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ISOLATION OF BACTERIA INVOLVED IN AMMONIUM UPTAKE TO DEVELOP A BIOFORMULATION FOR WASTEWATER TREATMENT

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ABSTRACT

Ammonium contamination in wastewater poses significant threats to aquatic ecosystems and public health. In this study, bacterial strains capable of ammonium uptake were isolated from seafood processing wastewater and evaluated for their potential application in producing bioformulations. From 25 strains isolated, 4 strains demonstrated ammonium removal efficiencies greater than 90% at an initial concentration of 100 ppm. The most efficient strain, WV5.7, achieved ammonium removal rates of 99% and 88.6% at 100 ppm and 200 ppm ammonium, respectively, after 24 hours of cultivation in mineral medium. Based on 16S-rRNA gene sequencing and biochemical characterization, strain WV5.7 was identified as *Pseudomonas protegens* WV5.7. Suitable conditions for ammonium uptake included aerobic cultivation at pH 7, with the temperature range from 28°C to 31°C, and salinity below 1%. Seven carrier materials were applied for storing strain WV5.7, including sugarcane bagasse, sawdust, coconut coir, soybean residue, talc, and mixtures of talc with bagasse or sawdust (1:1, w/w). Among these, talc powder and the sawdust-talc mixture were the most effective carriers, maintaining ammonium uptake activity and viable cell numbers reaching above 10^6 CFU/g after 180 days of storage. The addition of vitamin B_{12} to the bacterial suspension did not significantly enhance bacterial viability or activity. Bioformulations retained their ammonium removal capability in both mineral medium and a lab-scale domestic wastewater treatment model. These findings highlighted *P. protegens* WV5.7 as a promising candidate for the development of microbial products to treat ammonium-rich wastewater.

Keywords: Ammonium, bioformulation, isolation, *Pseudomonas protegens* WV5.7, wastewater treatment.

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INTRODUCTION

Seafood processing is one of the key economic sectors in the Mekong delta of Vietnam. However, this process generates a substantial volume of wastewater enriched with organic matters and nitrogen compounds, particularly ammonium (NH_4^+), which primarily originates from the breakdown of proteins in fish blood, visceral fluids, and other biological by-products (Funge-Smith & Briggs, 1998). At high concentrations, ammonium is not only toxic to aquatic organisms but also accelerates eutrophication, leading to reduced dissolved oxygen, promoting the proliferation of algal blooms, and causing mortality of aquatic life (Camargo & Alonso, 2006). In the nitrification process, ammonium is converted into nitrite (NO_2^-), nitrate (NO_3^-), and nitrous oxide (N_2O) - a greenhouse gas with a global warming potential. Moreover, when ammonium enters the human body, it can be converted into nitrite, which interferes with the oxygen-carrying capacity of red blood cells, leading to respiratory distress and severe complications involving the nervous and circulatory system (Fewtrell, 2004).

Conventional methods applied for ammonium removal, including ion exchange, chemical precipitation, biofiltration, and chlorination, are widely applied in wastewater treatment. However, these approaches often entail high operational costs and may generate by-products that pose risks of secondary pollution (Rittmann & McCarty, 2001). In contrast, microbial-based treatment has emerged as a sustainable and high-efficiency solution. This approach minimizes ecological impacts, offers operational flexibility, and is particularly cost-effective and environmentally friendly (Zhang et al., 2012).

Certain bacteria were able to absorb and utilize ammonium as a nitrogen source for growth. These bacterial strains were typically isolated from wastewater and belonged to various groups such as *Nitrosomonas*, *Nitrobacter*, *Bacillus*, *Acinetobacter*, and *Pseudomonas* (Han & Zhou, 2022; Madigan et al., 2019). The isolation, selection, and development of bio-products containing indigenous bacterial strains represent a promising approach to the biological treatment of contaminants such as ammonium. This strategy has been studied worldwide due to its sustainability, high efficiency, and environmental friendliness. The Mekong delta of Vietnam is a large region for culturing and processing seafood. However, no study on the production of bioformulation containing indigenous bacteria for the removal of ammonium in seafood processing wastewater has been reported. This study aimed at isolating indigenous bacterial strains able to efficiently uptake ammonium and selecting suitable carriers for the storage of bacteria, providing data for the development of a microbial formulation for ammonium removal in wastewater.

MATERIALS AND METHODS

Isolation of bacteria involved in ammonium uptake

Sampling: Three samples, including (i) wastewater sample of the influent, (ii) wastewater of the aeration tank, and (iii) activated sludge, were collected at the wastewater treatment system of Hai Sang seafood joint stock company (10°18'25.9"N, 105°30'12.4"E) in Thoi Thanh, Thoi Thuan, Thot Not, Can Tho. Each sample was taken along the tank at three points, each point spaced one meter apart. The three sub-samples were mixed thoroughly to get one representative sample. The characteristics of wastewater samples were illustrated in Table 1.

Enrichment of bacteria involved in ammonium uptake: For each sample, 5 mL of wastewater sample (or 5 g of activated sludge) was added to 45 mL minimal mineral medium (MM medium, 1.42 g Na_2HPO_4, 1.36 g KH_2PO_4, 98.5 mg $MgSO_4.7H_2O$, 5.75 mg $CaCl_2.2H_2O$, 3.2 mg Na_2-EDTA, 2.75 mg $FeSO_4.7H_2O$, 1.7 mg $MnSO4.H_2O$, 1.16 mg H_3BO_3, 1.15 mg $ZnSO_4.7H2O$, 0.24 mg $CuSO_4$, 0.24 mg $CoCl_2.6H_2O$, 0.1 mg MoO_3 and 1,000 mL H_2O, pH 7.0 ± 0.2 (Breugelmans, 2005) supplemented with 100 ppm NH_4Cl. The cultures were shaken at 200 rpm for one week at laboratory temperature (30

± 2ºC). After the 1st enrichment culture, the supernatants were transferred to pure MM medium, and the cultures were incubated under the same conditions. After repeated transfers, the cultures from the 3rd enrichment were settled for 30 minutes, and the supernatants were used for isolating ammonium-utilizing bacteria (Perry et al., 2002).

Table 1. Characteristics of domestic wastewater used in this study

Parameter	Unit	First wastewater sample		Second wastewater sample		QCVN 14: 2008/BTNMT (Column A)
		Non-sterilized	Sterilized	Non-sterilized	Sterilized	
pH	-	$7.87^{c} \pm 0.09$	$8.35^{b} \pm 0.13$	$7.05^{d} \pm 0.04$	$8.61^{a} \pm 0.01$	5–9
DO	mg/L	$0.60^{a} \pm 0.10$	$0.43^{a} \pm 0.06$	$1.17^{a} \pm 0.20$	$0.91^{a} \pm 0.11$	-
COD		$975.00^{a} \pm 5.77$	$855.00^{b} \pm 5.77$	$610.00^{c} \pm 5.77$	$575.00^{d} \pm 10.00$	75
TSS		$530.43^{a} \pm 26.43$	$483.15^{a} \pm 15.28$	$405.67^{b} \pm 24.01$	$324.00^{c} \pm 17.09$	50
PO_4^{3-}		$2.81^{a} \pm 0.26$	$2.67^{a} \pm 0.12$	0^{b}	0^{b}	6
NO_3^{-}		$2.57^{a} \pm 0.59$	$2.48^{a} \pm 0.66$	$1.52^{ab} \pm 0.16$	$0.96^{b} \pm 0.10$	30
NO_2^{-}		0	0	0	0	-
NH_4^{+}		$22.99^{a} \pm 1.08$	$21.29^{a} \pm 0.61$	$12.95^{b} \pm 0.49$	$9.69^{c} \pm 0.14$	5
Aerobic microbial count	$\times 10^6$ CFU/mL	$2.97^{a} \pm 0.25$	0^{b}	$1.67^{a} \pm 1.15$	0^{b}	-

Note: Within each row, means followed by the same letters are not significantly different based on statistical analysis at the 5% level of significance ($p < 0.05$). QCVN 14:2008/BTNMT (Column A): Vietnam National Technical Regulation on Domestic Wastewater.

Isolation of bacteria involved in ammonium uptake: The dilutions of the 3rd enrichment culture (10^{-1}-10^{-5}) were spread onto the surface of MM agar medium supplemented with 100 ppm ammonium, and the plates were incubated at 32 ºC. After 7 days, discrete colonies with distinct morphology were selected for bacterial isolation by streaking on the same medium. Subsequently, the colonies were further purified by repeated streaking on Tryptic Soy Agar plates (TSA, 30 g/L Tryptic Soy Broth (TSB), 15 g/L agar). The purity of the isolates was confirmed based on colony morphology and cell shape under the microscope.

Assessment of ammonium uptake efficiency

Bacterial suspension preparation: A single colony of each strain cultivated for 72 hours on TSA medium was inoculated into 4 mL of TSB and shaken at 200 rpm for 24 hours at laboratory temperature. The optical density ($OD_{600\ nm}$) of the culture was then adjusted to 0.7.

Experimental set up: 40 µL of the bacterial suspension was inoculated into 4 mL of MM medium supplemented with NH_4Cl to achieve ammonium concentrations of 25, 50, and 100 ppm. Two control treatments were (i) bacterial inoculation without ammonium supplementation, and (ii) ammonium supplementation without bacterial inoculation. The cultures were grown as mentioned above, each treatment was done in triplicate. After 24 hours of incubation, the residual ammonium concentration in the medium was quantified spectrophotometrically (Keeney & Nelson, 1982).

Identification of the selected strain involved in ammonium uptake

The bacterial strain exhibiting the highest ammonium uptake was selected for 16S-rRNA gene sequencing. Bacterial DNA was extracted following the DNA extraction protocol of Sambrook et al. (1989) and sequenced at Nam Khoa Service and Trading Co., Ltd., Ho Chi Minh City. The 16S-rRNA

gene was amplified using the primer pair 27F (5'-AGAGGTTTGATCC-TGGCTC-3') and 1492R (5'-TACGGTTACCTTGTTAACGAC T-3') (Frank et al., 2008). The PCR products were verified by electrophoresis on 1.5% agarose gel and sequenced using the Sanger method (Blazej et al., 2006). The 16S-rRNA gene sequence was compared for similarity with the corresponding gene sequences at the National Center for Biotechnology Information (NCBI) database using the BlastN tool. The 16S-rRNA gene was deposited at the NCBI to obtain the accession number.

Phylogenetic analysis of the 16S-rRNA gene sequences was performed using the selected strain and strains with the most similar sequences retrieved from GenBank. Sequence alignment was conducted with MUSCLE and manually refined. A neighbor-joining tree (Saitou & Nei, 1987) was constructed based on evolutionary distances calculated using the Kimura 2-parameter model in MEGA version 12 (Kumar et al., 2024). The robustness of the tree topology was evaluated by bootstrap analysis with 1,000 replicates (Felsenstein, 1985). Evolutionary distances were also estimated using the Maximum Composite Likelihood method (Tamura et al., 2004). Subsequently, biochemical analysis was used for species-level confirmation.

Effects of ammonium concentration, aeration, temperature, pH, and salinity on the ammonium uptake of the selected bacterial strain

Bacterial strain demonstrating efficient ammonium uptake was further examined for the effects of these factors on their ammonium absorption capacity. Bacterial suspension was prepared as described previously. Then, 40 µL of the bacterial suspension was inoculated into test tubes containing 4 mL of MM medium supplemented with ammonium at concentrations corresponding to each experimental condition. Control treatment was conducted similarly without bacterial inoculation. Each treatment was performed in triplicate. The residual ammonium in the culture medium was quantified after 24 hours of incubation.

Effect of ammonium concentration: Bacteria were cultured under aerated conditions in MM medium supplemented with ammonium concentrations of 200 and 300 ppm. The suitable ammonium concentration for bacterial growth was selected for subsequent tests.

Effect of aeration: Bacteria were cultured under two conditions, including (i) aeration on a rotary shaker at 200 rpm, and (ii) without aeration.

Effect of temperature: Bacteria were cultured (either being aerated or non-aerated based on the result of the previous experiment) at 28, 31, 34, 37, and 40ºC to determine the suitable temperature for ammonium uptake of the isolates.

Effect of pH: Bacteria were inoculated in MM medium supplemented with a suitable ammonium concentration based on the result of the previous experiment. pH of the medium was adjusted to 5, 6, 7, and 8 to assess the effect of pH on ammonium uptake of bacteria. Bacteria were grown either with or without aeration, and at a suitable temperature, depending on the results obtained from the above-mentioned experiments.

Effect of NaCl concentration: Based on the result of the previous experiments, bacteria were cultured in MM medium with selected ammonium concentration, aeration or not, temperature, pH and NaCl was added to the medium at concentrations of 1%, 2%, and 3% (w/v).

Selection of a suitable carrier for storing bacteria

Seven carriers, including bagasse, sawdust, coconut fiber, soybean hulls, talc powder, a mixture of talc powder and bagasse, and a mixture of talc powder and sawdust (1:1 ratio) (An et al., 2017; Oanh et al., 2022) were used to store the potential strain involved in ammonium uptake. These carriers were tested for the ability to maintain both bacterial viability and ammonium uptake during the storage period.

Carrier preparation: Bagasse, sawdust, coconut fiber, and soybean hulls were sun-

dried, ground into fine powder, and further dried for 48 hours at 65ºC. The carriers were prepared according to the method described by Oanh et al. (2022).

*Preparation of bacterial suspension***:** The bacterial suspension was prepared as described previously. The suspension was collected at the time point when bacteria reached the highest density. The density of bacterial suspension was adjusted to achieve 10^9 CFU/mL.

Inoculation of bacterial suspension into carriers: 0.5 mL of the bacterial suspension (approximately 10^9 CFU/mL) was inoculated into plastic bags containing carriers (5 g/bag). The moisture content was determined by a moisture analyzer (Ohaus MB120, USA). Preparations with moisture content below 20% were considered acceptable (Bharathi et al., 2004). The bags were sealed, labelled, and stored at laboratory temperature. Control treatments consisted of TSB medium and carrier. The experiment was arranged in a completely randomized block design with three replicates. After 30 days of storage, twenty-four bags, including seven carriers and control were randomly taken to determine the viability of bacteria. Each bag was suspended in 45 mL of sterile distilled water, shaken at 200 rpm for 30 minutes, and left to settle for 30 minutes. The supernatant was then used for viable cell enumeration on TSA medium by the drop-plating method. The ammonium absorption ability of the preserved bacteria was confirmed in MM medium supplemented with 100 ppm ammonium.

A carrier maintaining bacterial viability above 10^6 CFU/g and ammonium absorption was selected for the storage of bacteria for 180 days. Vitamin B_{12} (B_{12}) was also added to the bacterial suspension prior to inoculation into the selected carrier for examination of the bacterial viability enhancement and ammonium uptake after being preserved.

Experimental design for assessment of ammonium uptake in wastewater

After 90 and 180 days of storage, bacteria were pre-cultured in TSB medium and evaluated for their ammonium uptake in 50 mL of domestic wastewater. The experiment included 4 treatments as outlined in Table 2.

Table 2. Experimental setup for the evaluation of ammonium uptake in domestic wastewater

Treatment (T)	Description	Wastewater supplemented with ammonium to reach 100 ppm
T1	Bacterial suspension	Non-sterilized
T2		Sterilized
T3	Control (without bacterial inoculation)	Non-sterilized
T4		Sterilized

The experiment was done at 2 separate times using bacteria stored after 90 and 180 days in a suitable carrier for the evaluation of ammonium removal after storage. A domestic wastewater sample was collected at the drainage outlet of Tham Tuong canal, an area densely populated with plenty of food vendors, located at Mac Thien Tich street, Can Tho. Wastewater samples were taken at three locations, including at the beginning, middle, and end of the street. The samples were mixed to obtain one representative sample. The collected wastewater sample was filtered through a cloth of three layers (mesh size of 0.5 mm) to remove large solid particles prior to analysis.

RESULTS AND DISCUSSION

Isolation of bacteria involved in ammonium uptake

Among the three samples collected, 25 ammonium-absorbing bacterial strains were isolated, including 9 strains from activated sludge, 9 from the aeration tank, and 7 from influent water. The bacterial colonies exhibited round or irregular shape; color varied from white, yellow to orange; the colony margin was entire, lobate or serrated; elevation ranged from

flat to raised; surface was smooth. Among the isolates, 16 strains were Gram-negative and 9 were Gram-positive.

Assessment of ammonium uptake efficiency

The data showed differences in ammonium uptake among 25 strains when cultured in MM medium supplemented with 25, 50, and 100 ppm of ammonium after 24 hours of incubation. When cultured in MM medium supplemented with 25 and 50 ppm ammonium, most bacterial strains exhibited efficient ammonium uptake. Five strains, SH5.3, SH5.5, WV5.1, WV5.3, and WV5.7, demonstrated ammonium removal efficiencies exceeding 97% after 24 hours of incubation. When cultured in MM medium supplemented with 100 ppm ammonium, 20 out of 25 bacterial strains demonstrated an ammonium uptake efficiency greater than 70%, showing a statistically significant difference compared to that of the remaining strains and the uninoculated control treatment. Notably, strain WV5.7 showed the highest ammonium uptake efficiency (99%) at 100 ppm (Fig. 1).

Zhao et al. (2010) indicated that *Acinetobacter calcoaceticus* HNR, isolated from a biofilm membrane, could completely remove 120 ppm of ammonium within 48 hours of cultivation. In this study, WV5.7 demonstrated high ammonium uptake efficiency, reaching 100, 98, 99, and 88.6% at concentrations of 25, 50, 100, and 200 ppm, respectively, after 24 hours of growth. Compared to previous results, WV5.7 not only achieved high removal efficiency but also maintained stability across a wide concentration range, showing potential for application in wastewater treatment with fluctuating ammonium loads.

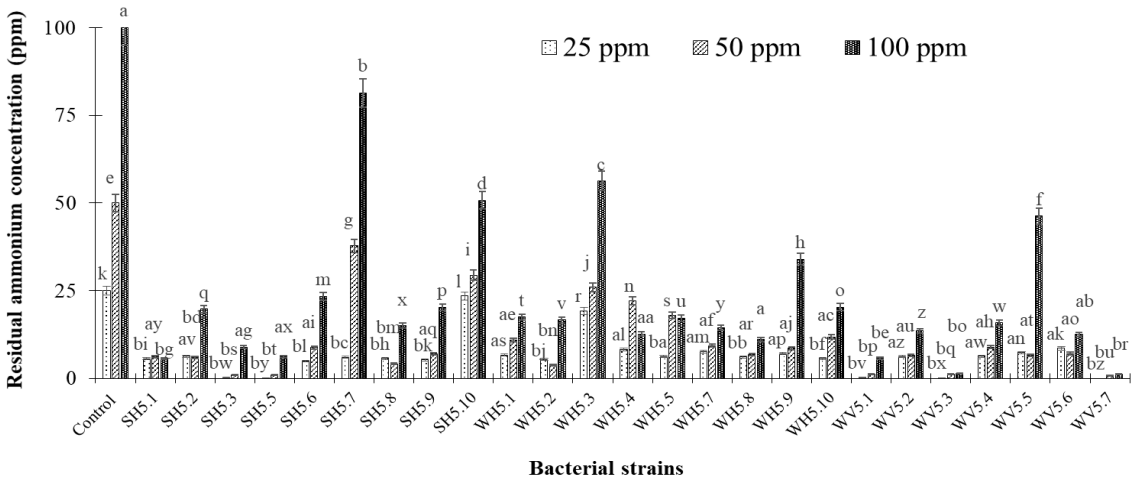

Figure 1. Ammonium uptake by bacteria cultivated for 24 hours in MM supplemented with ammonium (Treatments sharing the same letters are not significantly different at 5%)

Identification of strain WV5.7

Sequence analysis of the 16S-rRNA gene (accession number: PX472060) indicated that strain WV5.7 belongs to the genus *Pseudomonas* and shows the highest sequence similarity with the corresponding gene of the 24 strains, including type strains, of *Pseudomonas* species. Based on sequence alignment, the constructed neighbor-joining tree revealed that *Pseudomonas* sp. WV5.7 is the most related to *Pseudomonas protegens* CHAO^T (NR_114749) (Fig. 2). Comparing biochemical traits of WV5.7 and closely related species, including *Pseudomonas abietaniphila* BKME-9, *Pseudomonas saponiphila* DSM 9751^T, *Pseudomonas sesami* SI-P133^T, and *P. protegens* CHAO^T, showed that the biochemical profile of WV5.7 matched that of *P. protegens* CHAO^T. Both strains exhibited glucose and citrate assimilation; mannitol and glycerol fermentation; catalase, oxidase, gelatinase and

lipase activity. However, both had no signal for glucose, arabinose, sucrose, and lactose fermentation. The differences between them were starch fermentation and urease activity (Table 3). These data indeed confirmed that WV5.7 belongs to *P. protegens* and was designated as *P. protegens* WV5.7.

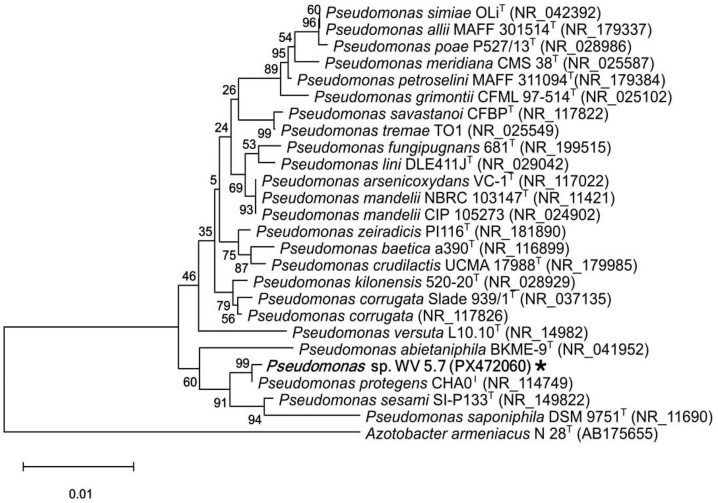

Figure 2. Neighbor-joining tree of *Pseudomonas* sp. WV5.7 and 24 strains including type strains of *Pseudomonas* species inferred from 16S-rRNA gene sequences (GenBank accession numbers of the sequences are given in parentheses next to each strain. Numbers at the nodes are percentage bootstrap values based on 1,000 resampled datasets. The 16S-rRNA gene sequence of *Azotobacter armeniacus* was used as the outgroup. The scale bar indicates a genetic distance of 0.01 nt substitutions per site. ^{T}Type strain)

Table 3. Comparison between biochemical traits of WV5.7 and *Pseudomonas* species

No.	Species	Biochemical characteristics														Reference
		Glucose assimilation	Glucose fermentation	Mannitol fermentation	Glycerol fermentation	Arabinose	Citrate assimilation	Starch fermentation	Urease	Catalase	Oxidase	Gelatinase	Lipase	Sucrose fermentation	Lactose fermentation	
1	*Pseudomonas abietaniphila* BKME-9	+	-	nd	+	+	nd	nd	nd	+	-	nd	nd	nd	nd	Mohn et al., 1999
2	*Pseudomonas saponiphila* DSM 9751^{T}	+	-	+	+	-	+	+	nd	+	+	+	+	+	-	Lang et al., 2010
3	*Pseudomonas sesami* SI-P133^{T}	nd	nd	nd	nd	nd	nd	nd	+	+	+	+	+	nd	nd	Madhaiyan et al., 2017
4	*Pseudomonas protegens* CHAO^{T}	+	-	+	+	-	+	nd	+	+	+	+	+	-	-	Ramette et al., 2011
	WV5.7	+	-	+	+	-	+	-	-	+	+	+	+	-	-	This study

Note: (-): negative; (+): positive; nd: not determined.

Pseudomonas protegens was shown to play a role in heavy metal resistance and plant protection. *P. protegens* S4LiBe and S5LiBe, isolated from heavy metal-contaminated agricultural wastewater, exhibited resistance to multiple metals, strong antagonism against diverse phytopathogenic fungi, insecticidal activity, and plant growth-promoting effects (Bensidhoum et al., 2016). Takeuchi et al. (2023) indicated that the root-colonizing fluorescent *P. protegens* produces a number of antibiotic secondary metabolites and extracellular enzymes contributing to the suppression of pathogen in the rhizosphere.

Effects of ammonium concentration, aeration, temperature, pH, and salinity on the ammonium removal of WV5.7

The results showed that when cultured in MM medium supplemented with 200 and 300 ppm ammonium, WV5.7 achieved ammonium removal efficiencies of 88.6% and 74%, respectively, after 24 hours of cultivation (Fig. 3A). According to Cao et al. (2023), *Alcaligenes aquatilis* AS1 achieved over 90% ammonium removal in livestock wastewater after 24 hours at an initial concentration of 200 mg/L. Therefore, an ammonium concentration of 200 ppm was applied for subsequent experiments.

After 24 hours of cultivation in MM medium supplemented with 200 ppm ammonium under both aerated and non-aerated conditions, WV5.7 exhibited significantly higher ammonium uptake under aerated conditions. The ammonium removal efficiencies were 88.75% and 59.65% under aerated and non-aerated conditions, respectively (Fig. 3B). These results indicated that aeration enhanced the ammonium uptake of WV5.7. Indeed, the presence of oxygen markedly improved ammonium absorption, which is consistent with the finding of Freyschmidt and Beier (2023) that aerobic ammonium-oxidizing bacteria (AOB) exhibited stronger biological activity under aerobic conditions.

Under aerated conditions, the pH of the culture medium also influenced the ammonium uptake of WV5.7. In medium adjusted with different pH, WV5.7 exhibited varying uptake efficiencies. At pH 5 and pH 6, WV5.7 showed lower ammonium uptake compared to those at pH 7 and pH 8. Notably, at pH 7, WV5.7 achieved the highest ammonium removal efficiency (93.95%), which was significantly higher than that at other pH conditions (Fig. 3C). The suitable ammonium uptake of strain WV5.7 was observed with a pH ranging from 7 to 8, aligning with the findings of Thandar et al. (2016) that *Nitrosomonas mobilis* Ms1 isolated from a wastewater treatment system was capable of efficiently removing ammonium under optimal conditions of pH 8 at 27ºC, and salinity below 3%.

Strain WV5.7 cultured in MM medium supplemented with 200 ppm ammonium (pH 7) under aerated conditions at temperatures of 28, 31, 34, 37, and 40ºC showed that ammonium uptake also varied depending on temperature. As the temperature increased, ammonium uptake efficiency decreased. The highest removal efficiency was observed at 28ºC and 31ºC, reaching 98.2% and 99%, respectively. At 34, 37, and 40ºC, ammonium uptake efficiency was 90.8, 73.3, and 39.9%, respectively (Fig. 3D). Among the five temperatures tested, WV5.7 achieved high ammonium removal efficiencies at 28, 31, and 34ºC. At 37ºC and 40ºC, ammonium uptake efficiency markedly declined, indicating that elevated temperatures negatively affected the ammonium absorption of this strain. According to Xu et al. (2025), *Klebsiella pneumoniae* LCU1 exhibited optimal ammonium removal efficiency at 30ºC, with a notable decline as the temperature increased to 37ºC.

Strain WV5.7 cultured in MM medium supplemented with 200 ppm ammonium (pH 7) under aerated conditions at temperatures ranging from 28ºC to 31ºC, NaCl added at concentrations of 0, 1, 2, and 3%, showed that ammonium uptake varied depending on NaCl concentration. As NaCl concentration increased, ammonium uptake decreased. Ammonium removal efficiency of WV5.7 was 98.1, 96.2, 92.6, and 83.6% at 0, 1, 2, and 3% NaCl, respectively (Fig. 3E). According to Dong et al. (2019), *Pseudomonas aeruginosa* HT1 demonstrated optimal ammonium metabolism at

a concentration of 50 ppm at pH 7.5 and salinity ranging from 1% to 3%. Furthermore, Fan et al. (2024) indicated that when the salinity of the environment exceeded 1.5%, the efficiency of ammonium removal decreased significantly. Our data indicated that WV5.7 exhibited high ammonium uptake under aerated conditions, neutral pH, and a low salinity environment. These findings showed that the physiological characteristics of WV5.7 align well with previous studies highlighting its potential application for ammonium treatment in wastewater, where pH, temperature, and salinity always fluctuate.

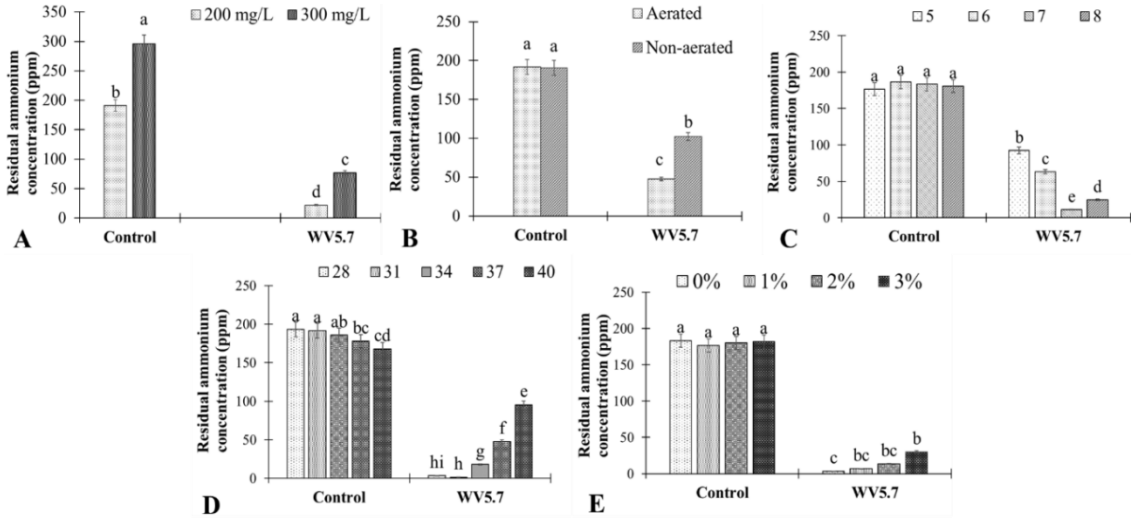

Figure 3. Factors influencing the ammonium uptake of WV5.7. A: Ammonium concentration; B: Aeration; C: pH; D: Temperature; E: NaCl concentration. Columns followed by the same letters indicate no statistically significant difference at 5% significance level ($p < 0.05$)

Selection of suitable carriers for the storage of WV5.7

Among seven carrier materials examined, talc powder and sawdust-talc mixture were able to maintain the viability of WV5.7 after 30 days of storage, with the cell viability reaching $(50 \pm 1.00) \times 10^6$ and $(20 \pm 0.75) \times 10^6$ CFU/g, respectively. The remaining five carriers, along with the corresponding control treatments, showed no detectable colony-forming units upon viability testing. Additionally, *P. protegens* WV5.7 preserved for 30 days still maintained ammonium uptake property after 24 hours of cultivation. When WV5.7 was stored in talc powder and a sawdust-talc mixture, ammonium removal efficiency was 92.05% and 91.73%, respectively (Fig. 4). Compared to the non-stored WV5.7, the strain achieved an ammonium absorption efficiency of 99%, which was slightly higher than that observed in the stored bacteria. Both talc powder and the sawdust-talc mixture maintained the viability and ammonium absorption of WV5.7.

Based on these data, the two carrier materials were used for further investigation of bacterial viability and performance over a 180-day storage period. B_{12} was also added to the bacterial suspension to evaluate its effect on viability. The viability of *P. protegens* WV5.7 stored in talc powder and sawdust-talc mixture after 180 days was presented in Table 4.

In general, the viability of *P. protegens* WV5.7 decreased over time in all treatments, however, significant differences were observed among the carrier materials. Talc powder with or without B_{12} was more effective in maintaining WV5.7 viability, showing statistically significant differences compared to the sawdust-talc formulations with or without B_{12}. The two tested carriers still maintained cell numbers above 10^6 CFU/g, indicating their

effectiveness in preserving bacterial viability (Table 4). During 180 days of storage, the ammonium uptake ability of the preserved bacteria was still maintained, reaching 90% to 94%, and showed a significant difference with the control treatment without bacterial inoculation. *P. protegens* WV5.7 with or without B_{12} stored in the two tested carrier materials had no significant difference in ammonium uptake during 180 days of preservation, indicating that talc and sawdust-talc were suitable carriers for this strain (Fig. 5). However, talc powder was more effective in maintaining WV5.7 viability and, as such, was identified as the most suitable carrier for the long-term storage of *P. protegens* WV5.7.

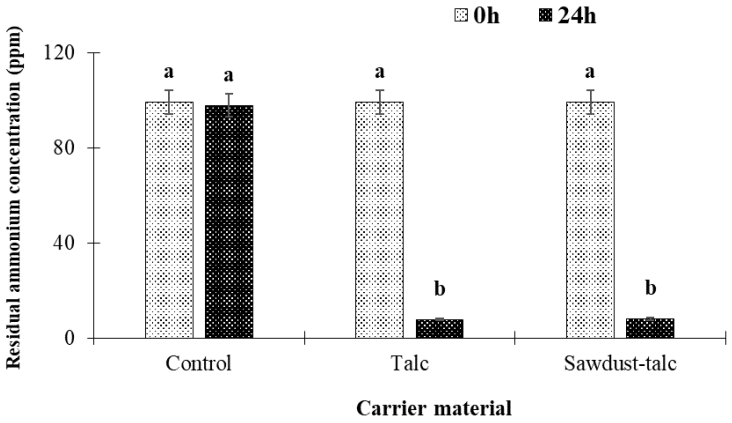

Figure 4. Ammonium absorption of *Pseudomonas protegens* WV5.7 after 30 days of storage. Columns followed by the same letters indicate no statistically significant difference at 5% significance level ($p < 0.05$)

Table 4. Viability of *Pseudomonas protegens* WV5.7 after 180 days of storage

Storage time (days)	Bacterial viability ($\times 10^6$ CFU/g bioformulation)			
	Talc	Talc + B_{12}	Sawdust-talc	Sawdust-talc + B_{12}
30	$56.67^{aA} \pm 15.28$	$63.33^{aA} \pm 5.77$	$23.33^{bA} \pm 5.77$	$26.67^{bA} \pm 5.77$
60	$43.33^{aAB} \pm 5.77$	$53.33^{aA} \pm 5.77$	$20.00^{bAB} \pm 0.00$	$23.33^{bAB} \pm 5.77$
90	$33.33^{aBC} \pm 5.77$	$36.67^{aB} \pm 5.77$	$16.33^{bABC} \pm 5.51$	$19.00^{bABC} \pm 1.73$
120	$25.33^{abBC} \pm 8.08$	$27.67^{aBC} \pm 4.04$	$13.33^{bBCD} \pm 1.53$	$14.67^{abBCD} \pm 5.51$
150	$20.33^{aC} \pm 1.53$	$19.67^{aCD} \pm 0.58$	$9.67^{bCD} \pm 3.06$	$9.00^{bCD} \pm 1.00$
180	$14.33^{aC} \pm 2.08$	$13.00^{aD} \pm 3.00$	$3.68^{bD} \pm 0.58$	$5.33^{bD} \pm 0.58$

Note: Within the same row, values followed by the same letters (e.g. a or b) and within the same column, values followed by the same capitalized letters (e.g. A, B or C) are not significantly different.

These findings are consistent with previous studies on bacterial viability in bioformulations. Wang et al. (2007) reported that vitamin B_{12} can stabilize cell membranes and enzymes, thereby prolonging bacterial survival under dry conditions and room temperature. Singh et al. (2020) demonstrated that bioformulation using an inorganic carrier such as talc maintains higher bacterial cell density compared to organic carriers that are more readily biodegradable, such as sawdust or agricultural residues. Oanh et al. (2022) found that *Rhodococcus* sp. XL6.2, a strain capable of degrading benzene, toluene and xylene (BTX), maintained over 90% removal efficiency after six months of storage in talc powder (with or without vitamin B_{12}). Importantly, there was no statistically significant difference in BTX degradation efficiency between the stored and non-stored cells when tested in MM medium supplemented with BTX, indicating that talc powder helps preserve microbial functional

stability during long-term storage. Moreover, Uyen et al. (2023) reported that *Comamonas* sp. PAN1.12 retained its phosphate absorption (100 ppm) after six months of storage in talc powder (with or without vitamin B_{12} addition to bacterial suspension). These results further confirm the role of talc powder in maintaining both viability and activity of bacteria stored in bioformulations. Our data demonstrated that the viability and property of WV5.7 stored in talc powder still remains and also highlighted the effectiveness of dry bioformulation, which are widely applied in environmental remediation practices.

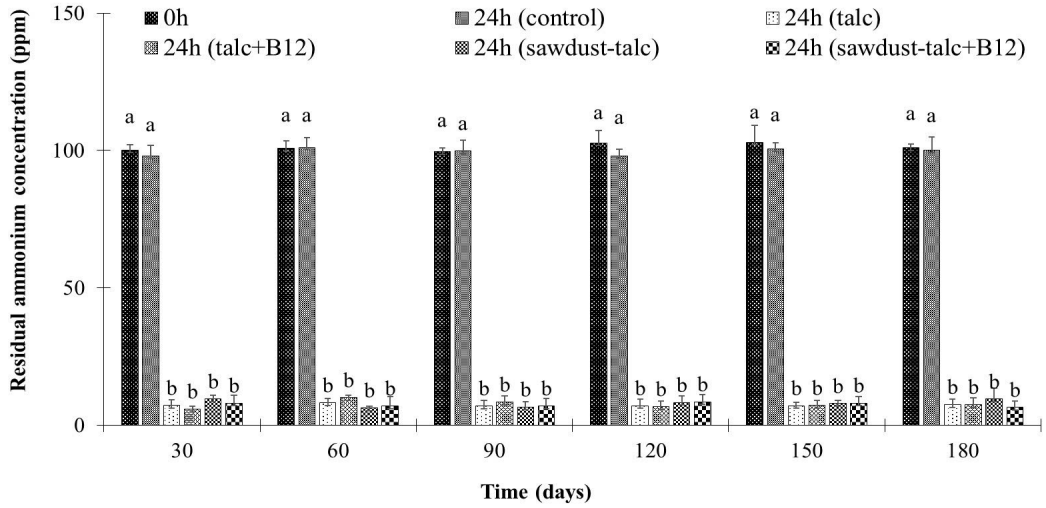

Figure 5. Ammonium absorption of *Pseudomonas protegens* WV5.7 after 180 days of storage. The preserved WV5.7 was cultivated for 24 hours in MM medium supplemented with 100 ppm ammonium. Control: Without WV5.7 inoculation. Columns followed by the same letters indicate no statistically significant difference at 5% significance level ($p < 0.05$)

Lab-scale application of *Pseudomonas protegens* WV5.7 stored in talc powder for the removal of ammonium from domestic wastewater

The collected wastewater exhibited a neutral to slightly alkaline pH, high concentrations of organic and inorganic substances. The total suspended solids (TSS), chemical oxygen demand (COD), and ammonium concentration exceeded the permissible limits for discharge into receiving water bodies, as regulated by QCVN 14:2008/BTNMT (Table 1). Following autoclave sterilization (121ºC, 20 minutes), several parameters of wastewater exhibited significant changes, notably a reduction in TSS and COD accompanied by a slight increase in pH. The decrease in TSS could be attributed to the destabilization of colloidal structures at high temperatures, resulting in sedimentation of suspended particles during sample cooling. In addition, high temperature could partially break down large organic molecules into less reactive compounds or volatilize certain labile organics, thereby reducing the COD value after sterilization.

The bacterial density assessment demonstrated that *Pseudomonas* sp. WV5.7 was capable of pronounced growth in domestic wastewater, under both sterilized and non-sterilized conditions. In treatments with WV5.7 inoculation, bacterial numbers increased until 24 hours of incubation (data not shown). Strain WV5.7 maintained ammonium uptake efficiency after 90 and 180 days of storage suggesting that carriers not only provided a stable surface for bacterial adhesion and growth, but also created a favorable microenvironment for maintaining microbial activity.

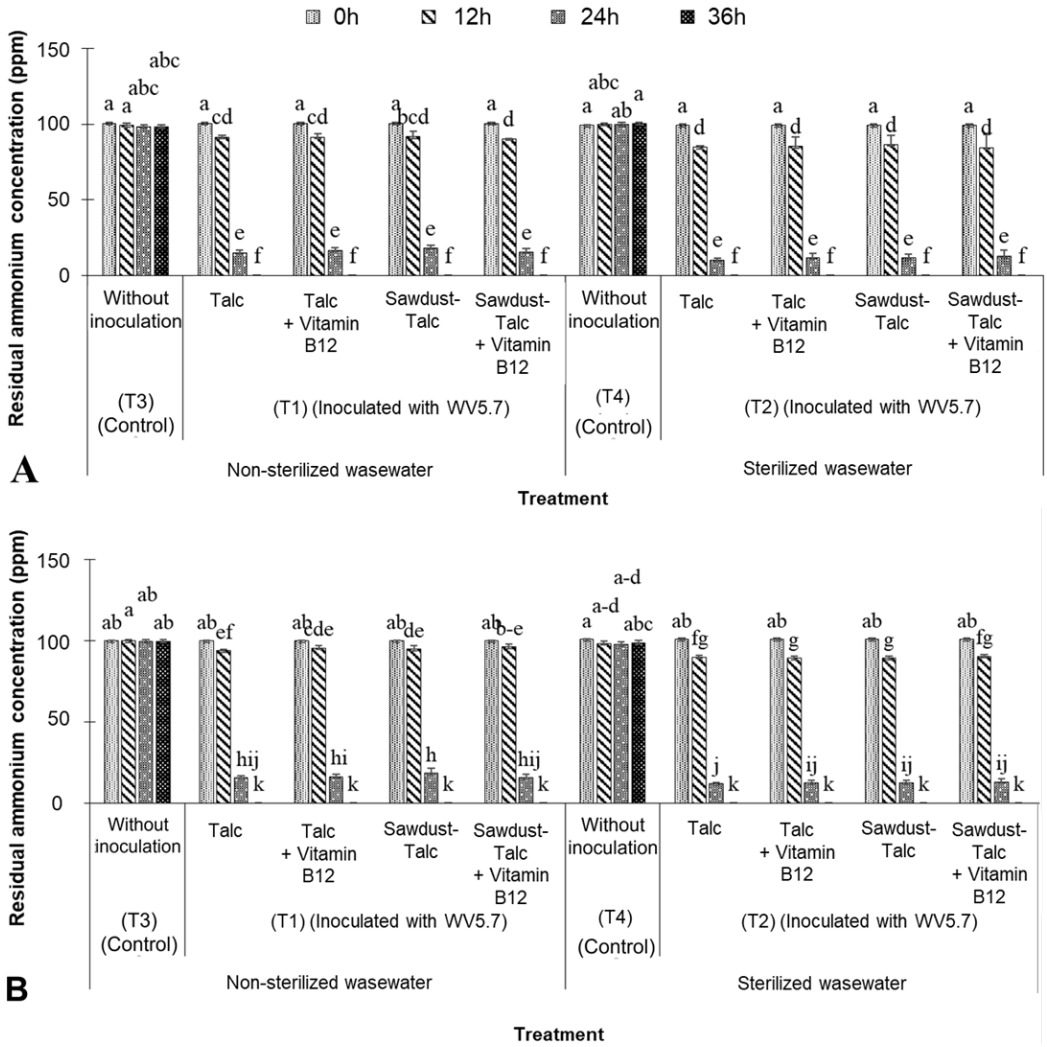

Figure 6. Ammonium uptake by *Pseudomonas protegens* WV5.7 after 90 (A) and 180 days (B) of storage. Values followed by the same letter are not significantly different at the 5% ($p < 0.05$)

The data revealed a remarkly decrease in ammonium concentration in treatments inoculated with *P. protegens* WV5.7. The strain demonstrated an ammonium removal efficiency of over 80% within 24 hours of incubation indicating that the effectiveness of WV5.7 in wastewater. The reduction in ammonium concentration may be attributed to heterotrophic mechanisms whereby the bacteria assimilated ammonium for biomass growth and protein synthesis.

When comparing non-sterilized wastewater (T1) and sterilized wastewater (T2) treatments, it was observed that bacteria exhibited better growth in sterilized wastewater due to the absence of competitors in the wastewater (data not shown). However, the ammonium removal rate was faster in the non-sterilized water samples which may be attributed to the presence of organic compounds and supportive microbial species that enhanced the metabolic environment for the introduced strain (Fig. 6). Additionally, after sterilization, pH values increased (ranging from 7.87 to 8.35 at 90 days and 7.05 to 8.61 at 180 days). The elevated pH likely negatively affected ammonium uptake since WV5.7 grows optimally at pH 7.

Our data demonstrated that the selected carriers supported bacterial growth and activity after 180 days of storage, despite a slight decline in cell density compared to these of the 90 days. This finding underscores the potential for long-term preservation of strain *P. protegens* WV5.7 for large-scale application.

CONCLUSION

From 25 bacterial strains capable of ammonium uptake isolated from seafood processing wastewater samples, 4 strains exhibited high ammonium removal efficiency, ranging from 90% to 94%. Strain WV5.7 achieved ammonium removal of 99 and 88.6% after 24 hours of cultivation in a MM medium supplemented with 100 ppm and 200 ppm ammonium, respectively. Based on 16S-rRNA gene sequencing and biochemical characterization, the strain was designated as *P. protegens* WV5.7. The suitable conditions for ammonium uptake in this strain were aerobic cultivation, pH 7, temperature ranging from 28ºC to 31ºC, and salinity below 1%. Talc powder and a sawdust-talc mixture (1:1 ratio) were effective carriers for maintaining bacterial viability and ammonium uptake after 180 days of storage. The addition of B_{12} to bacterial suspension prior to storage did not affect bacterial viability and ammonium uptake property. *P. protegens* WV5.7 retained ammonium uptake activity in domestic wastewater indicating that the strain can be further studied for bioremediation of ammonium-rich wastewater.

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