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Semicontinuous methanol biosynthesis by *Methylosinus trichosporium* OB3b

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Abstract

Methylosinus trichosporium OB3b is a methanotrophic bacterium containing methane monooxygenase, 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 batch type of methanol synthesis by *M. trichosporium* OB3b was terminated at ca. 6 mM of methanol, for MMO is inhibited by increasing methanol concentration. For prolonged methanol accumulation, a semicontinuous process was carried out. In this process, the reaction was repeated several times and the produced methanol was 36.1 μmol for 6 h.

Keywords: Methanol synthesis; Methanotroph; *Methylosinus trichosporium* OB3b; Methane monooxygenase; Methanol dehydrogenase; Cyclopropanol

1. Introduction

Direct oxidation of methane to methanol is one of the attractive chemical processes. Methane monooxygenase (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. To prevent further oxidation of methanol, the cell suspension was treated by cyclopropanol which was an irreversible inhibitor for MDH, leading to extracellular methanol accumulation as shown in Scheme 1 [1,2]. In this study, the improvement of methanol yield was accomplished by using a semicontinuous process.

2. Materials and methods

2.1. Materials

All the chemicals used were of the highest grade available and were used without further purification. Methane and nitrogen were pur-

Abbreviations: MMO, methane monooxygenase; pMMO, particulate methane monooxygenase; MDH, methanol dehydrogenase; FDH, formate dehydrogenase; PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*] quinoline-4,5-dione

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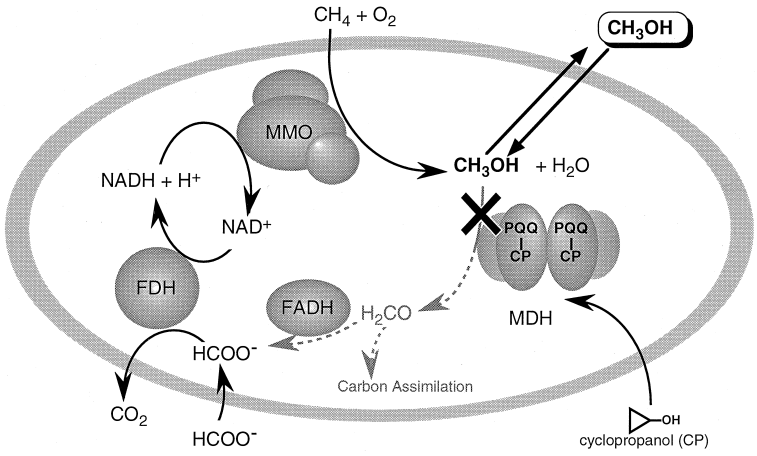

Scheme 1. The pathway of methanol synthesis with *M. trichosporium* OB3b. MMO: Methane monooxygenase, MDH: Methanol dehydrogenase, FADH: Formaldehyde dehydrogenase, FDH: Formate dehydrogenase, PQQ: Pyrroquinoline quinone.

chased from Fujiibussan (Tokyo, Japan). Tetrazotized *o*-dianisidine was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Naphthalene and copper sulfate pentahydrate were obtained from Kanto Chemical (Tokyo, Japan). Cyclopropanol was prepared by the method reported previously [1]. The other chemicals were purchased from Kanto Chemical.

2.2. Culture of M. trichosporium OB3b

M. trichosporium OB3b was kindly provided by Professor J.D. Lipscomb of University of Minnesota. *M. trichosporium* OB3b was cultivated as described by Fox et al. [3]. A nitrate mineral salts medium containing 1.25 μM $CuSO_4$ was used throughout this work. In this culture conditions, particulate MMO (pMMO) is predominantly produced. Cells were harvested in logarithmetic phase or initial stationary phase by centrifugation at $6800 \times g$ for 10 min, washed with 10 mM phosphate buffer (pH 7.0), and resuspended in the 10 mM phosphate buffer (pH 7.0).

2.3. Propene epoxidation assay

pMMO activity was measured by propene epoxidation [4]. pMMO activity was determined from the initial slope of a time course of propene oxide formation. Specific activity defined as the activity per total amount of dry cell in the sample.

2.4. Methanol synthesis by M. trichosporium OB3b

In batch reaction, methanol production by *M. trichosporium* OB3b was carried out as follows. The sample solution (3.5 ml) containing cell suspension treated with cyclopropanol and sodium formate (14.3 mM) in 12.9 mM phosphate buffer (pH 7.0) was introduced into a 10 ml Erlenmeyer flask with a screw cap. The flask was sealed with a Teflon-sealed septa, and then incubated for 5 min at 30°C. The reaction was initiated by injecting 2.5 ml of methane into the flask with a gas-tight syringe. The produced methanol was measured by gas chromatography using a Sorbitol 25%-Gasport B column (2 m × 3 mm i.d., GL Sciences) attached to a Hitachi 263-30 gas chromatograph (Hitachi, Tokyo, Japan) [oven temperature, 100°C; carrier gas, N_2; flow rate, 21.8 ml/min].

3. Results and discussion

3.1. Methanol synthesis in batch reaction

Methanol synthesis in batch reaction was carried out as described in Section 2. The reaction

mixture contains cell suspension treated with cyclopropanol and sodium formate in phosphate buffer (pH 7.0). When the cell suspension was treated by cyclopropanol, cyclopropanol combines with 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*] quinoline-4,5-dione (PQQ) which is the coenzyme of MDH, and MDH is deactivated, and methanol is accumulated.

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. Fig. 1 shows the time-course of methanol with *M. trichosporium* OB3b. By optimizing the reaction conditions, 6 mM of methanol was produced after 100 h, and the methanol accumulation was saturated [2]. When excess substrates such as methane, dioxygen and sodium formate were introduced, no further accumulation of methanol was observed (data not shown).

Fig. 2 shows the stability of activity of methanol synthesis at room temperature. The activity of methanol synthesis decreased with increasing an incubation time, and completely deactivated after 24 h. But, the activity of methanol synthesis remain at 100 h by 65% of a fresh cell, indicated that the saturation of methanol synthesis is caused by other reasons.

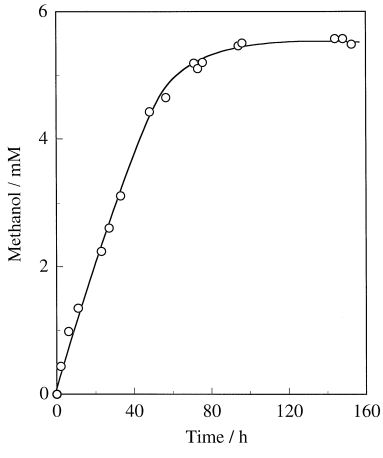

Fig. 1. The time course of methanol produced by *M. trichosporium* OB3b. The reaction mixture (3.5 ml) contains cell suspension treated with cyclopropanol, methane (112 μmol), oxygen (103 μmol) and sodium formate (14.3 mM) in 12.9 mM phosphate buffer (pH 7.0).

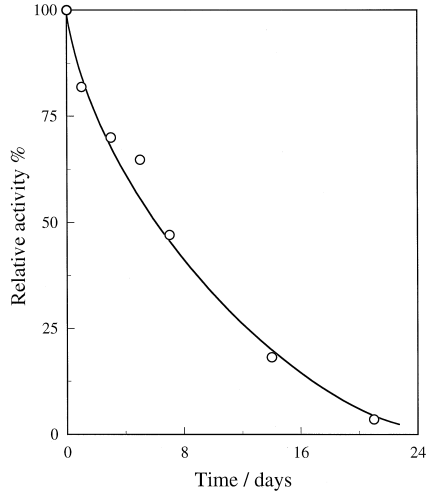

Fig. 2. Stability of the activity of methanol synthesis with *M. trichosporium* OB3b. The reaction mixture (3.5 ml) contains cell suspension treated with cyclopropanol, methane (112 μmol), oxygen (103 μmol) and sodium formate (14.3 mM) in 12.9 mM phosphate buffer (pH 7.0). Cell suspension (2.5 mg dry cell/ml) was stored at room temperature under anaerobic conditions.

3.2. Inhibition of methanol in methanol synthesis with M. trichosporium OB3b

When methanol concentration increased, the methanol synthesis with *M. trichosporium* OB3b was inhibited as shown in Fig. 3, and completely inhibited at 12.5 mM methanol. The result indicated that methanol inhibited methanol synthesis with *M. trichosporium* OB3b, i.e., methanol inhibits pMMO and/or formate dehydrogenase (FDH) in the cell.

Fig. 4 shows the effect of methanol on the propene epoxidation in the presence of cyclopropanol with *M. trichosporium* OB3b. When cyclopropanol was added to the reaction mixture MDH is deactivated, suggesting that only the pMMO enzyme reaction proceeds. As shown in Fig. 4, the rate of propene epoxidation was inhibited by increasing methanol concentration in the reaction mixture, and was completely

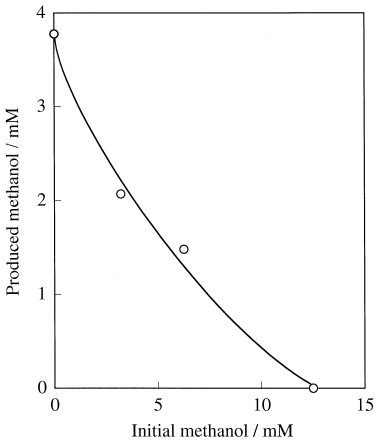

Fig. 3. Effect of methanol on methanol synthesis reaction with *M. trichosporium* OB3b. The reaction mixture (3.5 ml) contains cell suspension treated with cyclopropanol, methane (112 μmol), oxygen (103 μmol) and sodium formate (14.3 mM) in 12.9 mM phosphate buffer (pH 7.0).

inhibited at 10 mM methanol, showing methanol has an inhibitory effect on pMMO in *M. trichosporium* OB3b.

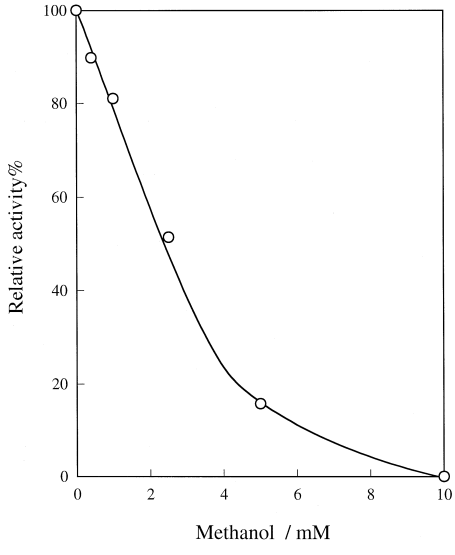

Fig. 4. Effect of methanol on propene epoxidation in the presence of cyclopropanol with *M. trichosporium* OB3b. The reaction mixture of propene epoxidation contains cell suspension (3.46×10^{-2} mg dry cell/ml), propene (112 μmol), oxygen (103 μmol) and cyclopropanol (44.1 μM) in phosphate buffer (12.9 mM), pH 7.0. The reaction mixture of methanol oxidation contains cell suspension (3.46×10^{-2} mg dry cell/ml), methanol and oxygen (103 μmol) in phosphate buffer (12.9 mM), pH 7.0. The reaction was carried out at 30°C.

As shown in Scheme 1, FDH catalyzes to supply NADH to pMMO by the combination of formate. Table 1 shows the effect of methanol on the FDH activity in the presence of cyclopropanol. The addition of methanol to reaction mixture inhibited pMMO activity but does not affect the activity of FDH. The results indicate that methanol inhibits only pMMO and does not affect the FDH in *M. trichosporium* OB3b.

These results indicate produced methanol has an inhibitory effect on methanol synthesis with *M. trichosporium* OB3b, and has much influence on the pMMO activity in vivo. As methanol inhibits the methanol synthesis, a semicontinuous methanol synthesis was tried to remove the produced methanol.

3.3. Methanol synthesis in semicontinuous reaction

In semicontinuous reaction, methanol production by *M. trichosporium* OB3b was carried out as follows. A standard 50-ml capacity ultrafiltration (UF) cell (Grace Japan, Amicon) was converted into a semicontinuous stirred reactor. The sample solution (17.5 ml) containing cell suspension treated with cyclopropanol and sodium formate (14.3 mM) in 12.9 mM phosphate buffer (pH 7.0) was introduced into the UF cell attached ultrafilter (Diaflo ultrafilter YM-100, Grace Japan, Amicon). The UF cell incubated for 5 min at 30°C, and the reaction was initiated by injecting 12.5 ml of methane into the UF cell with a gas-tight syringe. After incubation at 30°C for 90 min, the reaction mixture was filtrated by nitrogen pressure, lead-

Table 1
Effect of methanol on the formate dehydrogenase activity

Additives	pMMO activity (μmol/min/mg dry cell)	FDH activity (μmol/min/mg dry cell)
No additive	5.66	6.23
+Cyclopropanol +methanol	3.74	7.43

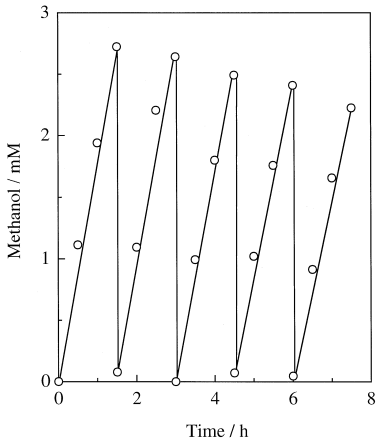

Fig. 5. Semicontinuous methanol synthesis with *M. trichosporium* OB3b. The reaction mixture (17.5 ml) contains cell suspension treated with cyclopropanol, methane (560 μmol), oxygen (515 μmol) and sodium formate (14.3 mM) in 12.9 mM phosphate buffer (pH 7.0).

ing to separation a produced methanol from cell suspension. The above procedure was repeated several times.

Fig. 5 shows the time dependence of methanol synthesis with *M. trichosporium* OB3b in the semicontinuous reaction. In this reaction, the methanol synthesis was repeated five times for 6 h and was a stationary rate of 3.17 μmol/h/mg dry cell for 6 h. When the methanol synthesis was repeated five times for 6 h, produced methanol was 36.1 μmol compared to 19.6 μmol in batch reaction under the same condition.

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