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ISOLATION AND IDENTIFICATION OF BACTERIA REMOVING NITRITE, NITRATE, AND AMMONIUM FROM BIOBALLS FILTER

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ABSTRACT

The presence of effective bacteria removing nitrite, nitrate, and ammonia in a recirculating aquaculture system (RAS) is necessary to attenuate their toxicity to fish. The research was conducted to find bacteria that can be cultured and reduce nitrite, nitrate, and ammonium. Sixteen bacterial colonies were isolated from bioballs of RAS biofilter and tested for their ability to reduce nitrite or nitrate concentrations. Using a simple indicator paper for nitrite and nitrate, four isolates that reduced nitrite and nitrate concentrations, namely K1NA3, K2NA3, CNA1, and PRO4NA1 were selected. The four isolates were then evaluated for the metabolism of nitrate, nitrite, and ammonium compounds using the spectrophotometry method. Results showed that the isolates K1NA3, CNA1, and PRO4NA1 reduced nitrite concentration but produced ammonium, whereas K1NA3 isolate was able to reduce nitrate concentration but produced both nitrite and ammonium. Experiments in reducing ammonium levels in the synthetic waste media showed the ability of four isolates to reduce ammonium levels after six days despite producing nitrite. Based on the 16S rRNA gene analysis, these isolates have a close relationship to *Pseudomonas otitidis* (K1NA3 and K2NA3), *Acinetobacter cumulans* (CNA1), and *Vogesella perlucida* (PRO4NA1).

KEYWORDS: ammonia removal; nitrogenous compounds; aquaculture bioremediation; biofilter bacteria; nitrification bacteria

INTRODUCTION

Excessive nitrogen-based waste in the form of ammonia, ammonium, nitrite, and nitrate can be harmful to ecosystems, both marine and freshwater, human, and animal health (Kumar *et al*., 2020). The accumulation of inorganic nitrogen is the main obstacle in an intensive aquaculture (Khangembam *et al*., 2017).

In aquaculture, bacteria play a role in the transformation of nitrogen compounds (Hu *et al*., 2013). This group of bacteria can convert N-organic compounds into ammonia (NH_3). Ammonia is then oxidized to nitrite (NO_2) and nitrate (NO_3) in the nitrification process (Stein & Klotz, 2016). The nitrate formed can be reduced to nitrogen gas (N_2) through denitrification or converted back to ammonia. The formation of nitrate into nitrogen gas is the ideal transformation in aquaculture systems (Rassamee *et al*., 2011). The utilization of indigenous microbes in the pond is considered an environmentally friendly method to accelerate nitrogen waste removal. The more active bacterial inoculum is usually in the form of a consortium (Barik *et al*., 2018). Each strain of bacteria has different metabolic capability in metabolizing nitrogen, mineralizing organic matter, and removing toxic compounds. (Dahiya & Mohan, 2016).

The development of wastewater treatment from recirculating aquaculture systems (RAS) to treat residual harmful nitrogenous waste compounds from RAS, in which bacteria play a role in nitrification and simultaneous nitrification-denitrification, is still needed (Shitu *et al*., 2021). Isolation of bacteria that reduce ammonia, nitrite, and nitrate levels is still considered relevant to be carried out in the context of controlling nitrogen waste on an industrial scale (Motamedi & Jafari, 2020). Screening of such bacteria is expected to increase the effectiveness of using indigenous bacteria to reduce nitrogen compound

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waste. This study aims to obtain bacterial colonies that have the potential to reduce the content of ammonium, nitrite, and nitrate.

MATERIALS AND METHODS

Briefly, the process of obtaining effective bacterial isolates begins with the isolation of bacteria from the bioballs filter. The isolates were then grown on 4 mL of NB media for 24 hours and centrifuged. The pellets obtained were added with 200 μL nitrite ($NaNO_2$) 5 mg/L or nitrate (KNO_3) 20 mg/L, and incubated for 24 hours. The decrease in nitrite or nitrate content in the supernatant indicated the capability of the isolates in removing nitrite or nitrate respectively and was estimated using indicator paper. Bacterial colonies capable of reducing nitrite and/or nitrate content were then selected for further analyses.

The supernatant containing nitrite or nitrate in which isolate is considered capable of reducing nitrite and/or nitrate was then measured using the spectrophotometer method to confirm the ability of the bacteria to reduce the levels of nitrite and nitrate. Ammonium production, as well as nitrate production in nitrite supernatant and nitrite production in nitrate supernatant, were also measured. The isolates capable of reducing nitrite or nitrate with low ammonium production were then selected for the ammonium reduction test on synthetic waste media by growing single or mixed isolates in the media containing ammonium based on a modified method of Du *et al*. (2015).

Isolation of Bacteria

Bacteria were isolated from bioballs used as a biological filter in a recirculating aquaculture system at the Research Center for Biology, National Research and Innovation Agency (BRIN), Cibinong, West Java. Samples from bioballs were taken at approximately 0.1 g by scraping the bioball cavity using a sterile spatula. The bacteria were grown on nutrient agar (NA) and nutrient broth (NB) media (Himedia) at room temperature.

Qualitative Measurement of Bacteria Activity to Reduce Nitrite and Nitrate Using Indicator Paper (Screening)

Bacterial isolates were grown in 4 mL of NB media for 24 hours with agitation at 115 rpm at room temperature. Cell suspension of 1 mL was centrifuged for five minutes at 10,000 rpm. The pellets were washed and added 200 μL of 5 mg/L $NaNO_2$ solution for the nitrite reduction test or 20 mg/L KNO_3 for the nitrate level test. Microtubes were agitated for 24 hours. The sample of 3 μL was placed on nitrite-nitrate test paper (JBL EasyTest, Germany) for 3-5 minutes to detect any color change on the test paper. Bacteria whose inoculum was positive for reducing nitrite or nitrate that showed pink to white color were selected for further testings.

Determination of Nitrite and Nitrate Metabolism

The ability of single isolates to decrease nitrite or nitrate in media, production of ammonium, and production of nitrate or nitrite in media containing nitrite or nitrate were observed to confirm the occurrence of nitrite and nitrate metabolic processes. Isolate cultures of 24 hours were centrifuged at 5,000 g; the pellet was washed with distilled water. The pellets were then resuspended with 2 mL of nitrite or nitrate with OD 600 nm of 3.75 and 2.26, respectively. Cultures were performed in microplate 12 wells, incubated at room temperature with shaking at 100 rpm. After six days of incubation, the nitrite, nitrate, and ammonium levels were measured using the spectrophotometer method. Experiments were performed in triplicate.

Determination of Ammonium Metabolism by Bacterial Consortia in Wastewater Synthetic Media

The procedure of this experiment is similar to the determination of nitrite and nitrate metabolisms method. Synthetic media is prepared according to a modified method by Du *et al*. (2015). In 1 L micro elements solution consisted of: 8.304 g Na_2-EDTA.$2H_2O$, 5 g $FeSO_4.7H_2O$, 0.215 g $ZnSO_4.7H_2O$, 0.495 g $MnSO_4$, 0.125 g, and $CuSO_4.5H_2O$. In 1 L synthetic waste solution consisting of 0.191 g NH_4Cl, 0.800 $NaHCO_3$, 0.081 KH_2PO_4, 0.214 $Na_2HPO_4.12H_2O$, 0.213 Na_2SO_4, 0.036 CaCl.$2H_2O$, 0.051 $MgCl_2.6H_2O$, and 1 mL microelements solution. Isolates experiment tested were PRO4NA1 (A); CNA1 and PRO4NA1 (B); and K1NA3, K2NA3, CNA1, and PRO4NA1 (C). OD 600 nm value each experiment was of 2.4.

Measurement of Nitrite Concentration (SNI 06-6989.9, 2004)

Measurement of nitrite concentration was performed using the azo staining method. A sample of 10 μL was added to 240 μL of distilled water into a 96-well microplate. The diluted sample was added with 5 μL of sulfanilamide acid and 5 μL of NED solutions and then homogenized. The mixture was allowed to stand for 10 minutes and then measured using a

microplate reader with a wavelength of 543 nm. The calibration curve was prepared by preparing a solution of $NaNO_2$ as a standard solution of nitrite with a concentration from 0 to 0.25 mg/L.

Measurement of Nitrate Concentration (EPA Method: 352.1, 1971)

The measurement of nitrate was carried out by the brucine sulfate colorimetric method. A sample of 40 μL was added with 360 μL of distilled water. The diluted sample was added with 80 μL of 30% NaCl solution, 400 μL of 75% sulfuric acid, and 20 μL of brucine sulfate reagent. The mixture was homogenized and heated at 95°C for 20 minutes. Then the absorbance was measured at a wavelength of 410 nm. The calibration curve was made by preparing a solution of KNO_3 as a standard solution of nitrate concentration from 0 to 2.5 mg/L.

Measurement of Ammonium Concentration (EPA Method: 350.2, 1974)

Measurement of ammonium concentration was done by using the Nessler method. The sample of 25 μL was added with 225 μL of distilled water in a 96-well microplate. The diluted sample was added with 2.5 μL Nessler A and 2.5 μL Nessler B, homogenized and allowed to stand for 10 minutes. The absorbance of the mixture was measured at a wavelength of 490 nm. The calibration curve was carried out by measuring the OD of a standard solution of ammonium with a concentration from 0 to 5 mg/L.

Identification of Bacteria

Selected bacteria were identified molecularly based on the partial 16S rRNA gene. The colony PCR technique was carried out by preparing the mixture (total 30 uL) consisting of 11 uL ddH2O (nuclease-free water), 15 uL Go Taq Green Master Mix 2x, 2 uL 10 pM forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), and 2 uL 10 pM reverse primer 1492R (5'-CGG TTACCTTGTTACGAC TT-3'). One bacterial colony grown on nutrient agar was picked up and loaded into the PCR mixture in the microtube. The PCR condition included the following steps: initiation at 94°C for five min; the 30 cycles of denaturing at 94°C for one min, annealing at 55°C for one min, and extension at 72°C for one min; and the final elongation at 72°C for one min. The 16S rRNA sequences of each bacterial isolate were compared with homologous sequences using the BLAST (Basic Local Alignment Search Tool: http://blast.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using Clustal X based on the neighbor-joining method.

RESULTS AND DISCUSSION

Isolate of Bacteria and Its Ability to Reduce Nitrate and Nitrite Content

There were 16 bacterial colonies visually observed and differed in morphology. The ability of each colony to reduce nitrate and nitrite content is presented in Figure 1 and Table 1. The white color of the band in Figure 1 indicated that the content of nitrate (band at the top) or nitrite (band at the bottom) decreased. From the data, it can be seen that there are four potential isolates, namely CNA1, K1NA3, K2NA3, and PRO4NA1. The ability to reduce nitrite and nitrate content was then confirmed using the spectrophotometer method.

In order to overcome poisoning due to nitrogen-based compounds, efforts are made to reduce the levels of ammonium, nitrite, and nitrate; which are then expected to become nitrogen molecules (N_2). Since the chemical reaction in the process of transformation of ammonium to nitrite, then to nitrate is reversible (Blum *et al*., 2018), it is necessary to know the potential of bacterial isolates in converting nitrite to nitrate and vice versa and converting ammonium to nitrite or nitrate and vice versa.

Nitrite Metabolism

It was confirmed that three isolates were able to decrease nitrite, namely K1NA3, CNA1, and PRO4NA1 (Figure 2). However, one of the products of nitrite metabolism is ammonium (Figure 3). In nitrite solution, K1NA3 isolate produced the highest ammonium. However, nitrite metabolism in these isolates did not produce nitrate (nitrate content was not observed, data not shown).

Nitrate Metabolism

Two isolates were confirmed to be able to reduce nitrate concentration, namely K1NA3 and PRO4NA1 (Figure 4). PRO4NA1 isolate was the highest in reducing nitrate but produced a higher concentration of nitrite than K1NA3 (Figure 5). In addition to producing nitrite, nitrate metabolism can produce ammonium, especially in K1NA3 isolate (Figure 6).

Ammonium Metabolism by Bacterial Consortia

Ammonium is an inorganic nitrogen compound resulting from the breakdown of protein. Considering that bacteria in an ecosystem contain nitrogenous waste that plays a role in the nitrogen cycle, which is usually complex, the ability of the bacterial consortium to remove ammonium and its metabolic pro-

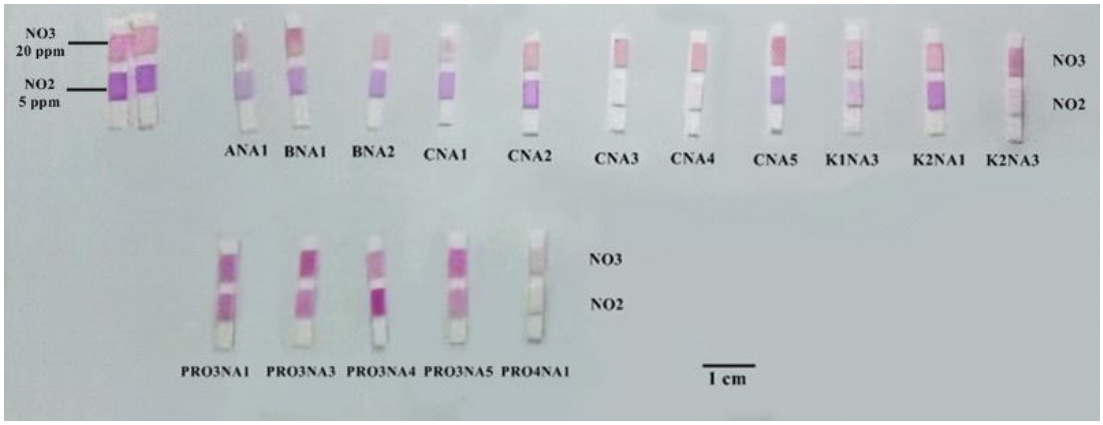

Figure 1. Nitrite nitrate indicator paper after dropping the sample.

Table 1. Nitrate and nitrite content in samples after inoculation with bacterial isolates

Isolate inoculated NO_3^- or NO_2^- sample	Colour of NO_3^- indicator paper after being wetted with NO_3 solution	Colour of NO_2^- indicator paper after being wetted with sample NO_2 solution
No isolate	+ +	+ +
ANA1	+ +	+
BNA1	+ + +	+
BNA2	+	+
CNA1	**-**	**+**
CNA2	+ +	+
CNA3	+	-
CNA4	+ +	-
CNA5	+ + +	+ +
K1NA3	**-**	**-**
K2NAI	+	+ +
K2NA3	**+ +**	**-**
PRO3NA1	+ +	+ +
PRO3NA3	+ + +	+
PRO3NA4	+	+ +
PRO3NA5	+	+
PRO4NA1	**-**	**-**

Notes: Presence of NO_3 is indicated by pink color, while NO_2 is purple (+ + +: strong, + +: medium, +: weak).
No detectable NO_2 or NO_3 is indicated by white color (-)

ducts is important to anticipate the accumulation of nitrogen compounds that can harm organisms such as nitrite, nitrate or even N_2O in the greenhouse effect (Blum *et al*., 2018). Figure 7, showed that isolates of PRO4NA1, B bacterial mixture (CNA1 and PRO4NA1) and the mixture of the four isolates could reduce ammonium levels in six days. These data illustrated that the application of these bacterial isolates to nitrogen waste of freshwater aquaculture could reduce ammonium contained within. However, the reduction was followed by the accumulation of nitrite (Figure 8), indicating the ineffectiveness of denitrification. The bacterial mixture of four isolates demonstrated the highest accumulation of nitrite. However, until day-6, no nitrate was observed (data not shown).

Identification of Bacterial Isolates

PCR amplification of four isolates (K1NA3, K2NA3, CNA1, and PRO4NA1) was successfully carried out based on observations on gel electrophoresis at around 1,500 bp. Partial sequencing of the 16S rRNA gene resulted in a sequence length ranging from 1,005-1,392 bp. Nevertheless, it provided an overview of genetic similarity information through alignment with a collection of rRNA sequences from NCBI Gen bank. BLAST analysis showed the percentage similarity of all isolates was 98.20%-99.75% (Table 2).

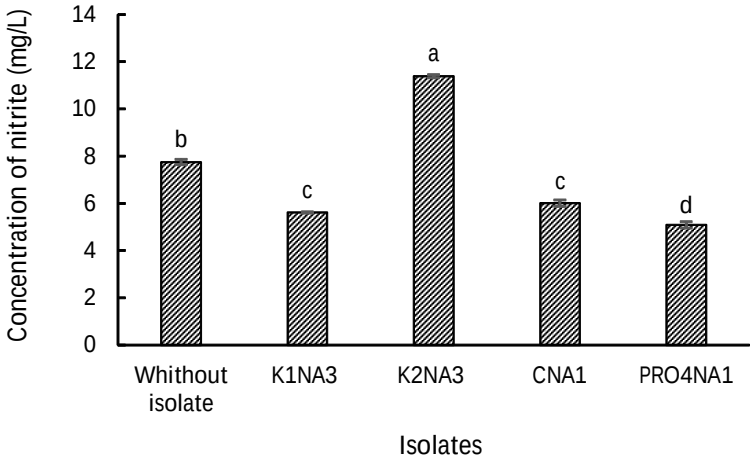

Figure 2. Nitrite content in nitrite media that has been inoculated with bacterial isolates. Different letters indicate significant differences (P < 0.05).

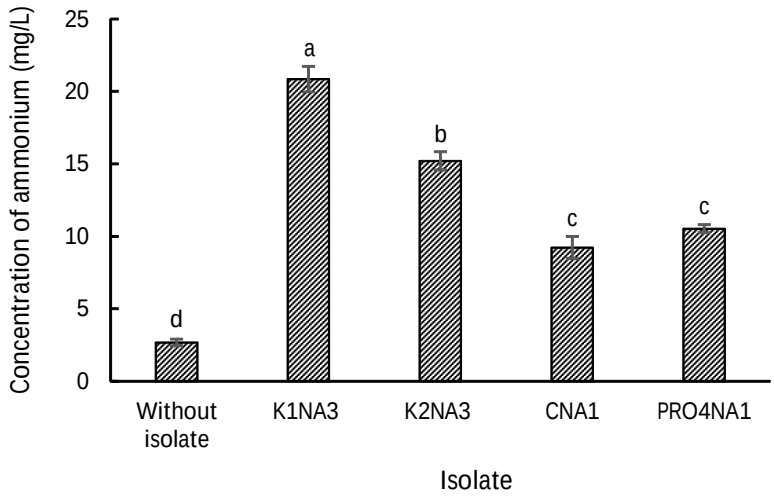

Figure 3. Ammonium content in nitrite media that has been inoculated with bacterial isolates. Different letters indicate significant differences (P < 0.05).

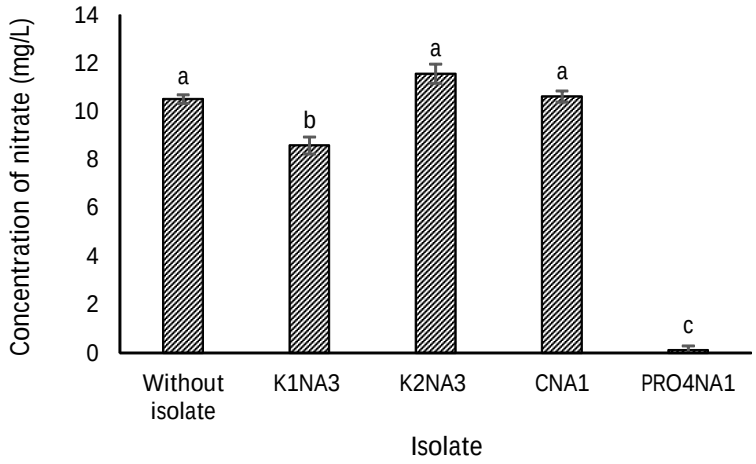

Figure 4. Nitrate content in nitrate media that has been inoculated with bacterial isolates. Different letters indicate significant differences (P < 0.05).

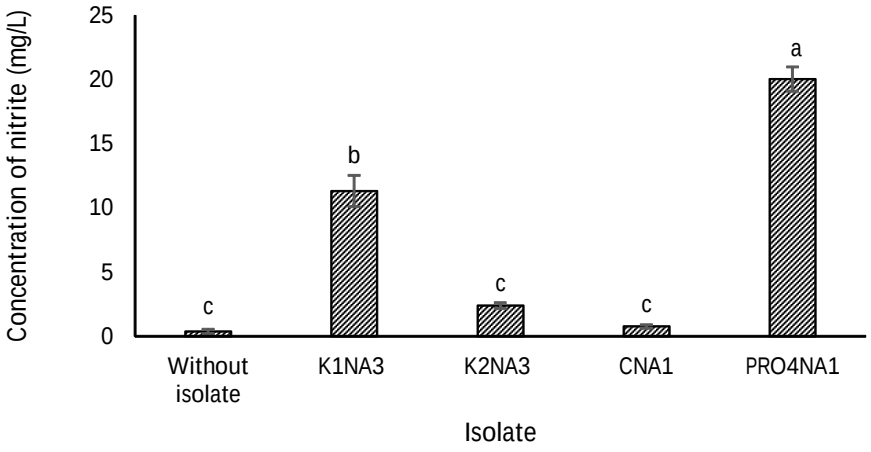

Figure 5. Nitrite content in nitrate media that has been inoculated with bacterial isolates. Different letters indicate significant differences ($P < 0.05$).

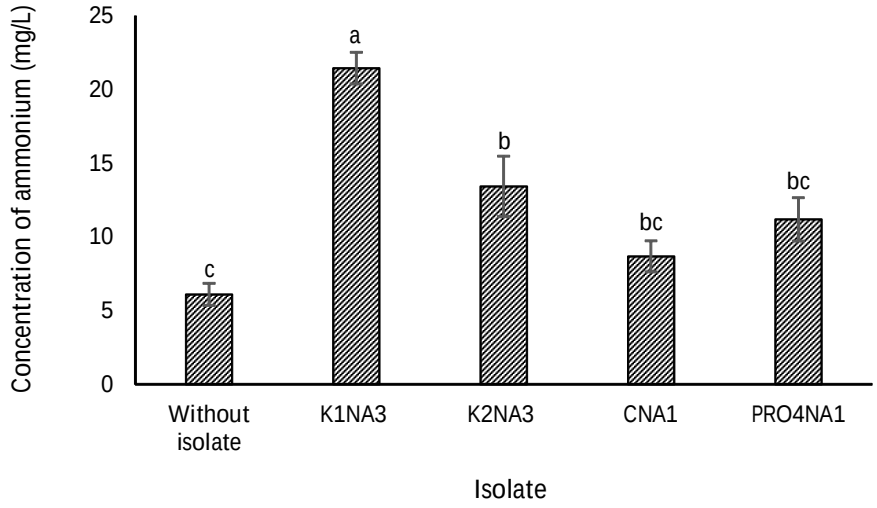

Figure 6. Ammonium content in nitrate media that has been inoculated with bacterial isolates. Different letters indicate significant differences ($P < 0.05$).

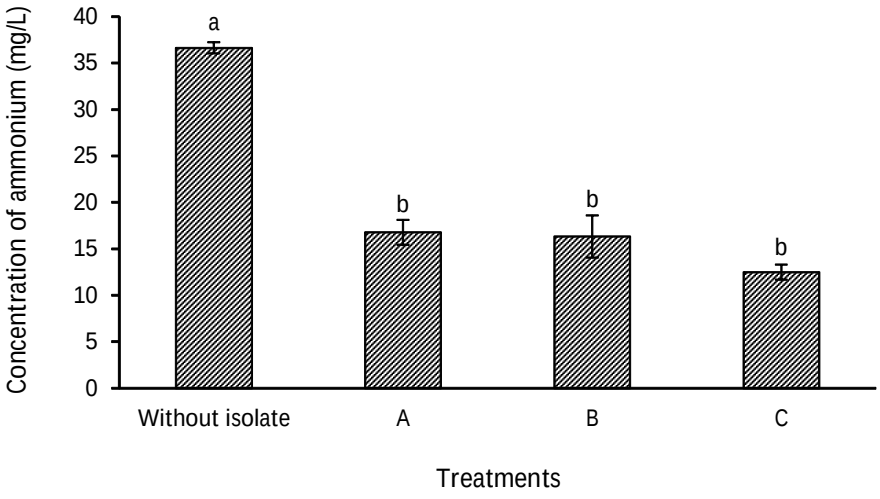

Figure 7. Ammonium content in synthetic media containing ammonium after being given isolates with six days incubation; (A) PRO4NA1 isolates, (B) CNA1 and PRO4NA1 isolates, (C) K1NA3, K2NA3, CNA1, and PRO4NA1 isolates. Different letters indicate significant differences ($P < 0.05$).

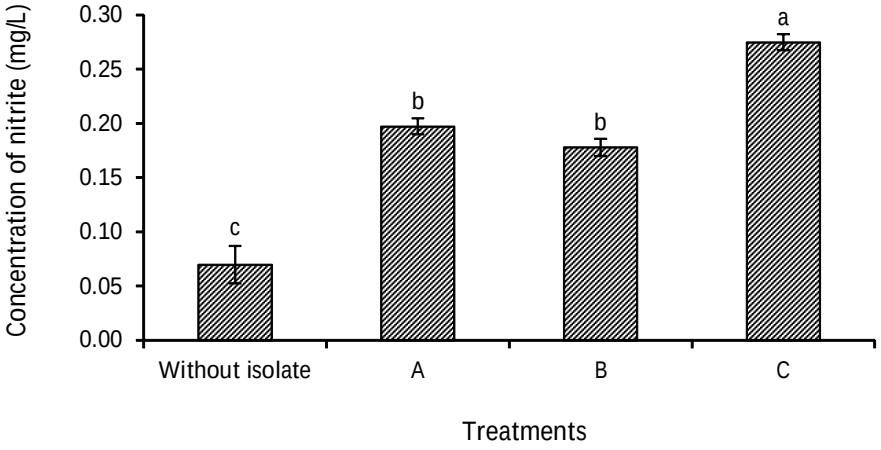

Figure 8. Nitrite content in synthetic media containing ammonium after being given isolates with six days incubation; (A) PRO4NA1 isolates, (B) CNA1 and PRO4NA1 isolates, (C) K1NA3, K2NA3, CNA1, and PRO4NA1 isolates. Different letters indicate significant differences ($P < 0.05$).

The phylogenetic analysis of 1,000 bootstraps showed that four isolates were grouped into three different clades (Figure 9), namely *Pseudomonas*, *Acinetobacter*, and *Vogesella*.

P. otitidis was first isolated and identified from the clinical specimens of infected human ears. The cells are rod-shaped, motile, and Gram-negative (Clark *et al*., 2006). According to Dahiya & Mohan (2016), *P. otitidis* is a potential bacterium for wastewater treatment. (Dados *et al*., 2015; Wu *et al*., 2009). *Pseudomonas* is a member of Gammaproteobacteria belonging to the group of denitrifying bacteria with diverse metabolic abilities (Dados *et al*., 2015). As reported previously, *P. otitidis* is used for wastewater treatment (Dahiya & Mohan, 2016), including contaminant removal processes, such as dye degradation and bioremediation in hydrocarbon-contaminated soils (Dados *et al*., 2015; Wu *et al*., 2009).

Vogesella perlucida is a Gram-negative bacteria and was firstly isolated from water samples from springs in Taiwan (Chou *et al*., 2008). Phylogenetic analysis showed that CNA1 is in the same clade as *Acinetobacter johnsonii* strain ATCC 17909^T, which indicates a close relationship with *Acinetobacter haemolyticus* strain ATCC 17906^T. *Acinetobacter* is a genus of bacteria from the Gamma proteobacteria group, widely distributed in various ecosystems with aerobic denitrification capabilities (Qin *et al*., 2019). These bacteria are able to reduce nitrate or nitrite to N_2 under aerobic culture conditions (Wang *et al*., 2020).

Phylogenetic analysis showed that the CNA1 isolate was in the same clade as *Acinetobacter johnsonii* strain ATCC 17909^T, which indicated a close relationship with *Acinetobacter haemolyticus* strain ATCC 17906^T (Figure 8). *Acinetobacter* is a genus widely distributed in various ecosystems with aerobic deni-

Table 2. Results of BLAST sequence analysis for 16S rRNA gene from NCBI gene bank

Isolate	The closest relationship strain [Accession number]	Homology (%)
K1NA3	*Pseudomonas otitidis* MCC 10330^T [NR_043289.1]	99.7
K2NA3	*Pseudomonas otitidis* MCC 10330 ^{T} [NR_043289.1]	98.9
CNA1	*Acinetobacter haemolyticus* ATCC 17906^T [NR_117622.1]	98.2
PRO4NA1	*Vogesella perlucida* DS-28^T [NR_044326.1]	99.75

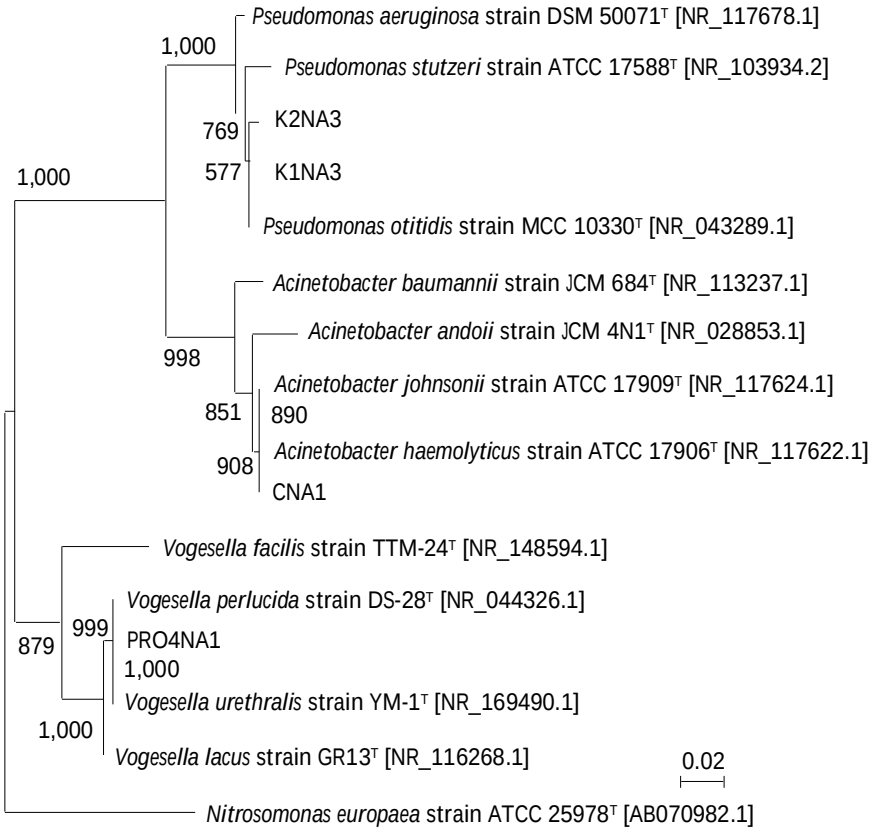

Figure 9. Phylogenetic tree of 16S rRNA sequences of four isolates obtained from bioball. Phylogenetic tree was constructed based on 16S rRNA gene sequencing by the neighbor-joining method. Distances were estimated according to the Kimura two parameter model with bootstrap percentages after 1,000 simulations. The bar represents a 2% estimated sequence divergence. *Nitrosomonas europaea* strain ATCC 25978T was used as an out-group.

trification capabilities (Qin *et al*., 2019), and can reduce nitrate or nitrite to N_2 under aerobic culture conditions (Wang *et al*., 2020). In this study, CNA1 isolates reduced nitrite levels, with a low accumulation of ammonium (Figure 2 and 3), but did not reduce nitrate levels (Figure 4). K1NA3 metabolizes nitrite and nitrate, as well as forms ammonium. According to Yang *et al*. (2012), members of *Pseudomonas* are strong denitrifiers. They have complete denitrification enzymes and can catalyze the reduction of nitrate to nitrogen. These bacteria are able to use oxygen on nitrite or nitrate ions when there is no free oxygen. This aerobic metabolism is shown by its ability to reduce nitrate to ammonium through nitrite (Dahiya & Mohan, 2016).

The use of indigenous bacteria in nitrogen-based wastes (ammonium, nitrite, and nitrate) control in aquaculture is still considered relevant. Basically, this waste control is to facilitate the conversion of ammonium to nitrite and/or nitrate to nitrogen gas. Bacteria that decompose ammonium, nitrite, and nitrate have an important role. By using indicator paper for nitrite or nitrate, the bacteria capable of removing nitrite and or nitrate are *Pseudomonas otitidis* (K1NA3 and K2NA3), *Acinetobacter cumulans* (CNA1), and *Vogesella perlucida* (PRO4NA1). Screening of active bacteria in diminishing nitrogen-based wastes was accelerated by using indicator paper (Figure 1 and Table 1). By using the spectrophotometer-based method, the bacteria are confirmed to be able to reduce the levels of nitrite (Figure 2) and nitrate (Figure 4). Diminishing nitrite content was followed by the formation of ammonium (Figure 3). This is due to the dissimilatory nitrite reduction to ammonium (DNRA) process (Stein & Klotz, 2016). Two isolates (K1NA3 and PRONA1) were able to metabolize nitrate (Figure 4) and producing nitrite (Figure 5) and ammonium (Figure 6). No isolate was observed to convert nitrite to nitrate indicating that the oxidation of nitrite to nitrate is not running smoothly. However, DNRA and the oxidation of ammonia to nitrite can be observed in a population.

The performance of isolates in removing ammonium in synthetic wastewater was briefly observed. Axenic cultures of PRONA1 had the lowest DNRA activity, as well as community cultures (CNA1 and PRONA1; and a mixture of K1NA3, K2NA3, CNA1, and PRONA1 should be PRO4NA1) and were able to reduce ammonium in synthetic wastewater after six days of incubation (Figure 7). Ammonium metabolism in mixed bacterial cultures yielded the highest nitrite (Figure 8). The nitrate content was not detected. The transformation of ammonium, nitrite, and nitrate into nitrogen gas is a complex reaction involving oxidoreductase enzymes of a population or community (Ge *et al*., 2012; Stein & Klotz, 2016; Zhang *et al*., 2019). Abiotic environmental factors such as pH, carbon sources, alkalinity, and oxygen could drive the reactions (Li *et al*., 2016; Dahiya & Mohan, 2016, Zhang *et al*., 2019; Ge *et al*., 2012).

CONCLUSIONS

Four bacterial isolates, namely K1NA3 and K2NA3, CNA1, and PRO4NA1, capable of reducing nitrite, nitrate, and ammonium, were successfully isolated from bioballs of a recirculating aquaculture system. Isolates K1NA3 and K2NA3 have the closest relationship to *Pseudomonas otitidis*; CNA1, and PRO4NA1 have a closer relationship to *Acinetobacter cumulans* and *Vogesella perlucida*, respectively. The isolates could be applied to enrich effective bacteria removing nitrogenous compounds in RAS to maintain good water quality and support sustainable fish farming. Further studies are still needed to ensure the role of these bacteria in nitrogenous waste control.

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