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Cupredoxin domain of particulate methane monooxygenase (pMMO) gene expression in recombinant *Escherichia coli*

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ABSTRACT

Aims: Particulate methane monooxygenase (pMMO) is an integral membrane protein that converts methane to methanol as the first step in the metabolic pathway of methanotroph bacteria. Methanotroph have a slow growth rate that make researcher have to develop an alternative approach by expressing the pMMO genes in *Escherichia coli*. However, it was very difficult to express all the pMMO encoded genes in *E. coli* and it is suspected that the protein might be toxic to *E. coli*. Therefore, this research tried another approach by expressing the active site of pMMO enzyme; cupredoxin domain of pmoB subunit encoded by *spmoB* gene.

Methodology and results: The *spmoB* gene from *Methylococcus capsulatus* (Bath) was expressed in *E. coli* BL21 (DE3) under T7 promoter and pET15b as the expression vector. Several modifications were made so this gene would be expressed in the cytoplasm. Expression analysis with SDS-PAGE showed that overexpression of this gene could be done at several concentrations of IPTG and incubation temperature. The *spmoB* gene expression produced a recombinant protein with a size approximately 38.9 kDa. Assay of spmoB protein activity showed that the amount of methanol accumulated during methane oxidation by the recombinant strain was 0.114 mmol/mL culture.h.

Conclusion, significance and impact study: We successfully expressed *spmoB* gene in *E. coli* BL21 (DE3) without high production of toxic compounds and it has methane oxidation activity. This result allowed further characterization of its potential applications.

Keywords: methanotroph, pMMO, *spmoB,* recombinant *Escherichia coli*

INTRODUCTION

Methanotroph are a group of bacteria that are able to grow by using methane as their source of carbon and energy (Hanson and Hanson, 1996). These bacteria have the metabolic oxidation pathway of methane to methanol which is catalyzed by methane monooxygenase (MMO) enzyme (Culpepper and Rosenzweig, 2012). The contribution of methane as a greenhouse gas is second only to carbon dioxide, and methane significantly absorbs more infrared radiation (Tol *et al.*, 2003). Therefore, methanotroph with MMO has the potential to reduce methane emission in the atmosphere. However, the application of methanotroph is hindered by their relatively slow growth rate and low cell density (Gou *et al*., 2006).

MMO enzymes have been the focus of intense study recently for its ability to oxidize methane and other substrates, including halogenated hydrocarbons (Semrau *et al.*, 2010). There are two types of MMO, a cytoplasmic complex; soluble methane monooxygenases (sMMO), and a membrane bound; particulate methane monooxygenases (pMMO). From all methanotroph that have been investigated, most produces pMMO and only some strains that have both sMMO and pMMO (Murrell *et al.*, 2000). pMMO is the dominant enzyme in methane oxidation in nature, but this enzyme has proven to be difficult to isolate (Lieberman and Rosenzweig, 2004). Some studies were then performed to clone and express the pMMO genes in *E. coli,* but the product(s) of pMMO encoding gene clusters seem to be toxic (Murrell *et al.*, 2000; Gou *et al.,* 2006).

pMMO is encoded by *pmoCAB* operon where *pmoC*, *pmoA*, and *pmoB* encode three pMMO polypeptide subunits respectively: γ subunit or pmoC (22 kDa), β or pmoA (24 kDa), and α or pmoB (47 kDa) (Ngunyen *et al.*, 1997; Lieberman and Rosenzweig, 2004). All three subunits form a complex trimeric structure $\alpha_3\beta_3\gamma_3$ (Lieberman and Rosenzweig, 2005; Hakemian *et al.*, 2008; Smith *et al.*, 2011a). Crystallography experiments on pMMO of *Methylococcus capsulatus* (Bath) showed

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that the active site allegedly contained in cupredoxin domain of pmoB subunit (47 kDa) (Lieberman and Rosenzweig, 2005). pmoB subunit consists of the N-terminal domain cupredoxin (spmoBd1) and C-terminal domain cupredoxin (spmoBd2) and is connected by two transmembrane helices. Both cupredoxin domain are in the periplasm and the only soluble domain (Culpepper and Rosenzweig, 2012). Initial 32 amino acid residues are thought to be a signal peptide (Semrau *et al.*, 1995). Cupredoxin domain coding gene in pmoB subunit, *spmoB* gene, is thought to be the pMMO active site coding gene. The evidence has been shown by Smith *et al.* (2011b) which expressed only *spmoB* gene in *E. coli* and it still had methane oxidation activity.

In this study, we expressed gene from *M. capsulatus* (Bath) that encode pmoB subunit in *E. coli* BL21 (DE3). The transmembrane helices and signal peptides sequence of this gene have been eliminated. *Methylococcus capsulatus* (Bath) was chosen because the whole genome information, including *pmoCAB* operon, had been known (Ward *et al.*, 2004). Structure and the active site information of pMMO had also been known (Lieberman and Rosenzweig, 2005; Smith *et al.*, 2011b; Culpepper and Rosenzweig, 2012). This gene was expressed under control of T7 promoter and pET15b as the expression vector in *E. coli* BL21 (DE3).

MATERIALS AND METHODS

Cloning of artificial *spmoB* gene

Manufacture of artificial *spmoB* genes was done through corporate services with Integrated DNA Technologies, Inc. (IDT). Gene sequence of *spmoB* from *M. capsulatus* (Bath) was obtained from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) website with the accession number of L40804.2. Then, artificial gene was amplified by polymerase chain reaction (PCR) (Gene Amp® PCR system 2720). Forward primer was spmoBd1F (5'-GGAATTCCATATGCACGGTGAGAAATCGCGG-3') with *Nde*I enzyme recognition sites (underlined sequence) and spmoBd2R (5'-CGGGATCCTTACATGAACGACGGGATCA-3') with *Bam*HI enzyme recognition sites (underlined sequence). The PCR reaction consisted of 5 U of DNA polymerase (LA-Taq Takara), 1 ng of artificial gene, 0.5 µM of spmoBd1 primer, 0.5 µM of spmoBd2R primer, 1× of buffer, and 0.25 µM of dNTPs. Amplification was done in 12 cycles. The PCR condition used was pre-denaturation (94 °C, 30 sec), denaturation (94 °C, 15 sec), annealing (55 °C, 15 sec), elongation (72 °C, 30 sec), and final elongation (72 °C, 5 min). The PCR products were purified and cloned into *E. coli* DH5α with the pGEM-T Easy vector (Promega, USA). Then, the coding region of *spmoB* was verified using DNA sequencing through corporate services with 1st Base, Malaysia.

Construction pET15b expression vector and transformation

spmoB gene fragments were obtained by cutting the recombinant pGEM-*spmoB* plasmid with *Nde*I and *Bam*HI restriction enzymes. The products were separated by 1% agarose gel and purified with Gel DNA Fragments Extraction Kit (Geneaid). pET15b plasmid which had also been digested with *Nde*I and *Bam*HI was ligated with *spmoB* gene. Ligation process was done using T4 DNA ligase (Invitrogen, USA). Then recombinant plasmid pET15b-*spmoB* was transferred to *E. coli* BL21 (DE3) competent cells by heat shock treatment at temperature 42 °C for 60 sec. Transformants of *E. coli* BL21 (DE3) were screened using resistance of ampicillin. The bacteria were spread on the Luria agar (LA) media which contain ampicillin 100 µg/mL. *E. coli* BL21 (DE3) colonies which carried recombinant plasmid were verified by colony PCR using spmoBd1F and spmoBd2R primers.

Expression analysis of *spmoB* gene

Escherichia coli BL21 (DE3) transformant were grown in 3 mL LB medium containing ampicilin 100 µg/mL for overnight at 37 °C. One hundred microliters of culture was used to inoculate 10 mL of LB and incubated at 27 °C or 37 °C. When the optical density of the cells have reached $OD_{600\ nm}$ of 0.6, IPTG was added to the culture with several final concentration (0.1 mM, 0.5 mM, and 1.0 mM) and one culture was uninduced. All of the cultures were incubated for 4 h post induction. Up to 100 µL of each culture was added into 30 mL sample buffer (60 mM Tris-HCl pH 6.8, 25% glicerol, 14,4 mM β-mercaptoethanol, 0.1% bromophenol blue) and heated at 100 °C for 10 min. Then each sample (10 µL) was loaded into the well 12.5% polyacrylamide gel. Electrophoresis was performed at a fixed voltage of 80 volts, 50 A for 3 h. Staining was done by Coomassie Brilliant Blue (CBB) for 30 min while destaining was by 10% acetic acid solution. Estimation size of the recombinant protein was calculated with value of relative mobility (Rf) and log molecular weight marker (BM). For comparison, the molecular weight of the spmoB recombinant protein was predicted based on the amino acid sequence through ExPASy software (http://web.expasy.org/compute_pi).

Assay of spmoB activity

Escherichia coli BL21 (DE3) transformant were cultivated in 3 mL LB medium containing antibiotic ampicillin 100 µg/mL for overnight at 27 °C. A total of 40 mL of the culture was used to inoculate 4 mL of LB medium containing antibiotic ampicillin 100 ug/mL and 10 µM $CuSO_4$ in a 12 mL tube. The cultures were incubated at 27 °C until the optical density of the cells have reached $OD_{600\ nm}$ of 0.6. Then 0.1 mM IPTG was added to the culture. The tube was closed with a rubber stopper, then the air in the head space was set so the composition was approximately 50% air and 50% methane. The culture were incubated at 27 °C for 24 h post induction. A total of 1 mL culture was taken to be measured the amount of

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methanol accumulation by SNP reagent method (Zhan *et al*., 2010).

RESULTS

Cloning of artificial *spmoB* gene

Amplification of *spmoB* artificial gene with spmoBd1F and spmoBd2R primer produced ± 900 bp amplicon (Figure 1). This gene was successful to be cloned into *E. coli* DH5α with the pGEM-T Easy vector (Promega, USA).

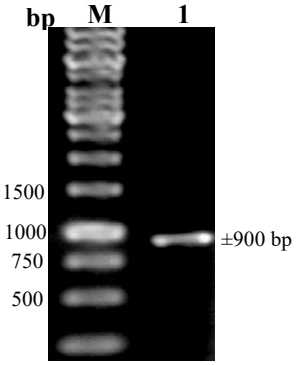

Figure 1: Visualization of *spmoB* amplicon in 1% agarose gel (w/v). M, marker 1 kb; 1, amplicon of *spmoB* artificial gene with the size ±900 bp.

Construction pET15b expression vector and transformation

spmoB artificial gene was designed to encode the pMMO spmoBd1 (amino acid residues 34-172) and spmoBd2 (amino acid residues 265-415) domains that were connected by 6 amino acids (Gly-Gly-Lys-Leu-Gly-Gly-Gly) (Figure 2). Recognition site of *Nde*I acts as replacement of start codon.

Expression analysis of *spmoB* gene and assay of spmoB activity

Overexpression of *spmoB* occured either at 27 °C or at 37 °C. IPTG with final concentration of 0.1 mM, 0.5 mM, and 1.0 mM could also induce *spmoB* gene expression (Figure 3). Molecular weight of spmoB was predicted to be 32.9 kDa by Expasy (Figure 4). Based on the calculation of relative mobility, spmoB recombinant protein was estimated to be 38.97 kDa. After 24 h IPTG induction, the amount of methanol in the culture was 2.736 mmol/mL. For the *E. coli* BL21 (DE3) non transformant, no product was found during the detection of spmoB activity.

DISCUSSION

Gene size of pmoB subunit in *M. capsulatus* (Bath) is 1245 bp. This gene consists of 96 bp peptide signal encoder (residues 1-32), 420 bp spmoBd1 encoder (residues 33-172), 279 bp two transmembrane helices encoder (residues 173-264), and 450 bp spmoBd2 encoder (residues 265-414). Then, modifications were made: removal of the signal peptide sequence and replacement of the two transmembrane helices sequence with 6 amino acids. Elimination of the 96 bp of peptide signal and 279 bp of two transmembrane helices sequences aimed to express *spmoB* in the cytoplasm, since there were many cases of membrane protein expression resulted toxicity in *E. coli* (Miroux and Walker, 1996). Elimination of 96 bp first sequence caused the disappearance of start codon. This start codon was replaced by *Nde*I recognition site that had ATG sequence so the translation could still be taken place. Transmembrane helices were also eliminated. Until now, the function of the transmembrane regions has not been elucidated (Culpepper and Rosenzweig, 2012). Amino acids Gly-Lys-Leu-Gly-Gly-Gly linker was used to replace the two transmembrane helices to connect residue 172 and 265. The glycine residues were selected because it could impart flexibility of the enzyme (Balasubramanian *et al.*, 2010). The *pmoB* gene, that was originally sized 1245 bp, shortened into 891 bp. Addition of some bases was the recognition sequences of *Nde*I and *Bam*HI.

We expressed the *spmoB* gene in *E. coli* BL21 (DE3). The advantages of using *E. coli* as the host organism are well known. It has unparalleled kinetics of fast growth, high cell density cultures are easily achieved, complex rich media can be made from readily available and inexpensive components, and transformation with exogenous DNA is fast and easy (Rosano and Ceccarelli, 2014). Then, compared to the relatively slow growth of *M. capsulatus* (Bath), the theoretical density limit of an *E. coli* culture liquid is estimated to be about 200 g cells/L (Lee, 1996; Shiloach and Fass, 2005).

Overexpression of *spmoB* gene in *E. coli* BL21 (DE3) with IPTG induction was successfully done. Several variants of IPTG final concentration and temperature of incubation were used to determine the effect on the expression. In this study, recombinant *E. coli* was able to express spmoB recombinant protein both at 27 °C and 37 °C. The effect of different final concentrations of IPTG on *spmoB* expression was examined and the result showed that 0.1 mM, 0.5 mM, and 1.0 mM IPTG could induce well the expression of recombinant gene. In several cases, incubation temperature greatly affects the expression of recombinant proteins in *E. coli* and the concentration of IPTG also can influence expression dramatically (Khlebnikov and Keasling, 2002; Gopal and Kumar, 2013). In this case, IPTG 0.1 mM could induce the expression as well as IPTG 0.5 mM and 1.0 mM. This result showed that IPTG 0.1 mM could bind almost all of the lac repressor. The enhancement of IPTG concentration would not give the difference of expression level anymore if all of the lac repressor has been binded.

Expression of *spmoB* gene produced a recombinant protein with a size approximately 38.9 kDa and it was different from ExPASy prediction. This difference was due to the addition of six histidine residues and some amino acids at upstream of the protein. The size of recombinant protein became smaller than the size of the pmoB subunit

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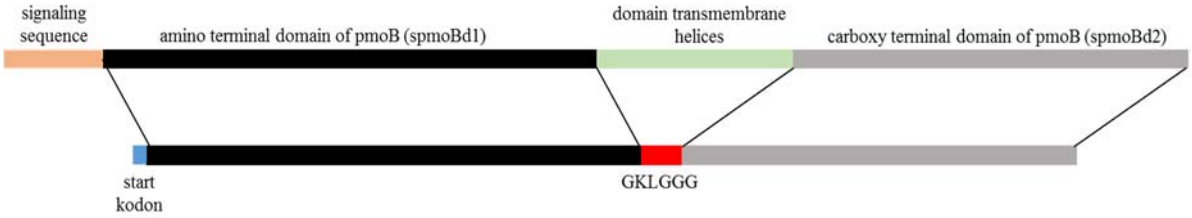


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1ATGCACGGTGAGAAATCGCAGGCCGCGTTCATGCGTATGCGTACC
M  H  G  E  K  S  Q  A  A  F  M  R  M  R  T
46 ATCCACTGGTACGACCTGAGCTGGTCGAAAGAGAAAGTCAAGATC
I  H  W  Y  D  L  S  W  S  K  E  K  V  K  I
91 AACGAGACCGTGGAAATCAAAGGCAAGTTCCACGTGTTCGAAGGC
N  E  T  V  E  I  K  G  K  F  H  V  F  E  G
136 TGGCCGGAAACGGTCGACGAACCGGATGTGGCGTTCCTGAACGTC
W  P  E  T  V  D  E  P  D  V  A  F  L  N  V
181 GGCATGCCGGGTCCGGTGTTCATCCGCAAGGAATCGTACATCGGC
G  M  P  G  P  V  F  I  R  K  E  S  Y  I  G
226 GGTCAGCTGGTGCCGCGTTCCGTACGTCTGGAAATCGGCAAGACC
G  Q  L  V  P  R  S  V  R  L  E  I  G  K  T
271 TATGACTTCCGGGTTGTCCTCAAAGCCCGTCGTCCGGGTGACTGG
Y  D  F  R  V  V  L  K  A  R  R  P  G  D  W
316 CACGTTCACACCATGATGAACGTCCAGGGCGGTGGACCGATCATC
H  V  H  T  M  M  N  V  Q  G  G  G  P  I  I
361 GGTCCCGGCAAATGGATCACCGTGGAAGGCTCCATGAGTGAATTC
G  P  G  K  W  I  T  V  E  G  S  M  S  E  F
406 CGCAACCCCGTCACCACCGGAAAGCTTGGAGGAGGAGCCGGCACC
R  N  P  V  T  T  G  K  L  G  G  G  A  G  T
451 ATGCGTGGCATGAAGCCGCTGGAACTGCCGGCGCCGACGGTATCG
M  R  G  M  K  P  L  E  L  P  A  P  T  V  S
496 GTGAAAGTGGAAGACGCCACCTACCGGGTACCGGGCCGCGCCATG
V  K  V  E  D  A  T  Y  R  V  P  G  R  A  M
541 CGGATGAAGCTGACCATCACCAACCACGGCAACAGCCCGATCCGG
R  M  K  L  T  I  T  N  H  G  N  S  P  I  R
586 CTGGGTGAGTTCTACACCGCCTCGGTGCGTTTCCTGGATTCCGAC
L  G  E  F  Y  T  A  S  V  R  F  L  D  S  D
631 GTGTACAAGGACACCACCGGCTATCCGGAAGACCTGCTGGCCGAA
V  Y  K  D  T  T  G  Y  P  E  D  L  L  A  E
676 GACGGCCTGAGCGTCAGCGACAACAGCCCGCTGGCTCCGGGTGAG
D  G  L  S  V  S  D  N  S  P  L  A  P  G  E
721 ACGCGCACGGTCGACGTGACGGCGTCCGACGCGGCGTGGGAAGTG
T  R  T  V  D  V  T  A  S  D  A  A  W  E  V
766 TACCGTCTGTCCGACATCATCTACGATCCGGACAGCCGTTTCGCC
Y  R  L  S  D  I  I  Y  D  P  D  S  R  F  A
811 GGTCTGCTGTTCTTCTTCGACGCCACTGGCAACCGCCAGGTCGTC
G  L  L  F  F  F  D  A  T  G  N  R  Q  V  V
856 CAGATCGACGCACCGCTGATCCCGTCGTTCATGTAA 891
Q  I  D  A  P  L  I  P  S  F  M  *
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Figure 2: Illustration of artificial gene design and *spmoB* gene sequence from *M. capsulatus* (Bath) that was used.

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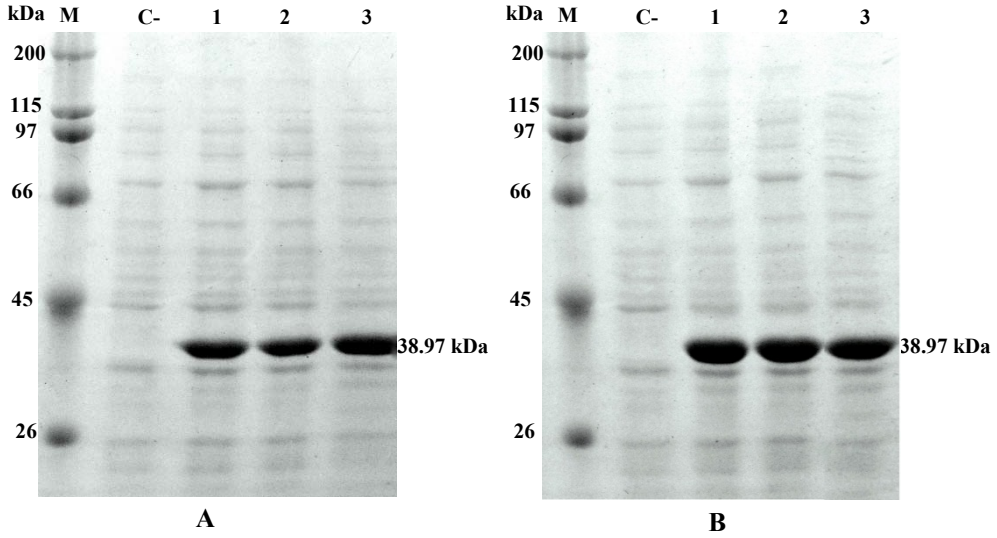

Figure 3: Analysis of *spmoB* gene expression by SDS-PAGE was resulting a recombinant protein with the size 38.97 kDa. A, incubation at 27 °C; B, incubation at 37 °C. M, Marker protein; C-, bacterial transformant was uninduced by IPTG; 1, induction of 0.1 mM IPTG; 2, induction of 0.5 mM IPTG; 3, induction of 1.0 mM IPTG.

```
        10         20         30         40         50         60
MHGEKSQAAF MRMRTIHWYD LSWSKEKVKI NETVEIKGKF HVFEGWPETV DEPDVAFLNV
        70         80         90        100        110        120
GMPGPVFIRK ESYIGGQLVP RSVRLEIGKT YDFRVVLKAR RPGDWHVHTM MNVQGGGPII
       130        140        150        160        170        180
GPGKWITVEG SMSEFRNPVT TGKLGGGAGT MRGMKPLELP APTVSVKVED ATYRVPGRAM
       190        200        210        220        230        240
RMKLTITNHG NSPIRLGEFY TASVRFLDSD VYKDTTGYPE DLLAEDGLSV SDNSPLAPGE
       250        260        270        280        290
TRTVDVTASD AAWEVYRLSD IIYDPDSRFA GLLFFFDATG NRQVVQIDAP LIPSFM
```

Theoretical pI/Mw: 6.11 / 32.93462

Figure 4: Isoelectric point (pI) and molecular weight measurement (Mw) prediction of spmoB recombinant protein by Expasy software.

(47 kDa) because of the transmembrane helices domain removal. This protein size is same as the size of spmoB recombinant protein reported by Balasubramanian *et al.* (2010).

The spmoB activity in *E. coli* BL21 (DE3) transformant was also assayed. Bacterial cells cultivated after 24 h IPTG induction in LB medium were used. The activity of the recombinant strain was 0.114 mmol/mL culture.h. For the *E. coli* BL21 (DE3) non transformant, no product was found during the detection of spmoB activity. This control experiment confirmed that the observed monooxygenase activity in the recombinant *E. coli* BL21 (DE3) was due to the cloned enzyme. The spmoB activity has not been optimal yet. This was presumably due to the strong promoter of T7 used in this study. In high level expression, rapid intracellular protein accumulation and expression of large proteins increase the probability of aggregation and leads to protein instability (Jonasson *et al.*, 2002; Palomares *et al.*, 2004). These buildups of protein aggregates are known as inclusion bodies.

In the end, our research succeeded to express recombinant protein spmoB in large quantity and it has methane oxidation activity. However, inclusion bodies was formed so purify and *in vitro* refolding steps have to be performed. The ability to express and purify the desired recombinant proteins in a large quantity allows for its biochemical characterization and the development of commercial goods.

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