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Potential of *Bacillus* sp. as a Nitrifying Agent in Coastal Ecosystems: Isolation and Characterization from Awur Bay Beach, Jepara, Indonesia

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Abstract

Coastal waters often experience nitrogen pollution, which can be harmful to aquaculture. One potential approach to mitigate this issue involves the use of biological agents that can transform nitrogen compounds through nitrification and denitrification. This study aimed to identify local nitrifying bacteria capable of adapting to marine environments and reducing the ammonia concentrations. Seawater and sediment samples were collected from the Awur Bay Beach in Jepara, Central Java, Indonesia.. Isolation and purification processes employed specific autotrophic media to cultivate ammonia-oxidizing bacteria (AOB). The measured parameters included the concentrations of nitrogen species, such as ammonia, nitrite, and nitrate, in the media before and after inoculation with the bacterial isolate. The incubation period was seven days. Nine pure isolates (four from seawater and five from sediment) were obtained and screened to identify bacteria that thrive at high ammonia concentrations. The results indicated that one potential isolate, S20-7, was identified as *Bacillus* sp. using 16S rRNA sequencing. *Bacillus* sp. demonstrated the ability to thrive in media with a salinity of 20‰ and reduced ammonia concentration by 37.19% from an initial concentration of 500 mg/L. This study highlights the potential of *Bacillus* sp. as a nitrogen waste degradation agent in coastal waters, particularly in wastewater treatment and aquaculture. Its ability to efficiently degrade ammonia nitrogen and nitrite, coupled with its tolerance to high concentrations of these compounds, makes it a valuable tool for improving water quality and mitigating the effects of nitrogen pollution in coastal ecosystems.

1. Introduction

The growth of the human population necessitates a corresponding increase in food availability to meet its fundamental needs. Aquaculture is a rapidly expanding sector in food production with significant potential to enhance food security (Braña *et al.*, 2021). Products from aquatic animals, including fish, crustaceans, mollusks, and other invertebrates, intended for direct human consumption or as fish and seafood, account for 15.3% of the total crude protein from animal sources globally (Boyd *et al.*, 2022). Intensification is pursued because of limited land and competition with other sectors. However, intensification increases the input (fish, shrimp, and feed), generating more waste (Wani *et al.*, 2025). Aquaculture waste is considered a global threat to aquatic ecosystems because of its impact on surrounding water bodies, which ultimately affects seawater quality.

Aquaculture waste is often associated with increased levels of various pollutants. These include organic carbon, suspended solids, phosphates, nitrogen species (ammonia, nitrite, and nitrate), chemical oxygen demand, and biological oxygen demand (BOD). High concentrations of phosphate and nitrogen can be toxic to aquatic organisms because they affect protein synthesis, enzyme activity, photosynthesis, oxidative stress responses, membrane permeability, and respiration (Hlordzi *et al.*, 2020). Nutrient accumulation from aquaculture discharges resulting from nitrogen pollution often occurs in coastal areas. High levels of nitrogen species (N) are caused by the accumulation of ammonia (NH_3), which is oxidized to nitrites (NO_2^-) and nitrates (NO_3^-). Excessive nutrient inputs to achieve aquaculture production targets can negatively affect the environment (Anwar *et al.*, 2021; Braña *et al.*, 2021; Yan *et al.*, 2025). High stocking density in intensive aquaculture systems increases the accumulation of uneaten feed and fish feces, which elevates the nutrient load and organic matter in the water. This deterioration in water quality subsequently reduces aquaculture productivity (Gao *et al.*, 2022).

Ammonia is present in water in two forms: ammonia (NH_3) and ionized ammonia (NH_4^+). Together, these are referred to as total ammonia nitrogen (TAN). Unionized N-NH_3 is more toxic to fish than N-NH_4^+ (Francis-Floyd *et al.*, 2022). The lipophilic nature of NH_3 enables rapid absorption by cultured organisms and disrupts their metabolism. The reactivity of N-NH_3 is responsible for ammonia toxicity because it can easily diffuse through the tissues into the circulatory system of aquatic organisms (Franklin & Edward, 2019; Pan *et al.*, 2023).

Exposure to N-NH_3 (ammonia) at concentrations above the tolerance limit can reduce growth and increase disease infection in aquatic organisms. Bioremediation is a sustainable approach to mitigate N–NH_3 in aquatic environments, with bacterial-mediated nitrification recognized as an effective method for maintaining water quality in aquaculture systems (Yan *et al.*, 2025). The N-NH_3 species in water are distributed according to the law of equilibrium in the form of NH_3 (ammonia) and NH_4^+ (ammonium) species. Nitrification involves two sequential oxidation processes: the first step is the oxidation of N-NH_4^+ to N-NO_2^-(nitrification) by ammonia-oxidizing bacteria (AOB). In the second step, nitrite (N-NO_2^-) is converted to N-NO_3^- (nitratation) by nitrite-oxidizing bacteria (NOB). Nitrate (N-NO_3^-) undergoes denitrification, a process that converts it to nitrogen gas (N_2), which is then released into the atmosphere (Mpongwana *et al.*, 2019; Rahimi *et al.*, 2020).

Ammonia degradation capacity was used to screen the bacteria involved in environmental nitrification. Therefore, screening for ammonia-oxidizing bacteria is necessary to address nitrogen waste in aquatic environments. This study aimed to identify local nitrifying bacteria capable of reducing ammonia concentrations. Subsequently, these isolates may have the potential to be used as biological agents to manage nitrogen pollution in water bodies on a larger scale.

2. Material and methods

2.1. Sample Collection

Seawater and sediment samples from marine environments were obtained using purposive sampling methods. Purposive sampling was applied by selecting the study site due to its proximity to intensive aquaculture ponds at the Science Techno Park, Diponegoro University area, where nitrogen pollution was suspected, making the location representative for assessing the impact on water quality. Seawater samples (1 L) were collected in plastic bottles and stored in cooling boxes. Sediment was collected using an iron shovel at a depth of approximately 10 cm, following the method of Peng *et al.* (2022), who stated that the highest nitrogen species content was found at depths of 1-10 cm. The samples were placed in Ziploc plastic bags, transported to the laboratory in a cool box, and stored at 4°C in a room before processing. The sampling location was the coastal waters of Awur Bay Beach, Tahunan, Jepara, Central Java, Indonesia (6°37'15.31″S, 110°38'17.74″ E) (Figure 1).

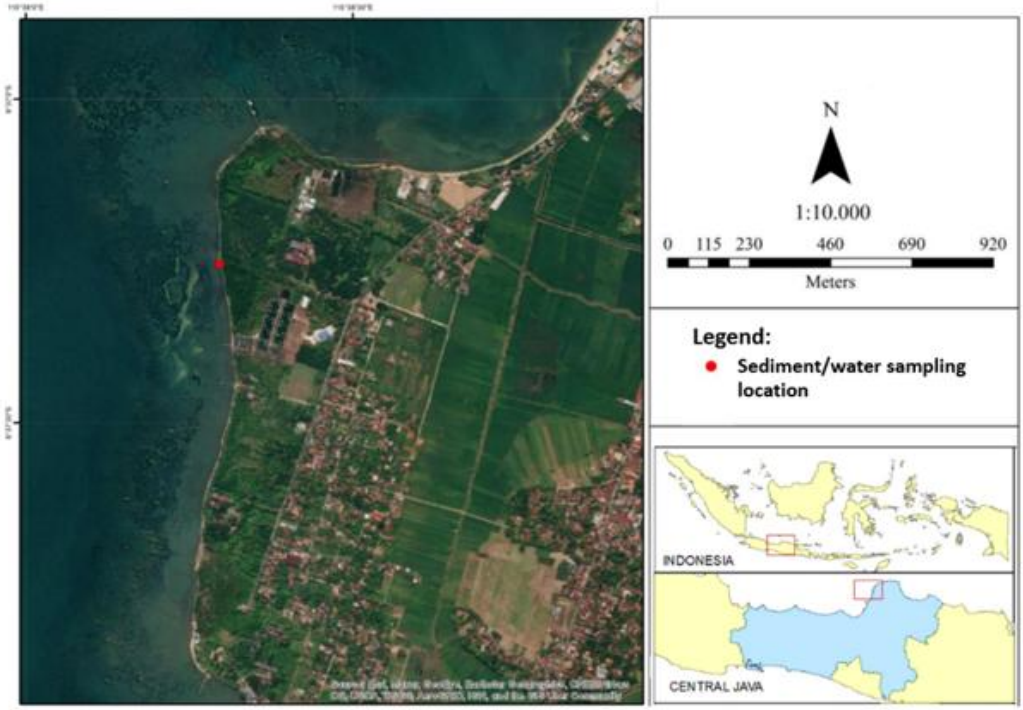


Figure 1. Map of sampling sites in Awur Bay, Jepara, Indonesia

2.2. Preparation of Bacterial Culture Media

The medium used for the bacterial screening process was a specific medium designed for the growth of Ammonia-Oxidizing Bacteria (AOB), commonly referred to as an autotrophic medium. The AOB medium was prepared following Shimaya & Hashimoto (2008), with modifications consisting of increased ammonium concentration and salinity adjustment. Specific nitrifying liquid media were used during the enrichment stage, and the composition per 1 L included 235 mg $(NH_4)_2SO_4$, 200 mg KH_2PO_4, 40 mg $MgSO_4$, 40 mg $CaCl_2$, 5 g Na_2CO_3, distilled water, and 5 mL Phenol Red (0.02% w/v) as pH indicators. The medium was prepared using distilled water with a salinity of 0‰. Additionally, to mimic natural habitat conditions, another medium was prepared using seawater with a salinity of 20‰. This procedure was used to examine the effects of salinity fluctuations on bacterial survival. Specific nitrifying agar media were used during isolation and purification. The agar medium was prepared by adding 20 g of agar to 1 L of a specific nitrifying liquid medium.

2.3. Bacterial Enrichment

A total of 10 g of sediment was weighed to obtain AOB. The sediment sample was suspended in Erlenmeyer flasks containing 500 mL of specific nitrifying liquid media

at two salinities (0‰ and 20‰). For seawater samples, up to 10 mL of seawater was added to the same enrichment medium. The pH of the medium was set to 8 at the start of the enrichment process. The media and sediment samples were homogenized and incubated for 14 days at room temperature (28-30°C) under dark and static conditions. During the incubation period, a color change from pink to yellow indicated a pH shift from alkaline to acidic, indicating bacterial growth.

2.4. Isolation and Purification of Bacteria

A volume of 1 mL of the bacterial sediment and seawater enrichment culture suspension, with an equivalent concentration of 0.5 McFarland (1.5 × 108 CFU/mL), was inoculated using the spread plate method on specific nitrifying agar media in Petri dishes inside a Laminar Air Flow cabinet. The bacteria were then incubated at room temperature (28-30°C) for 7 days (pH 8, in the dark, static conditions). Bacterial growth was observed during this period. Bacterial isolates with different morphologies grown on solid media were purified using a new AOB solid medium until a pure isolate was obtained. The bacterial isolates were macroscopically characterized by observing the color, edge, elevation, and shape of their colonies.

2.5. Increased Isolate Tolerance to Ammonia Concentration

A pure isolate from the AOB solid medium was inoculated into the liquid AOB medium using a sterile and aseptic technique with a sterile needle. The bacterial culture that successfully grew in the liquid AOB medium was centrifuged. The pellet obtained after centrifugation was transferred into a liquid AOB medium containing ammonium sulfate ($(NH_4)_2SO_4$) at an increased concentration of 500 mg/L (Kasmuri & Lovitt, 2018). The liquid bacterial culture was incubated at 28°C for 7 days (pH 8) in the dark and stirred using a magnetic stirrer. Visual observation of the color change in the liquid AOB media, from pink to yellow, was conducted to determine bacterial growth in the media with an increased concentration of ammonium. Liquid AOB medium without bacterial inoculation was used as a control.

2.6. Measurement of Ammonia, Nitrite, and Nitrate Levels

The concentrations of nitrogen species, such as ammonia (TAN), nitrite, and nitrate, were determined in the media before and after bacterial isolate inoculation. The bacterial isolates were grown in liquid AOB medium with an $(NH_4)_2SO_4$ concentration of 500 mg/L. Incubation was performed at 28°C and pH 8 in the dark (Marzan *et al.*, 2017). After a seven-day incubation period, the levels of ammonia, nitrite, and nitrate were evaluated to determine any reduction in their concentrations. The concentrations of ammonia, nitrite, and nitrate were determined following the Standard Methods (APHA, 2017), using the Nesslerization method for ammonia, the colorimetric method for nitrite at 543 nm, and the ultraviolet spectrophotometric method for nitrate at 220 nm. Measurements were conducted with a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan)

2.7. Identification of Bacteria

Molecular identification of the bacteria was performed by sending the most promising pure isolate, a nitrifying bacterium, for PT. Genetika Science, Indonesia. The 16S rRNA sequencing results of the isolate were compared with the nucleotide data of known bacteria in the GenBank database using the Basic Local Alignment Search Tool (BLAST). This comparison was conducted online using the website www.ncbi.nlm.nih.gov/BLAST/. Taxonomic identification was based on sequence similarity thresholds, with ≥97% identity applied for genus-level and ≥99% identity for species-level classification.

2.8. Data Analyses

This study employed a descriptive design to isolate and characterize nitrifying agents in coastal ecosystems from marine samples collected at Awur Bay Beach, Jepara, Indonesia. Data analysis was performed based on images and tables processed using Excel applications.

3. Results

3.1. Bacterial Growth on Enrichment and Isolation Media

Bacteria contained in sediment and seawater samples originating from the waters of Teluk Awur, Jepara, can grow on enrichment media (AOB liquid media). This can be seen from the results of visualization of bacteria in a Florence flask with a pink color on day 0 (Figure 2a) and after incubation for 14 days (Figure 2b), which turned yellowish. The color change shows a change in pH from alkaline to acidic, which indicates bacterial growth on the media. After the isolation process, several isolates were obtained, including white circles, as shown in Figure 3b.

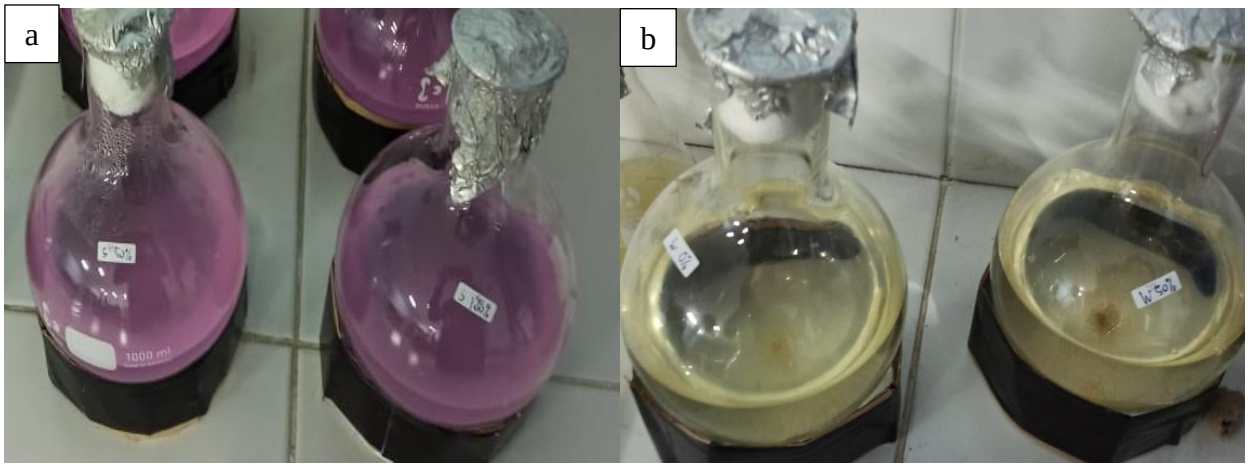


Figure 2. Bacterial enrichment from sediment and seawater: (a) 0-day and (b) 14-day incubation

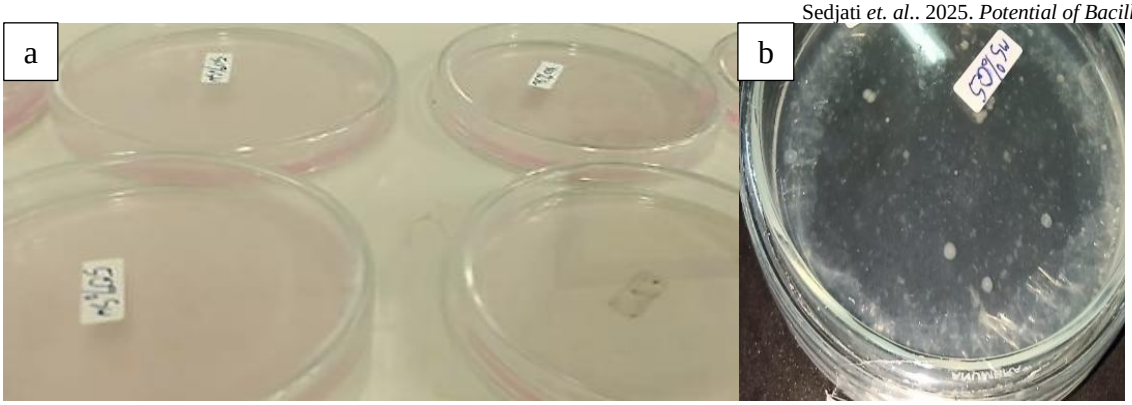


Figure 3. Bacterial isolation from sediment and seawater: (a) 0-day and (b) 14-day incubation

3.2. The Characterization and Tolerance to Ammonia Concentration of Bacterial Isolates

Through the screening process using selective AOB media, nine pure isolates were obtained, four from seawater and five from sediment. Based on the conditions of the nitrification media, four originated from media with 0‰ salinity and five from media with 20‰ salinity. The nine isolates exhibited different macroscopic morphological characteristics (Table 1). However, after culturing them in media with an increased concentration of ammonium sulfate up to 500 mg/L, only two isolates, W20-7 and S20-7, were obtained (Table 2).

Table 1. Pure isolates of Ammonia Oxidizing Bacteria and their macroscopic characteristics

Isolates code	Media salinity (‰)	Morphology of Bacterial Colonies			
		Color	Margin	Elevation	Form
Seawater bacteria					
W0-2	0	White	Lobate	Flat	Irregular
W0-4	0	White	Filamentous	Flat	Rhizoid
W20-5	20	White	Lobate	Flat	Irregular
W20-7	20	Yellowish-White	Filamentous	Flat	Filamentous
Sediment bacteria					
S0-1	0	White	Serrate	Flat	Filamentous
S0-4	0	Yellowish-White	Lobate	Raised	Circular
S20-1	20	White	Serrate	Flat	Irregular
S20-2	20	Yellowish-White	Undulate	Flat	Irregular
S20-7	20	White	Serrate	Flat	Irregular

Table 2. Growth of isolates on media with ammonium sulfate at 500 mg/L

Isolates code	Growth
Seawater bacteria	
W0-2	-
W0-4	-
W20-5	-
W20-7	+
Sediment bacteria	
S0-1	-
S0-4	-
S20-1	-
S20-2	-
S20-7	+

Denoted: (+) grow; (-) not grow

3.3. Levels of Ammonia, Nitrite, and Nitrate after Bacterial Isolate Inoculation

The spectrophotometry test showed that the culture media used had a measured ammonia concentration of 509.11 mg/L. Ammonia was supplied as a nitrogen source and substrate to evaluate the aerobic ammonia-oxidizing capacity of the bacterial isolates. Based on the conditions used in the enrichment, isolation, and purification steps during the screening process, the obtained isolates belonged to the group of aerobic nitrifying bacteria. The potential of the isolates was determined by measuring their ability to oxidize ammonia and produce nitrate, as shown in Figure 4. In particular, isolate S20-7 demonstrated the capability to reduce the ammonia concentration by 319.77 mg/L (37.19%), whereas W20-7 reduced it to 457.13 mg/L (10.21%).

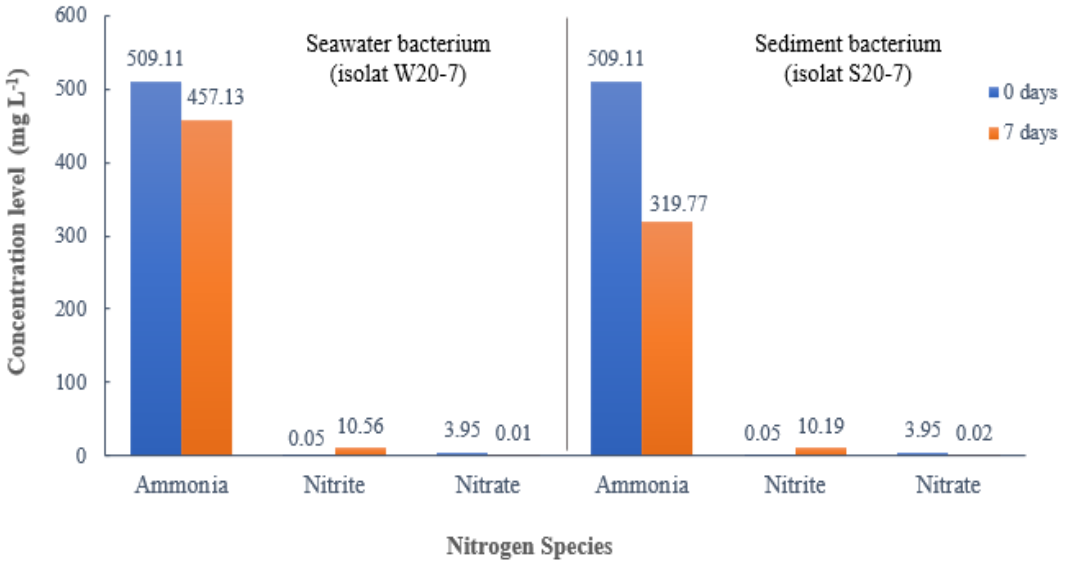
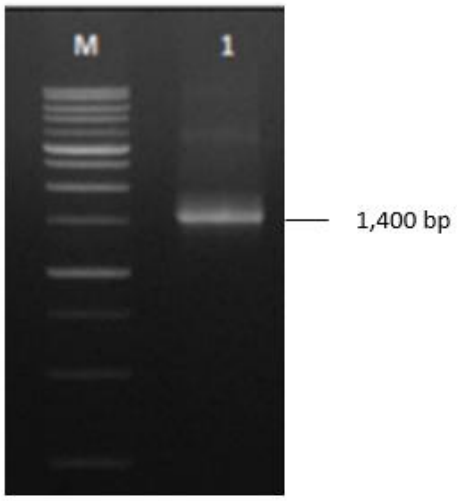


Figure 4. Ammonia (TAN; $N\text{-}NH_3 + N\text{-}NH_4^+$) reduction to Nitrite ($N\text{-}NO_2^-$)/ Nitrate ($N\text{-}NO_3^-$) of isolates W20-7 and S20-7

3.4. Molecular Identification of Potential Bacteria

Molecularly identified bacteria have the greatest potential to reduce ammonia concentrations, namely, sediment bacteria isolated with the code S20-7. The primers used in the polymerase chain reaction (PCR) amplification process were the 27F forward primer (5' AGA GTT TGA TCM TGG CTC AG 3') and reverse primer 1492R (5' TAC GGY TAC CTT GTT ACG ACT T 3'). The results of the analysis are presented in the form of nucleotide isolate sequences in FASTA format, as shown in Table 3.

Table 3. The results of amplification by PCR and partial nucleotide sequencing from the genome of S20-7 isolates

Gel photo of PCR product	Nucleotide sequencing results
	TGAGTAACACGTGGGCAACCTGCCTGTAAGAC TGGGATAACTTCGGGAAACCGAAGCTAATACC GGATAGGATCTTCTCCTTCATGGGAGATGATTG AAAGATGGTTTCGGCTATCACTTACAGATGGGC CCGCGGTGCATTAGCTAGTTGGTGAGGTAACG GCTCACCAAGGCAACGATGCATAGCCGACCTG AGAGGGTGATCGGCCACACTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTAGG GAATCTTCCGCAATGGACGAAAGTCTGACGGA GCAACGCCGCGTGAGTGATGAAGGCTTTCGGG TCGTAAAACTCTGTTGTTAGGGAAGAACAAGT ACGAGAGTAACTGCTCGTACCTTGACGGTACCT AACCAGAAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTATC CGGAATTATTGGGCGTAAAGCGCGCGCAGGCG GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTC AACCGTGGAGGGTCATTGGAAACTGGGGAACT TGAGTGCAGAAGAGAAAAGCGGAATTCCACGT GTAGCGGTGAAATGCGTAGAGATGTGGAGGAA CACCAGTGGCGAAGGCGGCTTTTTGGTCTGTA ACTGACGCTGAGGCGCGAAAGCGTG

The results of the BLAST analysis provide the names of bacteria with the degree of similarity of nucleotide rRNA isolates with nucleotide sequences of bacterial species according to the GenBank *database*. The molecular test results showed that the 16S rRNA genome fragment of isolate S20-7 was identified as *Bacillus* sp. The search results based on relatedness are presented in Table 4. The nucleotide sequence of isolate S20-7 had the highest percent identity value with three strains of *Bacillus* sp. Isolate S20-7 was most similar to *Bacillus* sp. strain BA4, with a percent identity of 97.76%.

Table 4. The closest similarity of the 16S rRNA sequences

Isolate code	Closest similarity (BLAST Analysis)	Accession Number	Query Cover (%)	Identity (%)
S20-7	*Bacillus* sp. strain BA4	MH518267.1	99	97.76
	Bacillus sp. strain VITHBRA037	MZ057737.1	99	97.74
	Bacillus sp. strain MAIDO-R9b-4	MW711431.1	99	97.55

4. Discussion

An inorganic medium devoid of organic compounds was used for the isolation process. This medium was appropriate for identifying autotrophic nitrifying bacteria. Nitrifying bacteria can oxidize ammonia aerobically. Commonly found species include Nitrosomonas, Nitrobacter, Nitrosospira, Nitrococcus, Nitrospina, and Nitrospira (Baskaran *et al.*, 2020; Rahimi *et al.*, 2020). However, the isolates identified by screening nitrifying bacteria from marine water sediments in this study were identified as *Bacillus* sp. The isolation unexpectedly yielded *Bacillus* sp., which can be ascribed to its metabolic versatility, including heterotrophic nitrification and aerobic denitrification, rapid growth, and adaptability to aerobic conditions. In contrast, autotrophic nitrifying bacteria typically exhibit slow growth and strict environmental requirements, rendering them less competitive under such conditions (Hong *et al.*, 2022). The high concentration of ammonium sulfate (500 mg/L) is suspected to be the reason autotrophic nitrifying bacteria were unable to survive. This finding aligns with that of Wardhani *et al.* (2017), who reported that most autotrophic nitrifying bacteria are unable to tolerate high concentrations of ammonia and organic matter. Rahimi *et al.* (2020) noted that the utilization of ammonia compounds as a nitrogen source is carried out by both autotrophic and heterotrophic nitrifying bacteria, such as *Bacillus* sp.

Bacillus belongs to a group of heterotrophic bacteria and can be considered a facultative mixotrophic ammonia-oxidizing bacterium that requires organic compounds as carbon sources. *Bacillus* sp. was successfully isolated from the sediment of intensive shrimp ponds in Bone Regency, South Sulawesi, Indonesia. The bacteria were cultured using AOB medium enriched with 1 g/L acetic acid as a carbon source and were able to reduce ammonia levels by 54-58% from the initial concentration of 51.3 mg/L at a temperature of 25°C for 30 days in the dark (Ardiansyah *et al.*, 2019). *Bacillus subtilis* strain ON358108 from Srinagar, India, also highlights the potential of this bacterium for ammonium nitrogen (NH_3-N) remediation (Mir & Rather, 2024). Several *Bacillus* strains exhibit enzymes associated with heterotrophic nitrification. Proteomic analyses have detected both ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR), suggesting the capability to oxidize ammonia to hydroxylamine and nitrite to nitrate (Mendoza *et al.*, 2019). Unlike classical nitrifiers such as *Nitrosomonas*, *Bacillus* does not rely solely on autotrophic pathways but instead performs heterotrophic nitrification while utilizing organic carbon sources. Moreover, certain strains can couple this process with partial denitrification, enabling flexible nitrogen removal under diverse environmental conditions (Li *et al.*, 2020; Liu *et al.*, 2023).

In the present study, the isolate S20-7 was found in marine sediments cultured in a medium with a salinity of 20‰. Throughout the screening and isolation process, the organic compound source in the nitrifying bacterial culture medium was solely derived from seawater and sediment samples, resulting in a very limited amount. Isolate S20-7 could only reduce ammonia concentration to a relatively small extent (37.19%), which was suspected to be due to suboptimal growth caused by the limited availability of organic carbon sources. The high concentration of ammonium in the medium (Figure 1) also contributed to this limitation of the study. According to Pasmionka (2021), ammonium concentrations above 100 mg/L can inhibit the nitrification rate of biofertilizers. Similar to the findings of Kasmuri & Lovitt (2018), the rate of ammonia oxidation decreased when the ammonia concentration in the medium exceeded 400 mg/L.

The genus *Bacillus* is a consortium of heterotrophic nitrifying bacteria that work optimally in media with an ammonia concentration of 350 mg/L and can reduce it by 96.28%. Moreover, Bacillus can reduce ammonia even at higher concentrations (up to 550 mg/L) than other genera. This consortium has the potential to be developed as an alternative biological agent for reducing high concentrations of ammonia (Wardhani *et al.*, 2017). According to Shalaby *et al.* (2020), ammonia is effectively consumed during the first five days of the incubation period, but its consumption rate decreases to the lowest level after 25 days of incubation.

The relatively modest reduction rate obtained in this study (37.19%) may be attributable to several limiting factors, including the comparatively short incubation period (7 days versus 30 days reported in other studies), restricted availability of organic carbon, and physiological stress imposed by elevated salinity and ammonium concentrations. These conditions likely constrained the metabolic activity of heterotrophic nitrifiers, resulting in lower efficiencies than those reported previously. Future investigations should explore strategies such as extending the incubation duration to facilitate microbial adaptation, supplementing with appropriate organic carbon sources to stimulate heterotrophic metabolism, and employing mixed bacterial consortia to enhance the stability and effectiveness of nitrogen removal processes under aquaculture conditions.

The use of Bacillus as a nitrogen pollution controller has several advantages. Bacillus sp. reduces ammonia levels without nitrite accumulation. Bacteria oxidize ammonia to nitrite and remove nitrogen through nitrification and denitrification processes (Janka *et al.*, 2022). The genus Bacillus performs heterotrophic nitrification and aerobic denitrification owing to AMO and nitrite oxidoreductase NXR, making it suitable for nitrogen waste degradation in intensive aquaculture systems (Mir *et al.*, 2024). It exhibits high tolerance to elevated ammonia and nitrogen concentrations, making it suitable for treating high ammonia, nitrogen, and nitrite concentrations in wastewater (Wang *et al.*, 2023). Bacillus spp. are potential biocontrol agents that can improve water quality in mariculture. This discovery has enabled the development of microbial agents for wastewater purification and environmentally friendly tropical aquaculture models to protect marine environments (James *et al.*, 2021; Ren *et al.*, 2021). Bacillus species have significant potential as nitrogen waste degradation agents in coastal waters, particularly for wastewater treatment. Their ability to degrade ammonia nitrogen and nitrite, coupled with their

tolerance to high concentrations, makes them valuable for improving water quality in coastal ecosystems.

5. Conclusions

Through the screening process, nine ammonia-oxidizing bacterial isolates were obtained from the Awur Bay Beach in Jepara. Among these, one isolate, S20-7, survived in a medium with an ammonia concentration of 500 mg/L. The isolate S20-7 was identified as a *Bacillus* sp. bacterium capable of adapting to a medium salinity of 20‰. Isolate S20-7 demonstrated the ability to oxidize ammonia and reduce its concentration in the medium by 37.19%, indicating its potential as a nitrogen waste degradation agent in coastal waters.

Ethics approval

No permits were required.

Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Author contributions

SS is responsible for formal analysis, data curation, project administration, funding acquisition, and responsible for writing the original draft. EY, and PGMP are contributed to investigation, conceptualization, resource acquisition, methodology, writing – review & editing.

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Declaration of competing Interest

None

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