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Optimization of Methanol Biosynthesis by *Methylosinus trichosporium* OB3b: An Approach to Improve Methanol Accumulation

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ABSTRACT

Methylosinus trichosporium OB3b is a methanotrophic bacterium containing methane mono-oxygenase, catalyzing hydroxylation of methane to methanol. When methane is oxidized, the product is subsequently oxidized by methanol dehydrogenase contained in the same bacterium. To prevent further oxidation of methanol, the cell suspension was treated by cyclopropanol, an irreversible inhibitor for methanol dehydrogenase, leading to extracellular methanol accumulation. However, the reaction was terminated at approx 3 h with a final methanol concentration below 2.96 mmol/g dry cell. The methanol production efficiency (the ratio of the produced methanol per methane consumption) was 2.90%. By selecting the culture conditions and the reaction conditions, the reaction continued for 100 h, resulting in a methanol concentration of 152 mmol/g dry cell. This level was 51 times higher than that of the conventional reaction, and the methanol production efficiency was 61%.

Index Entries: Methanol synthesis; methane monooxygenase; methanol dehydrogenase, cyclopropanol; *Methylosinus trichosporium* OB3b.

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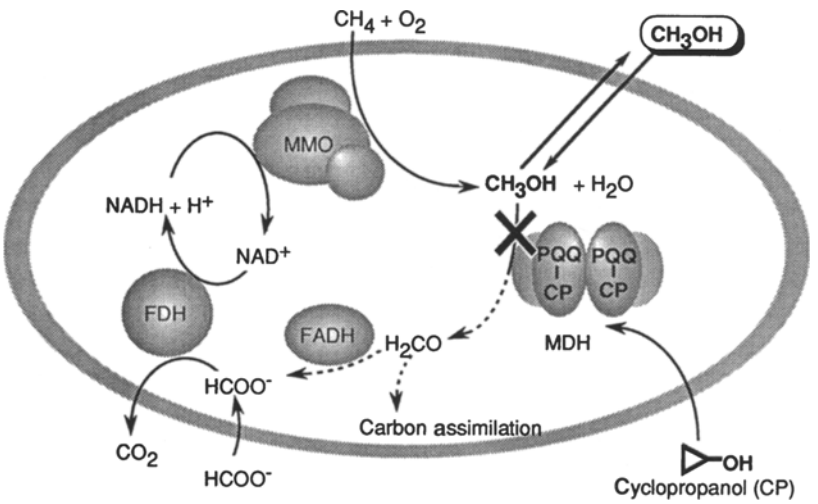

Scheme 1. The pathway of methanol synthesis with *M. trichosporium* OB3b. MMO, methane monooxygenase; MDH, methanol dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; PQQ, pyrroquinoline quinone.

INTRODUCTION

Direct oxidation of methane to methanol is highly attractive compared to the present process consisting of a two step process through syngas. Methane mono-oxygenase (MMO) catalyzes the single-step oxidation of methane to methanol according to the following equation:

$$CH_4 + O_2 + 2e^- + 2H^+ \rightarrow CH_3OH + H_2O$$

Purified MMO is not suitable in methanol synthesis reaction, because of the instability. Thus, the production of methanol from methane with methanotroph was tried. When the cell suspension was treated by cyclopropanol, which was an irreversible inhibitor for MDH, it led to extracellular methanol accumulation, as shown in Scheme 1 *(1)*. In this paper, we hope to describe the improvement of methanol yield by selecting culture and reaction conditions.

MATERIALS AND METHODS

Materials

All the chemicals used were of the highest grade available and were used without further purification. Methane and nitrogen were purchased from Fujiibussan (Tokyo, Japan). Tetrazotized *o*-dianisidine was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Naphthalene and copper sulfate pentahydrate were obtained from Kanto (Tokyo, Japan).

Cyclopropanol was prepared by the method reported previously *(2)*. The other chemicals were purchased from Kanto.

Culture of *M. trichosporium* OB3b

M. trichosporium OB3b was provided by J. D. Lipscomb of the University of Minnesota. *M. trichosporium* OB3b was cultivated as described by Fox et al. *(3)*, but the concentration of copper (Cu) in the medium was different, as indicated in the text. Cell densities were determined by measuring the absorbance at 660 nm. Cells were harvested in logarithmetic phase or initial stationary phase by centrifugation at 6,800*g* for 10 min, washed with 10 m*M* phosphate buffer, pH 7.0, and resuspended in the 10 m*M* phosphate buffer, pH 7.0.

Naphthalene Hydroxylation Assay

Naphthalene hydroxylation assay has been used as an indicator for soluble MMO (sMMO) activity potential in methanotrophs *(4)*. This assay is based on the ability of sMMO to oxidize naphthalene to 1-naphthol and 2-naphthol. Oxidation of naphthalene was performed as follows *(4)*. Oxidation of naphthalene was carried out in a 10-mL Erlenmeyer flask containing cell suspension (1.97 mg dry cell) and naphthalene (approx 10 mg) in 2 mL of 10 m*M* phosphate buffer, pH 7.0, at 30°C for 30 min. Naphthols (1-naphthol and 2-naphthol) as the reaction products were detected by the formation of purple diazo dyes from tetrazotized *o*-dianisidine.

Propene Epoxidation Assay

MMO activity was measured by propene epoxidation *(5)*. MMO activity was determined from the initial slope of a time-course of propene oxide formation. Specific activity was defined as the activity per total amount of dry cell in the sample.

Methanol Synthesis

Methanol production by *M. trichosporium* OB3b was carried out as follows. The sample solution (3.5 mL) containing the cell suspension was treated with cyclopropanol and sodium formate in phosphate buffer, pH 7.0, and was then introduced into a 10-mL screw-capped Erlenmeyer flask. The flask was sealed with a Teflon-sealed septa Funakoshi (Tokyo, Japan), and incubated for 5 min at reaction temperature. The reaction was initiated by injecting 2.5 mL of methane into the flask with a gas-tight syringe. Methanol was measured by gas chromatography using a Sorbitol 25% Gasport B column (4 m × 3 mm id, GL Sciences, Tokyo, Japan)

attached to a Hitachi (Tokyo, Japan) 263-30 gas chromatograph (oven temperature, 100°C; carrier gas [N_2] flow rate, 21.8 mL/min).

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli *(6)*. The separating gels contained 12% T (%T is the total concentration of monomer used to produce the gel) and 2.6% C, (%C is the percentage of the total monomer of crosslinking agent) and the stacking gels contained 3.0% T and 20% C. Phosphorylase b (M_r 97,400), serum albumin (M_r 66,200), ovalbumin (M_r 45,000), carbo-nic anhydrase (M_r 31,000), trypsin inhibitor (M_r 21,500), and lysozyme (M_r 14,400) were used as the reference proteins.

RESULTS AND DISCUSSION

Effect of Cu Concentration in Growth Medium

M. trichosporium OB3b was cultivated in mineral salts media containing cupric sulfate. As shown in Fig. 1, the growth rate of *M. trichosporium* OB3b and the maximum cell density decreased with increasing Cu concentration, up to 20 μ*M*. The maximum specific growth rate was 1.59×10^{-3}/min, and the maximum cell density was 0.235 mg dry cell/mL at 1.25 μ*M* of Cu concentration. *M. trichosporium* OB3b grew nicely at 1.25 μ*M* of Cu concentration, and grew slowly at 20 μ*M* of Cu concentration.

MMO activities of these bacteria were measured by propene epoxidation. MMO activity was directly related to the Cu concentration up to 20 μ*M*. The maximum MMO activity was 98.6 nmol of propene oxide formed/mg dry cell/mL at 20 μ*M* of Cu concentration. As shown in Table 1, *M. trichosporium* OB3b grown under 20 μ*M* of Cu concentration showed higher MMO activity than the cell grown at 1.25 μ*M* and 0.21 μ*M* of Cu concentration. However, *M. trichosporium* OB3b grew slowly at 20 μ*M* of Cu concentration. Thus, the methanol synthesis reaction was carried out by *M. trichosporium* OB3b grown in 1.25 μ*M* Cu concentration, which was the highest specific growth rate, and comparatively high MMO activity.

M. trichosporium OB3b expresses either sMMO or particulate MMO (pMMO), and the form expressed strongly depended on the concentration of Cu during the growth *(5)*. At low Cu concentration sMMO is expressed. Otherwise, pMMO is expressed.

MMO activities of these bacteria were measured by the naphthalene hydroxylation assay. Naphthalene hydroxylation assay has been used as an indicator for sMMO activity potential in methanotrophs *(4)*. This assay is based on the ability of sMMO to oxidize naphthalene to 1-naphthol and

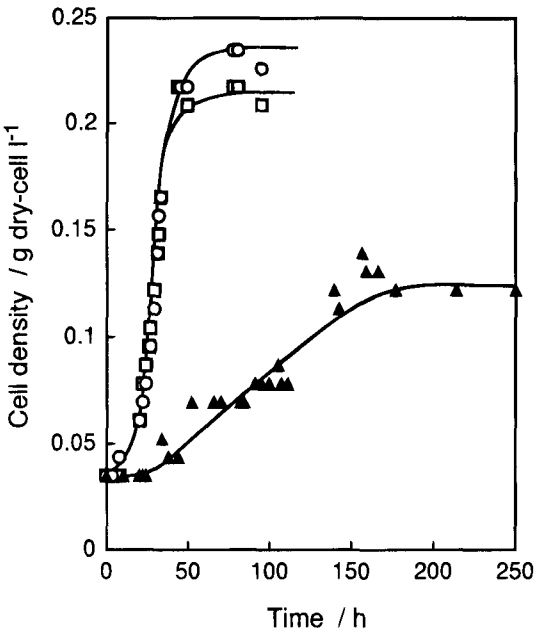

Fig. 1. Effect of Cu concentration on the growth of *M. trichosporium* OB3b. *M. trichosporium* OB3b was grown in the mineral salts medium at 30°C under methane-air (1:4 v/v%). The flask, were agitated on a rotary shaker table maintained at 120 rpm. Cu concentration in the medium: □, 0.21 μ*M*; ○, 1.25 μ*M*; ▲, 20 μ*M*.

Table 1
Effect of Cu Concentration in Medium on Specific Growth Rate and MMO Activity

Copper concentration in the medium / μM	Specific growth rate / min^{-1}	Naphthalene oxidation[a]	Propylene oxidation / μmol · min^{-1} · g dry - $cell^{-1}$
0.21	1.18×10^{-3}	+	27.0
1.25	1.59×10^{-3}	–	64.9
20	1.39×10^{-4}	–	98.6

[a]As evidenced by a purple color with the diazonium reagent.
+, color development; –, no color development.

2-naphthol. In this assay, only *M. trichosporium* OB3b grown in 0.21 μ*M* Cu concentration showed naphthalene hydroxylation (Table 1). *M. trichosporium* OB3b grown over 1.25 μ*M* Cu did not show naphthalene hydroxylation; however, the culture did show oxidation of propene in propene epoxidation assay. These results suggest that *M. trichosporium* OB3b grown in 0.21 μ*M* Cu concentration expresses mainly sMMO, and *M. trichosporium* OB3b grown over 1.25 μ*M* Cu concentration expresses only pMMO. In addition, it is known that two bands corresponding to polypeptides of molecular masses 46 and 26 kDa, seen on SDS-polyacrylamide gel of the membrane fraction,

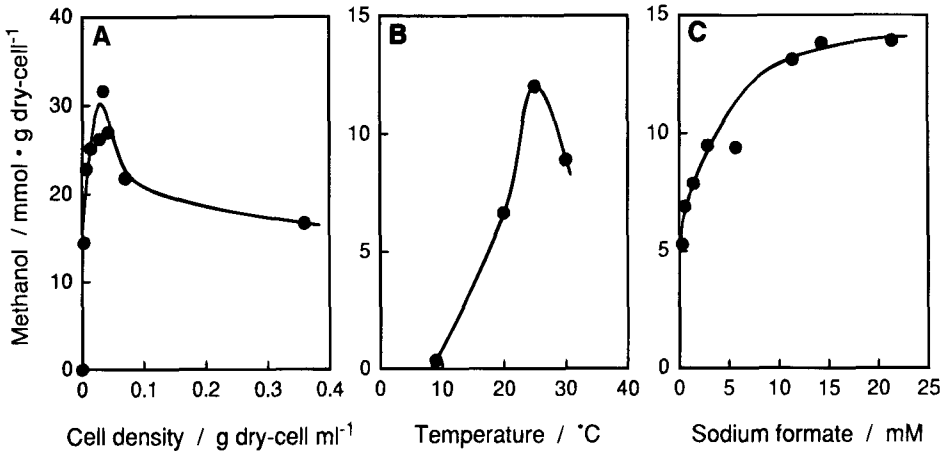

Fig. 2. Factors affecting methanol accumulation. **(A)** the effect of cell density; **(B)** the effect of temperature; **(C)** the effect of sodium formate concentration.

are implicated for pMMO *(5,7)*. The electrophoretic profile of the broken cells obtained from grown cells at 1.25 μ*M* of Cu concentration showed the same putative pMMO polypeptide bands. This result indicates that *M. trichosporium* OB3b grown over 1.25 μ*M* Cu expresses only pMMO.

Optimization of Methanol Biosynthesis

To optimize methanol accumulation by *M. trichosporium* OB3b, the effects of cell density, reaction temperature, concentration of sodium formate, concentration of phosphate buffer, pH 7.0, and concentration of cyclopropanol were examined.

The study on the effect of cell density on methanol accumulation by *M. trichosporium* OB3b indicated the optimum cell density to be 34.6 μg of dry cell/mL (Fig. 2A). At higher cell density, there was no further increase in methanol accumulation.

The effect of reaction temperature on methanol accumulation by *M. trichosporium* OB3b indicated the optimum temperature to be 25°C (Fig. 2B). Increasing the temperature results in a decrease in methanol accumulation, possibly caused by instability of the enzyme pMMO.

The relationship between concentration of sodium formate and methanol accumulation was studied (Fig. 2C). The methanol accumulation increased with increasing concentration of sodium formate, and was saturated by more than 14.3 m*M* sodium formate.

Mehta and coworkers reported that high concentration of phosphate ions (>80 m*M*) selectively inhibited MDH activity in the cells of *M. trichosporium* NCIB 11131 *(8)*. In the case of *M. trichosporium* OB3b, high concentration of phosphate buffer, pH 7.0, also inhibited MDH activity.

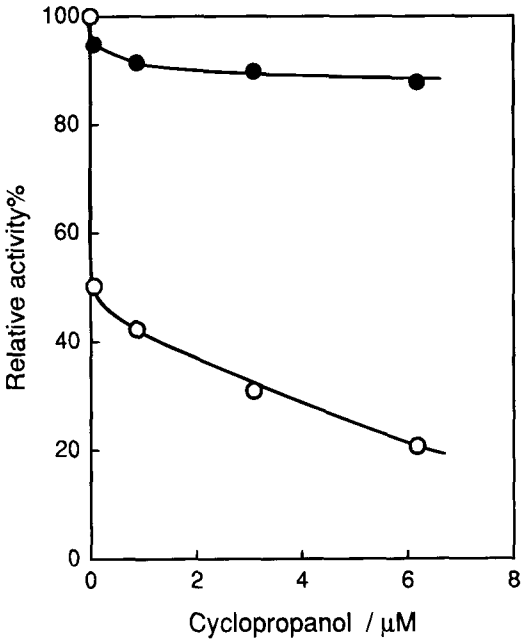

Fig. 3. Effect of cyclopropanol concentration on pMMO activity and MDH activity. The pMMO activity (●) was measured by propene epoxidation assay. The reaction mixture contains cell suspension (34.6 mg dry cell/mL), propylene (112 μmol), sodium formate (14.3 m*M*), and various concentrations of cyclopropanol in phosphate buffer (12.9 m*M*), pH 7.0. The MDH activity (○) was measured by methanol oxidation assay. The reaction mixture contains cell suspension (34.6 mg dry cell/mL), methanol (18 μmol), and various concentrations of cyclopropanol in phosphate buffer (22 m*M*), pH 7.0. The reaction was carried out at 30°C. The total volume of reaction mixture was 3.5 mL.

However, the methanol accumulation decreased with increasing concentration of phosphate buffer, pH 7.0. These results show that high concentration of phosphate ions inhibited not only MDH activity, but also pMMO activity in the cells.

To produce methanol by *M. trichosporium* OB3b, it is necessary to inhibit the oxidation of methanol by MDH contained in the same bacterium. In this study, the cell suspension was treated with cyclopropanol, which was an irreversible inhibitor for MDH (*2*).

Figure 3 shows the effects of cyclopropanol concentration on pMMO activity and MDH activity (the activities were measured by propene epoxidation and methanol consumption, respectively). In the presence of cyclopropanol, the MDH activity decreased with increasing cyclopropanol concentration. When the concentration of cyclopropanol was 6.18 μ*M* in the reaction mixture, the MDH activity decreased by 79.3%; however, pMMO activity was not affected. When the concentration of cyclopropanol was 6.18 μ*M*, the loss of pMMO activity was only 12%.

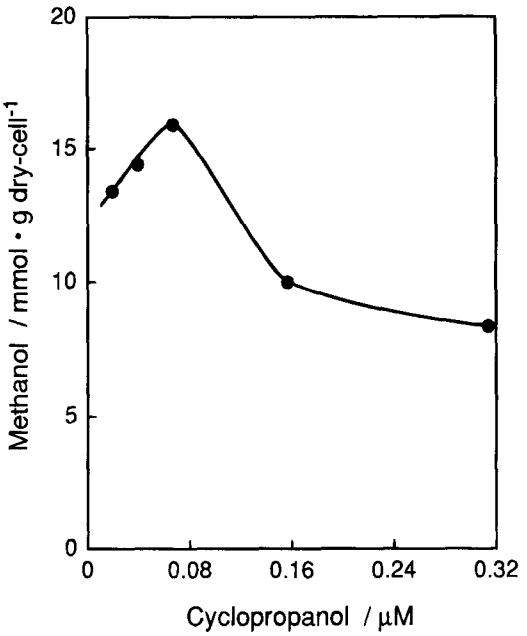

Fig. 4 Effect of cyclopropanol concentration on methanol accumulation. The reaction of mixture contains cell suspension (1.39 g dry cell/mL), methane (112 μmol), oxygen (103 μmol), and sodium formate (14.3 m*M*) in phosphate buffer (58.6 m*M*), pH 7.0. The reaction was carried out at 30°C.

Figure 4 shows the effect of cyclopropanol on methanol accumulation. The maximum methanol accumulation was observed with 0.67 μ*M* cyclopropanol in the reaction mixture. In this cyclopropanol concentration, the losses of MMO activity and MDH activity in *M. trichosporium* OB3b were 5.2 and 49.8%, respectively. Thus, when MDH activity in the cell remained more than 50%, the maximum methanol accumulation was observed. These results suggest that the MDH activity has a strong effect on the pMMO activity in vivo.

In previous studies, Tonge and coworkers *(9)* proposed that the physiological electron donor for pMMO in *M. trichosporium* OB3b was not NADH, but rather that the immediate donor was the CO-binding cytochrome *c*, being reduced in turn by the MDH. Andrew and coworkers *(10)* also reported that quinols were capable of directly reducing detergent-solubilized pMMO from *M. capsulatus* (Bath). The propene epoxidation by the cells that were not treated with cyclopropanol was measured in the presence of methanol instead of sodium formate. The rate of propene oxide formation was 116 μmol/min/g of dry cell, similar to the rate of methanol consumption (117 μmol/min/g of dry cell). This result suggests that in this system the rate of methane hydroxylation (the pMMO activity) is limited by the rate of methanol consumption (the MDH activity), and

Table 2
The Reaction Condition of Methanol Synthesis by *M. trichosporium* OB3b

Conditions	Conventional	Optimum
Cells	1.39 mg dry-cell/ml	3.46×10^{-2} mg dry-cell/ml
Sodium formate	14.3 mM	14.3 mM
Phosphate buffer (pH 7.0)	58.6 mM	12.9 mM
Cyclopropanol	251 μM	67.0 nM [a]
Temperature	30°C	25°C

[a] *M. trichosporium* OB3b was incubated with cyclopropanol at 20°C for 5 min before reaction.

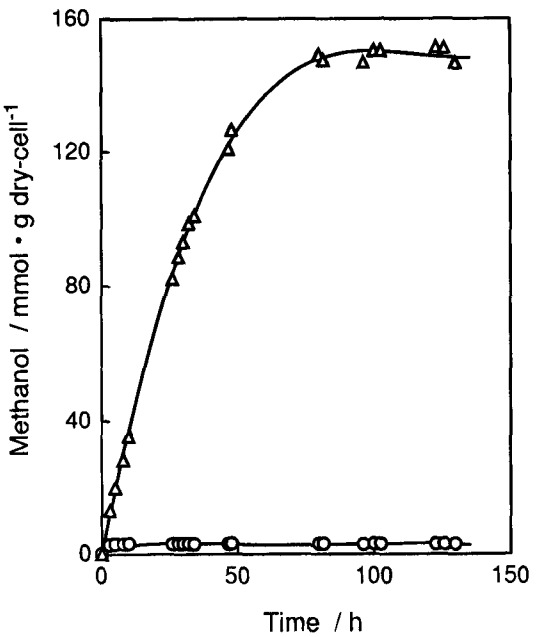

Fig. 5. Time-course of methanol produced by *M. trichosporium* OB3b. The methanol synthesis was carried out with conventional conditions (○), with optimum conditions (△). The reaction conditions were described in Table 2.

supports the contention that methanol itself is serving as the electron source for the pMMO.

Methanol Synthesis

Methanol synthesis was carried out with reaction conditions indicated in Table 2. By optimizing the reaction condition, the reaction continued for 100 h and the produced methanol was 152 mmol/g of dry cell, which is 51-fold higher than that of the conventional conditions (Fig. 5). In addition, the

methanol production yield per consumed methane, which was consumed by culture and methanol synthesis reaction, was 60.5% (compared to 2.9% under conventional conditions). By selecting the culture conditions and reaction conditions, methane hydroxylation activity in the cell was kept for a long time and the methanol production (the efficiency) dramatically increased.

ACKNOWLEDGMENTS

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