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Enhancement of methanol production from synthetic gas mixture by *Methylosinus sporium* through covalent immobilization

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HIGHLIGHTS

- CH_4 and CO_2 were efficiently used for methanol production by *Methylosinus sporium*.
- Covalent immobilization of *M. sporium* was more effective than adsorption.
- Methanol production was 6.7-fold enhanced by immobilization and optimization.

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ABSTRACT

Both methane (CH_4) and carbon dioxide (CO_2) are major greenhouse gases (GHGs); hence, effective processes are required for their conversion into useful products. CH_4 is used by a few groups of methanotrophs to produce methanol. However, to achieve economical and sustainable CH_4 reduction strategies, additional strains are needed that can exploit natural CH_4 feed stocks. In this study, we evaluated methanol production by *Methylosinus sporium* from CH_4 and synthetic gas. The optimum pH, temperature, incubation period, substrate, reaction volume to headspace ratio, and phosphate buffer concentration were determined to be 6.8, 30 °C, 24 h, 50% CH_4, 1:5, and 100 mM (with 20 mM $MgCl_2$ [a methanol dehydrogenase inhibitor]), respectively. Optimization of the production conditions and process parameters significantly improved methanol production from 0.86 mM to 5.80 mM. Covalent immobilization of *M. sporium* on Chitosan significantly improved the stability and reusability for up to 6 cycles of reuse under batch culture conditions. The immobilized cells utilized a synthetic gas mixture containing CH_4, CO_2, and hydrogen (at a ratio of 6:3:1) more efficiently than free cells, with a maximum methanol production of 6.12 mM. This is the first report of high methanol production by *M. sporium* covalently immobilized on a solid support from a synthetic gas mixture. Utilization of cost-effective feedstocks derived from natural resources will be an economical and environmentally friendly way to reduce the harmful effects of GHGs.

1. Introduction

The increasing concentration of greenhouse gases (GHGs) such as methane (CH_4) and carbon dioxide (CO_2) is a major global environmental concern. CH_4 is one of the most abundant natural gases, and it is an abundant energy source [1–6]; it has recently received more attention than CO_2 due to its significantly harmful environmental effects as a primary GHG. Thus, the direct conversion of CH_4 into less harmful, more useful products is a major challenge [7,8], and we need to explore the feasibility of processes that convert CH_4 into value-added products, such as methanol, to reduce the harmful GHG effects of CH_4 [8–10]. Methanol is a primary substrate for a broad range of chemical synthesis reactions, and it is used as a fuel in gasoline blends [8,11–14]. In addition to the low costs associated with methanol storage and transportation, it has a high energy density, which is about 400 times higher than that of CH_4. However, there are a number of problems associated with the chemical conversion of CH_4 to methanol, including high cost and low energy efficiency of the process. Compared to

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chemical methods, biological processes are more environmentally friendly and have the advantages of high conversion rates, selectivity, and low capital/energy costs [15–18]. Thus, efforts toward the development of methods for the biological conversion of CH_4 to methanol are recently gaining attention [9,18–20]. A special group of organisms, primarily known as methanotrophs, converts CH_4 to methanol [4]. Utilization of CH_4 as a carbon source for its assimilation into biomass occurs through a complex metabolic pathway with CO_2 as the end product. Initially, CH_4 is converted to methanol by methane monooxygenase (MMO), which is then metabolized to formaldehyde by methanol dehydrogenase (MDH). It is further metabolized to formate by formaldehyde dehydrogenase, and then finally to CO_2 by formate dehydrogenase [7], as shown below (Eq. (1)):

$$\text{Methane} \leftrightarrow \text{Methanol} \leftrightarrow \text{Formaldehyde} \leftrightarrow \text{Formate} \leftrightarrow \text{Carbon dioxide} \quad (1)$$

Methanotrophs belong to the Proteobacteria (α and γ classes) as well as a separate group known as the Verrucomicrobia, and these organisms are common in various environments, including soils, wetlands, sediments, fresh and marine waters, lakes, and peat bogs [8,21]. Methanotrophs encode MMOs, which are an oxidoreductases that oxidize diverse alkanes into primary alcohols, and are classified into three groups based on the type of MMOs they produce – (i) type I methanotrophs, which produce only particulate MMO (pMMO), (ii) type II methanotrophs, which produce both pMMO and soluble MMO (sMMO), and (iii) Type X methanotrophs, which produce MMOs with certain properties that are common in both Type I and II organisms [7]. pMMO is associated with the membrane-bound particulate enzyme system, whereas sMMO is located in a cytoplasmic complex. The expression of these enzymes is highly dependent on the copper (Cu) concentration in the growth medium. pMMO expression is dominant at higher Cu levels, and sMMO is dominant at lower Cu concentrations [22]. pMMO has a higher affinity for CH_4 oxidation than sMMO [23,24].

The bioconversion of pure CH_4 to methanol by various *Methylosinus trichosporium* strains, including IMV3011 [25], NCIB 11131 [26], and OB3b [19,27], has been widely studied. However, there are very few reports on methanol production by *Methylosinus sporium* strains that are focused on optimization of the process parameters and production conditions [20,28,29]. Here, the initial methanol production was quite low, with maximum yields in the range of 0.16–1.84 mM. Interestingly, methanol production is highly variable among different *M. sporium* strains, including B2119-B2123 (from CH_4) [28,29] and KCTC 22312 (from simulated gas mixture of CH_4 and CO_2) [20]. Thus, to improve methanol production from CH_4, the process parameters and production conditions need to be optimized. In this study, we evaluated the potential of using *M. sporium* to produce high levels of methanol from CH_4 through optimization of the growth conditions, production conditions, process parameters, and the concentration of MDH inhibitors. Furthermore, the stability and reusability was enhanced by covalent immobilization of the cells on different support materials. Overall, significant improvements were observed in methanol production from both pure CH_4 and synthetic gas mixture [CH_4 + CO_2 + hydrogen (H_2)]. These results suggest that *M. sporium* is a promising producer of methanol from both CH_4 and synthetic mixed gas similar to biogas.

2. Materials and methods

2.1. Organism and growth conditions

M. sporium DSMZ 17706 was obtained from the German Collection of Microorganisms and Cell Cultures and was grown on nitrate mineral salt (NMS) medium containing in (g L^{-1}) KH_2PO_4 (0.26), $Na_2HPO_4{\cdot}12H_2O$ (0.716), KNO_3 (1.0), $CaCl_2$ (0.20), $MgSO_4{\cdot}7H_2O$ (1.0), Fe-EDTA (0.38), and $Na_2MO_4{\cdot}2H_2O$ (0.026). Trace element solution (1 mL) was added to the medium containing (g L^{-1}) $ZnSO_4{\cdot}7H_2O$ (0.40), H_3BO_3 (0.015), $CoCl_2{\cdot}6H_2O$ (0.050), Na_2-EDTA (0.250), $MnCl_2{\cdot}4H_2O$ (0.020), and $NiCl_2{\cdot}6H_2O$ (0.010). The pH of the medium was adjusted to 7.0 using 1 M H_2SO_4 and 1 M NaOH. Millipore water (18 MΩ) was used in all the reagent preparations and for all measurements. All chemicals were of analytical grade and were purchased from Sigma–Aldrich or other commercial sources. The *M. sporium* strain was maintained by subculturing every 2 weeks, and was stored at 4 °C on NMS agar plates. The presence of contaminants was checked on R2 agar (Fluka, USA). Cells were cultivated in a 1-L flask (Duran-Schott, Germany) with an air-tight screw cap (Suba seal) containing 200 mL of NMS under an atmosphere containing 30% CH_4 and incubated at 30 °C on a rotary shaker (Lab Champion IS-971R, USA) at 200 rpm for 7 days. During cultivation, 30% CH_4 was added every other day. The effect of Cu^{2+} concentration (0–10 μM) on growth and sMMO activity (naphthalene oxidation) was evaluated under these same conditions. Cell growth was measured by determining the optical density (OD) at 595 nm with a UV/Vis spectrophotometer (JENWAY Scientific, UK). Fully grown cells were harvested by centrifugation (Gyrozen 1580 MGR, South Korea) at 10,000 rpm for 15 min at 4 °C, and then washed twice with phosphate buffer (20 mM, pH 7.0). Harvested cells were stored at 4 °C until use. The dry cell mass (DCM) was calculated after incubation for 48 h at 70 °C.

2.2. Methanol production

The effect of supplementation with two metal ions, Cu (1–10 μM) and Fe^{2+} (2.5–20 μM), on methanol production was studied in 20 mM phosphate buffer (pH 7.0). Batch culture experiments were performed in 120-mL serum bottles (Sigma–Aldrich, USA) containing 20 mL of medium and free cells at an initial density of 3 mg DCM mL^{-1}. A portion of the headspace air (30%) was replaced with CH_4 and the cultures were incubated at 30 °C with shaking (at 150 rpm) for 24 h.

2.2.1. Optimization of process parameters

Methanol production by *M. sporium* was evaluated at pH 5.5–8.0 in Na-acetate (pH 5.5) and phosphate (pH 6.0–8.0) buffers using 30% CH_4 as a feed with shaking at 150 rpm after incubation for 24 h. To evaluate the effect of incubation temperature on methanol production, cultures were incubated at different temperatures (25–40 °C) at optimum pH. The effect of different agitation rates (90–200 rpm) on methanol production was evaluated at optimum pH and temperature after 24 h of incubation.

2.2.2. Effect of MDH inhibitors and formate

First, different concentrations of phosphate buffer (10–120 mM, pH 6.8) were used to determine its effect on methanol production under optimum conditions. Then, to improve methanol production, different MDH inhibitors: (i) EDTA (0.1–1.5 mM), $MgCl_2$ (5–50 mM), NaCl (10–120 mM), and NH_4Cl (5–25 mM) were tested in phosphate buffer (100 mM, pH 6.8) using 30% CH_4 under optimum conditions after incubation for 24 h. In addition, a possible supportive role of formate (at 10–120 mM) for co-factor (NADH) regeneration during methanol production was evaluated.

2.2.3. Production profile

Methanol production was evaluated at different concentrations of CH_4 (10–50% head space) for up to 96 h of incubation under optimum conditions.

2.3. MDH activity and naphthalene oxidation

MDH activity was monitored spectrophotometrically as the change in absorbance at 600 nm induced by phenazine methosulfate-mediated reduction of 2,6-dichlorophenol-indophenol (DCPIP) as described previously [30]. The assay was performed in a 1-mL reaction volume containing $CaCl_2$ (10 mM), NH_4Cl (45 mM), phosphate buffer (0.3 M, pH 7.5), whole cell supernatant (5 mg DCM), DCPIP (0.13 μM), and phenazine methosulfate (3.3 μM). Naphthalene oxidation was used to evaluate relative sMMO activity using a modified assay as described previously [31]. First, naphthalene is oxidized to naphthol by sMMO. The presence of a purple-colored complex containing naphthol and tetrazotized *o*-dianisidine (Sigma–Aldrich, USA) was measured spectrophotometrically at 530 nm using an UV/Vis spectrophotometer. In brief, the assay was performed using a suspension containing 5 mg DCM of resting cells in phosphate buffer (50 mM, pH 7.0) containing sodium formate (2 mM). Then, a few naphthalene crystals were added, and the reaction was incubated at 30 °C with shaking at 180 rpm for 2 h. After incubation, 10 mg mL^{-1} tetrazotized *o*-dianisidine was added to observe the color changes.

2.4. Effect of the reaction volume to headspace ratio

The effect of the reaction volume to headspace ratio on methanol production was evaluated at 5 different ratios (1:1, 1:3, 1:5, 1:7, and 1:9). The experiment was performed under optimum conditions using 30% CH_4 as a feed. These ratios were achieved by varying the reaction volume from 12 mL to 60 mL in a 120-mL serum bottle.

2.5. Whole cell immobilization

2.5.1. Covalent immobilization

Amberlites (XAD-2, XAD-4 and XAD-7HP) and Duolite A-7 were purchased from Sigma–Aldrich (USA). Chitosan (90% deacetylated, crab shells) was purchased from Bio Basic Inc. (Canada). These different support materials were used for whole cell immobilization of *M. sporium* through adsorption and covalent methods. Each support (20 g) was washed two times with distilled water and then with phosphate buffer (50 mM, pH 7.0). These supports were functionalized with glutaraldehyde to add an aldehyde group to the particles as described previously [32]. The supports were separated by centrifugation at 4000 rpm for 30 min, and the residual glutaraldehyde was removed by washing three times with distilled water and then with phosphate buffer (20 mM). Whole cells were immobilized on the supports by loading 100 mg of DCM per g of support in phosphate buffer (50 mM, pH 7.0) with shaking at 100 rpm overnight at 4 °C. Unbound cells were separated by centrifugation at 4000 rpm for 20 min, and the immobilized cells were washed twice with phosphate buffer (20 mM, pH 7.0) containing $MgCl_2$ (5 mM) and stored at 4 °C until use. The immobilization yields (IYs) were calculated as follows (Eq. (2)):

$$\text{IY}\ (\%) = \text{amount of cells bound on the supports} / \text{total amount of cells loaded} \times 100 \quad (2)$$

2.5.2. Reusability

The reusability of both free and immobilized *M. sporium* was tested for methanol production under batch culture conditions for 24 h using 30% of CH_4 as a feed. After each cycle of methanol production, free and immobilized cells were separated by centrifugation and washed with phosphate buffer to use as inoculums in the next cycle. The methanol production efficiency was considered to be 100% in the initial (zero) cycle.

2.5.3. Scanning electron microscopy (SEM) of immobilized cells

The *M. sporium* cells covalently immobilized on Chitosan were transferred to Karnovsky's fixative solution for the primary fixation as described previously [33]. Then, the sample was dehydrated with graded ethanol (Duksan, South Korea; 30%, 50%, 70%, 80%, 90%, and 100%; for 10 min each at 4 °C), dried at room temperature for 12 h, and coated with Platinum. Images were analyzed using Field Emission SEM (JEOL, Japan).

2.6. Preparation of synthetic mixed gas and methanol production

A synthetic gas mixture was prepared by mixing pure CH_4, CO_2, and H_2 obtained from NK Co. Ltd. (Busan, South Korea) at a ratio of 6:3:1 and was used as the feed for methanol production up to 72 h by both free and covalently immobilized *M. sporium* cells under optimum conditions. Similar CH_4 content (30%) was maintained in the headspace during methanol production by dilution with air (1:1).

2.7. Analytical methods

The methanol concentration was analyzed via enzymatic oxidation by alcohol oxidase (Sigma–Aldrich, USA) instead of $KMnO_4$ as described previously [34]. The methanol concentration was analyzed using a gas chromatograph (GC; Agilent 7890A, USA) equipped with an HP-5 column (Agilent 19091J-413, USA) column and an FID detector. Helium was used as the carrier gas along with H_2 at a flow rate of 25 mL min^{-1} and air (300 mL min^{-1}). The oven temperature was initially maintained at 35 °C for 5 min, then raised to 150 °C at a rate of 5 °C min^{-1}, and then finally raised to 250 °C at a rate of 20 °C min^{-1}. Injector and detector temperatures were set at 220 °C and 250 °C, respectively.

3. Results and discussion

3.1. Methanol production

3.1.1. Effect of metals

First, the effects of Cu and Fe on methanol production were evaluated (Table A.1). Methanol production significantly improved from 0.86 mM to 1.14 mM as the Cu concentration was increased from 0 to 5 μM. Production decreased slightly (to 1.03 mM) at 10 μM Cu. Fe, at 10 μM, increased methanol production to 1.02 mM. Therefore, both metal ions affected methanol production, and these effects might be due to enhanced MMO activity [22]. Interestingly, a combination of Cu at 5 μM and Fe at 10 μM enhanced methanol production from 0.86 mM to 1.44 mM. Cu metal ions were previously shown to have a significant effect on the growth and MMO activity of *M. trichosporium* OB3b [22,27]. We also evaluated the effects of Cu on *M. sporium* growth and used it as inoculums for methanol production (Table 1). At 5 μM Cu, an increase in the specific growth rate, from 0.019 h^{-1} (at 0 μM Cu) to 0.021 h^{-1}, was observed, and at this Cu concentration, the methanol production yield increased to 2.52 mM. At 10 μM Cu, the specific growth rate decreased to 0.018 h^{-1}. The maximum specific growth rate of *M. sporium* at 5 μM Cu was lower than the value of 0.059 h^{-1} reported for *M. trichosporium* OB3b at the same concentration of CH_4 (30%) as a feed [27]. A similar effect of Cu concentration on the relative reduction in naphthalene oxidation was observed due to the predominance of pMMO [22].

3.1.2. Optimization of process parameters

The physiological properties of methanotrophs are strongly affected by various process parameters such as pH, temperature, and shaking during growth and methanol production. The effects

Table 1
Influence of Cu concentration on the growth of *M. sporium*.

Cu (μM)	Dry cell mass (mg mL^{-1})	Specific growth rate (h^{-1})	Naphthalene oxidation[a]	Methanol production (mM)
0	0.281 ± 0.021	0.019 ± 0.002	++++	1.44 ± 0.12
1	0.300 ± 0.023	0.020 ± 0.002	+++	1.65 ± 0.13
2.5	0.320 ± 0.025	0.020 ± 0.002	++	2.16 ± 0.17
5	0.358 ± 0.028	0.021 ± 0.002	+	2.52 ± 0.21
10	0.241 ± 0.020	0.018 ± 0.001	–	2.18 ± 0.19

[a] Intensity of purple color development: +ve (present) and −ve (absent).

of these conditions on methanol production by *M. trichosporium* strains have been well studied [26,35,36]. Here, we determined the optimum values of these parameters for methanol production from CH_4 by *M. sporium* (Fig. A.1). The effect of pH on methanol production is shown in Fig. A.1a. Maximum methanol production, 2.7 mM, was observed at pH 6.8. An decrease or increase in the pH significantly reduced methanol production. At pH 5.5 and 8.0, methanol production was reduced to approximately 12% and 28% of the maximum value, respectively. These results suggested that methanol production was optimal at pH values near neutral. Previous studies reported high methanol production at similar pH values for *Methylomonas* sp. Z201 [37], and at neutral pH for different strains of *M. sporium* (B2121 and KCTC 22312) [20,28]. In contrast, efficient methanol production was reported at significantly lower pH values of 6.3 and 6.4 for *M. trichosporium* strains OB3b and NCIB1113, respectively [26,27]. The optimum temperature and shaking conditions for methanol production by *M. sporium*, with a similar yield of 2.7 mM, were 30 °C and 150 rpm, respectively (Fig. A.1b and c). A temperature shift from 30 °C to 25 °C resulted in a slightly lower yield of 2.5 mM, whereas at 35 °C and 40 °C, the methanol yield was significantly reduced to 44% and 23% of the maximum, respectively. Likewise, very similar yields of 2.70 mM and 2.68 mM methanol were observed at shaking rates of 150 rpm and 180 rpm, respectively. At both lower (90) and (200) higher rpms, a decrease in methanol yield was observed. Lower optimum temperature of 28 °C was reported for methanol production by *M. sporium* strains B2120 in addition to a high shaking rate of 180 rpm [28,29]. These parameters are critical for maintaining high CH_4 and O_2 solubility and a specific ratio during methanol production. To produce 1 mole of methanol, one half mole of O_2 and 1 mole of CH_4 are required (Eq. (3)):

$$\text{Methane} + 0.5O_2 \rightarrow \text{Methanol} \quad (3)$$

3.1.3. Effect of MDH inhibitors on methanol production

First, the effect of phosphate buffer concentration on methanol production was evaluated (Fig. 1). An increase in phosphate buffer concentration from 20 mM to 100 mM resulted in an increase in methanol production from 2.70 mM to 3.66 mM. At a higher concentration (120 mM), a slight reduction in methanol production (3.48 mM) was observed. Previously, a significantly higher phosphate buffer concentration (500 mM) was reported for optimum methanol production by *M. trichosporium* OB3b [19]. Then, the effect of various inhibitors, including EDTA, $MgCl_2$, NaCl, and NH_4Cl, on methanol production was evaluated at the optimum buffer concentration (100 mM). The optimum concentration (mM) of these inhibitors were 1.0 for EDTA, 20 for $MgCl_2$, 80 for NaCl, and 15 for NH_4Cl (Fig. 2). In phosphate buffer, these inhibitors inhibited MDH activity by 38.7%, 32.5%, 30.2%, and 26.4%, respectively (Fig. 3a). In contrast, phosphate buffer (100 mM) alone only inhibited MDH activity by 17.6%. This inhibition is much higher than the 9.3% inhibition reported for *M. trichosporium* OB3b at the same concentration of phosphate [27]. However, 72.1% inhibition was

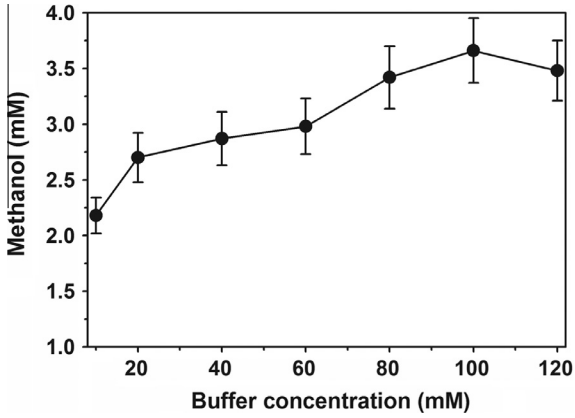

Fig. 1. Effect of phosphate buffer concentration on methanol production.

reported for *M. sporium* KCTC 22312 at a phosphate buffer concentration of 40 mM, with a maximum methanol production of 0.71 mM [20]. These inhibitors had mixed effects on methanol production at different concentrations, and after 24 h of incubation under optimal conditions, methanol production was increased to 4.20 mM from 3.66 mM. Among the inhibitors tested, the addition of $MgCl_2$ (20 mM) led to a maximum methanol production of 4.20 mM. Interestingly, greater inhibition (38.7%) of MDH activity in *M. sporium* induced in the presence of EDTA did not improve methanol production, which might be due to chelation of the metal ions present in the MMOs. A significantly higher methanol yield of 4.20 mM was observed than the maximum methanol production yield of 0.66 and 0.71 mM in presence of EDTA (50 μM) and buffer (40 mM) as a MDH inhibitors reported by *M. sporium* KCTC 22312, respectively [20]. Even, higher MDH inhibition up to 75.7%, did not significantly improve methanol production by *M. sporium* KCTC 22312.

3.1.4. Effect of formate

Formate had a positive effect on methanol production (Fig. 3b), and maximum methanol production (5.4 mM) was observed at 40 mM formate in presence of $MgCl_2$ (20 mM) and phosphate buffer (100 mM). Increasing the formate concentration to 120 mM did not significantly affect methanol production. Mehta et al. suggested that the methanol production rate is reduced over time due to the depletion of endogenous $NADH_2$ [26]. Thus, cofactor regeneration is necessary for maintaining a high-yield of bioconversion. Methanol biosynthesis by *M. trichosporium* OB3b has been evaluated at different Na-formate concentrations (10–80 mM) [19,27,36]. In these studies, a mixed response was observed with increased methanol production (1.2- to 4.7-fold higher). Here, we observed 1.3-fold higher methanol production at 40 mM Na-formate. The effect of Na-formate on methanol production by *M. sporium* was not reported previously.

3.1.5. Effect of the reaction volume to headspace ratio

Methanol production was evaluated at different ratios of reaction volume to headspace (1:1, 1:3, 1:5, 1:7, and 1:9) in a 120-mL serum bottle containing 30% CH_4 under optimized conditions. At different ratios, 3.63–5.40 mM methanol was produced (Table A.2), and the optimum reaction volume to headspace ratio was 1:5. Interestingly, higher ratios of 1:1 and 1:3 reduced methanol production by 33% and 23%, respectively. At lower ratios of 1:7 and 1:9, methanol production was consistent. These results suggested that a proper reaction volume to headspace ratio is necessary to achieve high methanol production. Methanol production

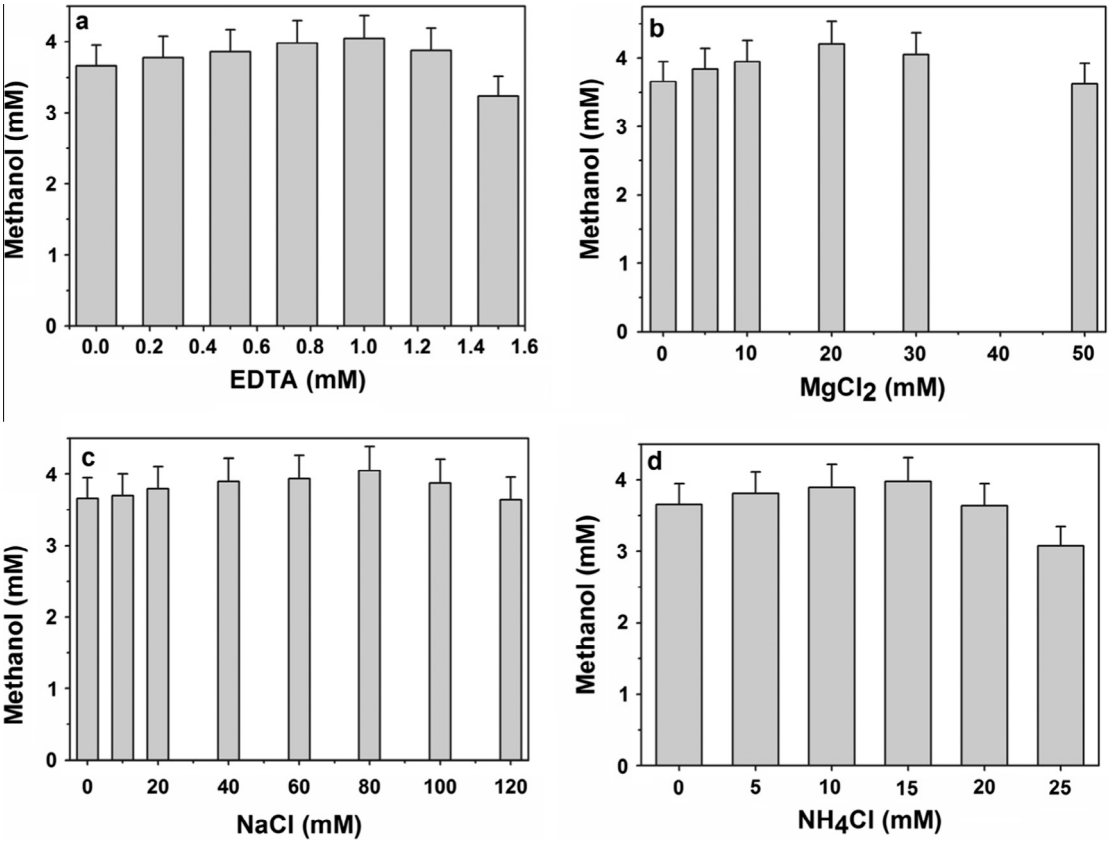

Fig. 2. Effect of MDH inhibitors in the phosphate buffer (100 mM) on methanol production: EDTA (a), $MgCl_2$ (b), NaCl (c), and NH_4Cl (d).

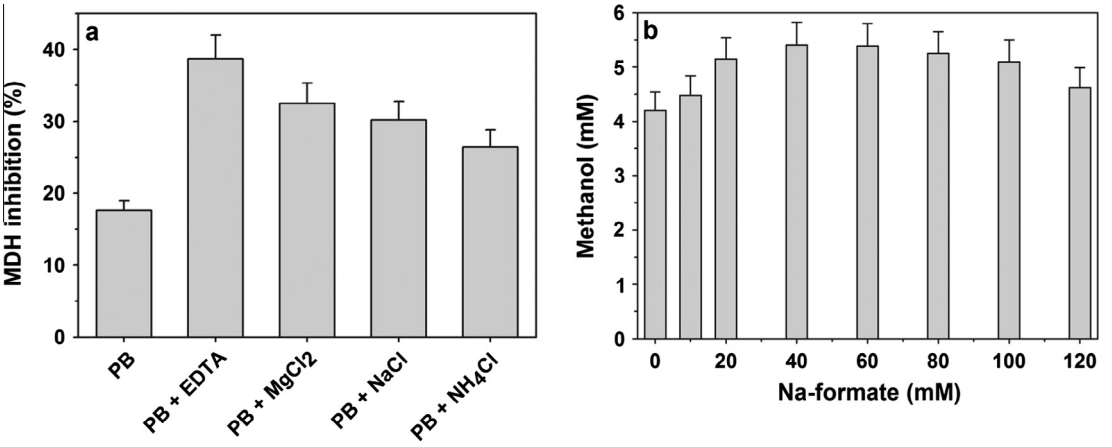

Fig. 3. Relative inhibition of MDH activity at optimum inhibitor concentration (a) and effect of formate concentration on the methanol production (b). Initial MDH activity was considered as 100% in at 20 mM phosphate buffer (PB).

by *M. trichosporium* OB3b was evaluated at ratios of 3:5 and 1:9 [38].

3.1.6. Methanol production profiles at different feed concentrations

At 10–50% CH_4, methanol production increased with increased incubation time (from 12 h to 24 h), and then significantly decreased at 96 h of incubation (Fig. 4). At 30% CH_4, methanol production significantly increased from 2.78 mM (at 10% CH_4) to 5.40 mM, and at higher concentrations of 40% and 50%, the yields were 5.74 mM and 5.80 mM, respectively, after incubation for 24 h. It was previously observed that, depending on the organisms and production conditions, increasing the CH_4 concentration generally increased methanol production [19,28,29]. In contrast, *M. sporium* KCTC 22312 was reported to produce significantly lower maximum yields CH_4 (0.60 mM) after 27 h of incubation [20]. This difference might be related to either further utilization of the produced methanol in subsequent metabolic pathways or feed inhibition [7,10,20].

3.2. Covalent immobilization of *M. sporium*

Previous experiments using *M. trichosporium* NCIB 111311 immobilized on DEAE cellulose suggested that covalent immobilization is more effective than adsorption [26]. Thus, covalent

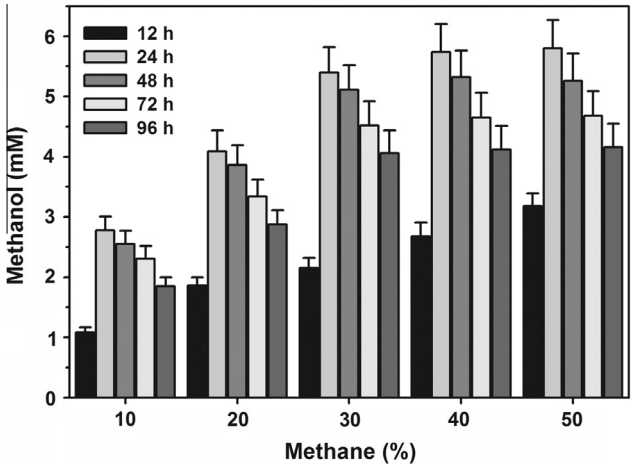

Fig. 4. Methanol production profile at different methane concentrations.

immobilization of *M. sporium* on different supports, including Amberlite XAD-2, XAD-4, and XAD-7HP as well as Duolite A-7 and Chitosan was evaluated (Table 2). The maximum IY of *M. sporium* was in the range of 17.4–33.5%. These cell-loading yields were much higher than those for adsorption under similar conditions, with IYs in the ranges of 7.15–13.4% (Table A.3). The methanol production efficiency of covalently immobilized cells was 64.9–85.4%. The production of adsorbed cells was significantly lower (40.7–69.3%) than that of free cells (100%). Among these supports, Chitosan-immobilized cells, through both adsorption and covalent immobilization, had the highest methanol production, with maximum cell loading of 13.4 and 33.5 mg of DCM g^{-1} of support, respectively. This efficient covalent immobilization of *M. sporium* on Chitosan was confirmed by SEM analysis (Fig. 5). The IYs and methanol production efficiencies of covalently immobilized *M. sporium* are much higher than those of *Methylomonas* sp. Z201 immobilized by adsorption on various activated carbon supports prepared from apricot shell, coconut shell, and coal [37]. Here, maximum cell loading and methanol production values of 8.5 mg of DCM g^{-1} of support and 67.6% were reported on pretreated activated carbon supports, respectively. In addition, *M. trichosporium* NCIB 111311 covalently immobilized on DEAE cellulose produced 0.40 and 2.10 μmol of methanol h^{-1} mg^{-1} compared to free cells, which produced 2.72 and 4.73 μmol of methanol h^{-1} mg^{-1} in 20 mM and 80 mM phosphate buffer under similar conditions [26]. Here, the methanol production efficiency of immobilized *M. trichosporium* NCIB 111311 was significantly lower, with values that were 14.7% and 44.4% of that produced by free cells, respectively. Covalent immobilization of *M. sporium* on different supports enhanced methanol production compared to that of free cells (Fig. A.2). Immobilized cells were stable on the supports for up to 96 h of incubation. Then, we evaluated the reusability of *M. sporium* immobilized on Chitosan using different immobilization methods (adsorption and covalently linked) under batch culture

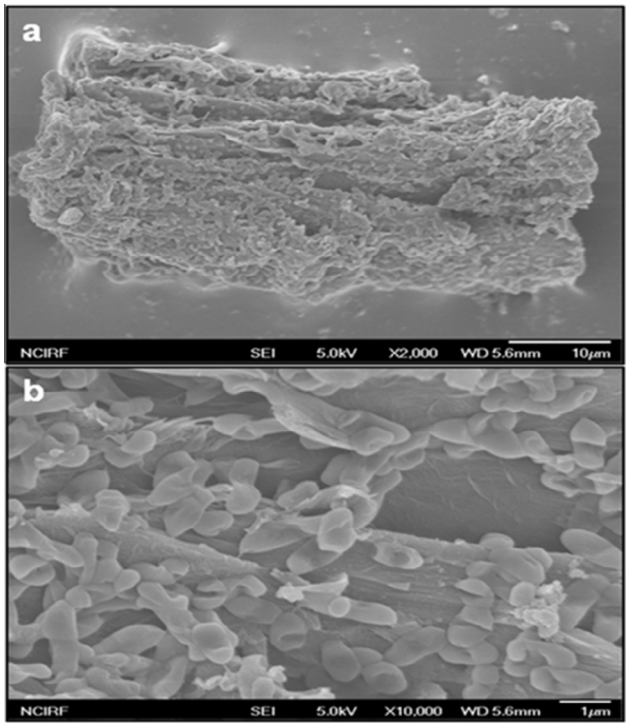

Fig. 5. SEM images of *M. sporium* covalently immobilized on Chitosan. (a) Single particle and (b) high resolution image.

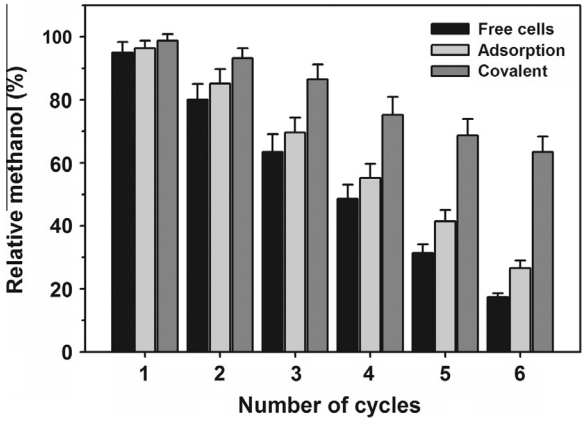

Fig. 6. Reusability of free and immobilized *M. sporium* cells. Initial methanol production was considered as 100%.

conditions (Fig. 6). After 6 cycles of reuse, *M. sporium* cells immobilized through adsorption and covalent methods retained 26.6% and 63.4% of their production efficiency, respectively. Under similar conditions, free cells yielded significantly lower methanol production rates of 17.3%. However, methanol production by a

Table 2
Methanol production by covalent immobilized *M. sporium*.

Support	Size (μm)	Surface area (m^2 g^{-1})	Immobilization yields (%)[a]	Relative methanol (%)[b]
Amberlite XAD-2	682	336	19.5 ± 1.4	70.8 ± 5.0
Amberlite XAD-4	564	725	17.4 ± 1.2	64.9 ± 4.7
Amberlite XAD-7HP	580	450	18.1 ± 1.3	73.5 ± 5.2
Duolite A7	600–800	36	27.2 ± 2.2	80.0 ± 5.5
Chitosan	45–165	180	33.5 ± 3.4	85.4 ± 4.6

[a] Cell loading was 100 mg of DCM g^{-1} of support.
[b] Free cells methanol production yields of 5.40 ± 0.42 mM was considered as a 100%.

Table 3
Comparison of methanol production by *Methylosinus sporium* strains.

Strain	Feed (%)	Immobilization		Mode	Temp. (°C)	pH	Yields (mM)	Reference
		Support	Method					
M. sporium B2119	CH_4 (22)	Polymer matrix	Encapsulation	Batch	28	7.0	1.68	[29]
M. sporium B2120							1.43	
M. sporium B2122							1.37	
M. sporium B2123							1.84	
M. sporium B2121		Free cells	–[a]				1.56	
		Polymer matrix	Encapsulation	Continuous			2.34	
		Free cells	–	Batch			0.35	[28]
		Polyvinyl alcohol	Encapsulation				1.94	
M. sporium KCTC 22312	CH_4:CO_2 (40)	Free cells	–		35	7.0	0.71	[20]
M. sporium DSMZ 17706	CH_4 (50)		–		30	6.8	5.80	This study
	CH_4:CO_2:H_2 (50)		–				5.80	
	CH_4 (30)	Chitosan	Covalent				4.61	
	CH_4:CO_2:H_2 (50)						6.12	

[a] Not applicable.

M. sporium strain B2119-23 encapsulated in polymeric matrix was reduced by more than 90% after three cycles of reuse [29]. These results suggested that covalent immobilization of *M. sporium* is a very effective approach for improved stability and reusability. This is the first report on covalent immobilization of *M. sporium*.

3.3. Methanol production from synthetic gas mixture

To evaluate the potential for the use of *M. sporium* for methanol production from a gas mixture similar to CH_4 anaerobic digester biogas, a synthetic gas mixture containing 60% CH_4, 30% CO_2, and 10% H_2 was prepared. Maximum methanol production by free and covalently immobilized *M. sporium* cells on Chitosan was 5.80 mM and 6.12 mM, respectively, using a synthetic gas mixture at a ratio of 1:1 with air under optimum conditions (Fig. 7). Methanol production from synthetic gas mixture by both free and immobilized *M. sporium* was more efficient than that from pure CH_4 (5.40 mM and 6.12 mM, respectively). Interestingly, methanol production by immobilized *M. sporium* (4.61 mM and 6.12 mM) was significantly higher than that produced by free cells (5.40 mM and 5.80 mM) after incubation for 24 and 48 h, respectively. Therefore, the addition of CO_2 and H_2 resulted in higher methanol production, as was described previously for *M. trichosporium* strains OB3b [35] and IMV3011 [25]. These methanol production yields by free and covalently immobilized *M. sporium* are 8.2- and 8.6-fold higher than the previously reported maximum yield of 0.71 mM by *M. sporium* KCTC 22312 from a simulated gas mixture ($CH_4 + CO_2$) [20]. Interestingly, lower methanol production (0.02 mM) was reported by *M. trichosporium* IMV3011 from a mixture of CH_4 and CO_2 [25]. Overall, covalent immobilization of *M. sporium* resulted in significantly higher methanol production from both pure CH_4 and synthetic gas mixture than the previous reports (Table 3).

The reduction in GHGs emission is a major global challenge due to the significant increase in their concentration through the anthropogenic activities [4,7,18,20]. In a previous study, 47% reduction in CH_4 emission (647 ton/year) from swine farms was achieved through the utilization of captured biogas in methanol production [39]. Therefore, high-yield conversion of CH_4 into methanol using methanotrophs seems to be a suitable approach for the reduction of GHGs [20]. Previously, 0.2 mM methanol was efficiently converted into formaldehyde using alcohol oxidase and catalase in 95% conversion rate [40]. Recent studies also suggested that methanol can be efficiently used as an economically feasible feedstock to produce formaldehyde, alternative fuels, higher alcohols, polymers, drugs, and vitamin precursors through construction of enzymatic cycles and genetic engineering of microorganisms [4,18,40–42]. Additionally, membrane bioreactor can significantly increase the accumulation of various metabolites [38,43]. Therefore, methanol production by methanotrophs using a membrane bioreactor can be a promising approach for further studies [44,45]. Our results should improve the understanding of methanol production from CH_4 in biological processes and set the stage for more practical investigations of methanotrophs as a biocatalyst for synthesis of economically feasible chemicals.

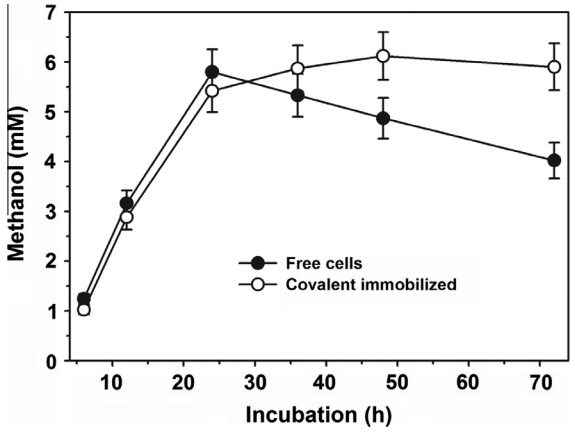

Fig. 7. Methanol production profile from synthetic gas mixture.

4. Conclusions

In this study, we evaluated biological production of methanol from both pure CH_4 and synthetic gas mixture ($CH_4 + CO_2 + H_2$) by free and immobilized *M. sporium* cells. Optimization of the process parameters and MDH inhibition improved methanol production by 6.7-fold. Covalent immobilization of the *M. sporium* cells on different support materials proved to be a very effective approach for improving stability during methanol production and provided significantly improved reusability compared to that previously reported encapsulation method. Utilization of a synthetic gas mixture as feed for methanol production was more efficient than utilization of pure CH_4. These promising results suggest that immobilized *M. sporium* can be used as a suitable candidate for methanol production from CH_4. Furthermore, the use of a natural mixed biogas or gas from anaerobic digesters will be cost effective for up-scaling methanol production and significant reduction of GHGs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apenergy.2016.03.022.

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