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Batch Conversion of Methane to Methanol Using *Methylosinus trichosporium* OB3b as Biocatalyst

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Recently, methane has attracted much attention as an alternative carbon feedstock since it is the major component of abundant shale and natural gas. In this work, we produced methanol from methane using whole cells of *Methylosinus trichosporium* OB3b as the biocatalyst. *M. trichosporium* OB3b was cultured on NMS medium with a supply of 7:3 air/methane ratio at 30°C. The optimal concentrations of various methanol dehydrogenase inhibitors such as potassium phosphate and EDTA were determined to be 100 and 0.5 mM, respectively, for an efficient production of methanol. Sodium formate (40 mM) as a reducing power source was added to enhance the conversion efficiency. A productivity of 49.0 mg/l·h, titer of 0.393 g methanol/l, and conversion of 73.8% (mol methanol/mol methane) were obtained under the optimized batch condition.

Keywords: Methane, methanol, methanol dehydrogenase, *Methylosinus trichosporium* OB3b, reducing power

Introduction

Recently, there has been a rapid increase in shale gas production using horizontal drilling and hydraulic fracturing technology in the United States [2]. Methane is the primary component in natural and shale gas up to 95%. Thus, methane has attracted much attention as the alternative chemical feedstock on the positive aspect, but in addition to being a greenhouse gas 25-times more harmful than carbon dioxide [12]. Methane is the resource of power stations at present and can be used as a basic raw material [19]. However, methane is difficult to transport from production sites in remote locations to the place where it is to be consumed, since methane is a gas at ambient temperatures (boiling point: –164°C) [20].

Conversion of methane to methanol has attracted enormous interest because methanol is easy to handle and has a higher energy density than methane. Methanol is a starting precursor for the production of various value-added chemicals [22]. If methane, the main component of natural gas, can be efficiently converted to liquid fuels, world reserves of methane could satisfy the demand for transportation fuels in addition to use in other sectors. However, the direct activation of strong C-H bonds in methane and conversion to desired products remain a difficult technological challenge. Thus, there are economic and technological demands for the development of an efficient conversion technology of methane to methanol. However, the direct conversion of methane to methanol is a technological challenge owing to the strong C-H bonds in methane [11].

Various chemical processes to produce methanol from methane have been successfully implemented on a commercial scale. However, some disadvantages of chemical processes are still unsolved. It depends on extremely energy-intensive unit processes with low conversion rates

and high capital costs [4]. As an alternative, direct biological methods can be employed to convert methane to methanol. Biological conversion is more selective and requires less energy since bioconversion is conducted at ambient temperatures [6]. Methane-to-methanol conversion using methanotrophic cells as the biocatalysts has been conducted to replace the chemical process [5, 13].

In metabolic assimilation of methane in methanotrophs, methane is converted to methanol by methane monooxygenase (MMO). Methanol is converted to formaldehyde by methanol dehydrogenase (MDH), which is then oxidized to formate and finally to carbon dioxide [15]. Thus, methanol is not a final product and is readily converted to formaldehyde, which is used to synthesize cell biomass *via* ribulose monophosphate (RuMP) or the serine pathway [10]. In order to accumulate methanol, suitable inhibitors for MDH such as phosphate [16], cyclopropanol [25], or a high concentration of NaCl [1] need to be added to the medium. In addition, NADH or NADPH is regenerated to supply a reducing power for methane oxidation [8]. Methanotrophs-catalyzed production of methanol from methane with high yield still remains a challenge. In this paper, we determined the optimal conditions and further improved batch methane-to-methanol conversion in high yield using *Methylosinus trichosporium* OB3b whole cells. Cultivation-relevant parameters, including air/methane ratio and copper concentration, were optimized. MDH activity was inhibited by adding various chemical inhibitors with optimal concentrations, and the optimal amount of formate was supplied to regenerate the reducing power for high methanol conversion.

Materials and Methods

Medium and Cultivation of *M. trichosporium* OB3b

M. trichosporium OB3b (NCIMB 11131) was cultured in a 600 ml joint flask with rubber septum containing 200 ml of NMS medium (1 g $MgSO_4 \cdot 7H_2O$, 1 g KNO_3, 0.2 g $CaCl_2 \cdot H_2O$, 0.0038 g Fe-EDTA, 0.0005 g $NaMo \cdot 4H_2O$ per liter); 1 ml of trace elements solution (500 mg $FeSO_4 \cdot 7H_2O$, 400 mg $ZnSO_4 \cdot 7H_2O$, 20 mg $MnCl_2 \cdot 7H_2O$, 50 mg $CoCl_2 \cdot 6H_2O$, 10 mg $NiCl_2 \cdot 6H_2O$, 15 mg H_3BO_3, 250 mg EDTA per liter), 2 ml of phosphate stock solution (26 g KH_2PO_4, 62 g $Na_2HPO_4 \cdot 7H_2O$ per liter), and 2 ml of vitamin stock (1×) (2 mg biotin, 2 mg folic acid, 5 mg thimine HCl, 5 mg Ca pantothenate, 0.1 mg vitamin B12, 5 mg riboflavin, 5 mg nicotinamide per liter) under various ratios of air:methane and copper concentration, at 30°C and 230 rpm [27]. Copper(II) was added for pMMO expression [17, 21]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify expression of sMMO and pMMO.

Methane-to-Methanol Bioconversion

M. trichosporium OB3b cells were harvested by centrifugation at 4°C and 8,930 ×*g* for 20 min. The supernatant was removed and the harvested cells were washed once with DDW and once with 20 mM potassium phosphate buffer (pH 7.0). The whole cells were resuspended in 20 mM sodium phosphate buffer (pH 6.3) and used for methane-to-methanol conversion. Reactions were conducted in a 12 ml serum vial containing 2 ml of 20 mM sodium phosphate buffer (pH 6.3) as a reaction medium, with gray butyl rubber septa and aluminum seal. The reaction solution contained various concentrations of sodium formate and various chemical inhibitors for MDH and *M. trichosporium* OB3b whole cells as the biocatalyst. Air and methane were supplied at a volume ratio of 7:3 and the reactions were conducted in a shaking incubator at 30°C and 230 rpm.

MDH Activity Analysis

The MDH activity was measured using the methanol oxidation test [10]. A serum bottle (working volume = 2 ml) containing 0.6 mg/ml *M. trichosporium* OB3b and initial methanol concentration of 0.1% (v/v) in 20 mM sodium phosphate buffer (pH 6.3) was incubated for 3 h at 30°C and 230 rpm. The remaining methanol was measured by GC to determine the amount of methanol converted to formaldehyde by MDH.

Effect of Methanol Concentration on MMO Activity Based on Propylene Epoxidation Assay

A modified propylene epoxidation assay was used to measure the pMMO activity [3]. Air and propylene at a volume ratio of 1:1 were supplied to 2 ml of 20 mM potassium phosphate (pH 7.0) containing 0–10% (v/v) methanol and *M. trichosporium* OB3b cells. All reactions were conducted in a 12 ml serum bottle with gray butyl rubber septa and sealed with aluminum seal and incubated at 30°C and 230 rpm. The amount of the remaining propylene and produced propylene oxide was determined by GC.

Analysis

Methane, propylene, and propylene oxide were analyzed using a Younglin 6500GC gas chromatograph equipped with a thermal conductivity detector and a HP-PlotQ capillary column. For methane analysis, nitrogen was used as the carrier gas with a 2 ml/min rate. The injector, detector, and oven temperatures were 250°C, 250°C, and 60°C, respectively. For propylene analysis, the oven temperature was increased from 100°C to 150°C. For propylene oxide analysis, the oven temperature was 160°C. For methanol analysis, a flame ionization detector was used. The injector and detector temperatures were 250°C. The oven temperature was 65°C for 1 min, and then increased to 115°C with a 5°C/min rate.

Results and Discussion

Effects of Air:Methane Supply Ratio and Copper Concentration on Cell Growth and MMO Expression

M. trichosporium OB3b uses methane as the carbon and

Table 1. Effect of air-to-methane ratio on cell growth of *M. trichosporium* OB3b.

Air:methane ratio	Dry cell weight (mg/ml)	Methane-to-biomass conversion (g biomass/g methane)	Specific growth rate (h^{-1})
8:2	0.16	0.18	0.036
7:3	0.21	0.47	0.044
5:5	0.22	0.33	0.039

Table 2. Effect of copper ion concentration on cell growth of *M. trichosporium* OB3b.

Copper concentration (μM)	Dry cell weight (mg/ml)	Methane-to-biomass conversion (g biomass/g methane)	Specific growth rate (h^{-1})
0	0.22	0.47	0.044
1	0.28	0.36	0.053
5	0.34	0.46	0.059
10	0.32	0.39	0.053
50	0.11	0.15	0.032

energy source *via* the oxidative metabolic pathway in the presence of oxygen. Methane and oxygen are key substrates for cell growth, and their supply ratio of air:methane is an important factor in the cultivation of methanotrophs. To find out the optimal air:methane ratio, *M. trichosporium* OB3b was cultivated at various air:methane ratio ranging from 8:2 to 5:5 in NMS medium. In the presence of 30% (v/v) methane, a higher methane-to-biomass conversion and specific growth rate could be achieved. When methane above 30% (v/v) was supplied, the final cell density was not affected. At the optimal condition of air:methane = 7:3, the final dry cell density (g cell/l), methane-to-biomass conversion (g biomass/g methane), and specific growth rate (h^{-1}) were 0.21, 0.47, and 0.044, respectively (Table 1).

The effects of copper concentration on *M. trichosporium* OB3b growth and the expression of MMO were examined in the presence of various concentrations of copper (from 0 to 100 μM) in the medium. The copper concentration is known to affect the relative expression level of soluble and particulate MMO. Generally, pMMO is expressed in the presence of copper ion [3, 14, 17, 21, 24]. Herein, we quantitatively evaluated the effects of copper concentration on batch cell growth, methane-to-biomass conversion, and specific growth rate. As shown in Table 2, the highest final batch cell growth of 0.34 g cell/l, methane-to-biomass conversion of 0.46 g biomass/g methane, and specific growth rate of 0.059 h^{-1} were obtained in the media containing 5 μM of $CuSO_4$. In this condition, the expression of sMMO was completely inhibited. As the copper concentration increased, the specific growth rate also increased up to 5 μM (Table 2). Further increase in copper concentration resulted in a decrease in cell growth. When *M. trichosporium* OB3b was cultured in the presence of 100 μM copper, there was no detectable cell growth and no change in methane concentration, indicating that the presence of high copper concentration above 100 μM in NMS media was inappropriate for the cultivation of *M. trichosporium* OB3b (data not shown).

Several studies have also demonstrated that sMMO is expressed only at low or no copper, and pMMO is preferentially expressed under high copper concentration [3, 9, 17, 18, 21, 23, 24]. Fig. 1 shows the expression of

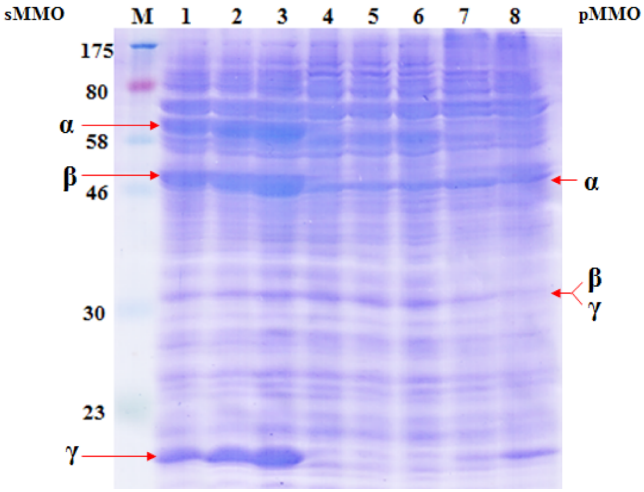

Fig. 1. SDS-PAGE analysis of *M. trichosporium* OB3b whole cell lysates.

The expected molecular masses of α, β, and γ subunits of sMMO and pMMO are indicated on the left-hand and right-hand sides of the gel, respectively. M, size marker in kDa; 1, air:methane = 8:2 without copper ; 2, air:methane = 5:5 without copper; 3, air:methane = 7:3 without copper; 4, 1 μM copper; 5, 5 μM copper; 6, 10 μM copper; 7, 50 μM copper; 8, 100 μM copper. The samples for lanes 4, 5, 6, 7, and 8 were obtained from cells grown at air:methane = 7:3.

sMMO and pMMO in the presence of various copper concentrations. In the absence of copper, the α, β, and γ subunits of sMMO were expressed and identified in lanes 1, 2, and 3, corresponding to the expected molecular mass of 60, 45.1, and 19.4 kDa, respectively. With respect to air and methane ratio, it did not affect the expression level of sMMO. Otherwise, in the presence of copper, the α, β, and γ subunits of pMMO were expressed and observed in lanes 4, 5, and 6, corresponding to the expected molecular mass of 46.9, 28.5, and 29 kDa, respectively. As the copper concentration was increased, the relative expression level of pMMO increased (Fig. 1). However, the expression of pMMO decreased when copper was supplied at more than 50 μM. For further experiments, 0.3 mg/l copper was added to the medium for high growth rate and pMMO expression.

Effect of Sodium Formate Addition on the Production of Methanol

Formate has been reported to increase methanol production by NADH regeneration [26]. For enhancement of methanol production using *M. trichosporium* OB3b, the effect of formate addition was investigated. The relative methanol production at various sodium formate concentrations ranging from 0 to 80 mmol/l are shown in Fig. 2. When 40 mM sodium formate was supplied, 0.232 g/l methanol was produced after 20 h and the methane-to-methanol conversion was 17.3%. Further increase in formate concentration did not increase the methanol production in the batch system. Further investigations need to be conducted to understand the saturation of methanol production in the presence of formate above 40 mM.

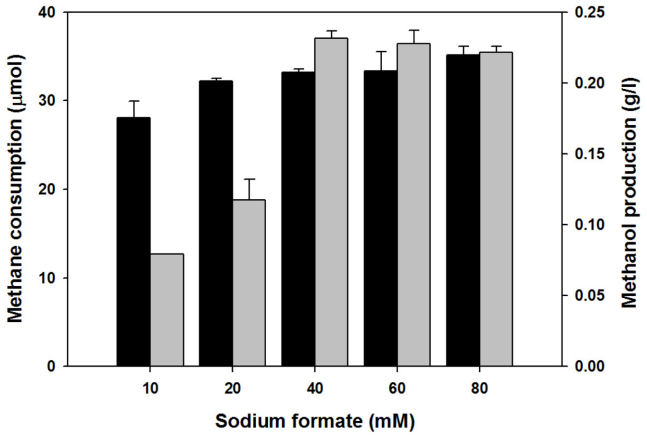

Fig. 2. Effect of sodium formate concentration on methane consumption and methanol production by growing and resulting cells of *M. trichosporium* OB3b (symbols: black bars, methane consumption; gray bars, methanol production).

Methane-to-Methanol Conversion Using Phosphate and EDTA as Methanol Dehydrogenase Inhibitor

For production of a higher methanol concentration, MDH needs to be inhibited. First, the effect of potassium phosphate addition on methanol production by *M. trichosporium* OB3b cells was investigated at various potassium phosphate concentrations (0–300 mM; Fig. 3A). As the potassium phosphate concentration increased, methane consumption continued to decrease, indicating that potassium phosphate might inhibit pMMO activity. In contrast, methanol production increased with an increase in potassium phosphate concentration up to 100 mM. The maximum amount of methanol was 0.309 g/l. However, methanol production decreased as potassium phosphate concentration was further increased to more than 100 mM. Methane-to-methanol conversion was also affected by potassium phosphate concentration. A 51.1% conversion was obtained with the addition of 100 mM potassium phosphate.

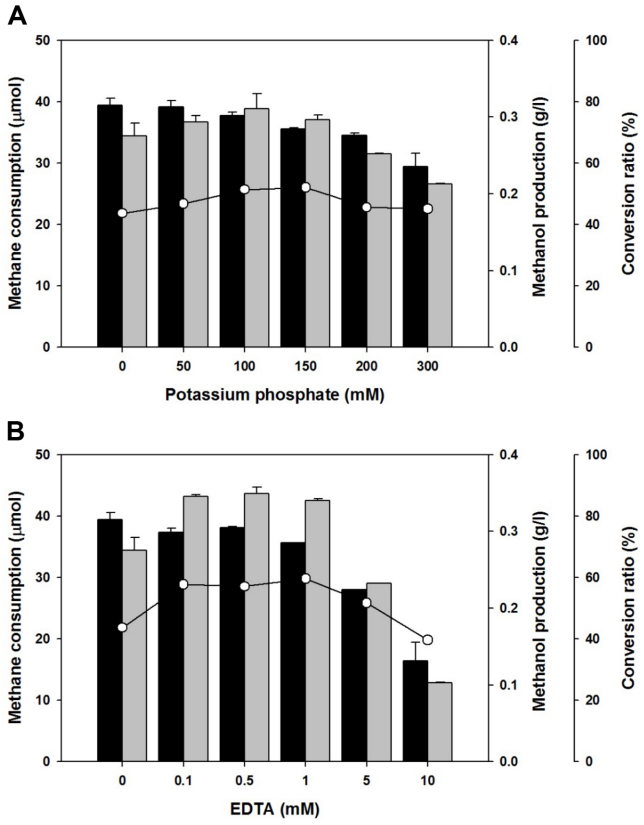

Fig. 3. Effects of potassium phosphate (**A**) and EDTA (**B**) as MDH inhibitors on methane consumption and methanol production by growing and resulting cells of *M. trichosporium* OB3b (symbols: black bars, methane consumption; gray bars, methanol production; line graph, methane-to-methanol conversion).

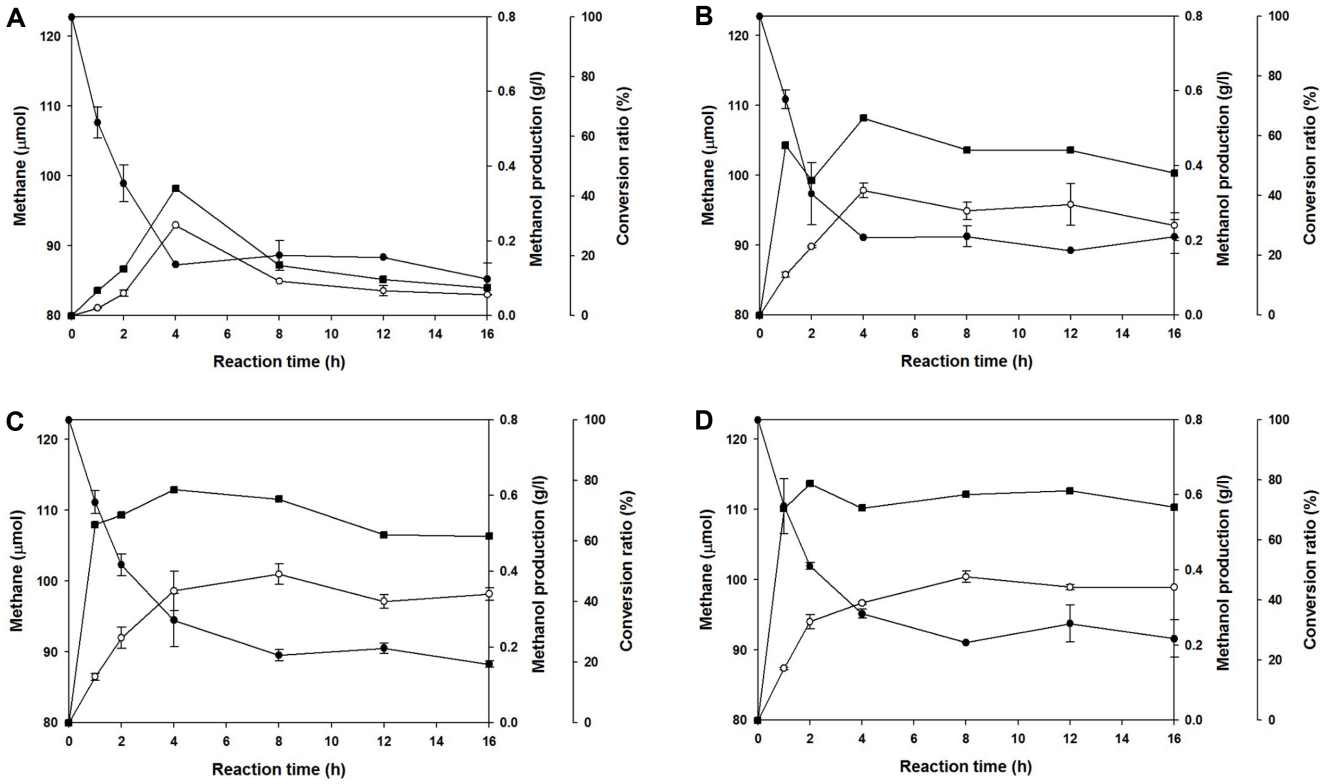

Fig. 4. Batch time course of methanol production at the optimized reaction condition by *M. trichosporium* OB3b.
Control experiment in the absence of formate and MDH inhibitor (**A**), and in the presence of 40 mM formate (**B**), 100 mM potassium phosphate (**C**), and 0.5 mM EDTA (**D**). The experiments of (**C**) and (**D**) were conducted in the presence of 40 mM formate (Symbols: ●, remaining methane; ○, produced methanol; ■, methane-to-methanol conversion).

In the case of EDTA, the methane consumption was also decreased as the EDTA concentration was increased (Fig. 3B). Methanol production increased with increase in EDTA concentration up to 0.5 mM, and then decreased with further increase in EDTA concentration. Approximately 0.349 g/l methanol was produced with a methane-to-methanol conversion of 57.1% in the presence of 0.5 mM EDTA.

The inhibitory effects of sodium formate, potassium phosphate, and EDTA on MDH activity were analyzed. There was no inhibitory effect of sodium formate on the MDH activity, but there was 9.3% and 15.2% inhibition of MDH in the presence of 100 mM potassium phosphate and 0.5 mM EDTA, respectively. This decrease of MDH activity allowed for methanol production.

Batch Methane-to-Methanol Conversion at the Optimized Condition

Methanol production in the absence of sodium formate and MDH inhibitors is shown in Fig. 4A. The maximum methanol concentration was 0.242 g/l at 4 h. With the addition of 40 mM sodium formate, methanol production was increased to 0.334 g/l during the same reaction time (Fig. 4B). However, the methanol concentration continued to decrease owing to subsequent methanol oxidation by MDH after 4 h.

Methanol production in the presence of MDH inhibitors is shown in Figs. 4C and 4D. The maximum methanol production levels were 0.393 and 0.382 g/l, and the methane-to-methanol conversion levels were 73.8% and 75.2% at 8 h in the presence of 100 mM potassium phosphate and 0.5 mM EDTA, respectively. The inhibitory effect of the presence of the produced methanol on MMO activity was also analyzed. Methanol at 0.4 g/l did not inhibit the propylene epoxidation rate of MMO (data not shown). The average volumetric productivity of methanol was 0.049 g/l/h. The average specific methanol productivity was 0.082 g/g cell/h, higher than the previously reported value of 0.00162 g/g cell/h [7, 26]. In conclusion, compared with the highest methanol production of 0.25 g/l in a simple batch reactor reported previously, a methanol concentration of up to 0.393 g/l was achieved in this study.

In terms of productivity, we obtained a 4.6-times higher productivity compared with the previously reported highest value.

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