ADVANCING BIOTECHNOLOGY IN THE 21st CENTURY
ENSURING SUSTAINABILITY AND SAFETY IN THE PURSUIT OF BIOTECHNOLOGY'S ECONOMIC BENEFITS
IBC 2001 Proceedings of The 2nd Indonesian Biotechnology Conference

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Using the *Nitrosomonas* and *Nitrobacter* bacteria to neutralize ammonia and nitrite in brackishwater

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1Chemical Engineering Department, Indonesian Institute of Technology
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**Abstract**

The presence of ammonia and nitrite decreases the resistance of organisms in brackishwater, making it necessary to neutralize them via nitrification process. In this study the nitrification process is done using bacteria from the group *Nitrosomonas* and *Nitrobacter* with a pH variation of 7-8.5 and DO variation of 4-7. After the nitrification process was done on a mud sample from a wudu shrimp farm pond, identification with the reseller, ironrod and diphenylamine reagent indicated the absence of ammonia and nitrite. This verifies the ability of the *Nitrosomonas* and *Nitrobacter* bacteria neutralize ammonia and nitrite. The nitrification process is optimal at a pH level of 8.5 and DO level of 7.

**INTRODUCTION**

Nitrogen is one of element that very essential inside the organisms growth because shaping the main element into the construction of protein. Nitrogen is in the waters able to have form as nitrogen gas (N₂), ammonia (NH₃), dissolved or ammonium nitrogen (NH₄⁺), nitrite (NO₂⁻), and nitrate (NO₃⁻) compound form.

In the waters that not polluted the ammonia is found in a sum of small relative. The consist of ammonia in the waters that not polluted usually no more 1.5 ppm (Environmental Health Criteria Ammonia, 1986).

The ammonia nitrogen in the waters exists as un-ionized ammonia (NH₃) and ammonium ion (NH₄⁺) and hydroxide ion in a temperature and pH dependent equilibrium as:

\[
\text{NH}_3 + \text{H}_2\text{O} \leftrightarrow \text{NH}_4^+ + \text{OH}^-
\]

Un-ionized ammonia is toxic to aquatic animals and its toxicity increases with increase pH and temperature (Alabaster and Halleviel, 1986).

For dealing with ammonia, waste is in the waters, an the introductory research was carried out about the method for biological removal of ammonia by varying the dissolved oxygen concentration and pH to get the optimum nitrification condition in the brackish waters.

The objective of this research is to be able to use ammonia-degrading bacteria that was isolated from shrimp farm pond mud. Nitrification Microbiology of Nitrification Nitrification is the conversion of ammonium to nitrate by microbial action. This process is carried out by two categories of microorganisms (Bitton, 1994):

1. Conversion of NH₄⁺ to NO₂⁻.
   - *Nitrosomonas* (e.g., *N. europaeae*, *N. oleocarbogenes*) oxidizes ammonium to nitrite via hydroxylamine (NH₂OH). Other ammonium oxidizers indicate in the table 1.
   - \[
   \text{NH}_4^+ + 0.5 \text{O}_2 \rightarrow \text{NH}_2\text{OH} + \text{H}^+
   \]
   - \[
   \text{NH}_2\text{OH} + \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}^+ + \text{H}_2\text{O} + \text{energy}
   \]
   - \[
   \text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{NO}_3^- + 2\text{H}^+ + \text{H}_2\text{O} + \text{energy}
   \]

2. Conversion of NO₂⁻ to NO₃⁻.
   - *Nitrobacter* (e.g., *N. agilis*, N. winogradskyi) converts nitrite to nitrate. NO₂⁻ + 0.5O₂ → NO₃⁻ + energy. Other nitrite oxidizers indicated in table 1. The oxidation of NH₄⁺ to NO₂⁻ and then to NO₃⁻.
NO$_3^-$ is an energy yielding process. Microorganisms utilize the generated energy to assimilate CO$_2$. Carbon requirements for nitrifiers are satisfied by carbon dioxide, bicarbonate, or carbonate. Nitrification is favored by the presence of oxygen and sufficient alkalinity to neutralize the hydrogen ions produced in the oxidation process.

Table 1. The different nitrifying bacteria (Mukkerjee, 1991)

<table>
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<tr>
<th>Genus</th>
<th>Converts</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosomonas</td>
<td>Ammonia to nitrite</td>
<td>Soil, freshwater, marine</td>
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<td>Nitrosospira</td>
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</tr>
<tr>
<td>Nitrosococcus</td>
<td>Nitrite to nitrate</td>
<td>Marine</td>
</tr>
</tbody>
</table>

Although autotrophic nitrifiers are predominant in nature, nitrification may be carried out by heterotrophic bacteria (e.g., Arthrobacter) and fungi (e.g., Aspergillus). These microorganisms utilize organic carbon sources and oxidize ammonium to nitrite. However, heterotrophic nitrification is much slower than autotrophic nitrification and, probably, does not have any significant contribution.

The factors which influence nitrification

In accordance with Barnes and Bliss (1986) some factors which influence the nitrification growth rate are:

**Temperature**

Nitrification growth rate can occur in the temperature area of 8 – 30°C. The reported optimum temperature about 30°C. A variety of kind of nitrification bacteria can still grow up the temperature around 40.0 – 50.0°C.

**Dissolved Oxygen**

Oxygen is utilized in the oxidation reaction carried out by nitrifying bacteria. The stoichiometric quantities of oxygen required are: 3.43 mg for nitrification of 1 mg NH$_4^+$. N and 1.14 mg for nitrification of 1 mg NO$_2^-$. N. By contrast with effect of high concentrations of their nitrogen substrates, the nitrifiers do not exhibit any inhibition at high DO concentration.

**pH**

The hydrogen ion concentration is the essential factor inside optimum pH nitrification for Nitrosomonas and Nitrobacter. The pH between of 7.5 and 8.5 ppm. The nitrification stoped in pH 6 or under 6. For avoiding the decreasing of consequence in the nitrification process often carried out the addition of Ca CO$_3$ along with aeration for releasing of CO$_2$ so that the pollution defended in alkali condition.

**Substrate Concentration**

The Nitrosomonas and Nitrobacter of sensitive toward substrate concentration highly. A review of many other studied has shown that Nitrosomonas is not inhibited by NH$_4^+$.N nitrogen concentration up to 100 mg/L. The Nitrobacter inside the pure culture of 8 – 16 mg/L N – NH$_3$ concentration, only a few of the decreasing of the rate of nitrification growth. The nitrite can hamper the rate of nitrification in the lowest concentration of 10 mg/L the nitrite is also obstructed by the free nitrite source and ammonia.

**Toxic and Inhibitory Substances.**

Nitrification is subject to inhibition by a wide variety of organic and inorganic chemicals. In general inhibitory substances have the large characteristic of toxic toward Nitrosomonas than Nitrobacter. Organic matter in wastewater is not directly toxic to
nitrifiers. Apparent inhibition by organic matter may be indirect and may be due to O₂ depletion by heterotrophes (Barres and Bliss, 1988). The most toxic compound to nitrifiers are cyanide, thiourea, phenol, aniline, and heavy metal (Silver, Mercury, Nickel, Chromium, Zinc and Copper).

**METHODOLOGY**

**Materials**

Microbe acclimatization media, (NH₄)₂SO₄, K₂HPO₄, NaHCO₃, Na₂CO₃, MgSO₄, FeSO₄, CaCl₂ and trace element. Medium nutrient, reagent Nessler, Trommsdorff, seawater. Sodium salislic, NH₄Cl, trisodium citric, 2-sodium nitropside, NaOH, dichloro isonicopic acid, sulfanilamide, NaNO₃, H₃PO₄, N(1-aphil ethilenediamine dihydrochloride), KNO₃, Cu-Cd, EDTA, NH₄OH, Alcohol.

**Isolation**

Material mud which is expected to contain *Nitrosomonas* group bacteria and *Nitrobacter* taken from shrimp dam in Tanjung Pasir Tangerang. Mud is entered into acclimatization media inside composition with ammonium nitrogen concentration 20 mg/L (Original from (NH₄)₂SO₄ and aeration for one month. During the time acclimatization is carried out substitusion the acclimatization media as many as two times. After acclimatization, carried out liquid interval toward bacteria culture become concentration by using reaction retort. Using aseptic technique, take one ml bacteria has been liquidated and enter to petri plate. Further adding media so that tryptone glucose yeast (PCA) which has been sterilization to the each of petri plate. After be cold in incubation to room temperature for 24 – 48 hours. The purification is carried out with method to scratch volumes from petri plate which will be isolation in plaque, further to be incubation more for 24 – 48 hours. The colony which has separated to be preserved in media so that sideways consist of ammonia. Media so that sideways to be subststituted every two weeks a times.

**Identification**

Microbe inclusion inside test tube ammonium sulfate broth and nitrite broth. Give label to suite. Shake the bottles during three minutes. The bottles incubation to room temperatur for seven days. After seven days, to one drop of the sulfuric acid and three drop reagent trommsdorff to petri plate, addition one drop the culture medium from ammonium sulfat, so mix to average. Blue – black color indicate any nitrite, if the nothing of colour alteration means the nothing of formation nitrite. Ammonia exam inside ammonium sulfat broth with sessler reagent exam to 3.5 days and 7. Take 1 mL culture and put on petri plate, further adding some drop of the nessler reagent while to be milked to average. If the nothing of colour change means the nothing ammonia and any nitrite. The alteration of colour which occurs to be able to be researched qualitatively. Pale yellow = a few of ammonia. The old yellow = middle Brown. Precipitate = a lot of ammonia. Exam nitrite residue inside nitrite broth carried out such as to the upper stage. If the nothing of blue – black colour, is carried out exam for nitrite namely addition one drop diphenilamine, two drop H₂SO₄ and one culture inside nitrite broth into petri plate. Blue – black colour shows any nitrate.

**The Cultivation Bacteria**

The isolation result bacteria will be used in the following experiment cultivate inside acclimation media with nitrogen amanium concentration about 10 mg/L, pH = 8, and DO = 7 mg/L. This cultivation can be used three days after the time of cultivation. The cultivation can be carried out the review every time due to use the bacteria.

**Dissolved Oxygen Variation**

The inside 4 a beakerglass of measuring 2L filled with acclimatization media each of 500 ml by ammonium nitrogen media about 10 mg/L, the pH managed to 8.5 with adding liquid HCL. The air current speed is
managed so that got the dissolved oxygen concentration (DO) to every beaker glass namely 4 mg/L, 5 mg/L, 6 mg/L and 7 mg/L. The Ammonia concentration, nitrite, and nitrate measured, further one ml of bacteria from the cultivation added into each of the beaker glass. The measurement of ammonia, nitrite, and nitrate are carried out every day for six days. Procedures used for such determination of ammonium, nitrite, and nitrate are indophenol blue for ammonium, based on reaction with sulfanilamide coupled to N(1-naphthyl) ethylene diamine for nitrite, and nitrate using cadmium reduction method.

**pH Variation**

Acclimatiation media has been suited the pH become 7; 7.5; 8; and 8.5 placed to inside the beaker glass each 500 mL. The Air current speed is managed so that dissolved oxygen concentration to every 7 mg/L beaker glass. The measurement of parameter, ammonia, nitrite, and nitrate carried out like in DO variation experiment.

**RESULT AND DISCUSSION**

Isolation and Identification

The usage of mire from shrimp pond location as sample assumed contain bacteria the group of *Nitrosomonas* and *Nitrobacter* based on the composition of chemistry. The accumulating mire on basing shrimp pond a great of original from weft rests much consist of protein and shrimp metabolism rest so possibly construct ammonia as a result of weft rest degradation in condition anaerob like the reaction indicated such as:

$$\text{HS-CH}_2\text{-CH(NH}_3\text{)-CO}_2\text{H} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{-CO}_2\text{H} + \text{H}_2\text{S} + \text{NH}_3$$

Ammonia constructed can be possibly occur nitrogen cycle participating in nitrification process, however a quite slowly process. In basis of this sample took from in to shrimp pond deeply location.

Acclimatiation is carried out for about one month by special tools for the group bacteria of *Nitrosomonas* and *Nitrobacter* with hoping other bacteria come across in the sample will be selection naturally. It possibly occurs for instrument composition used to consist of the inorganic materials that hoped the survive of life is autotrop bacteria. Bacteria autotrop is bacteria, which uses resource of carbon origin from CO₂ or inorganic carbon and nitrogen resource that come from ammonia or ammonium.

(NH₄⁺ or NO₃⁻) + CO₂ + Energy → Protein Cell (N-Organic)

Group bacteria *Nitrosomonas* and *Nitrobacter* include in to autotrop Bacteria.

For knowing the conversion in enzymatic from ammonia to be nitrite, nitrite become nitrate by microorganisms, so done qualitative exam chemistry. A result of identification resumed in Tabel 2.

A result of qualitative exam shows the decreasing of ammonia concentration, that can be seen in the change of colour of ammonium sulfate broth that the most colourless, after the addition of trommsdorf reagent.

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<td>Nitrite test</td>
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Exam toward nitrite broth without bacteria. The identification constructs it blue-black colour after the addition of trommidroph reagent, but the colour construct more transparency than nitrite broth exam without bacteria. The identification by diphenilamine show nitrite there, marked with constructing blue-black colour. (Tabel.3)

Table 3. The identification of enzimatic reaction by *Nitrosomonas* and *Nitrobacter* toward nitrite broth

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<td>Trommsdorff</td>
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<tr>
<td>Nitrate test</td>
<td>Blue-black</td>
<td>Diphenilamine</td>
</tr>
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Based on the observation, isolation result showed there is the group of *Nitrosomonas* and *Nitrobacter* to determine the condition of nitrification optimum.

Some factor can influence nitrification process are oxygen dissolved, pH, substrate concentration and any toxic composed. In this research is done the determining of optimum nitrification condition toward group *Nitrosomonas* and *Nitrobacter* bacteria result the isolation in brackishwater.

**The variation of oxygen dissolved**

The oxygen dissolved which is choise in the reseach to be in turning 4 - 7 ppm. The selective of turning is based on the requirement limit of water quality for brackishwater. The standard of nitrification that observed is ammonia concentration, nitrite, and nitrate.

![Ammonia concentration diagram](image)

**Figure 1. Change in ammonium concentration at pH 8 and Dissolved oxygen (DO) variation**

From the Figure 1 can be seen that when the dissolved oxygen increases, the rate of ammonium reduction is increased. It is concluded that this occurs because with the increase in dissolve oxygen concentration, the opportunity for the following reaction to occur is also increased:

\[
2 \text{NH}_3 + 3 \text{O}_2 \rightarrow 2 \text{NO}_2^- + 4 \text{H}^+ + 2 \text{H}_2\text{O} + \text{Energy}
\]

Beside the nitrification process, the reduction of ammonia in the solution coulds also becawse by aeration process.

Change of nitrite because the nitrification process occurring in dissolved oxygen concentration to be the variation shown on Figure 2.
As DO increases nitrite that is formed increase more quickly and after reaching maximum nitrite the decreases. This show the role of the *Nitrobacter* bacteria in degrading nitrite to nitrate. The accumulating nitrite can be caused acclimation *Nitrobacter* bacteria the longer than Nitrosomonas bacteria. So that if nitrite constructed recently, *Nitrobacter* bacteria not show activity yet. In the environment of Nitrosomonas nitrification also consist of a sum of the greater than *Nitrobacter*.

In general it can be seen that the change of nitrite to nitrate is faster when he nitrite concentration is at its maximum. This is because at this condition enough nitrite is available so that growth of *Nitrobacter* bacteria is faster. In dissolved oxygen concentration 4 ppm, increasing nitrate concentration most slowly. It can be caused the growth limit of *Nitrobacter* bacteria to occur in dissolved oxygen concentration under 4 ppm.

**pH Variation**

pH which is used in this research called 7 - 8.5 because this condition denote general rotation occur in shrimp pond. It is a range with nitrification optimum condition occurring in pH 7.5 - 8.5.
We observe that the role of pH is quite big in the process of ammonia degradation (Figure 4). When nitrification occurs, release of $H^+$ will occur:

$$2 \text{NH}_4^+ + 3 \text{O}_2 \rightarrow 2 \text{NO}_2^- + 4 \text{H}^+ + 2 \text{H}_2\text{O}$$

Whereas nitrification process can be occur well in the base condition. Thus a buffer condition is needed to neutralize the acidity. The observation done with a pH 7 show that the change of ammonia into nitrite occurs very slowly. This can be seen from ammonia concentration that remain on day 6. A too high pH condition can also decrease the nitrification effectiveness because this can cause the portion of ammonia not ionized to be increased and this condition can inhibition with the nitrifying bacteria. In this work the ammonia concentration used is not enough to result in unionized ammonia that can inhibition with nitrification. Unionize ammonia concentration that on inhibition with nitrisfection is 10 – 150 ppm.

With higher pH the maximum point an accumulation of nitrite concentration occurs faster. This condition indicates the difference in the rate ammonia change to nitrate.
Figure 6. Change in nitrate concentration at DO 7 and pH varied.

Figure 6, shows accumulation nitrate is lowest at pH 7. This occurs because the reaction of nitrite become nitrate can be inhibition in acid condition. Reaction: \( 2 \text{NH}_3 + 3\text{O}_2 \rightarrow 2\text{NO}_3^- + 4\text{H}^+ + 2\text{H}_2\text{O} + \text{energy} \), is accompanied by the release of \( \text{H}^+ \), so that the waters with a pH that relatively low ( pH ) will tend to become more acidic when nitrification occurs. In a relatively acidic condition the reaction: \( \text{HNO}_2 + \text{NO}_3^- + \text{H}^+ \) with move to left and nitrite will tend to change to \( \text{HNO}_3 \) so that the reaction: 

\[ 2\text{NO}_3^- + \text{O}_2 \rightarrow 2\text{NO}_3^- \text{ will be held up.} \]

CONCLUSION

The base on research done, the following conclusion can be made: Bacteria degrading ammonia can be isolated with a simple method using acclimatization media. This is for the bacteria *Nitrosomonas* and *Nitrirbacter*. The higher concentration of dissolve oxygen, the fastenitrification process occurs and the optimum condition occurs at DO 7. The nitrification process occurs faster in a base condition and optimal nitrification occurs at a pH of 8.5.

REFERENCES


