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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2017.52.58

Research Article
Indigenous *Halomonas* spp., the Potential Nitrifying Bacteria for Saline Ammonium Waste Water Treatment

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Abstract

Background and Objective: Toxic nitrogen compounds are one cause decreasing of shrimp production and water pollution. Indigenous *Halomonas* spp., isolated from Pacific white shrimp farm are benefitted for saline ammonium waste water treatment. This study aimed to isolate the heterotrophic-halophilic *Halomonas* spp. and investigate their ammonium removal efficiency. **Materials and Methods:** *Halomonas* spp., were isolated by culturing of samples collected from shrimp farm into modified Pep-Beef-AOM medium. Ammonium converting ability was tested and monitored by nitrite reagent. Ammonium removal efficiency was measured by the standard colorimetric method. Identification and classification of *Halomonas* spp., were studied by morphological, physiological and biochemical characteristics as well as molecular information. **Results:** There were 5 strains of heterotrophic-halophilic nitrifying bacteria including SKNB2, SKNB4, SKNB17, SKNB20 and SKNB22 were isolated. The identification result based on 16S rRNA sequence analysis indicated that all 5 strains were *Halomonas* spp., with sequence similarity values of 91-99 %. Ammonium removal efficiency of all strains showed a range of 23-71%. The production of nitrite was low detected of 0.01-0.15 mg-N L^{-1}, while the amount of nitrate was almost undetectable. **Conclusion:** This might suggest that the indigenous *Halomonas* spp., as nitrifying bacteria involved biological nitrification process for decreasing and transforming of ammonia. Due to being heterotrophic, halophilic and ammonium removing bacteria, these *Halomonas* spp., could be developed for use in treatment of saline ammonium waste water.

Key words: Toxic nitrogen compounds, indigenous *Halomonas* spp., saline ammonium waste water, ammonium removal efficiency, heterotrophic-halophilic nitrifying bacteria

Received: September 02, 2016 Accepted: October 19, 2016 Published: December 15, 2016

Citation: Yutthapong Sangnoi, Sunipa Chankaew and Sompong O-Thong, 2017. Indigenous *Halomonas* spp., the potential nitrifying bacteria for saline ammonium waste water treatment. Pak. J. Biol. Sci., 20: 52-58.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In aquatic ecosystems, the accumulation of nitrogen compounds such as ammonia, nitrite and nitrate are always found. These compounds come from the excretion of aquatic animals, exceed feeding (aquaculture) and discharge from terrestrial environments. Nitrogen compounds (especially ammonia and nitrite) with high concentration are most toxic to any aquatic organisms and can cause eutrophication in aquatic ecosystems[1,2]. Intensive aquaculture of marine shrimps in Thailand and other countries is much raised. Economic value of shrimp production is very high[3]. However, the effects of nitrogen compound that occurrence in shrimp ponds are serious problem for decreasing of shrimp production[1]. One of reducing method for nitrogen compounds in shrimp pond is partial changing of water. This method not only increases cost, but also leads to spread and exchange of shrimp pathogens between shrimp ponds and environments as well as makes the water pollution[1]. Therefore, treatment of nitrogen compounds in shrimp ponds without water exchanging is required for shrimp aquaculture. Another, mild way for nitrogenous waste water treating is nitrification process performed by nitrifying bacteria. Nitrification is a biological process involving the elimination of nitrogen compounds. This process is conducted in 2 steps, firstly, ammonia (NH_3) is converted to nitrite (NO_2^-) and, secondly, nitrite is transformed to nitrate (NO_3^-) which has low toxicity for organisms[4]. Nitrifying bacteria are key microorganisms that play an important role in the two steps of the nitrification process. Two different groups of nitrifying bacteria including Ammonium Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB) are involved in ammonium oxidation and nitrite oxidation, respectively. Bacteria with the group of *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus* and the group of *Nitrobacter*, *Nitrococcus*, *Nitrospira* are most commonly recognized as the groups of AOB and NOB, respectively[4]. However, these bacteria are autotroph. They are slow-growing and have less competition with other heterotrophic nitrifying bacteria. In addition, they also were inhibited by high concentration of ammonia[5]. As mention of problems by toxic nitrogen occurred in marine shrimp farm, the indigenous nitrifying bacteria which have the characteristics of nitrogen removal, fast-growing and salt-tolerance should be studied. The bacteria genus *Halomonas* has a unique salt-loving characteristic. They are commonly found in saline environments in both terrestrial and aquatic ecosystems[6,7]. Genus *Halomonas* is Gram-negative, heterotrophic, fast-growing and salt-tolerant bacteria. Moreover, there are reports indicated that *Halomonas* is nitrifying bacteria involving in nitrification process. However, most previous publications of *Halomonas* are focused on taxonomic and systematic studies for proposing the novel species[6,7]. The investigation on application for water improvement, especially saline waste water treatment from marine shrimp farm is very rare. Therefore, this study aimed to isolate indigenous heterotrophic-halophilic *Halomonas* spp. and to investigate their nitrogen removal efficiency for saline waste water.

MATERIALS AND METHODS

Isolation of nitrifying bacteria: A modified Pep-Beef-AOM liquid medium,which contained peptone 5 g, beef extract 3 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 and H_2O 1000 mL (adjusted pH to 7.0) was used to enrich and isolate the heterotrophic-halophilic nitrifying bacteria. Autoclaved isolation medium (100 mL) was inoculated with each 1% of water (v/v) and sediment (w/v) collected from Pacific white shrimp farm. Samples were incubated and shaken on a rotary shaker at 28°C, 150 rpm for 14 days. After incubation, the ammonium consumption was tested by the Griess-Ilosvay method. In brief, 1 mL of enrichment culture was spot-tested with 5-7 drops of nitrite reagent. A positive test with a red color reaction was spread on a freshly modified Pep-Beef-AOM agar medium. Purified cultures were obtained by repeated streaking on the isolation medium[8,9].

Identification and classification of nitrifying bacterial isolates: Morphological, physiological and biochemical characteristics of the isolates were measured. Gram's stain and vegetative cell morphology were studied under a light microscope (Olympus BX50). Scanning electron micrograph was examined by using scanning electron microscope (JEOL JSM-5800). Oxidase activity was tested on test strip (Merck) and catalase activity was tested with a 3% H_2O_2 solution. Salt requirement of the isolates was evaluated for the NaCl concentration ranging of 1-9% (w/v). The biochemical characteristics were determined by the commercial API 20E system (BIOMERIEUX). Genomic DNA of the isolates were extracted using Genomic DNA mini kit (Geneaid Biotech Ltd., Taiwan), in accordance with manufacturer instructions. The 16S rRNA gene was amplified by PCR (DNA Engine Dyad® Thermal Cycler, Bio-Rad, USA) using primers of 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and 1500R (5'-GTT ACC TTG TTA CGA CTT-3'). Each total 100 µL of PCR reaction mixture contained DNA template (15-20 ng), each primer (2.0 µmol), Taq polymerase (2.5 U), $MgCl_2$ (2.0 mM), dNTP (0.2 mM), 10x Taq buffer (10 µL) and ddH_2O.

The PCR amplification program followed, initial denaturation step (94°C for 3 min) and then 25 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and elongation (72°C for 2 min) and subsequently the final amplification step (72°C for 3 min). The PCR products were purified using a GeneFlowTM Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). Direct sequencing of the purified PCR products was performed on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA) by sequencing service provider. The 16S rRNA gene sequences of the isolates were compared with other microorganisms using the Basic Local Alignment Search Tool program (BLAST) within the GenBank/EMBL/DDBJ database. Phylogenetic tree of partial 16S rRNA gene sequences of the isolates and neighbor species was constructed by MEGA 6 program[10].

Ammonium removal efficiency of bacterial strains: Purified isolates of nitrifying bacteria were examined for the efficiency of ammonium removal. Bacterial cell suspension (1% v/v) of 10^9 CFU mL^{-1} was inoculated into 150 mL of modified Pep-Beef-AOM medium in which $(NH_4)_2SO_4$ was adjusted to 4 g. The cultivation flask was shaken at 160 rpm, 28°C for 5 days. After cultivation, the cultured broth medium was centrifuged at 3,500 rpm, for 40 min to remove cell suspension. Supernatant was collected and then the existent ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were measured by the standard colorimetric method[11].

RESULTS AND DISCUSSION

Isolation and characteristics of heterotrophic nitrifying bacteria: After several cycles of re-streaking on modified Pep-Beef-AOM agar medium, 5 strains of nitrifying bacteria including SKNB2, SKNB4, SKNB17, SKNB20 and SKNB22 were obtained. The characteristic features of the isolates were concluded in Table 1. The vegetative cell shapes of the strains are defined, strains SKNB2 and SKNB22 were short rod, SKNB4 was rod, whereas SKNB17 and SKNB20 were coccus shapes. Scanning electron micrograph of representative strain SKNB4 showed rod shape with blunt ends (Fig. 1). All strains were Gram negative bacteria. Oxidate test was positive for all strains, while catalase test was positive for strains SKNB2 and SKNB4 and negative for strains SKNB17, SKNB20 and SKNB22. They grew in optimal pH range of 6-9. All strains required salt for growth and tolerated salt for 1-9% NaCl (w/v). Strain SKNB2 grew well with salt concentration of 1-2% NaCl (w/v), whereas the other four strains grew with a higher salt concentration of 2-4% NaCl (w/v).

Table 1: Characteristics of *Halomonas* spp., strains

Characteristics	SKNB2	SKNB4	SKNB17	SKNB20	SKNB22
Isolation source	Shrimp farm water	Shrimp farm sediment	Shrimp farm water	Shrimp farm sediment	Shrimp farm water
Shape	Short rod	Rod	Coccus	Coccus	Short rod
Pigmentation	White	Cream-white	Cream-white	Cream-yellow	Cream-yellow
Gram's stain	Negative	Negative	Negative	Negative	Negative
Optimal pH	6-9	6-9	6-9	6-9	6-9
Optimal salt (%, w/v)	1-2	2-4	2-3	2-4	2-4
Oxidase	+	+	+	+	+
Catalase	+	+	-	-	-
β-galactosidase	-	-	+	+	-
Arginine dihydrolase	+	-	+	+	-
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	+	-	+	+	+
Citrate utilisation	+	+	+	+	-
H_2S production	-	-	-	-	-
Urea hydrolysis	-	-	-	+	+
Tryptophan deamination	+	+	+	+	+
Indole production	-	-	-	-	+
Acetoin production	+	+	+	+	+
Gelatin hydrolysis	+	-	-	-	+
Glucose fermentation	+	-	+	+	-
Mannitol	+	-	+	+	-
Inositol	-	-	-	-	-
Sorbitol	+	-	+	+	-
Rhamnose	+	-	+	+	+
Sucrose	+	-	+	+	-
Melibiose	+	-	+	+	+
Amygdalin	+	-	+	+	+
Arabinose	+	-	+	+	+

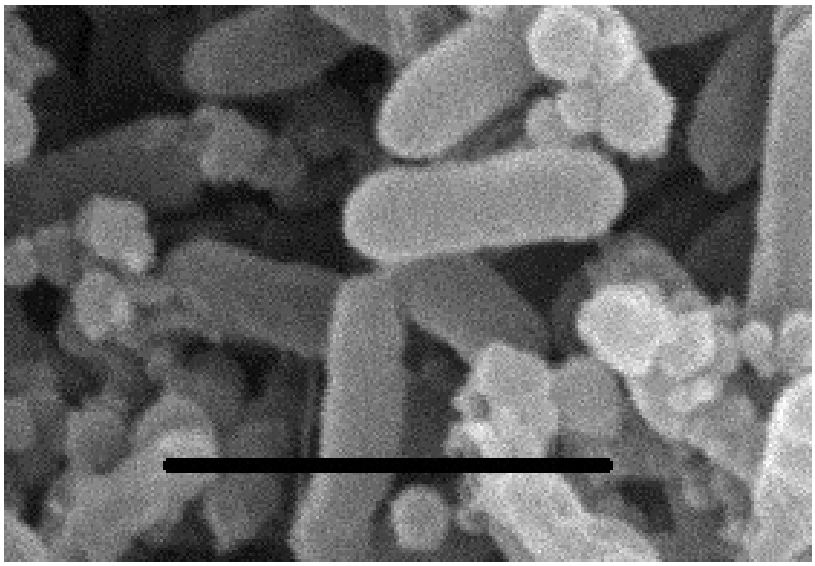

Fig. 1: Scanning electron micrograph of *Halomonas* sp., SKNB4. Bar = 2 µm

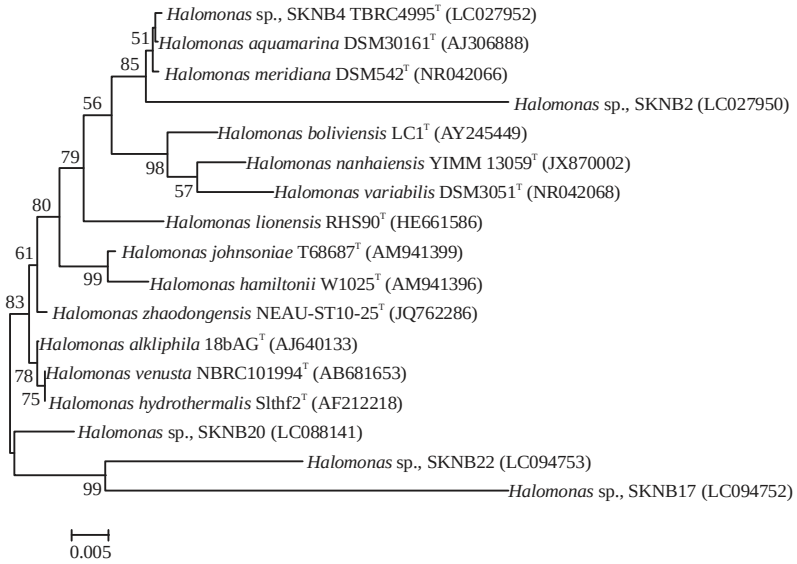

Fig. 2: Phylogenetic tree of partial 16S rRNA gene sequences of *Halomonas* spp., isolates and related species (Bar = 0.005)

Identification and phylogenetic tree analysis: The partial 16S rRNA gene sequence data of the isolates were analyzed and submitted at DNA Data Bank of Japan (DDBJ). The accession numbers for the 16S rRNA gene sequences of the strains were SKNB2, SKNB4, SKNB17, SKNB20 and SKNB22 are LC027950, LC027952, LC094752, LC088141 and LC094753, respectively. All partial sequences were blasted in DNA database (NCBI). The result showed similarities with the genus *Halomonas*. Strain SKNB4 showed a sequence similarity of 99% to *H. aquamarina*, while SKNB2 showed 94% similarity with *H. aquamarina*. Strains SKNB20, SKNB22 and SKNB17 showed species relatedness to *H. venusta* with sequence similarities of 96, 94 and 91%, respectively. The sequence similarity result was relevant with their phylogenetic tree analysis. Phylogenetic tree analysis demonstrated 2 clusters of the isolates. Strains SKNB4 and SKNB2 located in *H. aquamarina* branch. Whereas strains SKNB17, SKNB20 and SKNB22 formed a linage distantly separated from the clade of *H. venusta*, *H. hydrothermalis* and *H. alkaliphila* (Fig. 2).

Table 2: Ammonium removal efficiency of *Halomonas* spp., strains

Strain	Initial ammonia (mg-N L^{-1})	Final ammonia (mg-N L^{-1})	Ammonia removal (mg-N L^{-1})	Ammonia removal (%)	Nitrite (mg-N L^{-1})	Nitrate (mg-N L^{-1})
SKNB2	815.88±0.00^{c}	357.46±18.80^{c}	458.42±18.80^{d}	56.19±2.30^{c}	0.08±0.00^{c}	0.00±0.00^{b}
SKNB4	815.88±0.00^{c}	625.63±1.66^{a}	190.25±1.66^{e}	23.32±0.20^{d}	0.01±0.00^{d}	0.02±0.00^{a}
SKNB17	952.50±0.00^{a}	425.46±2.87^{b}	524.50±3.86^{c}	55.07±0.40^{c}	0.15±0.01^{a}	0.00±0.00^{b}
SKNB20	933.33±0.00^{b}	262.53±40.67^{d}	668.59±39.68^{a}	71.63±4.25^{a}	0.01±0.00^{d}	0.00±0.00^{b}
SKNB22	933.33±0.00^{b}	351.64±8.83^{c}	578.37±8.81^{b}	61.97±0.94^{b}	0.11±0.01^{b}	0.00±0.00^{b}

Ammonium removal efficiency: All five *Halomonas* spp., isolates showed a positive result for nitrite reagent testing. Then, the isolate's ammonium removal efficiency was tested by using standard colorimetric method. *Halomonas* spp., isolates exhibited an ammonium removal efficiency range of 23-71% under high initial ammonium concentration with a range of approximately 815-952 mg-N L^{-1}. Strain SKNB20 showed the highest ammonium removal ability followed by strains SKNB22, SKNB2, SKNB17 and SKNB4, which scored the amounts of 71.63, 61.97, 56.19, 55.07 and 23.32%, respectively (Table 2). While strain SKNB17 provided the highest nitrite production followed by strains SKNB22, SKNB2, SKNB4 and SKNB20 which amounts of 0.15, 0.11, 0.08, 0.01 and 0.01 mg-N L^{-1}, respectively. For nitrate production, only strain SKNB4 produced a very rare amount of nitrate with 0.02 mg-N L^{-1}.

Heterotrophic-halophilic nitrifying bacteria: The present study provided evidence that *Halomonas* spp., are nitrifying bacteria with heterotrophic-halophilic characteristics. These indigenous *Halomonas* spp., were isolated using organic medium (modified Pep-Beef-AOM) which consisted of peptone and beef extract. Likewise organic components, adding salt into isolation medium led to obtaining the halophilic bacteria. The salt requirement result indicated that all 5 strains of nitrifying bacteria needed salt for growth. Typical *Halomonas* spp., are proposed as halophiles because they strictly salt requirement. Other studies have reported the isolation of *Halomonas* spp., from various saline or hypersaline environments[12], however finding *Halomonas* spp., in ammonium contaminated environments have been scanted. Although, the marine shrimp farm contained ammonia, it also had a salinity factor that may have had more influence on *Halomonas* spp., isolation than the ammonium factor. Probably, the accumulation of ammonia in aquaculture farms may stimulate the ammonium removal function of *Halomonas* spp. The most salinity value of saline water used for culturing marine shrimp is ranging of 1-2.5% NaCl, which it aligned with optimal salt requirement of *Halomonas*. Therefore, the isolation and application of indigenous *Halomonas* spp., for ammonium elimination of saline ammonium waste water from shrimp farm is of particular interest. The biochemical result of catalase test indicated nitrifying bacterial isolates separated as two groups which are relevant with phylogenetic tree analysis. Based on sequence similarity results, all 5 isolates were identified as genus *Halomonas*. However, the similarity values of strains SKNB2, SKNB20, SKNB22 and SKNB17 to *H. aquamarina* and *H. venusta* were also low (96-91%). As well phylogenetic tree showed a separated lineage of these four strains from other related *Halomonas* spp. This might suggest that these four strains are possibilities to propose as new species of genus *Halomonas*. However, the data of cellular fatty acid profile, respiratory quinone and G+C content should be further study. While other *Halomonas* spp., were reported as denitrifying bacteria involving anaerobic nitrate reducing[7,13], there is rare reported of *Halomonas* spp., as heterotrophic nitrifying bacteria. Also, there is *H. campisalis* was proposed as involving both of nitrification and aerobic denitrification[14], the application study for using as saline waste water treatment has rarely.

Performance of ammonium removal ability: The highlight of research study indicated that indigenous *Halomonas* spp., strains have the significant abilities in two extreme conditions. Firstly, they have high performances of ammonium removal efficiencies for saline waste water and secondly, for high ammonium loaded waste water. These suggest that they are most important for helping of waste water treatment from marine shrimp and other coastal aquaculture industries. This study was different from previous investigations, indigenous *Halomonas* spp., exhibited performance of ammonium removal with high initial ammonium concentration around 815-952 mg-N L^{-1}. While, other studies reported that nitrifying bacteria have a good function in low ammonium concentration. For examples, *H. campisalis* has removed ammonia for about 71% with an initial ammonium concentration[15] of 140 mg-N L^{-1}, *Bacillus subtilis* has a reported ammonium removal efficiency of 58% with an initial ammonium concentration[8] around 104 mg-N L^{-1}, *Alcaligenes* sp., has exhibited a maximum ammonium removal of 95% with an initial ammonium concentration[9] about 400 mg-N L^{-1}, *A. denitrificans* has provided ammonium removal efficiency of 57% with an initial

ammonium concentration[15] around 105 mg-N L^{-1}, *Pseudomonas tolaasii* has showed ammonium removal efficiency of 93.6% with an initial ammonium concentration[16] of 209.62 mg-N L^{-1} and *Cupriavidus* sp., has performed ammonium removal efficiency of 99.68% with an initial ammonium concentration[17] of 100 mg-N L^{-1}. The point of initial ammonium concentration is very important for ammonium removal efficiency because high initial ammonium concentration has inhibited most nitrifying bacteria[5]. While, our indigenous *Halomonas* have grew well in high ammonium condition. Thus, indigenous *Halomonas* spp., may be a possibility to be developed as a microbial treatment product and applied for treating of high strength of ammonium waste water. Although, *Halomonas* spp., isolates showed high ammonium removal efficiency but also they provided low production of nitrite as well as nitrate production being almost undetectable (Table 2). This suggests that *Halomonas* spp., isolates could oxidize ammonia to nitrite better than oxidize nitrite to nitrate. Most ammonia may be consumed by cells for synthesis biomass[16] and only few converted to nitrite. With ammonium condition, the nitrogen consumption of heterotrophic *Halomonas* spp., may switch from organic to inorganic sources. This may be the reason that described why ammonia is most absent. However, another suggestion indicated that most of nitrite may convert to nitrate and further completely transformed to nitrogen gas by aerobic-denitrification process[14]. For confirming this suggestion, the amount of nitrogen gas that occurred during cultivation should bedetected. Generally, the complete nitrification consists of ammonium oxidizing and nitrite oxidizing processes. These processes involve groups of Ammonium Oxidizing Bacteria (AOB) and Nitrite Oxidizing Bacteria (NOB) which oxidize ammonia to nitrite and oxidize nitrite to nitrate, respectively. Based on the result, all *Halomonas* spp., isolates might be proposed as Ammonium Oxidizing Bacteria (AOB) or ammonium oxidizers by the reasons of obvious oxidizing ammonia to nitrite. The common genera of nitrifying bacteria, AOB such as *Nitrosomonas*, *Nitrosospira*, *Nitrosococcus* and *Nitrosolobus* are commonly reported as well as the NOB genera, such as *Nitrococcus*, *Nitromonas*, *Nitrospira* and *Nitrobacter*[3,4,18]. However, these AOB and NOB are autotrophic bacteria. They have very poor growth and limited competition to other heterotrophic bacteria in natural environments. Others have sensibility affected by chemicals, especially, high concentration of ammonia. In addition, high salinity always weakens the performance of biological process of nitrogen removal[19]. As autotrophic disadvantages, the indigenous heterotrophic, halophilic and high ammonium concentration tolerant *Halomonas* spp., may have high significant to be developed as microbial product for saline ammonium strength waste water treatment. Other *Halomonas* sp. and *H. aquamarina* have been suggested as beneficial probiotic for shrimp and have been successfully used to enhance growth and survival rates of shrimp larva[20,21]. Moreover, *H. aquamarina* has been claimed to be a non-pathogen, while its effective inhibitory property against pathogenic *Vibrio harveyi* has also been stated[21]. This evidence suggests that *Halomonas* spp., have other advantages like supporting growth of shrimps and pathogenic killing property. Accordingly, the application of *Halomonas* spp., as a water treatment microbial product would not only be beneficial for water quality, but also for aquatic animal health. Moreover, the combination of several *Halomonas* spp., may lead to enhance the efficiency of water improvement and support growth of aquatic animals. Therefore, the mixing of ammonium removal bacteria, *Halomonas* spp., should be highlighted for further study.

CONCLUSION

This study reported the isolation and ammonium removal ability of indigenous heterotrophic and halophilic nitrifying bacteria. All strains showed characteristics of salt-loving and ammonium removal ability with high ammonium condition. They were identified as *Halomonas* spp., while some of them have possibility to propose as new species. *Halomonas* spp., isolates were proposed as nitrifying bacteria with the reasons of their ammonium removal and nitrite producing abilities. They exhibited the performance of ammonium removal under high ammonium loaded condition. Therefore, the indigenous *Halomonas* spp., might develop as effective microbial product and apply for treating of saline ammonium waste water.

ACKNOWLEDGMENT

The researchers acknowledge the finance supported by budget revenue of Prince of Songkla University (NAT570385S).

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