**Methanol Biosynthesis from Methane Using *Methylosinus trichosporium* OB3b Grown in Medium Containing High Copper Concentration**

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Methanol production from methane in a batch reactor using whole cells of *Methylosinus trichosporium* OB3b grown in medium containing 50 μmol L⁻¹ copper was examined. Methanol productivity of the biocatalyst was 9.56 mmol g-dry cell⁻¹ h⁻¹, about 3-fold higher than that of the bacteria grown in medium containing 1.25 μmol L⁻¹ copper, indicating that methanol productivity of the methane bioconversion is improved by higher copper concentration in the growth medium. Methanol production almost ceased after 60-h reaction due to product inhibition. Therefore, repeated batch reaction was performed, in which the bacterial cells were collected after 24-h reaction and re-used in the next cycle. In the fourth cycle, 83 % of the methanol productivity was retained. About 856 mmol g-dry cell⁻¹ of methanol was obtained after 4 cycles, which was 3.7-fold higher than the methanol obtained in a single batch reaction for 96 h.

**Keywords**

Methane conversion, Methanol production, Biocatalyst, High copper concentration

1. Introduction

Methane is an abundant energy and carbon resource found in natural and shale gas deposits, and biogas waste produced by biological systems. The major use of methane is presently as a fuel, but considerable quantities are flared into the atmosphere despite the extensive potential as a carbon source. However, recent concerns about the environmental impacts and depletion of fuel resources have accelerated the requirement for the sustainable development goals (SDGs). Accordingly, alternative, more energy-efficient and highly selective processes of methane conversion to liquid, storable fuels and useful chemicals are desirable to establish more efficient utilization of methane resources. Methanol production is one important use of methane. Presently, methanol is produced from methane via syngas, a mixture of carbon monoxide and hydrogen. However, this methanol production process is not optimal as sustainable methanol production because of the high temperature and pressure conditions, and large CO₂ emissions as a by-product. Development of an alternative direct conversion process under mild conditions other than such indirect, high energy requirement processes is strongly desired.

One of the strategies for a sustainable methanol production from methane is the use of methane-utilizing bacteria (methanotrophs) as a biocatalyst. The bacteria utilize methane as the carbon and energy sources for growth at ambient temperature and pressure. Methanol is produced as an intermediate metabolite by methane monooxygenases (MMOs) enzymes by the bacterial metabolism. Such biological methane conversion is energy-efficient with high selectivity for methanol. Accordingly, industrial synthesis of methanol from methane using a methanotroph as a biocatalyst has been studied. *Methylosinus trichosporium* OB3b, which expresses particulate methane monooxygenase (pMMO) in the intracytoplasmic membrane of the bacterial cells, has been widely studied for this application. Efficient bacterial production of methanol from methane, requires two modifications to the biochemical reactions, inhibition of methanol dehydrogenase (MDH) and supply of electron donors, as shown in Fig. 1. MDH rapidly oxidizes the methanol produced by pMMO from methane. Cyclopropanol, NaCl, and EDTA have been used as inhibitors. Supply of electron donors is required because the physiological electron donor, nicotinamide adenine dinucleotide (NADH), is consumed during the oxidation of methane to methanol by pMMO, while metabolic synthesis is...
suppressed by the inhibition of MDH. Therefore, formate is supplied to the reaction mixture to regenerate NADH in the bacterial cells (Fig. 1)\(^{16}\). Formate is oxidized to carbon dioxide, coupled with NADH regeneration by formate dehydrogenase.

Methanol productivity of biocatalytic methane conversion in the batch reactor has reached 8.4 mmol g-dry cell h\(^{-1}\) by optimizing the reaction conditions and the reactor design (Table 1). However, methanol productivity should be increased to be over 30 mmol g-dry cell\(^{-1}\) h\(^{-1}\) for biocatalytic methanol production on the industrial scale. One of the strategies for increasing productivity is the use of methanotrophic bacterial cells expressing a high quantity and quality of pMMO. The quantity of pMMO expressed in the cells of \textit{M. trichosporium} OB3b depends on the copper concentration in the culture medium; with higher amount of pMMO expressed in the bacterial cells with higher copper concentration in the medium\(14,17\). High expression was achieved by culturing the bacteria in medium with copper concentrations up to 50 mmol L\(^{-1}\)\(^{14,17}\). Therefore, \textit{M. trichosporium} OB3b cultured with 50 mmol L\(^{-1}\) copper is expected to show superior methanol productivity in the bioconversion of methane. However, growth of bacteria in the presence of high copper concentrations is difficult due to the toxicity of copper. Recently, we established a way to overcome this difficulty by culturing the bacteria with cyanocobalamin\(17\).

The present study examined methanol production using whole cells \textit{M. trichosporium} OB3b cells grown under high copper concentration to assess the biocatalyst effectiveness for energy-efficient methanol synthesis from methane. \textit{M. trichosporium} OB3b was cultured in a medium containing 50 mmol L\(^{-1}\) copper with cyanocobalamin and applied the resting cells to methanol production from methane in a batch reactor. For comparison, methanol production was also performed using bacterial cells grown in culture mediums containing 1.25 mmol L\(^{-1}\) and 10 mmol L\(^{-1}\) copper. We expected that the bacteria grown in high copper concentration would achieve higher methanol productivity through methane bioconversion.

### 2. Materials and Methods

#### 2.1. Batch Culture of \textit{M. trichosporium} OB3b

\textit{M. trichosporium} OB3b was kindly donated by Professor T. Kamachi, Tokyo Institute of Technology, Tokyo, Japan. The bacteria were cultured using a method previously reported\(18\) with some modifications. The bacteria were stored at -80 °C in glycerol and transferred into 25 mL of nitrate mineral salt (NMS) medium containing 50 mmol L\(^{-1}\) CuSO\(_4\) and 100 mmol L\(^{-1}\) cyanocobalamin (Sigma-Aldrich, St. Louis, USA) in a 100 mL-Erlenmeyer flask with baffles. Note that not all CuSO\(_4\) added may be dissolved in the medium, although CuSO\(_4\) was added through a 0.45 μm sterile filter (Millex\textsuperscript{®} Syringe Filter Unit, Merck Millipore, Massachusetts, USA) to the medium after autoclave sterilization to avoid precipitation of copper and absence of copper precipitation was visually confirmed.

The flask was sealed with a silicone cap with a gas inlet tube fitted with a 0.45 μm sterile filter (Millex\textsuperscript{®} Vent Filter Unit, Merck Millipore, Massachusetts, USA) to the medium after autoclave sterilization to avoid precipitation of copper and absence of copper precipitation was visually confirmed.
USA). The pressure of the gas phase in the flask was reduced to 0.02 MPa (gauge pressure) using a hand pump. Then, methane gas was introduced as a carbon source from a balloon. The bacteria were cultured for 2 days in a rotary shaking incubator at 30 °C, 225 rpm, during which the bacterial growth entered the logarithmic growth phase. The pre-cultured bacteria were transferred to 1 L of NMS medium containing 50 mmol L⁻¹ CuSO₄ in a 5 L-Erlenmeyer flask. Methane gas was introduced into the gas phase as described above. Then, the bacteria were cultured for 3 days in the rotary shaking incubator at 30 °C, 150 rpm, during which the bacterial growth continued in the logarithmic growth phase. Bacterial growth was detected by measuring the turbidity of the culture spectrophotometrically at 660 nm.

The cultured cells were harvested by centrifugation (23,500 × g, 15 min, 4 °C) of the culture medium; the collected bacterial cells were suspended in 12.9 mmol L⁻¹ phosphate buffer, pH 7.0. The bacterial cells were then transferred to 1 L of NMS medium containing 50 mmol L⁻¹ KCl buffer was cooled to 4 °C and purged with nitrogen gas to remove dissolved oxygen. Approximately 30 g (0.5 g-wet cell mL⁻¹) of the frozen cell suspension of *M. trichosporium* OB3b stored at −80 °C was thawed and centrifuged to separate the bacterial cells (23,500 × g, 20 min, 4 °C). Approximately 15 g-wet weight cell of the collected bacterial cells were suspended in 30 mL of 25 mmol L⁻¹ MOPS (pH 7.0) buffer solution. This cell suspension was transferred to a 100 mL beaker, and DNase I, MgCl₂, 6H₂O, CuSO₄ 5H₂O, and benzamidine were added to final concentrations of 10, 4, 0.3, and 1 mmol L⁻¹, respectively. The suspension of bacterial cells was disrupted with an ultrasonicicator (Tomy Seiko Co., Ltd., Tokyo, Japan) on ice under a nitrogen atmosphere. The sonicated suspension was centrifuged (23,500 × g, 20 min, 4 °C) to remove unbroken cells. The supernatant was then centrifuged (170,000 × g, 90 min, 4 °C) and the membrane fraction containing pMMO was pelleted, then suspended in 25 mmol L⁻¹ MOPS buffer, pH 7.0, containing 0.5 mol L⁻¹ KCl, followed by ultra-centrifugation (170,000 × g, 90 min, 4 °C). Finally, the pellet was suspended in 25 mmol L⁻¹ MOPS buffer, pH 7.0, to protein concentration of about 20 mg mL⁻¹.

The membrane fraction was flash-frozen as granules with liquid nitrogen, and then stored at −80 °C. The total amount of protein in the membrane fraction was determined by the bicinchoninic acid (BCA) method using the BCA Protein Assay Kit (Bio-Rad, California, USA). Bovine serum albumin (BSA) was used as the standard protein for the assay.

The activity of pMMO in the membrane fraction was measured in a micro-reaction vessel capped with Mininert® valve (5 mL, Merck KGaA, Darmstadt, Germany) charged with 1 mL of the reaction mixture of 25 mmol L⁻¹ MOPS buffer, pH 7.0, containing 1 g-protein L⁻¹ of the membrane fraction and 10 mmol L⁻¹ of NADH. Using a gas-tight syringe, 1 mL of the gas phase in the vessel was withdrawn. Then, the reaction was initiated by introducing 1 mL of methane gas and performed in a water bath at 25 °C with stirring.

### 2.4. Quantification of pMMO in the Bacterial Cells

Expression level of *M. trichosporium* OB3b intracellular proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples for the analysis were prepared by extracting proteins from the bacterial cells using EzBactYeast Crusher (ATTO Corp., Tokyo, Japan). The concentration of the extracted protein was measured using the BCA protein assay kit (Thermo Fisher Scientific K.K., Tokyo, Japan). The protein concentration was adjusted to 2.5 g-protein L⁻¹ by adding a Tris-HCl buffer (pH 7.8). Then, 6 μL of the cell lysate were transferred into an Eppendorf tube and 19 μL of electrophoresis...
buffer (EzRun, ATTO Corp., Tokyo, Japan) was added for dilution. Then, 25 μL of Laemmli Sample Buffer (2 ×) (Bio-Rad, California, USA) was added, suspended, and left at room temperature for 5 min. The electrophoresis sample solution and 5 μL of molecular weight markers (All Blue Standards, Bio-Rad, California, USA) were loaded onto 15 % polyacrylamide gel (e-PAGEL, Bio-Rad, California, USA), and electrophoresis was performed at 40 mA for 75 min. After electrophoresis, the gel was stained with Coomassie brilliant blue (Bio-Safe™ Coomassie G-250 Stain, Bio-Rad, California, USA).

The gel images were analyzed using ImageJ, to measure the staining intensity of each protein, and the distribution of pMMO in the total intracellular proteins was calculated. The amount of each protein separated by electrophoresis was calculated from the distribution and amount of protein in the electrophoresis sample. The estimation assumed that the band intensity and the protein amount were proportional.

3. Results and Discussion

3.1. Methanol Biosynthesis from Methane Using *M. trichosporium* OB3b Grown in High Copper Concentration Medium

The activity of *M. trichosporium* OB3b grown in the medium containing high copper concentration for methanol biosynthesis from methane was examined using the resting cells of the bacteria. The reaction conditions reported previously were chosen for the biosynthesis because the amount of methanol obtained in a batch reactor was relatively high (~152 mmol g-dry cell⁻¹)⁸. Bacterial cells grown in medium containing 50 μmol L⁻¹ and 10 μmol L⁻¹ of copper were used as the biocatalysts prepared at high copper concentration, and the cells grown in medium containing 1.25 μmol L⁻¹ copper as the conventional biocatalyst.

The time course of methanol production from methane in a batch reactor is shown in Fig. 2. Bacterial cells grown with 50 μmol L⁻¹ of copper produced methanol at a constant rate for about 30 h after the reaction started, then the rate decreased gradually. Methanol production almost ceased around the reaction time of 60 h, due to the inhibition of pMMO by the produced methanol⁹. Reaction for 96 h obtained 234 mmol of methanol per g of dry-cell, and maximum methanol concentration in the reaction mixture reached about 8 mmol L⁻¹.

Methanol production rate in the initial stage and total amount of methanol were lower using bacterial cells grown with 10 μmol L⁻¹ and 1.25 μmol L⁻¹ of copper ions (Fig. 2). Table 2 summarizes the methanol productivity (mmol-methanol g-dry cell⁻¹ h⁻¹) in the initial stage of the biosynthesis, the total amount of methanol (mmol-methanol g-dry cell⁻¹), and the maximum meth-

| Copper concentration in the growth medium [μmol L⁻¹] | Methanol productivity (mmol g-dry cell⁻¹ h⁻¹) | Amount of methanol (mmol g-dry cell⁻¹) | [mmol L⁻¹] |
|---------------------------------|---------------------------------|---------------------------------|-------------|
| 1.25                           | 3.07 ± 0.02                     | 149 ± 11                         | 5.21 ± 0.39 |
| 10                             | 5.57 ± 0.08                     | 167 ± 10                         | 5.84 ± 0.35 |
| 50                             | 9.56 ± 0.04                     | 227 ± 13                         | 7.95 ± 0.46 |

a) Determined from the reaction period where the production rate of methanol was constant.
b) Amount of methanol produced by 98-h reaction in 3.5-mL reaction mixture.
c) Results are expressed as means ± SD of triplicate experiments (p < 0.01 versus each data by t-test).

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The maximum methanol concentration in the reaction mixture increased with higher copper concentration in the growth medium of the biocatalyst (Table 2), although the concentration is known to be limited due to product inhibition of the biocatalyst9). Previous studies shown in Table 1 indicated that the maximum methanol concentration tended to increase with higher cell density in the reaction mixture. In contrast, the methanol synthesis experiments in this study were performed with the same cell density. Therefore, the increase in maximum methanol concentration in our experiment is caused by higher expression of pMMO in the bacterial cells.

3.2 Quantity and Quality of pMMO in the Bacterial Cells

High expression of pMMO in the bacterial cells of M. trichosporium OB3b is expected to result in high activity. To confirm this, the membrane fraction was prepared from the bacterial cells, and the pMMO content and activity for methanol production from methane were measured. The pMMO distribution among total proteins in the membrane fraction depended on the copper concentration in the growth medium of the bacteria. The protein distributions showed statistically significant differences (p<0.01) as shown in Table 3. The membrane fraction of bacteria prepared with 1.25 μmol L⁻¹ copper contained about 28 % of the total proteins as pMMO. The pMMO distribution increased with higher copper concentration, and reached to about 58 % in the bacteria prepared with 50 μmol L⁻¹ copper. Therefore, the amount of pMMO in a single bacterial cell increased by about 2.1 times as the copper concentration in the growth medium increased from 1.25 to 50 μmol L⁻¹.

Furthermore, the specific activity of pMMO (mol-methanol mg-protein⁻¹ min⁻¹) depended on the copper concentration (Table 3). pMMO activities in the different samples showed significant differences (p<0.01) as shown in Table 3. The membrane fraction prepared from the bacteria grown with 1.25 μmol L⁻¹ copper, had about 18 mmol-methanol mg-protein⁻¹ min⁻¹ of pMMO activity, whereas the membrane fraction prepared from the bacteria grown with 50 μmol L⁻¹ copper had activity of about 48 mmol-methanol mg-protein⁻¹ min⁻¹ in. Therefore, the pMMO activity increased about 2.7 times as the copper concentration in the growth medium increased from 1.25 to 50 μmol L⁻¹. The increase in pMMO activity was higher than the increase in pMMO distribution, indicating that the copper affects not only the quantity of pMMO but also the quality of pMMO in a bacterial cell. The same effect of copper was reported in another methane-oxidizing bacterium, Methylococcus capsulatus (Bath)30). The increase in the quantity and quality of pMMO induced by copper ions contributes to the increase in biocataytic performance for methanol production from methane.

The increase in methanol productivity of the biocatalyst (3.1-fold) was higher than that in pMMO activity in the membrane fraction (2.7-fold). One of the reasons is that the amount of bacterial membrane also increased with higher copper concentration in the growth medium of the bacteria. Alternatively, the difference is due to the loss of pMMO activity during the preparation of the membrane fraction from the bacterial cells.

3.3 Repeated Batch Methanol Production from Methane

Methanol production almost ceased as the amount of methanol produced in the batch reactor increased, which is known to be caused by inhibition of pMMO by the produced methanol31). Therefore, the removal of the product from the reaction mixture would be a useful technique for maintaining methanol productivity during the methane conversion. To confirm this, repeated batch reaction was carried out. A single reaction was carried out for 24 h. After the reaction, the bacterial cells were collected from the reaction mixture using a
centrifuge filter, and re-used in the next batch reaction.

**Table 4** summarizes the amount of biocatalyst collected from the reaction mixture and the methanol productivity during 4 cycles of the repeated batch reaction. More than 80% of the biocatalyst was collected by the centrifugation filter from the reaction mixture in each cycle. Furthermore, over 90% of methanol productivity was retained in the reaction cycle, but was reduced gradually by repeating the cycle. Such decrease in productivity was probably due to disruption of the bacterial cells and the protein structure of pMMO during the methanol synthesis reaction and collection of the biocatalyst\(^{(3,18)}\). Over the 4 cycles (total 96 h), ~856 mmol g-dry cell\(^{-1}\) of methanol was obtained, 3.7-fold higher than the amount of methanol in the batch reaction for 96 h. As reported previously\(^{(3)}\), the total amount of methanol produced from methane was improved by using repeated batch reaction to prevent the product inhibition of pMMO.

### 4. Conclusion

As we expected, *M. trichosporium* OB3b grown in medium containing high copper concentration had improved methanol productivity from methane biconversion in a batch reactor. The increase in the methanol productivity was due to higher quantity and quality of pMMO in the bacterial cells. The methanol productivity of the biocatalyst prepared in growth medium containing 50 μmol L\(^{-1}\) copper was 9.56 mmol g-dry cell\(^{-1}\) h\(^{-1}\), which was the highest reported productivity in the methane conversion to methanol in batch reactors (**Table 1**). By optimizing the design of batch reactors and the reaction conditions such as methane to air ratio in the gas phase of the reactor, concentration of formate, and inhibitors for MDH, as suggested in previous studies, the methanol productivity of *M. trichosporium* grown in medium containing 50 μmol L\(^{-1}\) copper would approach the required performance of biocatalyst for methane conversion to methanol in industrial use.

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要旨
高濃度の銅含有培養液で調製したメタン資化細菌 Methylosinus trichosporium OB3b によるメタンからのメタノール合成

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メタン資化細菌 Methylosinus trichosporium OB3b による常温常圧でのメタン転化反応におけるメタノール生産性を高めるため、50 μmol L⁻¹の銅を含む培地で調製した細菌細胞を生体触媒として用いた。回分反応器におけるメタン転化反応のメタノール生産性は9.56 mmol g-dry cell⁻¹ h⁻¹であり、従来法で用いられた1.25 μmol L⁻¹銅を含む培地で調製した生体触媒と比べて約3倍に向上した。この生産性向上は、銅により細菌細胞内の銅含有メタンモノオキシゲナーゼ発現量と酵素活性の増大に起因する。ただし、生体触媒の生成物阻害により反応開始後60時間で反応はほぼ停止した。そこで、24時間の反応後に生体触媒を回収し、次の反応に再利用することで、メタノール合成反応を4回繰り返し行った。その結果、得られたメタノール量は約856 mmol g-dry cell⁻¹に達し、96時間の回分反応と比べて約3.7倍に増大した。