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The effectiveness of methanotrophic bacteria and *Ochrobactrum anthropi* to reduce CH_4 and N_2O emissions and to promote paddy growth in lowland paddy fields

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ABSTRACT

Aims: Paddy field is one of the sources of greenhouse gasses such as methane (CH_4) and nitrous oxide (N_2O), which causes global warming and other negative effects in agricultural sector. An alternative to optimize paddy productivity and reduce emissions of CH_4 and N_2O is by using methanotrophic bacteria and *Ochrobactrum anthropi* BL2.
Methodology and results: This study consisted of two parts, i.e. positive control and experimental treatments. Positive control consisted of 250 kg/ha NPK inorganic fertilizer NPK (15:15:15) (100% of the recommended normal dose) without any methanotrophic bacteria. Meanwhile the experimental treatment consisted of 50 kg/ha inorganic fertilizers NPK (20% of the recommended normal dose) with methanotrophic bacteria (*Methylocystis rosea* BGM 1, *M. parvus* BGM 3, *Methylococcus capculatus* BGM 9, *Methylobacter* sp. SKM 14) and N_2O reducing bacteria (*Ochrobactrum anthropi* BL2). Using weight indicator of 1000 grams, all the bacteria are capable of increasing paddy productivity by 42.07%, compared to conventional method which can only increase the productivity by 2.51% (Cepy and Wangiyana, 2011). The increasing productivity and growth of paddy plants were due to the nitrogen fixation activity of *M. rosea* BGM 1, *M. capculatus* BGM 9, and *Methylobacter* sp. SKM 14. In the experimental treatment using bacteria, the emission of CH_4 and N_2O was reduced with the highest CH_4 and N_2O sinks of 24018.8 mol CH_4/day/ha and 68.48 mol N_2O/day/ha, respectively. However, the positive control treatment with 100% of the recommended fertilizer dose showed the highest CH_4 and N_2O emissions which were up to 74346.45 mol CH_4/day/ha and 26.21 mol N_2O/day/ha, respectively.
Conclusion, significance and impact study: All the methanotropic bacteria and *O. anthropi* BL2 are significantly increase paddy production, compared to positive control treatment. The addition of bacteria in paddy fields results in CH_4 and N_2O sinks.

Keywords: Methanotrophs, *Ochrobactrum anthropi*, CH_4 emission, N_2O emission, paddy field

INTRODUCTION

Rice is one of the most important basic needs in Asian countries, especially in Indonesia. To fulfill the demand on rice, it is essential to increase paddy productivity using various methods such as applying fertilizer, improving irrigation system, and controlling pesticide use. Intensive pesticide use can bring about negative effect to environment because either it can restrict soil microbial community or can lead to genetic mutation to soil microbes. Inorganic fertilizers will increase greenhouse gas emission as the cause of global warming and climate change which can directly affect agricultural sectors (Arth and Frenzel, 2000).

Concentration of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O) is continuously increasing in the atmosphere. CH_4 is potential to cause global warming 26 times higher than that of CO_2 while nitrous oxide gas 298 times higher than that of CO_2 (IPCC, 2007; Millar *et al.*, 2010).

The oxidation of CH_4 by methanotrophic bacteria and the reduction of N_2O by *Ochrobactrum anthropi* BL2 are potentially good methods to reduce CH_4 and N_2O emissions from paddy fields. There are four methanotrophic bacterial isolates, i.e. BGM 1, BGM 3, BGM 9, and SKM 14 which are highly capable of oxidizing CH_4 (Hapsari, 2008). Astuti (2009) identified the isolates as *Methylocystis rosea, M. parvus*, *Methylococcus capsulatus* and *Methylobacter* sp., while *O. anthropi* BL2 is a denitrifying bacterial species which is potential to reduce N_2O emission. The species was isolated from paddy fields in Tangerang, Banten, Indonesia by Setyaningsih *et al.* (2010). Based on the study, it is known that *O. anthropi* BL2 is capable of reducing N_2O up to 5.41 µmol/mL (Setyaningsih *et al.*, 2010).

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Methanotrophic bacteria and *O. anthropi* BL2 were tested as methane oxidizing agent and N_2O reducing agent, respectively, in a green house (Muttaqin, 2012) and in high land paddy field (Pingak *et al.*, 2014; Sutanto *et al.*, 2014). The application of the bacteria in field is capable of reducing CH_4 up to 20% (Pingak *et al.*, 2014; Sutanto *et al.*, 2014). *Methylococcus capsulatus* BGM 9 and *Methylobacter* sp. SKM 14 are not only capable of oxidizing methane, but also capable of fixing N_2 because of their nitrogenase activity (Maisaroh, 2009).

The growth of paddy plant with biofertilizer and 200 kg/ha NPK fertilizers (14.2%) was better than control which was with 300 kg/ha NPK fertilizers (Hadianta *et al.*, 2014). *Methylococcus* c*apsulatus* BGM 9 and *Methylobacter* sp. SKM 14 are also capable of degrading organochlorine pesticides in paddy field (Nurhasanah, 2009). Therefore, this study determined the effectiveness of *O. anthropi* BL2 as reducing agent of N_2O, while mtehanotrophic bacteria as methane oxidizing and paddy growth-promoting agents in lowland paddy field.

MATERIALS AND METHODS

Culturing bacterial isolates

Methanotrophic bacterial isolates, i.e. *M. rosea* BGM 1, *M. parvus* BGM 3, *M. capculatus* BGM 9, and *Methylobacter* sp. SKM 14 were cultured in Nitrate Mineral Salts (NMS) media with the addition of 1% methanol prior to incubation at room temperature for 7 days, while *O. anthropi* BL2 was cultured in denitrification media prior to incubation at room temperature for 3 days. All bacterial cultures should reach minimum cell density of 1×10^6 cell/mL.

Seedling preparation

Seeds of paddy variety IR64 were germinated for 24 h in the dark because by doing so auxin hormon can promote the germination faster, in condition to 50-60% humidity, for 24 h (Weerakoon *et al.*, *2*008), prior to being sowed in the field for 20 days to make the seedlings grow and ready for further planting.

Planting and the measurement of plant growth parameters and gas fluxes

The study was conducted in 1000 m^2 paddy field in two treatments, i.e. positive control (T1) and bacterial application (T2). Positive control treatment used 100% of the recommended dose of inorganic NPK fertilizer (250 kg/ha) (15:15:15) (Permentan, 2007), while inorganic NPK fertilizer was given on day after planting (DAP) 21. T2 used bacterial soaking treatments (1.5 L *M. rosea* BGM 1*,* 2.5 L *M. parvus* BGM 3*,* 3.5 L *M. capculatus* BGM 9, 3 L *Methylobacter* sp. SKM 14 and 3.5 L *O. anthropi* BL2) for 15-30 min prior to planting and 20% of the recommended dose of inorganic NPK fertilizers (50 kg/ha) (15:15:15) was given on DAP 21. Each bacterial culture should cell culture density minimum of 1×10^6 cell/mL. For T1 and T2, inorganic NPK fertilizer was given only once until reaching harvest period. The treated seedlings were planted with distance of 25 cm^2. Each T1 and T2 used one paddy terrace and 10 paddy clump of each were obtained by random sampling. Experimental design was counted manually.

Paddy growth was observed at DAP 21, 42, 63, and 84. Observation was conducted on plant height, number of tiller per cluster, number of panicles per cluster, and color leaf. The score of leaf color in this study was 2-5 (score 2-3 require 15 kg/ha urea fertilizer, score 3-4 10 kg/ha urea fertilizer, score 4-5 0 kg/ha urea fertilizer), indicating that the fertilizer was sufficiently provided. Harvest parameters were shoot's dry weight, root's length, root's dry weight, productive tillers per plant, grains per panicles, weight of 1000 grains and hollow grains (Agriculture Department, 2003).

Methane and N_2O fluxes were measured using manual sampling by placing the closed chamber of 50 × 50 × 100 cm^3 in size on paddy field. Methane and N_2O gases were collected at time zero (t_0) of the chamber installation and 5 h (t_5) after the installation at DAP 21, 42, 63, and 84. The effectiveness of the treatment was measured based on paddy height, number of tiller per cluster, number of panicles per cluster, and leaf color; while the paddy productivity based on the shoot's dry weight (g), root's dry weight (g), root's height (cm), productive panicles, grains per panicles (grains), weight of 1000 grains (g), and hollow grains (%).

RESULTS

Plant growth and harvest results

The average of paddy height in bacterial treatment was 12.50% higher than that of the positive control treatment (Figure 1). Number of tillers per plant in bacterial treatment was also higher than that of positive control and increased along with plant age (Figure 2). The average increasing number of tillers per plant from DAP 21, 42, 63 and 84 of the bacterial treatment was 23.76% higher than that of the positive control.

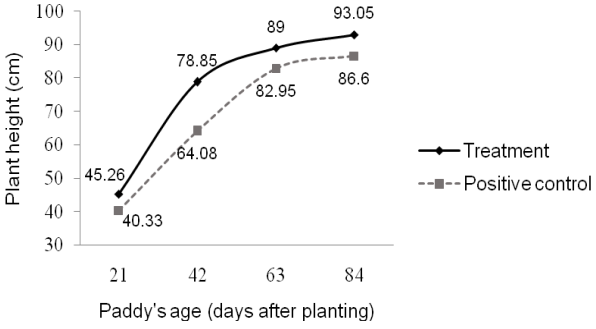

Figure 1: The paddy's height (cm) of T2 with methanotrophic bacteria and *Ochrobactrum anthropi* BL2 and of T1 without bacteria in low land paddy field.

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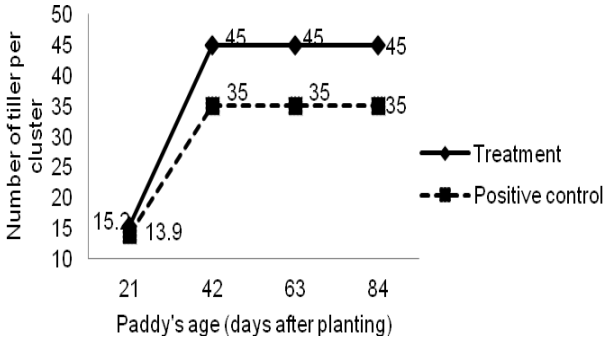

Figure 2: The number of tiller per cluster of T2 with methanotrophic bacteria and *O. anthropi* BL2 and of T1 without bacteria in low land paddy field.

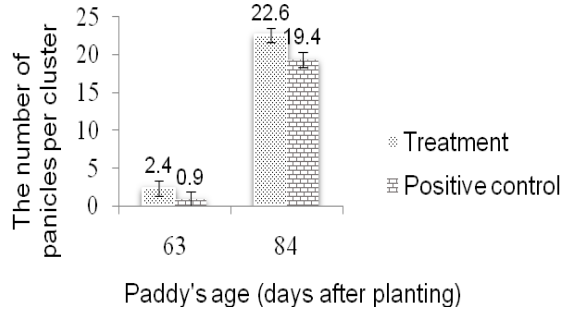

Figure 3: The number of panicles per tiller of T2 with methanotrophic and *O. anthropi* BL2 bacteria and of T1 without bacteria in lowland paddy field.

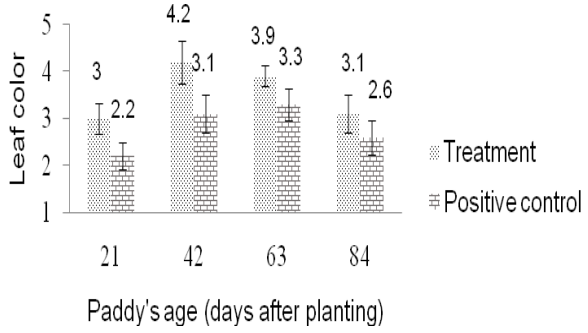

Figure 4: Score of green color of leaf of T2 with methanotrophic bacteria and *O. anthropi* BL2 and of T1 without bacteria addition in lowland paddy field. (Score 2-3 requires 15 kg/ha urea fertilizer, score 3-4 10 kg/ha, score 4-5 0 kg//ha).

The panicle appearance was initially observed at DAP 63. The number of panicles per plant in bacteria treatment was higher, i.e. up to 16.49%, compared to that of positive control (Figure 3).

The sufficiency of NPK fertilizer was shown by the green color of leaf until DAP 42. On DAP 63 and 84, the leaf color started to degrade because almost all nutritions have been used to fill the panicles. Positive control treatment showed leaf color level lower than T2. The green color of leaf in T2 paddy increased up to 35.92% compared with positive control in each observation (Figure 4).

The average of shoot's dry weight (g), root's dry weight (g), root's length (cm), and productive panicles in T2 plant were 67.60%, 143.32%, 37.05%, and 24.46% higher than that of the positive control, respectively. In addition, grains per panicles (grains) and the weight of 1000 grains (g) in T2 were higher than that of the positive control, i.e. 48.28% and 42.07%, respectively. The hollow grains decrease in T2 was higher than that of the positive control plants (Table 1).

Methane (CH_4) and nitrous oxide (N_2O) fluxes

Methane and N_2O fluxes on DAP 21, 42, 63, and 84 indicated that higher emissions values belonged to positive control field. Methane emission on the positive control field was up to 74346.45 mol CH_4/day/ha and T2 field showed CH_4 sink up to 1278.16 mol CH_4/day/ha (Figure 5). Similar trend was also showed in N_2O fluxes where the emission of the positive control field increased and T2 field showed N_2O sink up to 23.54 mole N_2O/day/ha (Figure 6).

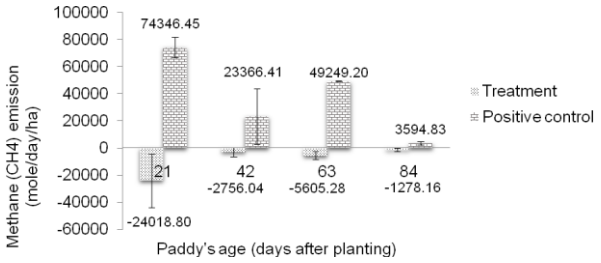

Figure 5: Methane emission of T2 with methanotrophic bacteria and *O. anthropi* BL2 and of T1 without bacteria in low land paddy field.

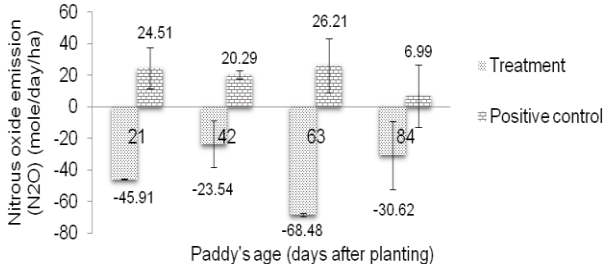

Figure 6: Nitrous oxide (N_2O) emission of T2 with methanotrophic bacteria and *O. anthropi* BL2 and of T1 without bacteria in low land paddy field.

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Table 1: Paddy yield of T2 with methanotrophic bacteria and *Ochrobactrum anthropi* BL2 that was higher than that of T1 without bacteria in low land padddy field. The paddy was harvested in 98 days after planting.

	Shoot's dry weight (g)	Root's dry weight (g)	Root's height (cm)	Productive panicles	Grains per panicles (grains)	Weight of 1000 grains (g)	Hollow grains (%)
T2	45.32 ± 4.5	17.86 ± 1.8	23.3 ± 0.7	17.3 ± 1.2	72.91 ± 5.6	30.19 ± 1.8	2.65 ± 1.2
T1	27.04 ± 2.9	7.34 ± 1.0	17 ± 1.15	13.9 ± 1.4	49.17 ± 3.8	21.25 ± 0.9	9.49 ± 3.5

Note: ± Standard Error

DISCUSSION

Increase in plant growth

The growth percentage and harvest component of T2 was higher than T1 eventhough positive control was given 100% recommended dose of inorganic NPK fertilizer and T2 was given 20% inorganic NPK fertilizer. The parameters between positive control (T1) and treatment with methanotrophic bacteria (T2) were different because of *M. capculatus* BGM 9 and *Methylobacter* sp. SKM 14. In paddy, those bacteria take part in methane oxidation and nitrogen fixation process, because they have *nifH* and *nifD* gene which codes nitrogenase reductase enzyme (Hanson and Hanson, 1996; Hadianta *et al.*, 2014).

Methylococcus capculatus BGM 9 and *Methylobacter* sp. SKM 14 are capable of acumulating ammonia of 47 µM and 15 µM in *in vitro* condition, respectively (Sagala, 2009). However, bacteria culture mixed of *M. rosea* BGM 1 and *Methylobacter* sp. SKM 14 has nitrogenase activity of 11.27 µmol/h/mL culture in *in vitro* condition (Chatrina, 2010).

Nitrogen is one of chlorophyll components. The amount of chlorophyll pigment is indicated by leaf's green color. Chlorophylls take a major role in photosynthesis process which produces energy that will eventually improve the development of productive grains. Photosynthesis is highly important in grain filling process. However, roots also are important to absorb nutrients for organs and productive grains development. A large number of roots can improve plants ability to absorb nutrients and thus, plant growth such as height and tiller productivity can increase and resulting in the increase in the number of tiller, panicles, and grains (Wangiyana *et al.*, 2009)

Estimation of the weight of 1000 grains (g) of T2 increased 42.07%. Another study reported 53% of the same variable which is considered a quite high value. Such high value is produced because of 200 kg/ha NPK fertilizer and biofertilizers (Hadianta *et al.*, 2014), while this study only used 50 kg/ha of NPK fertilizer, methanotrophic bacteria, and *O. anthropi* BL2.

Macalady *et al*. (2002) reported that nitrogen fixation is a process to convert nitrogen into ammonium and then convert it to amino acids, nucleic acids, and proteins. The outcome of which can be used for plant growth. Nitrogen as one of chlorophyll components which is involved in photosynthesis process that increases the amount of productive grains and protein percentage, also functions to form substantial components of plant organs (Watanabe and Kitagawa, 2000; Chaturvedi, 2005; Netto *et al.*, 2005).

Methane and nitrous oxide fluxes

Negative value indicates that metanotrophic bacteria, i.e. *M. rosea* BGM 1, *M. parvus* BGM 3, *M. capculatus* BGM 9, and *Methylobacter* sp. SKM 14 use methane as the source of carbon and energy for their growth. The highest methane sink of T2 was 24018.80 CH_4 mole/day/ha, while other studies reported that methanotrophic bacteria can oxidize methane (methane sink) up to 19.57 mmol/m^2.h (Pingak *et al.*, 2014) and 16.72 kg C ha^1/day (Sutanto *et al.*, 2014), while *M. rosea* BGM 1, *M. parvus* BGM 3, *M. capculatus* BGM 9, and *Methylobacter* sp. SKM 14 in *in vitro* condition can oxidize methane up to 969.62 mole/day, 4186.25 mole/day, 66556.82 mole/day, and 25654 mole/day, respectively (Hapsari, 2008).

Methanotrophic bacteria as methane sink (Dubey, 2005) are capable of oxidizing methane in paddy field. Dedysh *et al.* (2005) also reported that methanotrophic bacteria take important role in the world's methane cycle. The methanotrophic bacteria are capable of using methane as carbon and energy sources through methane monooxygenases (MMO) with oxidation process (Semrau *et al*., 2010)

Increasing emission of nitrous oxide in T1 occurred due to the availability of inorganic fertilizers in paddy fields, in addition to from N_2O flow from natural environment (Dong *et al*., 2009). The value of N_2O emission in T2 was negative because *O. anthropi* BL2 with its N_2O reductase enzyme can reduce N_2O become N_2. Nitrous oxide was reduced up to 68.48 N_2O mole/day/ha, while another study reported that N_2O can be reduced up to 127.19 µmol/m^2.day (Pingak *et al.*, 2014). *Ochrobactrum anthropi* BL 2 is capable of reducing N_2O up to 5.41 µmol/mL in *in vitro* condition (Setyaningsih *et al.*, 2010).

Shapleigh (2006) reported that *O. anthropi* is a Gram-negative bacterium that capable of reducing N_2O. Based on this study result, methanotrophic bacteria *M. rosea* BGM 1, *M. parvus* BGM 3, *M. capculatus* BGM 9, and *Methylobacter* sp. SKM 14, as well as *O. anthropi* BL2 can be used as biological fertilizers in low land paddy field. In addition, methanotrophic bacteria and *O. anthropi* BL2 are highly potential to decrease greenhouse gasses.

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CONCLUSION

Methanotrophic bacteria *M. rosea* BGM 1*, M. parvus* BGM 3, *M. capculatus* BGM 9, *Methylobacter* sp. SKM 14, and *O. anthropi* BL2 are capable of increasing paddy's growth and reducing CH_4 and N_2O emission. The use of methanotrophic bacteria and *O. anthropi* BL2 were significantly increase rice production per 1000 grains weight (g), i.e. up to 42.07% higher than positive control treatment (T1). The addition of methanotropic bacteria and *O. anthropi* BL2 into paddy fields was capable of sinking CH_4 and N_2O up to 1278.16 mole CH4/day.ha and 23.54 mole N_2O/day.ha, respectively.

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