Biotechnology and Bioprocess Engineering 15: 476-480 (2010)
DOI 10.1007/s12257-010-0039-6

RESEARCH PAPER

Optimization of Lab Scale Methanol Production by *Methylosinus trichosporium* OB3b

Hee Gon Kim, Gui Hwan Han, and Si Wouk Kim

Received: 4 February 2010 / Revised: 5 March 2010 / Accepted: 5 March 2010

Abstract *Methylosinus trichosporium* OB3b is a methanotrophic bacterium containing particulate methane monooxygenase (MMO), which catalyzes the hydroxylation of methane to methanol. The methanol is further oxidized to formaldehyde by methanol dehydrogenase (MDH). We developed a novel compulsory circulation diffusion system for cell cultivation. A methane/air mixture (1:1, v/v) was prepared in a tightly sealed gas reservoir and pumped into a nitrate mineral salt culture medium under optimal conditions (5 μM $CuSO_4$, pH 7.0, 30°C). Cells were harvested, washed, and resuspended (0.6 mg dry cells/mL) in a 500 mL flask in 100 mL of 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl and 1 mM EDTA as MDH inhibitors, and 20 mM sodium formate. A single 12 h batch reaction at 25°C yielded a final concentration of 13.2 mM methanol. The use of a repeated batch mode, in which the accumulated methanol was removed after each of three 8 h cycles over a 24 h period, showed a productivity of 2.17 μmol methanol/h/mg dry cell wt. Finally, a lab-scale reaction performed using a 3 L cylindrical reactor with a working volume of 1 L produced 13.7 mM methanol after 16 h. Our results identify a simple process for improving the productivity of biologically derived methanol and, therefore the utility of methane as an energy source.

Keywords: methane monooxygenase, methanol dehydrogenase, *Methylosinus trichosporium* OB3b, NaCl, EDTA

Hee Gon Kim, Gui Hwan Han, Si Wouk Kim*
Department of Environmental Engineering, BK21 Team for Biohydrogen Production, Chosun University, Gwangju 501-759, Korea
Tel: +82-62-230-6649; Fax: +82-62-225-6040
E-mail: swkim@chosun.ac.kr

1. Introduction

The benefit of methane as an energy source is that it produces few atmospheric pollutants and generates less carbon dioxide per unit energy than oil or coal. However, the use of methane is hindered by difficulties with storage and transportation. These problems can be solved by the conversion of methane to methanol. Methanol is used as a fuel for heat engines and fuel cells and can be converted by dehydration to dimethyl ether (DME), a good diesel fuel with a higher octane rating than regular diesel fuel. Methanol is produced exclusively from methane, by processing syngas under conditions of high temperature and pressure. In recent years, however, investigators have explored the biological production of methanol under ambient conditions.

Methanotrophic bacteria are able to grow aerobically using methane as a sole source of carbon and energy. The first step in the metabolism of methane is its conversion to methanol by the enzyme methane monooxygenase (MMO, EC 1.14.13.25). Methanol is converted subsequently to formaldehyde by methanol dehydrogenase (MDH, EC 1.1.99.8), which is coupled to the electron transport chain at the level of cytochrome c_L [1]. Formaldehyde is assimilated into biomass or oxidized to carbon dioxide, thereby providing reducing power for MMO. The production of methanol using a methanotrophic bacterium requires that oxidation of methanol be prevented, so that methanol can be excreted into the medium [2,3].

It is well known that NaCl inhibits electrostatic interaction between MDH and cytochrome c_L [1]. We have optimized methanol biosynthesis in *M. trichosporium* OB3b by using high NaCl concentrations to create conditions inhibiting MDH activity, while retaining MMO activity [4]. The high salt concentration inhibits the transfer of

electrons from MDH to cytochrome c_L that occurs during the oxidation of methanol, resulting in the excretion of methanol to medium. However, at concentrations above 100 mM, NaCl significantly disrupts cell structure, including the slime layer and the intracytoplasmic membrane (ICM), where particulate MMO (pMMO) binds. Dithiothreitol, phenylhydrazine, iodoacetate, cyclopropane, cyclopropanol, and EDTA act as MDH inhibitors [1,5-7]. Among them, cyclopropanol is very difficult to be synthesized chemically and maintain its stability under aerobic condition. However, EDTA can be obtained easily and inhibits the activity of metalloprotein like MDH as a metal chelator. In this study, we have further improved methanol production by introducing EDTA as an MDH inhibitor, allowing us to reduce the concentration of NaCl and its deleterious effects on cell morphology. In addition, we developed a repeated batch technique for the production of lab-scale batches of methanol.

2. Materials and Methods

2.1. Bacteria and cultivation conditions

All chemicals were analytical grade and were used without further purification. Methane and propane were obtained from Sigma Chemical Co. (USA). *M. trichosporium* OB3b was cultured in a modified nitrate mineral salt (NMS) medium containing 5 μM $CuSO_4$. Under these conditions, cells synthesize pMMO as the predominate form of the enzyme. Cells were cultured in a 500 mL Erlenmeyer flask containing 50 mL of medium at 30°C with a gas-tight seal. Air in the flask headspace was replaced with a methane/air mixture (1:1, v/v).

To achieve lab-scale production of methanol, cells were mass-produced in a compulsory circulation diffusion system as described by Kim *et al*. [8]. The pre-cultured cells were inoculated into a 5 L Erlenmeyer flask containing 3 L of medium. The methane/air mixture, maintained in a separated gas reservoir, was forced into the flask by an air pump.

2.2. Enzyme assay

pMMO activity was determined by measuring the amount of propylene oxide (PO) produced from the epoxidation of propylene [9]. PO concentration was determined by gas chromatography (Shimadzu GC-17A, Japan), using a 25% Sorbitol Gasport B (60/80) column (3 mm × 4 m) under the following conditions: column temperature = 80°C; injector temperature = 100°C; detector temperature = 100°C; and carrier gas (He) flow rate = 30 mL/min. MDH activity was assayed spectrophotometrically at 30°C by a two-dye linked assay system, in which PES was used as an artificial primary electron acceptor, and 2,6-dichlorophenol indophenol (DCPIP) as a terminal acceptor. The reaction mixture (3 mL) contained 100 mM Tris-HCl buffer (pH 9.0), 13.4 mM methanol, 1.1 mM PES, 0.04 mM DCPIP, 1 mM KCN, and 15 mM NH_4Cl. Reactions were initiated by the addition of the MDH, and the reduction of DCPIP was measured by monitoring the decrease in the absorbance at 600 nm [10].

2.3. Methanol production by *M. trichosporium* OB3b

Methanol biosynthesis by *M. trichosporium* OB3b in a batch reaction was carried out as follows. Cells were harvested in the middle stationary phase by centrifugation at 15,000 × *g* for 10 min, washed with 12.9 mM phosphate buffer (pH 7.0, standard buffer), and resuspended in the same buffer. The cell density was adjusted to 0.6 mg dry cells/mL. The sample solution (100 mL), treated with 100 mM NaCl, 1 mM EDTA, and 20 mM sodium formate, was introduced into a 500 mL Erlenmeyer flask with a screw cap. After the flask was incubated for 5 min at 25°C, the reaction was initiated by replacing 200 mL of air in the head-space of the flask with the same volume of methane/air mixture (1:1, v/v).

Methanol was also produced in a repeated batch mode in a 500 mL flask. In this process methanol was removed periodically to prevent the accumulation of high concentrations, which can retard further methanol biosynthesis. After an 8 h reaction, 100 mL of the reaction mixture was centrifuged with sterilized tubes at 15,000 × *g*, to separate the newly produced methanol from the reaction suspension. In the following reaction, the harvested cells were resuspended in the 12.9 mM phosphate buffer and introduced into the 500 mL flask. Thereafter, 20 mM sodium formate was included as a reducing agent for pMMO, and the methane mixture was introduced to the flask. The above procedure was repeated several times.

Lab-scale methanol production was designed in a 3 L reactor with 1 L working volume. The reaction was carried out in a compulsory circulation diffusion system consisting of cylindrical reactor (ø 110 × h 400 mm), portable rubber gas bag, mixed gas reservoir containing gas bubbler and silica-gel trap (Fig. 1). The 1 L reaction mixture contained 100 mM NaCl, 1 mM EDTA, 20 mM sodium formate, and 0.6 mg dry cells/mL. A limited volume of fresh methane/air mixture was introduced continuously from a rubber gas bag into the gas reservoir. Thereafter the mixture was forced by an air pump through micro pores prepared in the bottom of reactor and dispersed evenly in the reaction mixture. Part of methane mixture in the reactor was fed back into the gas reservoir, mixed with fresh methane and re-circulated into the reactor, until fresh methane in the gas bag was consumed completely. This system was set up within an incubator at 25°C. Produced methanol was

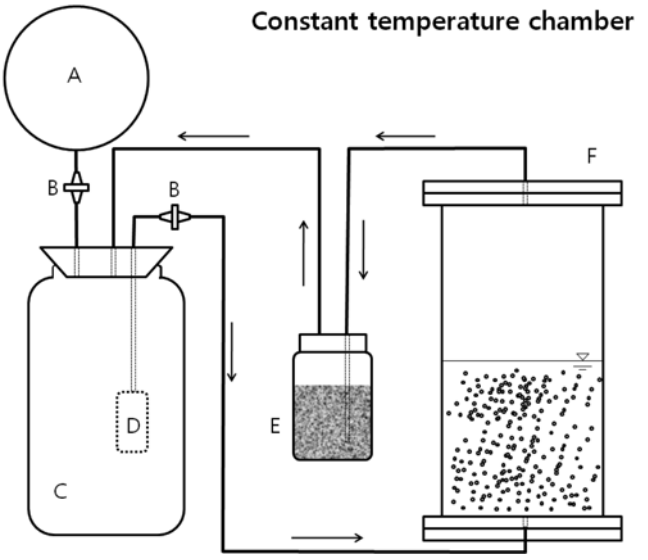

Fig. 1. Schematic diagram of lab-scale compulsory circulation diffusion system for methanol production. This system was set up in an incubator at 25°C. A, rubber gas bag; B, air filter; C, gas reservoir; D, air pump; E, silica-gel trap; F, 3 L reactor. Arrow indicates flow of methane/air mixture (1:1, v/v).

concentrated by evaporation (BÛCHI Rotavapor R-205, Switzerland).

2.4. Electron microscopy

NaCl-induced changes in *M. trichosporium* Ob3b cell morphology were observed using transmission electron microscopy (TEM), as described previously [11]. Briefly, the cells in the each reaction mixtures were initially fixed with glutaraldehyde, added to the medium at a final concentration of 2% (v/v). The cells were then subjected to an additional fixation using 1% (w/v) OsO_4 in 0.1 M cacodylate buffer, and washed two times with water. After dehydration in a series of alcohols, the cells were embedded with Epon resin and polymerized at 60°C for 18 h. Sections of 70 ~ 80 nm thickness were prepared using an ultramicrotome (LKB 2128 Ultratome; LKB) and viewed with a JEOL JEM-2000F X2 TEM operating at 80 kV.

3. Results and Discussion

3.1. Effect of NaCl on cell morphology and enzyme activity

Our previous study established the optimal conditions for methanol biosynthesis by *M. trichosporium* OB3b: 200 mM NaCl, 20 mM sodium formate, 0.6 mg dry cells/mL, 12.9 mM phosphate buffer (pH 7.0), at 25°C [4]. Under these conditions, *M. trichosporium* OB3b accumulated a maximum concentration of 7.7 mM methanol after a 36 h reaction. To observe the effect of NaCl on cell morphology, the ultra-structural features of *M. trichosporium* OB3b were observed by TEM. At a concentration of 100 mM, NaCl did not affect the normal cell morphology. Higher concentrations, however, caused the slime layer to disappear and disrupted the intracytoplasmic membrane structure (Fig. 2). We found that MDH and pMMO activities were reduced by 55 and 30%, respectively, in the presence of 200 mM NaCl, but were reduced by only 25 and 20%, respectively, at 100 mM NaCl. To preserve the activity of pMMO, which is very unstable under aerobic conditions, the purification procedure should be done under anoxic conditions. The reduction in pMMO activity seen with high NaCl concentrations might be as a result of disruptions in the slime layer that allow direct contact of pMMO with oxygen. At the same time, NaCl inhibits the transfer of electrons from MDH to cytochrome c_L in the process of methanol oxidation. These results indicate that the optimum NaCl concentration for stable methanol production should be lower than 200 mM.

3.2. Methanol production using NaCl and EDTA as MDH inhibitors

To reduce the concentration of NaCl below the 200 mM level used in our previous work, EDTA was added as a co-inhibitor of MDH. Because of EDTA is known to inhibit the transfer of electrons from MDH to cytochrome c_L, by reacting with lysyl residues on MDH [12]. Fig. 3 shows the effects of different concentrations of EDTA and NaCl on the production of methanol in a 20 mL batch reactor. In

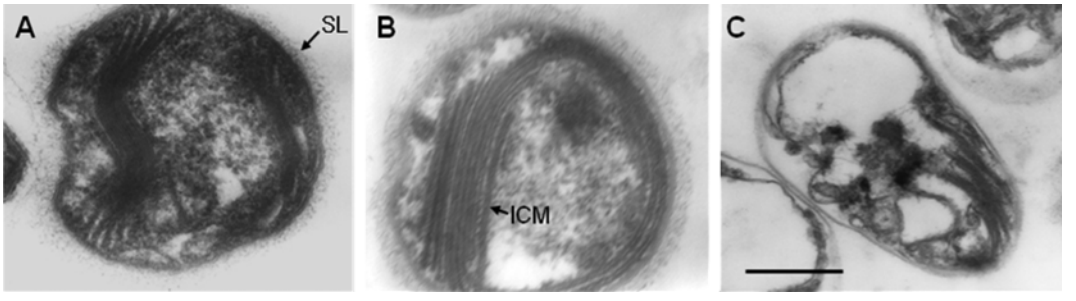

Fig. 2. Morphological changes of intracytoplasmic membrane (ICM) and slime layer (SL) of *M. trichosporium* OB3b by NaCl. A, control; B, 100 mM NaCl; C, 200 mM NaCl. Scale bar indicates 0.5 μm.

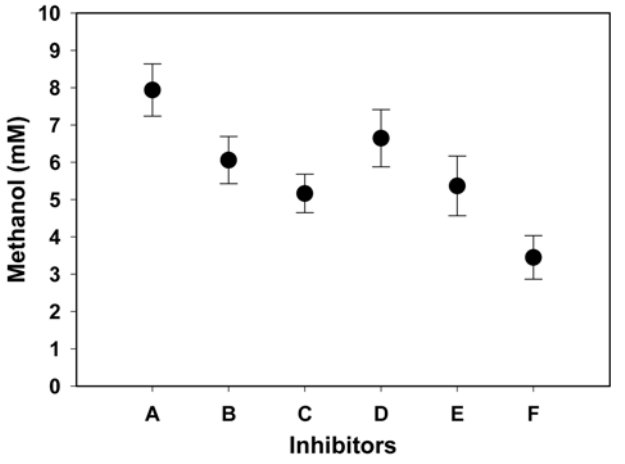

Fig. 3. Effects of NaCl and EDTA concentrations on methanol production. The reaction mixture (6 mL) contained 0.6 mg dry cells/mL, 20 mM sodium formate, 12.9 mM phosphate buffer (pH 7.0), NaCl and EDTA in the following concentrations. A, 1 mM EDTA + 100 mM NaCl; B, 2 mM EDTA + 100 mM NaCl; C, 3 mM EDTA + 100 mM NaCl; D, 1 mM EDTA + 200 mM NaCl; E, 2 mM EDTA + 200 mM NaCl; F, 3 mM EDTA + 200 mM NaCl.

this experiment, the highest methanol production was achieved using 100 mM NaCl and 1 mM EDTA. There was a concentration-related decrease in methanol production when EDTA was increased to 2 and 3 mM. This is likely due to the effects of EDTA on metalloprotein enzymes such as MDH and pMMO, whose activities are inhibited by the metal chelator.

Based on this result, a batch reaction for methanol synthesis from methane was carried out in the 500 mL flask.

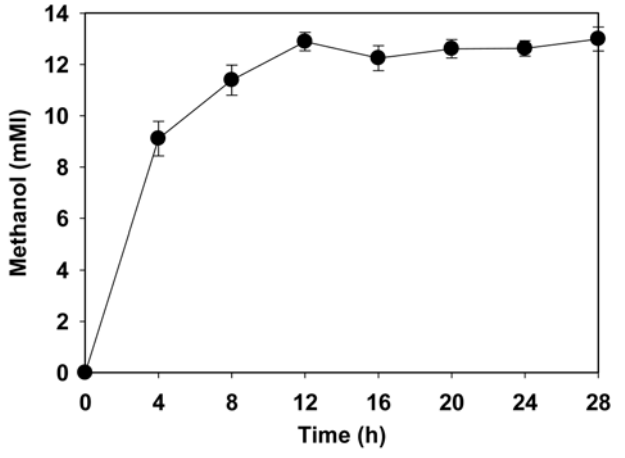

Fig. 4. Time-dependent methanol production by *M. trichosporium* OB3b in a 500 mL flask. The reaction mixture (6 mL) contained 0.6 mg dry cells/mL, 20 mM sodium formate, and various concentrations of NaCl and EDTA in 12.9 mM phosphate buffer (pH 7.0). The reaction was carried out at 25°C. The reaction was initiated by replacing 200 mL of air in the head-space of the flask with the same volume of methane/air (1:1, v/v).

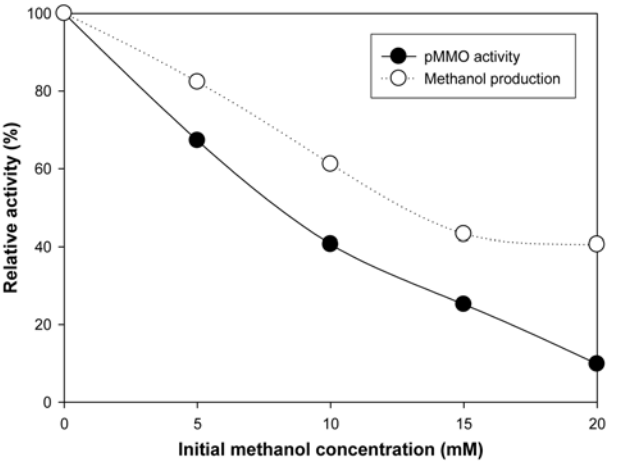

Fig. 5. Effect of initial methanol concentration on propene epoxidation (●) and methanol production (○).

The concentration of methanol in the cell suspension (100 mL, 0.6 mg dry cells/mL) treated with 100 mM NaCl, 1 mM EDTA, and 20 mM sodium formate peaked at 13.2 mM methanol after 12 h, and remained steady thereafter (Fig. 4).

3.3. Effect of accumulated methanol on further methanol production

It has been reported that the presence of a low concentration of methanol improves methane oxidation in methanotrophic bacteria, whereas a high concentration of product (methanol) competitively inhibits MMO [13]. Fig. 5 shows the effect of initial methanol concentration on pMMO activity in *M. trichosporium* OB3b, in the presence of 100 mM NaCl and 1 mM EDTA. Increasing the initial methanol concentration from 5 to 20 mM produced concentration-dependent decreases in both the pMMO-catalyzed epoxidation of propene and the conversion of methane to methanol, indicating that high concentrations of methanol have an inhibitory effect on pMMO. The results show that methanol synthesis was inhibited by approximately 60% at an initial methanol concentration of 20 mM. This result indicates that the removal of methanol accumulated in the reaction mixture is required in order to increase the overall methanol production.

3.4. Optimization of lab scale methanol production

Fig. 6 shows the time-dependent synthesis of methanol by *M. trichosporium* OB3b in the repeated batch mode. Three sequential 8 h cycles of methanol synthesis and removal were performed over a 24 h period. While there was a decrease in methanol production for the second and third cycles, productivity was 2.17 μmol methanol/h/mg dry cell wt. Thus, methanol production by the repeated batch method affords higher overall yields than the single batch

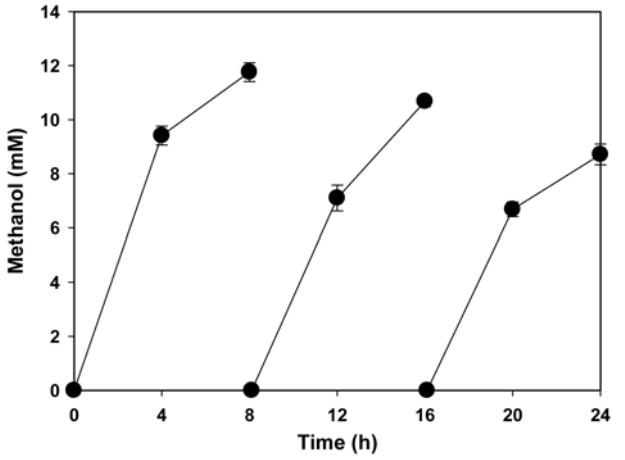

Fig. 6. Methanol production in the repeated batch mode. Methanol synthesis and removal was repeated three times at 8 h intervals for 24 h.

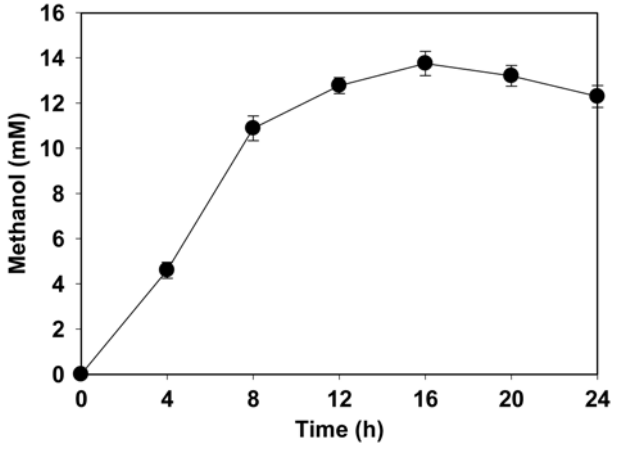

Fig. 7. Lab scale methanol production in a 3 L cylindrical reactor with a working volume of 1 L.

method.

After establishing the optimal reaction conditions in a 500 mL flask, a larger lab-scale methanol production was carried out using a 3 L cylindrical reactor with a working volume of 1 L. There was no significant difference in the rate of cell growth between lab-scale reactor and flask (data not shown). Under optimal reaction conditions, the 3 L reactor yielded a methanol concentration of 13.7 mM after 16 h (Fig. 7).

4. Conclusion

In this study, we have introduced two new approaches for increasing the biological production of methanol by *M. trichosporium* beyond those obtainable using previously defined methods. The introduction of EDTA helps preserve cell morphology, by reducing the requirement of NaCl in the reaction mixture, and increases methanol production from a single reaction. The use of repeated batch methodology increases the overall yield of methanol from a given cell biomass. These results provide straightforward means for increasing the production of biological methanol and for increasing the overall utility of methane as an energy source.

References

1. Anthony, C. (1986) Bacterial oxidation of methane and methanol. *Adv. Microb. Physiol.* 27: 113-210.
2. Takeguchi, M., T. Furuto, D. Sugimori, and I. Okura (1997) Optimization of methanol biosynthesis by *Methylosinus trichosporium* OB3b: An approach to improve methanol accumulation. *Appl. Biochem. Biotechnol.* 68: 143-152.
3. Furuto, T., M. Takeguchi, and I. Okura (1999) Semicontinuous methanol biosynthesis by *Methylosinus trichosporium* OB3b. *J. Mol. Catal. A: Chem.* 144: 257-261.
4. Lee, S. G., J. H. Goo, H. G. Kim, J. -I. Oh, Y. M. Kim, and S. W. Kim (2004) Optimization of methanol biosynthesis from methane using *Methylosinus trichosporium* OB3b. *Biotechnol. Lett.* 26: 947-950.
5. Mark, A. C., M. H. Kevin, A. P. Roy, and W. J. Colin (1984) The effect of EDTA and related chelating agents on the oxidation of methanol by the methylotrophic bacterium, *Methylophilus methylotrophus*. *Eur. J. Biochem.* 138: 611-615.
6. Johannes, F. J., H. K. Simon, P. E. J. Verwiel, A. J. Jacob, C. M. Antonia, and A. D. Johannis (1989) On the mechanism of inhibition of methanol dehydrogenase by cyclopropane-derived inhibitors. *Eur. J. Biochem.* 184: 187-195.
7. Shimoda, M. and I. Okura (1991) Selective inhibition of methanol dehydrogenase from *Methylosinus trichosporium* (OB3b) by cyclopropanol. *J. Mol. Catal.* 64: L23-L25.
8. Kim, H. G. and S. W. Kim (2006) Purification and characterization of a methanol dehydrogenase derived from *Methylomicrobium* sp. HG-1 cultivated using a compulsory circulation diffusion system. *Biotechnol. Bioproc. Eng.* 11: 134-139.
9. Burrows, K. J., A. Cornish, D. Scott, and I. J. Higgins (1984) Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b. *J. Gen. Microbiol.* 130: 3327-3333.
10. Cox, J. M., D. J. Day, and C. Anthony (1992) The interaction of methanol dehydrogenase and its electron acceptor, cytochrome c_L in methylotrophic bacteria. *Biochim. Biophys. Acta* 1119: 97-106.
11. Kim, H. G., G. H. Han, C. Y. Eom, and S. W. Kim (2008) Isolation and taxonomic characterization of a novel type I methanotrophic bacterium. *J. Microbiol.* 46: 45-50.
12. Dales, S. L. and C. Anthony (1995) The interaction of methanol dehydrogenase and its cytochrome electron acceptor. *Biochem. J.* 312: 261-265.
13. Jensen, S., A. Prieme, and L. Bakken (1998) Methanol improves methane uptake in starved methanotrophic microorganisms. *Appl. Environ. Microbiol.* 64: 1143-1146.