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The Effect of Biofertilizer on The Diversity of N2O Reducing Bacteria in Paddy Fields of Sukabumi, Indonesia

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Abstract

Some of the methanotrophic bacteria and N_2O reducing bacteria have been proven to be able to support the plant growth and increase the productivity of paddy. However effect of the methanotrophic and N_2O reducing bacteria application as a biofertilizer to indigenous N_2O reducing bacteria is still not well known yet. The aim of this study was to analyze the diversity of N_2O reducing bacteria in lowland paddy soil based on a *nosZ* gene. Soil samples were taken from lowland paddy soils in Pelabuhan Ratu Sukabumi, West Java, Indonesia. There were two treatments for the paddy field soil, ie. biofertilizer-treated field 20% fertilizer (50 kg/ha) with the addition of biofertilizer and 100% fertilizer. PCR amplification of *nosZ* gene was successfully conducted using *nosZF* and *nosZR* primer pair. Denaturing Gradient Gel Electrophoresis (DGGE) process was conducted at 150 V for 5.5h. There were three differences *nosZ* bands were sequenced. The phylogenetic analysis showed that they were close to uncultured bacteria. Microbial diversity in the biofertilizer-treated field was higher than that of in the 100% fertilizer-treated field. The biofertilizer treatment has higher in microbial diversity than that of applied non-biofertilizer paddy fields. This research might have impact in the application of biofertilizers due to the emission of N_2O as a green house gas from paddy fields farming activity. The biofertilizer has great potential application in sustainable environmental friendly agriculture systems.

How to Cite

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INTRODUCTION

Application of inorganic nitrogen fertilizer by farmers over years tends to increase in order to improve soil fertility and agricultural production. The excessive application of N fertilizers likes NPK and urea may have negative impacts to the environment. This application lead to the increasing of N_2O (nitrous oxide) emission from the nitrification and denitrification processes. The agricultural landfield is become the largest source of N_2O emissions to the atmosphere (Smith *et al.*, 2010). N_2O has potential effect of greenhouse gas 310 times higher than CO_2 (Forster, 2007). The concentration of N_2O in the atmosphere has been increased by 20% since pre-industrial era (Montzka *et al.*, 2011). N_2O emissions are influenced by nitrogen fertilizer application rate. The number of applied nitrogen fertilizer are correlates with the increasing of N_2O emissions (MacKenzie *et al.*, 1998). Submerged paddy fields system provides anaerobic or oxygen limited condition supports denitrification process (Chen *et al.*, 2012).

Denitrification is gradual reactions to reduce nitrate into N_2 gas. Nitrate and nitrite are used by denitrifiers as an electron acceptor in a microoxic or anoxic environment (Knowles,1982). There are four important enzymes in the denitrification process, which are nitrate reductase which convert NO_3^- into NO_2^-, nitrite reductase which convert NO_2 to NO, nitric oxide reductase which convert NO to N_2O, and nitrous oxide reductase which convert N_2O into N_2. Uncomplete denitrification process generates the formation of N_2O gas as the end product of nitrate reduction (Canfield *et al.*, 2010).

The alteration of N_2O into N_2 need nitrous oxide reductase enzyme encoded by *nosZ* gene. Nitrous oxide reductase is the key enzyme in the N_2O reduction in the denitrification process (Jung *et al.*, 2013). This enzyme can be used to mitigate N_2O emissions because the enzyme is able to catalyze N_2O reduction into N_2 (Orellana *et al.*, 2014). The reduction of N_2O into N_2 in the terrestrial environments is done by denitrifiers using nitrous oxide reductase enzymes (NosZ) (Zumft & Kroneck, 2007). The potential of microbial communities to reduce N_2O into N_2 can be predicted by determination of *nosZ* genes abundance using metagenomic analysis (Henry *et al.*, 2006). Analysis of the microbial diversity in the ecosystem samples is important to improve the knowledge of metabolisms, biodiversity, genetic resources and understanding the function and roles of the microbes in the environments (Fakruddin & Mannan, 2013).

One of metagenomic approach which allows analyzing the diversity of microbial communities, especially denitrifier communities in paddy soil samples is *Denaturing Gradient Gel Electrophoresis* (DGGE) methods. The principle of this technique is a separation of same length DNA fragments that have different sequences of base pairs. The DGGE technique has been successfully used to determine the complex microbial community diversity (Muyzer & Smalla, 1998). The study on the diversity of denitrifying bacteria encoding *nosZ* gene in the paddy fields using the metagenomic approach in Indonesia is very limited. Therefore, this research aim was to determine the bacterial diversity of denitrifiers in paddy fields, based on the *nosZ* gene through metagenomic analysis approach. These research is expected becoming a part of mitigation leading to alleviate N_2O emission from paddy fields, so there are need study about the diversity of denitrifier community which able to reduce N_2O into N_2 due to nitrogen anorganic fertilizer applications. These research also expected becoming basic information in order to make biofertilizer contained denitrifier which able to reduce N_2O emission from paddy fields.

METHODS

Biofertilizer composition and soil sampling

Biofertilizer used was containing methanotrophic bacteria *Methylocystis rosea* BGM 1, *Methylocystis parvus* BGM 3, *Methylococcus capulatus* BGM 9, *Methylobacter* sp. SKM 14, and N_2O reducing bacteria *Ochrobactrum anthropi* BL2 (Table 1). Paddy cultivar used was IR64, which mostly applied by farmers in Java. The experiment was conducted in Citarik, Pelabuhan Ratu Sukabumi West Java, Indonesia. There were two treatments of biofertilizer or fertilizer in the experiment i.e. 20% dose of fertilizer (50 kg/ha fertilizer with biofertilizer) and 100% chemical fertilizer. The standard dose of fertilizer was according to Ministry of Agriculture Regulation (Permentan, 2007). Soil sampling was performed three times i.e. at 0 days after planting (DAP), 60 DAP and 120 DAP. The soil sample was taken randomly from 5 different location of paddy field and then mixed together become one part in order to get the DNA extract.

DNA extraction and quantification

Soil DNA extraction was conducted using the Soil DNA Extraction Kit (TIANGEN, Beijing, CN), following the protocol as suggested by the producer. Total DNA genome was extracted

Table 1. Reference of isolates which used as biofertilizer

Code of isolate	Source	Species	References
BGM1	Paddy field in Bogor	*Methylocystis rosea*	(Hapsari, 2008; Astuti, 2009)
BGM3	Paddy field in Bogor	*Methylocystis parvus*	(Hapsari, 2008; Astuti, 2009)
BGM 9	Paddy field in Bogor	*Methylococcus capsulatus*	(Hapsari, 2008; Astuti, 2009)
SKM14	Paddy field in Sukabumi	*Methylobacter* sp.	(Hapsari, 2008; Astuti, 2009)
BL2	Paddy field in Bogor	*Ochrobactrum anthropi*	(Setyaningsih, 2010)

then quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA).

Amplification of *nosZ* gene

The extracted DNA was amplified by PCR T1-Thermocycler (Biometra, Goettingen, DE). Amplification was performed using primer pairs of *nosZ*-1126F genes with GC clamps at the 5 'end and *nosZ*R. The sequences of *nosZ*-1126F (: 5 'CCG CCG CGC CGC GGC GCG GCGGCG GGG GGGGGGG-GGGCTBGGGCCRTT-GCA GCA CGG-3') and the sequence of the reverse *nosZ*R was 5'-GAAGCGRTCCTTSGA-RAACTTG-3' (Throback *et al.*, 2004). The PCR mix of 25μL was prepared containing 2μL primers with a concentration of 10 pmol, 12.5 μL of KAPA 2G Robust Hotstart Readymix 2× (KAPA Biosystems, USA), 4 μL of DNA template (~100 ng) and 6.5 μL of Nuclease-free water (NFW). The PCR conditions were used based on modification of the Touchdown PCR system with conditions of initial denaturation at 94 °C for 2 min denaturation at 94 °C for 1 min, annealing at 60 °C for 45 sec, elongation at 72 °C for 1 min, for 10 first cycles annealing temperatures was lowered 0.5°C per cycle, and to the continuing 25 cycles of PCR the start at 94 °C for 1 min, and annealing 53 °C for 45 sec, elongation 72 °C for 4 min. The expected size of PCR product is about ~500 bp. The PCR products were run on 1% agarose gel electrophoresis. Gel staining using ethidium bromide 0.1%, and then visualized using the G:BOX Gel Documentation (Syngene, Frederick, USA).

DGGE analysis of *nosZ* genes

DGGE was performed with D-Code Universal Mutation Detection System (Bio-Rad, Hercules, CA, US). A total of 25 μL of DNA template (20 μL + 5 μL PCR amplicon loading dye) was migrated on a 8% polyacrylamide gel. The 8% polyacrylamide was made from Acrylamide-Bisacrylamide (37:5). The denaturants used are urea and formamide. The denaturant gradient was 70% (high denaturant): 40% (low denaturant). 100% denaturant was made of a mixture of 7 M urea and 40% (v / v) formamide.

The DGGE process was carried out at 60 °C, voltage of 150 V for 5.5 h Polyacrylamide gel was stained with ethidium bromide (EtBr) of 0.1% for 15 min and visualized by using G: BOX Gel Documentation (Syngene, Frederick, USA). The images were then analyzed using G: BOX and CLIQS 1D software (Total Lab, GB). The DNA bands were then cut and put in 100 mL sterile nuclease-free water then eluted and stored at 4 °C overnight for further analysis.

Abundance based on OTU (Operational Taxonomic Unit) obtained from each treatment

OTU, which represent abundance of bacterial community determined based on the band volume of DGGE polyacrylamide gel which had analysed by CLIQS 1D software followed by determination of OTU by PAST 3 software

Reamplification of *nosZ* gene amplicon

A total of ~50 ng of the eluted DNA was amplified by using primer pairs *nosZ*F and *nosZ*R and without GC clamps. Mastermix PCR conditions were set up similar to the previous experiment. The DNA amplicons were sent to a laboratory providing sequencing services.

Phylogenetic Analysis

The sequences of *nosZ* gene amplicons were analyzed using ChromasPro (Technelysium, AU) for assembly and trimming process. The sequences were then compared to the database in GeneBank by using BLAST-N (Basic Local Alignment Sequence Tools for nucleotide) (blast.ncbi.nlm.nih.gov). Phylogenetic analysis was performed by using MEGA 6.0 software (Tamura *et al.*, 2013) and the phylogenetic tree was constructed using neighbor-joining method with 1000x bootstrap according to the Bayesian Information Criterion (BIC) score.

RESULTS AND DISCUSSION

DGGE profiles of *nosZ* gene diversity

The DGGE profiles of the samples show variety DNA bands indicated the differences of *nosZ* gene diversity. There were five thick bands

of *nosZ* gene amplicon that always appeared in all samples of DGGE. DGGE profiles of the samples showed variety DNA bands indicating the differences of *nosZ* gene diversity. Samples of biofertilizer application showed more diverse *nosZ* gene diversity indicating the diversity of N_2O reducing bacteria. Interpretation analysis trough CLIQS 1D program showed the increasing number of bands from the beginning day of planting (DAP) up to 120 DAP (Figure 1).

According to OTU obtained from each treatment showed that there were different type of *nosZ* gene abundance as shown in OTU diagram from the K0 to P120 treatment (Figure 2). The OTU of 0 DAP showed that P0 treatment had higher abundance and diversity compared with K0, at the 60 DAP showed that the abundance of K60 are higher than P60, but the diversity are lower in K60. In the 120 DAP as a last treatment showed that those high abundance in K120 but low in diversity compared with P120 that lower in abundance but higher in diversity. The most dominan OTU appeared in 11, 12, 2, 1, 6, 7 compared with other different bands which appeared in different percentage (Table 2).

Clustering analysis showed that diversity of N_2O reducing bacterial community during the vegetative phase of paddy was different with that of the generative phase. The diversity of N_2O reducing bacteria on 0 DAP of both the biofertilizer and 100% fertilizer treatment was very close resemblance, similar to the N_2O reducing bacteria diversity on 120 DAP of the both treatments.

There are three *nosZ* gene within the microbial genomes were successfully amplified using primers *nosZ* from seven chosen band derived from six paddy field soil samples. The BLAST-N of the three succesfully sequenced bands indicated that the nucleotide sequences had a close similarity with the *nosZ* gene sequence of uncultured bacteria (Table 3). These results were confirmed by the results of BLAST-X that the amino acid sequences were similar with that of nitrous oxide reductase enzyme (Table 4).

nosZ gene diversity of N_2O reducing bacteria.

The thickness of the bands showed the abundance of microbes, the thicker and clearer bands mean more abundant species, because the band in the gel will be formed when cell density of the species has at least 10^6 cells/g soil (Gelsomino *et al.*, 1999). The abundance of bacterial community which represent from visualization of band volume and the OTU result showed the fluctuation in each treatment (Figure 1). The abundance and diversity of bacterial commu-

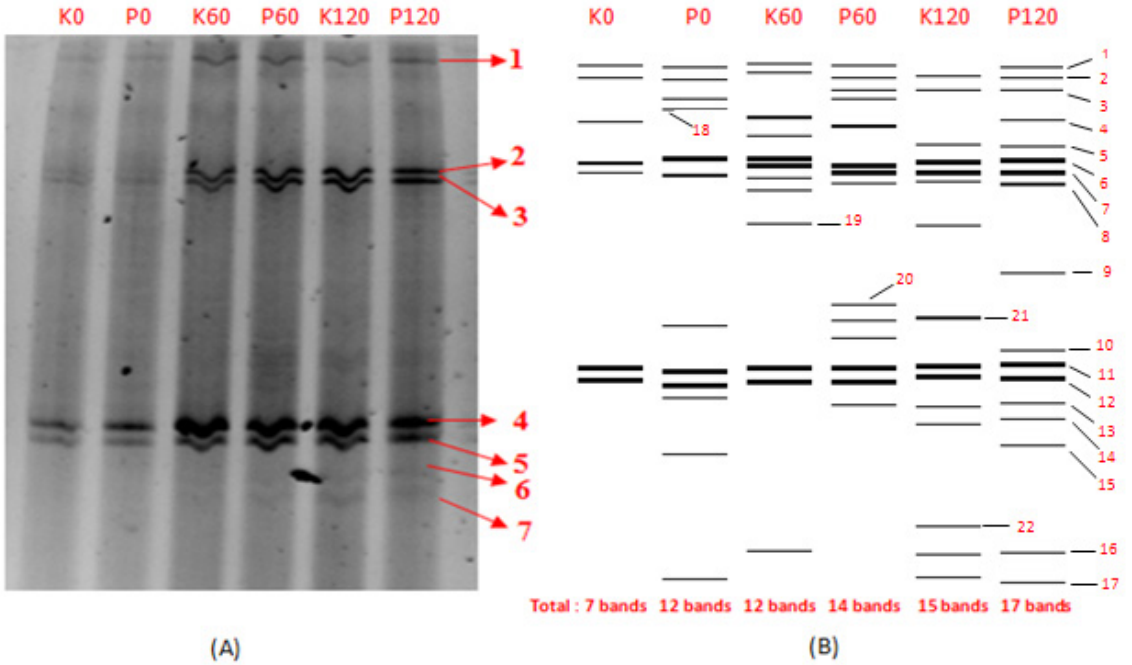

Figure 1. (A) *nosZ* gene pattern from DGGE profile with seven (red numbered) chosen band that encised for re-PCR. (B) Gel doc G: BOX photo result and interpretation based visualization on CLIQS 1D program (K0: control 0 DAP P0: treatment 0 DAP (day after plantation), K60: control 60 DAP, P60: treatment 60 DAP, K120: control 120 DAP, P120: treatment 120 DAP). The number of bands represent the variety species of microbial diversity types and OTU (Operational Taxononomic Unit) which obtained from each treatments

Table 2. Rank of the *nosZ* gene abundance based on OTU from soil sample

Treatment	OTU amount	OTU that frequently appeared in each treatment	Dominan OTU Percentage	Frequently Appeared Percentage
K0	7	11(38%),12(20%), 2(10%),1(9%),7(8%),6(9%)	11,12,2,1,7,6	94
P0	12	11(37%),12(14%), 7(8%),2(8%),1(8%)	11,12,7,1	75
K60	12	11(36%), 2(16%),1(12%), 12(10%), 6(9%),7(7,5%)	11,2,1,12,6,7	90,5
P60	14	11(25%),2(17,6),12(13%),7(11%),6 (8,5%),1(7,5%)	11,2,12,7,6,1	82.5
K120	15	11(28%),2(26%),7(14%),12(12%), 6(3%)	11,2,7,12,6	83
P120	17	11(27%),12(17%),7(15%),6(13%),2 (7,5%),1(7%)	11,12,7,6,2,1	86.5

Table 3. BLAST-N result and from re-PCR band

Description	E- value	Identity Percentage	Accession Number
Uncultured bacterium partial *nosZ* gene	8e-125	86	FR8658653.1
Uncultured bacterium isolate DGGE gel band PZ6E nitrous oxide reductase (*nosZ*) gene, partial cds	3e-157	88	AY577576.1
Uncultured soil bacterium clone JR125p4c20 nitrous oxide reductase catalytic subunit (*nosZ*) gene, partial cds	1e-142	88	DQ387547.1

Table 4. BLAST-X result and from re-PCR band

Description	E- value	Identity Percentage	Accession number
Nitrous oxide reductase uncultured denitrifying bacterium	1e-72	84	ACC76853.1
Putative nitrous oxide reductase (uncultured bacteria)	7e-62	92	ADR1049.1
Nitrous oxide reductase uncultured denitrifying bacterium	1e-81	91	ACC76853.1

nity might resulted from different growth phase of paddy plant. The diversity of N_2O reducing bacteria communities in paddy field soil tends to increase during vegetative phase of paddy plant growth, these were supported by clustering analysis result. However there were microbial diversity fluctuations in the soils affected by some factors such as soil physical and chemical properties, addition of fertilizer and soil treatment systems (Sylvia, 2005). The biofertilizer containing i.e. methanotroph bacteria and nitrate reducing bacterium *Ochrobactrum anthropi* were able to increase the growth of paddy plant. Bacterial isolates used has been shown to promote the growth of paddy due to their ability to fix N_2 (Pingak, 2013; Bintarti, 2014). Application of the biofertilizers that affecting microbial communities was found in the paddy field, denitrifying bacteria encoding *nosZ* gene.

The temporal shift of microbial community in the rhizosphere could be caused by organic molecules released from roots due to growth phase changes of paddy plant (Aulakh *et al.*, 2001). Root exudates of photosynthate compounds can provide nutrients to increase the microbial populations (Bai *et al.*, 2000). Paddy plants require nitrogen during vegetative phase until tillering and panicle formation stage (Dobermann & White, 1999). The surface soil condition more oxic at the end of planting due to decreasing of irrigation, but deep inside the soil is still anoxic. The increasing diversity of bacterial communities is probably because of at the end phase of growing paddy, the content of nitrogen-based nutrient in the soil is still abundant and partially absorbed by root plants which made increasing of bacterial encoding *nosZ* genes population due to denitrification process. In the other hand, application of this biofertilizer also decreasing levels of N_2O emissions from initial 24.51 to 6.99 mol/day/ha at the end of the treat-

ment which conducted at Sukabumi paddy fields soil (Sukmawati, 2016). This research showed the correlation between high bacterial diversity encoding *nosZ* with decreasing N_2O emissions from paddy fields. In the other hand, this biofertilizer composition also made high fluctuation diversity of *pmoA* gene encoding methane monooxygenase enzyme which consumes methane especially in vegetative phase of paddy growth (Sutanto, 2014). However in *nifH* gene diversity, both of those experiments in Sukabumi paddy field were no fluctuation of diversity and tend to be low in diversity (Hadianta, 2014). Both of those experiment were conducted in Sukabumi paddy field. The diversity of bacteria in the control treatment was high expressed, however it did not to determine to affect reduction of N_2O emission because the gene was not actively expressed. Whether the *nosZ* genes in the field actively expressed analyzed based on presence of mRNA of the genes can be done (Perez *et al.*, 2014).

From the shift of bacterial diversity of each sample, it can be assumed that the bacterial communities diversity at the vegetative phase is different from the diversity at the generative phase (Figure 2). Based on the phylogenetic tree analysis, there were found that three DGGE bands were close with uncultured bacteria (Figure 3).

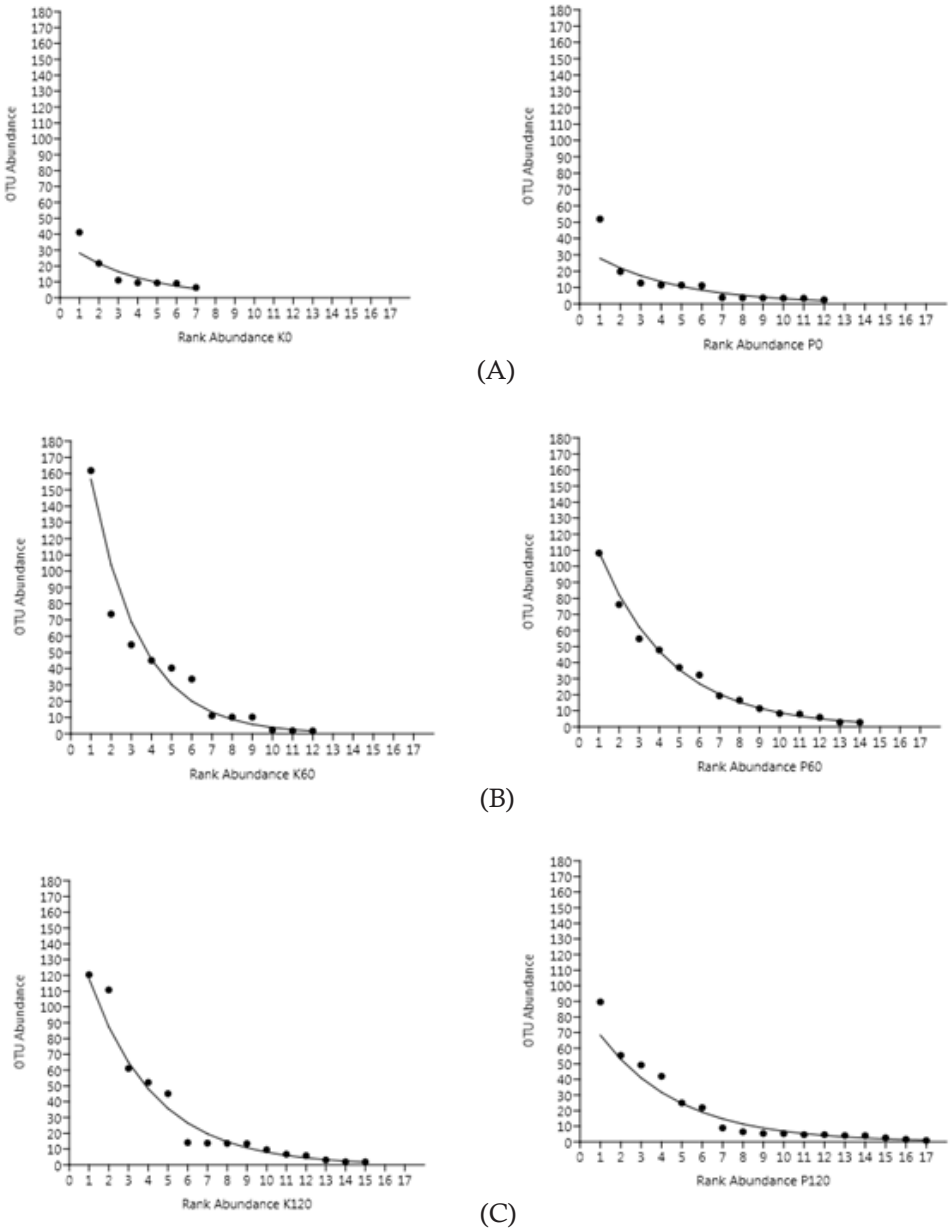

Figure 2. OTU diagram obtained from each day of treatment (A) 0 DAP, (B) 60 DAP, and (C) 120 DAP showing the curve that represent abundance of bacterial communities

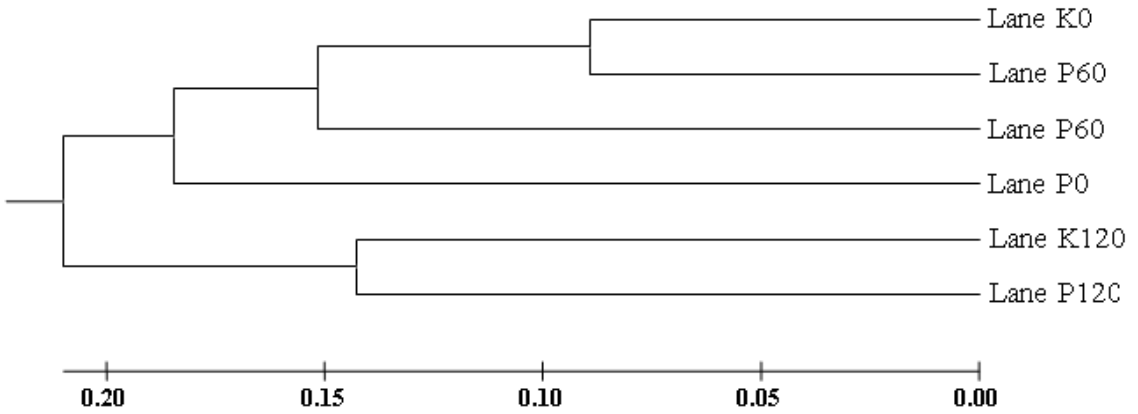

Figure 3. Clustering analysis based on each lane of DGGE polyacrylamide gel

This result also supported with BLAST-X analysis result which proves that the amino acid sequences were similar with that of nitrous oxide reductase enzyme. Based on BLAST-P result with amino acid sequences proved that protein domain was related with Tat conserved protein domain. The twin-arginine translocation (Tat) protein export system is present in the cytoplasmic membranes of most bacteria and archaea and has the highly unusual property of transporting fully folded proteins (Palmer & Berks, 2012). Tat in *nosZ* gene has function in the functional respiratory system of N_2O in the periplasmic compartment, Tat translocon is used to transport apoenzyme from cytoplasm to the periplasm.

CONCLUSION

There is variation in diversity of bacterial *nosZ* gene in paddy field soil samples taken from Pelabuhan Ratu, Sukabumi by using DGGE profiles. The majority of *nosZ* gene in the samples has similarity with uncultured bacteria. The *nosZ* gene detected diversity is higher in the biofertilizer application paddy fields. Similarly the diversity of the *nosZ* gene at generative phase is higher than that of at generative phase during paddy cultivation.

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