Isolates of nitrifying and denitrifying bacteria activities that derived from catfish, *Pangasius* sp. culture pond

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Abstract. Nitrifying and denitrifying bacteria are very fruitful for controlling water quality in fish culture because they have a role in converting toxic chemical compounds into non-toxic compounds. The purpose of this experiment is to determine the isolates of nitrifying and denitrifying bacteria in terms of oxidizing ammonia, accumulating total nitrite, and accumulating total nitrate activities. Eight isolates of bacteria consist of four nitrifying bacteria (NP1, NP2, NP3, and NP4), and four denitrifying bacteria (DP1, DP2, DP3, and DP4) were inoculated with the density of $10^2$ CFU/ml. The result showed that NP1, NP2, NP3, and NP4 isolates have almost similar ability to oxidize ammonium of 99.57, 99.37, 99.89, and 99.83%, respectively. The NP1-NP4 isolates on the total nitrite accumulated were 0.06, 0.05, 0.06, and 0.06%, while the total nitrate accumulated were 1.56, 1.65, 2.08, and 1.65%, respectively. The isolates of DP1, DP2, DP3, and DP4 can reduce total nitrate by 99.9, 100.0, 99.9, and 99.9%, respectively, while accumulated total nitrite was 0.01, 0.04, 0.00, and 0.00%. Isolates of NP2 and DP2 are recommended for controlling chemical compounds in the pond water.

Keywords: catfish; culture; denitrifying; nitrifying bacteria

1. Introduction
Catfish (*Pangasius* sp.) culture has a prospective to develop and increase the income, especially in Sumatera Island involves Jambi, Palembang, Riau, Lampung, and Borneo Island such as South and Central Borneo. Most of the culture technique is intensive. Intensive culture's negative impacts are the waste by-product from cultivation activity such as uneaten food, fesses, urine, and sludge, which affect the water quality and aquatic organisms, including fish [1]. The intensive culture waste contains rich nitrogen compounds, i.e., ammonia ($NH_3$), nitrite ($NO_2^-$), and nitrate ($NO_3^-$), which affect water quality, and several such as ammonia and nitrite are toxic for aquatic organisms. Inappropriate water quality due to high containing inorganic nitrogen compounds (i.e., ammonia and nitrite) that harmful to fish culture and has resulting inhibit growth even died; thus, pond productivity is low [2]. Therefore, the addition of nitrifying and denitrifying bacteria into the pond to degrade the nitrogen compounds are needed.

Water quality can be improved through bioremediation. Bioremediation is a contaminant degrading process via biological process using microorganisms of metabolism capability [3]. One of the bioremediation processes is the addition of nitrifying and denitrifying bacteria that play an essential role in nitrogen compound degradation. Nitrification is a process of ammonium oxidation to hydroxylamine, then continued by the oxidation process to nitrite and nitrate [4]. The denitrification is the final step of the nitrogen removal process through the biological process with nitrate as an acceptor electron [5].
This research aims to determine nitrifying and denitrifying bacteria isolates in ammonium and nitrite oxidation and nitrate reduction activities.

2. Method

Fourth isolates of nitrifying bacteria (NP1, NP2, NP3, NP4) and fourth isolates of denitrifying bacteria (DP1, DP2, DP3, DP4) were isolated and characterized [6]. These isolates were inoculated with the density of $10^2$ CFU/ml.

2.1. Total plate count (TPC) nitrifying and denitrifying bacteria.

Two loops of isolates were inoculated into 100 ml of nitrifying and denitrifying media. Incubation was conducted in a shaker incubator for ± 3 hours at 37ºC. The OD (optical density) was measured using a spectrophotometer at a wavelength of 600 nm. The expected OD of isolates in nitrification media was 0.2, and denitrification media was 0.035. The OD was obtained from several trials. If the OD has been reached, then dilution is carried out in the nitrifying and denitrifying media. The dilutions are 1:1 (3 ml of the isolate is inoculated on 3 ml of nitrifying and denitrifying media), 1:2, 1:4, 1:8, and 1:16. The OD was measured using a spectrophotometer. In addition, dilution was carried out in physiological NaCl solution for plating on nitrification and denitrification media. 1 ml of the isolate was added to 9 ml of physiological NaCl (dilution $10^1$), then diluted to $10^3$. A total of 0.1 ml was taken and put on the media for nitrification and denitrification with the total plate count method to see how many colonies were grown. The growing colonies were then counted (30-300 colonies) and multiplied by the dilution factor with CFU units = colony-forming units [6].

2.2. Cultivation of nitrifying and denitrifying bacterial isolates

Growth patterns of nitrifying bacteria isolates (NP1, NP2, NP3, NP4) and denitrifying bacteria (DP1, DP2, DP3, DP4). The bacterial isolates were grown in nitrifying and denitrifying liquid media at room temperature (28-31ºC) for two days as the test isolates' inoculant. A total of 1 ml of the culture was inoculated into 25 ml of nitrifying and denitrifying media, with 4.65 mM glucose concentration. Incubation was carried out at room temperature above a swaying incubator at a speed of 75 rpm for eight days. Then every two days, the optical density (OD) value was analyzed using a spectrophotometer. The OD value profile can show the growth pattern of nitrifying and denitrifying bacterial isolates. The results of the isolation are used as the basis for determining the time of observation in the selection test for the ability to use ammonia.

2.3. Activity of nitrifying bacteria

The concentrations of ammonium, nitrite, and nitrate in the filtrate were measured using a spectrophotometer. The percentage of oxidizing of ammonium concentration and the forming of nitrite and nitrate compounds were calculated as follows [7].

Percentage of oxidized ammonium:

$$[AO] = \frac{[AK-AP]}{[AK]} \times 100$$

Information:
AO = Percentage of oxidized ammonium
AK = Concentration of ammonium at control
AP = Concentration of ammonium at media that containing bacteria inoculum

Percentage of nitrate forming:

$$[PNA] = \frac{[NT-NK]}{[AK-API]} \times 100$$

Information:
PNA = Percentage of nitrate forming
NT = Concentration of nitrate at the treatment (with bacteria inoculum)
NK = Concentration of nitrate at the control (without bacteria inoculum)
AK = Concentration of ammonia at the control
AP = Concentration of ammonia at the treatment

Percentage of nitrite forming

\[
[PNI] = \frac{[\text{NIT}-\text{NI}]}{[\text{AK}-\text{AP}]} \times 100
\]  

Where:

PNI = Percentage of nitrite forming
NIT = Concentration of nitrite at the treatment (bacteria inoculum)
NI = Concentration of nitrite at control (without bacteria inoculum)
AK = Concentration of nitrite at ammonia (control)
AP = Concentration of ammonia at the treatment

2.4 Activity of denitrifying bacteria

The number of nitrate forming can be calculated using formula:

\[
\text{[NO}_3^- \text{]} \text{ reduction} = [\text{NO}_3^-] \text{ control} - [\text{NO}_3^-] \text{ inoculum}
\]

\[
\% \ [\text{NO}_3^- ] \text{ reduction} = \frac{[\text{NO}_3^-] \text{ reduction}}{[\text{NO}_3^-] \text{ control}} \times 100
\]

The number of nitrite forming can be calculated using formula:

\[
\text{[NO}_2^- ] \text{ formed} = [\text{NO}_2^-] \text{ treatment} - [\text{NO}_2^-] \text{ control}
\]

\[
\% \ [\text{NO}_2^- ] \text{ formed} = \frac{[\text{NO}_2^-] \text{ treatment} - [\text{NO}_2^-] \text{ control}}{[\text{NO}_2^-] \text{ reduction}} \times 100
\]

Gas (end product) can be calculated using formula:

\[
[\text{Gas}] = [\text{NO}_3^-] \text{ reduction} - [\text{NO}_2^-] \text{ formed} - [\text{NH}_3] \text{ formed}
\]

\[
\% \ [\text{GAS}] = \frac{[\text{Gas}]}{[\text{NO}_3^-] \text{ reduction}} \times 100
\]

3. Result and discussion

3.1 Result

The activity of bacteria isolates on ammonium oxidized and nitrite and nitrate accumulated is presented on table 1. Four isolates of nitrifying bacteria (NP1, NP2, NP3, and NP4) have the capability in oxidizing ammonium and accumulating nitrite and nitrate. Table 1 shows that all of the isolates are the same in oxidizing ammonium, which was 99% (about 15 µM/hour). The nitrite accumulation at isolate NP3 and NP4 were the highest (1.21 µM), while the lowest was found at NP2 (0.88 µM). The nitrate accumulation at NP3 (38.8 µM and 2.08%) was the highest, where the lowest was found at NP1 (29.1 µM and 1.56%). The activity of denitrifying bacteria isolates in reducing nitrate and accumulating nitrite is presented in Table 2. Isolates (DP1, DP2, DP3, and DP4) of denitrifying bacteria (table 2) showed that DP2 was the highest on total reducing nitrate (58804.03 µM and 100%), then followed by DP1 (58793.43 µM and 99.9%), DP4 (58792.13 µM and 99.9%), and DP3 (58787.63µM and 99.9%). The total nitrate accumulation was highest at DP2 (58804.03 µM and 100%), then followed by DP1 (58793.43 µM and 99.9%), DP4 (58792.13 µM and 99.9%), and DP3 (58787.63 µM and 99.9%).
accumulation at DP2 (21.57 µM and 0.04%) was the highest, then followed by DP1 (5.78 µM and 0.01%), DP3 (1.63 µM and 0.00%), and DP4 (1.46 µM and 0.00%).

Table 1. Activity of nitrifying isolate bacteria on ammonium oxidation and accumulation of nitrite and nitrate.

| No | Isolate code | Ammonium oxidizing activity | Nitrite accumulation | Nitrate accumulation |
|----|--------------|----------------------------|---------------------|---------------------|
|    |              | Total oxidized ammonium    | Total oxidized      | Total nitrite        | Total nitrate          |
|    |              | (µM)                      | oxidizing activity  | accumulation (µM)   | accumulation (µM)      |
|    |              | (%)                       | (µM/hour)           | (%)                 | (%)                  |
| 1  | NP1          | 1861.12                   | 99.57               | 15.51               | 1.08                 | 0.06                 | 29.1                | 1.56               |
| 2  | NP2          | 1857.46                   | 99.37               | 15.48               | 0.88                 | 0.05                 | 30.6                | 1.65               |
| 3  | NP3          | 1867.16                   | 99.89               | 15.56               | 1.21                 | 0.06                 | 38.8                | 2.08               |
| 4  | NP4          | 1865.91                   | 99.83               | 15.55               | 1.21                 | 0.06                 | 30.7                | 1.65               |

Table 2. Activity of denitrifying bacteria isolates on nitrate reduction and nitrite accumulation.

| No | Isolate code | Nitrate reduction activity | Nitrite accumulation |
|----|--------------|----------------------------|---------------------|
|    |              | Total reduced nitrate      | Total accumulated   |
|    |              | (µM)                      | nitrite (µM)        |
|    |              | (%)                       | (%)                 |
|    |              | Nitrate reduction          | Nitrite             |
|    |              | activity (µM/hour)         | accumulation (%)    |
| 1  | DP1          | 58793.43                   | 99.90               | 489.95              | 5.78                 | 0.01               |
| 2  | DP2          | 58804.03                   | 100.00              | 490.03              | 21.57               | 0.04               |
| 3  | DP3          | 58787.63                   | 99.90               | 489.90              | 1.63                 | 0.00               |
| 4  | DP4          | 58792.13                   | 99.90               | 489.93              | 1.46                 | 0.00               |

3.2. Discussion

As an autotroph, nitrifying bacteria can utilize carbon dioxide as an organic carbon source [8]. On the other hand, autotroph nitrifying bacteria's energy can be derived from ammonium or nitrite oxidation, which has relatively slow growth compared to heterotroph nitrifying bacteria [9]. The autotroph nitrifying bacteria are slow in growth because the propagation time needs 7-8 hours, while in situ conditions, the propagation occurs 26 hours [8]. Moreover, Nitrosomonas sp. is nitrification process will be inhibited by light for 10 minutes, but it can be solved with dark conditions for 3-4 hours. Light exposure affects nitrifying bacteria in the stationary phase, and bacteria can grow in media containing ammonium.

In the anoxic condition, heterotroph bacteria can use nitrate as a terminal electron acceptor with the existence of carbon and energy sources [10]. The capability of isolates itself may cause the fluctuation of nitrate reduction at the present experiment. It might be influenced by the contribution of enzymes in the denitrification process, such as nitrate reductase (NAR), nitrite reductase (NIRs and NIRk), periplasmic nitrate reductase (NAP), nitric oxide reductase (NOR), and nitrous oxide reductase (NOS). The present experiment shows that the DP2 isolate has the highest nitrate reduction, then following DP1, DP3, and DP4 in anaerobic condition. This condition suggests that the NAR enzyme's existence that active in the anaerobic condition is better than that of in aerobic condition in terms of NAP enzyme expression [11]. The differences among isolates on nitrate reduction can be influenced by the environment or metabolism activity. The number of nitrate reductions is also influenced by electrons on available carbon compound oxidation in denitrification media. Electron produced by oxidation carbon compound from NADH and FADH2 molecules plays a role as electron donor on chain respiration. Denitrifying bacteria use nitrate as the final electron acceptor in anaerobic conditions [12].

The addition of nitrifying bacteria increases nitrite and nitrate oxidation capability in pond water, but too high nitrification process may cause imbalance; thus, the addition of denitrifying bacteria is needed.
to reduce nitrate to nitrogen gas (N\textsubscript{2}) and reduce nitrite concentration in the water [13]. Nitrification consisted of two steps that involve two groups of bacteria. The first group is bacteria that oxidize NH\textsubscript{4}\textsuperscript{+} to NO\textsubscript{2} where the second group is to oxidize NO\textsubscript{2} to NO\textsubscript{3} [14]. Therefore, to keep water quality in the pond culture in stable condition, the addition of nitrifying and denitrifying bacteria should be balanced. The number of bacteria and the timing are also important.

4. Conclusions

The capability of isolates in terms of reduction, accumulation, and oxidation of chemical compounds such as ammonium, nitrite, and nitrate is influenced by isolates themselves, enzyme activity, and environmental conditions such as aerobic or anaerobic condition. According to the capability of reduction, accumulation, and oxidation, NP2 and DP2 isolates can be recommended as probiotics.

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