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Potential of East Java Indigenous Anammox Microbes from Selorejo Reservoir as Degrading Agents of Ammonia Wastewater

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Anammox (*Anaerobic Ammonium Oxidation*) microbes play a role in the process of changing from ammonium ions to nitrogen gas with nitrite as an electron acceptor. Eventhough most of the Indonesian territorial are rivers and sea which very suitable for Anammox microbial growth, the research about Anammox microbes in Indonesia are very limited. One of the waters that may be inhabited by Anammox microbes is the Selorejo Reservoir which is expected to be inhabited by Anammox *Cadindatus brocadia* microbes. This study aims to determine the presence of Anammox microbes and their activities in the waters of the Selorejo Reservoir using the *Anaerobic Bottle Bath Reactor* method. The stages in this research include: (1) sample preparation (2) Anammox microbes analysis using *NGS* (3) isolation of bacteria with an *Anaerobic Bottle Reactor* (4) testing bacterial activity in reducing ammonia levels using Colorimetry. The results of this study showed that Next Generation Sequencing (NGS) analysis detected an indigenous Anammox microbial of *Cadindatus kuineneia stutgartensis* with an abundance of 0,1% of the total microbes present. In addition to anammox microbes, there are also other microbes that play a role in the nitrogen cycle, which are nitrification and denitrification bacteria, namely *Nitrospira, Nitrosococcus, Nitrobacter, Nitrosomonas,* and *Pseudomonas*. The Average of Ammonia during batch experiments is 0.10%/Day

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Waste has become the main problem of global environmental pollution, one of which is in the East Java Province of Indonesia, the number of polluting agents in river water flows makes the state of water in an area experience eutrophication, damage to water ecosystems, and even health problems caused by nitrite and nitrate levels [1-2]. In Indonesia, precisely in East Java, there are several companies, namely MSG companies, pharmaceuticals, and tanneries that have by-products in the form of company waste with the potential to contain excess nitrogen, according to research by Ali et al 2013 wastewater from this company has the potential to contain excess nitrogen [3].

In general, the bioremediation process of ammonia-rich waste can be carried out conventionally by nitrification and denitrification [4], in practice this method requires high energy and considerable costs, so recently the modern biological process is to use the Anammox (*Anaerobic Ammonium Oxidation*) process, as a substitute for the modern physical process that has been discovered before, namely by means of adsorption that is considered less effective [3].

The Anammox process (*Anaerobic Ammonium Oxidation*) works by lowering the level of ammonium in waste to nitrogen gas which in the process involves anammox bacteria that utilize nitrites as electron receivers in anoxic condition (without oxygen), or more commonly known as anaerobic [5-6]. This process is not costly, or in another cost-effective [7-9], which is because it does not require a lot of external organic carbon consumption, and does not use oxygen, because the process is an Anaerobic process [5, 10-12].

The Anammox (Anaerobic Ammonium Oxidation) process is assisted by Anammox bacteria, but there is very little research on Anammox bacteria carried out in Indonesia, although research on biodegradation of ammonia-rich waste with indigene

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bacteria in Indonesia has never been carried out, but some research results show that the use of indigenous bacteria can increase the rate of degradation without causing adverse impacts and is environmentally friendly. This study aims to obtain Anammox indegen Java Tiwur bacteria and find out its activity in reducing ammonia levels from samples taken from the Selorejo Reservoir which has the potential to contain Anammox bacteria because there are many hyacinths there as a sign that the nitrogen nitrate content there is quite high [13], and is a fresh water that is adaptive to anammox bacteria from *Cadindatus brocadia* [3]. Activity testing was carried out using a UV-Vis spectrophotometer.

EXPERIMENT DETAILS

Equipment

This Research was carried out using analitycal balance, waterbath shaker, Sentrifugator, *Next Generation Sequencing* (NGS), and UV-Vis spectrophotometer.

Materials

Water samples and sediment samples from Selorejo Reservoir Ngantang Malang, $MgCl_2$, NH_4HCO_3, KH_2PO_4, Na_2-EDTA.$2H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $Na_2MoO_4.2H_2O$, $NiCl_2.6H_2O$, Na_2SeO_3, H_3BO_4, $FeCl_2.4H_2O$, aquades, gas nitrogen, fenol, natrium nitroprusida, $Na_3C_6H_5O_7$, NaOH, natrium hipoklorit, asam sulfanilat, 1-naftil etylendiamin dihidroklorida, $Fe(NH_4)_2.(SO_4)_2.6H_2O$, brusin sulfat ,blue ice and also filter paper.

Procedures

Preliminary Test of Water Samples

Sample testing is carried out at the sampling location using an ammonia test kit, nitrite test kit, nitrate test kit, and pH meter.

Sediment Sample Testing with Next Generation Sequencing (NGS)

Testing the potential presence of bacteria with NGS begins with test sampling, genomic extraction of DNA, amplification of DNA isolation results using primary barcodes, sequencing the results of DNA amplification using NGS, and taxonomic identification by comparing data banks. In assessing the potential presence of anammox microbes, it begins with isolating genome DNA from samples at point 3, namely the Kwayangan river in the Selorejo Reservoir using the NucleoSpin Soil Kit Macherey-Negel. The second process is to determine the concentration of DNA using the NanoDrop Spectrophotometer and the Qubit flouro-meter. These results were further amplified by comparing Kits data from Oxford Nanopore Technology. The results of DNA amplification will be sequenced using GridION which is operated using MinKNOW software version 20.06.9. the next process is basic invocation using high accuracy Guppy 4.0.11 software [14], the next step is the visualization of genetic sequencing data from FASTQ files using NanoPlot (De Coster et al. 2018). The results of the visualization were read and classified using the Centrifuge Classifier (Kim et al. 2016), which had previously had bacterial index and Archae data from the centrifuge website, analysis and visualization were carried out using Pavian and Krona Diagrams.

Sediment Sample Preparation

Botol reactor is filled with synthetic waste solution that has been made in 3/4 bottles, then as many as 5 g of sediment samples from Selorejo Reservoir are put in bottles, bottle closed using rubber caps, tightened rubber caps again using aluminum cover, and put in shaker water bath, after that, a set of nitrogen gas tools is installed, nitrogen gas is inserted into the reactor bottle using a syringe that has been assembled with a nitrogen gas tool, and punched another hole in the bottle cap using a syringe to keep the gas pressure in the bottle stable. The pressure in the reactor bottle must be the same as the pressure conditions outside the system, this will make it easier to calculate the volume addition of nitrogen gas, allowed the device to operate, and tested once a week [15].

Testing the Activity of Anammox Bacteria in Reactor Bottles

In testing bacterial activity, it is carried out by taking a solution in a reactor bottle using a syringe, then testing the content of ammonia, nitrites and also nitrogen gas in the sample, repeated this test once a week, bacterial activity will be seen with reduced levels of ammonia and nitrites as well as the addition of nitrates and the volume of nitrogen gas in the sample.

RESULT AND DISCUSSION

Sampling Location

Selorejo Reservoir is the place chosen as the place for sampling in the implementation of this study because in previous studies it has been explained that in fresh waters it can be overgrown by anammox bacteria of the genus *Cadindatus brocadia* [16]. The sampling location was carried out at three points in the Selorejo Ngantang Reservoir with the help of the Global Positioning System (GPS) in areas suspected to be benthic zones, namely Kali Pinjal, Kali Konto, and Kali Kwayangan. The three points are shown in Figure 1.

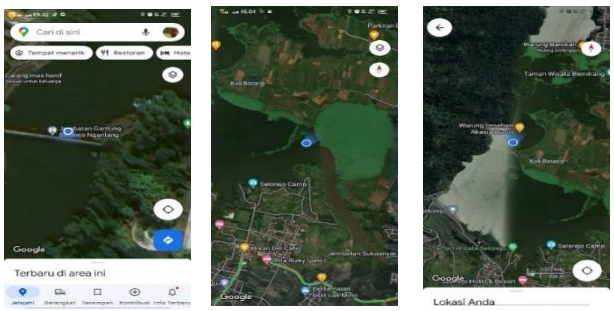

Figure 1. Sampling point a. Kali Pinjal b. Kali Konto c. Kali Kwayangan.

Table 1. Preliminary test results.

Parameter	Point 1 (Suspension Bridge / Kali Pinjal)	Point 2 (Kali Konto)	Point 3 (Kali Kwayangan)
pH	7	7	7
Nitrite	0	0	0,1
Nitrate	2	2	2
Ammonia	5	10	10

Testing Water Quality Parameters

Anmmox bacteria can be found in areas with high nitrogen content, so it is necessary to test nitrogen levels in waters as a concrete step, it is necessary to test water contamination parameters composed of pH, Nitrite levels, Nitrate levels, and also ammonia levels, this is because Anammox bacteria need nutrients to survive with high nitrogen levels, the first thing carried out in this study was testing water quality parameters from samples that will be made as a reference for the survival of bacteria and knowing the quality of water in the Selorejo Reservoir. In testing water parameters, pH, Nitrite, Nitrate and Ammonia levels were tested as shown in Table 1.

From the data obtained from the Nitrate content from the Selorejo Reservoir, it turns out that >0.2 mg / L which means that the waters of the Selorejo Reservoir have undergone Eutrophication, although Nitrate does not have high toxicity properties, but high nitrate levels can cause hyacinth growth which is difficult to control, on the other hand, nitrites levels can cause eutrophication (the state of *algae* growth which is difficult to control) and ammonia in the selorejo reservoir is quite high, this can cause disruption of the ability of fish and shrimp in the process of oxygen absorption because nitrites will react more strongly with hemoglobin from the body of fish and shrimp, as a result of which the mortality rate of shrimp and fish is getting higher [17], this is also strengthened by research data of Sayekti *et al.* (2015) [18] which says that the waters of the selorejo reservoir are in a mildly polluted state.

Microbes NGS Readings

Anammox bacteria today have been found in artificial ecosystems including *Cadindatus jettenia, Cadindatus brocadia, Cadindatus kuinenia stuttgartensis, Cadindatus scalindua,* and *Cadindatus anammoxo-globus* [19]. The results of the NGS reading of the abundance of microbes in the selorejo reservoir read 2141 microbes, consisting of 39 philums, namely *Proteobacter* 45.97%, *Firmicutes* 20.87%, *Acidobakter* 15.19%, *Plantomicetes* 8.446%, and a *Actianobakter* 3.116% and 6.408% other *phylums*. 73 classes namely *Alphaproteobacter* 21.69%, *Betaproteobakter* 7.551%, *Gammaproteobacter* 7.468%, *Deltaproteobacter* 8.675, *Bacilli* 15.41%, *Clostrida* 5.476%, *Acidobacter* 13.54%, *Vicinamidbacter* 2.107%, *Plantomycetia* 6.872%, *Citinophagia* %, and 1.373% other classes. 157 orders with 20 orders that have an abundance above 0.5%. From this data, a krona diagram is obtained as Figure 2.

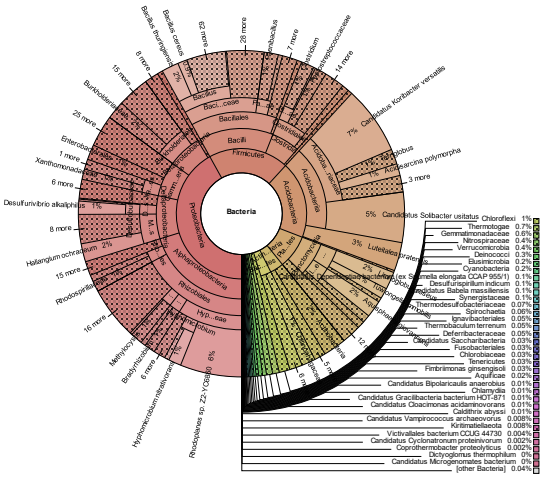

Figure 2. Microbial data from NGS.

Bacteria in the selorejo reservoir from samples tested with NGS (*Next Generation Sequencing*) have obtained results as Figure 2, the bacteria in the Selorejo Reservoir are dominated by bacteria from the proteobacter phylum, because the samples used are samples from sediments [20-21]. Proteobacter is a phylum that produces NOB bacteria (*Nitrite Oxidation Bacteria*), AOB bacteria (Ammonium Oxidation Bacteria) where these bacteria are very useful in the process of nitration or the process of oxidizing Nitrite to nitrates and nitrification or the change of ammonia into nitrites, in addition to that the Proteobacter phylum is also a donor of DB (*Denitrification Bacteria*) bacteria including P*seudomonas* and *Vibrio* which serves for the denitrification process or the process of reducing nitrates to nitrogen gas. The NOB group of bacteria is *Nitrospira, Nitrobacteria, Nitrotoga, Nitrococcus, Nitrospina, and Nitrolanceace* [22], the results of the NOB bacterial NGS test are Nitrobacteria and also Nitrospira found in *Betaproteobacter*. The AOB group of bacteria includes *Nitrosomonas, Nitrosococcus, Nitrospira, nitrosolobus*, and *Nitrosobrio*, with the results of NGS samples obtained *nirosococcus*, *Nitrospira*, and *Nitrosomonas* bacteria, as well as nitrogen fixation bacterium in the form of *Azoto-bacteriaceace*, *Cyanobacteria*, and *Clostridium*. Bacteria – these bacteria are useful in the nitrification process [22].

Anammox bacteria are the main microbes expected in the Anammox process (*Anaerobic Ammonium Oxidation*) from several Anammox bacteria that have been present in the Selorejo Reservoir obtained Anmmox bacteria from *Cadindatus kueninenia stutgartensis* amounting to 0.1% of the total bacteria in the Selorejo Reservoir shown in Figure 3, this is because bacteria of this genus often appear in sediments [20].

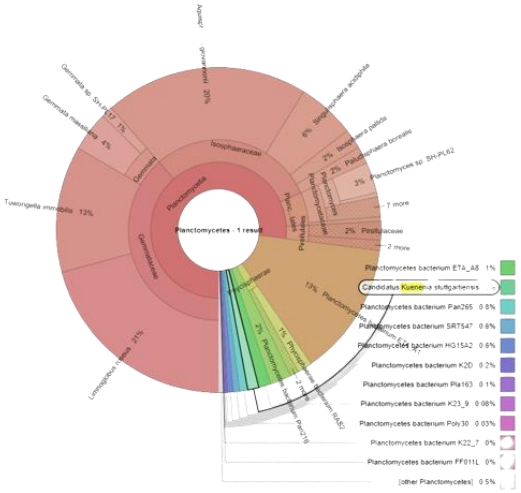

Figure 3. Anammox bacteria from NGS readings.

Anammox Microbes Activity in Reactor Bottle

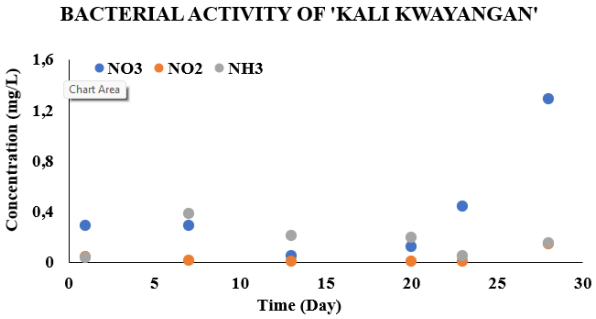

Figure 4. Bacterial activity

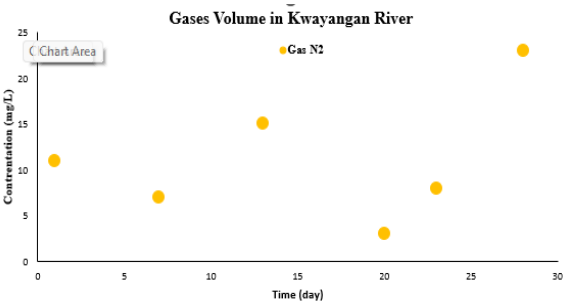

Figure 5. Bacterial activity of increased Nitrogen Gases

The picture above shows that on the first day which is the result of effluent after the operation of the reactor 24 hours and it is produced that the levels of NO_3, NO_2, NH_3 and Gas N_2 are quite high, this is due to the need for microbial adjustment in the reactor with the reactor environment containing synthetic waste with high nitrogen levels. On day 7 the sample decreased the nitrate, nitrite, and gas nitrogen, but ammonia increased due to the death of some microbes from philum proteobacter so that the yield of ammonia was high. On the 13th day there was a decrease in ammonia and nitrate levels as a result of the presence of denitrifying bacteria Pseudomonas and Vibrio in samples that produced nitrites by redefining nitrates in the absence of external carbon, while on the 20th day there was an increase in nitrates and gas nitrogen as well as a decrease in nitrite and ammonia levels by 0.143mg / L and 1,534mg / L as a sign of early division of Anammox bacteria. On the 23rd day, it showed the activity of Anammox bacteria which was characterized by an increase in nitrate and gan nitrogen levels in line with a decrease in ammonia and nitrite levels which are the main foods of Anammox bacteria, on day 28 the sample showed an increase in all parameters this occurred as a result of the color of the test sample being too brown even though it had been centrifuged for 30 minutes with a shock of 1500rpm. This results in a high UV-Vis spectrophotometer reading so that the concentration is high, because the more concentrated a test sample is, the result of the absorbent reading in the UV-Vis Spectrophotometer will be high.

East Java indiegen anammox bacteria produced from the Selorejo Reservoir managed to reduce Ammonia by 0.71% in a week, or 0.1% per day.

SUMMARY

Anammox indigen bacteria in East Java have been successfully found from the Selorejo Reservoir from *Cadindatus kuenineia stutgartensis* using NGS amounting to 0.1% of the total bacteria present. From 28 days of testing, it was found that the activity of reducing ammonia and nitrites levels and increasing nitrate levels, which is the working system of Anammox bacteria. Anammox Bacteria from this study reducing Ammonia by 0.71% in a week, or 0,1% per day.

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