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Optimization of methanol biosynthesis from methane using *Methylosinus trichosporium* OB3b

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Abstract

Methylosinus trichosporium OB3b oxidized methane to methanol in the presence of a high concentration of Cu^{2+}. Further oxidation of methanol to formaldehyde was prevented by adding 200 mM NaCl which acted as a methanol dehydrogenase H inhibitor. The bacterium, 0.6 mg dry cell ml^{-1}, in methane/air (1:4, v/v) at 25 °C in 12.9 mM phosphate buffer (pH 7) containing 20 mM sodium formate and 200 mM NaCl accumulated 7.7 mM methanol over 36 h.

Introduction

Methanotrophic bacteria can grow aerobically on methane as a sole source of carbon and energy. The first two enzymes involved in methane oxidation are methane monooxygenase (MMO) and methanol dehydrogenase (MDH) (Anthony 1986). MMO oxidizes methane to methanol, and MDH catalyzes the oxidation of methanol to formaldehyde. *Methylosinus trichosporium* OB3b is a methanotrophic bacterium and contains two forms of MMOs: a soluble- (sMMO) and a membrane-bound particulate (pMMO) one whose syntheses depends on growth conditions. *M. trichosporium* OB3b growing on methane in Higgins nitrate mineral salt (NMS) medium (Fox *et al.* 1990) containing 5 μM Cu^{2+}, produced only pMMO (Murrell *et al.* 2000, Nielson *et al*. 1997, Takeguchi & Okura 2000).

Dithiothreitol, phenylhydrazine, metal chelating agents, iodoacetate, $MgCl_2$, high concentrations of phosphate, cyclopropane, and cyclopropanol act as MDH inhibitors (Anthony 1986, Carver *et al*. 1984, Frank *et al*. 1989, Shimoda & Okura 1991). Among these cyclopropanol has the highest inhibitory effect and has been used as an inhibitor for the experimental production of methanol from methane (Takeguchi *et al.* 1997, Furuto *et al.* 1999). The cyclopropanol, however, is very difficult to synthesize chemically and to maintain its stability under aerobic conditions.

The primary electron acceptor of MDH is cytochrome c_L. The two proteins interact by electrostatic interactions which can be disrupted by high salt concentrations (Cox *et al*. 1992), thereby preventing further oxidation of methanol to formaldehyde. In this paper, we have identified optimal conditions that inhibit MDH activity but retain MMO activity by using NaCl, resulting in the production of methanol from methane in high yield using *M. trichosporium* OB3b.

Materials and methods

Bacterial strain, cultivation conditions, and cell free extract preparation

Methylosinus trichosporium OB3b, provided by Dr I. Okura of the Tokyo Institute of Technology, was cultivated in the modified NMS medium containing 5 μM $CuSO_4$ under 30% (v/v) methane/70% (v/v) air at 30 °C. Cells were harvested in the early stationary phase by centrifugation at 15 000 × *g* for 10 min, washed once with 12.9 mM phosphate buffer (pH 7) and resuspended in the same buffer. Cells in the suspension were then disrupted by sonic treatment (10 s ml^{-1}) and centrifuged at 15 000 × *g* for 30 min, and the resulting supernatant was used for MDH and pMMO assays.

Enzyme assays

pMMO activity was determined by measuring the amount of propylene oxide produced following propylene epoxidation (Burrows *et al.* 1984). The activity was calculated from the initial slope of a time course of propylene oxide formation. MDH activity was measured by a cytochrome *c*/DCPIP-linked assay system (Cox *et al.* 1992). Horse cytochrome *c* and 2,6-dichlorophenol indophenol (DCPIP) were used as artificial primary and terminal electron acceptors, respectively. The reduction of DCPIP was measured at 600 nm.

Methanol biosynthesis

The determination of methanol produced in the batch reaction was carried out as follows. The sample (5 ml), which contained a cell suspension treated with appropriate amounts of NaCl and sodium formate in 12.9 mM phosphate buffer (pH 7), was introduced into a 20 ml bottle. The bottle was sealed with a Teflon septa and incubated for 5 min at 30 °C. The reaction was initiated by replacing 3 ml air in the head-space of the bottle with 3 ml methane using a gas-tight syringe. Methanol concentration in the reaction mixture was determined after 36 h by gas chromatography using a 25% sorbitol Gasport B (60/80) column (3 mm × 4 m, GL Sciences, Japan). Conditions were: column 80 °C, injector 100 °C, detector 100 °, carrier gas He at 30 ml min^{-1}.

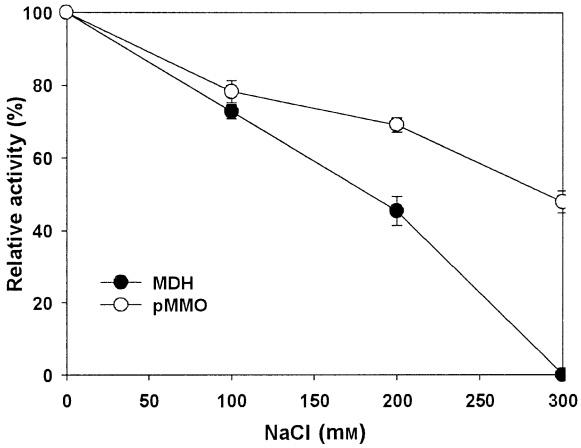

Fig. 1. Effect of NaCl on the MDH and pMMO activities. MDH activity (●) was measured by the cytochrome c_L/DCPIP-linked assay system. pMMO activity (○) was determined by the propylene epoxidation assay procedure as described in Materials and methods. All values are average of three independent determinations.

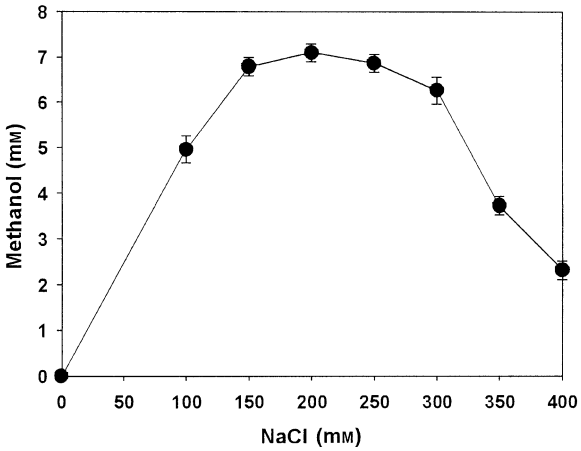

Fig. 2. Effect of NaCl on the methanol accumulation. The reaction mixture contained cell suspension (0.6 mg dry cell ml^{-1}), sodium formate (20 mM), and various concentrations of NaCl in 12.9 mM phosphate buffer (pH 7). The reaction was carried out for 36 h at 25 °C by injecting methane gas (3 ml) into a 20 ml reaction bottle containing 5 ml of reaction mixture. All values are average of three independent determinations.

Results and discussion

Effect of NaCl on the pMMO and MDH activities

As shown in Figure 1, both MDH and pMMO activities decreased concomitantly with an increase in NaCl concentration in the assay mixture. To achieve a high yield of methanol, MDH should be inhibited but pMMO has to maintain a high activity. With 300 mM NaCl MDH activity was completely inhibited but pMMO lost only half of its original activity. However,

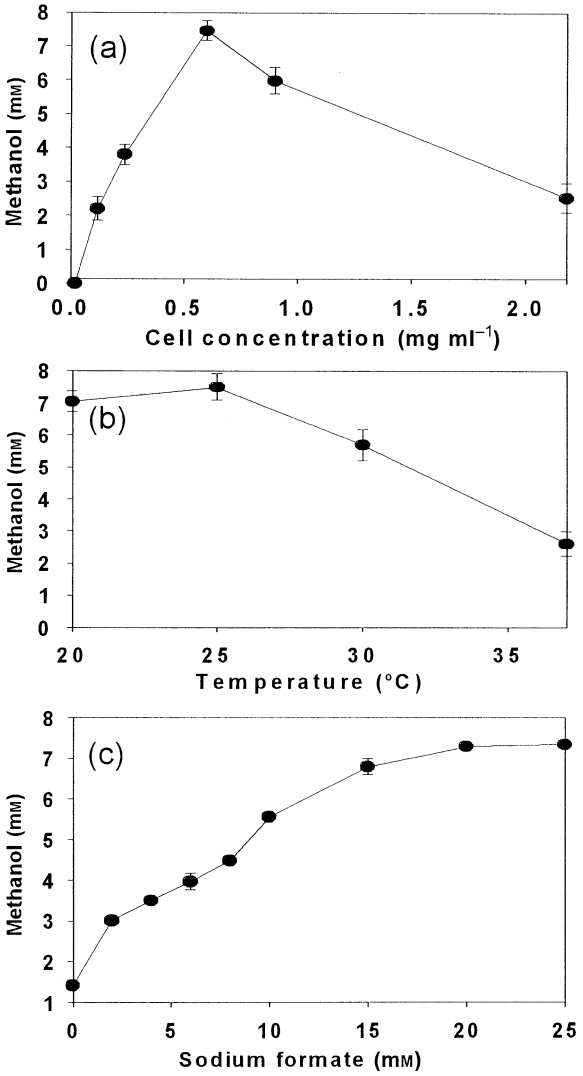

Fig. 3. Factors affecting methanol accumulation. (a) Cell density, (b) temperature, and (c) sodium formate. All values are average of three independent determinations.

as shown in Figure 2, the optimal concentration of NaCl for methanol accumulation was 200 mM. Where 7 mM methanol accumulated over 36 h.

Optimization of methanol biosynthesis

To establish the optimal reaction conditions for methanol synthesis by *M. trichosporium* OB3b, several parameters such as the cell density, reaction temperature, and sodium formate concentration were examined.

As shown in Figure 3a, methanol accumulation increased as cell density increased up to 0.6 mg dry cell ml^{-1}. *M. trichosporium* OB3b produced most methanol at 25 °C (Figure 3b) above which possibly pMMO became unstable. Accumulation of methanol also increased with an increase in the concentration of sodium formate up to 20 mM (Figure 3c). Under the optimal conditions, *M. trichosporium* OB3b accumulated 7.7 mM methanol over 36 h incubation (Figure 4).

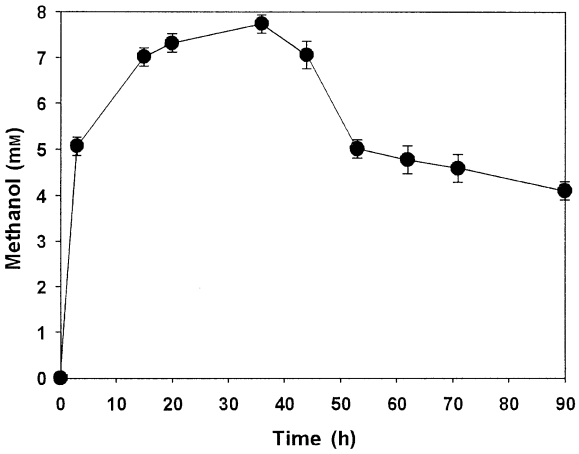

Fig. 4. Time-dependent methanol production by *M. trichosporium* OB3b. The methanol biosynthesis was carried out under the optimal conditions described in Results and discussion. All values are average of three independent determinations.

As an inhibitor acting on MDH, NaCl has advantages over cyclopropanol in that it is cheaper and stable. It may therefore open a door for cheap and efficient biological production of methanol from methane using methanotrophic bacteria.

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