Isolation and identification of nitrifying bacteria from tilapia (Oreochromis sp.) pond in Sleman Yogyakarta Indonesia

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Abstract. This research aims to isolate and identify autochtonous nitrifying bacteria from tilapia pond in Sleman Yogyakarta Indonesia for future application in aquaculture practices in the region. Bacteria were isolated using a nitrification medium. Bacterial characterization was carried out by non-pathogenic test to tilapia (Oreochromis sp.), and nitrification activity test in a single bacterial fermentation medium for 9 days. Bacterial identification was carried out based on the colony and cell morphologies, biochemical tests, and molecular analysis using the 16S rRNA and gyrB genes. A total of 15 isolates of nitrifying bacteria were obtained. Four non-pathogenic isolates obtained the highest nitrification activity on the sixth day of incubation, with nitrate production of 17.26-21.54 ppm. Two selected bacteria, isolates A2 and A3, have colony morphology that is milky white, smooth surface, circular shape, entire edge, and convex elevation. Both bacteria are short rods, Gram-negative, non-motile, produce catalase, fermenting glucose, sucrose, and lactose, and do not produce oxidase, ornithine decarboxylase, indole, and H2S. Molecular analysis showed that the two isolates had the highest similarity (99.28% and 99.34%) to Klebsiella spp.

1. Introductions
Fisheries industries need fast-growing aquaculture products to meet the world food demands. Asia contributes to 90% of the global aquaculture products. The requirement for animal protein increases as the human population grows [1]. It shows that aquaculture production is continually growing [2]. As an illustration, aquaculture production in Indonesia in 2013 reached 13.3 million tons and in 2017 has reached 16.1 million tons [3]. In the third quarter of 2018, aquaculture production in Indonesia has reached 13,17 million tons [4]. Meanwhile, during April-Juni 2020 KKP estimated aquaculture production in the middle of the Covid-19 pandemic reached 450 thousand tons.

The growing aquaculture production encourages the intensification of fish farming technology. The fast-growing aquaculture production will require more inputs that increased waste from the production system. Intensive aquaculture systems produce large wastewater that is rich in total nitrogen [5]. The excess nitrogen in aquaculture ponds is harmful to fish hence the conversion process from inorganic nitrogen (ammonia and nitrite) to unharmful substances (nitrate) is needed [6][1]. Intensive and semi-intensive aquaculture will
result in increased stocking density, feeding, and wastewater, especially nitrogen in ammonia form, which
degrades the water quality [7]. Intensive aquaculture systems are required to meet market needs, but the
practice is at risk of fish stress and the emergence of disease. Hence, intensification with fish health
management steps needs to be applied to support the fish growth and health condition [8].

Ammonia in water comes from the leftover fish food and feces of aquatic organisms. Excessive ammonia
level leads to physiological problems and is toxic for the fishes. That problem could be solved by improving
the water quality using the microorganisms that can parse and remodel it to become harmless substances.
This biological process is often called bioremediation. One of the materials for bioremediation is nitrifying
bacteria [6]. Nitrifying bacteria are bacteria that are capable of changing ammonium to nitrate through two
aerobic steps [9]. Nitrifying bacteria can be isolated from fish farming ponds sediments [10] and can be
used for increasing bioremediation activity in fish farming ponds. Applying nitrifying bacteria is proven to
increase the water quality, especially in controlling the rate of ammonia and nitrite in fish farming ponds
[11]. Accordingly, this research aims to isolate and identify autochtonous nitrifying bacteria from the tilapia
pond in Sleman Yogyakarta Indonesia for future application in aquaculture practices in the region.

2. Materials and Methods

This research was conducted in six months (September 2020 - February 2021) in the Laboratory of Fish
Health and Environment, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada.

2.1 Sediment sampling and water quality measurement

Samples were taken from tilapia fish farming pond at Mina Ngremboko Pond in Bokesan, Sindumartani,
Ngemplak, Widodomartani, Sleman, Yogyakarta, Indonesia. Tilapia pond sediment was taken from the 10-
15 cm depth using water pipes and stored in a 250 ml plastic bottle. All of the samples were put in a
styrofoam cooler. At the same time, pH, temperature, and Dissolved oxygen concentration in the pond were
measured.

2.2 Bacterial isolation

Nitrifying media was made based on Rodina (1972)[12], and the isolation of nitrifying bacteria steps was
following Fei et al. (2020)[13]. Briefly, the samples from different 3 points were homogenized, then 1
gram of sample was enriched and cultured in a liquid medium on a 90 rpm shaker at 28°C for 7 days. After
the incubation, 5 ml samples were incubated on the same media using a shaker at 28°C at 90 rpm for 7 days.
A total of 1 loop bacterial isolate was inoculated and purified on nitrification agar by using the quadrant
method[14] for 24 hours of incubation at 28°C. The next step was preserving the bacteria in Tryptone soy
broth medium (TSB) with 30% of glycerol at -25°C and TSB with 3% trehalose at -80°C.

2.3 Non-pathogenicity test

The Nila tilapias which were used were about 11 cm in length and were about 44 grams. Pure cultures were
taken to take a non-pathogenic test by intraperitoneal injection to tilapia (5 fishes/bacterial strain) with a
dose of 5x10^5 CFU/fish and the mortality was observed for 7 days. The Nila tilapias which were used were
about 11 cm in length and were about 44 grams. This injection method allows the bacteria to hit the target
organ quickly. The process should be done carefully to avoid hitting the internal organs which can cause
bleeding. Control fish were injected with PBS (Phosphate Buffer Saline Solution). Infected tilapia and
control tilapia were observed and maintained for a week by controlling the environmental condition in which
the dimension of the water was 40 x 30 x 20 cm^3 in a container which the dimension was 40 x 30 x 24 cm^3
and the stocking density of tilapia was 5 fishes/container. Bacteria that did not produce disease signs or fish
mortality were claimed as non-pathogenic bacteria.
2.4 Nitrification activity test
A conversion rate of ammonia to nitrate by the bacteria was tested using a nitrifying liquid medium with the composition that has been mentioned above. Bacteria were inoculated to the medium for 3 days incubation. Then, 100 µl of culture were taken to be inoculated on 5 ml of nitrifying liquid medium. After 24 hours of incubation, 5 ml of culture was inoculated into 100 ml of nitrifying liquid medium and it was incubated for 9 days. Nitrate concentrations in the medium were monitored daily.

Nitrate measurement was conducted by using an API test kit and observed spectrophotometrically. A total of 5 ml of the sample was put in a tube from the API kit then was added with 10 drops of the first nitrate test solution and homogenized. After a while, 10 drops of the second nitrate test solution were added, followed by 5 minutes of mild mixing to assure the stable color of the mixture. A light to dark yellow color indicates that there is nitrate in the mixture and it also shows the intensity of nitrate level.

A standard nitrate cure was made by preparing a standard nitrate solution using KNO₃ and water. 100 mg/L nitrate was prepared to dissolve 721.8 mg KNO₃ in 100 ml of distilled water. The solution was diluted gradually to get a series of standard solutions with decreasing concentrations. A total of 1 ml of the mixture was taken and put in a cuvette to measure its absorbance.

2.5 Identification of selected bacteria
Identification was done on selected bacteria with high nitrification ability. Bacteria identification was made by phenotypic and molecular methods. Phenotypic identification was conducted by morphological and biochemical examinations, which include Gram staining, and test on catalase, oxidase, oxidative or fermentative, Motility Indol Ornithine, and Triple sugar iron agar media.

Molecular bacterial identification was made based on the 16S rRNA and gyrB genes. The primer used for 16S rRNA genes analysis were 27F (5'-AGA GTT TGA TCA CTG GCT CAG- 3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') while the primer for gyrB genes were 3F (5'-TCC GGC GGT CTG CAC GGC GT-3') and 14R (5'-TTG TCC GGG TTG TAC TCG TC-3'). Amplification was organized for 30 cycles in which DNA template was denatured at 96°C for a minute, annealing primer at 55°C for a minute, and extension primer at 72 °C for 2 minutes for 16S rRNA primers. Meanwhile, the gyrB primer was amplified for 35 cycles in which DNA template was denatured at 94 °C for a minute, annealing primer at 55°C for a minute, and extension primer at 72 °C for 2 minutes according to[5][14]. DNA from PCR amplification was analyzed by 1% agarose gel electrophoresis for 15 minutes.

A DNA sequencing was conducted to determine the sequence of nucleotide bases (adenine, guanine, cytosine, dan thymine). First Base (PT. Genetika Sains Indonesia) service was involved in this stage. Sequencing results were in a form of a dendrogram containing information regarding the sequence of the potential nitrogenous bases of bacteria. The nitrogen base sequences were analyzed using BioEdit application and other applications named BLAST (Basic Local Alignment Search Tool) to find out the species similar sequence in Gene Bank at NCBI. The sequence results were the sequences of other species that have been registered in the Gene Bank in which the similarities with the other species can be detected. The gene sample sequences were merged with the reference sequences from the BLAST results and were traces using Clustal W Multiple Alignment to create a phylogenetic tree. The phylogenetic tree construction was made using software named Molecular Evolutionary Genetics Analysis (MEGA) version 10.0 also using the Neighbor-Joining method in which the bootstrap value is 1000 to determine the link of the sequence results to several bacterial strains in which the similarity value is above 97%.
3. Result and Discussion

3.1. Sampling
Samples were taken at 11:00 WIT (Western Indonesia Time) in a tilapia pond belongs to Mina Ngremboko fish farmer from 3 (three) spots (near the inlet, near the outlet, and at the center of the pond). The pond contained tilapia fry has been raised in the pond for 36 days. Water quality measurement results were 32.4 °C for water temperature, 15.5 ppm for dissolved oxygen, and 7.1 for pH parameters.

3.2. Purification, morphological observation, isolated storage
A total of 15 pure bacterial isolates were obtained, namely A1, A2, A3, A4, A5, B1, B2, B3, B4, B5, B6, B7, B8, B9, and B10. The color of those fifteen bacterial isolates was a milky white color, had a smooth surface, a circular shape, an entire edge, and a convex elevation. The most striking difference between them was the size, in which A1, A3, B1, B4, B7, and B10 were the smallest colony, while A2, A5, B2, B5, B6, and B8 having a medium size, and A4, B3, and B9 were the biggest colony. All of those pure bacterial isolates were preserved in TSB with 30% glycerol in a refrigerator at -40 °C and in TSB with 3% trehalose in a refrigerator at -80 °C.

3.3. Non-pathogenic test
Survive fish did not have disease signs. Accumulation of daily fish mortality and final fish survival rate is shown in Table 1.

| Isolate | Cumulative Mortality at Day Post Infection | Total Mortality (fish) | Total mortality (%) | Survival rate (%) |
|---------|------------------------------------------|------------------------|---------------------|-------------------|
| A1      | 4 4 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| A2      | 0 0 0 0 0 0 0 0 0 0 100                 |                        |                     |                   |
| A3      | 0 0 0 0 0 0 0 0 0 0 100                 |                        |                     |                   |
| A4      | 0 0 0 0 0 0 0 0 0 0 100                 |                        |                     |                   |
| A5      | 0 0 0 0 0 0 0 0 0 0 100                 |                        |                     |                   |
| B1      | 0 0 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| B2      | 0 0 1 1 2 3 4 4 4 80 20                |                        |                     |                   |
| B3      | 0 0 0 1 1 3 4 4 4 80 20                |                        |                     |                   |
| B4      | 0 0 2 2 3 4 4 4 4 80 20                |                        |                     |                   |
| B5      | 2 4 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| B6      | 1 3 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| B7      | 4 4 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| B8      | 3 4 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| B9      | 2 2 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| B10     | 0 2 4 4 4 4 4 4 4 80 20                |                        |                     |                   |
| C       | 0 0 0 0 0 0 0 0 0 0 100                 |                        |                     |                   |
3.4. Nitrification activity

![Figure 1. Nitrification process in test medium.](image)

The nitrifying ability of the bacteria is shown in Figure 1. Nitrification activity of the four strains increased to its peak on the sixth day. The highest accumulation of nitrate within the nitrification medium of each bacterium on the sixth day ranged from 17.26 to 21.54 ppm. The amount of nitrate within the nitrification medium decreased on the ninth day.

Isolate A2 and A3 showed the best nitrification activity which then was identified by observing the morphology cell, biochemical tests, and molecular identification.

3.5. Phenotypic characters of selected nitrifying bacteria

Cell morphology observation and biochemical tests on isolates A2 and A3 are presented in the following table.

| Characters    | A2 isolate | A3 isolate | Klebsiella<sup>6</sup><sup>[35]</sup> |
|---------------|------------|------------|--------------------------------------|
| Gram          | Negative   | Negative   | Negative                             |
| Shape         | Short rod-shaped | Short rod-shaped | Rod-shaped                           |
| Motility      | -          | -          | -                                    |
| Catalase      | +          | +          | +                                    |
| Oxidase       | -          | -          | -                                    |
| Decarboxylation| -         | -          | -                                    |
| Ornithine     | -          | -          | -                                    |
| Indole        | -          | -          | -                                    |
| OF            | Fermentative | Fermentative | Fermentative                       |
| TSIA          | A/A        | A/A        | A/A                                 |
| Acid from Glucose | +      | +          | +                                    |
| Acid from Sucrose | +      | +          | +                                    |
| Acid from Lactose | +       | +          | +                                    |
| Gas           | +          | +          | +                                    |
| H<sub>2</sub>S | -          | -          | -                                    |

3.6. Molecular identification

PCR product of A2 and A3 isolates based on the 16S rRNA and gyrB are shown in figure 2. The result of DNA visualization shows the PCR yield size is 1.500 bp on 16S rRNA and 1.100 bp on gyrB.
3.6.1. 16S rRNA gene phylogenetic tree

The results of DNA sequencing were read using the BLAST system. The reading result will show several species therefore it is important to know the degree of kinship through the phylogenetic tree. The result of the 16S rRNA gene sequencing is 1.500 bp. The BLAST result shows that the isolate has similarities above 99%. The isolate A2 is similar to *Klebsiella variicola* strain ABG7, *K. quasipneumoniae* subsp. similipneumoniae strain 2437, *Klebsiella* sp. strain VITRSJ2, dan *K. pneumoniae* strain IOB-L. Meanwhile, isolate A2 is similar to *Enterobacteriaceae bacterium* CK-2, *K. pneumoniae* strain IOB-L, *Klebsiella* sp. strain VITRSJ2, dan *K. quasipneumoniae* subsp. similipneumoniae strain. The phylogenetic tree was created based on BLAST results using the “MEGA X” software. The following figure is the result of the phylogenetic tree based on the BLAST result.
3.6.2. **Gryase B gene phylogenetic tree**
The result of Gyrase B gene sequencing is 1,100 bp in size. The result of the Basic Local Alignment Search Tool (BLAST) shows that each isolate has a similarity which is above 97%. Isolate A2 is similar to *K. quasipneumoniae* strain S15-2, *E. bacterium* S05, *Klebsiella* sp. LY, dan *K. pneumoniae* KAM260. It is found that isolate A3 had the similarity which is above 99% and it is similar to *K. quasipneumoniae* strain L22, *E. bacterium* S05, *Klebsiella* sp. LY, dan *K. pneumoniae* KAM260. A phylogenetic tree was created based on BLAST results using “MEGA X” software. The following figure is the result of the phylogenetic tree based on the BLAST result.
Figure 5. Phylogenetic tree based on gyrB gene sequencing using the Neighbor-Joining.

Throughout this study, the nitrifying bacteria had been isolated which generated 15 isolates that were obtained from tilapia pond sediment in which the quality of the water was in a good category; the temperature was 32.4 °C, pH 7, and DO 15.45 ppm. Within high DO concentration, nitrite formation occurs quickly. The final result of the nitrification process is the nitrite formation in which high DO will help to make the formation faster and it leads to a faster nitrification process[15].

One of the parameters that support the success of aquaculture is the condition of microbes in the water. Those microbes have a role in biochemical cycles in the water that is used in aquaculture. The abundance of the bacteria in fish farming media shows a good number of yields which is indicated along with the fish farming period. The predominant bacteria which grow in fish farming media are 11 genera which include Enterobacter, Bacillus, Streptococcus, Acinetobacter, Kuthria, Eubacterium, Pseudomonas, Corynobaeterium, Alcaligenes, Staphylococcus, and Listeria [16].

Nitrogen (N) is one of the important nutrients and it is widely found in soil either organic or inorganic soil[17]. Therefore, it is found many nitrifying bacteria in sediments. Bacterial sampling which is obtained from fish farming media should be taken from the sediment since it contains more bacteria than in the air.
column[18]. The sediments contain an abundant population of microorganisms[19]. Nitrification is a two-steps oxidation of ammonium to nitrate. The first step of the nitrification process is the oxidation of ammonium to produce nitrite, air, and hydrogen ions which then nitrite is oxidized to nitrate. In the other words, ammonium is oxidized to nitrate, water, and hydrogen ions. Nitrate is usually not toxic. The nitrification process is assisted by nitrifying bacteria. Nitrifying bacteria can oxidize ammonia to nitrate which, then, is converted to nitrate[20].

Nitrifying bacteria, in this research, has been tested for pathogenicity. Four isolates were confirmed to be non-pathogenic, while the other 11 isolates were pathogenic which caused the mortality of 75% of the fish samples. Those four non-pathogen bacteria had a high nitrifying activity which was around 17.26-21.54 ppm. Nitrate levels increased until the sixth day and it decreased until the ninth day. The increase in nitrate level indicated a nitrification process. Up until the sixth day, it indicated a proper bacterial growth characteristic even though the ammonium was the only source of nitrogen as the nutrient. This illustrated its metabolic flexibility in carrying out nitrification reactions[21]. This nitrification activity can effectively control the nitrogen compound pollution; hence it can be used to eliminate pollution in soil and water due to the accumulation of organic matter[23]. Nitrate on nitrification process which was produced will face a phase of increasing, stagnant and decreasing[24]. In this research, the nitrate decreased starting on the sixth day until the ninth day. The decrease was caused by the nutrient contained in the medium which was slowly run out and the beginning of the denitrification process (the use of nitrate by bacteria which was converted into N₂ gas). The flexibility of bacterial metabolism plays a role in the making of denitrification reactions. Furthermore, the two best isolates (A2 and A3) were identified through cell morphology observation, biochemical tests, and 16S rRNA gene followed by gyrB[22].

The two selected nitrifying bacteria, in this study, were identified as Klebsiella. The genus Klebsiella belongs to the family Enterobacter, order Enterobacteriales, class Gammaproteobacteria, phylum Proteobacteria, and kingdom Bacteria[23]. The result of 16S rRNA gene sequencing in both isolates had a similarity level above 99%. Those isolates had high similarity to several species within the same genus, Klebsiella sp. The similarity which is above 97% indicates a different genus while it is under 95% indicates a different genus[24]. In this research, all of the samples had the similarity which is above 99% and it indicates that it is from the same genus. A phylogenetic tree was created to determine the link between isolates and suspected bacteria. Isolate A2 had the tight link with K. varicola and K. quasipenmoniae while isolate A3 had the tight link with Klebsiella sp. The sequencing result of gen gyrB showed the same result. The gen gyrB result for all the isolates had a similarity which was above 97%. Those two isolates possessed high similarity to several species. A phylogenetic tree of the gyrB gene was created to determine the link between the two isolates. Gyrase B (gyrB) is the gene encoding the B subunit of DNA gyrase. The result of the gyrB gene illustrated that it matched the result of the 16S rRNA gene. In addition, the gyrB gene was able to show a higher capacity to differentiate between species. Therefore, the gyrB gene can be used as a useful target to identify bacterial species and strains[25]. Isolate A2 had the tight link with K. quasipneumoniae while isolate A3 had the tight link between E. bacterium and K. pneumoniae. It can be concluded that 16S rRNA and gyrB, isolates A2 and A3 have a tight link with Klebsiella sp. Up until this stage, the two types of Klebsiella are still at a level of suspicion therefore it can be stated that Klebsiella that is obtained from this study is Klebsiella spp.

The biochemical test illustrated that this bacterium was non-motile and it proved a positive response to the catalase test and it produced gas. Meanwhile, it showed a negative response to oxidase test, H₂S, ornithine decarboxylation, and indole. The TSIA test illustrated A/A or lactose, sucrose, glucose fermenter. In the O/F test, fermentative acid was obtained with a yellow color formed in both aerobic and anaerobic tubes. The characteristic of this biochemical test indicated that this bacterium belongs to Klebsiella bacteria[26][27]. Up until now, the biochemical test is unable to differentiate species K. pneumoniae, K. quasipneumoniae, and K. varicola, at the same time[28]. Mingyue et al. (2015) also argued that it is very
difficult to differentiate *K. pneumoniae*, *K. quasipneumoniae*, and *K. variicola* using biochemical and phenotypic tests. In addition, *K. pneumoniae* has a tight link to *K. quasipneumoniae*.[29] Klebsiella is a Gram-negative, non-porous, and non-motile bacterium. It is capable to ferment lactose and acid, it shows the negative results of oxidase, and it possesses thick polysaccharide capsules which extend out in which bacterial colonies become shiny and slimy. Klebsiella is rod-shaped in which the diameter is 0.3-1 mm and 0.6-6 mm. These bacteria are facultative anaerobes.[30] These bacteria can also produce hydroxylamine oxidase (HAO), periplasmic nitrate reductase (Nap), Nitrite reductase (NIR) which play an important role in heterotrophic nitrification and aerobic denitrification. Pal *et al.* (2014) added that *Klebsiella* sp. can perform heterotrophic nitrification and aerobic simultaneously. *Klebsiella* sp. is a bacterium that is capable to utilize NH$_3$ – N, NO$_2$ – N, and NO$_3$ – N as the sole source of nitrogen.[31]

*K. pneumoniae* is non-pathogenic when it lives in soil, water, and plants.[32] However, these bacteria can be categorized as opportunistic pathogens which means they are pathogenic when the host’s immune system is experiencing a decline and is influenced by unfavorable environmental factors.[33] The opportunistic pathogens are microorganisms that can cause disease in hosts experiencing atypical environment stressors or having impaired immune function.[34] Das *et al.* (2017) explained that tilapias that had *K. pneumoniae* intraperitoneal injection at a dose of 1.05 x 10$^6$ CFU/ml for each fish showed a mortality of 50%.[35] *K. pneumoniae* was found in goldfish with lesions. However, it was not caused by *K. pneumoniae*. It was caused by the infection that did not have good handling which made *K. pneumoniae* entered through the lesion from the fish and worsened the illness.[36] Previously, Dasklov *et al.* (1998) explained that rainbow trout fish intraperitoneal was injected with *K. pneumoniae* at a dose of 10$^4$ CFU/ml/for each fish. After 7 days, it is found that the fish mortality was caused by the previous infection from several pathogenic bacteria in which there were lesions in the fish fins.[35] In the present study, a dose of 5 x 10$^5$ CFU/ml/fish was injected for the healthy fish and it turned out to produce 100% survival in isolates A2, A3, A4, and A5. Similar results also found in Mahon *et al.* (2011) statements.[32] The previous study demonstrated that at least 10$^5$ CFU obtained an improved complement bactericidal activity.[37]

*Klebsiella* is a common genus to be isolated from the water, sediment, plants, animals (including the fish), and the human body. Klebsiella can produce capsules and slime layers.[37] Polysaccharide capsules lay surround the Klebsiella to protect itself from the phagocytic activity and it is considered the most important virulence factor of Klebsiella.[39] Those six species of Klebsiella (*K. granulomatis*, *K. michiganensis*, *K. oxytoca*, *K. pneumoniae*, *K. quasipneumoniae*, dan *K. variicola*) are opportunistic pathogens that are found in the digestive tract of fish hosts.[38] *K. pneumoniae* is closely linked to *K. quasipneumoniae*.[40] It is commonly found in sediments and aquatic environments. The virulence of Klebsiella is originating from the environment which is still unknown.[41]

The present study found a heterotrophic bacteria with strong nitrification activity. Although nitrification activity was originally known to be done by autotroph bacteria, recent finding demonstrated that heterotrophic bacteria able to remove ammonia faster than autotrophic bacteria. One reason is that heterotrophic bacteria regenerated faster by utilizing ammonia as an energy source to multiply cells. The decrease in ammonia level partly occurred due to the use of ammonia by the heterotrophic process of bacterial biosynthesis which produces bacterial biomass. Ammonia oxidizing bacteria oxidize ammonia with the assist of an integral membrane enzyme, ammonia monooxygenase, followed by periplasmic membrane enzymes and hydroxylamine oxidoreductase. Nitrite oxidizing bacteria carried nitrite oxidoreductase which oxidizes nitrite to nitrate.[31] Referring to the result of the present study that the dose of *Klebsiella* spp. 5 x 10$^5$ CFU/ml is safe to be applied to healthy fish, further research is needed to make Klebsiella probiotics with a bacterial density of 5 x 10$^5$ CFU/ml or less. A previous study demonstrated that *Klebsiella* spp. is opportunistic pathogen that infect the host when the host is experiencing a stress condition and is influenced by unfavorable environmental factors.[33] Accordingly, it is important to keep maintaining the fish immunity as well as protecting the environment and the fish would not be stressed[34].
Another scenario for application on the nitrifying bacteria from the present study is to apply the bacterial strain in wastewater treatment or filtration facility, not in aquaculture pond. Klebsiella grows well on ammonium, nitrite, and nitrate culture media as the sole source of nitrogen[42]. This indicates that Klebsiella potentially performs nitrification and denitrification simultaneously. *K. pneumoniae* was inoculated into wastewater with a concentration NH$_4^+$ at a dose of 27 mg/L. After 24 hours, the concentration of NH$_4^+$ did not change until 24 hours later. The incubation of NH$_4^+$ concentration decreased to 22 mg/L after 48 hours. The concentration of NH$_4^+$ was at 11 mg/L after 72 hours and it was completely removed within 120 hours. Wastewater is filtered using physical and biological filters. Physical filters can be done by filtering wastewater through some rocks, coir, sand, and others. After doing that filtration, it is filtered using the biological filtration method in which utilizes nitrifying bacteria such as *Klebsiella* sp. *Klebsiella* spp. has shown that it can decompose nitrogen in the waste disposal tank according to[31]. Then, the purified water is given UV light to kill Klebsiella in the water. Heterotrophic bacteria can oxidize N compounds. According to the research on several strains, it has shown that heterotrophic nitrifying bacteria work using a similar mechanism to NH$_3$-oxidizing bacteria. Klebsiella is a heterotrophic bacterium that can remove ammonia quickly. Klebsiella can utilize ammonia as an energy source to multiply cells[42].

It is interesting to consider which enzyme or which genes are involved in nitrification and denitrification activities by the bacteria in the present study. hence, further investigation is required. It is demonstrated by other studies that ammonia monoxygenase, hydroxylamine oxidoreductase, and nitrite oxidoreductase are important enzymes for nitrification[43]. Meanwhile, in the denitrification process, nitrate is reduced to nitrite by nitrate reductase which, then, is changed to nitric oxide by nitrite reductase and nitric oxide reductase can convert nitrate to nitrogen oxide which, then, is converted to N$_2$. A set of genes that are involved in the nitrification and denitrification processes takes ammonia monoxygenase, nitrite reductase, nitrate reductase, nitrite oxide reductase, and assimilatory nitrate reductase. The subunit-A ammonia monoxygenase gene played a role in the isolation and characterization of nitrifying bacteria, *Nitrosomonas marina*, *N. nitrosa*, and *Nitrosospira*[43]. Nitrification reaction is a sequential aerobic oxidation reaction from ammonium to nitrate involving hydroxylamine and nitrite as intermediates by ammonia monoxygenase, hydroxylamine oxidoreductase, and nitrite oxidoreductase. Likewise, in the denitrification process (the process of converting nitrate to nitrogen gas) nitrate is reduced to nitrite by nitrate reductase which is, then, converted to nitric oxide by nitrite reductase, nitric oxide reductase can convert nitrate to nitrogen oxide which is then converted into N$_2$ gas. A set of genes that are involved in the nitrification and denitrification processes takes ammonia monoxygenase, nitrite reductase, nitrate reductase, nitrite oxide reductase, and assimilatory nitrate reductase[43]. A variety of ammonia monoxygenase and nitrogen fixation gene was used to identify and the result found out that the dominant sequence is similar to *K. pneumoniae*[44]. Klebsiella is a heterotrophic bacterium that potentially performs nitrification and denitrification simultaneously. In that study, nir, nar, nor, and nos genes were verified by PCR to monitor nitrification and denitification activities involving Klebsiella. The result of that study study indicates the presence of nitrite reductase, respiration, hydroxylamine reductase, and nitric oxide reductase in which this gene sequence was conserved in the genus Klebsiella. The similarity of the species with *K. pneumoniae* is 99-100%[43].

4. Conclusion
4.1 Fifteen isolates of nitrifying bacteria were successfully collected; four among them were non-pathogenic and potential for aquaculture
4.2 The bacterial isolates have the highest nitrification activity on day six of incubation, with 17.26-21.54 ppm of nitrate products in a fermentor.
4.3 Phenotypic and molecular identification based on the 16S rRNA and gyrB genes of two selected isolates (A2 and A3 found the closest similarity to *Klebsiella* spp.
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