Asia-Pacific Journal of Science and Technology: Volume: 29. Issue: 06. Article ID.: APST-29-06-11. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research Department,
Khon Kaen University, Thailand

Isolation and nitrogen removal efficiency of halophilic heterotrophic nitrifying bacteria, *Alcaligenes* strains SRNB23 and SRNB35

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Received 27 November 2023
Revised 1 May 2024
Accepted 13 May 2024

Abstract

The halophilic heterotrophic nitrifying bacteria play a significant role in mitigating the toxicity of inorganic nitrogen compounds, namely ammonia, nitrite, and nitrate in shrimp production systems. Two strains of heterotrophic nitrifying bacteria, namely strain SRNB23 and strain SRNB35, were obtained from sediment samples collected from a pacific white shrimp farm. The microorganisms were classified as *Alcaligenes faecalis,* with a similarity value ranging from 91% to 98%. The findings indicated that the ammonium removal effectiveness of SRNB23 and SRNB35 was 91.75% and 91.21%, respectively. The optimal proportion of the SRNB23 and SRNB35 mixture was determined to be 30% SRNB23 and 70% SRNB35. The observed ratio exhibited an ammonium removal effectiveness of 59.72%. As a result, sodium citrate has been identified as the preferred carbon source for SRNB23, whereas sucrose has been found to be the best carbon source for SRNB35. Ammonium sulfate was shown to be the most effective nitrogen source for both strains. The C/N ratio of SRNB23 and SRNB35 was found to be 16 and 2, respectively, indicating their respective ideal conditions. Subsequently, an analysis was conducted on both individual bacterial strains and bacterial mixtures in order to assess their efficacy in the treatment of wastewater originating from a shrimp farm, characterized by an ammonium concentration ranging from 461 to 467 mg-N/L. The findings indicated that the combination of SRNB23 and SRNB35 exhibited the most notable efficacy in removing ammonium, with an efficiency rate of 63.07%.

Keywords: *Alcaligenes faecalis*, Ammonium oxidizing bacteria, Ammonium removal, Nitrogen removal, Halophilic heterotrophic nitrifying bacteria

1. Introduction

The marine shrimp farming industry in Thailand constitutes a substantial agricultural sector. However, the restoration sector is characterized by a lack of security. Thailand's position as the leading exporter of shrimp has been relinquished due to the mortality rates observed in shrimp populations [1]. The development and spread of disease in shrimp are significant factors contributing to shrimp mortality and a substantial decline in production. To mitigate the incidence of illnesses in shrimp, farmers employ closed system aquaculture techniques characterized by minimal or negligible water exchange. In a closed system, the accumulation of waste resulting from shrimp excreta and food wastage commonly occurs within the pond. In particular, the presence of inorganic nitrogen compounds, such as ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-), poses a hazardous threat to shrimp. Consequently, the shrimp experience elevated stress levels, weakened immune systems, increased susceptibility to disease, and ultimately succumb to mortality. Hence, the elimination of ammonia and nitrite is of utmost significance in shrimp aquaculture systems as it serves to safeguard the shrimp population against potential toxicities [2]. The utilization of biological processes for the removal of nitrogenous chemicals in wastewater is widely recognized and implemented, making biological nitrogen removal a prevalent approach in this regard. Biological nitrogen removal methods can be categorized into two distinct stages of the nitrogen cycle. The initial

phase involves autotrophic nitrification occurring in aerobic settings. The subsequent phase involves denitrification through the process of heterotrophic denitrification, which occurs in an anaerobic environment. The nitrification process refers to the biological mechanism wherein ammonia is converted into nitrite, a reaction facilitated by ammonia oxidizing bacteria (AOB). The AOB is classified among the taxonomic groups *Nitrosomonas*, *Nitrosospira*, *Nitrosococcus*, *Nitrosolobus, Nitrosovibrio*, and *Nitrosogloea*. The conversion of nitrite to nitrate is facilitated by nitrite-oxidizing bacteria (NOB). The NOB is comprised of *Nitrobacter winogradskyi*, *N. agilis*, *Nitrospira gracilis*, and *Nitrococcus mobilis*. The growth rate of autotrophic nitrifying bacteria is often limited due to its sluggish nature. The species in question exhibits low competitive ability and is unable to withstand elevated levels of ammonium concentrations. Nitrogen removal necessitates the implementation of both aerobic and anaerobic procedures, as indicated by several studies [3-6]. The rate at which the nitrification process occurs is influenced by the activity of nitrifying bacteria and can be influenced by several operational and environmental parameters, including the levels of organic carbon and nitrogen. In order to enhance the performance of nitrifying bacteria and optimize the efficiency of wastewater treatment, it is necessary to investigate and optimize the various elements that influence the nitrogen removal process [7]. Previous studies have shown that when carbon-to-nitrogen (C/N) ratios are high, heterotrophic bacteria tend to be more abundant than nitrifying bacteria, resulting in a reduction in ammonium removal [8].

Currently, there is a significant emphasis among scientists on studying heterotrophic organisms that exhibit the ability to perform nitrification and denitrification in aerobic environments. Heterotrophic nitrifying bacteria has the ability to eliminate ammonia, nitrite, and nitrate from their environment. As a result, there is a notable increase in growth and a significant degree of competitiveness. Additionally, it was found that it possesses the ability to withstand elevated concentrations of ammonia in comparison to autotrophic nitrifying bacteria. The heterotrophic nitrifying bacteria, including *Bacillus*, *Halomonas*, *Pseudomonas*, and *Alcaligenes*, have been identified by various researchers [4,9-15].

The primary goal of this study was to identify and isolate heterotrophic nitrifying bacteria that possess the capacity to effectively eliminate elevated levels of ammonium concentration. In addition to examining the optimization of carbon and nitrogen sources, this study also evaluated the impact of the carbon-to-nitrogen (C/N) ratio on nitrogen removal efficiency.

2. Materials and methods

2.1 Medium

The modified Pep-Beef AOM was prepared by combining the following ingredients: 5.0 g of peptone, 3.0 g of beef extract, 2.0 g of $(NH_4)_2SO_4$, 0.75 g of K_2HPO_4, 0.25 g of NaH_2PO_4, 0.03 g of $MgSO_4$, 0.01 g of $MnSO_4$, and 17.8054 g of tri-sodium citrate. These ingredients were dissolved in 1,000 mL of sea water with a salinity of 22 ppt (prepared by mixing distilled water and sea salt). This modified medium was utilized for the purpose of enriching salt-tolerant heterotrophic nitrifying bacteria [13,14].

A high ammonium medium was prepared for the purpose of investigating the efficacy of salt-tolerant heterotrophic nitrifying bacteria in removing ammonium. The medium consisted of the following components: peptone (5.0 g), beef extract (3.0 g), $(NH_4)_2SO_4$ (4.0 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). These components were dissolved in 1,000 mL of sea water with a salinity of 22 ppt [13,14].

2.2 Isolation of heterotrophic nitrifying bacteria

The bacterial strains SRNB23 and SRNB35, which exhibit heterotrophic characteristics, were obtained through isolation from sediment samples collected from a *Litopenaeus vanamei* farm. The Pep-Beef-AOM medium was employed to enrich and isolate heterotrophic nitrifying bacteria. A quantity of sediment weighing one gram was introduced into a volume of 100 mL of modified Pep-Beef-AOM medium. The mixture was then subjected to agitation on a rotary shaker operating at a speed of 170 revolutions per minute, while maintaining a temperature of 28ºC. This process was carried out for a duration of 28 days. The nitrogen oxidizing activity was assessed at regular intervals of 3 days using the Griess-Ilosvay method. Specifically, 5-7 drops of nitrite reagent were added to 1 mL of suspension medium and allowed to react for 1 min. A positive result for nitrogen oxidation was shown by a change in the suspension's color to red. The positive sample was diluted by a factor of 10 and subsequently transferred to a modified Pep-Beef-AOM agar medium, which served as an isolation medium. The acquisition of a genetically homogeneous population of heterotrophic nitrifying bacteria was achieved through repetitive streaking on the isolation medium, as documented in previous studies [10,11,13,14].

2.3 Morphological and Biochemical Analysis

The morphological and biochemical properties of the isolates were assessed. The examination of Gram's staining and cell morphologies was conducted using a light microscope (Olympus BX50). Catalase activity was assessed through the utilization of bubble production in a 3% hydrogen peroxide (H_2O_2) solution during biochemical testing. The process of oxidizing N, N-dimethyl-1, 4-phenylene diammonium dichloride was detected by employing a test strip manufactured by Merck [13,14].

2.4 Identification of heterotrophic nitrifying bacteria

The genomic DNA of heterotrophic nitrifying bacteria was isolated from a cell solution using the Genomic DNA micro kit (Geneaid Biotech Ltd., Taiwan). The amplification of the 16S rRNA gene was conducted using polymerase chain reaction (PCR) employing the DNA Engine Dyad® Thermal Cycler, manufactured by Bio-Rad in the United States. The 16S rRNA gene universal primers 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5'-GGTTACCTTGTTACGACTT-3') were employed for this purpose. The PCR amplification process was conducted in the following manner: an initial denaturation step at 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 2 min. This was followed by a final extension step at 72ºC for 3 min, and the samples were then stored at 4ºC. Following the amplification process, the agarose gel electrophoresis technique was employed to analyze the PCR results. The PCR products underwent purification using a GeneFlowTM Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan) as described in previous studies [13,14]. The sequencing service provider conducted direct sequencing of the purified PCR products using an ABI Prism® 3730XL DNA Sequence instrument (Applied Biosystems, Foster City, California, USA). The 16S rRNA gene sequences were subjected to comparison with other microbes using the Basic Local Alignment Search Tool program (BLAST: http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The construction of a phylogenetic tree, based on partial 16S rRNA gene sequences of the isolates and neighboring species, was performed using the MEGA 11 program [16].

2.5 Ammonium removal efficiency of heterotrophic nitrifying bacteria

The effectiveness of ammonium removal in a high ammonium media was assessed in a small-scale flask experiment. A 1% volume per volume (v/v) cell suspension of bacteria in modified Pep-Beef AOM was introduced into a 150 mL culture of high ammonium medium. The mixture was then agitated at a speed of 170 revolutions per minute (rpm) and maintained at a temperature of 28ºC. The study investigated the ammonium removal effectiveness of mixed strains comprising SRNB23 and SRNB35 at various ratios i.e. 30:70, 40:60, 50:50, 60:40, and 70:30. Following a culture period of 5 days, the bacterial cells were separated using centrifugation at a speed of 3,500 revolutions per minute for a duration of 40 min. After the collection of the supernatant, the concentrations of ammonium (NH_4^+), NO_2^-, and NO_3^- were quantified [17].

2.6 Optimization of carbon and nitrogen sources, and C/N ratio

2.6.1 Carbon source

The isolate was cultured in a high ammonium medium supplemented with various carbon sources, namely 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 ($C_{12}H_{22}O_{11}$), which were provided individually as the sole carbon source in the high ammonium medium [18]. The quantity of $(NH_4)_2SO_4$, which served as the source of nitrogen (N), was held constant throughout the experiment. The starting concentration of ammonia was maintained at a consistent range of 790-800 mg-N/L. The medium was subjected to autoclaving at a temperature of 121°C for a duration of 15 min. A volume of 1.5 mL of bacterial starter obtained from an enrichment culture was introduced into 250 milliliter shaker flasks holding 150 mL of medium. The flasks were then subjected to shaking at a speed of 170 revolutions per minute and maintained at a temperature of 28ºC for a duration of 5 d. After the completion of cultivation, the suspension underwent centrifugation at a speed of 3,500 revolutions per minute for duration of 40 min in order to eliminate bacterial cells [19]. The supernatant was collected, and afterwards, the amounts of ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-) were determined using the usual method [17]. Subsequently, the carbon source present in the chosen medium was identified as the most effective in achieving the highest ammonium removal efficiency. Therefore, it is recommended that this carbon source be utilized as the ideal choice for future investigations.

2.6.2 Nitrogen source

The strain was cultured on a high-ammonium medium, whereby several nitrogen sources were used as substitutes. The starting concentration of ammonium was maintained at a fixed range of 800-820 mg-N/L, while the corresponding carbon source was appropriately selected. Two distinct nitrogen sources utilized in the study were ammonium sulfate ($(NH_4)_2SO_4$) and ammonium chloride (NH_4Cl). The media underwent autoclaving at a temperature of 121°C for a duration of 15 min. The aforementioned cultural conditions were then described. Following a 5-day incubation period, the supernatant was collected via centrifugation and subsequently quantified [17,19]. The medium with the highest nitrogen removal efficiency was identified as the ideal nitrogen source [10,11].

2.6.3 C/N ratio

The selection of optimal carbon and nitrogen sources was undertaken to investigate the ideal carbon-to-nitrogen ratio. The C/N ratio was manipulated to five different levels (0, 2, 4, 8, and 16) in the experiment [19]. This was achieved by maintaining a constant amount of $(NH_4)_2SO_4$ as the nitrogen source at a concentration of 800-830 mg-N/L, while introducing sodium citrate for SRNB23 and sucrose for SRNB35 as the respective carbon sources. The media underwent autoclaving at a temperature of 121°C for a duration of 15 min. The culture condition remained consistent throughout. The analysis of nitrogen removal effectiveness in the supernatant was conducted subsequent to the processes of culture and centrifugation [17,19].

2.7 Nitrogen removal in wastewater

The ammonium concentration of the wastewater collected from the shrimp pond was seen to have increased following a 3-day fermentation period using shrimp feed. Following a period of 72 hours, the wastewater underwent sterilization using autoclaving, wherein it was subjected to a temperature of 121°C for a duration of 15 min. A 1% (v/v) concentration of cell suspension containing single strains SRNB23 and SRNB35, as well as a mixture of SRNB23 and SRNB35 at a ratio of 30:70, together with a control group (without bacteria), were introduced into a 4 L volume of high ammonium wastewater. Aeration was provided continuously over the 14-day trial duration. On a daily basis, a volume of 200 mL of wastewater was gathered and subjected to analysis in order to determine the concentrations of ammonium, nitrite, and nitrate [17,20].

2.8 Statistical methods

The application of analysis of variance (ANOVA) was utilized to examine the characteristics of water, specifically ammonia, nitrite, and nitrate. Prior to performing the analysis, the data was subjected to appropriate transformations as required. The determination of the difference between the treatment means was conducted utilizing Duncan's novel multiple range test (DMRT) method with a significance level of 95% ($P<0.05$). The data was presented in the form of Mean ± SE (standard error mean) using a statistical analysis application, specifically the R program.

3. Results and discussion

3.1 Identification and characterization of heterotrophic nitrifying bacteria

Following enrichment and subsequent streaking on a modified Pep-Beef-AOM agar medium, two strains of heterotrophic nitrifying bacteria, namely SRNB23 and SRNB35, were successfully isolated. The colony color observed on the modified Pep-Beef-AOM medium for SRNB23 and SRNB35 was identified as creamy (Figure 1A) and orange (Figure 1C), respectively. Under microscopic examination, the morphology of both strains revealed characteristics consistent with Gram-negative bacteria. Specifically, they had a rod-shaped structure and did not display any evidence of endospore formation (Figure 1B, 1D). The findings from biochemical analyses, specifically the catalase and oxidase tests, indicated that both strains exhibited positive and negative results, respectively (Table 1). According to the examination of the 16S rRNA sequence, it was determined that SRNB23 and SRNB35 belong to the *Alcaligenes* species. The nucleotide sequences of SRNB23 and SRNB35 were deposited in the Genbank database with accession numbers LC274887 and LC274888, respectively. The results of the blast alignment analysis indicated that SRNB23 exhibited the maximum similarity value of 98% when compared to the *A. faecalis* strain MVSV7T. In contrast, SRNB35 was shown to be phylogenetically distinct from *A. faecalis* (Figure 2). Based on the observed low similarity value of 92%, there is a high probability that SRNB35 represents a newly discovered species or maybe a unique genus. This inference is supported by the phylogenetic analysis, which revealed a significant genetic difference between SRNB35 and *A. faecalis* [21]. However, in order

to propose the classification of a new bacterial species or genus, additional analytical evidence is required. The dataset encompasses the complete length of the 16S rRNA gene sequence and includes DNA-DNA hybridization. Furthermore, further investigations are necessary to explore biochemical analysis and chemotaxonomic factors, such as respiration quinone, fatty acid profile, G+C mol composition, and other relevant characteristics.

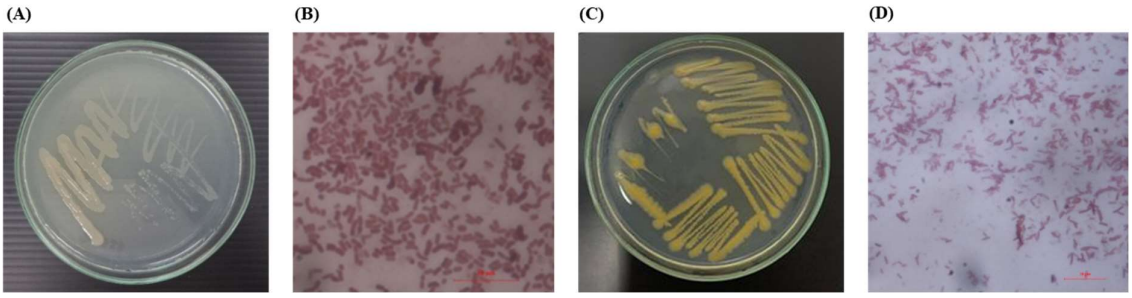

Figure 1 The morphology and vegetative cells of heterotrophic nitrifying bacteria; Colony of SRNB23 (A) and SRNB35 (C), Vegetative cells of SRNB23 (B) and SRNB35 (D) (Bars = 10 µm).

Table 1 Phenotypic characteristics of nitrifying bacteria isolates.

Characteristic	SRNB23	SRNB35
Isolation source	Sediment	Water
Shape	Rod	Rod
Pigmentation	Cream	Orange
Gram's stain	Negative	Negative
Endospore forming	-	-
Oxidase	-	-
Catalase	+	+

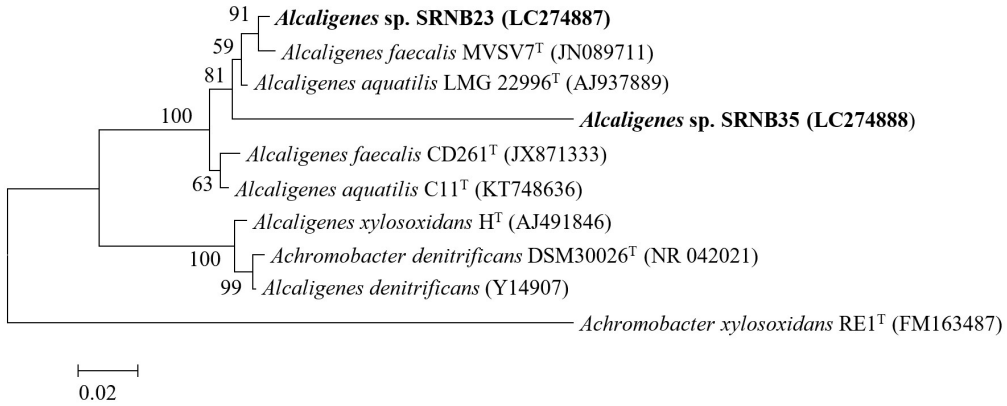

Figure 2 Phylogenetic tree of partial 16S rRNA gene sequences of heterotrophic nitrifying bacteria and related species (Bar = 0.02).

3.2 Efficiency of ammonium removal under high ammonium medium

As part of an analysis on the efficacy of nitrogen removal, measurements were taken to determine the quantities of ammonia, nitrite, and nitrate. The findings indicated that the individual strains of *Alcaligenes* sp. SRNB23 and *Alcaligenes* sp. SRNB35 had ammonium removal efficiencies of 91.75% and 91.21%, respectively (Figure 3A). The nitrite levels produced by SRNB 23 and SRNB 35 were recorded as 0.05 and 0.20 mg-N/L, respectively (Figure 3B). The findings align with prior research, which indicated that *Alcaligenes spp.* exhibited a strong capacity for ammonium removal. In a study conducted by Lu et al. [11], it was found that *A. faecalis* WT14 achieved a maximum ammonium removal rate of 95% when exposed to high ammonium concentrations of approximately 400 mg-N/L. The researchers achieved a maximum ammonium removal efficiency of 95%, which subsequently declined to 60% when the ammonium content ranged from 700 to 1,600 mg-N/L. The ammonium removal efficiency exhibited the lowest value of 43% when subjected to a high ammonium concentration of 2,000 mg-N/L. According to a study conducted by Joo et al. [4], it was found that *Alcaligenes faecalis* no.4 had a significant capacity for ammonium removal, particularly at elevated levels of ammonium concentration (1,050

mg-N/L throughout a time frame of 68 hours). The treatment of high-ammonia-nitrogen wastewater involved the utilization of immobilized cells of *Alcaligenes* sp. TD-94 and *Paracoccus* sp. TD-10 [22]. The removal of ammonium nitrogen was primarily attributed to biodegradation, accounting for 90.27% of the overall removal, while adsorption contributed 9.73% to the process. The immobilized cells were employed for the treatment of authentic high-ammonia-nitrogen wastewater, resulting in ammonium removal rates of 75.21 mg/L per day. The removal efficiency achieved a value of 99.27% following a 48-hour reaction period. According to Zhang et al. [22], while the effectiveness of a single strain in reducing ammonia levels is evident, it has been observed that it only has a limited ability to convert ammonia to nitrite. However, it is unable to complete the nitrification process by converting nitrite to nitrate. Hence, an investigation was conducted to assess the efficacy of the mixed culture.

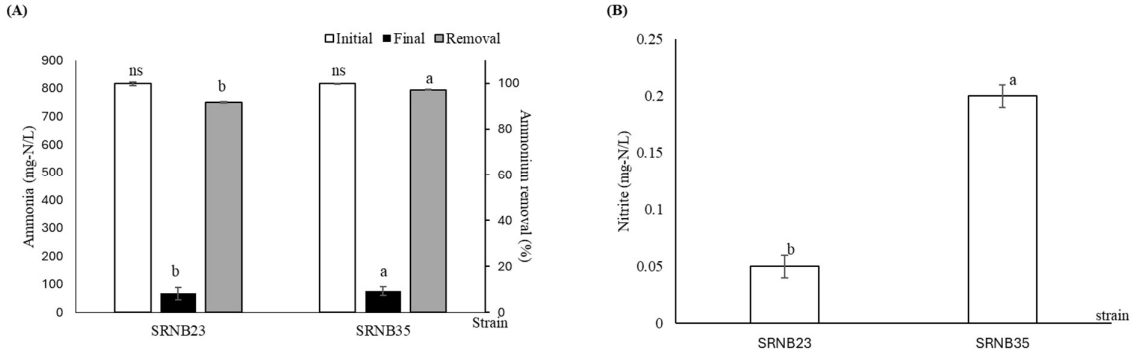

Figure 3 Nitrogen removal efficiency; (A) Ammonium removal efficiency, (B) Nitrite concentration.

Based on the findings of the inquiry, it was seen that the optimal ratio of mixed strains, specifically *Alcaligenes* sp. SRNB23 and SRNB35, at a C/N ratio of 30:70 resulted in the maximum reduction of ammonia, reaching a percentage of 66.77% (Figure 4A). Additionally, this ratio also led to the production of a nitrite concentration of 0.18 mg/L, as depicted in (Figure 4B). The concentration of ammonia can be reduced by utilizing several ratios, namely 50:50, 40:60, 60:40, and 70:30. These ratios result in reductions of ammonia concentration by 59.72%, 53.72%, 52.21%, and 47.68%, respectively. Additionally, the nitrite compositions associated with these ratios are 0.21, 0.25, 0.27, and 0.23 mg-N/L, respectively. Nevertheless, the measurement of nitrate concentration was not conducted in this study. According to the study by Joo et al. [23], which investigated the ammonium removal rate in a mixed culture of *A. faecalis* no.4 and L1, it was higher compared to a single culture. The findings of this study demonstrated that the co-cultivation of bacteria resulted in a greater efficacy in ammonia removal compared to the use of a single bacterial strain. Furthermore, varying combinations of strains yield distinct capacities for ammonium removal. Hence, the utilization of mixed culture demonstrates the capacity to achieve maximum efficiency. Additionally, it is necessary to examine suitable combining ratios.

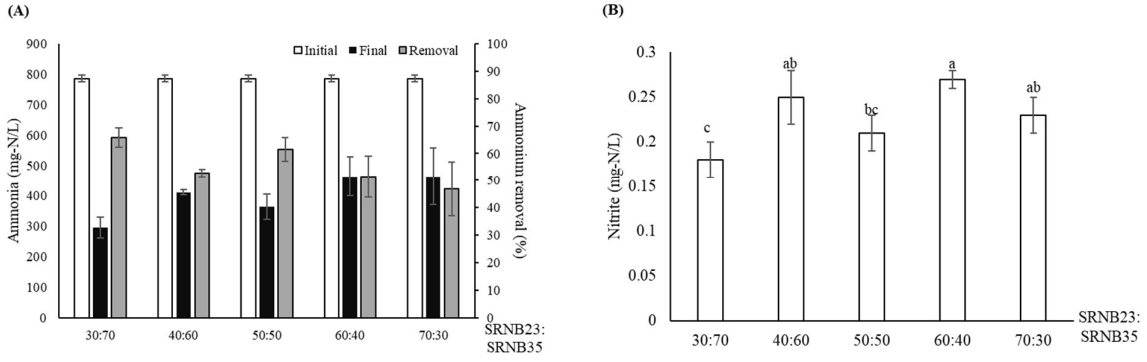

Figure 4 Efficiency of ammonium removal of ratio of mixed strain between SRNB23:SRNB35; (A) Ammonium removal efficiency, (B) Nitrite concentration.

3.3 Optimization of carbon and nitrogen sources and C/N ratio

3.3.1 Carbon and nitrogen sources

The study utilized *Alcaligenes* sp. SRNB23 and SRNB35, which are heterotrophic nitrifying bacteria, to investigate the optimal carbon sources for ammonia removal. The initial ammonia concentration ranged from 800 to 802 mg-N/L. The strain SRNB23, which utilized sodium citrate as its carbon source, exhibited an ammonium removal efficiency of 82.47% (Figure 5A). Additionally, it demonstrated a peak nitrite concentration of 0.16 mg-N/L, as depicted in Figure 5C. In contrast to other carbon sources such as glucose, sodium acetate, sodium succinate, and sucrose, this resulted in ammonium removal rates below 50%. A notable impact of sodium succinate usage was observed in the elimination of ammonium, resulting in a 4.68% reduction. In contrast, it was shown that sucrose exhibited the highest percentage with up to 59.31% (Figure 5B) in terms of ammonium elimination and produced nitrite 0.07 mg-N/L (Figure 5D) in the case of SRNB35. The efficacy of heterotrophic bacteria in ammonia removal has been observed to vary greatly depending on the carbon sources used [5,10,11]. According to Zhao et al. [6], heterotrophic nitrifying bacteria exhibit variability in their utilization of carbon sources, potentially impacting the extent of nitrogen reduction during the nitrification process. According to Lu et al. [11], sodium citrate proved to be an effective carbon source for *A. faecalis* WT14, resulting in a maximum ammonium removal rate of 98%. In accordance with this, the utilization of sodium succinate and sodium citrate as the principal carbon sources resulted in significantly enhanced ammonium removal efficiency in *A. faecalis* strain NR and *Vibrio diabolicus* strain SF16, with respective values of 91.8% and 92.2% [5,18,24].

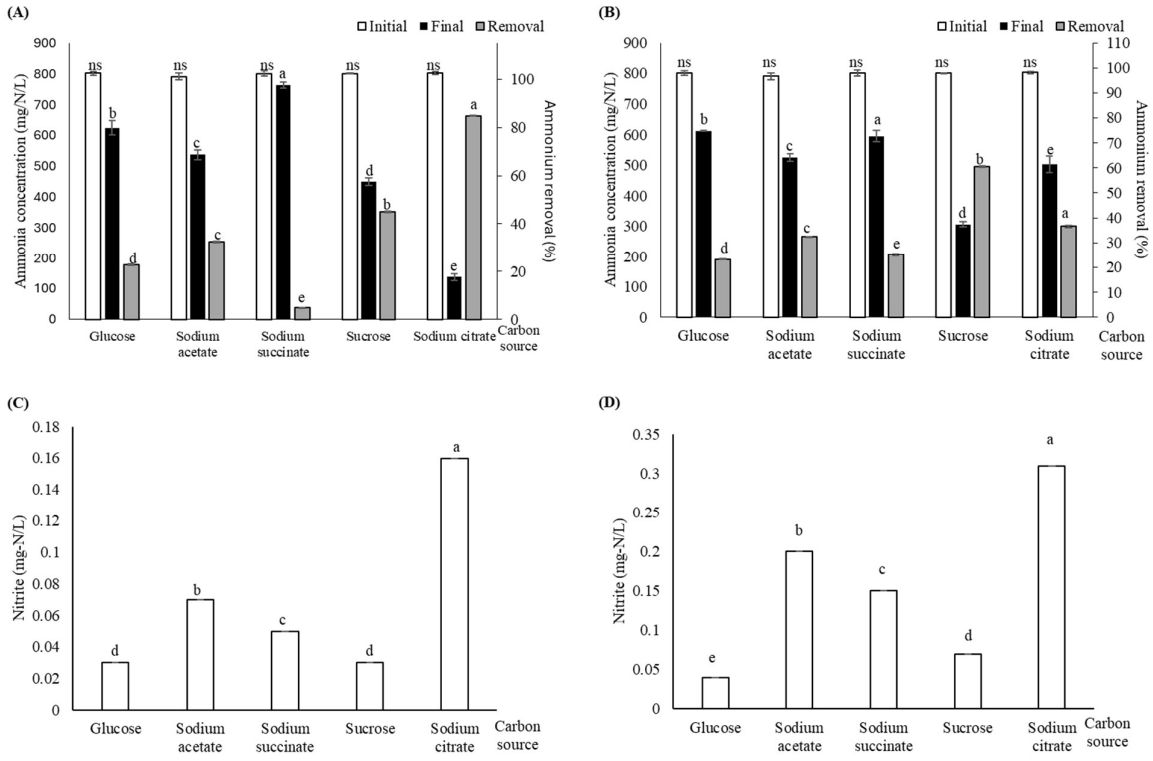

Figure 5 The optimization of carbon source; (A), (C) Ammonium removal efficiency and nitrite production of *A. faecalis* SRNB23, respectively, (B), (D) Ammonium removal efficiency and nitrite production of *A. faecalis* SRNB35, respectively.

The investigation focused on the nitrogen source requirements of the two species, with consideration given to variations in carbon sources. In order to accommodate the distinct carbon source preferences of *Alcaligenes* sp. SRNB23 and SRNB35, sodium citrate and sucrose were employed as the corresponding carbon sources. Based on the findings, it was observed that ammonium sulfate exhibited the highest efficacy as a nitrogen source for both strains of heterotrophic nitrifying bacteria. In the present study, it was observed that strain SRNB23 and SRNB35 had the ability to reduce ammonia levels by 78.13% (Figure 6A) and 67.49% (Figure 6B), respectively. Ammonium sulfate showed nitrite concentration of SRNB23 and SRNB35 was 0.77±0.01 mg-N/L (Figure 6C) and 1.00±0.12 mg-N/L (Figure 6D), respectively. The findings of this study align with the tests conducted by Lu

et al. [11], wherein they observed the ammonia reduction efficiency of *Alcaligenes* sp. W14 when exposed to various nitrogen sources. Consistent with the findings, it was shown that ammonium sulfate had the greatest efficacy in reducing ammonium levels. Therefore, it may be concluded that sodium citrate and ammonium sulfates were identified as the most suitable carbon and nitrogen sources, respectively, for the heterotrophic nitrifying bacterium *Alcaligenes* sp. W1. The report concurred with the findings of this investigation, which indicated that *A. faecalis* SRNB23 demonstrated sodium citrate and ammonium sulfate to be suitable donors of carbon and nitrogen. In their study, Yang et al. [10] investigated the impact of four distinct carbon sources (acetate, glucose, sodium acetate, and succinate) and ammonium sulfate as a nitrogen source on the ability of *Bacillus subtilis* A1 to remove ammonia. The results indicated that there were no statistically significant changes seen ($p>0.05$) in the ammonia removal capacity of *B. subtilis* A1 across the different carbon sources tested.

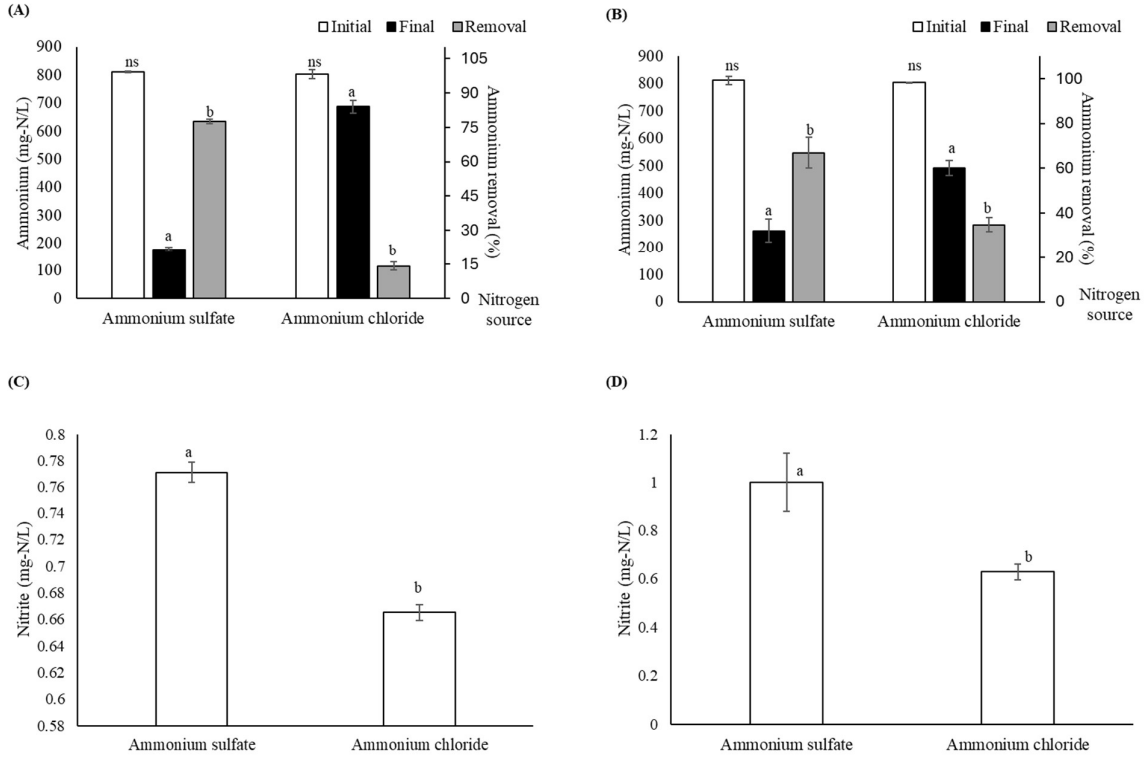

Figure 6 The optimization of nitrogen source; (A), (C) Ammonium removal efficiency and nitrite production of *A. faecalis* SRNB23, respectively, (B), (D) Ammonium removal efficiency and nitrite production of *A. faecalis* SRNB35, respectively.

3.3.2 C/N ratio

Sodium citrate and sucrose were employed as carbon sources, selected based on their suitability for providing adequate carbon and nitrogen. Ammonium sulfate is employed as a nitrogen source for two strains of *Alcaligenes* spp. The study determined that the ideal C/N ratio for SRNB23 was 16, resulting in a 75.30% reduction in ammonia (Figure 7A) and converted to nitrite as 0.05±4.19 mg-N/L (Figure 7C). This was closely followed by a C/N ratio of 8, which achieved a 71.93% reduction in ammonia and nitrite concentration was produced 0.03±4.02 mg-N/L. The findings from the SRNB35 study revealed that the C/N ratio of 2 exhibited the best efficiency in ammonia removal, achieving a rate of 52.60% (Figure 7B). This was followed by C/N ratios of 0 and 4, which resulted in corresponding ammonia removal rates of 33.67% and 31.00%, respectively. As the concentration of nitrite increases 0.07±0.03 mg-N/L at C/N ratio of 2 and followed by C/N ratios of 0 and 4, the nitrite concentration was detected as 0.12±0.00 and 0.12±0.00 mg-N/L, respectively (Figure 7D). This study focused on optimizing the C/N ratio of the *A. faecalis* strain NR. The study revealed that this strain achieved ammonium removal of 19.2 mg/L within a 48-hour period when the C/N ratio was set at 5 [6]. A comparable outcome was previously documented, indicating that *Acinetobacter junii* YB did not utilize ammonium when the carbon-to-nitrogen ratio was 5. According to Ren et al. [25], it was shown that *A. faecalis* strain no. 4 demonstrated a significant capability for ammonium removal at a C/N ratio of 10, as previously observed by Joo et al. [4]. A high C/N ratio can have a substantial impact as it enhances the quantity of organic matter available to heterotrophic nitrifying bacteria.

Nevertheless, it is evident that the enhancement of the C/N ratio does not lead to a perpetual improvement in the ability of heterotrophic nitrifying bacteria to eliminate nitrogen. Furthermore, according to Joo et al. [4] and Yang et al. [26], it was observed that when the C/N ratio exceeded 10, the growth rate of heterotrophic nitrifying bacteria was shown to be dependent on carbon sources, such as the generation of bioflocs [27].

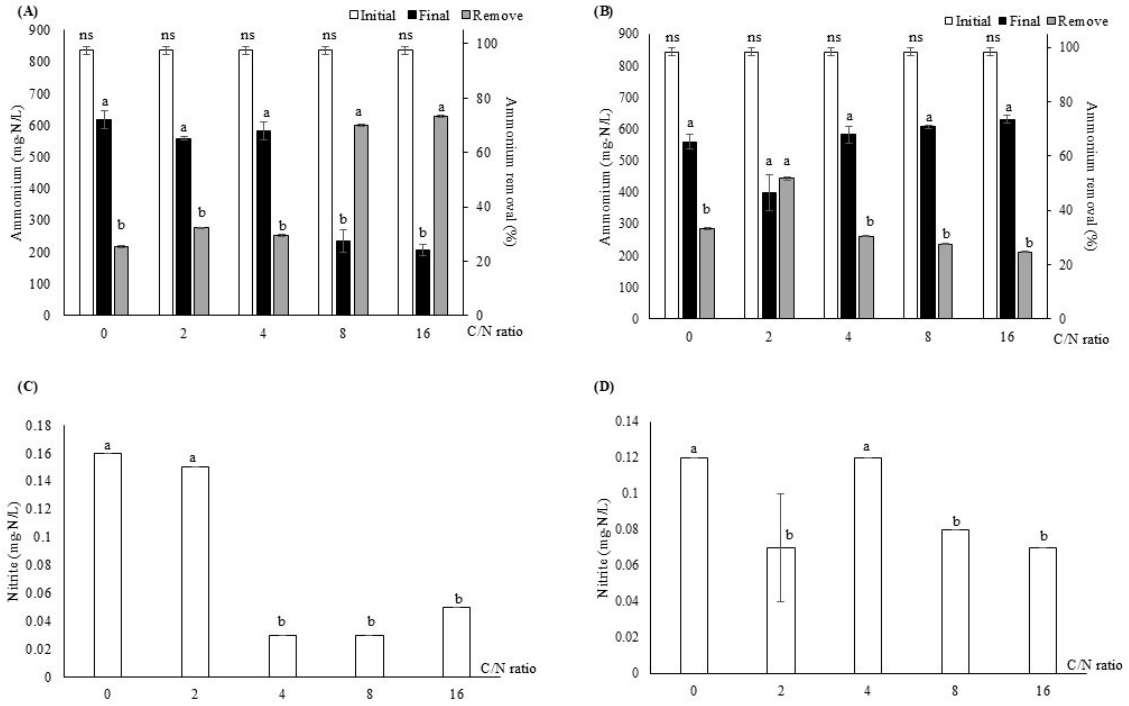

Figure 7 The optimization of C/N ratio; (A), (C) Ammonium removal efficiency and nitrite production of *A. faecalis* SRNB23, respectively, (B), (D) Ammonium removal efficiency and nitrite production of *A. faecalis* SRNB35, respectively.

3.4 Nitrogen removal in wastewater

The findings from the analysis of the wastewater treatment indicate that the mixed culture exhibited the best effectiveness in removing ammonium, with a rate of 63.07%. This was followed by the single cultures of SRNB35 and SRNB23, which had ammonium removal efficiencies of 57.43% and 56.45%, respectively (Figure 8A). The results of the nitrite production analysis indicated that all treatments exhibited an increase in nitrite concentration, with the exception of the mixed culture treatment, which demonstrated a reduction in nitrite concentration from 0.15 to 0.40 mg-N/L. The strain SRNB35 exhibited the most notable efficiency in removing nitrite, with levels decreasing from 0.14 to 0.09 mg-N/L (Figure 8B). The results indicate that the efficiency of nitrate production exhibited an increase in the treatments involving single cultures SRNB23 and SRNB5, as well as the control group, starting from day 0. However, the mixed culture treatment had a slight drop of around 3.14% compared to day 0 (Figure 8C).

The beginning concentration of ammonia in the experiment conducted to remove ammonium from shrimp aquaculture wastewater was approximately 450 mg-N/L, which was half of the initial concentration of ammonium in the flask-scale experiment (900 mg-N/L). According to Guo et al. [12], the initial ammonia demand had a notable effect on the efficacy of heterotrophic bacteria in ammonia removal.

The phenomenon of co-metabolism allows for enhanced efficiency in the removal of ammonia by mixed cultures, as compared to single cultures. Dhanasiri et al. [28] reported that the utilization of nitrifying bacteria derived from AOB and NOB in combination proved to be highly effective in regulating ammonia concentrations inside zebrafish culture systems. The application of heterotrophic bacteria in conjunction with *Halomonas aquamarina* and *Shewanella algae* as probiotics in white shrimp hatcheries resulted in notable improvements in the survival rate and weight gain of shrimp fry. Furthermore, the bacteria *Vibrio harveyi*, which is known to be a significant disease in shrimp, was impacted by these probiotics [29].

The present study observed variations in the nitrite levels among four treatment groups, potentially indicating differences in the nitrification process. Over the course of the 14-day experiment, a consistent rise in nitrite levels was observed, which corresponded with a decline in ammonia levels. However, it is worth noting that this trend was not observed in the experimental treatment including the suspension of mixed culture. The bacterial consortium employed in this research investigation exhibits the highest efficacy in mitigating ammonium concentrations. In a broad sense, the oxidation of ammonium content can be efficiently achieved through nitrification processes facilitated by AOB, specifically *Nitrosomonas* and *Nitrosospira*. For example, when ammonium concentrations are low. According to Bitton [30], AOB demonstrated a notable capacity for ammonia oxidation. Nevertheless, if the initial concentration of ammonia in this experiment exceeded the normal threshold, it is possible that the nitrification reaction, which converts ammonia to nitrite, may not have reached completion. Furthermore, the process of aerobic denitrification has the potential to convert nitrite into nitrogen gas. Several heterotrophic bacteria, including *Alcaligenes denitrificans*, *A. faecalis*, *Bacillus subtilis*, *Thioshaera pantotropha*, and *Ochrobactrum grignonense*, exhibit this particular trait [9,10].

The experiment involving mixed culture had the highest concentration of nitrate. The nitrification process involves the oxidation of inorganic nitrogen compounds, specifically ammonia to nitrite and nitrite to nitrate. The process of converting nitrite to nitrate is facilitated by the activity of certain bacteria such as *Nitrobacter* and *Nitrococcus* [31]. Nevertheless, it is worth noting that nitrate has the potential to undergo conversion into nitrogen gas by an aerobic denitrification reaction facilitated by some heterotrophic bacteria [9,10,32].

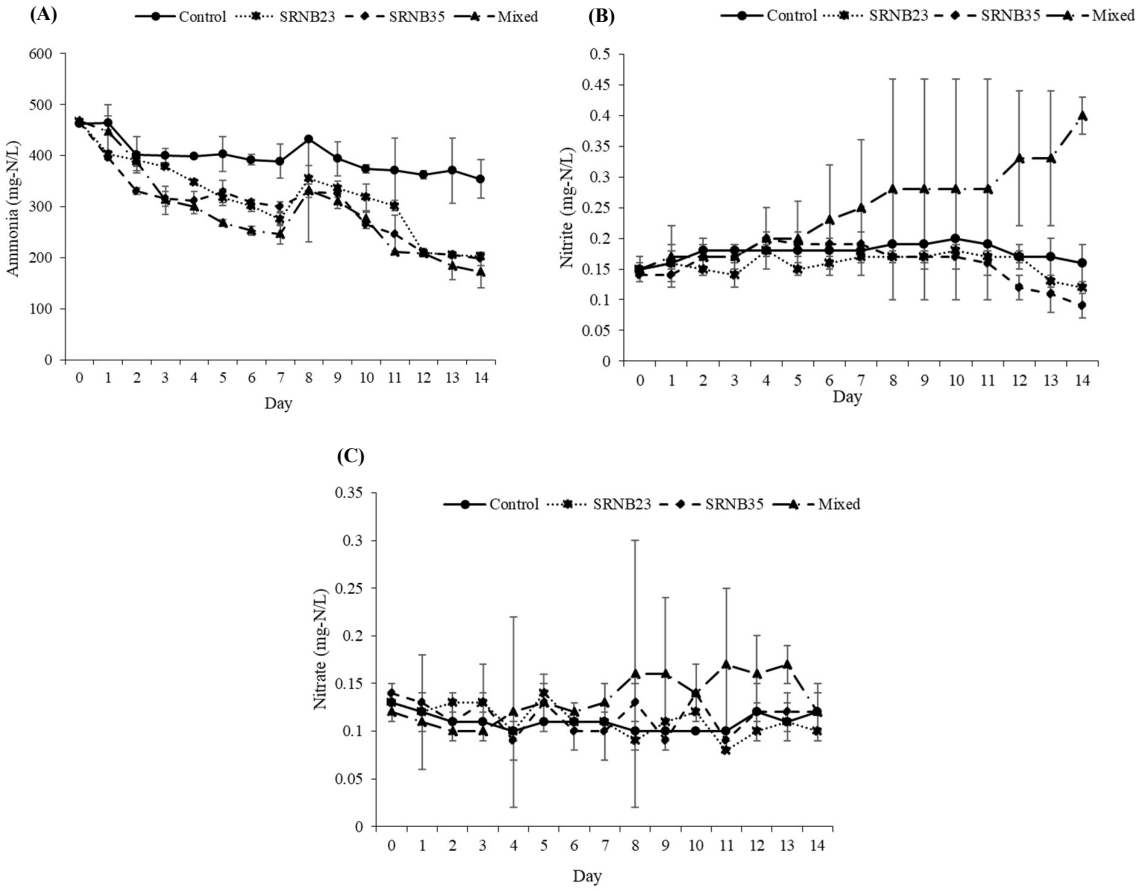

Figure 8 The potential of sterile shrimp wastewater to remove ammonium; (A) ammonia, (B) nitrite, (C) nitrate.

These *Alcaligenes faecalis* SRNB23 and SRNB35 can grow in saline water. In cases of high temperature and/or high pH and low salinity in shrimp ponds, ammonia is highly toxic; however, the bacterial isolates had the benefit of removing high ammonium concentration. On the other hand, reducing inorganic nitrogen in shrimp ponds, could also be used to treat a variety of wastewaters, including municipal, industrial, and hospital sources, since they have the ability to eliminate ammonia at high concentrations and tolerate a wide range of salinities.

4. Conclusion

The present study successfully identified and isolated two heterotrophic nitrifying bacteria, namely strain SRNB23 and SRNB35, from the bottom sediment of Pacific white shrimp cultivation. Based on the study of the 16S rRNA sequence, it was determined that SRNB23 and SRNB35 can be classified as *Alcaligenes* spp. Both strains exhibited a notable capability for ammonium removal, with an approximate efficiency of 91%. The optimal mixing ratio for *Alcaligenes* spp. SRNB23 and SRNB35 was determined to be 30:70. The observed ratio exhibited an ammonium removal effectiveness of 66.77%. Sodium citrate and sugar were identified as the appropriate carbon sources for SRNB23 and SRNB35, respectively. Ammonium sulfate serves as a suitable nitrogen source for both strains. The combination of SRNB23 and SRNB35 exhibited the highest efficacy in removing ammonium from shrimp wastewater, with an efficiency of 63.07%. The substance exhibits proficient ammonia removal capabilities and demonstrates a significant capacity for nitrification, resulting in the conversion of ammonia into nitrites and nitrates. Moreover, there is potential for enhancing and applying the output of heterotrophic nitrifying bacteria in order to enhance water quality in saline water or marine shrimp aquaculture in the future.

5. Acknowledgements

The authors would like to acknowledge the Agricultural Research Development Agency (ARDA) (CRP6105020740).

6. References

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