Denitrification activity of isolate CD I-III in nitrate broth medium with carbon source variation and identification using VITEK-2

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Abstract. Denitrification is a reducing process of nitrate into nitrogen gases. Bacteria are able to do this process in high nitrate and low oxygen concentration. Fresh sediment from Cideng River, Jakarta, was incubated in Nitrate Broth medium then spread on Potato Dextrose Agar. Nine isolates was grown separately and inoculated in Oxidative/Fermentative medium to determine the denitrification ability. One isolate, named Isolate CD I-III, showed a positive result of denitrification activity. This isolate was inoculated in a Nitrate Broth medium with carbon source variations for 19 days to optimize its denitrification process. Parameters measured for indicating the denitrification activity were nitrate concentrations by the Spectrophotometric method and the bacterial viability by the total plate count method. This isolate was also identified by using the VITEK-2. The result revealed that glucose and glycerol as carbon source variations gave a significant difference (α = 0.05) in nitrate concentrations, whilst there was no difference in bacterial viability. The isolate CD I-III was identified by the VITEK-2 as \textit{Escherichia hermannii} with 97\% probability.

1. Introduction
Nitrate (NO$_3^-$) is one form of nitrogen molecules that is often found in the environment as a product of the nitrogen cycle. The maximum limit of nitrate level in water is 10 g / L. Many water streams across the city contain nitrate level that exceeds their maximum limit and becomes toxic to living things. Several factors can affect the increasing nitrate levels in water, including contamination from septic tanks in the soil, excessive use of high nitrogen fertilizers, and unprocessed disposal of agricultural and livestock wastes.\cite{1}

Increased nitrate levels can give a negative impact on both the environment and human. Increasing nitrate levels can cause eutrophication, which is the increasing amount of aquatic plants due to the abundance of nutrients within an environment. Eutrophication leads to decreasing dissolved oxygen levels and may kill the aquatic organism. In humans, increased nitrate levels may cause methemoglobinemia, a condition where the body becomes blue due to lack of oxygen caused by the conversion of excess nitrate ions in the body. This process will produce nitrite which binds hemoglobin in the blood so that the oxygen that is bound by hemoglobin to be delivered throughout the body is reduced. \cite{2,3}
Denitrification is the process of converting nitrate to nitrogen gas. The process is important because it can balance the composition of nitrogen by removing it in gas form to the environment only in anaerobic conditions, where nitrate is used as the last electron acceptor in the process of metabolism of microorganisms. It process occurs in 4 stages, namely reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$) by nitrate reductases, reduction of nitrite (NO$_2^-$) to nitric oxide (NO) by nitrite reductases, reduction of nitric oxide (NO) to nitrous oxide (N$_2$O) by nitric oxide reductases, and reduction dinitrogen oxide (N$_2$O) becomes nitrogen gas (N$_2$) by dinitrogen oxide reductases.[4]

The research aims to isolate and identify bacterial microorganisms that play a role in the denitrification process in Cideng River, Central Jakarta. In addition, this study also aims to determine the effect of additional carbon sources on denitrification activities.

2. Materials and Methods
The study was conducted from July 2017 to April 2018 in Microbiology Laboratories, Center for Environmental Technology, Agency for the Assessment and Application of Technology, Serpong. Isolation of microorganisms is carried out in the Cideng River stream, Central Jakarta. Identification of microorganisms was carried out at the General Hospital of Tangerang Regency, Banten.

2.1. Sampling and analysis of water quality
Samples were taken in the form of river water and sediment. Samples were taken from three locations consists of two outlet points and one point in the center of the river flow. Water samples are taken directly from the outlet area using water sampler or bucket and dipper then transferred to the plastic box. Sediment samples were taken using the Eikman grab then stored in a plastic jar. All samples were placed in the coolbox containing ice packs.[5]

Environmental parameters were also measured including dissolved oxygen (DO), pH, temperature, electrical conductivity, and total dissolved solid (TDS). Dissolved oxygen measured by DOmeter. pH measured by pH meter. The temperature measured using a thermometer. Electrical conductivity measured using conductometer. The number of dissolved solids measured using TDS meter.[6]

2.2. Isolation and characterization of denitrifying bacteria
Denitrifying microorganism isolation starts with inoculating 1 mL of sediment samples and 1 mL of river water samples on 20 mL of Nitrate Broth medium containing 5 g/L nitrate in a screw tube. The inoculum incubated under anaerobic conditions in a glass jar for three days at room temperature.[5] After three days, the inoculum streaked on Plate Count Agar (PCA) medium using quadrant streak method and incubated at 30ºC for 48 hours. Colonies that grow with different colors and textures purified by inoculation on PCA medium and incubated in incubator at 30ºC for 48 hours.

Each isolate was tested for metabolic ability using a fermentative oxidative test (O / F). The O / F test will determine the metabolic ability of microorganisms in aerobic and anaerobic conditions. The samples inoculated in two test tubes containing O / F basal medium then incubated under aerobic and anaerobic conditions. Anaerobic conditions are created by liquid paraffin addition over the medium. Then inoculum was incubated in an incubator at 37ºC for 24-48 hours. Denitrification bacterial isolates were shown by changing the color of the medium from blue to yellow in aerobic conditions, but there was no change in anaerobic conditions.[7]

Denitrification bacterial isolates were characterized based on morphology and staining. Observation of colony morphology was carried out by observing the shape, color, and edges of colonies in ± 48-hour-old isolates growing on PCA medium. Simple staining using crystal violet dye to determine the shape of bacterial cells and Gram staining to determine the structure of the bacterial cell wall.[8]

2.3. Optimization of denitrification of isolates with the addition of carbon sources
Optimization is carried out by adding carbon sources in the form of glucose or glycerol in Nitrate Broth medium containing 10 g / L of nitrate. Pure isolate inoculated into 100 mL Nitrogen Broth
medium in Erlenmeyer and incubated anaerobically for five days in room temperature. Amount of 90 mL incubated isolates then inoculated into 810 mL modified Nitrate Broth medium in a 1000 mL plastic bottle. The anaerobic atmosphere within plastic bottles was made by spraying CO₂ gas into the tube for 5 minutes, then sealed using a plastic valve. The incubation was continued for 18 days. Measurement of nitrate levels and bacterial counts was carried out seven times with a 3-days interval using a 10 mL syringe.

Nitrate measurement carried out using the colorimetric method. 0.25 mL of sample was added with 0.5 mL of salicylic acid solution, then homogenized with vortex and incubated for 30 minutes. Then the sample was added with 5 mL 5 M NaOH solution and homogenized using vortex. The sample was incubated for 1 hour; then the nitrate content was measured using a spectrophotometer at a wavelength of 406 nm.[9]

Measurement of the number of bacteria was carried out using the Total Plate Count (TPC) method. The TPC method begins by performing dilution in the test sample to a concentration of 10⁻⁴. Furthermore, the diluted sample was inoculated on Plate Count Agar (PCA) medium in a petri dish with a spreading technique. Then, the inoculum in the petri dish was incubated in an incubator at 30ºC for 48 hours. The number of colonies growing was calculated.[10]

2.4. Identification using VITEK-2

Identification of CD I-III bacterial isolate started with inoculation into MacConkey Agar medium then incubated at ± 36ºC for 18-24 hours. The culture then suspended with 3 mL of sterile saline solution in a 12 x 75 mm glass tube and concentrations of 0.5-0.63 McF. Glass tube and an identification card was placed on the diskette and connected using a special hose. The diskette was inserted into the Vitek-2 Compact tool and the identification process will be completed within ± 10 hours and will be stored directly in the computer (Environment Protection Agency 2016: 7-11).

3. Results & Discussion

3.1. Water quality

Observation of the water quality of the Cideng River is undertaken at three different points. The conditions found when observing indicate that the Cideng River has a blackish-brown color, lots of garbage floating on the surface, and has several silting points across the stream. Analysis of river water quality generally shows that the pH of the water 7.3-8.4, temperature 25ºC, electrical conductivity 384-558 µS / cm, DO 0.27-1.15 ppm and TDS of 332-395 mg / L (Table 1).

| Environmental Parameter          | Unit       | Location I | Location II | Location III |
|---------------------------------|------------|------------|-------------|--------------|
| pH                              |            | 8.4        | 7.5         | 7.3          |
| Temperature                     | ºC         | 25         | 25          | 25           |
| Electrical Conductivity         | µS/cm      | 558        | 416         | 384          |
| Dissolved Oxygen (DO)           | ppm        | 0.81       | 0.27        | 1.15         |
| Total Dissolved Solid (TDS)     | mg/L       | 395        | 337         | 332          |

Data in the Table 1 shows that the Cideng river can be categorized as polluted due to the colored water conditions, the pH value exceeds the standard, and the DO value is below the reference value. However, this condition still allows for the life of bacteria, especially denitrification bacteria. This is shown in the temperature and DO values that are highly favored by denitrification bacteria. The temperature in the river is considered ideal for optimal bacterial growth, while low DO values can cause bacteria to use nitrates in metabolism so that the denitrification process is optimal.[8]
3.2. Isolation and Characterization

River water samples and fresh sediments were incubated anaerobically in Nitrate Broth medium using screw tubes containing Durham tube to catch air bubbles. Durham tubes that contain air bubbles indicate that denitrification has taken place. After being incubated for three days, an air bubble was caught in the Durham tube which indicated that the denitrification process had occurred. Furthermore, all samples in the screw tube were inoculated on PCA medium in a petri dish using the quadrant streak method to obtain a single colony. Amount of nine types of single colonies were found and then isolated in different Petri dishes containing PCA medium.[6]

3.3. Oxidative/fermentative test result

The nine single isolates tested for their type of metabolism using the O / F test. Denitrification bacteria are not only aerobic bacteria because under normal conditions they are able to use oxygen as the last electron receiver, but also can survive under anaerobic conditions only if there is nitrate in their environment. In addition, fermentative oxidative tests can also distinguish denitrification bacteria with common fermentative bacteria which only reduce nitrate (NO₃⁻) to NH₄⁺ ions.[6] The nine isolates tested, only isolates with CD I - III code were proven to be oxidative bacteria. This is because there is a color change from blue to yellow on a medium that is not given paraffin, while there is no change in color on the medium given paraffin. These results indicate that CD I-III isolates are able to metabolize under aerobic conditions and cannot denitrify under anaerobic conditions due to the absence of nitrate in the medium.

3.4. Isolate characterization

Morphological characterization was carried out by streaking on Plate Count Agar (PCA) medium. Isolate CD I-III has a rounded shape, milky white, flat edge and shiny surface. The results of simple staining using Crystal Violet dye showed that bacterial isolates have a round shape (cocci), but can form colonies in the form of staphylococcus or streptococcus. Gram staining results indicate isolates including Gram-negative bacteria because they can absorb Safranin dyes.[8]

3.5. Optimizing the ability of denitrification with the addition of carbon sources

The addition of carbon sources is known to optimize bacterial growth as it is expected to reduce nitrate levels more rapidly. Glucose could be metabolized by both aerobic and anaerobic bacteria so that it can be used for denitrification which only occurs in low oxygen conditions. Glycerol has low prices on the market so it has the potential to be tested as an alternative carbon source for the denitrification process. [12] [13]

Isolates CD I-III were grown in a 1000 mL volume reactor containing Nitrate Broth medium with a nitrate content of 10 g / L and additional glucose or glycerol as carbon source. Parameters measured are nitrate concentration to determine the amount of nitrate that has been successfully reduced and the number of bacteria to determine the bacterial growth.

3.6. Nitrate Concentration

The addition of the glycerol group experienced the fastest decrease in nitrate content during observation, followed by groups without the addition of carbon sources and glucose groups in a row (Figure 1). The addition of glycerol shows the most rapid decrease in nitrate due to nitrate reduction reaction occurs in the cell membrane simultaneously with the oxidation of glycerol-3-phosphate by the enzyme glycerol-3-phosphate dehydrogenase so that the denitrification process by bacteria takes place more efficiently than other carbon sources.[14][15]
Figure 1. Decreasing in NO$_3$-N concentration in nitrate broth medium with variation of carbon sources.

The treatment without additional carbon sources showed the second nitrate reduction activity after the addition of glycerol. The Nitrate Broth medium contains peptone and meat extract as a source of nutrition for bacteria. Peptone consists of amino acids and inorganic salts as a source of nitrogen for bacteria. Meat extract is a processed decoction of animal tissue that functions as a source of nitrogen and organic acids for bacteria. Both materials provide abundant nutrition so that the bacteria were able to metabolize even under anaerobic conditions although without additional carbon sources.[16][17]

The addition of glucose treatment group showed the lowest nitrate reduction activity. It caused by an imbalance of carbon and nitrogen ratios that inhibit the synthesis of cyclic adenosine monophosphate (cAMP) as a signal to begin the process of glucose metabolism. Inhibited glucose metabolism causes the use of nitrate as the last electron acceptor to be small, so that the nitrate reduction activity tends to be low.[18][19]

3.7. Bacterial Count
The number of bacterial inocula inoculated into fermenters is about 1.12 - 1.38 x 10$^8$ CFU / mL. The growth activity causes an increase in the number of bacteria from the 3rd - 12th day. The treatment without the addition of carbon sources has the highest increase in the number of bacteria followed by the addition of glucose and glycerol respectively (Figure 2).

Figure 2. CD I-III bacterial growth in nitrate broth medium with variation of carbon sources.
The treatment without the addition of carbon sources showed the highest growth activity and a number of bacteria. Nitrate Broth medium contains peptone and meat extract as a source of nutrients that support bacterial growth. In addition, another factor that supports bacterial growth is the preference of bacteria that will metabolize nutrients from the simplest to complex forms. This is because both glucose and glycerol require the conversion process into simple molecular forms before can be metabolized, while peptone and meat extracts in the Nitrate Broth medium have been adjusted so that they can be directly used by bacteria as a nutrient source [8].

3.8. Identification
CD I-III bacterial isolates were identified using VITEK-2 as Escherichia hermannii from the Enterobacteriaceae group with a probability level of 97%. Escherichia hermannii described as rod-shaped Gram-negative bacteria, facultative anaerobes, ability to reduce nitrate to nitrite, and is able to metabolize various types of carbon sources such as glucose, maltose, mannitol, and mannose. Based on these characteristics, E. hermannii allegedly has nitrate reduction ability because it has the Nitrate Reduction (nar) gene that helps reduce nitrate from the environment.[20]

4. Conclusion
Denitrification bacteria has successfully isolated from Kali Cideng, Jakarta Pusat. The bacteria proved that it could perform denitrification activity in Nitrate Broth medium with or without additional carbon source and identified as Escherichia hermannii.

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