MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 7, No 2, June 2013, p 45-50

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.7.2.1

Nitrous Oxide Reduction Activity of Denitrifying *Ochrobactrum anthropi* Isolated from Rice Field

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Nitrous oxide (N_2O) is one of the principal greenhouse gases. Differences in soil microbial community composition affect N_2O emission. *Ochrobactrum anthropi* BL1 and BLN1 isolated from rice field in Tangerang, Banten, Indonesia can grow on and reduce N_2O. This study investigated the patterns of N_2O reduction activity and growth of *O. anthropi* BL1 and BLN1 on denitrification media and also examined the ability of BLN1 strain to reduce N_2O in flooded rice soil. Nitrous oxide reduction activity and growth of strains BL1 and BLN1 occurred simultaneously, indicating that the bacteria used N_2O for growth. BL1 and BLN1 showed the same specific growth rate, but the N_2O reduction rate of BLN1 was higher than that of BL1. Increase of the N_2O concentration in the surface water of flooded soil without BLN1 isolate six hours after the addition of NO_3^- was significantly greater than the surface water from soil that had been inoculated with the isolate.

Key words: N_2O, *Ochrobactrum anthropi*, reduction, rice field

Dinitrogen oksida (N_2O) merupakan salah satu gas rumah kaca utama. Perbedaan komposisi komunitas mikrobia dalam tanah mempengaruhi emisi N_2O. *Ochrobactrum anthropi* BL1 dan BLN1 yang diisolasi dari sawah di daerah Tangerang, Banten, Indonesia dapat tumbuh dengan mereduksi N_2O. Penelitian ini mengkaji pola pertumbuhan dan aktivitas reduksi N_2O dari *O. anthropi* BL1 dan BLN1 dalam media denitrifikasi serta kemampuan galur BLN1 mereduksi N_2O di tanah sawah tergenang. Aktivitas reduksi N_2O dan pertumbuhan galur BL1 dan BLN1 berlangsung serentak, menunjukkan bahwa bakteri menggunakan N_2O untuk tumbuh. Nilai kecepatan pertumbuhan spesifik antara BL1 dengan BLN1 sama, sedangkan kecepatan reduksi N_2O galur BLN1 lebih tinggi dari pada BL1. Peningkatan konsentrasi N_2O dalam air permukaan tanah tergenang setelah enam jam penambahan NO_3^- di tanah tanpa isolat BLN1 lebih besar secara signifikan dari pada di tanah yang ditambah isolat.

Kata kunci: N_2O, *Ochrobactrum anthropi*, reduksi, sawah

Denitrification is defined as the dissimilatory reduction of nitrate (NO_3^-), or nitrite (NO_2^-) to nitrous oxide (N_2O) or nitrogen gas (N_2) (Hayatsu *et al*. 2008). Denitrification is part of the bioenergetic apparatus of bacterial cell. The complete denitrification leads to N_2 formation while the incomplete process produces N_2O (Zumft 1997). Nitrous oxide is one of the principal greenhouse gases. Concentration of N_2O in the atmosphere increased from 270 in pre-industrial era to 319 ppb in 2005. The primary driver of N_2O increase is microbial production in expanding and fertilized agricultural land (Forster and Ramaswamy 2007). Differences in soil microbial community composition affect N_2O emission (Holtan-Hartwig 2000).

The species *Ochrobactrum anthropi* was described first by Holmes *et al*. (1988) as the sole species of *Ochrobactrum*. Since then, some other species of *Ochrobactrum* had been discovered (Kämpfer *et al*. 2007). *O. anthropi* is a Gram negative, rod shaped, motile, and obligately aerobic bacteria (Holmes *et al*. 1988). *O. anthropi* are widely distributed in diverse habitat including human body (Holmes *et al*. 1988), activated sludge taken from reclaimed land (Song *et al*. 2002), rice field water (Reche and Fiuza 2005), and wastewater treatment plant (Zuo *et al*. 2008). Some *O. anthropi* strains have been identified as denitrifier (Sung *et al*. 2002; Doi *et al*. 2009). Nevertheless, N_2O reduction activities of these strains have not been investigated.

Setyaningsih *et al*. (2010) reported that *O. anthropi* BL1 and BLN1 could grow on N_2O as a sole electron acceptor. In 5 days, BL1 and BLN1 reduced N_2O up to

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4.09 and 3.91 μmol mL^{-1} cultures respectively. To confirm and further characterize N_2O reduction capacity of the isolates, this study investigated the patterns of N_2O reduction activity and growth of *O. anthropi* BL1 and BLN1 on denitrification media. This study also examined the ability of BLN1 strain to reduce N_2O in flooded rice soil. N_2O reducing denitrifier maybe able to be used as N_2O emission controller in rice field.

MATERIALS AND METHODS

Bacterial Strains and Media. The bacterial strains used in this study were isolated from rice field in Tangerang, Banten, Indonesia and have been characterized previously (Setyaningsih *et al*. 2010). Experiments were conducted using denitrification media containing $CH_3COONa \cdot 3H_2O$ 2.72 g L^{-1}, K_2HPO_4 0.80 g L^{-1}, KH_2PO_4 0.30 g L^{-1}, NH_4Cl 0.40 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.40 g L^{-1} (Barford *et al*. 1999), and yeast extract 3 g L^{-1}.

Soil. Soil samples were obtained from the top 20 cm of a paddy field. The soil was air-dried, broken up, sieved (2 mm) and thoroughly mixed. The particle size distribution was 51 % clay, 34 % silt, and 15 % sand.

Nitrous Oxide Reduction Activity and Bacterial Growth Patterns. N_2O reduction activity and bacterial growth patterns on denitrification media were observed using N_2O as a sole terminal electron acceptor. Experiments were carried out in anaerobic condition with 10.1 mL sterile media in the 20.1 mL test tubes with rubber stoppers. Media were flushed with N_2 for 10 minutes. N_2O was injected into each test tube. N_2O concentration dissolved in the media at the beginning of the experiment was 12.75 mM. Inoculum was prepared by anaerobic growth in media supplemented with $NaNO_3$ (1.275 g L^{-1}). Cells from exponential phase inoculum culture were twice washed by centrifugation in sterile distilled water and resuspended in fresh media without NO_3^-. After inoculation, cultures were incubated at 30 °C with rolling. Periodically, N_2O were sampled from the headspace of test tubes by gas-tight syringes and measured by gas chromatography with a Shimadzu 14A ECD gas chromatograph equipped with Q Porapak column. Carrier gases were methane (5%) and argon (95%) and the operating temperatures were as follows; detector 340 °C, column 35 °C, injector 200 °C. Growth of bacteria was monitored based on the culture's turbidity using nephelometer EEL Unigalvo DS29.

N_2O concentration in solution was calculated with the formula: $y = \alpha * x$ (solution volume/headspace volume), where y was amount of the dissolved N_2O (μmol) in a closed system, α was solubility of N_2O expressed as dissolved N_2O (mL N_2O mL^{-1} H_2O), and x was amount of N_2O in the flask headspace (μmol) (Carter 1993). The specific growth rate was calculated with the formula $\mu = (\Delta \ln N)/\Delta t$, where N was the cells number per milliliter and t was the corresponding time (hours) (El Hassan *et al*. 1985). Reduction rate of N_2O (v_{red}) was calculated by the analogous equation $v_{red} = \Delta S/\Delta t$ where S was dissolved N_2O concentration.

N_2O Reduction in Rice Soil. N_2O reduction in rice soil experiment was carried out using rice soil flooded with distilled water in close system. Two hundreds and twenty grams of soil was filled into the 603 mL bottle with rubber stopper. Soil thickness in the bottle was 6 cm. Soil was flushed with N_2 for 15 min to minimize the residual NO_3^- and incubated for 2 d. Flushing the soil with N_2 was repeated to remove the N_2O that might have been produced from residual NO_3^-. Distilled water was added up to 2 cm above the soil surface. The flooded soil was incubated for 7 d with the bottle cap opened to mimic the rice field condition. The soil pH was measured. 0.6 mmol NO_3^- was added as $NaNO_3^-$. *O. anthropi* BLN1 inoculum was added as suspension in sterile distilled water (5 x 10^8 CFU). After NO_3^- and inoculum addition, the bottle was closed with rubber stopper. Control was prepared without bacterial inoculum. The headspace gas was sampled every 3 h to determine N_2O concentration. After gas sampling from the headspace, bottle cap was opened. Surface water sample for dissolved N_2O analysis was taken using syringe and transferred into test tube containing 100 μL of formaldehyde (38% (v/v)) and capped with rubber stopper. Water samples in the test tubes were shaken vigorously and N_2O concentration in the headspace was measured using Shimadzu 14A ECD gas chromatograph with Q Porapak column. Carrier gas used was N_2 and the operating temperatures were as follows; detector 350 °C, column 60 °C, and injector 150 °C. The emission value was calculated by subtracting the N_2O concentration in the headspace at the certain time by the initial N_2O concentration .

RESULTS

Nitrous Oxide Reduction Activity and Bacterial Growth. Fig 1 reported the N_2O reduction activity and growth of BL1 and BLN1 bacterial strains. The slopes of curves indicate the specific growth rate (μ) and N_2O reduction rate (v_{red}) (Table 1). BL1 and BLN1 strains showed the same μ. However BLN1's v_{red} was higher

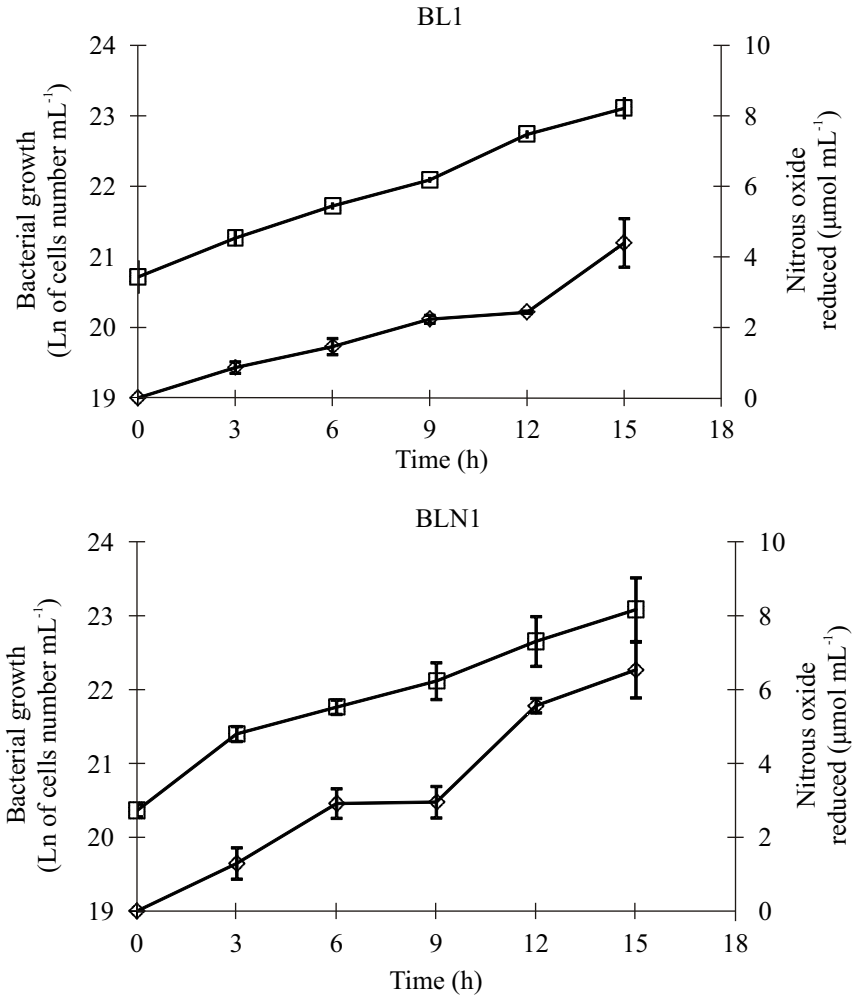

Fig 1 Nitrous oxide reduction activity and bacterial growth of strains BL1 and BLN1. Bars represent standard error, calculated from three experiments. -□-:bacterial growth; -◇-:nitrous oxide reduced.

Table 1 Nitrous oxide reduction rate (v_{red}) and specific growth rate (μ) of strains BL1 and BLN1

Strain	v_{red} (μmol mL^{-1} h^{-1}) ± SE	μ (h^{-1}) ± SE
BL1	0.26 ± 0.03	0.17 ± 0.02
BLN1	0.43 ±0.03	0.17 ±0.03

SE: standard error.

than BL1's. The μ:v_{red} ratio of strains BL1 and BLN1 were 0.65 and 0.45, respectively. This ratio expresses the efficiency of each bacterial strain in utilizing N_2O as electron acceptor.

Nitrous Oxide Emission from Rice Soil. The amount of N_2O in the headspace of bottle filled with rice soil at the beginning of experiment was 6.86 nmol. There was the fluctuation of the amount of N_2O in the headspace during 9 h observation (Fig 2). Nevertheless, the N_2O emission did not show any significant difference between soil incubated with BLN1 strain or without bacterial strain after 3, 6, and 9 h of incubation (Table 2). The concentration of N_2O in the surface water at the beginning of the treatment was 0.70 nmol L^{-1}. The concentration of N_2O in the surface water increased sharply for 6 h following the addition of NO_3^- in soil without isolate. However, the concentration started to go down after 6 h of incubation (Fig 3). Increase of the concentration of N_2O in the surface water of flooded soil after incubation was significantly greater for soil without BLN1 isolate than soil with the addition of isolate (Table 3).

DISCUSSION

Nitrous oxide reduction activity and growth of

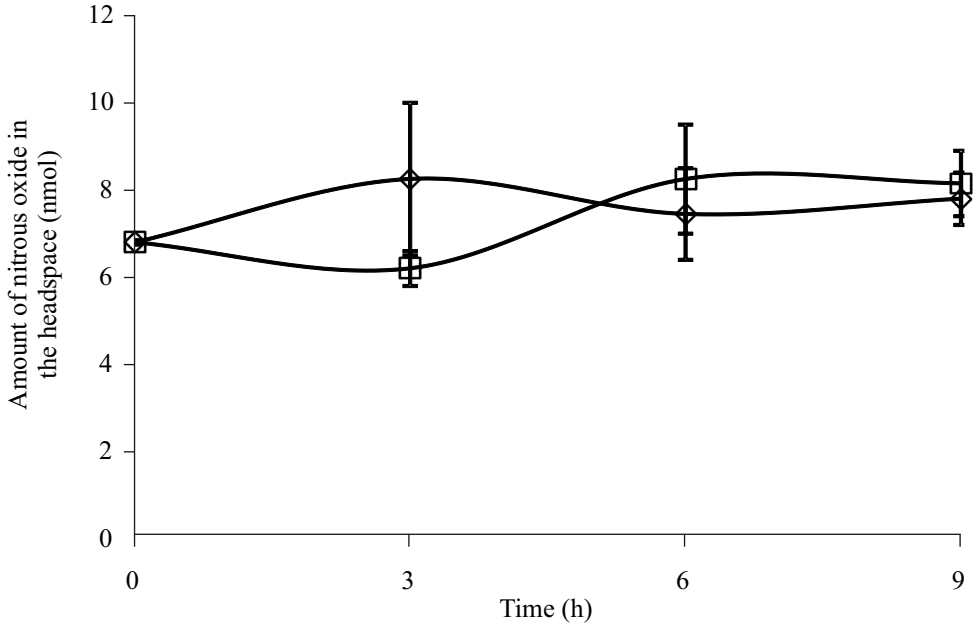

Fig 2 Amount of N_2O in the headspace without and with BLN1 inoculation following the addition of 0.6 mmol of NO_3^-. Bars represent standard error. ⊟: without isolate; ◇: with isolate.

Table 2 Emission of N_2O with and without BLN1 inoculation following the addition of 0.6 mmol of NO_3^-

Time (h)	Without isolate (nmol m^{-2})	With isolate (nmol m^{-2})
3	0.30 a	-0.11 a
6	0.12 a	0.29 a
9	0.20 a	0.28 a

All of the numbers followed by the same alphabet indicates no significant difference in the analysis of variance with α=0.05.

strains BL1 and BLN1 occurred simultaneously, indicating that the bacteria used N_2O for growth. *O. anthropi* BL1 and BLN1 had high ability to grow on and reduce N_2O. They grew well for 15 h on N_2O. Snyder *et al*. (1987) found that *Pseudomonas aeruginosa* PAO1 and P1 lost the ability to grow on N_2O after 1-3 h due to the loss of N_2O uptake activity. *P. aeruginosa* P2 also exhibited a loss of N_2O uptake activity but the loss was not as extensive as strains PAO1 and P1. P2 could still grew at slower rate until N_2O became exhausted after 8 h. Synthesis and destruction of enzyme could control N_2O reduction activity in denitrification bacteria. Bacterial strains having the ability to reactivate or synthesize new enzyme could grow longer on N_2O.

The increase of N_2O in the surface water for 6 h following the addition of 0.6 mmol of NO_3^- indicated an NO_3^- reduction activity of denitrifying bacteria in the flooded soil. The reduction of NO_3^- could be done by the native denitrifiers and the inoculants. Nevertheless addition of the inoculants increased the activity of N_2O reduction. The concentration of N_2O in the surface water of flooded soil decreased after six h of incubation since N_2O went through further reduction to become N_2. In spite of the increasing N_2O concentration in the surface water of flooded soil without isolate at the sixth hour, N_2O released into the headspace did not go through any significant rise, then. According to Heincke and Kaupenjohan (1999), the speed of N_2O dissolving in the water and escaping into the atmosphere depended, among others, on the turbulence and the speed of water current. If there was a current with sufficient aeration, then the dissolved N_2O would go in to the atmosphere in several min. If N_2O stayed in the ground water sufficiently long then there would be sufficient time for N_2O to be reduced to N_2.

BLN1 strain is expected to maintain its high ability to reduce N_2O in the environment among the bacterial community. BLN1 strain could use NO_3^-, NO_2^-, and O_2 (Setyaningsih 2011) in addition to N_2O as the terminal electron acceptor so that its sustainibility in the environment could be supported. According to Martin

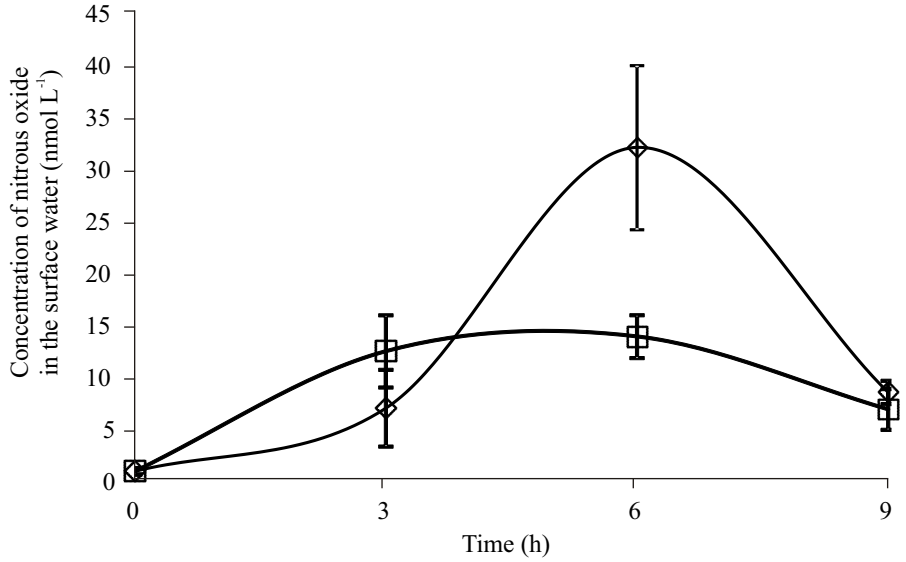

Fig 3 Concentration of N_2O in the suface water without and with BLN1 inoculation following the addition of 0.6 mmol of NO_3^-. Bars represent standard error. ⊟: without isolate; ◇: with isolate.

Table 3 Increase of the concentration of N_2O in the surface water with and without BLN1 inoculation following the addition of 0.6 mmol NO_3^-

Time (h)	Without isolate (nmol m^{-2})	With isolate (nmol m^{-2})
3	6.07 a	11.53 a
6	31.12 b	12.94 a
9	7.59 a	5.95 a

All of the numbers followed by the same alphabet indicates no significant difference in the analysis of variance followed by Duncan's multiple range test with α=0.05.

et al. (1988) the population of denitrifying bacteria had persistent and stable characteristics. In conclusion, our results seems to indicate that the BLN1 strain could be used to reduce the emission of N_2O from the rice fields.

ACKNOWLEDGMENT

This research was supported by BPPS scholarship grant from DIKTI to the first author. Thanks also due to DB Nedwell, emeritus professor of Department of Biological Sciences, University of Essex, Colchester, United Kingdom for his great support and advice.

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