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ISOLATION AND CHARACTERIZATION OF METHANOTROPHIC BACTERIA FROM RICE FIELDS

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ABSTRACT

Methane is a greenhouse gas capable of depleting the ozone layer. Rice fields are significant sources of atmospheric methane. The application of chemical fertilizer in rice fields increases the methane emission. Methanotrophic bacteria has a unique ability as it can utilize methane as a source of carbon and energy. This research was able to isolate and characterize successfully the methanotrophic bacteria from rice fields in Bogor and Sukabumi, in West Java, Indonesia. Methane oxidation was determined through Gas Chromatography and it shows that all isolates performed methane oxidation activity. The highest methane oxidation activity was performed by BGM 9 isolate. And the DNA amplification of BGM 9 genome was performed by a single band of *mmo*X in the size of 500 bp and three bands of *pmo*A in the size of 1000, 750 and 500 bp respectively.

Key words: Methanotrophic bacteria, methane oxydation, methane monooxygenase, rice field.

INTRODUCTION

Rice fields In Indonesia are the backbone of rice production as well as a source of methane (CH_4) emission to the atmosphere. Methane is a greenhouse gas and has potency to deplete ozone layer. The contribution of methane in the atmosphere is approximated to 15-20 % of the total greenhouse gas effect and its concentration in the atmosphere is increasing by approximately 1 % per year (Mossier 1998).
It has been estimated that the methane production in the rice fields is about 575 Tg $year^{-1}$ (Hanson & Hanson 1996). Chemical fertilization in the rice fields increases methane emission. Application of ammonium sulphate fertilizer, as much as 140 kg

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ha^{-1} can increase methane emission. Setyanto *et. al* (1999) reported that emission of methane by application of ammonium sulphate fertilizer was 164 - 175 kg CH_4 ha^{-1}. Indonesia is the third biggest contributor of methane emission from rice fields after China and India, it is estimated 7.08 % of the total world of methane emission from rice fields (Sass *et al*. 2000).

Measurements of methane emission in rice fields indicates that methane was oxidized in oxidation layer of soil sediment by mathanotrophic bacteria (Conrad & Rothfus 1991). Methane oxidation was also found in the rhizosphere area (Whalen 2005). Methane oxidation activity in rice fields was up to 80 % of methane produced by methanogenic archaebacteria (Conrad & Rothfus 1991).

Methanotrophic bacteria has a unique ability as it can utilize methane as a source of carbon and energy. There are three groups of methanotrophic bacteria; type I, type II, and type X methanotrophs. The genera included in type I are *Methylomonas, Methylobacter*, type II methanotrophs are *Methylosinus* and *Methylosystis*, and type X methanotroph is *Methylococcus capsulatus* (Hanson & Hanson 1996). Several methanotrophic bacteria of type II (four species of *Methylosinus*) and type X (*Methylococcus capsulatus*) were able to degrade TCE (trichloroethylene). Methanotrophic bacteria can also degrade methyl bromide and methyl fluoride. Mixed cultures of methanotrophic bacteria could degrade 2- and 4-chloro-biphenyl and 4-hydrroxy-2-chlorobiphenyl. The type II and type X methanotrophs have also ability to fix N_2 (Hanson & Hanson 1996).

Potential methanotrophic bacteria have great application purposes for environmental friendly and sustainable agriculture system. They have the abilities to reduce methane emission to the atmosphere, to degrade halogenated aliphatic compounds in the polluted soils and groundwater, and to fix N_2. However, information about the activity and diversity of these bacteria from Indonesian rice fields is very limited. Therefore, this research project was conducted to determine the activities and diversity of these bacteria from Indonesian rice fields using microbiological and molecular techniques, so that the results and information can be used to support the achievement of environmental friendly and sustainable agricultural system program of rice fields in Indonesia.

MATERIALS AND METHOD

Sample Collection

Sediments and water samples of rice fields were collected using sediment core from several areas in Bogor and Sukabumi, West Java, Indonesia.

Isolation of methanotrophic bacteria

Isolation of methanotrophic bacteria was conducted using enrichment technique in NMS (Nitrate Minral Salts) media (Hanson 1998). One ml of both upper layer of sediment and water was inoculated into 50 ml NMS medium in 100 ml bottles capped with butyl rubber septum. The bottle headspace will be filled up with 50 % of methane and 50 % of air. Incubation was held on shaker for 5-6 days in room temperature.

Enrichment of type II methanotrophic bacteria that have ability to fix N_2 will be carried out in the same media without nitrate but with gas composition in the headspace such as of 20 % air, 5 % CO_2, 25 % N_2 and 50 % methane (Hanson 1998).

Isolates were purified by using a streak plate technique with NMS agar, containing 100 mg/l cycloheximide dan 10 mg/l amphoterisin B (Hanson 1998). The plates were incubated for 5-6 days in an anaerobic jar with similar gas composition as mentioned above. The pure isolates was characterized based on cell morphology and physiological assays.

Determination of Methane Monooxygenase Activity

Qualitative Determination of MMO enzymes activity was determined using a colony plate assay based on naphthalene oxidation stained with orto-dianisidine and the activity of MMO was measured using Gas Chomatograph (Shimadzu 17A; Shimadzu, Kyoto, Japan) (Hanson 1998).

Genomic DNA Isolation of Methanotrophic bacteria

Genomic DNA of selected isolates was extracted using alkaline lyses methods (Sambrook *et al.* 1989). The extracted DNA was used as a template for amplification of *mmo*X gene using *Polimerise Chain Reaction* (*PCR*) technique. PCR machine (Perkin elmer PCR GeneAmp, USA).

Amplification of *pmo*A *and mmo*X genes

Specific primers of A189F (GGNGACTGGGACTTCTGG**)** and mb661 (CCGGMGCAACGTCYTTACC) were used in amplifying *pmo*A gene (Bourne *et al* 2001). While specific primers of *mmo*XA (ACCAAGGARCARTTCAAG) and *mmoXB (*TGGCACTCRTARCGCTC) were used in amplifying *mmo*X gene (Auman *et al.* 2000). PCR amplification reactions were performed in 25 µl reaction mixtures. Individual reagents and their concentrations were as follows: 1X PCR Buffer, 1.5 mM $MgCl_2$, 1.5 units of *taq* DNA polymerase, 20 µg of bovine serum albumin, 200 µmol of dNTP, 20 pmol each primer, and 1 µl of template DNA. Taq pol was added after the initial denaturalization step at 96°C for 5 minutes, followed by 30 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. A final extension period of 5 minutes at 72°C was observed (Bourne *et al.* 2001).

RESULTS AND DISCUSSION

Isolates of Methanotrophic Bacteria

Isolation of methanotrophic bacteria from 7 samples of rice field in Bogor and Sukabumi provided 37 isolates, i.e. 15 isolates were isolated from the rice fields in Bogor and 22 isolates were isolated from the rice fields in Sukabumi (Table 1). Colony morphology of the isolates on NMS media were of different colors such as white, yellow. pink, and orange. The growth rate and colony development on NMS agar also varied. It took up to 14 days to develop a colony with diameter of 2 mm.

Table 1. Bacterial Isolates isolated from rice fields in Bogor and Sukabumi

Origin of Sampel	Sampel Code	Number of isolate	Isolate Code
Bogor	BG1	10	BGM 1, BGM 2, BGM 3, BGM 4, BGM 5, BGM 6, BGM 7, BGM 8, BGM 9, BGM 10
	BG2	5	BGM 11, BGM 12, BGM 13, BGM 14, BGM 15
Sukabumi	SK1	3	SKM 1, SKM 2, SKM 18
	SK2	3	SKM 3, SKM 4, SKM 19
	SK3	7	SKM 5, SKM 6, SKM 7, SKM 8, SKM 9, SKM 10, SKM 20
	SK4	5	SKM 11, SKM 12, SKM 13, SKM 17, SKM 21
	SK5	4	SKM 14, SKM 15, SKM 16, SKM 22

Methanotrophic bacteria have different pigments, some of them had yellow and brown pigments (Hanson & Hanson 1996), others did not have or they have white pigment, pink (Eller & Frenzel 2001) and reddish orange (Holt *et al.* 1994). This methanotropic bacteria was slow growing and the optimum growth on NMS media was 14 days of incubation.

Qualitative Determination of sMMO Activity

Qualitative assay of activity sMMO found that BGM 8 isolate performed activity of sMMO (Fig. 1). Positive result of the assay was indicated by the change in color from white/cream into violet after treatment using naphtalena and o-dianisidin. Brusseau *et al.* (1990) reported that type II methanotrophs having sMMO could convert napthalene into naphtol. This reaction is an indicator that merthanotrophic bacteria could degrade thrichloroethilene.

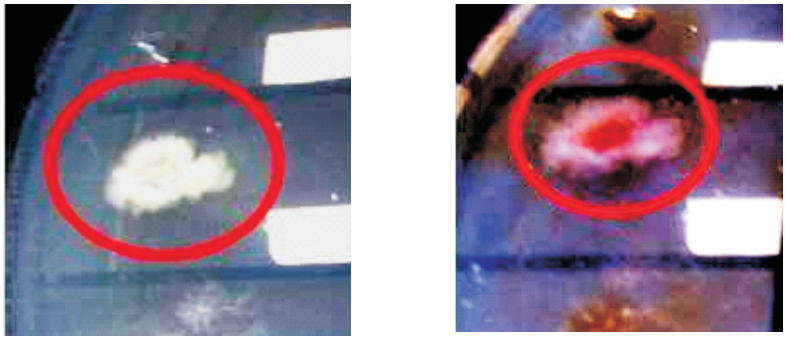

Figure 1. Positive results of sMMO assay performed by BGM 8 isolate (a) before assay (b) after assay

Copper (Cu^{2+}) could regulate MMO biosynthesis in methanotrophs. The bacteria will produce pMMO if they were grown in media containing high Cu^{2} (Zahn & Dispirito 1996). pMMO enzyme was found in all methanotrophic bacteria (Hanson & Hanson 1996). But, in low Cu^{2+} condition, some methanotrophic bacteria such as *Methylosinus trichosporium* OB3b (Lipscomb 1994) and *Methylomonas* (Shigematsu *et al.* 1999) will produce sMMO. Graham *et al.* (1992) reported that concentration of 1-2 μM Cu^{2+} could inhibit synthesis of sMMO in *M. trichosporium* OB3b, *M. methanica* (ATCC 35067) and Type II methanotrophs DG1 species.

Activity of Methane Oxydation

Methane analysis results showed that all isolates have ability to oxidize methane (unpublished data). The best isolate was chosen based on methane oxydation activity and bacterial growth on NMS agar. Six isolates *i.e.* BGM 1, BGM 2, BGM 3, BGM 9, BGM 12, dan SKM 14) were selected for further experiment including. More detail analysis of methane oxydation activity carried out showed that BGM 9 has the highest activity of methane oxydation, followed by SKM 14 (Fig. 2).

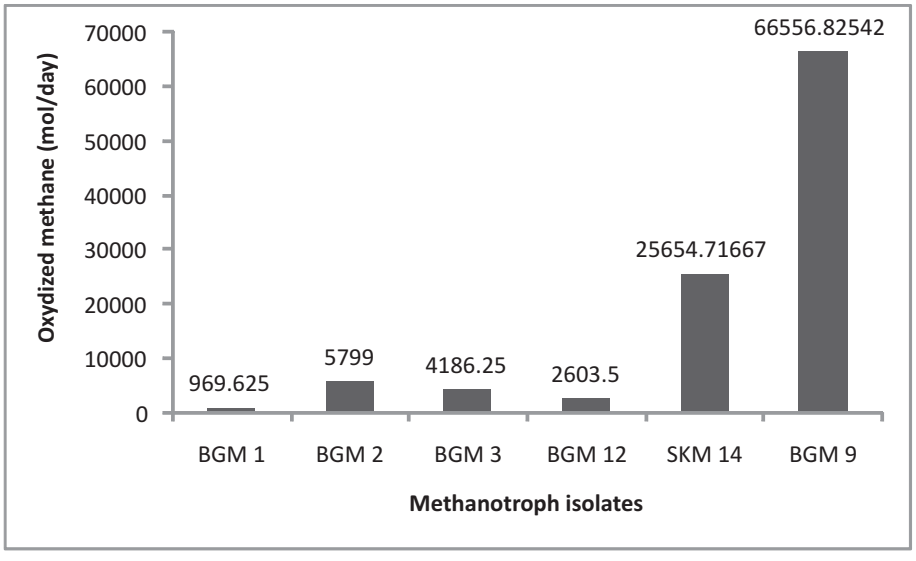

Figure 2. Methane oxydation activity of six methanotroph isolates .

Phenotive and Physiological Characteristics of BGM 9 and SKM 14 isolates

Based on microscopic observation, bacterial cells of BGM 9 and SKM 14 isolates were Gram negative, rod, and motile. Results of physiological assay showed that the isolates have positive raction on H_2S production and urease assays. But they showed negative result for oxydase, indole and glucose assays.

Phenotive characterization of BGM 9 and SKM 14 isolates indicated that the isolates are closely related with methanotrophic bacteria type II. Holt *et al.* (1994) in *Bergey's Manual of Determinative Bacteriology* grouped this bacteria into Gram negative eubacteria. *Methylobacterium* was described as a bacterium with rod cell, gram negative, motile, slow growing, pink until orange in colour of the colony, optimum growth at 25-30°C, and aerobic obligate. Characteristics of SKM 14 isolate were very similar with *Methylobacterium*.

Amplification of *pmo*A dan *mmo*X Genes

Amplification of *pmo*A dan *mmo*X genes from selected isolates showed that *mmo*X of BGM 9 was performed on a single band of amplified DNA in the size of 500 bp. This band size was tipically for *mmo*X gene amplification band (Fig. 2). Auman *et al.* (2000) reported that the size of *mmo*X gene PCR product using *mmo*XA and *mmo*XB specific primers was 510 bp. This results confirmed that BGM 9 isolate has sMMO enzyme. However aplification of *pmo*A was performed on three bands of amplified DNA in the size of 1000, 750 and 500 bp respectively (Fig. 3). These results indicated that the isolates have *pmo* gene, however this *pmo* gene PCR product still need to be investigated more detail to determine which band is the spesific band for *pmo*A gene.

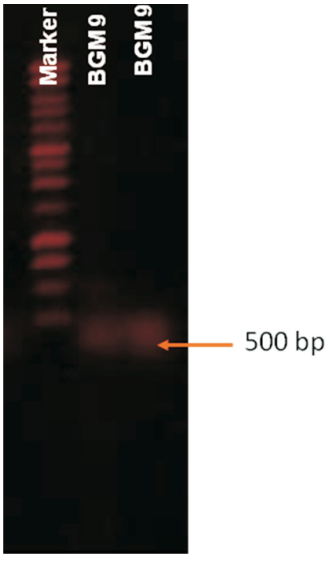

Figure 2. Aplification results BGM 9 using *mmo*X specific primer of BGM 9 DNA genome.

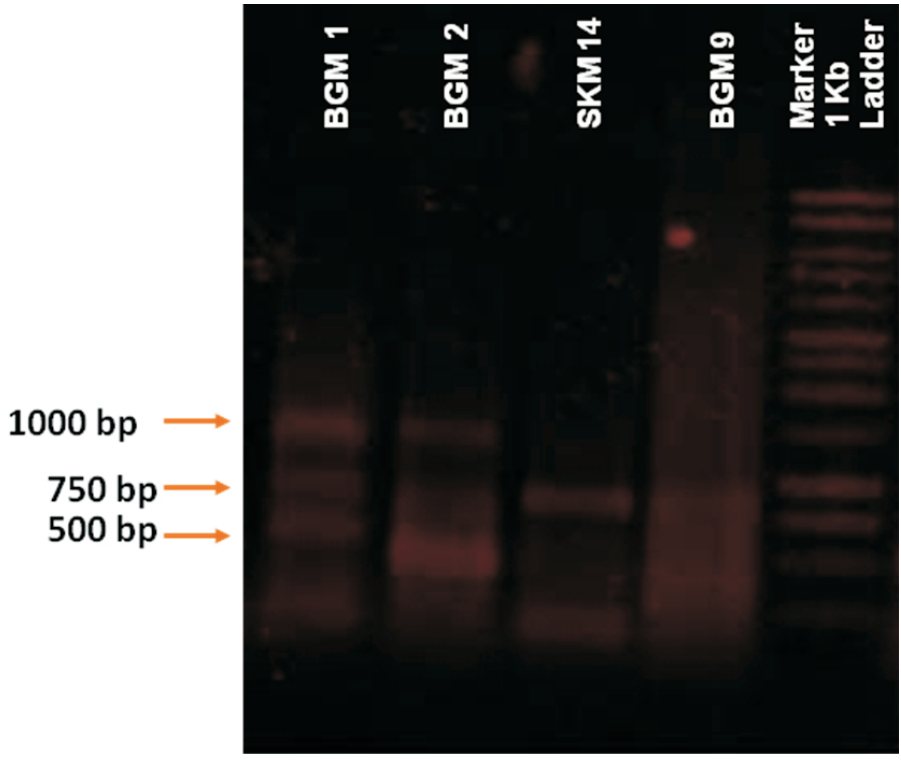

Figure 3. Aplification results using *pmo*A specific primer of BGM9, SKM14, BGM2 and BGM1 DNA genome.

CONCLUSION

As many as 37 isolates of methanotrophic bacteria were isolated from rice fields in Bogor and Sukabumi, West Java. The highest activity of methane oxydation was performed by BGM 9 isolate. This isolate also had the characteristics activity of sMMO enzymes. BGM 9 was performed on a single band of amplified *mmo*X DNA in the size of 500 bp and aplification of *pmo*A was performed on three bands of amplified DNA in the size of 1000, 750 and 500 bp respectively. More detail molecular study including identification based on 16S rRNA sequences should be conducted to determine the diversity of methanotrophic bacteria in Indonesian rice fields.

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