

ISSN 0216-5023 (printed)  
ISSN 1907-770X (electronic)

# BIOTROPIA

The Southeast Asian Journal of Tropical Biology

Vol. 16 No. 2 December 2009



Published by  
SEAMEO BIOTROP  
Southeast Asian Regional Centre for Tropical Biology  
Bogor, Indonesia  
[www.biotrop.org](http://www.biotrop.org)

## EDITORIAL BOARD

- Editor-in-Chief : Dr. Bambang Purwantara  
Members : Prof. Dr. Abu Bakar Bin Saleh : Dr. Irdika Mansur, M.For.Sc.  
: Dr. Zohrah binti Haji Sulaiman : Dr. Sri S. Tjitrosoedirdjo  
: Dr. Hartrisari Hardjomidjojo : Dr. Okky S. Dharmaputra  
Managing Editors : Mr. Jahja Kartana  
: Mr. Iwan Setiawan, PM  
: Ms. Tika Tresnawati

## REVIEWERS for Vol. 16 No. 2

- Dr. Ika Mariska, Research Institute for Agricultural Biotechnology and Genetic Resources (Balitbiogen), Bogor, Indonesia  
Prof. Dr. Dondin Sajuthi, Primate Research Center, Bogor, Indonesia  
Prof. Dr. Iswandi Anas, Department of Soil Sciences, Bogor Agricultural University (IPB), Darmaga, Bogor 16680, Indonesia  
Dr. Bahagiawati, Research Institute for Agricultural Biotechnology and Genetic Resources (Balitbiogen), Bogor, Indonesia  
Dr. Widodo, Department of Plant Protection, Bogor Agricultural University (IPB), Darmaga, Bogor 16680, Indonesia  
Dr. Lisdar M. Sudirman, Department of Biology, Bogor Agricultural University (IPB), Darmaga, Bogor 16680, Indonesia  
Dr. Diah Iswanti, Department of Chemistry, Bogor Agricultural University (IPB), Darmaga, Bogor 16680, Indonesia  
Dr. Sri Hendrastuti Hidayat, Department of Plant Protection, Bogor Agricultural University (IPB), Darmaga, Bogor 16680, Indonesia  
Dr. Hilman Affandi, SEAMEO BIOTROP, Jl. Raya Tajur Km. 6, Bogor, Indonesia  
Dr. Soekisman Tjitrosemito, Department of Biology, Bogor Agricultural University (IPB), Darmaga, Bogor 16680, Indonesia  
Dr. Soetrisno Sukimin, SEAMEO BIOTROP, Jl. Raya Tajur Km. 6, Bogor, Indonesia

BIOTROPIA The Southeast Asian Journal of Tropical Biology is a scientific publication of SEAMEO BIOTROP published biannually. It contains scientific articles in any one of the areas of tropical biology. Contribution in the form of technical papers, review articles and short communication are also considered for publication. A guide for authors is found on the inside back cover.

## SUBSCRIPTION RATE

US\$ 15.00 per issue or US\$ 25.00 for two issues

## REMITTANCE

For international subscription kindly remit your payment through IMO (International Money Order) or telegraphic transfer in US Dollars, payable to SEAMEO BIOTROP, to Bank BNI 46 Bogor Branch, Jalan Ir. H. Juanda, Bogor, West Java, Indonesia, Acct. No. 3890624 or for Indonesian currency (rupiahs) remit to Acct. No. 3893863 at the similar bank.

Orders should be addressed to:

SEAMEO BIOTROP, Jalan Raya Tajur Km. 6, PO Box 116, Bogor 16001, Indonesia  
Email: [iru@biotrop.org](mailto:iru@biotrop.org) or visit our home page: <http://journal.biotrop.org>

Cover Photo:

*Fusarium* sp. microscopic view under 400x objective (source: [www.globalcyran.com](http://www.globalcyran.com))



# ISOLATION AND CHARACTERIZATION OF METHANOTROPHIC BACTERIA FROM RICE FIELDS

IMAN RUSMANA<sup>1</sup> and ALINA AKHDIYA<sup>2</sup>

<sup>1</sup>*Departement of Biology, Bogor Agricultural University, Jl. Agatis, Darmaga, Bogor, Indonesia*

<sup>2</sup>*Indonesian Research Center for Agricultural Biotechnology and Genetic Resources Research and Development,*

*Agency for Agricultural Research and Development, Department of Agriculture, Jl. Tentara Pelajar, Cimanggu, Bogor, Indonesia*

## 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 *mmoX* in the size of 500 bp and three bands of *pmoA* 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<sub>4</sub>) 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<sup>-1</sup> (Hanson & Hanson 1996). Chemical fertilization in the rice fields increases methane emission. Application of ammonium sulphate fertilizer, as much as 140 kg

\*Corresponding authors : rusmana13@yahoo.com

ha<sup>-1</sup> can increase methane emission. Setyanto *et. al* (1999) reported that emission of methane by application of ammonium sulphate fertilizer was 164 - 175 kg CH<sub>4</sub> ha<sup>-1</sup>. 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 archaeobacteria (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-hydroxy-2-chlorobiphenyl. The type II and type X methanotrophs have also ability to fix N<sub>2</sub> (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<sub>2</sub>. 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 amphotericin 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 Chromatograph (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 *mmoX* gene using *Polymerase Chain Reaction (PCR)* technique. PCR machine (Perkin elmer PCR GeneAmp, USA).

### Amplification of *pmoA* and *mmoX* genes

Specific primers of A189F (GGNGACTGGGACTTCTGG) and mb661 (CCGGMGCAACGTCYTTACC) were used in amplifying *pmoA* gene (Bourne *et al.* 2001). While specific primers of *mmoXA* (ACCAAGGARCARTTCAAG) and *mmoXB* (TGGCACTCARTARCGCTC) were used in amplifying *mmoX* gene (Auman *et al.* 2000). PCR amplification reactions were performed in 25  $\mu$ l 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  $\mu$ g of bovine serum albumin, 200  $\mu$ mol of dNTP, 20 pmol each primer, and 1  $\mu$ l 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 Samples	Sample 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 methanotrophic 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 naphthalena and o-dianisidin. Brusseau *et al.* (1990) reported that type II methanotrophs having sMMO could convert naphthalene into naphthol. This reaction is an indicator that methanotrophic bacteria could degrade trichloroethylene.

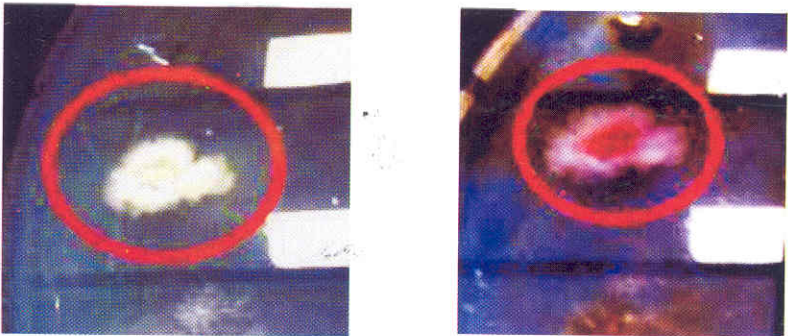


Figure 1. Positive results of sMMO assay performed by BGM 8 isolate (a) before assay (b) after assay

Copper ( $\text{Cu}^{2+}$ ) could regulate MMO biosynthesis in methanotrophs. The bacteria will produce pMMO if they were grown in media containing high  $\text{Cu}^{2+}$  (Zahn & Dispirito 1996). pMMO enzyme was found in all methanotrophic bacteria (Hanson & Hanson 1996). But, in low  $\text{Cu}^{2+}$  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  $\mu\text{M}$   $\text{Cu}^{2+}$  could inhibit synthesis of sMMO in *M. trichosporium* OB3b, *M. methanica* (ATCC 35067) and Type II methanotrophs DG1 species.

### Activity of Methane Oxidation

Methane analysis results showed that all isolates have ability to oxidize methane (unpublished data). The best isolate was chosen based on methane oxidation 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 oxidation activity carried out showed that BGM 9 has the highest activity of methane oxidation, followed by SKM 14 (Fig. 2).

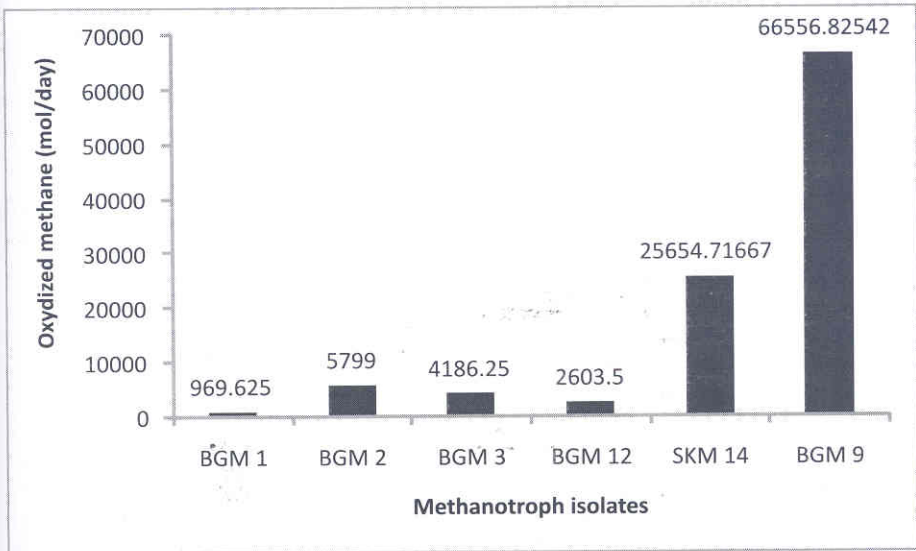


Figure 2. Methane oxidation activity of six methanotroph isolates .

### Phenotype 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 reaction on H<sub>2</sub>S production and urease assays. But they showed negative result for oxydase, indole and glucose assays.

Phenotype 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 *pmoA* dan *mmoX* Genes

Amplification of *pmoA* dan *mmoX* genes from selected isolates showed that *mmoX* of BGM 9 was performed on a single band of amplified DNA in the size of 500 bp. This band size was typically for *mmoX* gene amplification band (Fig. 2). Auman *et al.* (2000) reported that the size of *mmoX* gene PCR product using *mmoXA* and *mmoXB* specific primers was 510 bp. This results confirmed that BGM 9 isolate has sMMO enzyme. However aplication of *pmoA* 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 *pmoA* gene.

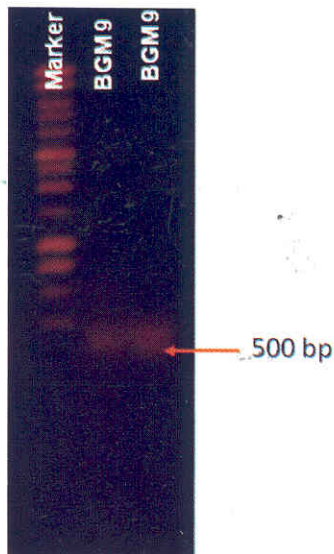


Figure 2. Amplification results BGM 9 using *mmoX* specific primer of BGM 9 DNA genome.



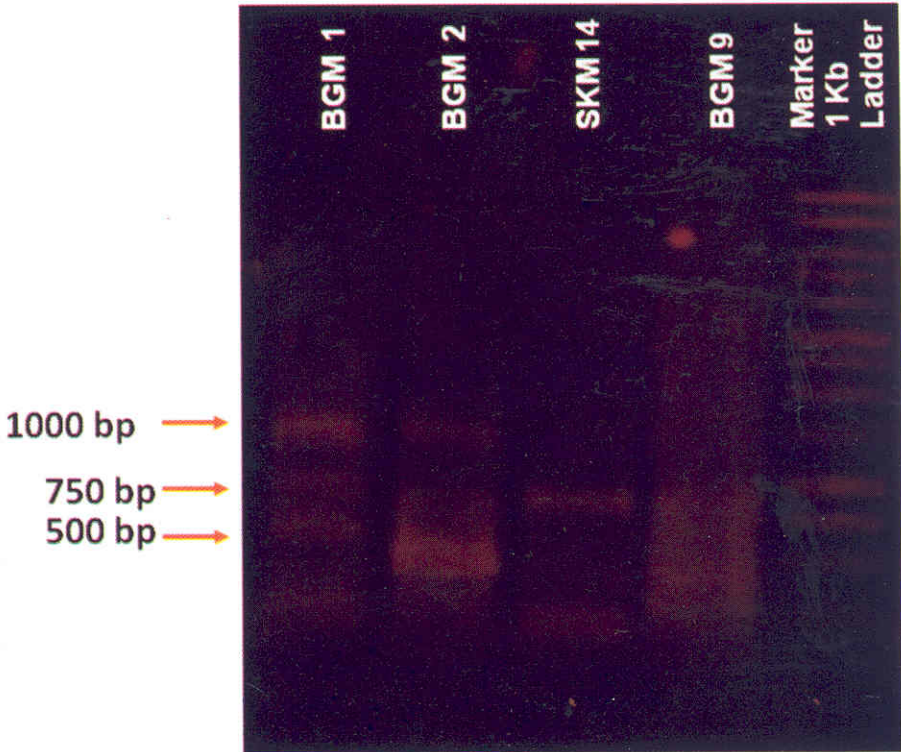


Figure 3. Amplification results using *pmoA* specific primer of BGM9, SKM14, BGM2 and BGM1 DNA genome.

## 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 oxidation 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 *mmoX* DNA in the size of 500 bp and amplification of *pmoA* 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.

## ACKNOWLEDGEMENT

This study was supported by a research grant of DIPA SEAMEO BIOTROP in 2008.

## REFERENCES

- Brusseau G.A., Tsien H.C., Hanson R.S. and L.P. Wackett. 1990. Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monoxygenase activity. *Biodegradation*, 1: 19-29.
- Bourne D.G., McDonald I.R. and J.C. Murrell. 2001. Comparison of pmoA PCR primer sets as tools for investigating methanotroph diversity in three Danish soils. *Applied and Environmental Microbiology*, 67: 3802-3809.
- Conrad R. and F. Rothfus. 1991. Methane oxidation in the soil surface layer of a flooded rice field and the effect of ammonium. *Biology and Fertility of Soils*, 12: 28-32.
- Eller G. and P. Frenzel. 2001. Changes in activity and community structure of methane-oxidizing bacteria over the growth period of rice. *Applied and Environmental Microbiology*, 67 (6): 2395-2403.
- Graham D.W., Korich D.G., Leblanc R.P., Sinclair N.A. and R.G. Arnold. 1992. Applications of a Colorimetric Plate Assay for Soluble Methane Monoxygenase Activity. *Applied and Environmental Microbiology*, 58(7): 2231-2236.
- Hanson R.S. 1998. Ecology of methylotrophic bacteria. *In: Burlage R.S., Atlas R., Stahl D., Geesey G. and G. Dayler (eds). Techniques in Microbial Ecology: 137-162. Oxford University Press. Oxford.*
- Hanson R.S. and T.E. Hanson. 1996. Methanotrophic bacteria. *Microbiological Reviews*, 60: 439-471.
- Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. and S.T. Williams. 1994. *Bergey's Manual of Determinative Bacteriology*. 9<sup>th</sup> Edition. Lippincott William & Wilkins: a Wolter Kluwer Co.
- Lipscomb J.D. 1994. Biochemistry of soluble methane monoxygenase. *Annual Review of Microbiology*, 48: 371-399.
- Sambrook J.E., Fritsch E. and T. Maniatis. 1989. *Molecular Cloning: a Laboratory Manual*. 2<sup>nd</sup> Edition. Cold Spring Harbor Laboratory Press, New York.
- Setyanto P., Suharsih, Wihardjaka A. dan A.K. Makarim. 1999. Pengaruh pemberian pupuk anorganik terhadap emisi gas metan pada lahan sawah. *In: Partohardjono S., Soejitno J. dan Hermanto. Menuju Sistem Produksi Padi Berwawasan Lingkungan: 36-43. Pusat Penelitian dan Pengembangan Tanaman Pangan, Bogor.*
- Shigematsu T., Hanada S., Eguchi M., Kamagata Y., Kanagawa T. And R. Kuran. 1999. Soluble methane monoxygenase gene clusters from trichloroethylene-degrading *Methylomonas* sp. strains and detection of methanotrophs during in situ bioremediation. *Applied and Environmental Microbiology*, 65: 5198-5206.
- Zahn Z.A. and A.A. Dispirito. 1996. Membrane-associated methane monoxygenase from *Methylococcus capsulatus* (Bath). *Journal of Bacteriology*, 178: 10181-1029.

A Publication of the Southeast Asian Ministers of Education Organization  
Southeast Asian Regional Centre for Tropical Biology  
SEAMEO BIOTROP  
Bogor, Indonesia

Vol. 16 No. 2

December 2009

CONTENTS

Research Papers

- Distribution and Diversity of *Fusarium* Species Associated with Grasses in Ten States Throughout Peninsular Malaysia  
NUR AIN IZZATI, M.Z., SITI NORDAHLIAWATE, M.S., NOR AZLIZA, I. and SALLEH, B. .... 55 - 64
- Discrimination of Two Species of Orangutans (*Pongo* sp.): A Rapid Protocol for Rehabilitation Centres and Zoos  
DYAH PERWITASARI-FARAJALLAH ..... 65 - 70
- Isolation and Characterization of Methanotrophic Bacteria from Rice Fields  
IMAN RUSMANA and ALINA AKHDIYA ..... 71 - 78
- Development of Antibody to *Ralstonia solanacearum* and Its Application for Detection of Bacterial Wilt  
YADI SURYADI ..... 79 - 87
- Genetic Variations Among *Aquilaria* Species and *Gryinops versteegii* Using Amplified Fragment Length Polymorphism Markers  
NURITA TORUAN-MATHIUS, DEWI RAHMAWATI and ANIDAH ..... 88 - 95
- Exopolysaccharide Production and Its Bioactivities of the Edible *Pleurotus ostreatus* in Submerged Culture  
IWAN SASKIAWAN ..... 96 - 104

ISSN 1907-770X



9 771907 770013