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Research article

Exploring the Efficacy of *Bacillus oceanisediminis* Ba9 from Asian Seabass Cage Sediment in Saline Wastewater Treatment

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Abstract: Preventing toxicity in aquaculture systems from ammonia and nitrite is important. This study isolated the salt-tolerant Bacillus sp. strain Ba9 from bottom sediment under an Asian Seabass (*Lates calcarifer*) cage cultivating at Koh Yor, Songkhla, Thailand. Morphological characteristics showed that strain Ba9 was rod-shaped, endospore-forming, and Gram-positive. Strain Ba9 grew well at a salinity of 1.5 to 4.0% NaCl. The catalase test of the isolate was positive, while the oxidase test was negative. Based on 16S rRNA gene sequencing data and phylogenetic tree analysis, strain Ba9 was identified as *B. oceanisediminis* with 97% similarity (strain HQB337^T). The result showed that the ammonium removal efficiency of Ba9 in a high ammonium medium was 64.24%. The nitrite and nitrate production were 0.10% and 0.08%, respectively. Consequently, sucrose had been the optimal carbon source for Ba9, which showed ammonium removal was 61.05%. Ammonium sulfate is the most suitable for ammonium oxidation, with 50.53% for the nitrogen source. The optimal C/N ratio of strain Ba9 was 8, with 71.15% ammonia removal. For wastewater improvement, strain Ba9 was inoculated into artificial wastewater for 14 days. The result showed that the ammonium removal efficiency of Ba9 was 96.87%. In addition, the biochemical oxygen demand (BOD) removal efficiency of Ba9 was 90.86%. From this result, the salt-tolerant *B. oceanisediminis* Ba9 has a high potential as a microbial product for water quality management in marine aquaculture.

Keywords: Salt-tolerant *Bacillus* sp.; *B. oceanisediminis*; ammonium removal; BOD; water treatment

1. Introduction

A major element of conventional wastewater treatment processes is nitrogen removal. In an aquaculture pond, genuinely dangerous nitrogen compounds include ammonia (NH_3) and nitrite (NO_2^-). One of the most efficient methods for removing nitrogen from wastewater is a microbiological process (nitrification and denitrification). In principle, aerobic nitrification and anaerobic denitrification are performed to remove nitrogen compounds [1-3]. Most of the microbial population in nitrification are autotrophic nitrifying bacteria. Ammonia-oxidizing bacteria in the members of genera *Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosolobus*, and *Nitrosovibrio* can transform ammonium into nitrite. Nitrite-oxidizing bacteria, including genera *Nitrobacter, Nitrospira*, and *Nitrococcus*, can convert nitrite to nitrate [4-5]. Then, nitrate is converted to nitrogenous gas because of the denitrification process carried out

by heterotrophic denitrifying microorganisms. Besides, nitrate can be utilized by algae. Moreover, separating aerobic and anaerobic phases, the prolonged life cycle of autotrophic nitrifying bacteria results in a long starting period for nitrogen removal. The specific growth rates of autotrophic nitrifiers are weak, and their biomass is sensitive to toxic substances such as heavy metals and pH [6-7]. Recently, it has been found that nitrification and denitrification can be carried out simultaneously in one system [8], thus overcoming the problems existing in the traditional biological nitrogen removal process.

In addition to autotrophic nitrifying bacteria, a large variety of heterotrophic bacteria can oxidize ammonia, which is important to the natural nitrogen cycle [9]. A previous report showed that the heterotrophic nitrification process outperformed the autotrophic problem regarding ammonia removal [10]. Since heterotrophic nitrifying bacteria have more phylogenetic diversity than their autotrophic counterparts, they can better adapt to their conditions [11]. Several studies have shown that some *Bacillus* can control nitrogenous waste in aquaculture. In a study by Thurlow *et al*. [12], they reported that catfish pond water treated with *Bacillus velezensis* AP193 had reduced levels of nitrate-nitrogen (by 75%) and total nitrogen (by 43%). In a similar finding, Lalloo *et al*. [13] noted decreased nitrate and nitrite in synthetic pond water following *Bacillus* treatment. Regardless of the form, relatively low concentrations of nitrite and nitrate have been recorded. As an illustration, increased nitrate, nitrite, and decreased nitrite-N have been seen following *Bacillus* treatment [13-18]. It has been reported that *Bacillus* can reduce or modulate ammonia toxicity. In particular, aquaculture research has found that *B. subtilis, B. megaterium*, and *B. amyloliquefaciens* reduced ammonia levels in shrimp ponds [16-17,19]. Aquaculture constantly makes use of *Bacillus* spp. They have been developed as a treatment to clean water, enhance growth rates, protect against disease, and increase the immune system. [12,20-23].

In many investigations, Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) were lowered when *Bacillus* species were used as water quality modulators in aquaculture. Better feed utilization by fish may be related to this; as a result, less organic material decomposes using Dissolved Oxygen (DO), and potentially, *Bacillus* species require less DO to break down organic material efficiently. For instance, *B. megaterium* effectively lowered the BOD of major carp's pond water [18]. Reduced BOD (above 90%) was again recorded by Reddy *et al*. [24] in *Bacillus* (*B. subtilis, B. mojavensis*, and *B. cereus*) treated ponds. Similarly, a mix of *B. cereus* and *Aeromonas veronii* decreased BOD after effluent treatment [25]. Reduced COD levels were recorded in *B. subtilis, B. megaterium,* and *Bacillus* sp. YB1701 [18, 26] Therefore, elements including organic nutrition, DO, salinity, and application methods influence the efficiency of *Bacillus* in biological treatment.

This study aims to isolate and determine salt-tolerant *Bacillus* sp. based on morphological, biochemical, and 16S rRNA sequence information. Additionally, the purpose is to measure their nitrogen removal efficiency and biochemical oxygen demand in synthetic salinity wastewater.

2. Materials and Methods

2.1 Isolation and screening of *Bacillus* sp.

Salt-tolerant *Bacillus* sp. was isolated from sediment samples collected under a cage of Asian Sea bass (*Lates calcarifer*) culture at Koh Yor, Songkhla province, South Thailand. One gram of the sample was dissolved into 0.7% NaCl and heat shock on a water bath at 80 ^{o}C for 20 min, followed by a cold shock into room temperature water [27]. The suspension of the sample was ten-fold diluted, and then 0.1 ml was pipetted onto nutrient agar (NA) (HiMedia, India) + 0.7% NaCl. Purified strains were obtained after 3-4 times of re-streaking. The single colony was used to determine morphological and biochemical. The Gram staining and cell morphology were examined under a light microscope (Olympus BX50). Catalase activity was tested by bubble formation in 3% H_2O_2 solution. Oxidase activity was tested on a test strip (Merck) to observe the oxidation of *N,N*-dimethyl-1, 4-phenylene diammonium dichloride. Optimal salt requirement of 0-4.0% NaCl (w/v) was examined. One milliliter of bacterial cells was inoculated in tubes containing 9 ml of nutrient broth (NB) (HiMedia, India), with the addition of NaCl in the range of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 g/100 ml.

2.2 Identification of heterotrophic nitrifying bacteria

The Genomic DNA mini kit (Geneaid) was used to extract the genomic DNA of the *Bacillus* species. The 16S rRNA gene universal primers 27F (5'-AGAGTTTGATCATGGCTCAG-3') and 1492R (5′-TACCTTGTTACGACTT) were used to amplify the 16S rRNA genes by PCR [28-30]. The PCR amplification protocol proceeded as follows: initial denaturation (94°C for 3 minutes), 30 cycles of denaturation (94°C for 1 minute), annealing (50°C for 1 minute), extension (72°C for 2 minutes), final extension (72°C for 3 minutes), and storage (4°C for hold). The PCR result was examined using 1% agarose gel electrophoresis after amplification. The PCR products were purified using a GeneFlowTM Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). The sequencing service provider directly sequenced the purified PCR products on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, California, USA). The 16S rRNA gene sequence was compared with other microorganisms using the Basic Local Alignment Search Tool program (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). A phylogenetic tree of partial 16S rRNA gene sequences of the isolates and neighboring species was constructed by the MEGA 11 program [31]. A bootstrap value was performed with 1,000 replicates, and the phylogenetic tree was determined using neighbor-joining, maximum parsimony, and maximum likelihood.

2.3 Ammonium removal efficiency of *Bacillus* sp.

The efficacy of ammonium removal in a high ammonium medium was evaluated on a flask scale. About 1.5 ml (1% v/v) cell suspension of Ba9 cultivated in modified Pep-Beef AOM (peptone 5.0 g, beef extract 3.0 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 and tri-sodium citrate 17.8054 g in seawater (22 ppt) 1,000 mL) was inoculated into 150 ml of high ammonium medium (modified Pep-Beef AOM, which adjusted $(NH_4)_2SO_4$ to 4 g). This was used to examine the ammonium removal efficiency of *Bacillus* sp. and shaken at 170 rpm, 28°C. After 5 days of cultivation, the bacterial cells were removed by centrifugation at 3,500 rpm for 40 minutes [28-30]. After collecting the supernatant, the amounts of ammonium (NH_4^+) and nitrite (NO_2^-) were determined by the colorimetric method, and the concentration of nitrate (NO_3^-) was measured by the cadmium reduction column method [32].

2.6 Optimize carbon and nitrogen sources, C/N ratio, and salt tolerance.

2.6.1 Carbon source

The isolate was cultivated in a high ammonium medium supplemented by different carbon sources: sodium citrate ($C_6H_5Na_3O_7$), sodium acetate (CH_3COONa), glucose ($C_6H_{12}O_6$), sodium succinate ($C_4H_4Na_2O_4$), and sucrose ($C_{12}H_{22}O_{11}$) providing as a single carbon source in high ammonium medium [33]. The amount of $(NH_4)_2SO_4$ (N source) was fixed (the initial concentration of ammonia was set at 790-800 mg-N/L). Then, the medium was autoclaved at 121 °C for 15 min. The 1.5 ml of 10^9 cfu/ml bacterial starter from enrichment culture was inoculated in 250-mL shaker flasks containing 150 mL medium and shaken at 170 rpm, 28°C for 5 days. At the end of cultivation, the suspension was centrifuged at 3,500 rpm for 40 minutes to remove bacterial cells. The supernatant was collected, and then the concentrations of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were measured following the standard method [32]. Then, the highest ammonium removal efficiency was obtained, and the carbon source contained in the selected medium was suggested as the optimal carbon source for further study.

2.6.2 Nitrogen source

The strain was cultivated in a high ammonium medium with different nitrogen sources substituting (the initial concentration of ammonium was fixed at 800-820 mg-N/L) with the appropriate carbon source. Two different nitrogen sources were ammonium sulfate [$(NH_4)_2SO_4$] and ammonium chloride (NH_4Cl). All media were autoclaved at 121 °C for 15 min. The cultural conditions were mentioned above. After incubation for 5 days, the supernatant was obtained through centrifugation and measured. The medium that showed the highest nitrogen removal efficiency was cited as the optimal nitrogen source [34-35]

2.6.3 C/N ratio

Optimal carbon and nitrogen sources were chosen to study the optimal C/N ratio. The C/N ratio was adjusted to 0, 2, 4, 8, and 16 by fixing the amount of $(NH_4)_2SO_4$ (the N source) at 800-830 mg-N/L and adding sucrose as the carbon source. At 121 °C for 15 minutes, all media were autoclaved. The cultural condition was the same as above. Nitrogen removal efficiency in supernatant was analyzed after cultivation and centrifugation, followed by the standard method [32].

2.6.4 Optimization of salt requirement of Bacillus sp.

A single *Bacillus* sp. colony was taken from the plate, inoculated in NB with 0.85% NaCl (w/v), and then incubated at 28°C 180 rpm for 24 hr. NaCl was added at concentrations of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 % (w/v) to the media for the salinity requirement study. Test flasks were incubated at 28°C 180 rpm for 24 hr. After incubation, the supernatant was also used to measure cell density by a spectrophotometer (NanoPhotometer, IMPLEN, Germany) at a wavelength of 600 nm [27].

2.7 The efficient removal of *Bacillus* sp. in the sterilization of synthetic wastewater.

The salinity wastewater was increased by fermenting with an Asian seabass diet (Profeed, Thailand) at 0.5% w/v for 5 days. After 5 days, the salinity wastewater was sterilized by autoclave at 121°C for 15 min. *Bacillus* sp. was tested for the efficiency of BOD and nitrogen removal. Seventy milliliters of bacterial suspension (10^9 cfu/ml) was inoculated into 7 L of prepared wastewater and aeration was given throughout the trial period for 14 days. Every day of cultivation, 200 ml of wastewater was collected and evaluated for the amounts of ammonia, nitrite, and nitrate [32]. The wastewater was collected, and its BOD concentration was measured every 7 days of cultivation [36].

2.8 Statistical methods

The analysis of variance (ANOVA) was applied to the parameters of ammonia, nitrite, nitrate, and BOD. Before analysis, the data was transformed as necessary. The difference between the treatment means was determined using Duncan's new multiple range test (DMRT) method at 95% significance ($P < 0.05$). Data was expressed as Mean ± SE (standard error mean) using a statistical analysis R program.

3. Results and Discussion

3.1 Characterization and its biochemical properties.

Gram-stained morphological analysis of Ba9 demonstrated that the bacterial isolate was Gram-positive, rod-shaped, and endospore-forming (Figure 1). The biochemical characteristics of catalase and oxidase tests were positive and negative, respectively. The information on the Ba9 is shown in Table 1. A blast search result of the partial 16S rRNA gene sequence of Ba9 revealed 97% similarity to *B. oceanisediminis* HQB337^T (KT758497). Also, the identification result of the partial 16S rRNA gene sequence was relevant to the phylogenetic tree analysis (Figure 2). This tree demonstrated the evolutionary branches of strain Ba9 and *B. oceanisediminis* HQB337^T (KT758497) were closely related (Figure 2). However, more identification results of Ba9 should be performed by full-length 16S rRNA gene analysis.

Table 1. Morphology and biochemical test of *Bacillus oceanisediminis* Ba9.

Strain	Colony color	Gram	Shape	Endospore-forming	Catalase tests	Oxidase tests
Ba9	Creamy	Positive	Rod	Positive	Positive	Negative

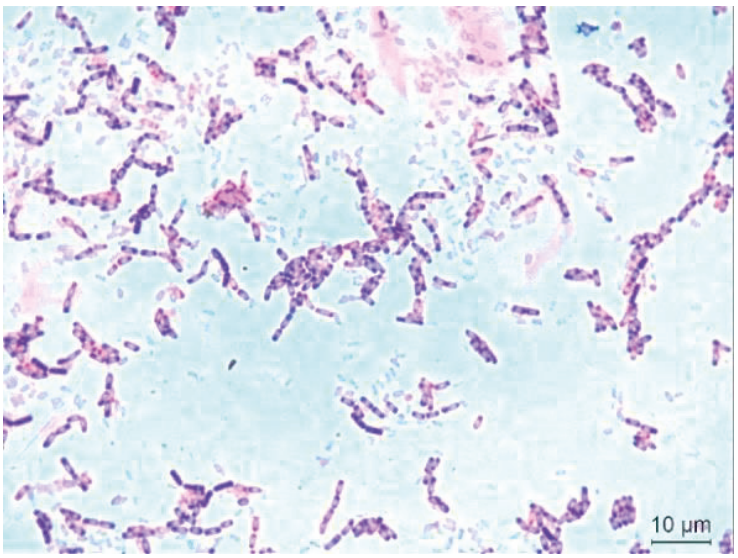

Figure 1. Vegetative cell morphology of *Bacillus* sp. Ba9.

3.2 Efficiency of ammonium removal in a high ammonium medium.

The concentrations of ammonia, nitrite, and nitrate were measured as part of a nitrogen removal efficiency analysis. The results showed that the single culture of the *Bacillus* sp. Ba9 had an ammonium removal efficiency of 64.24%. Nitrite and nitrate were produced by Ba9 from 0.00 mg-N/L to 0.01 and 0.08 mg-N/L, respectively (Figure 3). According to previous studies, the result suggested that *B. subtilis* A1 provided an excellent ability for ammonium removal. Lu and co-workers [35] reported a maximum ammonium removal of another heterotrophic bacterium, *Alcaligenes faecalis* WT14, for 95% in high ammonium concentration treatment (about 400 mg-N/L). However, the ammonium removal efficiency of *A. faecalis* WT14 decreased to 60% when an ammonium concentration increased to 700-1,600 mg-N/L. Also, the report of Xiao *et al*. [37] that demonstrated the efficacy of *Bacillus subtillis* AYC for nitrogen removal proceeded that had initial ammonium 10 mg-N/L, after 10 min the concentration of ammonium dramatically decreased, after 30 min, the ammonium concentration was low and remained to steadiness. The lowest ammonium removal efficiency was 43% when the high ammonium concentration was 2,000 mg-N/L. Other reports indicated that *Alcaligenes faecalis* no.4 had a high efficiency of ammonium removal at high ammonium levels (1,050 mg-N/L in 68 hrs.) [38]. Moreover, the immobilized cells of *Alcaligenes* sp.TD-94 and *Paracoccus* sp. TD-10 for treating high-ammonia-nitrogen wastewater was reported. Their contributions of biodegradation and adsorption to ammonium nitrogen removal were 90.27% and 9.73%, respectively. Bacterial immobilized cells were used to treat genuine high-ammonia-nitrogen wastewater, and the removal rates of ammonium reached 75.21 mg/L per day and removal efficiency of 99.27% after 48 hours of reaction. [23].

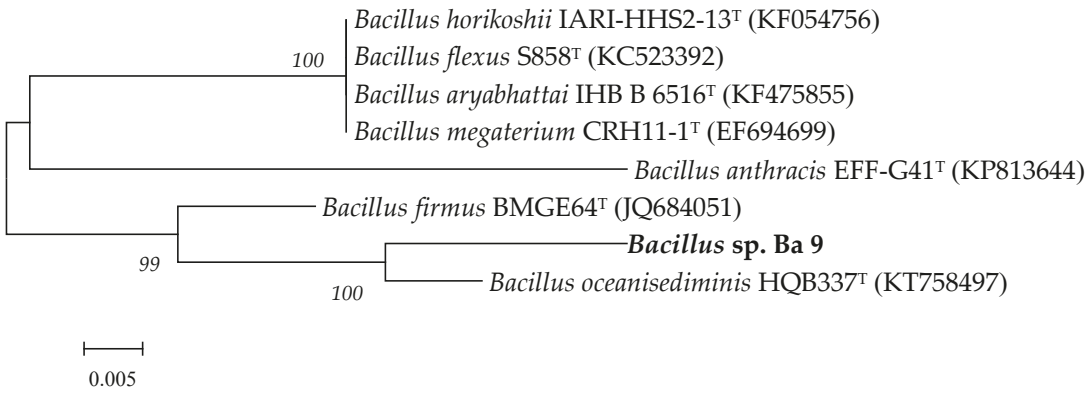

Figure 2. Phylogenetic tree of partial 16S rRNA gene sequences of *Bacillus* sp. Ba9 and related species (Bar = 0.005).

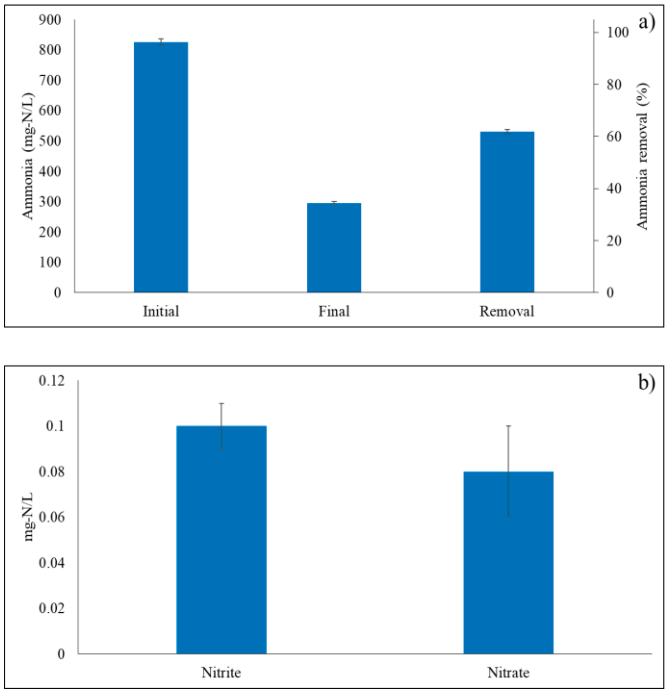

Figure 3. Nitrogen removal efficiency, a) ammonium removal efficiency, and b) nitrite and nitrate concentration (mg-N/L).

3.3 Optimize carbon and nitrogen sources, C/N ratio, and salt tolerance.

3.3.1 Carbon and Nitrogen Sources

Bacillus sp. Ba9 was employed during the research to determine the most effective carbon sources for ammonium removal (initial ammonia of 789-893 mg-N/L). Strain Ba9 using sucrose as a carbon source had ammonium removal of 61.05% (Figure 4a) and a nitrite content of 0.02 mg-N/L (Figure 4b). In comparison, other carbon sources such as glucose, sodium acetate, sodium succinate, and sodium citrate had given ammonium removal of 46.14, 39.76, 50.83 and 56.56%, respectively. The study differed from Zeng and co-workers [39], which illuminated that growth and ammonium removal were optimum for glucose, followed by citrate, sucrose, acetate, and starch. A publication suggested sodium citrate was an excellent carbon source for *A. faecalis* WT14 with a maximum of 98% ammonium removal [35]. Yang *et al*. [34] reported the four different carbon sources (acetate, glucose, citrate, and succinate); the result showed that all the tested carbon sources supported *B. subtillis* A1 had a percentage of ammonium removal up to 50.

As the nitrogen source requirement, *Bacillus* sp. Ba9 was studied based on sucrose as a carbon source. In contrast, ammonium sulfate and chloride were used as different nitrogen sources. The result showed that ammonium sulfate was the most effective nitrogen source for *Bacillus* sp Ba9. In this condition, strain Ba9 could decrease ammonia by 50.53% (Figure 5a), whereas nitrate production of these two nitrogen sources was non-significant (Figure 5b). This study result was consistent with the experiment by Lu *et al*. [35], which reported the ammonium reduction efficiency of *Alcaligenes* sp. W14 using different nitrogen sources. In agreement with the results, ammonium sulfate decreased total ammonium, having the highest effectiveness. Thus, it may be said that sodium citrate and ammonium sulfate were the optimum carbon and nitrogen sources for the heterotrophic nitrifying bacterium *Alcaligenes* sp. W1. The report was agreeable with this study, which *Bacillus* sp. Ba9 had sucrose and ammonium sulfate as appropriate carbon and nitrogen sources. In contrast, Yang *et al*. [34] mentioned that when four different carbon sources (acetate, glucose, sodium acetate, and succinate) and ammonium sulfate were used for investigation, *Bacillus subtilis* A1 was capable of removing ammonia but did not show significant differences (P>0.05).

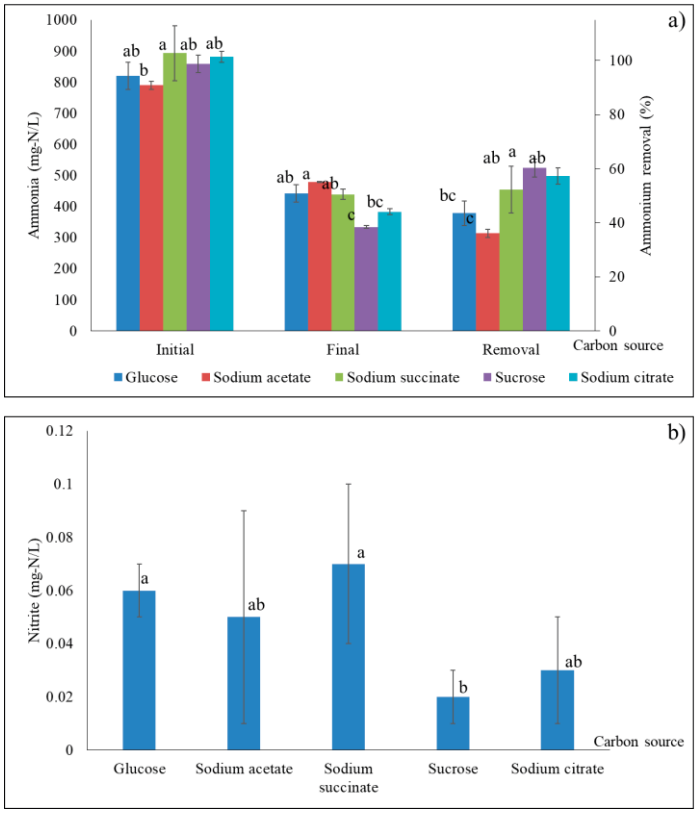

Figure 4. The optimization of the carbon source of *Bacillus* sp. Ba9, a) ammonium removal efficiency, b) nitrite production.

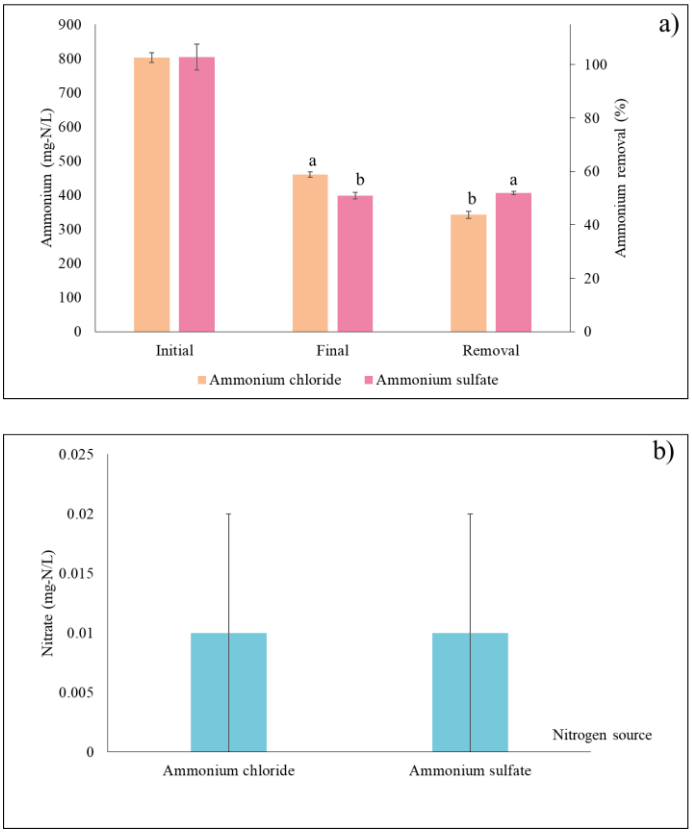

Figure 5. The optimization of the nitrogen source of *Bacillus* sp. Ba9, a) ammonium removal, b) nitrate production.

3.3.2 C/N ratio

Based on the above result, sucrose and ammonium sulfate were used as carbon and nitrogen sources, respectively. The optimum C/N ratio of Ba9 was 4, which can eliminate ammonia by 42.55%. This is followed by C/N ratios 12, 8, 2, and 0, which can remove ammonia for 37.84, 37.78, 32.53, and 19.94, respectively (Figure 6a). The result showed that the ammonium removal efficiency was low when the C/N ratios were lower or higher than 4. In the case of nitrite production, C/N ratio 2 showed the highest amount rather than ratio 4 (Figure 6b). This suggested that ratio 2 may be suitable for converting ammonia to nitrite. The C/N ratio result in this study was related to the optimization of C/N of *A. faecalis* strain NR, which had the ammonium removal of 19.2 mg/L at C/N ratio 5 in 48 hrs. [3]. The optimum C/N ratio of bacteria may depend on species or strains. While *A. faecalis* strain no. 4 showed high-efficiency ammonium removal at C/N ratio 10 [35]. A high C/N ratio can have a significant effect because it improves the volume of organic matter provided to the heterotrophic nitrifying bacteria. However, the optimal C/N ratio shows that the heterotrophic nitrifying bacterial capacity to remove nitrogen cannot be continuously improved by increasing the C/N ratio [33,38]. In addition, it has been reported that the most suitable was 10, which has the high percentage of ammonium removal by *B. subtillis* AYC showed at C/N ratios 10, 20, and 30 were 93.55%, 94.19%, and 96.77%, respectively [37]. Additionally, it became apparent that with a C/N ratio higher than 10, the heterotrophic nitrifying bacteria required carbon sources for growing like a biofloc formation.

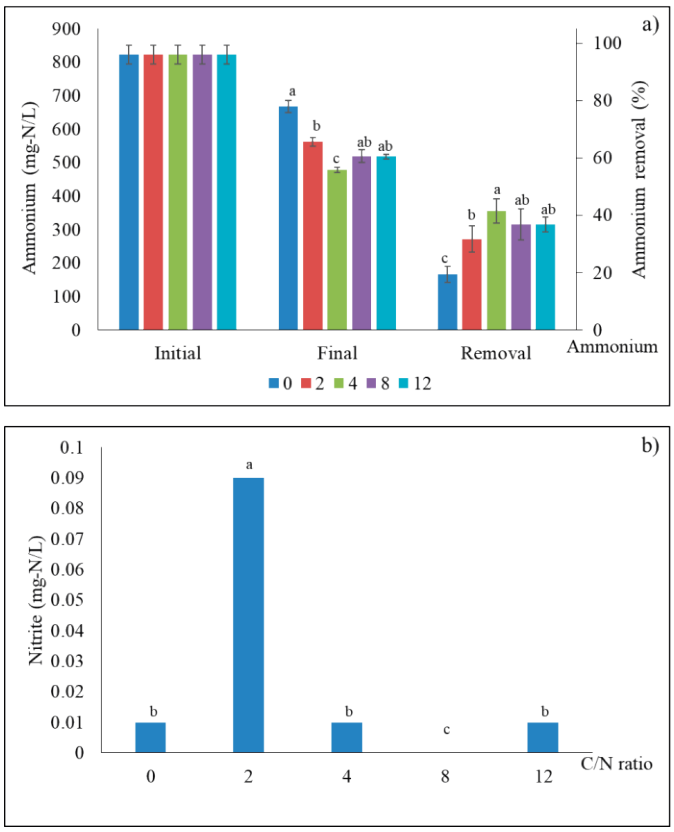

Figure 6. The optimization of C/N of *Bacillus* sp. Ba9, a) ammonium removal efficiency, b) nitrite production.

3.3.3 Optimization of salt tolerance of Bacillus sp. Ba9

The optimal salinity requirement for *Bacillus* sp. Ba9 was tested at 9 levels of salinities: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0% NaCl (w/v). The result found that Ba9 could grow well at a salinity range of 1.5 to 4.0% NaCl (w/v) (Figure 7). Purivirojkul *et al*. [27] described that *B. pumilius, B. sphaericus*, and *B. subtilis* may grow in environments with salinities ranging from 0% to 10% NaCl (w/v). Bacterium *B. oceanisediminis* was isolated from South China Sea sediments by Zhang *et al*. [40] and can grow at salt concentrations within 0 and 13% NaCl

(w/v), with the optimum 0.5% NaCl (w/v). Moreover, *B. aerius* NY6 could grow at up to 6% NaCl (w/v) (optimum 2% NaCl (w/v)), and *B. maritimus* may survive in conditions as high as 7% NaCl (w/v) (optimum 5% NaCl) [41-42].

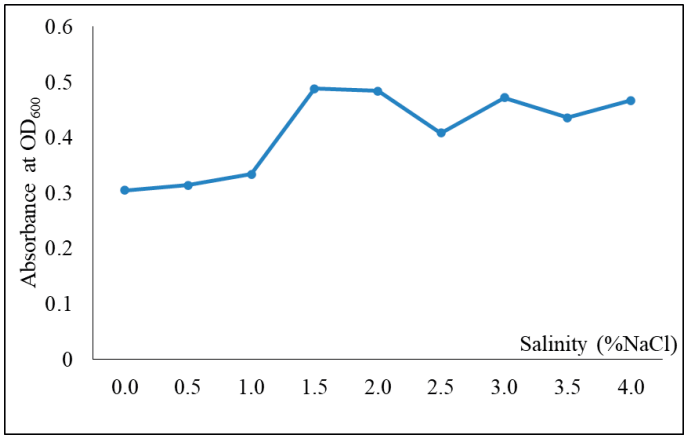

Figure 7. The growth rate of *Bacillus* sp. Ba9 at different salinity levels.

3.4 The efficiency of *Bacillus* sp. Ba9 to improve synthetic wastewater.

The ammonium removal efficiency in sterilized synthetic wastewater showed that *Bacillus* sp. Ba9 can remove ammonia from 191.38 ± 0.02 mg-N/L to 5.99 ± 1.12 mg-N/L (96.87%) on day 14 (Figure 8a-b). There is a report of tilapia wastewater treatment belonging to *Pseudomonas* sp. HBf01 and *Acinetobacter baumannii* HHf01 have an ammonium removal efficiency after 48 h for 67.9% [43]. The efficiency of nitrite production showed that all the treatments were decreased on day 7, and nitrite concentration increased on day 14. *Bacillus* sp. Ba9 had the highest nitrite production from 0.02 to 0.14 mg-N/L (Figure 8c-d). Another publication described that ammonia was reduced by 41.02% by *B. methytotrophicus* L7 of the nitrification effectiveness for 9 days, with ammonia levels beginning at 146.71 mg-N/L and ending at 38.29 mg-N/L [44]. The results showed that after 14 days of the experiment, all trials showed an increase in nitrate concentration. By day 14, *Bacillus* sp. Ba9 had a nitrate concentration of 0.23 ± 0.01 mg-N/L (Figure 8e-f). The result of nitrogen removal can prove that *B. oceanisediminis* Ba9, a member of the heterotrophic nitrifying bacteria group, had a higher ammonium removal efficiency at high ammonium levels than autotrophic nitrifying bacteria [45]. The mix of two strains, *B. cereus* PK5 and *B. subtilis* PK8, had ammonium removal efficiency at 72.0% (initial 50.0 ± 1.5 mg-N/L) [46]. It has been reported to be used *Bacillus* sp. in shrimp aquaculture (*Litopenaeus vannamei*) by *Bacillus* sp. N31 effectively reduced ammonium, nitrite, and nitrate for 86.3%, 89.3%, and 89.4%, respectively (nitrogen concentration starts at 250 mg-N/L) [47]. In the case of BOD, the efficiency of BOD removal on days 0, 7, and 14 was determined (the initial was 2636.86 ± 0.00 mg/L). The result showed a tendency to decrease in all trials except the control experiment. At the end of the experiment (14 days), *Bacillus* sp. Ba9 can remove BOD for 90.86%, while the control trial had removed BOD for about 15.77% (Figure 9). In several studies, the BOD was lower when *Bacillus* species were applied as water quality stimulators in aquaculture compared to controls. As a result, less organic matter dissolves using DO, and probably less DO is needed for *Bacillus* species to dissolve organic matter efficiently. This is possibly related to fish that consume their diet more successfully. For instance, *B. megaterium* effectively lowered the BOD of significant carp in pond water relative to the control [18]. Reduced BOD (above 90 %) was again recorded by Reddy *et al*. [24] in *Bacillus (B. subtilis, B. mojavensis*, and *B. cereus*) treated ponds. Similarly, a mix of *B. cereus* and *Aeromonas veronii* decreased BOD after effluent treatment [25]. These results provided strong evidence that *Bacillus* spp. is effective for BOD removal.

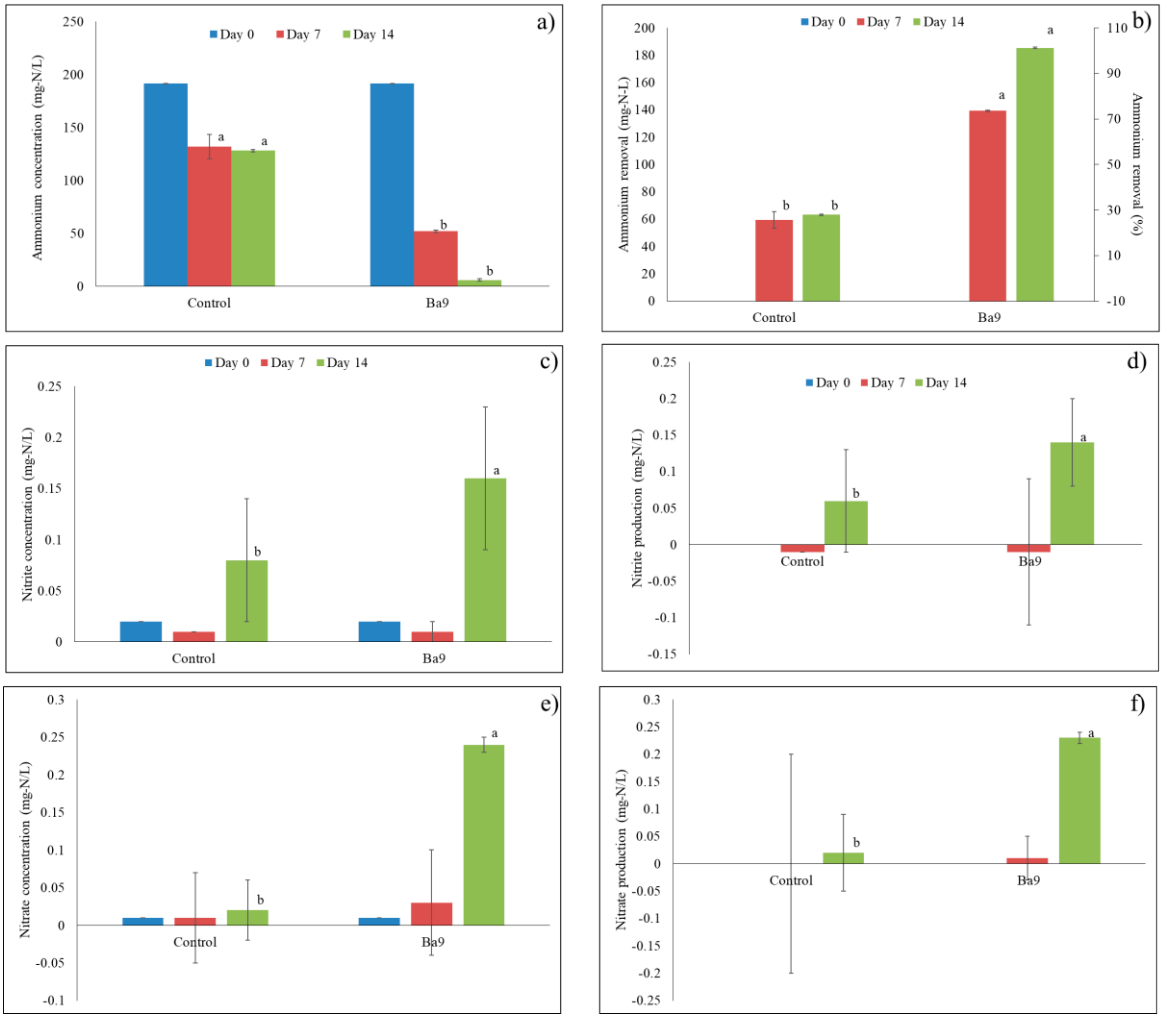

Figure 8. The efficiency of *Bacillus* sp. Ba9 for improving synthetic wastewater.

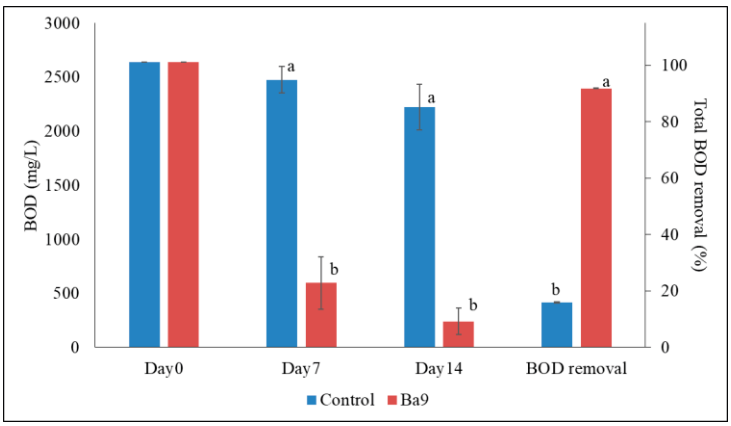

Figure 9. Biochemical oxygen demand (BOD) removal efficiency in synthetic wastewater of *Bacillus* sp. Ba9

4. Conclusions

Following these studies, *Bacillus* sp. strain Ba9 was unusually salt-tolerant heterotrophic nitrifying bacteria. According to the measurements of nitrogen removal efficiency. *Bacillus* sp. Ba9 was effective for oxidizing ammonium and reducing BOD. Therefore, it might be a significant opportunity for improvement in sectors including marine aquatic animals and saline wastewater treatment.

5. Acknowledgements

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