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ISOLATION OF INDIGENOUS DENITRIFYING BACTERIA FROM THE ODOROUS REGION OF AN LAYER FARM AND ITS POTENTIAL AS NITRATE REDUCING AGENT

Agus Fitriyanto Nanung¹, Amalia Ditasari¹, Erwanto Yuny¹, Pertiwiningrum Ambar¹, Triatmojo Suharjono¹, Bachruddin Zaenal¹, Wihandooyo¹, Tomoyuki Nakagawa², Takashi Hayakawa²

¹Faculty of Animal Science Gadjah Mada University Indonesia, ²Faculty of Applied Biological Sciences Gifu University Japan

INTRODUCTION

Poultry production within the agricultural sector represents the high source of CH₄, NH₃, and CO₂ emissions and possibly have a huge potential for greenhouse gas (GHG) mitigation. Unpleasant odor is generally produced by the animal from the poultry farm industry, and may cause environmental problems such as ammonia emission in high concentration which can decrease the productivity of poultry and endanger of person health who is living in the farm or people community who is living nearby the poultry industries. Alteration of volatile ammonia into non-volatile form is a general biological treatment process including of nitrification and denitrification processes. Some technical and management for mitigating of NH₃, CH₄ and CO₂ emissions from poultry operating systems have been suggested in the previous investigation (Borhan et al., 2011, Fredeen et al., 2013). Volatilization of ammonia is a critical issue due to it represents a loss of fertilizer value, and it can significantly impact the environment, becomes one of the pathways for N loss from poultry operations. Ammonia could be deposited from the atmosphere and may be beneficial to plants nutrient sources for growth, but when excess N is stored in N-sensitive ecosystems, this may impact adverse effect (Borhan et al., 2011).

High ammonia concentration may cause many environmental emission problems because of their odor, toxicity and contribution to acid rains (Zeng et al., 2012). Potential consequences regarding with high concentrations of both oxidized and reduced forms of N in the environment include: (1) vegetation or ecosystem changes due to higher concentrations of N (2) respiratory diseases caused by exposure to high ammonia (3) decreasing of water quality and eutrophication of surface water bodies resulting in harmful algal blooms (4) nitrate contamination of drinking water (5) soil acidification through nitrification and leaching (6) N saturation of forest soils and (7) climatic changes associated with increases in nitrous oxide (N₂O) (Ndegwa et al., 2008).

Several specific potential control strategies for NH₃ mitigation from poultry production facilities are include changing animal feed, renovating or redesigning barns and other facilities, cleaning the exhaust air, aerobic composting for treating manure as raw material, and improvement of fertilizer application to agriculture farming field. Among these treatments, biological additives using microorganisms has attracted big attention from people for the ease of utilization, faster action, and lower cost expenses (McCrory and Hobbs, 2001, Satoh et al., 2004).

This ammonia removing treatment is based on the transformation of volatile N to non-volatile N comprising of nitrification and denitrification systems by nitrifying and denitrifying bacteria.

An important process involved in the Nitrogen cycle stated as Nitrification. In many ecosystems, ammonia-oxidizing bacteria (AOB) and archaea (AOA) have the ability to oxidize NH₃ to nitrite (NO₂). NO₂ is further oxidized to nitrate (NO₃) by nitrite oxidizing bacteria (NOB). Recently, the major grouping of AOB belongs to the subclass Beta-proteobacteria has been isolated and observed to be involved in nitrification system such as Pseudomonas spp. (Zhang Jibing et al., 2011), Alcaligenes faecalis (Joo et al., 2005), Bacillus methylotrophicus (Zhang Qing-Ling et al., 2012), Arthrobacter spp. (Verstraete and Alexander, 1972), and another group of bacteria such as Nitrosomonas spp., Nitrosococcus spp., Nitrosospira spp., Nitrosovibrio spp., Nitrosolobus spp. (Spieck et al., 2005). Besides bacteria and archaea, fungi are another kind of decomposer for which the role and development are not clear in the nitrification- denitrification system occurs in the composting process, the conventional biological to treat animal feces. It is believed that the composting self-heating pile may reach a temperature too extreme for their survival. They would thus be eliminated during the thermophilic stage and recovered when the temperature decreases (Zeng et al., 2012).

The objective of this paper is to isolate and to observe the capability of a microorganism reduce nitrate, as well as the development of nitrifying-denitrifying microorganisms, to reduce unpleasant odor from poultry farming activities.
MATERIALS AND METHODS

Media and Culture
The stock meat extract medium which consists of 1 g meat extract, 1 g microbiological peptone, and 0.5 g NaCl was made by diluted with 70 ml distilled water in beaker glass. The pH adjustment was into 7.2 with NaOH or H2SO4, and final volume was adjusted to 100 ml. The Stock of meat extract medium was always preserved in -20℃. The Stock of 5% NaNO3 was made by diluted of 5 g NaNO3 in 100 ml ionic water and maintained in 4℃. Furthermore, for screening ammonium-responsive microorganisms a 1/100 of stock meat extract medium with 1.5% agar added by 5% NaNO3. Cultures were performed for 7 d at 30℃. The liquid culture was carried out using 100 ml of 1/100 nutrient broth with 500 mg l⁻¹ NaNO3 in 250 ml-Erlenmeyer flasks and cells were grown aerobically at about 30℃ with a reciprocal shaker (120 rpm). Bacterial growth was monitored at Optical Density (OD) 600.

Screening of ammonium-responsive microorganisms
Indigenous isolates initially obtained from ammonia high emitted area of the poultry farm around Daerah Istimewa Yogyakarta. Nitrification-denitrification was suggested occur in this place. One gram samples of soil collected from various spots were suspended in 9 ml sterile distilled water and diluted appropriately. A portion of the cell suspension was spread on a 1/100 nutrient agar plate with 5% NaNO3. Colonies appearing on the plate were picked and purified. Each purified colony was inoculated on an agar plate with and without 5% NaNO3. Microorganisms displaying a peculiar growth on the agar with NaNO3 were selected as ammonium-responsive microorganisms. Isolates were then purified by plating on 1/100 nutrient broth (0.01% yeast extract, 0.01% polypeptone, and 0.005% NaCl, pH 7.2) supplemented with 5% NaNO3 and incubated at 30℃ for 48 h in aerobic condition.

Assessment of growth and ammonium reduction
To investigate the effect of NaNO3 addition on growth and the ability of the strain in reduce nitrate shaking culture experiments were conducted. A portion of 100 ml 1/100 meat extract medium was made in 250 ml Erlenmeyer shaking flask, containing 5% NaNO3. Periodically, 1 ml culture was prepared for measurement of cell density by spectrophotometer OD600, and another 1 ml culture was taken for nitrate analysis. NO3 was analyzed by Brusin method.

RESULTS AND DISCUSSION

Results of isolation on nitrate reducing bacteria
As environmental conditions for the living of strain microorganisms in soil are usually nutritionally low, a portion of 1/100 nutrient agar was used for screening nitrate stressor-resistant strains. The selected media was added with 5% NaNO3 used for screening "high nitrate stressor resistance-strain." This medium will screen the isolate which has not ability of a counter system with the stressor of NaNO3 will not grow in this selected medium. We have isolated a soil bacterium, which located at the high odorous region of the poultry farming area. This nitrate resistant strain were screened at four different places from 2 poultry farms at around Yogyakarta City. One spot was chosen at the poultry farm of Surya Indah Maguwoharjo, and another spot was at Bangun Desa Kaliurang at Yogyakarta.

The strain which showed good growth in solid and liquid culture was submitted to this present study. The pictures of colony morphology of selected strain were described in Fig. 1.

One of the important steps in screening microorganisms from the environment is a method to purify the colonies in agar medium which contain specific substrate to obtain a single colony. Because in nature, the habitat of the strain microorganisms is living together with other strains and purification is obligatory steps in the screening of new isolate microbes. Fig. 1 shows us the colony of purified strains. As describe in Fig. 1, almost all the strain has white and light brown colors. Strain microorganisms employed in nitrification and denitrification system are usually has white or yellow color (Joo et al., 2005, Zhang Jibing et al., 2011, Zhang Qing-Ling et al., 2012). The purified strain was then preserved, and maintenance in slant agar medium containing 5% mg l⁻¹ NaNO3 and put in refrigerate temperature.

Table 1 shows the colony number, code of selected strains, some morphological characters including colony shape, color, colony edge, and cell shape of the strain isolated from various places at nitrate high contain areas. The largest number of the colony were obtained from the soil at the Bangun Desa Kaliurang Farm, which observed 3.2 x 10⁵ CFU/mg. Furthermore, the colony number obtained from the soil around excreta dump at the same
farm was observed $1.8 \times 10^5$. From Surya Indah Maguwoharjo Layer Farm, soil and excreta sample were also investigated and resulted in the number of colonies $2.0 \times 10^3$ and $7.2 \times 10^4$, respectively. Moreover, colors of the strain colonies were observed dominant in white and light brown (Fig. 1). The ecology of denitrifying bacteria at deeper places was observed luck in some bacteria strains than at top soil location. The dominant bacteria employ for denitrification process are usually categorized as aerobic strain, this indicates that the denitrification system often occurs at the more atmospheric area.

**Growth profiles in liquid medium**

When the liquid medium was added by NaNO$_3$, there were additional nitrogen sources in the medium for growing. Four selected strains were given different response for NaNO$_3$ addition at the different concentration to the growth profiles (Fig. 2). Comparing to the control, the addition of 5% - 7.5% of NaNO$_3$ has improved the acceleration of exponential phase level of Strain TS1. Growing with the addition of 5% NaNO$_3$ showing the best growth profile of Strain TS1. However, the addition of 10% of NaNO$_3$ showed the same pattern with the control. Strain TS2, TB1 and TB2 have given a similar growth pattern in counter the high concentration of NaNO$_3$ addition. The addition of 10% NaNO$_3$ prolongs the adaptation phase of both three strains. The strains growth did not show the prohibition effect by the addition of 10% NaNO$_3$. In precise dose, it seems that additional nitrate will promote the growth of screened strains. Otherwise, for some strains, when the NaNO$_3$ added in very high concentration, it will poison the growth and alter the growth profiles.

**Growth profiles in solid medium**

Research in Fig. 3 was performed to know more details the ability of strains for growing on solid agar medium with supplementation of NaNO$_3$. In that figure, the comparison of colony diameter growing with and without NaNO$_3$ was compared. We tried to find the strain with ‘nitrate stimulated growth’ by the addition of NaNO$_3$ on agar medium. The results had shown that almost all of the strains have similar growth profile when growing on a solid agar medium with the addition of NaNO$_3$ at different concentration. To elucidate the ability of bacteria strains in counter 10% NaNO$_3$, research was continued by observing the colony diameter on agar medium of all strains after 5 d cultivation. Comparing with control, growing with 10% NaNO$_3$ was not affecting the colony diameter of all the strains.

**Ability in decreasing nitrate concentration**

Reduction of nitrate by individual strain in liquid medium was observed, and the percentage of nitrate reduction in detail was written in Table 2. All of the strain has the ability to denitrify nitrate at a different level. Strain TS1 reduced nitrate to 64.91% of 84.12 mg l$^{-1}$ from initial NaNO$_3$ added in the medium. Strain TS2 oxidized 65.59% of 82.23 mg l$^{-1}$ initial concentration in the medium and became the highest NaNO$_3$ reduction among the other strains. Reduction ability of Strain TB1 and TB2 was observed 65.01% and 63.00% from 84.16 and 81.04 mg l$^{-1}$ initial NaNO$_3$, respectively. The culture was performed for 24 hours only. The reduction of ammonia by individual strain may continue by the prolonged of the cultivation period.

**CONCLUSION**

All the 4 selected strain isolated from the odorous region of the poultry farming area around Yogyakarta City can counter the stressor of NaNO$_3$ in solid and liquid medium. The strains also have able to reduce the NaNO$_3$ concentration during the cultivation period. Strain TS2 proved has the best ability in reducing NaNO$_3$ concentration in liquid medium comparing with the other isolated strain. Therefore, TS2 is a promising candidate for the extensive application on the various pollution control system especially odor reduction agent. Research dealing with a gas form of ammonia is under investigation.

**ACKNOWLEDGMENT**

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**KEYWORD**: bacteria isolation, denitrifying bacteria, manure, nitrate, odorous region
Fig. 1 Colony of selected strain. (A) Strain TS1 from Surya Indah Maguwoharjo Farm; (B) Strain TS2 from Surya Indah Maguwoharjo Farm; (C) Strain TB1 from Bangun Desa Kaliurang; and (D) Strain TB2 from Bangun Desa Kaliurang.

Fig. 2 Growth of isolated strains in nitrate supplemented medium at different concentration. (A) Strain TS1; (B) Strain TS2; (C) Strain TB1; (D) Strain TB2. All strains were cultivated in 1/100 dilution meat extract medium added by 0% (white circle); 2.5% (grey circle); 5% (black circle); 7.5% (white square); 10% (black square) of NaNO₃.
Fig 3. The growth of selected strain in solid medium with and without various NaNO₃ concentrations after cultivated at 30°C for 5 d. Medium was prepared by 1/100 dilution of meat extract stock with 1.5% agar.

Table 1. Description of selected strain (sample location, colony number, selected strains, and morphology) appear on agar after suitable dilution from 1 g of soil sample taken from different places of dairy farming area around Yogyakarta City.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sample Location</th>
<th>Colony Number</th>
<th>Obtained Strain</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soil around excreta dump at Surya Indah Maguwoharjo Layer Farm</td>
<td>2.0 x 10³</td>
<td>TS1</td>
<td>Circle White Flat Bacillus</td>
</tr>
<tr>
<td>2</td>
<td>Excreta at Surya Indah Maguwoharjo Layer Farm</td>
<td>7.2 x 10⁴</td>
<td>TS2</td>
<td>Circle White Flat Coccus</td>
</tr>
<tr>
<td>3</td>
<td>Excreta at Bangun Desa Kaliurang Farm</td>
<td>3.2 x 10⁵</td>
<td>TB1</td>
<td>Circle White Wave Coccus</td>
</tr>
<tr>
<td>4</td>
<td>Soil around excreta dump at Bangun Desa Kaliurang Farm</td>
<td>1.8 x 10⁵</td>
<td>TB2</td>
<td>Circle White Wave Coccus</td>
</tr>
</tbody>
</table>

Table 2. Nitrate decreasing ability by selected strains

<table>
<thead>
<tr>
<th>Bacteria Strain</th>
<th>Nitrate Concentration (ppm) (hour)</th>
<th>Nitrate decreasing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>TS 1</td>
<td>84,12</td>
<td>87,39</td>
</tr>
<tr>
<td>TS 2</td>
<td>82,23</td>
<td>85,16</td>
</tr>
<tr>
<td>TB 1</td>
<td>84,16</td>
<td>83,75</td>
</tr>
<tr>
<td>TB 2</td>
<td>81,04</td>
<td>88,48</td>
</tr>
</tbody>
</table>
REFERENCES