SOLUBLE METHANE MONOOXYGENASE ACTIVITY OF METHANOTROPHIC BACTERIA ISOLATED FROM RICE FIELD

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ABSTRACT

ROTUA CITRA A. LUMبان GAOL. Soluble Methane Monooxygenase Activity of Methanotrophic Bacteria Isolated from Rice Field. Supervised by IMAN RUSMANA and ALINA AKHDIYA.

Methanotrophic bacteria have soluble methane monooxygenase (sMMO) enzyme known for its capability in catalyzing many pollutants transformation. The aim of this research was to measure sMMO enzyme’s activity of methanotrophs isolated from rice fields. Three methanotrophic isolates (BGM 1, BGM 9, and SKM 14) were successfully grown and purified. Soluble methane monooxygenase (sMMO) activity was screened using Colorimetric Plate Assay. Growth curve and sMMO’s activity in oxidizing naphthalene into naphthol was measured. The results showed that only BGM 9 isolate showed highest sMMO activity (16.3 µM/hour).

Key words: characterization, soluble methane monooxygenase, methanotrophic bacteria, rice field

ABSTRAK

ROTUA CITRA A. LUMبان GAOL. Aktivitas Soluble Methane Monooxygenase Bakteri Metanotrof dari Lahan Persawahan. Dibimbing oleh IMAN RUSMANA dan ALINA AKHDIYA.

Bakteri metanotrof memiliki enzim soluble methane monooxygenase (sMMO) yang diketahui mampu mengkatalisis transformasi berbagai polutan. Tujuan penelitian ini adalah mengkarakterisasi aktivitas enzim sMMO dari isolat-isolat bakteri metanotrof asal sawah di Bogor dan Sukabumi. Tiga isolat bakteri metanotrof (BGM 1, BGM 9, dan SKM 14) berhasil ditumbuhkan dan dimurnikan. Enzim sMMO diuji dengan metode kolorimetrik. Kurva pertumbuhan bakteri dan aktivitas sMMO dalam mengoksidasi naftalen menjadi naftol diamati pada penelitian ini. Hasil pengamatan menunjukkan bahwa hanya isolat BGM 9 yang menunjukkan aktivitas tertinggi sMMO (16.3 µM/jam).

Kata kunci: karakterisasi, soluble methane monooxygenase, bakteri metanotrof, sawah
SOLUBLE METHANE MONOOXYGENASE ACTIVITY OF METHANOTROPHIC BACTERIA ISOLATED FROM RICE FIELD

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Minithesis
In partial fulfillment of the requirement for Bachelor Degree of Science in Department of Biology Faculty of Mathematics and Natural Sciences Bogor Agricultural University

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Bogor, August 2012

Rotua Citra A. Lumban Gaol
CURRICULUM VITAE

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Author also took part in many college event, such as Pesta Sains Nasional, Biology on Experiment (BOX), Workshop dan Seminar Artikel Ilmiah Populer, Kebaktian Awal Tahun Ajaran PMK IPB, and many others. During the study, the author received Peningkatan Prestasi Akademik (PPA) scholarship. Beside that, author has also took part on several competitions, recorded her accomplishment becoming 3rd winner of IPB’s students creativity program (PKM) Generation in 2009 and others.

Author assisted the practical class for Biology in 2012. During July 2010, author did the field study entitled Potential of Microalgae as Biodiesel Fuel. In July until August 2011, author did field work entitled Microbiological Quality Control of “Jahe Wangi” Beverage Product in PT Sido Muncul Semarang.
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INTRODUCTION

Background

Methane has become one of the most important greenhouse gases contributing to global warming. According to International Panel on Climate Change, IPCC (1992), methane is third after CO$_2$ and chlorofluorocarbon (CFC) on its contribution to global warming. About 18,000 years ago, atmospheric concentration of methane was only 0.35 ppmv, but it has been increasing from 0.75 ppmv to 1.7 ppmv in the latest 300 years (Lelieveld et al. 1993). In this concentration, methane leads into the increasing of global temperature which is up to 1.3°C. Although methane’s concentration is not as high as CO$_2$ concentration in atmosphere, methane’s ability to absorb irradiation of infrared is higher than CO$_2$. Reemission of the energy from absorbed radiation is the one causing global warming. Reducing atmospheric methane gas will mitigate global warming up to 60%. Hence, methane gas’s reducing will affect 20-60 times more effective in lowering global warming’s potential than CO$_2$ (Hogan et al. 1991).

One of the biological agent which is known of its ability in decreasing methane’s emission is methanotrophic bacteria. They commonly live in wetlands, including rice fields (Hanson & Hanson 1996). Methanotrophic bacteria oxidize methane to methanol using unique enzyme called Methane Monooxygenase (MMO) which consists of two different types of MMO, they are soluble MMO (sMMO) and particulate MMO (pMMO). All known methanotrophs are capable of forming pMMO when grown in the presence of copper (Dalton 1992). However, only several types of methanotrophs contain sMMO. *Methylococcus* and *Methylosinus* are two examples of genera known of their sMMO activity. Meanwhile, few species of genera *Methylomonas* and *Methylcystis* are able to produce sMMO enzyme (McDonald et al. 1997).

Beside methane oxidation activity, MMO enzyme also has capability in oxidizing hydrocarbon compounds. Particulate MMO (pMMO) can oxidize only short hydrocarbon chain like alkane, alkene, and ammonia. In other hand, sMMO can oxidize many kinds of hydrocarbon chain like alkane, alkene, and aromatic compounds (Colby & Dalton 1978). Thus, sMMO is more effective in catalyzing transformation of pollutants of halogenated hydrocarbon compounds such as trichloroethylene (TCE) (McDonald et al 1997).

Exploration of methanotrophs and characterization of the bacteria and its enzyme are very important as the first step of development of this bacteria to eradicate methane’s emission and other pollutants in rice fields as their habitat.

Objectives

The objective of this research was to measure sMMO enzyme’s activity of methanotrophs isolated from rice fields.

MATERIALS AND METHOD

Time and Place

This research was conducted on January-July 2012 in Microbiology Laboratory of Department of Biology, Bogor Agricultural University, Indonesia.

Materials

Materials used in this research were methanotrophic bacterial isolates from several rice fields in Bogor and Sukabumi, Nitrate Mineral Salts (NMS) medium containing MgSO$_4$.7H$_2$O 1.0 g/L, CaCl$_2$.6H$_2$O 0.2 g/L, KNO$_3$ 1.0 g/L, KH$_2$PO$_4$ 0.272 g/L, Na$_2$HPO$_4$ 4.0 g/L, Na$_2$EDTA 0.5 g/L, FeSO$_4$.7H$_2$O 0.2 g/L, H$_3$BO$_4$ 0.03 g/L, CoCl$_2$.6H$_2$O 0.02 g/L, ZnSO$_4$.7H$_2$O 0.01 g/L, MnCl$_2$.4H$_2$O 3.0 mg/L, Na$_2$MoO$_4$.2H$_2$O 3.0 mg/L, NiCl$_2$.6H$_2$O 2.0 mg/L, and CaCl$_2$.2H$_2$O 1.0 mg/L, methanol, Bacto Agar, naphthalene crystal, o-dianisidine, sodium format, mineral water, aquadest, autoclave, syringe, laminar cabinet, bottle, micropipettes, and other materials for routine laboratory analysis.

Methods

Purification of methanotrophic bacteria

Isolates of methanotrophic bacterial isolates from several rice fields in Bogor and Sukabumi. Nitrate Mineral Salts (NMS) medium containing MgSO$_4$.7H$_2$O 1.0 g/L, CaCl$_2$.6H$_2$O 0.2 g/L, KNO$_3$ 1.0 g/L, KH$_2$PO$_4$ 0.272 g/L, Na$_2$HPO$_4$ 4.0 g/L, Na$_2$EDTA 0.5 g/L, FeSO$_4$.7H$_2$O 0.2 g/L, H$_3$BO$_4$ 0.03 g/L, CoCl$_2$.6H$_2$O 0.02 g/L, ZnSO$_4$.7H$_2$O 0.01 g/L, MnCl$_2$.4H$_2$O 3.0 mg/L, Na$_2$MoO$_4$.2H$_2$O 3.0 mg/L, NiCl$_2$.6H$_2$O 2.0 mg/L, and CaCl$_2$.2H$_2$O 1.0 mg/L, methanol, Bacto Agar, naphthalene crystal, o-dianisidine, sodium format, mineral water, aquadest, autoclave, syringe, laminar cabinet, bottle, micropipettes, and other materials for routine laboratory analysis.
Colorimetric plate assay for screening of soluble methane monoxygenase activity

Soluble methane monoxygenase activity was assayed using a slightly modified method on methane-air atmosphere (25% to 50%) described by Graham et al. (1992). Plates containing methanotrophs were incubated at 30°C under a 50% methane-air atmosphere for 7 to 21 days. Plates were removed carefully, and methanotrophs colonies were screened for sMMO activity by the following procedure. A few naphthalene crystals were sprinkled on the lid of the plate, and the plate was stored inverted at 30°C for 15 minutes. The plates were then opened and sprayed with 5 mg/ml o-dianisidine, for 2 to 3 s. The lid was replaced with a new one, and the plate was stored for 15 min in the presence of the dye. If naphthol was formed by the colonies, a purple-red color appeared as the color of colonies.

Growth Curve of Methanotrophs

One loop of pure bacteria colony were inoculated into 50 ml NMS medium under an atmosphere 1:1 air/methane. Cultures were then incubated on 100 rpm shaker at room temperature. Growth of bacteria was observed by monitoring culture’s optical density using spectrophotometer at 620 nm wavelength every two days during 10-14 days of incubation.

Quantitative measurement of sMMO activity

A slightly modified version of the naphthalene oxidation assay of Burlage et al. (1998) was used to quantitatively measure sMMO activity. The modification is on the wavelength of spectrophotometry (540 nm to 525 nm) when bacterial optical density was measured. One milliliter culture was transferred in screw-cap test tubes, 1 ml of prefiltered saturated naphthalene solution and 25 mM sodium formate were added to each aliquot. The samples were prepared in triplicate. The reaction mixtures were incubated at 200 rpm on a rotary shaker at 25°C for 1 to 3 h. A sterile medium control as blanks were also prepared and processed in the same way as the tests. After incubation, 100 µl of freshly prepared tetrazotized-o-dianisidine solution (4.21 mM) was added to each tube. The color of the reaction product was stabilized by adding 0.4 ml glacial acetic acid after 1 minute. The solution was immediately monitored by recording the A_{525} by spectrophotometry after centrifugation at 5000 rpm for 5 minutes. The intensity of diazo-dye formation is proportional to the naphthol concentration (1-naphthol).

RESULTS AND DISCUSSION

Purification of methanotrophic bacteria.

Three methanotrophic isolates (BGM 1, BGM 9, and SKM 14) were successfully grown and purified. Time of isolate’s growth also varied between 12-14 days. At the final stage of bacterial growth, diameter of a single colony reached 2-4 mm (Table 1). Bacterial growth rate between those three isolates was not significantly different. Compared to BGM 1 and BGM 9, SKM 14 isolate grew fastest.

After grown on NMS medium, those three isolates showed differences in color (Table 1, Figure 1, 2, 3). BGM 1 was white-colored, BGM 9 was cream-colored, and SKM 14 was cream-orange-colored. These results are in agreement with those of Hapsary (2008) who found the same differences in isolates color. Murrel & Dalton (1992) reported that methanotrophic bacteria produce many kinds of coloring pigment, either soluble or insoluble pigment. Soluble pigment is more frequently found, for instance, white, cream, and orange. It means coloring pigment in BGM 1, BGM 9, and SKM 14 isolates belong to soluble pigment. Moreover, according to Murrell & Dalton (1992), beside genetic factor, pigmentation is also affected by culture’s age and physiological stage of bacteria.

Table 1. Colony morphology of methanotrophic Bacteria on NMS Agar

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Days Incubation</th>
<th>Diameter of colony</th>
<th>Morphology</th>
</tr>
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<tbody>
<tr>
<td>BGM 1</td>
<td>12 days</td>
<td>2 mm</td>
<td>White, convex, smooth, circular</td>
</tr>
<tr>
<td>BGM 9</td>
<td>12 days</td>
<td>2 mm</td>
<td>Cream, convex, smooth, circular</td>
</tr>
<tr>
<td>SKM 14</td>
<td>10 days</td>
<td>4 mm</td>
<td>Cream orange, convex, smooth, circular</td>
</tr>
</tbody>
</table>
BGM 9 and SKM 14 isolates were chosen for this research because of their highest methane oxidation activity (66,556 mol/day and 25,654 mol/day) among six methanotrophic isolates that were originally isolated and measured by Hapsary (2008). However, BGM 1 was chosen as the comparative isolate because of its lowest methane oxidation activity (969 mol/day). Soluble methane monooxygenase enzyme was known for its capability in catalyzing methane oxidation more effective than particulate methane monooxygenase. Therefore, methane oxidation activity of the bacteria is one of several indicators in detecting sMMO’s existence. It was the reason why methane oxidation activity became an important factor when choosing methanotrophic isolates that would be used in this research.

Identification of BGM 1, BGM 9, and SKM 14 was conducted by Astuti (2009) using 16s rRNA gene sequencing. BGM 1 has 74% similarity with *Methylocystis rosea*, BGM 9 has 85% similarity with *Methylococcus capsulatus*, and SKM 14 has 65% similarity with *Methylobacter* sp.

According to Tsien et al. (1989) the expression of sMMO is inhibited by Cu(II) concentrations as low as 0.25 µM. The copper concentration for sMMO repression depends on several factors such as the ambient oxygen tension and species identity. Thus, to optimize expression of sMMO, those three isolates were grown on copper-free NMS agar. In this research, a balanced atmosphere of methane and air on medium’s headspace is due to methanotroph’s dependency on methane as the sole carbon, energy source and oxygen in air as their natural condition (Hanson & Hanson 1996). The results showed that all isolates were successively grown on NMS medium and utilized methane as the sole carbon source.

**Colorimetric plate assay for Soluble Methane Monooxygenase activity.**

A procedure of colorimetric plate assay described by Graham *et al.* (1992) was used to qualitatively detect sMMO’s activity. After conducted with the assay, only BGM 9 formed purple-red color whereas BGM 1 and SKM 14 did not show any color development (Appendix 1). The results indicated only BGM 9 possessed sMMO activity (Table 2).

This method was based on observations that (i) sMMO in bacteria catalyzes the transformation of naphthalene to 1-naphthol and (ii) naphthol formation can be observed using colorimetric assay by the addition of certain aromatic diazo compounds to the reaction mixture (Parsons *et al.* 1955). The conversion of naphthalene to naphthol has been used as an indicator of trichloroethylene degradation potential in methanotroph (Brusseau *et al.* 1990). In this research, orto dianisidine was used as an aromatic diazo compound as well as a coloring agent. O-dianisidine turned BGM 9 isolate’s...
color into purple-red in the presence of naphthol. This was as a result of sMMO activity in BGM 9 in order to oxidize naphthalene into 1-naphthol. NMS medium used for bacterial growth was copper-free. This manner was due to Graham et al. (1992) who reported that sMMO activity was not expressed in the presence of copper in the NMS medium.

Table 2. Screening of sMMO activity (based on Colorimetric Plate Assay)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Result</th>
</tr>
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<tbody>
<tr>
<td>BGM 1</td>
<td>Negative</td>
</tr>
<tr>
<td>BGM 9</td>
<td>Positive</td>
</tr>
<tr>
<td>SKM 14</td>
<td>Negative</td>
</tr>
</tbody>
</table>

The results of sMMO activity in this research also supported Hapsary (2008) who reported that BGM 9 performed highest methane oxidation (66556 mol/day) than SKM 14 and BGM 1 which was only 25654 mol/day and 969 mol/day. The high difference of methane oxidation activity between BGM 9 and two other isolates supports sMMO activity assay which BGM 9 was the only isolate expressing sMMO.

As stated by Graham et al. (1992) it is also possible to distinguish between methanotrophic organisms that able and unable to converse cyclohexane to cyclohexanol by using the colorimetric plate assay procedure mentioned. This is because cyclohexane reactivity represents the ability to transform several persistent chemical pollutants, for instance trichloroethylene (Tsien et al. 1990).

Growth Curve of Methanotrophs

Growth of selected isolate bearing sMMO activity (BGM 9) was observed by measuring optical density (OD) of bacterial culture during 12 days incubation at room temperature on 100 rpm’s shaker. Figure 4 illustrated OD measurement of the isolate’s growth. The result apparently showed an increasing bacterial OD until day 7, stagnancy OD from day 8 until day 12. Based on this result, BGM 9 isolate exhibited some growth dynamics: from day 0 until day 4, bacterial cells initially adjust to the new NMS medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase) from day 5 until day 8. When their growth becomes limited, the cells are in stationary phase, from day 9 until day 12. A slight increasing OD measurement (only 0.08) represented slow diffusion rate of BGM 9. Bacterial growth rate appears to depend on a complex relationship involving wide variety of factors, such as the size of inoculum, nutritional condition (pH medium, temperature, etc), time necessary to recover from physical damage or shock in the transfer, time required for synthesis of essential coenzymes or division factors, and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium (Pelczar & Chan 1981)

Figure 4. Growth Curve BGM 9 isolate

Quantitative measurement of sMMO activity

From the foregoing result, it was apparent that BGM 9 beared sMMO enzyme. In order to show sMMO activity, minor modification of quantitative measurement of sMMO activity method described by Burlage et al. (1998) was used. This method utilized naphthalene oxidized to 1-naphthol as indicator of sMMO activity. Therefore, standard curve of 1-naphthol was drawn (Appendix 2) as the parameter of 1-naphthol concentration formed from naphthalene oxidation.

As stated in Table 3, sMMO enzyme of BGM 9 isolate was capable of forming 1-naphthol from naphthalene oxidation. Peak of enzyme’s activity of bacterial population was observed in the end of exponential phase (16.3 µM/hour), whereas in other phase (lag, stationary, and death phase) was observed only in range 8.12-11.45 µM/hour.
Table 3. Naphthalene oxidation rates of BGM 9 isolate

<table>
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<tr>
<th>Day</th>
<th>Naphthalene oxidation rate (µM/hour)</th>
<th>Phase</th>
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</thead>
<tbody>
<tr>
<td>3</td>
<td>10,383</td>
<td>Lag</td>
</tr>
<tr>
<td>6</td>
<td>16,347</td>
<td>Exponential</td>
</tr>
<tr>
<td>8</td>
<td>11,447</td>
<td>Stationary</td>
</tr>
<tr>
<td>12</td>
<td>8,117</td>
<td>Stationary</td>
</tr>
</tbody>
</table>

These results were in agreement with Koh et al. (1993) reporting that sMMO enzyme’s peak activity of bacterial population was detected in the end of exponential phase. However, an interesting phenomenon was found if we observe sMMO activity in day 3. At third day of incubation (lag phase), bacterial population exposed a very low level but in contrary the sMMO activity was pretty high (10,383 µM/hour). Based on this result, it can be concluded that sMMO activity per cell methanotroph appears the highest number at lag phase because concentration of methane and nitrogen is still abundant at that phase.

In this research, a sterile medium as blank was also processed in the same way as the test known as sMMO negative. Activity of sMMO was influenced by several factors such as inoculum size, pH, temperature, growth time, copper-free nutritional condition, nitrogen level, aeration of methane and oxygen. Habitats lacking copper but abundant methane and oxygen provide a niche for sMMO producing methanotroph (Dalton & Leak 1985).

CONCLUSION

From BGM 1, BGM 9, and SKM 14, only BGM 9 isolate was found expressing sMMO enzyme which bacterial population showed highest activity represented with naphthalene oxidation rates 16,347 µM/hour.

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APPENDIXES

Appendix 1. Colorimetric plate assay for screening of sMMO activity. A and C: BGM 1 and SKM 14 after assay. No color development on colonies within red circle known as negative sMMO activity. B: BGM 9 after assay. Color turned into purple-red on colonies within red circle as a result of sMMO activity.
Appendix 2 1-Naphthol standard curve

\[ y = 0.0034x + 0.5315 \]

\[ R^2 = 0.9856 \]

Optical Density (A)

Concentration (ppm)

10 0.561
20 0.608
30 0.635
40 0.673
50 0.691
60 0.745