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Diversity and Kinetics of Bacterial Nitrification Isolated from Soil of Rubber and Oil Palm Plantation in Jambi Indonesia

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ABSTRACT

Nitrification is one of the important process in the nitrogen cycle. Nitrification is done by two groups of bacteria i.e. ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Land use change of forest to oil palm and rubber plantation would affect bacterial community. The aims of this study were to determine the diversity of nitrifiers in soil samples of oil palm and rubber plantation and to measure the kinetics of the nitrification rate activity of the isolates. The highest abundance of AOB and NOB was found in soil sample from oil palm plantations. A total of 74 AOB and 72 NOB was isolated from soil of rubber and oil palm plantations. From 74 AOB, one isolate C4.7 had the highest ammonium oxidation activity, it was up to 230.7 μM day^{-1}. While from 72 NOB, one isolate C14.14 had the highest nitrite oxidation, it was up to 42.2 μM day^{-1}. The kinetics of Vmax and Km values in this study were calculated using Lineweaver-Burk plots. The Vmax of ammonium oxidation of C4.7 isolates was of 24.3 μM mL^{-1} hr^{-1} with the Km was 161.8 μM. The Vmax of nitrite oxidation of C14.14 isolates was of 5.1 μM mL^{-1} h^{-1} with the Km was 100 μM.

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INTRODUCTION

Indonesia's tropical rainforest area in ranks third (after Brazil and Zaire) [32], but Indonesian forest area continues to decrease over time. Since 1996, Indonesia's deforestation has been the highest in the world, estimated at 2 million ha per year. Indonesia's oil palm plantation produced 44% of palm oil worldwide in 2011 and indicated the potential to double its oil palm area from 9.7 million ha in 2009 to 18 million ha by 2020 [15]. While the rubber plantations in Indonesia increased from 2001 to 2005 up to 8.7% [26].

Similar to other areas in Indonesia, Jambi also has a vast tropical rainforest that reached 2.1 million hectares [12]. However, it also includes the forest areas that have been converted. It means that the forests function has been shifted because these forests have had land management permit. Today, the oil palm and rubber plantation in Jambi Province has increased very significantly thus many forest areas are cleared [1]. Rubber and oil palm as plantation crops are export commodities that are able to contribute to the improvement of Indonesia's foreign exchange [7]. Increased of plantation crops production causes increase in land clearing per annum.

While oil palm and rubber plantation have caused an economic boom in the producing regions, it has also attracted criticism on environmental. The oil palm and rubber as a plantation tree is held responsible to deforestation, increased greenhouse gas emition, and reduction of biodiversity by fragmentation, disturbance, and destruction of natural habitats [17,21]. Futhermore Fu *et al.* [16] reported that land use change has drastically lowered the availability of N, P, and K elements shortly after forestland logging. Larisa *et al*. [22] reported a change in the composition of bacterial communities on forest land which has been converted into oil palm plantations. Handayani and Prawito [19] also reported that the diversity and population of soil microflora which include bacteria, fungi, and *Actinomicetes* are higher in forestland compared to land that has been deforested. Furthermore, change in forest land function into oil palm plantations shows the change of soil chemical properties such as pH, organic C, cation exchange capacity, total N, and organic substances [25].

Hadianta [18] and Masrukhin [24] have studied how the abundance and kinetics rates of nitrification, denitrification, and DNRA on land that has undergone land use change in Jambi's forests, but they did not use pure culture isolate, thus it becomes interesting to be studied. Nitrification as one of the processes that play an

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important role in the nitrogen cycle in nature is also suspected to undergo a change due to land use change. Nitrification is an oxidation process that occurs in the consortium, involving two different groups of chemolithotroph bacteria which are ammonium-oxidizing bacteria (AOB) that catalyze the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), the process is called nitritation and NO_2^- oxidizing bacteria (NOB) that catalyze the oxidation of NO_2^- to nitrate (NO_3^-), the process is called nitratation [13]. Nitrification bacteria are generally considered to be obligate chemolithoautotrophs, which means that nitrification is their only source of energy and they can only acquire C through fixation. The coupling between nitrification need yields energy to the nitrifying microorganisms and growth of the nitrifiers (assimilation of C, N, etc. into new microbial biomass, which requires energy) in natural environments [34]. The aims of this study were to analyze the diversity of isolates on land that has been undergone land use change into oil palm and rubber plantations and measure the activity of nitrification kinetics rate in isolates that have the highest nitrification activity so that is expected to be input for government policy making in permitting the opening of palm oil plantation and rubber.

Methods:

Isolation of Nitrifying Bacteria:

The sample was originated from Lubuk Kepayang Village, Air Hitam District, Sarolangun Regency with geographically located 102^o 03’ 39”-103^o 13’ 17” East Longitude and 01^o 53’39”-02^o 46’24” South Latitude, code LK and Bungku Village, Bajubang District, Batanghari Regency with geographically located 102^o 30’-104^o 30’ East Longitude and 1^o 15’-2^o 20’ South Latitude, code Bungku, Jambi Indonesia. The isolation process of nitrifying bacteria was conducted by direct spread plate method using AOB and NOB media [6]. Bacteria were incubated for 6 days at 31^{o}C. After 6 days, the number of colonies was calculated using TPC on plates containing colonies between 30-300 *cfu* mL^{-1}. Each separated colony with different appearance was purified by the quadrant method using AOB and NOB media to obtain pure single colony. Pure single colony was then Gram staining tested.

Selection of Nitrifying Bacteria:

Selection of AOB and NOB was conducted by preparing 6 day old isolates on AOB and NOB broth media. Broth culture media were centrifuged in 12 x 1.5/2 mL rotor, *Microcentrifuge MiniSpin*® with 12000 rpm rotor speed for 15 minutes to obtain supernatants. Furthermore, supernatant samples were measured ammonium and nitrite oxidation activity using Eaton *et al*. [14] method. Isolate with highest ammonium and nitrite oxidation activities would be examined for its kinetics of nitrification activity.

Kinetics of ammonium and nitrite oxidation activities:

Inoculum for the test was prepared by growing the isolates in 50 mL AOB and NOB broth media, then incubated in the shaking incubator at 80 rpm and 31^{o}C for 6 days. Bacterial culture was made into pellets with centrifugation at 12000 rpm for 15 minutes. Pellets were separated and resuspended with AOB medium without ammonium and NOB medium without nitrite. 2 mL of bacterial culture was inoculated into 50 mL AOB media with ammonium concentrations of 500, 1000, 1500 and 2000 µM while NOB media with nitrite concentrations of 100, 500, 1000, and 1500 µM. After that, they were incubated for 6 days in the shaking incubator at 80 rpm and 31^{o}C. Each treatment was repeated three times. Control of each treatment was made without bacterial culture inoculation. Measurement of ammonium and nitrite oxidation activities was conducted after 12 hours by the analysis of ammonium and nitrite Eaton *et al*. [14]. The basic of kinetics of ammonium and nitrite oxidation was calculated with Michaelis-Menten kinetics equation using the Lineweaver-Burk plots [37].

Analysis of Ammonium Content:

Ammonium concentration was determined by spectrophotometric method. Broth culture medium was centrifuged at 12000 rpm for 10 minutes to obtain supernatant. 2 mL of supernatant was added with 0.08 mL of 11.1% phenol-alcohol, 0.08 mL of 0.5% nitroprusside, and 0.2 mL of mixture 20% of technical hypochlorite and alkaline citrate (1:4), then allowed to stand for 1 hour in a dark room. After each reagent addition, the mixture was stirred with vortex. Reagent addition would produce blue color. The color formed was then read for its absorbance at 640 nm wavelength [14]. The absorbance values obtained were converted using standard curve to obtain the concentration units (µM).

Analysis of Nitrite Content:

Nitrite concentration was determined by spectrophotometric method. Broth culture medium was centrifuged at 12000 rpm for 10 minutes to obtain supernatant. 2 mL of supernatant was added added with 0.08 mL of nitrite reagent and allowed to stand for 10 minutes. After reagent addition, the mixture was vortexed. Reagent addition would produce pink to purplish color. The color formed was then read for its absorbance at 540 nm wavelength [14]. The absorbance values obtained were converted using standard curve to obtain the concentration units (µM).

RESULTS AND DISCUSSION

Results:

The abundance of AOB and NOB:

The abundance of AOB in oil palm and rubber plantations was divided into two soil depth strata, namely 5 and 10 cm depth strata (Fig 1 and 2). Generally, all 5 cm stratum soil samples showed higher abundance of AOB and NOB compared to 10 cm stratum and total abundance of AOB and NOB in 5 cm and 10 cm strata of soil samples from oil palm plantation have higher abundance of AOB and NOB (Fig 1 and 2) compared to soil samples from rubber plantation.

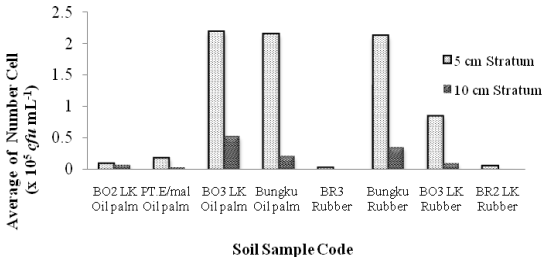


Fig. 1: The average abundance of AOB (Ammonium Oxidizing Bacteria) in the soil originates from oil palm and rubber tree plantations.

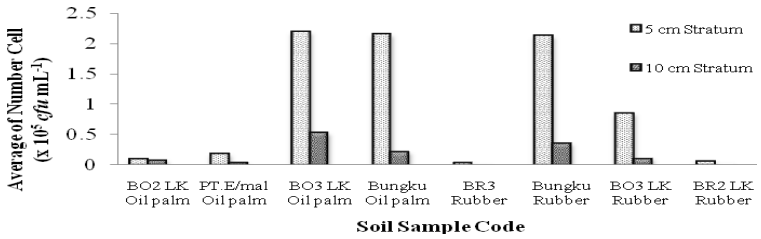


Fig. 2: The average abundance of NOB (Nitrite Oxidizing Bacteria) in the soil originates from oil palm and rubber plantations.

Isolation and Morphological Characterization and Oxidation Activity of AOB and NOB:

146 isolates derived from rubber and oil palm plantations were isolated, consisted of 74 AOB and 72 NOB. The number of isolates that represented every soil strata was diversely originated from each soil sample (Table 1).

According to ammonium oxidation activity, there was one isolate suspected as AOB which grown on autotrophs media. Isolate with the highest ammonium oxidation activity (230.7 μM day^{-1}) was obtained from the activity of C4.7 AOB isolate (Table 2).

According to nitrite oxidation activity, there was one isolate suspected as NOB which grown on autotrophs media. Isolate with the highest nitrite oxidation activity (42.2 μM day^{-1}) was obtained from the activity of C14.14 AOB isolate (Table 3). According to ammonium and nitrite oxidation activities, there were 2 isolates suspected as AOB and NOB which grown on autotrophs media. Isolates with the highest ammonium and nitrite oxidation activities were then characterize their morphological features using the Gram staining (Table 4).

Table 1: Isolation results and selection of AOB and NOB activities in oil palm and rubber plantations.

No	Soil sample code	Number of isolate				Average of Oxidation activity (μM day^{-1})			
		AOB		NOB		Ammonium (AOB)		Nitrite (NOB)	
		Soil Depth Strata (cm)				Soil Depth Strata (cm)			
		5	10	5	10	5	10	5	10
1	Bungku Rubber	-	15	1	11	-	37.7	35.6	32.7
2	BO2 LK Oil Palm	7	8	13	3	79.4	64.5	34.7	35.4
3	PT.E/mal Oil Palm	5	5	8	5	69.7	34.6	32.8	33.1
4	BO3 LK Oil Palm	1	7	3	1	7.9	26.6	36.9	33.4
5	Bungku Oil Palm	1	2	7	3	5.7	125.7	33.7	36.7
6	BR3 Rubber	4	6	3	2	21.8	56.3	33.0	36.5
7	BO3 LK Rubber	6	-	8	4	108	-	35.9	29.1
8	BR2 LK Rubber	1	6	-	-	1.4	40.5	-	-

Table 2: Five AOB isolates from oil palm and rubber plantations with the highest ammonium activities.

No.	Soil sample code	Isolate code	Ammonium oxidation activity (μM day^{-1})
1	BR2 LK Rubber 10	C3.8	185.0
2	BO3 LK Rubber 5	C4.7	230.7
3	BO2 LK Oil palm 5	C4.9	157. 9
4	PT.E/mal Oil palm 5	C9.6	230.0
5	BO3 LK Rubber 5	C9.10	184.3

Table 3: Five NOB isolates from oil palm and rubber plantations with the highest nitrite activities.

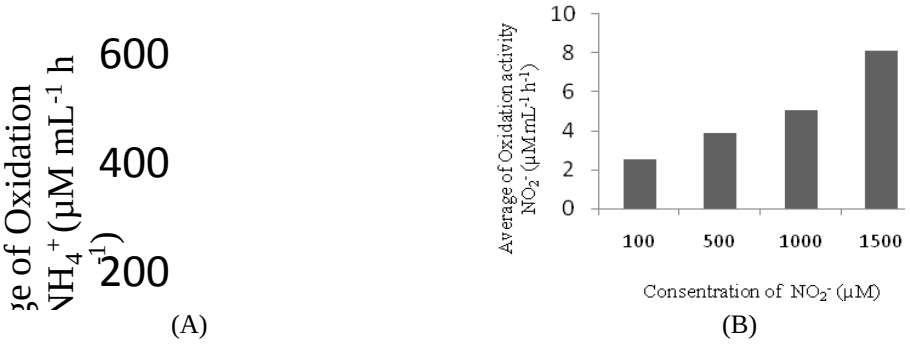
No.	Soil sample code	Isolate code	Nitrite oxidation activity (μM day^{-1})
1	BO3 LK Rubber 10	C10.1	41.0
2	BO2 LK Oil palm 5	C10.3	41.0
3	PT.E/mal Oil palm 10	C11.3	41.4
4	BO3 LK Rubber 5	C11.6	40.6
5	Bungku Oil palm 10	C14.14	42.2

Table 4: Morphological characterizations of AOB and NOB.

Isolate Code	Bacterial Morphology				Stain Gram	Cell shape
	Elevation	Colony peripheral	Shape	Colour		
C4.7	raised	Flat and firmly	Medium round	Pale yellow	Negative	Short rod
C14.14	Convex	Flat and firmly	Small round	Yellowish beige	Negative	Rod

Kinetics of Ammonium and Nitrite Oxidation Activity:

According to kinetics analysis using Lineweaver-Burk plots, on AOB C4.7 isolate was found the decrease rate of maximum ammonium compound (Vmax) at 24.3 μM mL^{-1} h^{-1} with Km value of 161.8 μM. While on the kinetics analysis of NOB C14.14 isolate it was showed the decrease Vmax at 5.1 μM mL^{-1} h^{-1} with Km value of 100 μM.



(A) (B)

Fig. 4: The relationship between the concentrations of different substrates with oxidation rate (A) C4.7 and (B) C14.14 isolates.

Discussion

The high rate of deforestation and forest degradation as a result of land use change has reduced the role of tropical forests in Indonesia. Situmorang *et al*. [30] reported that during 2009-2010, Indonesia has lost 0.48 million ha of forest. Decreased of canopy cover, understorey, brown waste (*serasah*), also plant roots and microbial diversities are affected by forest use change into rubber and oil palm plantations. Soils in Jambi are generally dominated by ultisols that have low fertility nature, characterized by acidic pH (3.1-5), <35% alkali saturation, low cation exchange capacity (<16 cmol/kg), and high Al saturation ranges from 37-78% which is very vulnerable to the N loss due to leaching [33].

The abundance of AOB and NOB on each soil sample (Table 1 and 2) was divided into two soil strata, namely 5 and 10 cm soil depths. Abundance of AOB and NOB on each soil samples was different, generally at 5 cm depth each soil sample has higher abundance than at 10 cm depth. Oxygen availability was suspected as one of the causes of the abundance. Nitrifying bacteria use ammonia/ammonium and nitrite as an electron donor and oxygen as a final electron acceptor [29] thus the oxygen presence becomes one of the prerequisites for an optimum growth of nitrifying bacteria.The highest abundance of AOB and NOB in soil samples was obtained from oil palm plantation. The soil pH was suspected to influence the activity of nitrifying bacteria. Soil pH below 6.5 causes slow nitrification activity [27]. Agustiyani *et al*. [3] also reported that ammonium oxidizing bacteria have an optimum growth activity between pH 7-8. More acidic soil reaction occurs on rubber forest and rubber tree plantation in Jambi than natural forest. However, the pH of soil in palm oil plantation is higher

than the land planted with rubber trees, even compared to the forest. Low base leaching and intensive liming action is probably cause [33]. In addition, less abundant nitrifying bacteria also can be caused by several factors, such as secondary metabolites compounds derived from root exudates and dissolved substances in the soil such as nitrapyrin, acetylene, chlorate and cycloheximide which can inhibit the growth of nitrifying bacteria [11,35]. Rubber trees can produce monoterpenes as secondary metabolite [39]. As one the group of terpenes, monoterpenes ($C_{10}H_{16}$) can impact interactions between organism including soil organism [5] Monoterpenes can inhibit net mineralization of nitrogen and net nitrification [37]. Neem oil extracted from the *Azadirachta indica* leaves can also inhibit the nitrification process [4].

There were 74 AOB and 72 NOB that isolated from the soils of rubber and oil palm plantations. Among 74 AOB C4.7 isolates, there was one isolate which has the highest ammonium oxidation ability at 230.7 μM day^{-1} of the total ammonium added to the media. While among 72 NOB C14.14 isolates, there was one isolate which has the highest nitrite oxidation ability at 42.2 μM day^{-1} of the total nitrite added to the media. The low ability of ammonium and nitrite oxidation can be caused by a limited substrate in the soils that have undergone land use changes.

Reduction content of organic matters tends to occur on soil of land use change. Total organic matters content decreased successively from the forest, into the rubber forest, rubber tree plantation, and the lowest is in the oil palm plantation [33]. In addition, Cesylia [10] also reported that carbon stock in the monoculture rubber forest tends to be lower compared to the natural forest. Changes in this organic matter content are clearly visible, especially in the top soil layer (0-10 cm). Degradations of soil organic matters after 4 years on rubber tree plantation and palm oil plantation are 0-30% and 0-40%, respectively [8].

Microscopic morphology of C4.7 isolate after Gram staining showed Gram negative, rod-shaped bacteria. Ammonia-oxidizing bacteria belong to Gram negative with rod-shaped cells (length 0.6-4 μm), ellipsoid, spherical, and spiral. Cells are non-motile and motile with polar to subpolar or peritrichous flagella. All species activities run under aerobic conditions, the optimum growth temperature of 25-30ºC, inactive at of 4ºC, the optimum pH ranges from7.5-8.0, colonizing on the media such as gravel, sand, and other synthetic media, requiring oxygen to convert inorganic compounds as a source of energy, and require CO_2 as a carbon source. Its reproduction ratio is very slow (generation time at 20-40 hours) [20]. While C14.14 isolate is a Gram negative rod shaped bacteria. Nitrite-oxidizing bacteria is a rod shaped, pleomorphic, Gram negative, and usually non-motile [20]. The growth of NOB is slow; the generation time varies from 8 hours to several days. The growth rate is controlled by the substrate concentration, temperature, pH, light, and oxygen concentration. Most of the NOB grow well at nitrite concentrations between 2-30 mM, pH 7.5-8.0 and 25-30ºC [31].

Nitrification activity carried out by AOB and NOB is due to the role of the enzyme catalyzing the activity. AOB have two important enzymes, namely ammonium monooxygenase (AMO) which is located in integral membrane and plays role in oxidizing NH_4^+/NH_3 into NH_2OH (hidroxylamine). Furthermore, there is hydroxylamine oxidoreductase (HOA) enzyme, located in the periplasmic membrane which has a role in transforming NH_2OH into NO_2^-, whereas NOB use nitrite oxidoreductase enzymes to oxidize NO_2^- into NO_3^- [23]. These three enzymes can catalyze reactions at a rate of millions of reactions per second. Reaction rate depends on the solution condition and substrate concentration. An increase in the substrate concentration tends to increase its activity. To determine maximum rate of an enzymatic reaction, substrate concentration is increased until the rate of product formation is constant.

An increase in the substrate concentration will increase V (rate) until the maximum velocity/speed value (Vmax) achieved (Fig 4) . If further increase in substrate concentration did not increase the reaction rate, enzyme is considered as saturated by the substrate. Michaelis-Menten equation shows the relationship between initial reaction velocity [V] with the substrate concentration [S]. Km value can be used in determining the affinity level of enzyme-substrate (ES) and is an indicator of E-S complex bond strength or an equilibrium constant for the dissociation of E-S complex into E and S [28]. Small Km value means steady E-S complex and high enzyme affinity to the substrate, vice versa. Km value of the enzyme varies greatly depending on the type of substrate, environmental conditions and ionic strength [38].

Vmax of C4.7 isolate was 24.3 μM mL^{-1} h^{-1}, which means that during the optimum condition, HOA and AMO enzymes could convert NH_4^+ substrate into NO_2^- as much as 24.3 μM mL^{-1} per hour. Whereas the Vmax of C14.14 isolate was 5.1 mM mL^{-1} h^{-1}, which means that during optimum condition, nitrite oxidoreductase enzyme could transform NO_2^- substrate into NO_3^- as much as 5.1 μM mL^{-1} per hour. Vmax of C4.7 AOB isolate was greater than C14.14 isolate, thus if both isolates were cultured in the same environment, nitrite accumulation would occur due to the non-occurenceof equilibrium between these two processes. If the accumulation of nitrite was occured, the nitrite ions would be absorbed into the blood, and when they were in contact with erythrocytes, nitrite would oxidize Fe^{+2} in hemoglobin (Hb) into Fe^{3+} to form methaemoglobin (MetHb). 30-40% of MetHb contents in the blood can cause clinical symptoms, and if its contents reach 80-90%, it will cause death in livestock, fish, and humans [9]. In addition, nitrite that goes into the human body can lead to the formation of N-nitrosamines which can cause cancer in the digestive tract [2].

Km is the substrate concentration required by an enzyme to reach half of its maximum rate. Each enzyme has a different Km value for each substrate, and this fact can show the binding strength of the substrate to the enzyme. Km values obtained in this study for C4.7 and C14.14 isolates were 161.8 μM and 100 μM, respectively. On C4.7 and C14.14 isolates, nitrite oxidoreductase enzyme, AMO, and HOA have low enzyme affinity to NO_2^- substrate thus the reaction equilibrium was directed to the formation of enzyme-substrate complex resulting in a low yield of product.

Conclusion:

The highest abundance of AOB and NOB on area that has undergone changes in land use, originated from oil palm plantation on 5 cm stratum depth from the soil surface. 74 AOB and 72 NOB were isolated from the rubber and oil palm plantations. Among 74 AOB C4.7 isolates, there was one isolate which has the highest ammonium oxidation ability at 230.7 μM day^{-1} of the total ammonium added to the media. While among 72 NOB C14.14 isolates, there was one isolate which has the highest nitrite oxidation ability at 42.2 μM day^{-1} of the total nitrite added to the media. The maximum rate (Vmax) of C4.7 isolate was 24.3 μM mL^{-1} h^{-1} with Km value of 161.8 μM while the maximum rate (Vmax) of C14.14 isolate was 5.1 μM mL^{-1} h^{-1} with Km value of 100 μM.

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