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CONTENTS

1–7 ABILITY OF NITRATE REMOVAL AND GROWTH BEHAVIORS OF ISOLATED BACTERIA FROM DAIRY FARM AT TROPICAL AREA
—NANUNG AGUS FITRIYANTO, FEBRI INDRI YANI, YUNI ERWANTO, SUHARJONO TRIATMOJO AND AMBAR PERTIWININGRUM

9–13 URBAN CONTAMINATION BY ZOONOTIC HELMINTHES EGGS IN VLADIVOSTOK, RUSSIA
—T. V. Moskina, L. V. Zheleznova and A. V. Ermolenko

15–25 SURVIVAL OF LACTOBACILLUS ACIDOPHILUS TISTR1338 IN BILE SALT STRESS CONDITIONS
—S. Thaweessang and B. Leenanon

27–32 THE EFFECT OF COW FAT LEVEL ON THE QUALITY OF FERMENTED CATFISH SAUSAGES (CLARIASSP)
—Nursyam, H., Sukoso and Yuniarta

33–41 MICROBIOLOGICAL AND HEAVY METAL CHARACTERIZATION OF SOIL FROM AN OPEN HOSPITAL WASTES DUMPSITE IN ENUGU, NIGERIA
—Eze, Chukwuduba Thank God and Amaeze and Nnamdi Henry

43–49 UPTAKE OF Pb, Cr, Mn and Zn BY VEGETABLES FOUND GROWING IN THE VICINITY OF ESISI OPEN DUMPSITE IN WARRI
—(MRS.) HELEN ATEKI RUIRUI AND F. E. OKIEIMEN

51–59 EVALUATING SEASONALITY AND PATHOGENICITY OF AEROMONAS IN KOREA USING ENVIRONMENTAL DNA
—Jonathan J. Fong, Hae-Jin Cho, Myung Soo Park and Young Woon Lim

61–64 PHYSICO-CHEMICAL AND BACTERIOLOGICAL ANALYSIS OF OSMANABADI GOAT MILK
—Rajesh Kumar Sah, Satyasamparnna Raut, A.M. Chappalwar, C.D. Bhong and V.V. Deshmukhi

65–67 IMPACTS OF INSECTICIDE DELTAMETHRIN ON INTEGUMENT OF FRESH WATER LABEO ROHITA
—Arunka Gumasta, Shashi Bal B. Shrivastava and H. Maini

69–72 OPTIMIZATION OF GENOMIC DNA EXTRACTION FROM THE LEAF TISSUES OF JATROPHA SPECIES FOR MOLECULAR STUDIES
—V.D. Mendhulkar and Hena Hayat

73–80 MARINE YEAST: A POTENTIAL CANDIDATE FOR BIOTECHNOLOGICAL APPLICATIONS- A REVIEW
—Anwesha Sarkar, K. V. Bhaskara Rao

81–86 EVALUATION OF PHYTOCHEMICAL, NUTRITIONAL AND ANTIOXIDANT ACTIVITY OF SEEDS OF SAM KATHAL (ARTOCARPUS CHAPLASA)
—S.C. Biswas, Nibedita B. Dutta and Hirishkesh Sarmah

87–90 GENETIC DIVERSITY OF COTTON LEAFHOPPER POPULATIONS, AMRASCA BIGUJTULBA BIGUJTULBA (ISHIDA) IN MAJOR COTTON GROWING REGIONS OF SOUTH INDIA
—Vimala, M. Bheemanna, Rajesh Chowdary and Srividhaya Reddy

91–95 EFFECT OF VITAMIN C DIETARY SUPPLEMENTATION ON GROWTH AND SURVIVAL OF GREY MULLET, MUGIL CEPHALUS (LINNAEUS, 1758) FRY
—Samir Swain, N.K. Chandra, J.K. Sondaray, P.B. Sawant and E.M. Chhanda Pranadarsini

97–101 PHYSICO CHEMICAL PROPERTIES OF WATER FROM DIFFERENT WATER SOURCES OF MIDLAND LATERITE HILL ECOSYSTEM OF KASARAGOD DISTRICT OF KERALA, INDIA
—P. M. Biebi Razeena and Mini M.

103–109 BIOCONVERSION OF STARCHY POTATO WASTE TO ETHANOL BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (SSF) PROCESS USING ASPERGILLUS NIGER MTCC 281 AND SACCHAROMYCES CEREVISIAE MTCC 170
—Dey Pinki and Wangyal Lhakpa

119–119 REVIEW ON ANTI QUORUM SENSING ACTIVITY IN PSEUDOMONAS AERUGINOSA USING PLANTS EXTRACTS
—N. Banu and R. Nancy Immaculate Mary

121–125 A REVIEW OF RESEARCH ON ACTINOMYCETES IN RAJASTHAN
—Meeta Masand, Gaurav Sharma and Ekta Menghani

127–131 VISUALIZATION OF APOPTOTIC NETWORK USING BIOINFORMATICS TOOL
—Rashmi Rameshwar, Shilpa S. Chapatgaonkar and T.V. Prasad

133–135 OPTIMIZATION OF LIPASE PRODUCTION BY PSEUDOMONAS AERUGINOSA ISOLATED FROM AN OIL INDUSTRY UNIT
—B.N. Shukla, N.D. Pandya and P.V. Desai

(Continued on Inside Back Cover)
CONTENTS

137–147 OPTIMIZATION OF MEDIA BY RESPONSE SURFACE METHODOLOGY FOR THE REDUCTION OF CHROMIUM BY BACILLUS SP.
—Renganathan Kasimani, Renganathan Seenivasagan, Sadasivam Munraj, Chellappan Balagurunathan and Krishnan Sundar

149–154 ACCEPTABILITY OF COMBINED FOOD GROUP BISCUITS FOR REDUCING MICRONUTRIENT DEFICIENCY
—Charis K. Ripnar, Uma Devi S. Hiremath and Anitha, S.

155–164 OCCURRENCE AND ELIMINATION OF HELMINTH EGGS AT DIFFERENT STAGES IN THE SEWAGE TREATMENT PLANTS IN THE VHEMBE DISTRICT, SOUTH AFRICA
—Amitou Samie, Phindile Ntekele, Ahmed I. Yagi and Ali El Bakri

165–173 THE IMPACT OF MALODOUR EXPOSURE ON THE PSYCHOLOGICAL WELL-BEING OF A COMMUNITY IN TRINIDAD
—Tasha Ragooobar, Wayne Ganpat and Kern Rocke

175–180 DEGRADATION OF AFLATOXIN B₁, USING THE HERBAL DRUG, LIV-52
—Visenuo Aiko and Alka Mehta

181–184 A STUDY ON EFFECTS OF INOCULUM MEDIUM AND FED BATCH CULTIVATION FOR POLYHYDROXYALKANOTES SYNTHESIS BY PSEUDOMONAS PUTIDA MTCC 102 TYPE B
—Natarajan Ahilananth and Subramanian Ramalingam

185–192 ISOLATION AND PRODUCTION OF BACTERIAL ACIDOHERMOPHILIC AND ORGANIC SOLVENT TOLERANT Xylanase
—Radhika Kashyap, Monika and Sandeep Tripathi

193–196 ALKALINE AMYLASE PRODUCTION BY SUBMERGED FERMENTATION BY BACILLUS SP.
—S. Priyadarshini and P. Ray

197–201 IMPROVEMENT OF HOMOLOGOUS RECOMBINATION EFFICIENCY IN E. COLI
—S.M. Amin Marashi, S. Yaslianjfard, M. Erfanmanesh, P. Afroghi and E. Kalantar

203–211 HUMAN PATHOGENIC BACTERIA ASSOCIATED WITH FIELD GROWN TOMATO AND RADISH
—Indu Gaur, P. D. Sharma and P. K. Paul

213–218 ISOLATION, CHARACTERIZATION AND SCREENING OF ALKALINE PROTEASE PRODUCING ALKALIPHILIC BACTERIA FROM THE POLLUTED HABITATS
—S. Jeevanendra, P. Palavu and S. Ram Reddy

219–224 RESPONSES OF PHOTOSYNTHETIC PIGMENTS IN ANABAENA CYLINDRICA TO SHORT TERM EXPOSURE OF COPPER, CADMIUM, AND LEAD
—Wan Nurul Aisyah Wan Jusoh, Ling Shing Wong and Mee Kin Chai

225–229 A STUDY OF BIODEGRADATION OF PAPER WASTES BY USING BACTERIA ISOLATED FROM THE SOIL
—Dhuha Mahdi Jabir and Mustafa Mahdi Jabir
ABILITY OF NITRATE REMOVAL AND GROWTH BEHAVIORS OF ISOLATED BACTERIA FROM DAIRY FARM AT TROPICAL AREA

NANUNG AGUS FITRIYANTO*, FEBRI INDRI YANI1, YUNY ERWANTO1, SUHARJONO TRIATMOJO1 AND AMBAR PERTIWININGRUM1

Faculty of Animal Science, Gadjah Mada University, Yogyakarta, Indonesia, Jl. Fauna No. 3 Jl. Kampus UGM Bulaksumur, Yogyakarta (55281)

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Key words: Ammonia emission, Bacterial growth behavior, Biological treatment, Nitrate removal, Nitrification-denitrification

Abstract—Dairy farm and other livestock industry are usually generate odor by animal. It possibly cause environmental problems such as ammonia emission in high concentration which can reduce productivity of dairy cows and other animals as well as endanger the health of person who living inside the farm or community who living nearby livestock industry. The transformation of volatile ammonia to non-volatile form are a primary biological treatment process comprising of nitrification and denitrification processes. The aim of this research was to isolate bacteria which have the ability in reducing nitrate through denitrification process from the dairy farm in the tropical area. Bacteria strains were screened from the soil and observation of the potential of these strains in reducing nitrate was performed. This research was first conducted in isolation of bacteria which has the ability in reducing nitrate. Four bacteria which able to reduce nitrate were successfully isolated from the soil sample. Strain SP1 which isolated from the soil at the dairy farm of Faculty of Animal Science UGM has the ability for better growth in liquid and solid medium with the addition of nitrate in high concentration. The colony diameter of Strain SP1 growing at different concentration of nitrate 0; 2.5; 5; 7.5 and 10% were observed 0.876; 0.793; 0.952; 0.492 and 0.015 cm respectively. All strains have optimal ability to reduce nitrate in the liquid medium. Based on the observation of nitrate reduction in the liquid medium, Strain SP1 reduced 63.48%, Strain SP3 reduced 63.38%, Strain SP4 reduced 62.47% and Strain SP2 has reduced 62.03%. Some physiological characteristics of four denitrification strains are investigated as follow: Strain SP2 and SP4 could produce catalase enzyme while SP1 and SP3 could not produce the same enzyme. Strain SP1, SP2, and SP3 were observed as Gram-negative bacteria while SP4 was Gram-positive. Strain SP1 and SP3 have coccus shape while SP2 and SP4 are observed as Bacillus shape. The conclusion of this research was isolate bacteria from the soil around the dairy farm have ability to reduce nitrate and have a better growth at 7.5% nitrate containing medium.

INTRODUCTION

Dairy production within the agricultural sector represent the high source of CH₄, NH₃, and CO₂ emissions and possibly have a huge potential for greenhouse gas (GHG) mitigation. Unpleasant odor are generally produced by the animal from the dairy farm industry, and may cause environmental problems such as ammonia emission in high concentration which can decrease productivity of dairy cows and endanger of person health who living in the farm or people community who living nearby the dairy cows 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 dairy operating systems have been suggested in the former 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 dairy 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

*Corresponding author’s email: nanungagusfitriyanto@ugm.ac.id (N A Fitriyanto)
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 (N$_2$O) (Ndegwa et al., 2008).

Several specific potential control strategies for NH$_3$ mitigation from animal 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 (McCory 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 ability to oxidize NH$_3$ to nitrite (NO$_2$). NO$_2$ is further oxidized to nitrate (NO$_3$) by nitrite oxidizing bacteria (NOB). Recently, the major grouping of AOB belongs to the subclass Betaproteobacteria has been isolated and observed to be involved in nitrification system such as 
Pseudomonas
spp. (Zhang et al., 2011), 
Alcaligenes faealis (Joo et al., 2005), 
Bacillus methyloptrophicus (Zhang et al., 2012), 
Arthrobac ter spp. (Verstraete and Alexander, 1972), and another group of bacteria such as 
Nitrosomonas spp., 
Nitrosococcus spp., 
Nitrosospira spp., 
Nitrosovibrio spp. and 
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 dairy farming activities.

**MATERIALS AND METHODS**

**Media and Culture**

The stock meat extract medium consist of 1 g meat extract, 1 g microbiological peptone, and 0.5 g NaCl and made by diluted with 70 ml distilled water in beaker glass. Then PH adjustment into 7.2 with NaOH or H$_2$SO$_4$ and final volume was adjusted to 100 ml. The Stock of meat extract medium was always preserved in -20°C. The Stock of 5% NaNO$_3$ was made by diluted of 5 g NaNO$_3$ in 100 ml ionic water and maintained in 4°C. Furthermore, for screening ammonium-responsive microorganisms a 1/100 of stock meat extract medium with 1.5% agar added by 5% NaNO$_3$. Cultures were performed for 7 d at 30°C. The liquid culture was carried out using 100 ml of 1/100 nutrient broth with 500 mg l$^{-1}$ NaNO$_3$ in 250 ml-Erlenmeyer flasks and cells were cultured aerobically at about 30°C 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 dairy 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% NaNO$_3$. Colonies appearing on the plate were picked and purified. Each purified colony was inoculated on an agar plate with and without 5% NaNO$_3$. Microorganisms displaying a peculiar growth on the agar with NaNO$_3$ 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°C 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 concentration analysis. NO₃ was analyzed by Brusin method.

RESULTS AND DISCUSSION

Results of isolation on nitrate reducing bacteria

As environmental conditions for living of strain microorganisms in soil are usually nutritionally low, a portion of 1/100 nutrient agar was used for screening nitrate-resistant strains. Selected media was added to 5% of NaNO₃ used for screening “high nitrate stressor resistance-strain”. This medium made the isolate which has not ability of counter system with the stressor of NaNO₃ will not grow in this selected medium. We have isolated a soil bacterium, which located at high odorous region of the dairy farming area. This nitrate resistant strain were screened at four different places from 2 dairy farms at around Yogyakarta City. One spot was chosen at the dairy farm of Faculty of Animal Science, Gadjah Mada University, and another spot was at PT Sarijani dairy farm at Yogyakarta.

The strain which showed real 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 step 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 the nature, habitat of the strain microorganisms are living together with other strains, and purification is obligatory steps in 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 (Zhang et al., 2012, Joo et al., 2005, Zhang et al., 2011). The purified strain was then preserved and maintenance in slant agar medium containing 5% mgL⁻¹ NaNO₃ and put in refrigerator temperature.

Table 1 shows us 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 highest number of the colony were obtained from the soil at the dairy farm at Faculty of Animal Science UGM, observe 1.6 x 10⁶ CFU/mg. Furthermore, the colony number obtained from PT Sarijani dairy farm at different depth position (10 cm, 15 cm and 20 cm) were observed 1.46 x 10⁶, 1.21 x 10⁶ and 1.17 x 10⁶ respectively. Furthermore, colors of the strain colonies were dominant in white and light brown (Fig. 1). It indicates that the ecology of denitrifying bacteria at deeper places was luck in number of bacteria strains than at top soil location. Since the dominant bacteria employ for denitrification process are usually categorized as aerobic strain, this indicates that the denitrification system often occur

Fig. 1. Colony of selected strain. (A) Strain SP1; (B) Strain SP2; (C) Strain SP3; and (D) Strain SP4
When the liquid medium was added by NaNO₃, there was additional nitrogen sources in medium for growing. Four selected strains were given different response for NaNO₃ addition at the different concentration to growth profiles (Fig. 2). Comparing to the control, the addition 2.5% - 15% of NaNO₃ has improved the exponential phase level of Strain SP1. Thus, the different nitrate concentrations have not changed the growth profile of Strain SP1. Strain SP2 and SP3 have given a similar growth pattern in counter the high concentration of NaNO₃ addition. The addition of 15% NaNO₃ prolongs the adaptation phase of both strains. The strain SP2 did not show the prohibition effect by the addition of 10% NaNO₃ while the prohibition effect was observed in strain SP3. Comparing to the control, Strain SP3 was growing slowly, linear to the increasing of nitrate concentration. The addition of NaNO₃ in the liquid medium did not affect the growth profiles of Strain SP4 (Fig. 2). In precise dose, it seems that additional nitrate will promote the growth of screened strains. Otherwise, for some strains, when the NaNO₃ added in very high concentration, it will poisoning the growth and alter the growth profiles.

Research in Fig. 4 was performed to know more

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>Dairy farm at Faculty of Animal Science UGM (soil sample at 8 cm in depth)</td>
<td>1.6 x 10⁶</td>
<td>SP1</td>
<td>Circle</td>
</tr>
<tr>
<td>2</td>
<td>PT Sarijani dairy farm (soil sample at 10 cm in depth)</td>
<td>1.46 x 10⁶</td>
<td>Sr2</td>
<td>Circle</td>
</tr>
<tr>
<td>3</td>
<td>PT Sarijani dairy farm (soil sample at 15 cm in depth)</td>
<td>1.21 x 10⁶</td>
<td>Sr3</td>
<td>Circle</td>
</tr>
<tr>
<td>4</td>
<td>PT Sarijani dairy farm (soil sample at 20 cm in depth)</td>
<td>1.17 x 10⁶</td>
<td>Sr4</td>
<td>Circle</td>
</tr>
</tbody>
</table>

Fig. 2. Growth of isolated strains in nitrate supplemented medium at different concentration. (A) Strain SP1; (B) Strain SP2; (C) Strain SP3; (D) Strain SP4. 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.
details the ability of strains for growing on solid agar medium with supplementation of NaNO₃. In that figure, the comparison of colony diameter growing with and without NaNO₃ was compared. We tried to find the strain with ‘nitrate stimulated growth’ by the addition of NaNO₃ on agar medium. The results had showed that almost all of the strains have similar growth profile when growing in a solid agar medium with the addition of NaNO₃ at different concentration. To elucidate the ability of bacteria strains in counter 10% NaNO₃, research was continued by observing the colony diameter on agar medium of all strains after 5 d cultivation. Comparing with control, growing with 5% NaNO₃ was not affecting the colony diameter of all the strains; furthermore prohibition effect was appearing in addition of 7 and 10% NaNO₃.

Reduction of nitrate by individual strain in liquid medium was observed, and data was shown in Fig. 3, and the percentage of nitrate reduction in detail was written in Table 2. All of the strain has ability to denitrify nitrate at a different level. Strain SP1 reduced nitrate to 63.48% of 83.35 mgL⁻¹ from initial NaNO₃ added in the medium. Strain SP2 oxidized 62.03% of 84.26 mgL⁻¹ initial concentration in the medium and become the highest NaNO₃ reduction among the other strains. Reduction ability of Strain SP3 and SP4 was observed 63.38% and 62.47% from 84.21 and 84.71 mgL⁻¹ initial NaNO₃ respectively. The culture was performed for 24 hours only. The reduction of ammonia by individual strain may continued by the prolonged of the cultivation period.

Recently, many strains have observed as denitrifying bacteria such as Pseudomonas sp. ASM 2-3 (Kariminiaae-Hamedaani et al., 2004, Zhang et al., 2011), Paracoccus denitrificans (Vacková et al., 2011), and Bacillus sp. (Yang et al., 2011, Zhang et al.,

Table 2. Nitrate reduction in liquid medium

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Nitrate measurement in medium (mgL⁻¹)</th>
<th>Nitrate Oxidation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>4 h</td>
</tr>
<tr>
<td>SP1</td>
<td>83.352</td>
<td>87.271</td>
</tr>
<tr>
<td>SP2</td>
<td>84.256</td>
<td>78.427</td>
</tr>
<tr>
<td>SP3</td>
<td>84.206</td>
<td>82.950</td>
</tr>
<tr>
<td>SP4</td>
<td>84.708</td>
<td>87.070</td>
</tr>
</tbody>
</table>

Fig. 3. Growth (black circle) and nitrate removal (black square) at liquid medium by isolated strains. Shaker cultivation (120 rpm) was performed at 30°C in 1/100 meat extract medium. (A) Strain SP1; (B) Strain SP2; (C) Strain SP3; and (D) Strain SP4
The growth profile and NaNO₃ reduction ability are showing differences one from the others. The process of ammonia production and volatilization are well known occur in the animal productions especially in manure storage or treatment structures, animal buildings, and during or after land application of manure. Some bacterial activities involving N substrates may generate ammonia production. The enzyme urease found in the animal feces rapidly hydrolyses urea and uric acid into NH₄⁺-N when urine is mixed with the feces. Consequently, an unpleasant odor will be generated. Undigested protein from a feed by the animal was also suggested to be sources of ammonia.

Some strategies for ammonia emission from animal production facilities have been performed by many researchers is by changing animal diet, redesigning or renovating barns, cleaning the exhaust air from buildings, treating manure as raw material for aerobic composting, and improving the application of manure to agriculture farming land. Among them, biological additives employ microorganisms has attracted attention of their ease of uses, fast in action, and relatively lower expenses. This ammonia removing treatment is based on the transformation of volatile N to non-volatile N comprising of nitrification and denitrification processes by chemoautotrophic nitrifying and denitrifying bacteria. We have isolated microorganisms showing a better growth under ammonia stressor in liquid medium and strain was proved to have ability to reduce NaNO₃ concentration. This effort will be continued by observing the ability of strain to reduce gas form of NaNO₃ and developing consortium microbes which consist of nitrification employed strain such as ammonia reduction strains, nitrate and nitrate oxidation strains and denitrification strain.

**CONCLUSION**

All the 4 selected strain isolated from the odorous region of the dairy farming area around Yogyakarta City can counter the stressor of NaNO₃ in solid and liquid medium. The strains also have able to reduce the NaNO₃ concentration during the cultivation period. Strain SP2 proved has a best growth with NaNO₃ supplementation and has best ability in reduce NaNO₃ concentration in liquid medium comparing with the other isolated strain. Therefore, SP2 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.

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