undetectable. This suggested that these four isolates slightly could or could not convert nitrite to nitrate. Another possibility, nitrate could convert to free nitrogen via aerobic-denitrification process. Measurement of nitrogen gas in experimental flasks should be further studied to prove an assumption. The phenomenon of less nitrite and nitrate might indicate that ammonium was used for cell growth more than being conversed to nitrite. In contrast, another study of *B. subtilis* showed good nitrate production [12]. Two steps of nitrification process were carried on by two groups of nitrifying bacteria. First step, ammonium was oxidized to nitrite by ammonium oxidizing bacteria (AOB). Second step, nitrite was oxidized to nitrate by nitrite oxidizing bacteria (NOB). Therefore, all four isolates in this study might have a possibility to be ammonium oxidizing bacteria (AOB) instead. They are especially expected to be development of effective ammonium oxidizers for saline wastewater treatment. However, in order to complete a nitrification process, the NOB should be further isolated and observed the nitrogen removal abilities. Some studies suggested that using of nitrifying consortia would be beneficial and good for water quality improvement [2, 27-28]. Therefore,

the isolates of this study should be investigated further for combining as nitrifying consortia in order to help for the improvement of water quality in shrimp farm.

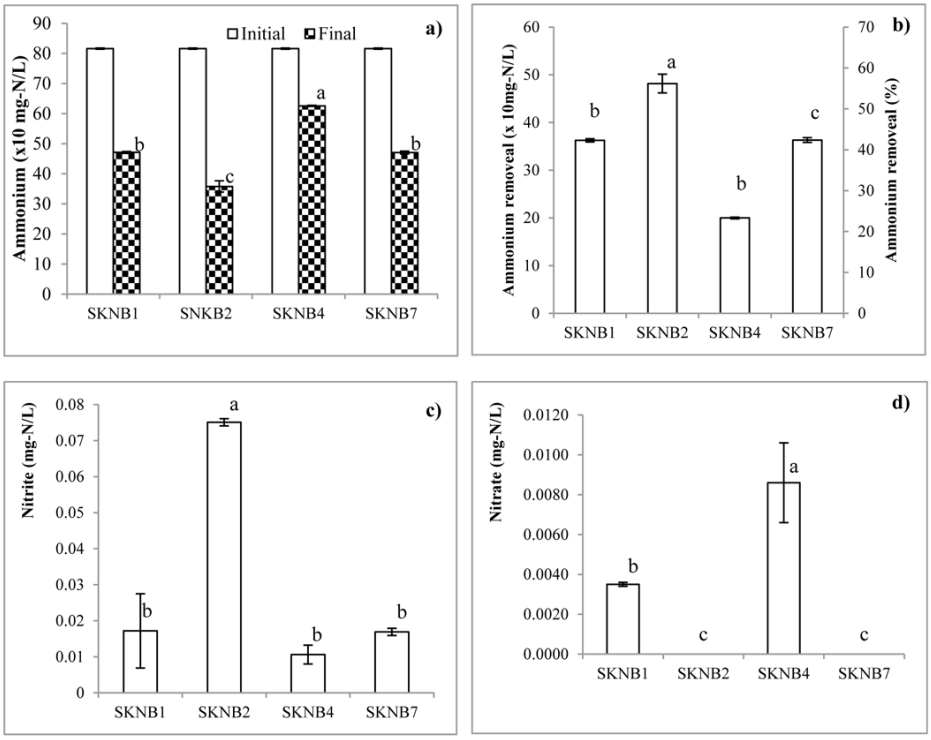

Figure 3. Nitrogen removal efficiency; a) Initial and final ammonium concentration (mg-N/L), b) Ammonium removal efficiency (%), c) Nitrite concentration (mg-N/L), d) Nitrate concentration (mg-N/L).

4. CONCLUSIONS

These finding suggest that the nitrifying bacterial isolates including genera *Bacillus*, *Halomonas*, and *Psychrobacter* were salt-tolerant heterotrophic nitrifying bacteria. The result of nitrogen removal efficiency indicated that all isolates could be the ammonium oxidizing bacteria (AOB) which they responded to high ammonium concentration. So, they might benefit for treatment of high ammonium loaded saline wastewater.

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