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Isolation and Characterization of Ammonia and Nitrite Removing Bacteria in Thoothukudi Region

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ABSTRACT

Aquaculture has rapidly expanded as a key contributor to global seafood production but faces significant challenges due to the accumulation of toxic nitrogenous wastes, particularly ammonia and nitrite, which pose threats to aquatic organisms and overall ecosystem health. This study aimed to isolate and characterize ammonia- and nitrite-degrading bacterial strains from shrimp aquaculture wastewater to identify potential candidates for bioremediation. Samples collected from shrimp farming ponds in Thoothukudi, Tamil Nadu, India were enriched and screened, leading to the isolation of fifteen distinct bacterial strains. Of these, seven strains showed effective ammonia degradation, and four demonstrated significant nitrite degradation. Morphological and molecular characterization identified predominant species from *Burkholderia* and *Brevibacillus* genera, notably *Burkholderia cepacia, Burkholderia paludis, Burkholderia territorii,* and *Brevibacillus borstelensis.* These isolates exhibited robustness under varied environmental conditions, making them ideal candidates for practical application in wastewater treatment. The use of 16S rRNA gene sequencing confirmed their identities, underscoring their potential roles in enhancing bioremediation practices. The study highlights the importance of specific bacterial consortia in mitigating nitrogenous waste in aquaculture, promoting sustainability through effective biological wastewater management.

INTRODUCTION

Aquaculture contributes nearly 50% of global seafood production (FAO, 2021) but faces environmental challenges due to nitrogenous waste accumulation, primarily ammonia (NH_3) and nitrite (NO_2^-). These compounds are toxic to aquatic organisms, causing physiological stress, gill damage, reduced growth rates, increased disease susceptibility, and mortality (Boyd and Tucker, 2012; Chen and Ni, 2012). In natural ecosystems, microbial communities regulate nitrogen through nitrification and denitrification (Jiang *et al*., 2021). However, aquaculture intensification disrupts this balance due to high organic loads from uneaten feed, fish excretion, and organic matter decomposition, leading to harmful nitrogen buildup. Effective wastewater treatment is essential for maintaining water quality and sustainability in aquaculture.

Microbial bioremediation is an eco-friendly and cost-effective approach to nitrogenous waste management. Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) play a crucial role in this process by converting ammonia into nitrite and then into nitrate, which is less toxic and can be assimilated by aquatic plants or removed through denitrification (Prosser *et al*., 2019). Nitrification is a two-step aerobic process: ammonia oxidation is carried out by AOB such as *Nitrosomonas* and *Nitrosospira* (Kim *et al*., 2006), which use ammonia as an electron donor and oxygen as an electron acceptor, producing nitrite. Nitrite oxidation is performed by NOB like *Nitrobacter*, *Nitrospira*, and *Burkholderia*, which convert nitrite into nitrate (Daims *et al*., 2001). The efficiency of nitrification depends on environmental factors such as pH, temperature, dissolved oxygen, and salinity (Liu *et al*., 2020). The presence of specific microbial consortia is also critical. Li *et al*. (2016) demonstrated that *Burkholderia cepacia* and *Bacillus proteolyticus* exhibit strong ammonia degradation in aquaculture biofilters, highlighting the importance of isolating efficient nitrogen-removing bacteria. Traditionally, *Nitrosomonas* and *Nitrobacter* have

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been studied as key nitrifiers, but research has revealed other genera such as *Burkholderia*, *Bacillus*, *Agrobacterium*, and *Rhizobacterium* also contribute to ammonia and nitrite oxidation (Liu *et al*., 2020). Kim *et al*. (2006) reported that *Burkholderia paludis* and *Burkholderia territorii* exhibited rapid nitrite oxidation, making them potential candidates for bioremediation. Similarly, *Brevibacillus borstelensis* has been identified as an important ammonia remover in wastewater treatment plants (Wei *et al*., 2018). Molecular tools such as 16S rRNA sequencing, metagenomics, and fluorescence in situ hybridization (FISH) have enhanced bacterial identification and classification (Daims *et al*., 2001). This study aims to isolate and characterize ammonia- and nitrite-degrading bacteria from shrimp aquaculture wastewater, contributing to improved bioremediation strategies for sustainable aquaculture.

MATERIALS AND METHODS

Sample collection and enrichment

Water and sediment samples were collected from aquaculture wastewater sources, including fish and shrimp ponds in Thoothukudi, Tamil Nadu, India. Sampling focused on high organic load areas near discharge points and was conducted early morning before routine activities (John *et al*., 2020; Aswiyanti *et al*., 2021). Samples were transported on ice and stored at 4°C until processing. Wastewater was filtered using Whatman No.1 filter paper and enriched in a mineral salt medium (K_2HPO_4 1 g/L, $MgSO_4$ 0.5 g/L, $CaCl_2$ 3.5 g/L, $FeSO_4$ 0.05 g/L, $MnSO_4$ 0.3 g/L, NaCl 0.5 g/L) with ammonium sulfate for ammonia degraders and potassium nitrite for nitrite degraders (John *et al*., 2020). Enrichment was performed at 30±2°C in an orbital shaker at 200 rpm for three days. Serial dilutions (10^{-1}–10^{-5}) were spread-plated on mineral salt medium (MSM) agar for primary screening of ammonia- and nitrite-utilizing bacteria.

Isolation of nitrate and ammonia degrading bacteria

Bacterial isolation was conducted using an enrichment culture technique. Wastewater (10 mL) or sediment (10 g) samples were inoculated into mineral media containing ammonium sulphate for AOB and potassium nitrite for NOB. Cultures were incubated at 30±2°C with continuous shaking (90 rpm) for seven days, followed by serial subculturing every seven days to promote bacterial growth (Huang *et al*., 2017). Once visible turbidity was observed, serial dilutions were plated on mineral medium agar with ammonium sulphate for AOB and potassium nitrite for NOB. Plates were incubated at 28°C for 48 h, and colonies were selected based on morphology. Pure isolates were obtained through repeated quadrant streaking (Elbanna *et al*., 2012; Gould and Lees, 1960).

Screening of bacteria for nitrate and ammonia degradation

To confirm the ability of the isolates to degrade ammonia and nitrite, primary screening was performed using phenol red indicator plates. The selected isolates were inoculated onto mineral medium agar supplemented with phenol red (0.02%), and a colour change from red to yellow indicated ammonia oxidation (John *et al*., 2020). Similarly, nitrite oxidation potential was assessed by inoculating the bacterial isolates into nitrite calcium carbonate medium (NCCM), where degradation efficiency was determined using the diphenylamine test. Only those isolates that demonstrated significant colour changes were selected for further characterization (Gould and Lees, 1960).

Morphological characterization of isolates

Morphological characterization of isolates was conducted to categorize bacterial isolates based on colony and cellular features, aiding in preliminary identification of ammonia-degrading bacteria. Isolates were streaked on nutrient agar and incubated at 30°C for 24–48 h, with colony characteristics such as size, shape, elevation, margin, surface texture, and color recorded. Gram staining was performed using the crystal violet method to differentiate Gram-positive and Gram-negative bacteria (Aswiyanti *et al*., 2021). The KOH string test was also used for further confirmation, distinguishing Gram-negative bacteria by their viscous string formation in a 3% KOH solution (Saha *et al*., 2013).

Molecular characterization of isolates

Molecular characterization of bacterial isolates was conducted using polymerase chain reaction (PCR) and DNA sequencing, essential for precise species-level identification (Huang *et al*., 2017). DNA was extracted using a commercial kit (Qiagen or Thermo Fisher Scientific) and assessed for quality and concentration via a nanodrop spectrophotometer. The *16S rRNA* gene, a conserved marker for bacterial identification (Saha *et al*., 2013), was amplified using universal primers 16SF (5'-AGAGTTTGATCMTGGCTC-3′) and 16SR (5'-AAGGAGGTGWTCCARCC-3′). PCR was performed in a 50 µL reaction containing 10-50 ng genomic DNA, 10 p mol of each primer, 10 µL 5X PCR buffer, 1.5 µL 10 mM dNTP mix, 1.5 µL 25 mM $MgCl_2$, and 0.5 µL Taq polymerase. The cycling conditions included an initial denaturation (94°C, 5 min), 35 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min), followed by a final extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide, confirming the expected ~1500 bp band. The purified PCR products were sent to

a sequencing facility for Sanger sequencing using the same primers used for PCR amplification. The obtained sequencing data were analyzed using the BLAST tool in NCBI GenBank to match the sequences with known bacterial species. The sequences were compared to the reference database, and the closest match was used to identify the bacterial species (Huang *et al*., 2017; Saha *et al*., 2013).

Phylogenetic analysis

For phylogenetic analysis, the obtained *16S rRNA* gene sequences were aligned using clustal omega and MEGA X software to generate a multiple sequence alignment (MSA). A phylogenetic tree was constructed using the neighbor-joining (NJ) method or the maximum likelihood (ML) method, and bootstrap values were calculated to assess the reliability of the tree (Aswiyanti *et al*., 2021).

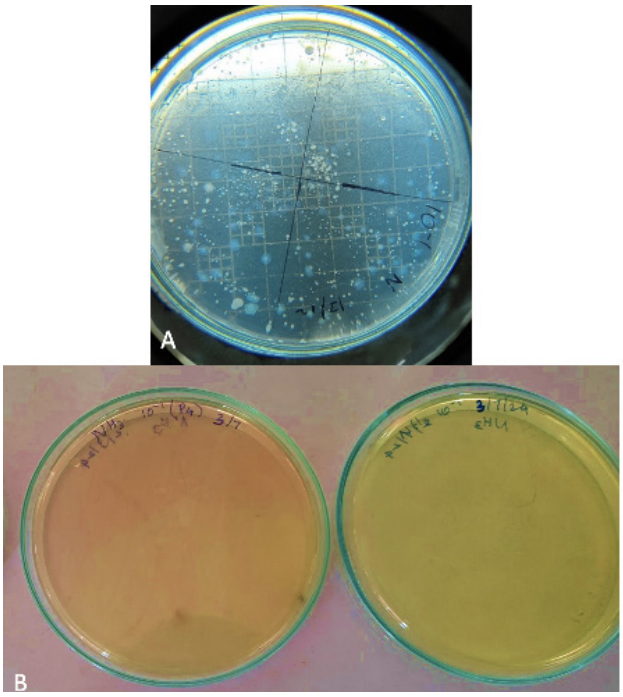

Fig. 1. Enumeration of NH3 utilizing bacteria (A) and screening of bacteria (yellow discoloration).

RESULTS

Enumeration and screening of ammonia and nitrite utilizing bacteria

Enumeration of bacteria colonies were done using colony counter in which the serially diluted sample plated 10^{-1} had 206 CFU, 10^{-2} had 164 CFU, 10^{-3} had 123 CFU, 10^{-4} had 81 CFU and 10^{-5} had 56 CFU. Fifteen well grown and morphologically distinct bacterial colonies (N_1-N_{15}) were isolated and purified from all the plate. Further slants were prepared and stored at 4 °C. For secondary screening of ammonia degrading bacteria, these fifteen isolates were inoculated in mineral medium agar plates with 0.05% phenol red indicator and incubated at 32±2°C for 24 h and seven isolate plates colour were changed to yellow around the colonies (Fig. 1). Instantaneously secondary screening of nitrite degraders, these fifteen isolates were tested in Griess-Ilosvay reagent and incubated at 30±2°C for 24 h and four isolates showed the colour changes occurrence around the colonies.

Morphological characteristics

Morphologically 80% bacteria are rod-shaped while remaining were cocci shaped about 20%; 30% were Gram-positive and 70% Gram-negative (Fig. 2). The colour of the grown bacteria in the mineral salt media were opaque, creamy white and pale yellow. Isolates (N_2 -N_{10}) texture were smooth and mucoid whereas N_1 were dry and wrinkled texture (Table I). Majority of the ammonia and nitrite degraders were motile. The most of the isolates belonged to facultative anaerobic and some were aerobic.

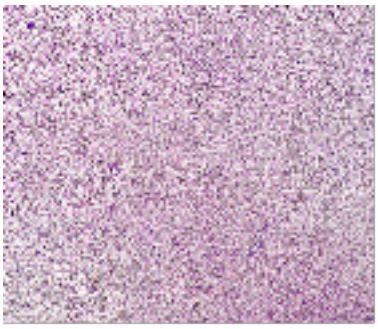

Fig. 2. Gram staining showing purple (positive).

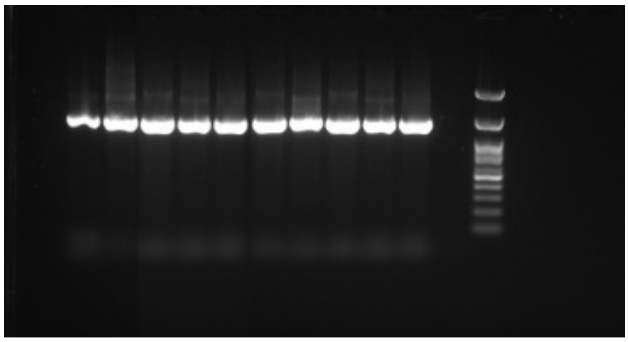

Fig. 3. 16S rRNA gene of selected bacteria(N1-N10).

Sequence analysis of ten *16S rRNA* gene isolates (Fig. 3) indicated 99% similarities where N_1 was *Brevibacillus*

borstelensis, N_2 was *Burkholderia cepacia* strain BS, N_3 was *Burkholderia paludis*, N_4 was *Burkholderia territorii*, N_5 was *Burkholderia seminalis,* N_6 was *Burkholderia cenacepacia*, N_7 was *Agrobacterium pusense*, N_8 was *Rhizobacterium* sp, N_9 was *Bacillus proteolyticus* and N_{10} was *Staphylococcus hominis.* The sequence similarity of *Brevibacillus borstelensis* was 95.89%*, Burkholderia cepacia strain BS* was 97.49%*, Burkholderia paludis* was 97.74%*, Burkholderia territorii* was 96.52%*, Burkholderia seminalis* was 96.78%*, Burkholderia cenacepacia* was 98%*, Agrobacterium pusense* was 97.33%*, Rhizobacterium* sp. was 95.80%, *Bacillus proteolyticus* was 94.07%*, Staphylococcus hominis* was 95.37% (Table II). The phylogenetic tree based on *16S rDNA* gene sequences of the isolates and phylogenetically related bacteria confirmed. The phylogenetic relationships among the identified isolates were analyzed using hierarchical clustering, as represented in the dendrogram (Fig. 4). The clustering pattern suggests that isolates N_7 and N_8 share a close genetic relationship, forming a distinct clade, while N_{10} appears as the most divergent among the analyzed isolates. Similarly, isolates N_3, N_4, and N_5 grouped together, indicating their phylogenetic similarity. Isolates N_2 and N_6 also clustered, signifying a shared evolutionary origin. Notably, N_9 and N_1 formed a separate branch, suggesting their genetic distinctness from other clusters. These phylogenetic relationships provide insights into the evolutionary lineage of the isolates, which may have implications for their functional roles in the studied environment.

DISCUSSION

Nitrate and ammonia degrading bacteria

Our findings align with recent studies on the role of *Bacillus* spp. in nitrogen removal in aquaculture (Ma *et al*., 2020). Predominantly isolating *Burkholderia* species, we identified ten bacterial strains capable of nitrite degradation, with *Brevibacillus borstelensis*, *Burkholderia cepacia*, and *Burkholderia paludis* exhibiting high affinity for ammonia and nitrite degradation. Similar to Ma *et al*. (2020), who reported *Bacillus subtilis* demonstrating aerobic denitrification under varying aquaculture conditions, our isolates displayed robustness across pH, temperature, and salinity ranges, reinforcing their suitability for aquaculture applications. Additionally, our results parallel Aswiyanti *et al*. (2021), where *Klebsiella* strains showed high nitrification activity (17.26–21.54 ppm nitrate production by day six). Our *Burkholderia* isolates demonstrated comparable nitrification potential, confirming their bioremediation role in aquaculture systems. These findings emphasize the significance of *Burkholderia* and *Bacillus* species in maintaining water quality and preventing nitrogen toxicity in aquaculture.

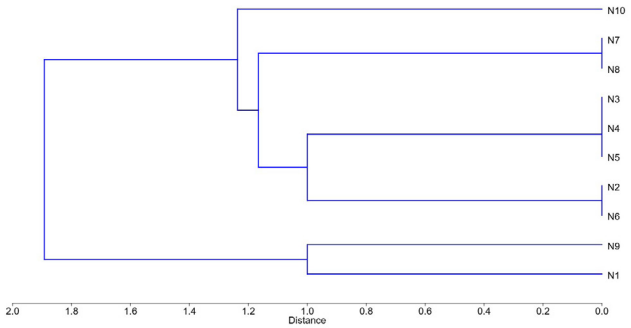

Fig. 4. Phylogenetic tree based on *16S rRNA* gene sequencing. For details of isolates N -N_{10}, see Table I.

Table I. Morphology characteristics of 10 isolates.

Isolates	Color	Colony morphology			Cell Characteristics	
		Shape	Texture	Elevation	Shape	Gram
N_1	Off-White	Slightly curved	Wrinkled	Flat	Cocci	Positive
N_2	Creamy to yellowish	Circular	Mucoid	Convex	Rod	Negative
N_3	Creamy to pale yellow	Circular	Smooth, mucoid	Raised	Rod	Negative
N_4	Creamy white	Circular	Smooth, mucoid	Raised	Rod	Negative
N_5	Creamy white	Circular	Smooth, mucoid	Raised	Rod	Negative
N_6	Creamy white	Circular	Smooth, mucoid	Raised	Rod	Negative
N_7	Creamy white	Circular	Smooth, mucoid	Convex	Rod	Negative
N_8	Off-white to yellowish	Circular	Smooth, mucoid	Raised	Rod	Negative
N_9	Opaque white	Irregular	Dry, mucoid	Raised	Rod	Positive
N_{10}	White to creamy white	Circular	Smooth, mucoid	Raised	Cocci	Positive

N_1 was *Brevibacillus borstelensis*, N_2 was *Burkholderia cepacia* strain BS, N_3 was *Burkholderia paludis*, N_4 was *Burkholderia territorii*, N_5 was *Burkholderia seminalis,* N_6 was *Burkholderia cenacepacia*, N_7 was *Agrobacterium pusense*, N_8 was *Rhizobacterium* sp., N_9 was *Bacillus proteolyticus* and N_{10} was *Staphylococcus hominis.*

Table II. Partial sequences analysis of *16S rRNA* gene sequences of the isolates.

Isolate	Most related Organisms	Ss(%)	QL
N_1	*Brevibacillus borstelensis*	95.89	1433
N_2	*Burkholderia cepacia strain BS*	97.49	1371
N_3	*Burkholderia paludis*	97.74	1373
N_4	*Burkholderia territorii*	96.52	1405
N_5	*Burkholderia seminalis*	96.78	1326
N_6	*Burkholderia cenacepacia,*	98	1370
N_7	*Agrobacterium pusense*	97.33	1392
N_8	*Rhizobacterium sp.*	95.80	1352
N_9	*Bacillus proteolyticus*	94.07	1303
N_{10}	*Staphylococcus hominis*	95.37	1384

Ss(%), sequence similarity; QL, query length.

Secondary screening revealed seven ammonia-degrading and four nitrite-degrading isolates, with *Burkholderia* and *Brevibacillus* species identified as the most promising for aquaculture applications. Similar to Aswiyanti *et al*. (2021), where *Klebsiella* spp. exhibited strong bioremediation potential, our findings highlight the suitability of these strains for wastewater treatment. Our screening method, using pH indicators to detect ammonia oxidation, aligns with Elbanna *et al*. (2012), who utilized phenol red and methyl orange for visual acidification detection. Both studies confirm the effectiveness of pH indicator-based methods as a rapid alternative to labour-intensive chemical analyses for identifying nitrifying bacteria.

Morphological characteristics

Our isolates were predominantly motile and facultative anaerobes, similar to *Bacillus* strains identified by Qingshan *et al*. (2020), which thrive under aerobic conditions and tolerate variable pH, temperature, and salinity. *Burkholderia* species were the most prevalent, with 70% of isolates being Gram-negative and 80% exhibiting motility, aligning with Aswiyanti *et al*. (2021), who reported *Klebsiella* spp. as non-motile, Gram-negative, and facultative anaerobes in aquaculture. Our findings also parallel Kouki *et al*. (2011), who identified *Bacillus* and *Exiguobacterium* as dominant mixotrophic ammonia-oxidizers in wetlands, while our isolates included *Burkholderia*, *Brevibacillus*, *Agrobacterium pusense*, and *Staphylococcus hominis*. The predominance of motile, facultative anaerobic bacteria (80%) supports their role in wastewater nitrification, as seen in Elbanna *et al*. (2012), where motility was a key trait for nitrification efficiency. These results emphasize the importance of motility in enhancing nitrogen compound degradation in aquaculture wastewater treatment.

Molecular characteristics

Our study aligns with Saha *et al*. (2013) in utilizing 16S rDNA sequencing for precise bacterial identification, confirming *Nitrosomonas*, *Nitrobacter*, and *Burkholderia* species in wastewater samples. Molecular techniques proved essential for distinguishing nitrifying bacteria, supporting rapid detection in aquaculture systems where timely intervention is critical for water quality management. *Burkholderia cepacia* and *Burkholderia paludis* exhibited 97–99% sequence similarity with known strains, reinforcing their wastewater treatment potential. Our findings on genetic diversity align with Kouki *et al*. (2011), who clustered 35 isolates into 10 phylogenetic groups in wetlands. Additionally, *Brevibacillus borstelensis* showed 99% sequence similarity with previously identified strains, consistent with Elbanna *et al*. (2012), where *Bacillus* and *Brevibacillus* were confirmed as ammonium oxidizers. These results highlight the reliability of molecular techniques in characterizing nitrifying bacteria in wastewater environments.

CONCLUSION

This study successfully isolated and characterized *Burkholderia* and *Brevibacillus* species from shrimp aquaculture wastewater, highlighting their efficiency in ammonia and nitrite degradation. Molecular characterization via 16S rRNA sequencing confirmed their taxonomic placement among nitrifying bacteria. The isolates demonstrated robustness across varying pH, temperature, and salinity, making them suitable for practical application in aquaculture. Their morphological and physiological traits, including motility and facultative anaerobic nature, align with known nitrifiers. Integrating these strains into biofiltration and bioremediation strategies can enhance water treatment efficiency, reduce nitrogen toxicity, and improve aquaculture sustainability.

DECLARATIONS

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Generative AI or AI-assisted Technology Statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Statement of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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