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Potential of Immobilized Whole-Cell *Methylocella tundrae* as a Biocatalyst for Methanol Production from Methane

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Methanol is a versatile compound that can be biologically synthesized from methane (CH_4) by methanotrophs using a low energy-consuming and environment-friendly process. *Methylocella tundrae* is a type II methanotroph that can utilize CH_4 as a carbon and energy source. Methanol is produced in the first step of the metabolic pathway of methanotrophs and is further oxidized into formaldehyde. Several parameters must be optimized to achieve high methanol production. In this study, we optimized the production conditions and process parameters for methanol production. The optimum incubation time, substrate, pH, agitation rate, temperature, phosphate buffer and sodium formate concentration, and cell concentration were determined to be 24 h, 50% CH_4, pH 7, 150 rpm, 30°C, 100 mM and 50 mM, and 18 mg/ml, respectively. The optimization of these parameters significantly improved methanol production from 0.66 to 5.18 mM. The use of alginate-encapsulated cells resulted in enhanced methanol production stability and reusability of cells after five cycles of reuse under batch culture conditions.

Keywords: Biocatalyst, immobilization, methane, methanol, *Methylocella tundrae*, whole-cell immobilization

Introduction

Climate change, environmental problems, and the need to manage the depletion of fossil fuels are issues that the world faces today, and the utilization of alternative energy instead of fossil fuels is the only solution. Several alternative energy sources have been evaluated, including alcohols, hydrogen, and methane (CH_4) [1, 11–13, 19–21, 23, 28, 29, 33, 34, 37, 46]. Among these energy sources, CH_4 has received considerable attention owing to its greenhouse gas nature. To overcome this problem, CH_4 can be converted into useful products such as methanol. Methanol is a convenient liquid fuel, and can be easily stored, distributed, transported, and dispensed. Methanol is one of the best energy carriers and can be readily synthesized from CH_4, both chemically and biologically [10, 43]. However, biosynthetic methanol production is more highly considered owing to the low energy consumption and clean technology involved in the process.

Biosynthetic methanol production is performed by methanotrophs, which can utilize CH_4 as their sole carbon and energy source [2, 41]. Methanotrophs are categorized into three types (I, II, and X) based on their carbon assimilation pathway, cell morphology, membrane arrangement, and 16S rRNA sequences. As organisms that play a role in methanol biosynthesis from CH_4, methanotrophs possess a unique enzyme: methane monooxygenase (MMO). This enzyme has the ability to activate the stable C-H bond in CH_4 and oxidizes it to form methanol. In its oxidation mechanism, MMO splits an oxygen molecule into two single oxygen atoms and introduces one of these oxygen atoms into CH_4. The oxidation of CH_4 into methanol is the

first step of the metabolic pathway of methanotrophs [40]. However, methanol is further oxidized to formaldehyde in the metabolic pathway; therefore, high methanol production using methanotrophs remains a challenge.

The bioconversion of CH_4 into methanol has been studied using different types and strains of methanotrophs, including *Methylosinus trichosporium* [5, 9, 32], *Methylococcus capsulatus* [8], *Methylocaldum* sp. [39], *Methylocystis bryophila* [30], and *Methylosinus sporium* [45]. The methanotrophs were cultured in Higgins nitrate mineral salt (NMS) medium, and then harvested at the exponential phase to obtain active cells. These cells were then used to increase methanol productivity. In order to achieve higher methanol production, several parameters were optimized, including the concentration of phosphate, the type of methanol dehydrogenase inhibitors used, the concentration of formate, and the cell density.

In this study, the potential of *Methylocella tundrae* as a biocatalyst for methanol biosynthesis was evaluated. The strain used is a type II methanotroph that uses the serine cycle for carbon assimilation. However, to our best knowledge, no studies have reported methanol production using *M. tundrae*. The growth conditions, physical process parameters, production conditions (including the effect of the sodium phosphate buffer, sodium formate [HCOONa], and cell concentration) were optimized. In order to prevent the further oxidation of methanol into formaldehyde, which is catalyzed by methanol dehydrogenase, we screened four chemicals (magnesium chloride ($MgCl_2$), sodium chloride (NaCl), ammonium chloride (NH_4Cl), and ethylenediaminetetraacetic acid (EDTA)) as inhibitors of methanol dehydrogenase activity and investigated the effectiveness of these inhibitors on methanol production. Furthermore, the whole-cell immobilization of *M. tundrae* was used to improve the stability and reusability of methanol productivity. Whole-cell immobilization was performed by cell entrapment in alginate beads under mild conditions.

Materials and Methods

Strains and Culturing Conditions

M. tundrae (DSMZ 15673) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The strain was cultured in NMS medium, which was modified to the following composition (g/l): KNO_3 (1.0), $MgSO_4{\cdot}7H_2O$ (1.0), $CaCl_2{\cdot}2H_2O$ (0.2), Fe-EDTA (0.38), $Na_2MoO_4{\cdot}2H_2O$ (0.026), $Na_2HPO_4{\cdot}12H_2O$ (0.716), and KH_2PO_4 (0.26). A trace element solution (1 ml), which contained $ZnSO_4{\cdot}7H_2O$ (0.4), H_3BO_3 (0.015), $CoCl_2{\cdot}6H_2O$ (0.05), Na-EDTA (0.02), $MnCl_2{\cdot}4H_2O$ (0.02), and $NiCl_2{\cdot}6H_2O$ (0.01), was added to the medium [31]. Copper and iron were additionally added in the form of $CuSO_4$ and $FeSO_4$ to give a specified working concentration [30, 31]. All chemicals were of analytical grade and were purchased from Sigma-Aldrich (USA), DaeJung Chemicals and Metals (South Korea), and Junsei Chemical (Japan). Pure CH_4 was purchased from NK Co. (South Korea). The seeds were cultivated in 120 ml serum bottles (Sigma-Aldrich, USA) containing 20 ml of modified NMS medium and were capped with teflon-coated rubber butyl stoppers (Wheaton) and sealed with aluminum crimp seals (Supelco, USA). Twenty percent of the headspace air in the serum bottle was replaced by CH_4 of the same amount. The seed cultures were incubated at 30°C and 150 rpm in a shaking incubator (VS-8480 Vision Scientific). For laboratory-scale production of methanol, one percent of the seed cultures was introduced into a 1 L Erlenmeyer flask (Duran-Schott, Germany) containing 400 ml of modified NMS medium and 20% of CH_4 with a gas-tight seal (Suba-Seal) as described previously [30]. The production cultures were kept at 30°C and 150 rpm in a shaking incubator (Lab Champion IS-971R, USA). Cell growth was measured by analyzing the optical density at 600 nm with a UV/Vis spectrophotometer (Jenway Scientific, UK) [4, 14]. The specific growth rate (μ) of *M. tundrae* was determined using a method described previously [39]. The strain was maintained by subculturing on NMS agar plates, as described previously [30].

Production of Methanol

Methanol production was performed in a batch system with the following steps. Cells were harvested in the middle of the exponential phase by centrifugation (Gyrozen 1580 MGR, South Korea) at 4°C and 11,200 ×*g* for 15 min [6, 17, 26, 27]. The harvested cell pellets were washed twice with distilled water and 20 mM sodium phosphate buffer (pH 7.0). Furthermore, harvested cells were resuspended in the same buffer and kept at 4°C [16]. The reactions were conducted in a 20 ml vial containing 2 ml of 20 mM sodium phosphate buffer (pH 7.0) as a reaction medium. The reaction mixture contained various concentrations of cells, sodium phosphate buffer (pH 7.0), methanol dehydrogenase (MDH) inhibitors, and HCOONa. CH_4 was injected into the vials using a syringe. The vials were incubated at a specified incubation time, temperature, and agitation rate.

Methanol Dehydrogenase (MDH) Activity Analysis

MDH activity was determined using phenazine methosulfate (PMS) and 2,6-dichlorophenol-indophenol (DCPIP) as electron acceptors [30, 31]. The assay mixture contained 50 μl of $CaCl_2$, 50 μl of NH_4Cl, 350 μl of Phosphate buffer, 530 μl of whole cells, 10 μl of PMS, and 10 μl of DCPIP. The reaction was initiated by the addition of PMS, and MDH activity was measured by monitoring the decrease of DCPIP using a UV/Vis spectrophotometer at 600 nm.

Optimization of Whole-Cell Immobilization

For whole-cell immobilization by cell entrapment in alginate beads, the alginate and cell loading concentrations were optimized for methanol production in the ranges of 1–4% (w/v) and 1–5 mg

of DCM, respectively. Cells were mixed with a sodium alginate solution and the resulting mixture was extruded dropwise using a syringe into 200 ml of 1.5 M $CaCl_2$ solution for the preparation of beads, as described previously [6]. The beads were hardened in $CaCl_2$ solution for 2 h at 25°C and cells containing alginate beads were washed with saline solution to remove any excess calcium ions and loosely bound cells. The alginate beads with immobilized cells of *M. tundrae* were used for methanol production under optimum conditions for incubation (24 h), CH_4 (50% (v/v)), temperature (30°C), pH (7.0), agitation rate (150 rpm), and inoculum (18 mg DCM/ml). The reusability of free and immobilized cells was performed up to five cycles of reuse.

Analytical Methods

Methanol concentration was spectrophotometrically analyzed by alcohol oxidase protocol at 412 nm using a UV/Vis spectrophotometer as described previously [30, 31]. The methanol concentration was also detected using a gas chromatography Agilent 7890A system equipped with a flame ionization detector and an HP-5 column (Agilent 19091J-413). Hydrogen was used as the carrier gas with a rate of 25 ml/min. The injector and detector temperatures were 220°C and 250°C, respectively. The oven temperature was 35°C for the first 5 min, which was then increased to 150°C with an increase rate of 5°C/min [30, 31]. All experiments and assays were performed in triplicates.

Results and Discussion

Substrate Specificity of *M. tundrae*

M. tundrae has been reported as a unique methanotroph owing to its ability to utilize single-carbon and multi-carbon compounds as an energy source [3]. Several single-carbon and multi-carbon compounds were examined as a substrate for *M. tundrae* (Table 1). This strain was able to grow on C-1, C-2, and C-3 compounds as a sole carbon and energy source; however, CH_4 was still the most suitable substrate for growth of this strain and gave the highest growth rate value of 0.027 h^{-1}.

According to Dedysh *et al.* [3], *M. tundrae* can grow in a temperature range of 4–30°C and a pH range of 4.2–7.5, and is highly sensitive to salt stress. Moreover, the liquid cultures exhibit homogeneous turbidity. For the methanol production process, *M. tundrae* was cultivated in NMS liquid medium with a pH of 6.8 at 30°C, and was harvested in the middle of the exponential phase. As a sole carbon and energy source, the ratio of CH_4 to air is an important factor for the cell growth of methanotrophs. High concentrations of CH_4 inhibit cell growth because they decrease the amount of oxygen [38], and therefore the optimum ratio of CH_4 to air should be used. In this experiment, we cultivated *M. tundrae* under the CH_4-to-air ratio of 2:8.

Table 1. Substrates utilization and growth rate of *Methylocella tundrae*.

Substrates (%)	μ (h^{-1})
CH_4 (20)%	0.027 ± 0.002
Methanol (0.05)%	0.018 ± 0.001
Ethanol (0.05)%	0.018 ± 0.001
Glycine (30)%	0.016 ± 0.001
Acetone (0.05)%	0.025 ± 0.002
Glycerol (0.04)%	0.023 ± 0.001
Ethyl acetate (0.05)%	0.019 ± 0.002

Influence of Feed and Physical Process Parameters on Methanol Production

At the beginning of the optimization process, we monitored the change in methanol production based on incubation time under different CH_4 concentrations as a substrate in a reaction system (Fig. 1). In the 0–96 h range of incubation time, methanol began to accumulate after 6 h and continued to increase in concentration until 24 h, and then tended to slowly decrease and remained steady from the incubation time of 48–96 h. The highest concentration of methanol, 2.00 mM, was achieved at 24 h in 50% CH_4. The change in methanol production based on pH, agitation rate, and temperature was also monitored (Fig. S1). The optimum pH for methanol production was in the range of 6.5–7.5 in phosphate buffer. The methanol concentration increased from 0.66 to 1.98 mM when the pH increased

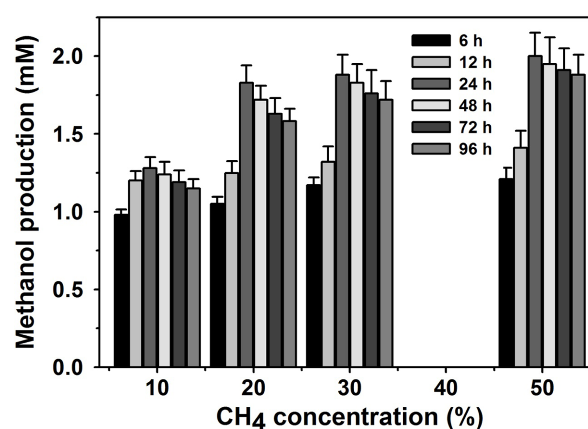

Fig. 1. Effect of CH_4 concentration at different incubation times. The reaction was conducted in the presence of 5 μM of Cu, 10 μM of Fe, and 20 mM phosphate buffer (pH 7.0) as the reaction medium. CH_4 as substrate was supplied at different concentrations (●) 10%, (○) 20%, (▼) 30%, and (△) 50% (v/v). Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

from 4.5 to 6.5. The methanol concentration remained stable (1.96–2.00 mM) in the pH range of 6.5–8.5 (Fig. S1A). The agitation rate and temperature also affected methanol production. The methanol production increased from 1.83 to 2.00 mM when the agitation rate was increased from 50 to 150 rpm, and fell to 1.89 mM when the agitation rate was over 150 rpm (Fig. S1B). It was predicted that this result was due to incomplete mass transfer. The same trend was also observed for temperature; the methanol concentration decreased slowly from 2.00 to 1.72 mM when the reaction was conducted at over 30°C (Fig. S1C).

Effect of MDH Inhibitors on Methanol Production

To further optimize the process, the inhibitory effects of phosphate buffer and MDH inhibitors on methanol production were evaluated. First, methanol was produced in different phosphate concentrations between 0 and 125 mM (Fig. 2A). Methanol increased from 2.00 to 3.00 mM when the sodium phosphate buffer concentration increased from 0 to 100 mM. However, increasing the concentration of sodium phosphate buffer over 100 mM produced lower methanol concentrations. Previously, Duan *et al.* [5] and Yoo *et al.* [45] showed that high concentrations of sodium phosphate buffer could reduce the methanol productivity of *M. trichosporium* OB3b, and they found that the optimum concentrations were 400 mM and 40 mM, respectively. In this case, a sodium phosphate buffer can be used as an inhibitor for methanol oxidation, which is catalyzed by MDH, and 100 mM of sodium phosphate buffer inhibited MDH activity by 16.87%. In order to achieve higher methanol production, it is necessary to add chemicals into the reaction system to inhibit MDH activity. Several MDH activity inhibitors ($MgCl_2$, NaCl, NH_4Cl, and EDTA) of different concentrations were screened for use in methanol production (Table 2). The optimum concentration (mM) of these inhibitors was 50 for $MgCl_2$, 50 for NaCl, 10 for NH_4Cl, and 1.0 for EDTA in the presence of 100 mM sodium phosphate buffer, respectively. Among the inhibitors screened, the addition of $MgCl_2$ into the reaction system produced the highest methanol concentration. The methanol concentration was 3.24 mM at 50 mM of $MgCl_2$. Surprisingly, the greatest inhibition to MDH activity was not given by $MgCl_2$; the greatest inhibition was shown by 1.0 mM EDTA (33.63%). However, the addition of EDTA into the reaction system did not show significant improvement on methanol production. This might be due to the use of EDTA as a chelating agent [25, 44].

According to the biosynthetic pathway of methanotrophs, the bioconversion of CH_4 to methanol is an NADH-dependent reaction. One mole of NADH is required to oxidize one mole of CH_4 into methanol. This process is related to electron transfer in the oxidation system. The use of reductase or MMOR in soluble MMO will react with NADH to release an electron, which is then transferred to hydroxylase to facilitate CH_4 hydroxylation and to produce methanol [18]. NADH is generated in the bioconversion of formaldehyde into formate, and the further bioconversion of formate into carbon dioxide by formaldehyde dehydrogenase and formate dehydrogenase, respectively. The addition of formate to the oxidation system of CH_4 into methanol is necessary as a co-substrate for NADH generation and for maintaining a high rate of bioconversion. In this study, various HCOONa concentrations ranging from 0 to 150 mM

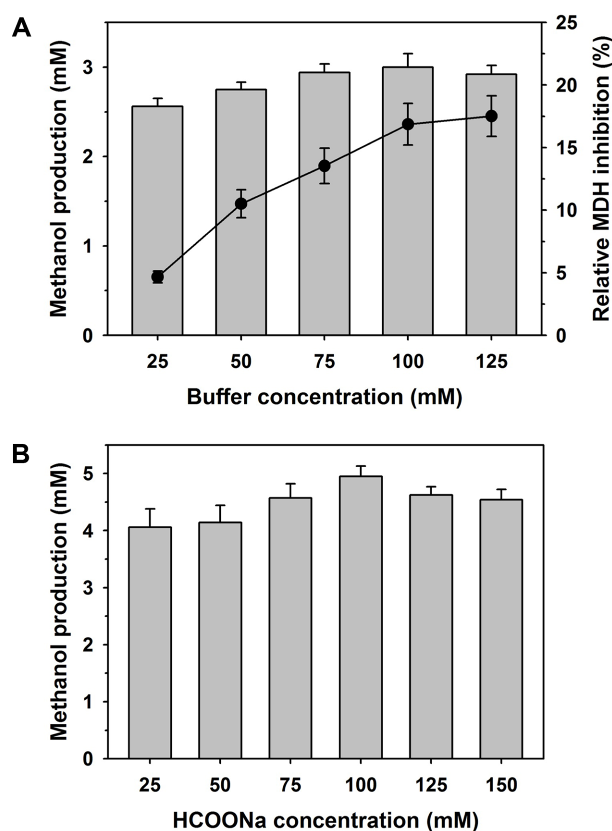

Fig. 2. Effects of buffer salts on methanol dehydrogenase activity.
(**A**) Effect of sodium phosphate buffer concentration on methanol production. Control: 20 mM phosphate buffer; the initial MDH activity was considered as 100% at 20 mM sodium phosphate buffer. (Symbols: gray bars, methanol production; line graph, relative MDH inhibition). (**B**) Effect of HCOONa on methanol production. The reaction was conducted in the presence of sodium phosphate buffer (pH 7.0). CH_4 as substrate was supplied at 50% (v/v). Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

Table 2. Effect of MDH inhibitors on methanol production.[a]

MDH inhibitors (mM)	Methanol (mM)	Relative MDH inhibition (%)
Magnesium chloride ($MgCl_2$)		
5	3.52 ± 0.13	27.04 ± 2.41
10	3.64 ± 0.22	27.97 ± 1.11
25	3.71 ± 0.18	28.50 ± 1.55
50	3.82 ± 0.18	29.35 ± 2.11
75	3.75 ± 0.26	28.8 ± 1.98
Sodium chloride (NaCl)		
10	3.29 ± 0.12	25.58 ± 1.98
25	3.38 ± 0.09	26.28 ± 1.23
50	3.66 ± 0.16	28.46 ± 1.55
75	3.53 ± 0.22	27.45 ± 1.11
100	3.42 ± 0.12	26.59 ± 2.41
Ammonium chloride (NH_4Cl)		
5	3.26 ± 0.12	25.55 ± 1.63
10	3.55 ± 0.26	27.82 ± 1.23
25	3.41 ± 0.09	26.72 ± 1.11
50	3.33 ± 0.09	26.09 ± 2.11
75	3.31 ± 0.12	25.94 ± 1.55
EDTA		
0.1	3.27 ± 0.09	29.69 ± 1.55
0.5	3.32 ± 0.12	32.17 ± 1.97
1.0	3.47 ± 0.22	33.63 ± 2.41
1.5	3.19 ± 0.12	28.92 ± 2.11

[a]The reaction mixture contained 5 μM of Cu, 10 μM of Fe, and 100 mM of phosphate buffer (pH 7.0).

were used to investigate its effect on methanol production using *M. tundrae*. Fig. 2B shows that the addition of sodium formate into the reaction system increased methanol production (~1.3-fold higher), and the highest methanol concentration (4.95 mM) was obtained at 100 mM of HCOONa. However, further increasing the HCOONa concentration did not have significant effects on methanol concentration. This phenomenon might be caused by methanol accumulation in the reaction system, which inhibits the activity of the enzyme [7, 15, 22, 32].

Effect of Cell Concentration on Methanol Production

The cell concentration also had an effect on methanol production. The amount of methanol produced in 24 h increased from 3.86 to 4.95 mM as the cell concentration was increased from 3 to 12 mg/ml (Fig. 3). A higher cell

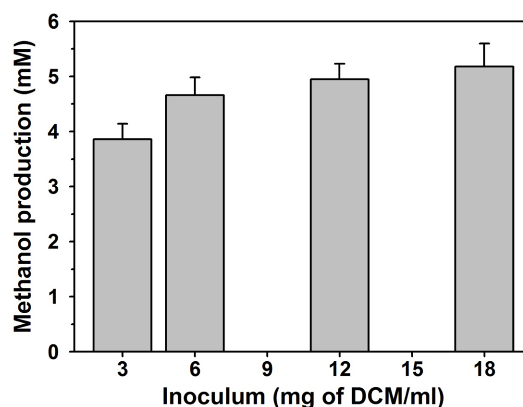

Fig. 3. Effect of cell concentration on methanol production.
The reaction was conducted in the presence of 100 mM of sodium formate, 100 mM of phosphate, 50 mM of $MgCl_2$, 5 μM of Cu, 10 μM of Fe, and 20 mM phosphate buffer as the reaction medium. CH_4 as substrate was supplied at 50% (v/v). Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

concentration did not further increase in methanol production, although the methanol concentration did increase from 4.95 to 5.18 mM after we increased the cell concentration to 18 mg/ml. High methanol production was reported at 17 g of DCM/l for *M. trichosporium* OB3b and higher concentrations of methanol could be accumulated using high cell densities in the presence of a higher phosphate concentration [5]. For achieving high methanol accumulation in the reaction system, the amount of cells in the reaction medium should be proportional to the phosphate concentration, which is related to the inhibition of MDH activity [42].

Whole-Cell Immobilization and Methanol Production

Very few reports are available on methanol production by immobilized methanotrophs. The use of covalently immobilized *M. trichosporium* NCIB 11131 on DEAE-cellulose and encapsulated cells of *M. sporium* B-2121 in poly(vinyl alcohol) cryogel has been shown to enhance the stability of methanol production efficiency compared with the use of free cells [24, 36]. In this study, the cell entrapment of *M. tundrae* in alginate beads was evaluated. The encapsulation of whole cells through alginate seems a promising approach to cell immobilization owing to its high efficiency and its biocompatible nature [6, 35]. Cells were entrapped in alginate beads to prevent the risk of cell damage and contamination.

Initially, the effects of different concentrations of alginate (1–4% (w/v)) on methanol production by encapsulated cells were evaluated. The 2% sodium alginate was optimum for

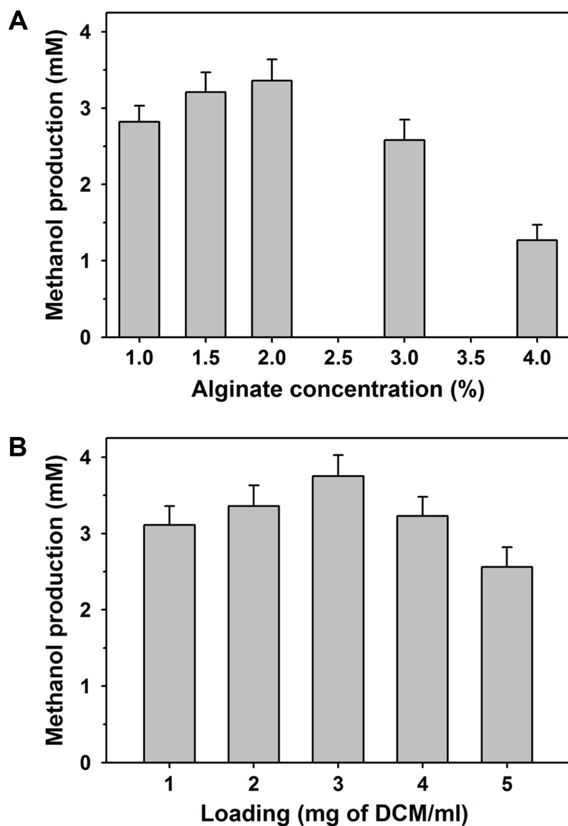

Fig. 4. Effect of (**A**) sodium alginate concentration and (**B**) cell loading on methanol production by immobilized whole cells.
The reaction was conducted in the presence of 100 mM of sodium formate, 100 mM of phosphate, 50 mM of $MgCl_2$, 5 µM of Cu, 10 µM of Fe, and 20 mM phosphate buffer as the reaction medium. CH_4 as substrate was supplied at 50% (v/v). Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

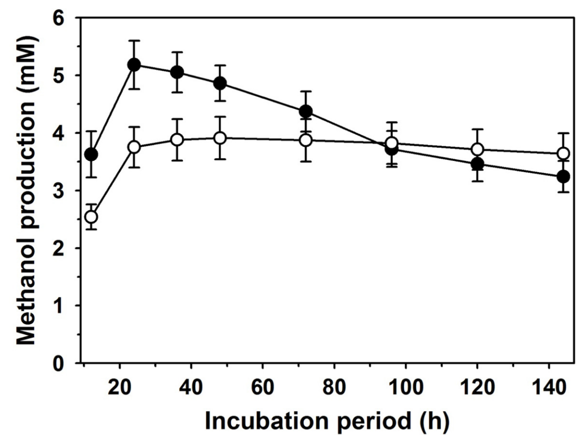

Fig. 5. Whole-cell immobilization of *M. tundrae* in alginate beads.
Methanol production of free (●) and immobilized cells (○). the reaction was conducted in the presence of 100 mM of sodium formate, 100 mM of phosphate, 50 mM of $MgCl_2$, 5 µM of Cu, 10 µM of Fe, and 20 mM phosphate buffer as the reaction medium. CH_4 as substrate was supplied at 50% (v/v). Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

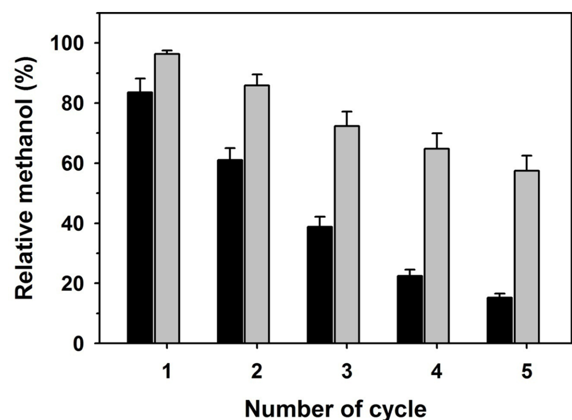

Fig. 6. Reusability of free (black bars) and immobilized cells (gray bars).
The relative production of methanol by the cells in the initial time was define as 100%. The reaction was conducted in the presence of 100 mM of sodium formate, 100 mM of phosphate, 50 mM of $MgCl_2$, 5 µM of Cu, 10 µM of Fe, and 20 mM phosphate buffer as the reaction medium. CH_4 as substrate was supplied at 50% (v/v). Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

a maximum methanol production of 3.36 mM at a cell loading of 2 mg DCM/ml (Fig. 4A). Here, a significantly lower methanol production of 1.27 mM at a 4% sodium alginate concentration was observed owing to the high rigidity of the prepared beads, as described previously [6]. Furthermore, the influence of the cell loading concentration on bead preparation at 2% sodium alginate was evaluated. As an increase in cell loading from 1 to 3 mg DCM/ml resulted in an increase in methanol production from 3.11 to 3.75 mM (Fig. 4B), a subsequent decrease in methanol production of 2.56 mM was observed at a cell loading of 5 mg DCM/ml. After the optimization of sodium alginate and cell loading concentrations, immobilized cells resulted in a maximum methanol production efficiency of 72.4% as compared with free cells (5.18 mM) at a CH_4 concentration of 50%. Immobilized *M. tundrae* cells resulted in stable methanol production up to an incubation time of 144 h in comparison with free cells, which resulted in a lower methanol production of 3.24 mM under similar conditions (Fig. 5). For the most cost-effective process, the reusability of the immobilized whole cells is an important aspect. The reusability of immobilized *M. tundrae* cells in alginate beads for methanol production was investigated. After five

cycles, the immobilized cells retained about 57.5% (2.16 mM) of their methanol production efficiency as compared with 15.2% (0.79 mM) by free cells (Fig. 6). Here, an approximately 3-fold higher methanol production was observed by immobilized cells compared with free cells, which suggests that the immobilization of *M. tundrae* is an effective method to improve the stability and reusability of cells.

In conclusion, *M. tundrae*, a type II methanotroph, has good potential as a biocatalyst in the production of methanol from CH_4 under normal conditions. The biocatalytic synthesis of methanol from CH_4 was performed using both free and immobilized cells. The optimization of the process parameters and MDH inhibitors improved methanol production from 0.66 to 5.18 mM for free cells. Furthermore, the immobilized cells demonstrated enhanced stability and reusability compared with free cells. Immobilized cells retained 57.5% of their methanol production efficiency after five cycles. The results show a promising use of technology for a low energy consumption process of methanol biosynthesis for industrial applications.

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