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Research article

Bacillus vietnamensis SF-1, a Promising Heterotrophic Nitrifier for Saline Wastewater Treatment

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Abstract: Shrimp farming wastewater regularly contains significant quantities of organic matter and inorganic nitrogen. Additionally, shrimp are extremely toxic to inorganic nitrogen, such as nitrite and ammonia. Ammonia removal is necessary for shrimp culture to improve water quality. Heterotrophic-nitrifying bacteria, genus *Bacillus*, are a kind of bacteria that effectively eliminate ammonia during the nitrification and aerobic denitrification processes. *Bacillus vietnamensis* SF-1 demonstrated 69.44% ammonium removal efficiency in a high ammonium medium. The nitrite production was 0.24 mg-N/L, and the nitrate concentration was 0.14 mg-N/L. The suitable carbon source of *B. vietnamensis* SF-1 was sucrose, which had ammonium removal at 57.39%, while a suitable nitrogen source was ammonium sulfate, which had ammonium removal at 46.15%. The C/N ratio at 8 showed the highest ammonium removal of 71.15%. An optimal salinity range for strain SF-1 growth was 2.0 to 4.0% NaCl (w/v). B. vietnamensis SF-1 was added to synthesis wastewater for 14 days to improve wastewater. The study found that the efficacy of ammonium removal of *B. vietnamensis* SF-1 was 94.77%. Nitrite and nitrate increased from 0.02-0.09 and 0.01-0.27 mg-N/L. The result showed considerable potential for the salt-tolerant *B. vietnamensis* SF-1 to improve water quality in coastal aquaculture.

Keywords: Heterotrophic nitrifying bacteria; ammonium removal; *Bacillus vietnamensis* SF-1; C/N ratio

1. Introduction

The wastewaters include significant quantities of nitrogenous organic compounds, including ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-), which endanger the ecological balance and cause eutrophication in aquatic systems. For this reason, removing nitrogenous wastewater is essential for maintaining the aquaculture ecosystem [1]. Microbiological processing is one of the best techniques for eliminating nitrogen from wastewater. In principle, aerobic nitrification and anaerobic denitrification [2-4]. Microorganisms known as autotrophic nitrifying and heterotrophic denitrifying perform nitrification and denitrification, respectively, constituting traditional biological nitrogen removal processes [5]. Furthermore, autotrophic nitrifying bacteria have an extended life cycle and weak growth rates, which means that their biomass yields are sensitive to pH, heavy metals, toxic substances, and high ammonia concentrations. These factors make it difficult for traditional technology to use autotrophic nitrifying technology [6-7]. For this reason, there are practical limits to the conventional biological nitrogen removal approach.

According to a previous investigation, the autotrophic problem was less effective in removing ammonia than the heterotrophic nitrification process [8]. A new and efficient process for removing nitrogen from wastewater has been made possible by the heterotrophic nitrification-aerobic denitrification bacteria. These microorganisms have the potential for nitrification as well as denitrification at the same time [1, 9]. As a result of the higher phylogenetic variety relative to their autotrophic different versions, heterotrophic nitrifying bacteria are more adapted to their surroundings [10]. Investigation has demonstrated that *Bacillus* species can manage nitrogenous waste in aquaculture. Nitrate levels in catfish pond water treated by *B. velezensis* AP193 were found to have been reduced by 75% and total nitrogen levels by 43% in research conducted [11]. *Bacillus* treatment has reported a similar observation about decreasing toxic inorganic nitrogen compounds in synthetic water [12]. Both nitrite and nitrate have been found in relatively few concentrations. For example, *Bacillus* treatment resulted in increased nitrite and nitrate. *Bacillus* has reportedly been shown to improve the toxicity of ammonia. Specifically, studies on aquaculture have discovered that *B. subtilis*, *B. megaterium*, and *B. amyloliquefaciens* decreased ammonia levels [12-18]. *Bacillus* spp. is widely used in aquaculture. They have been developed to treat diseases, boost immune systems, improve growth rates, and improve water quality [11, 19-23]. Although coastal aquaculture is a significant sector, most of the current research on the microorganisms that contribute to heterotrophic nitrification and aerobic denitrification has been focused on freshwater sewage. There are few studies on the biological treatment of wastewater with high salinity. Therefore, if we can find a salt-tolerant heterotrophic nitrifier that can eliminate salty wastewater. It will increase the aquacultural efficiency and reduce the cost of coastal aquaculture.

This study aims to determine the optimization of carbon sources, nitrogen sources, and C/N ratio of salt-tolerant *B. vietnamensis* SF-1. Additionally, it will measure the efficiency of nitrogen removal in synthetic sewage.

2. Materials and Methods

2.1 Stock of heterotrophic nitrifying bacteria strain SF-1

The heterotrophic nitrifying bacterium, *Bacillus* strain SF-1, was obtained from the bottom sediment in the seabass cage in Songkhla Lake, Thailand, and was identified as *B. vietnamensis* SF-1 [24]. Bacterial cells were stored in a 25% glycerol at −20 °C, inoculated with a modified Pep-Beef-AOB medium, and incubated at 28 °C at 170 rpm for three days [25-27].

2.2 Ammonium removal efficiency of *B. vietnamensis* SF-1 in high ammonium level

A high ammonium medium was used to examine the efficiency of ammonium removal; 1.5 ml of *B. vietnamensis* SF-1 was inoculated in a flask containing 150 ml of medium. Then the flasks were shaked at 170 rpm at 28 °C. After 5 days of culturing, the supernatant was separated and collected by centrifugation at 3,500 rpm for 40 minutes [25, 26]. The concentrations of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were measured [28].

2.3 Optimization of carbon source, nitrogen source, C/N ratio, and salt-requirement

2.3.1 Carbon source

Five different carbon sources (glucose ($C_6H_{12}O_6$), sucrose ($C_{12}H_{22}O_{11}$), sodium acetate (CH_3COONa), sodium citrate ($C_6H_5Na_3O_7$) and sodium succinate ($C_4H_4Na_2O_4$)) were each added to the high-ammonium medium used for cultivating the isolate [5, 27]. The starting concentration of ammonia was around 790–880 mg-N/L, and ammonium sulfate was fixed as a nitrogen source. The 1.5 ml of *B. vietnamensis* SF-1 from the enrichment culture was inoculated into a 150 mL medium. The cultures were shaken in an incubator at 170 rpm, 28°C for 5 days. [27] To remove the bacterial cells, the suspension of cultures was centrifuged at 3,500 rpm for 40 minutes at the end of cultivation. Following standard methods, the concentrations of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) in the supernatant were determined [28]. When a result was obtained, the carbon source with the highest effectiveness was suggested to study further.

2.3.2 Nitrogen source

Ammonium sulfate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl) were used as different sources of nitrogen. The initial ammonium concentration had been set to 800-820 mg-N/L, supplementing a suitable carbon source. The cultural conditions were followed as mentioned above. The supernatant was harvested and measured after 5 days of incubation. The optimum nitrogen source was recognized when the medium had the highest nitrogen removal efficiency [27, 28].

2.3.3 C/N ratio

The C/N ratio was studied using the optimal of carbon and nitrogen sources. By adjusting the ammonium concentration to around 820 to 840 mg-N/L. The level of C/N ratio was adjusted to 0, 2, 4, 8, and 16. The cultural condition, supernatant collection, and nitrogen removal determination were done above [27, 28].

2.3.4 Optimization of the salt requirement of B. vietnamensis **SF-1**

1% (v/v) of seed solution, *B. vietnamensis* SF-1 from the enrichment culture, was inoculated in modified Pep-Beef-AOB medium, which had 9 levels of NaCl concentrations as 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0% (w/v) for study. Then incubated at 28^{o}C, 170 rpm for 24 hours. A spectrophotometer was also used to measure the cell density at a wavelength of 600 nm using the supernatant after 24 hours of cultivation [27, 29].

2.4 The effectiveness of *B. vietnamiensis* SF-1 for nitrogen removal in the sterilizing of synthetic wastewater

Synthetic wastewater (a single usage), using 0.5% w/v shrimp feed for three days during fermentation, boosted the ammonium level in the salty wastewater. The synthetic wastewater was autoclaved at 121°C for 15 minutes after left for five days. Four liters of high synthetic wastewater were inoculated with 1% (v/v) of *B. vietnamensis* SF-1 cell suspension, and a control group received no bacterial inoculation. The experiment was extended to 14 days at room temperature, keeping aeration given during that period. Wastewater of 200 ml was kept every 7 days, ammonium, nitrite, and nitrate concentrations were analyzed [24, 25, 28].

2.5 Analytical methods

The concentration of ammonia and nitrite was analyzed using the colorimetric method. The nitrate concentration was determined by cadmium reduction column (ion chromatography) [28].

2.6 Statistical methods

The variance analysis (ANOVA) will be applied to water, ammonia, nitrite, and nitrate parameters. Duncan's new multiple range test (DMRT) method at 95% significance ($P < 0.05$) was used to analyze the difference in treatment using the R program.

3. Results and Discussion

3.1 Morphology of heterotrophic nitrifying bacterium *B. vietnamensis* SF-1

After re-streaking on modified Pep-Beef-AOB medium, *B. vietnamensis* SF-1 was obtained. The color of the colony on the solid medium was yellow-orange. *B. vietnamensis* SF-1 was rod-shaped, Gram-positive, and produced an endospore (Figure 1).

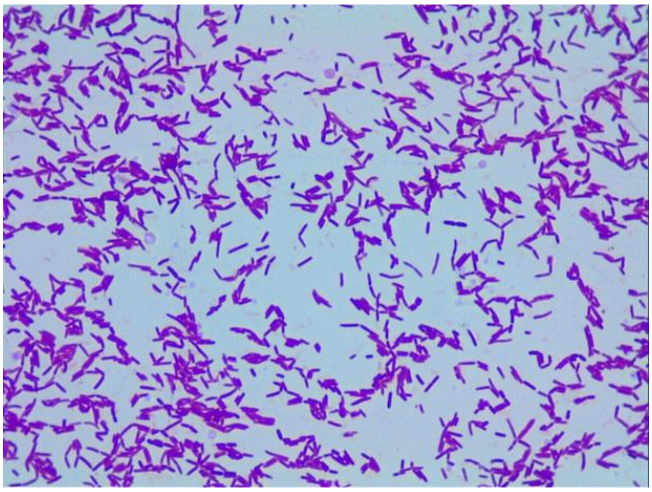

Figure 1. Cell morphology of *B. vietnamensis* SF-1.

3.2 Ammonium removal efficiency in high ammonium condition

The investigation of the nitrogen removal efficiency included ammonia, nitrite, and nitrate concentrations. According to the results, *B. vietnamensis* SF-1 demonstrated an ammonium removal efficiency of 69.44%, reducing the concentration from 825.16 ± 11.22 mg-N/L to 252.17 ± 8.00 mg-N/L. *B. vietnamensis* SF-1 can produce a nitrite concentration of 0.24 ± 0.01 mg-N/L and a nitrate concentration of 0.14 ± 0.03 mg-N/L (Figure 2). The result, which was consistent with additional previous studies, indicated that *B. subtilis* A1 had high ammonium removal abilities [30]. Furthermore, the study of Xioa et al. [31] shows that *B. subtilis* AYC's had effective nitrogen removal at an initial ammonium concentration of 10 mg-N/L. The ammonium concentration dropped rapidly after 10 minutes and steadily decreased after 30 minutes. The lowest ammonium removal efficiency was 43% at a high ammonium concentration of 2,000 mg-N/L. In another study, *Alcaligenes faecalis* no. 4 showed excellent ammonium removal efficiency at heavy ammonium concentrations (1,050 mg-N/L in 68 hours) [32].

3.3 Optimization of carbon source, nitrogen source, C/N ratio, and salt requirement

3.3.1 Optimal carbon and nitrogen sources

An essential component of heterotrophic nitrification capacity was thought to be the carbon supply. During the study, sucrose was used to determine the optimal carbon source for ammonium removal, exhibiting a statistically significant difference from the other four carbon sources ($p<0.05$). Ammonium removal was 57.39% (Figure 3). At the same time, other carbon sources, sodium citrate, sodium succinate, glucose, and sodium acetate, provided ammonium elimination of 52.99%, 47.32%, 42.60%, and 36.47%, respectively. The study was related to the efficiency of *B. oceanisediminis* Ba9 to remove ammonia when sucrose is the carbon source [27], and the red yeast, *Sporidiobolus pararoseus* Y1, demonstrated an ammonium removal rate of 62.08%, which has sucrose as a carbon source. Additionally, *B. subtilis* A1 was provided as acetate, glucose, citrate, and succinate as carbon sources; the percentages of ammonium removed were up to 50 percent within 60 hours (60.3 ± 1.2%, 60.8 ± 2.4%, 59.7 ± 1.0%, and 59.8 ± 2.3%, respectively) [30]. The various carbon sources have demonstrated the effectiveness of heterotrophic bacteria in eliminating ammonia well. According to the study, different carbon sources have the potential to be used by heterotrophic nitrifying bacteria, which might have an impact on the amount that nitrogen decreases throughout the nitrification process [30, 33, 34]. The other research results demonstrated that glucose and sodium succinate could effectively support the development of *B. methylotrophicus* L7, demonstrating effective nitrification capabilities. There was a 48.00% and 38.40% decrease in total ammonium, respectively [35]. Additionally, using citrate, glucose, and L-malate as the carbon source for 24 hours raised the ammonium removal efficiency of *Alcaligenes faecalis* strain NR to 98.9%, 94.9%, and 94.7%, respectively [34]. When succinate was used as a carbon source, *Acinetobacter junii* YB can remove ammonium entirely in 25 hours [36]. Consequently, sucrose was utilized in additional research as a suitable carbon source for *B. vietnamensis* SF-1.

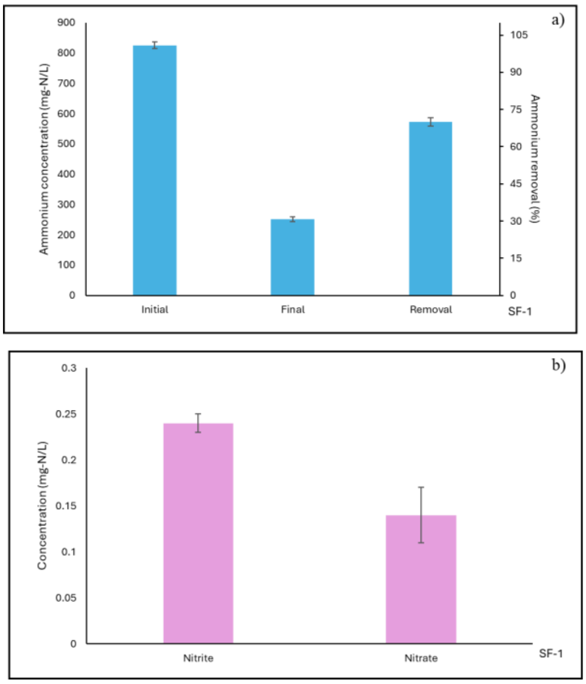

Figure 2. Nitrogen removal efficiency of *B. vietnamensis* SF-1; a) Ammonium removal efficiency, b) Nitrite concentration (mg-N/L).

Ammonium sulfate was the most effective nitrogen source for B. vietnamensis SF-1 in the nitrogen source. This bacterial strain can remove ammonia for 46.15% (Figure 4). This investigation's results aligned with our previous study regarding *B. oceanisediminis* Ba9, which utilized sucrose and ammonium sulfate as carbon and nitrogen sources, respectively, with an ammonium reduction efficiency of 50.53% [27]. Yang et al. [30] reported the effect of ammonium sulfate as a nitrogen source and 4 different carbon sources (glucose, acetate, sodium acetate, and succinate) on the ability of *B. subtilis* A1 to remove ammonia. The study results showed that carbon sources did not affect the ammonia elimination efficiency of B. subtilis A1. Considering different nitrogen sources, Lu et al. [33] studied the ammonium removal of the bacterium *Alcaligenes* strain W14. According to the data, ammonium sulfate was very effective at reducing ammonium. *Alcaligenes* strain W1 consequently selected sodium citrate and ammonium sulfates as optimal carbon and nitrogen sources. Also, with other research, ammonium sulfate was optimal for *B. tropicalis* L2, which has ammonium removed within 24 hours to 95.08%, and the maximum ammonium removal of 98.37% was observed at 36 hours [37]. Consequently, the report agreed with this study that *B. vietnamensis* SF-1 preferred sucrose as a carbon source and ammonium sulfate as a nitrogen source.

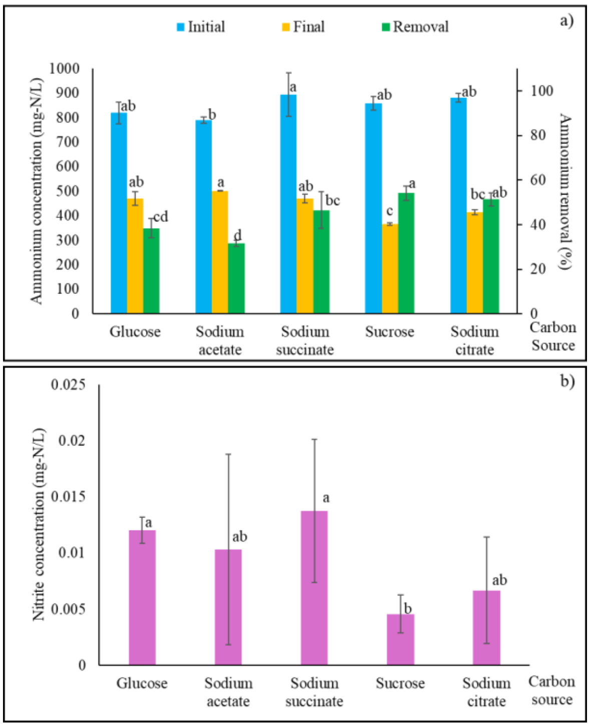

Figure 3. Optimal carbon source of *B. vietnamensis* SF-1, as well as a) ammonium removal efficiency and b) nitrite production.

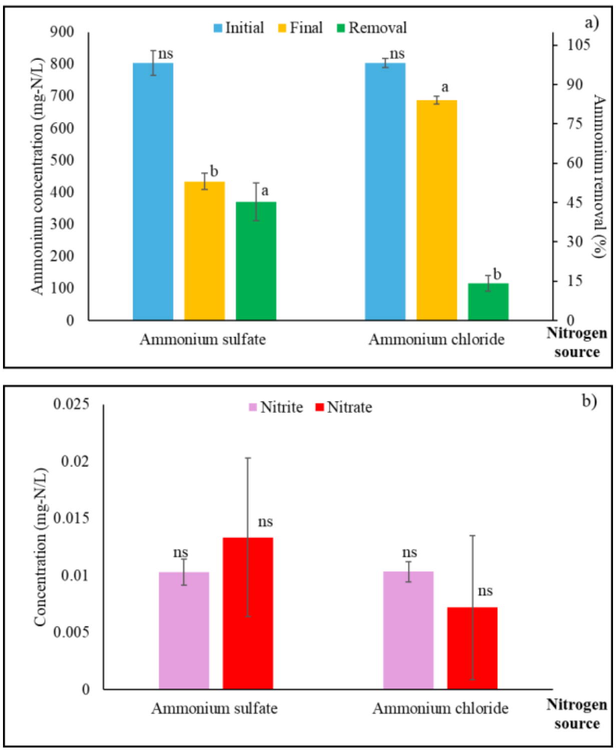

Figure 4. Optimal nitrogen source of *B. vietnamensis* SF-1; a) Ammonium removal, b) Nitrate production.

3.3.2 The optimal C/N ratio

The optimal C/N ratio is a significant regulator that has a major impact on the capability of heterotrophic nitrification-aerobic denitrification bacteria [38, 39]. Sucrose and ammonium sulfate were used as carbon and nitrogen sources for *B. vietnamensis* SF-1. The C/N ratio optimum of SF-1 was found to be 8, which can eliminate ammonia for 71.15%, subsequent by 4, 2, 16, and 0, which had ammonium removal of 65.93%, 62.09, 57.14, and 44.41%, respectively. Meanwhile, C/N ratio 4 has the highest production of nitrite and nitrate concentration (Figure 5). The C/N ratio at 0 showed the lowest ammonium removal. This result indicated carbon is important for heterotrophic nitrifying bacteria. The study has been related to the result that C/N at 9 was the most effective nitrogen removal condition for *B. tropicalis* L2 [37]. At a C/N ratio 5, *A. faecalis* NR demonstrated ammonium decreasing at 19.2 mg/L in 48 hours [34]. *Acinetobacter junii* YB showed a similar result, with ammonium being used at a C/N ratio of 5 [36], whereas *A. faecalis* no. 4 removed ammonia with good efficiency when the C/N ratio was 10 [32]. Furthermore, it became obvious that C/N ratios reached up to 10. The heterotrophic nitrifier needs carbon for development, comparable to biofloc production [40]. Furthermore, Xiao et al. [31] suggested that the most appropriate C/N ratio at 10 had the highest e ammonium removal efficiency by *B. subtillis* AYC. Another study revealed the mixer of *Pseudomonas geniculata* ATCC 19374 and *B. cereus* EC3 could remove ammonia of 99.84% at a C/N ratio of 12, which was higher than the C/N ratio 8 (92.8%) [41]. Research demonstrated effective nitrogen removal efficiency at a low C/N ratio, which is optimal for practical applications [42].

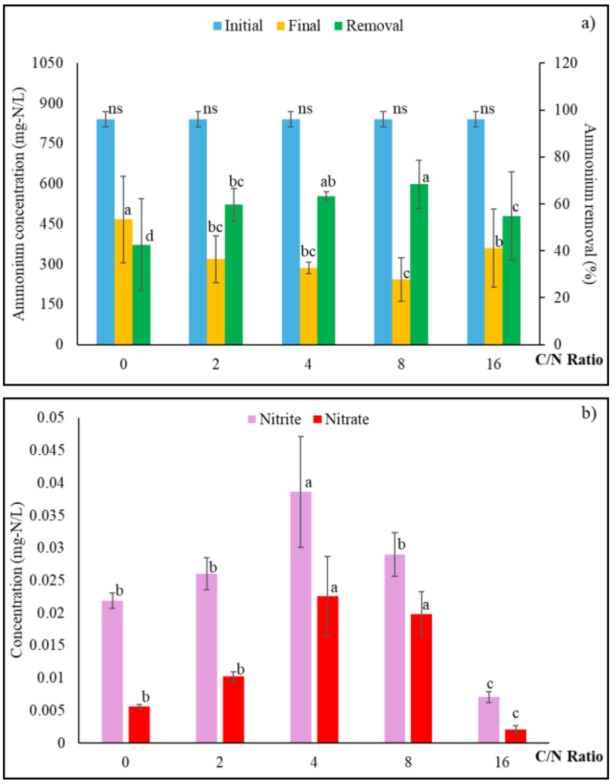

Figure 5. Optimal C/N of *B. vietnamensis* SF-1; a) Ammonium removal efficiency, b) Nitrite production.

3.3.3 Salt Requirement of B. vietnamensis **SF-1**

Salinity showed a major factor impacting nitrification; even strains grown in marine environments were inhibited by high salinity [43]. The optimum salinity test for *B. vietnamensis* SF-1 was studied at 9 different salinity levels from 0 to 4.0% NaCl (w/v). It demonstrated *that B. vietnamensis* SF-1 *can grow at the salinity range* from 2 to 4.0% NaCl (w/v) (Figure 6). According to Noguchi et al. [44], halotolerant *B. vietnamensis* 15-1 can reproduce in 15% NaCl, with 1% NaCl providing the best conditions for growth and development. *B. methylotrophicus* L7 could have been characterized as a halotolerant bacterium since it eliminated more than 58.70% of ammonium from 0-30% NaCl [35]. *B. oceanisediminis* is capable of developing at concentrations of salt between 0 and 13% NaCl (w/v), the optimal conditions being 0.5% NaCl (w/v) [45], *B. oceanisediminis* Ba9 grows thoroughly at 1.5-4.0% NaCl (w/v) (optimum 1.5-2.0% NaCl (w/v)) [27], while other reports suggested that *B. maritimus* may tolerate situation as high as 7% NaCl (w/v) (optimum at 5%), *B. aerius* NY6 can grow at up to 6% (optimum at 2%) [46, 47]. This salt-resistant property can effectively treat low-salinity and high-salinity aquaculture wastewater.

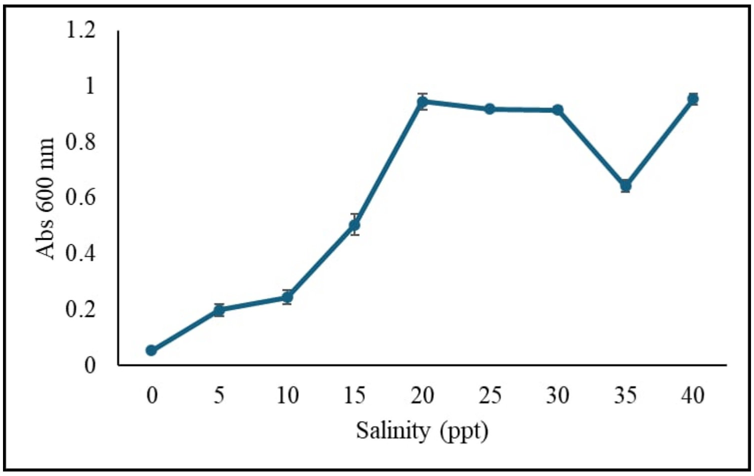

Figure 6. Growth profile of *B. vietnamensis* SF-1 at different salinity levels.

3.4 The efficiency of *B. vietnamensis* SF-1 for synthetic sewage treatment

The efficacy of ammonium removal in sterilizing sewage demonstrated that removing ammonia by *B. vietnamensis* SF-1 had a high ammonium removal efficiency (94.77%). Nitrite productivity dropped slightly from day 0 to day 7 (0.02-0.01 mg-N/L) and rose significantly from the 7 to 14 experiment date. From 0 to 14, the efficiency of nitrate production tends to increase with a quantity of 0.01-0.27 mg-N/L (Figure 8). The initially determined concentration of ammonium in the experiment to remove ammonium from shrimp aquaculture wastewater was around 191.38 mg-N/L, which was one-quarter of the initial concentration of ammonium in the flask-scale experiment (825 mg/L). According to Guo et al. [48], the initial ammonium demand significantly impacted the performance of heterotrophic bacteria in ammonium removal. For treating *B. oceanisediminis* Ba9 can remove ammonia for 96.87% (from 191.38 ± 0.02 mg-N/L to 5.99 ± 1.12 mg-N/L) and produce nitrite from 0.02 to 0.14 mg-N/L [27] when *B. methytotrophicus* L7 can decreased ammonia from 146.71 mg-N/L to 38.29 mg-N/L (41.02%) for 9 days [35]. *B. vietnamensis* SF-1, a heterotrophic nitrifier, showed higher ammonium removal efficiency at high ammonium concentrations than autotrophic nitrifying bacteria, as demonstrated by the nitrogen removal result [49].

The present investigation observed variances in nitrite and nitrate levels among treatment groups, which might indicate variation in the nitrification process. During the 14-day experiment, nitrite levels continuously got up and dropped, whereas the nitrate concentration progressively increased, corresponding to a decrease in ammonia levels. *B. vietnamensis* SF-1 efficiently converted ammonia to nitrite and continually nitrite to nitrate. In general terms, ammonium content can potentially be effectively oxidized by nitrification processes promoted by AOB (Ammonia-Oxidizing Bacteria), such as *Nitrosomonas* and *Nitrosospira.* AOB showed extraordinary ammonia oxidation potential. At the same time, the conversion of nitrite to nitrate is facilitated by nitrite-oxidizing bacteria (NOB). The NOB comprises *Nitrobacter winogradskyi, N. agilis, Nitrospira gracilis*, and *Nitrococcus mobilis*, all of which are members of this group. However, because the initial concentration of ammonia in this experiment was higher than the standard limit, the nitrification process that converts ammonia to nitrite could not have been completed [50, 51]. Kim et al. [52] found that combining B. subtilis PK8 and B. cereus PK5 led to 72% ammonium removal (initial ammonium 50.0 ± 1.5 mg-N/L). *Bacillus* sp. N31 can decrease ammonium, nitrite, and nitrate by 86.3, 89.3, and 89.4%, respectively (initial ammonium 250 mg-N/L) [38]. The nitrogen removal process can demonstrate that *B. vietnamensis* SF-1 belongs to the heterotrophic nitrifier.

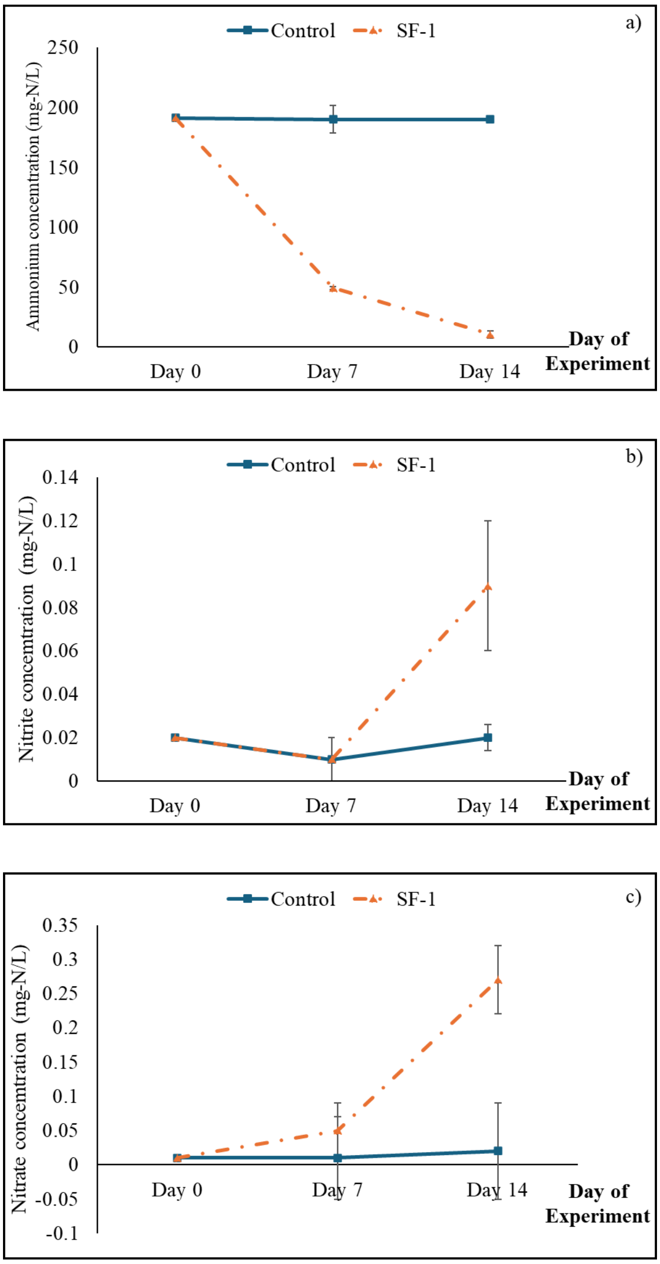

Figure 7. The efficiency of *B. vietnamensis* SF-1 removal in synthetic wastewater.

4. Conclusions

This study summarizes that *B. vietnamensis* SF-1 is an extraordinarily salt-tolerant heterotrophic nitrifying bacterium. It effectively reduces ammonia when sucrose is used as a carbon source and ammonium sulfate is used as a nitrogen source. Optimal C/N ratio of *B. vietnamensis* SF-1 is 8. *B. vietnamensis* SF-1 can grow over a wide salinity range. This study demonstrated by nitrogen removal efficiency studies that *B. vietnamensis* SF-1 was effective at oxidizing ammonium to nitrite and nitrate. As a result, there may be enormous prospects for expansion in sectors such as marine shrimp farming and saline wastewater treatment. In the future, *B. vietnamensis* SF-1 may be employed to sustain water quality for cultivating brackish and marine shrimp aquaculture. Controlling water quality by *B. vietnamensis* SF-1 will help reduce the cost of marine shrimp aquaculture.

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