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Production of Methanol from Methane by Encapsulated *Methylosinus sporium* S

Sanjay K. S. Patel[1†], Jae-Hoon Jeong[1†], Sanjeet Mehariya[1], Sachin V. Otari[1], Bharat Madan[1], Jung Rim Haw[2], Jung-Kul Lee[1*], Liaoyuan Zhang[1,3*], and In-Won Kim[1*]

[1]*Department of Chemical Engineering, Konkuk University, Seoul 05029, Republic of Korea*
[2]*Department of Materials Chemistry and Engineering, Konkuk University, Seoul 05029, Republic of Korea*
[3]*Key Laboratory of Biopesticide and Chemical Biology, Ministry of Education, College of Life Sciences, Gutian Edible Fungi Research Institute, Fujian Agriculture and Forestry University, Fuzhou, Fujian Province, 350002, P.R. China*

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*Corresponding author
Phone: +82-2-450-3508;
Fax: +82-2-458-3504;
E-mail: inwonlam@naver.com, zliaoyuan@126.com

[†]These authors contributed equally to this work.

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Massive reserves of methane (CH_4) remain unexplored as a feedstock for the production of liquid fuels and chemicals, mainly because of the lack of economically suitable and sustainable strategies for selective oxidation of CH_4 to methanol. The present study demonstrates the bioconversion of CH_4 to methanol mediated by Type I methanotrophs, such as *Methylomicrobium album* and *Methylomicrobium alcaliphilum*. Furthermore, immobilization of a Type II methanotroph, *Methylosinus sporium*, was carried out using different encapsulation methods, employing sodium-alginate (Na-alginate) and silica gel. The encapsulated cells demonstrated higher stability for methanol production. The optimal pH, temperature, and agitation rate were determined to be pH 7.0, 30°C, and 175 rpm, respectively, using inoculum (1.5 mg of dry cell mass/ml) and 20% of CH_4 as a feed. Under these conditions, maximum methanol production (3.43 and 3.73 mM) by the encapsulated cells was recorded. Even after six cycles of reuse, the Na-alginate and silica gel encapsulated cells retained 61.8% and 51.6% of their initial efficiency for methanol production, respectively, in comparison with the efficiency of 11.5% observed in the case of free cells. These results suggest that encapsulation of methanotrophs is a promising approach to improve the stability of methanol production.

Keywords: Methanotroph, *Methylosinus sporium*, immobilization, methane bioconversion

Introduction

Anthropogenic activities have led to an exorbitant increase in greenhouse gas (GHG) emissions worldwide, which, in turn, has increased awareness about the utilization of alternative energy for sustainable development [5, 7, 10, 42, 44]. Several studies have demonstrated the potential of other energy sources, such as hydrogen (H_2), methane (CH_4), and alcohols, as promising alternatives to fossil fuels [3, 11, 12, 15, 23, 26, 27, 32, 34, 35, 38]. Among these, CH_4, a potent GHG associated with several environmental issues, is receiving major attention. One potential strategy for curbing CH_4 emissions is to transform the gas to methanol, which can then be used as fuel or raw material for the production of organic solvents [5, 7, 42]. Several efforts have been undertaken to increase the cost effectiveness and sustainability of the bioconversion process, attracting the attention of industries [4, 8, 9, 17, 28, 29, 33, 44, 45]. Methanotrophs, which utilize CH_4 as a major carbon source, play an essential role in the global carbon cycle by limiting the escape of this greenhouse gas into the atmosphere. These microorganisms inhabit soils, wetlands, sediments, fresh and marine waters, lakes, and peat bogs, where they act as biofilters, thus reducing CH_4 emissions into the atmosphere [2, 19]. Even though the CH_4 concentration in upland soils remains at atmospheric levels or below, the diverse range of methanotrophs present in such habitats is sufficiently active to reduce CH_4 and respond to the changes in temperature, soil moisture, and nitrogen availability [2, 14].

Methanotrophs possess the unique ability to utilize CH_4

as the carbon source and act by oxidizing the gas to methanol with methane monooxygenase (MMO), which is then converted to formaldehyde by the activity of methanol dehydrogenase (MDH). Following this, formaldehyde is converted to formic acid in a reaction catalyzed by formaldehyde dehydrogenase, followed by conversion of formic acid to CO_2 by formate dehydrogenase [5, 31]. MMO is a metalloenzyme belonging to the oxidoreductase class of enzymes, with the ability to oxidize alkanes to primary alcohols. Type I methanotrophs, including *Methylobacter*, *Methylomicrobium*, and *Methylocaldum*, produce only the particulate form of the enzyme (pMMO). However, Type II methanotrophs, including *Methylosinus*, *Methylocella* and *Methylocystis*, produce both soluble (sMMO) and particulate (pMMO) forms of MMO. The Type X methanotrophs possess certain properties common to both Type I and II methanotrophs [5, 29, 41].

Methylosinus sporium is an obligate, aerobic methanotroph, which primarily utilizes C1 carbon sources such as CH_4, carbon dioxide (CO_2), and methanol as growth substrates [28, 44]. *M. sporium* belongs to the Type II class of methanotrophs, and is known to produce both sMMO and pMMO [1, 44]. Whereas sMMO is synthesized in the cytoplasm and is expressed at low concentrations of copper in the medium, pMMO is membrane-bound and is expressed at higher copper concentrations [31, 43]. One of the distinctive features of *M. sporium*, compared with other *Methylosinus* species, is its ability to produce a water-soluble, brown-black-colored pigment [1]. The inhibitors of MDH, such as phosphate buffer, ammonium chloride, ethylenediaminetetraacetic acid, magnesium chloride ($MgCl_2$), and sodium chloride, offer significant advantages to improve methanol production [4, 8, 17, 28, 31, 40, 43]. Additionally, immobilization is widely used to improve the properties of cells [16–18, 20, 24, 25, 30, 37, 39]. In this study, the methanol production potential of immobilized *M. sporium* cells was evaluated, using different encapsulation methods, such as sodium-alginate (Na-alginate) and silica gel. The results obtained suggest that an appropriate encapsulation strategy may offer significant advantages for methanol production, such as improved cell stability and the potential for reuse of the immobilized cells.

Materials and Methods

Materials

The microbial strains *M. sporium* (DSMZ 17706), *Methylomicrobium (Mb.) album* (ATCC 33003), and *Mb. alcaliphilum* (DSMZ 19304) were procured from the German Collection of Microorganisms and Cell Cultures (DSMZ) and American Type Culture Collection (ATCC). The reagents alginic acid (sodium salt), pluronic (P-123) triblock polymer (poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)), polyethylene glycol, and tetraethylorthosilicate (TEOS) were purchased from Sigma-Aldrich Pvt. Ltd. (USA). All other chemicals used were procured from different commercial sources. High-purity gases were purchased from NK C. Ltd (South Korea).

Growth Conditions

Organisms were cultured in nitrate mineral salt (NMS) medium (composition (g/l): 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 (composition (g/l): $ZnSO_4 \cdot 7H_2O$ (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) (pH 7.0)). The cells were cultivated in 1 L flasks with air-tight screw caps, containing 200 ml of NMS with 20% CH_4. The flasks were incubated at 30°C on a rotary shaker (Lab Champion IS-971R, USA) at 200 rpm for up to 7 days [31]. During the process of cultivation, 20% CH_4 was added to the flasks every other day. 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), as described previously [13, 21, 22, 31, 36]. The dry cell mass (DCM) was calculated after incubation at 70°C for 48 h, and the specific growth rate (μ) of *M. sporium* was determined using the method described previously [31, 40]. The strains were maintained on NMS agar plates at 4°C, and were revived by subculturing after every 2 weeks. To check for possible contaminants, the R2 agar plate was used (Fluka, USA).

Methanol Production

Production of methanol in batch cultures was evaluated in 120 ml serum bottles (Sigma-Aldrich, USA) containing 20 ml of phosphate buffer (100 mM) with 10 µM of Fe(II) and 5 µM of Cu(II). Initial inoculum of 1.5 mg DCM/ml was added to the reaction mixture. Pure CH_4 (20%) was used as a feed and the cultures were incubated at 30°C, 150 rpm for 24 h [28, 31].

Effect of Inoculum Size

Different inoculum sizes, in the range of 0.5 to 12 mg of DCM/ml of the reaction mixture, were evaluated for methanol production, using the 20% CH_4 as the feed, followed by incubation at 30°C for 24 h with shaking at 150 rpm.

Whole Cell Encapsulation of *M. sporium*

Encapsulation of *M. sporium* was performed using Na-alginate and silica gel-based systems. For the Na-alginate bead entrapment method, whole cells were prepared using 2% (w/v) alginate and a loading concentration of 3 mg of DCM/ml was used, as described previously [6, 17]. Briefly, washed cells were mixed with Na-alginate solution, and the resulting mixture was extruded drop

wise using a syringe into 200 ml of 1.5 M $CaCl_2$ solution for the preparation of beads. Thereafter, the cells containing Na-alginate beads were washed with saline solution to remove any loosely bound cells. The silica gel-based encapsulation of whole cells was performed using synthetic precursor solution (20 ml), which is a mixture of TEOS/P-123/H_2O/ethanol/HCl/glycerol (molar ratio: 1/0.015/5.3/18.1/0.3/1.13; pH 5.0) and fresh culture (1 mg of DCM/ml, 40 ml), as described previously [20]. Briefly, fresh inoculum was mixed with the precursor solution, followed by incubation at 30°C for 2 h with continuous stirring. Thereafter, the encapsulated cells were washed with distilled water and buffer solution to remove any unbound cells. These encapsulated cells were then stored at 4°C.

Optimization of Process Parameters

Methanol production by encapsulated *M. sporium* was evaluated in the presence of MDH inhibitor (20 mM $MgCl_2$) and Na-formate (40 mM) at pH 6.4–7.5 in phosphate buffer (100 mM) using 20% CH_4 as feed, with incubation for 24 h at 150 rpm [31]. The ability of encapsulated *M. sporium* cells to produce methanol was evaluated at elevated temperatures (25°C to 40°C). For this purpose, cultures were incubated at different temperatures (25°C, 30°C, 35°C, and 40°C) for 24 h, with agitation at 150 rpm. Furthermore, the influence of agitation rates (100 to 200 rpm) on methanol production was evaluated at optimum pH and temperature after 24 h of incubation.

Reusability

The reusability of immobilized *M. sporium* cells for methanol production was tested under batch culture conditions for 24 h, using 20% of CH_4 as feed under optimum conditions. After each cycle of methanol production, free and immobilized cells were separated by centrifugation and washed with phosphate buffer, to be used as inoculum for the next cycle. The methanol production efficiency was taken as 100% in the initial (zero) cycle.

Analytical Methods

Cell growth was measured in terms of optical density at 595 nm using a UV/Vis spectrophotometer (Jenway Scientific, UK) [13, 36]. Methanol concentrations in liquid samples were analyzed using a gas chromatography (GC) system (Agilent 7890A, USA) equipped with an HP-5 column (Agilent 19091J-413, USA), connected to a FID detector. Helium was used as a carrier gas along with H_2, at a makeup flow of 25 ml/min and air (300 ml/min), as described previously [28, 31].

Results and Discussion

Screening of Methane-Oxidizing Bacteria

Type I methanotrophs, *Mb. album* and *Mb. alcaliphilum*, were evaluated for their methanol production potential, at an initial inoculum of 1.5 mg DCM using 20% CH_4 as feed (Table S1). These strains produced 0.043 and 0.022 mM methanol, respectively. The Type II methanotroph *M. sporium* exhibited enhanced methanol production (0.94 mM) under similar conditions. The methanol production observed here was considerably higher than the methanol production of 0.71 mM reported in *M. sporium* KCTC 22312 from a simulated biogas mixture of CH_4 and CO_2 [44].

Effect of Feed Concentration on Growth of *M. sporium*

The effect of CH_4 concentration on the growth of *M. sporium* was evaluated using CH_4 in the range of 10–50% as feed, followed by incubation for 7 days, under similar conditions as described previously [28, 31]. Initially, an increase in the specific growth rate (μ) of *M. sporium* from 0.017/h to 0.023/h was obtained with an increase in the CH_4 concentration from 10% to 20% in the head space (Table 1). This was followed by a decrease in μ to 0.18/h at 50% CH_4. Since the maximum specific growth of *M. sporium* (0.023/h) was obtained at 20% CH_4 and beyond, this concentration was used for the preparation of inoculum.

Effect of Inoculum Size on Methanol Production

The cell concentration significantly influences the methanol production by methanotrophs [8, 17, 28, 31, 39, 44]. The methanol production by *M. sporium* was evaluated using 20 mM $MgCl_2$ as a MDH inhibitor and 40 mM of sodium-formate in phosphate buffer (100 mM), pH 7.0 [28]. Methanol production increased with an increase in the inoculum size up to 24 h, followed by a decline (Fig. 1). An improvement in methanol production from 1.75 to 4.52 mM was observed with an increase in the concentration of cell inoculum from 0.5 to 12 mg of DCM/ml, using 20% CH_4 as a feed (Fig. 1). Similar trends have been reported in *M. sporium* using raw biogas and synthetic gas mixture (CH_4:CO_2:H_2) [28]. In this study, methanol production was found to be significantly higher than reported in earlier studies on *M. sporium* B2121, where an increase in yield from 0.22 to 1.94 mM was obtained upon increasing the inoculum size from 35 to 105 mg of cells/ml [37, 39]. In contrast, a lower inoculum of

Table 1. Effect of different CH_4 concentrations used as feed on the growth rate of *M. sporium*.

Feed (%)	Specific growth rate (h^{-1})
10	0.017 ± 0.001
20	0.023 ± 0.002
30	0.021 ± 0.002
40	0.020 ± 0.002
50	0.018 ± 0.001

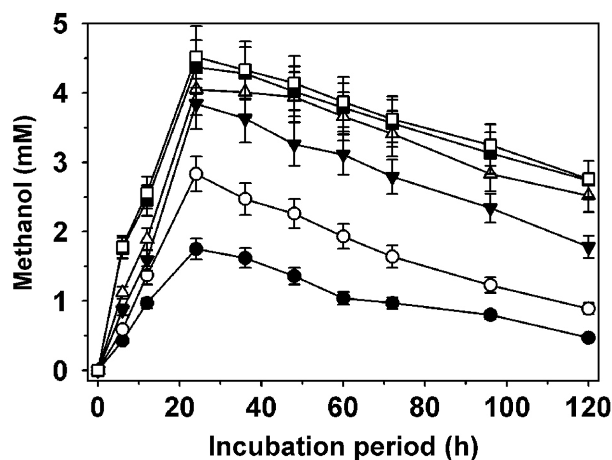

Fig. 1. Effect of inoculum size on methanol production.

The reaction was performed in 120 ml serum bottles with a working volume of 20 ml, using different inoculum sizes of *M. sporium* (mg of DCM/ml): ● (0.5), ○ (1.0), ▼ (1.5), △ (3.0), ■ (6.0), and □ (12). The reaction mixture comprised Cu (5 µM), Fe (10 µM), $MgCl_2$ (20 mM), and sodium-formate (40 mM) in phosphate buffer (100 mM, pH 6.8). The reaction was incubated at 30°C for 120 h with an agitation rate of 150 rpm using 20% CH_4 as feed. Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

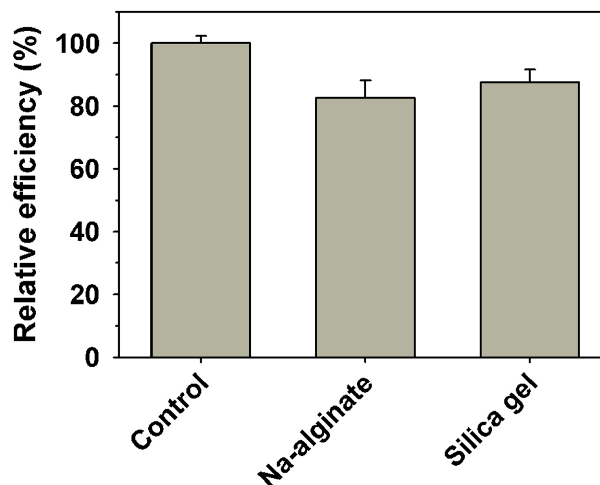

Fig. 2. Relative methanol production efficiency of encapsulated *M. sporium* cells.

Under batch culture conditions, methanol production was evaluated in phosphate buffer (100 mM, pH 6.8) containing Cu (5 µM), Fe (10 µM), $MgCl_2$ (20 mM), sodium-formate (40 mM), and inoculum (1.5 mg of DCM/ml) after incubation at 30°C for 24 h with an agitation rate of 150 rpm using 20% CH_4 as feed. Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

M. sporium KCTC 22312 (0.05 mg of cells/ml) resulted in higher methanol yields of up to 0.71 mM, using 50% synthetic simulated biogas (CH_4 and CO_2) as a feed [43].

Whole Cell Immobilization

Whole cell immobilization methods, such as encapsulation, aid in the improvement of stability of cells [6, 39]. Very few reports have described the immobilization of methanotrophs through encapsulation methods [17, 37, 39]. In the present study, the encapsulation of *M. sporium* through two different methods using Na-alginate and silica gel has been carried out. The methanol production efficiencies of Na-alginate- and silica gel-encapsulated cells were found to be 82.6% and 87.3% higher than that of the free cells (producing 3.84 mM methanol), respectively (Fig. 2). The *M. sporium* cells immobilized through silica gel and Na-alginate encapsulation methods are shown in Fig. 3. The methanol production obtained was remarkably higher than that reported in other encapsulated strains of *M. sporium* (B2119-B2123) and *M. trichosporium* (B2117 and B2118), which had methanol yields in the range of 1.37–1.97 mM [39]. A lower methanol production (1.94 mM) was reported in *M. sporium* B2121 encapsulated through polyvinyl alcohol [37]. Similarly, Na-alginate-immobilized *Methylocella tundrae* cells exhibited a lower methanol production efficiency of 72.4% using higher inoculum (3 mg of DCM/ml) and 50% CH_4 as feed [17].

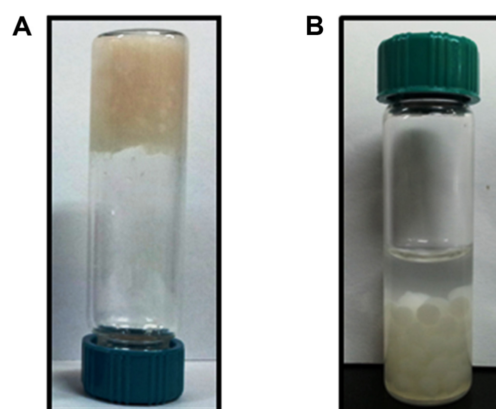

Fig. 3. Whole cell immobilization of *M. sporium* using (**A**) silica gel and (**B**) sodium-alginate beads.

Silica gel encapsulation of cells was performed using synthetic precursor solution (20 ml) comprising TEOS/P-123/H_2O/ethanol/HCl/glycerol (molar ratio: 1/0.015/5.3/18.1/0.3/1.13; pH: 5.0) and fresh culture (1 mg of DCM/ml, 40 ml). Similarly, 2% (w/v) of Na-alginate and a loading concentration of 3 mg of DCM/ml of *M. sporium* we used for the preparation of Na-alginate beads.

Effect of Physical Process Parameters

In methanotrophs, optimization of pH could stimulate higher cell growth and methanol production owing to modulation of the activity of MMO, which is inhibited at low and high pH values [8, 17, 18, 29]. To test this hypothesis, the effect of varying the pH and temperature on methanol production by free and encapsulated *M. sporium* was studied. At pH values of 6.4 and 7.5, low methanol production of 48.1% and 41.5% was recorded in free cells, respectively. The optimal pH for maximum methanol production (3.84 mM) was 6.8, with an incubation period of 24 h (Fig. 4A). Encapsulation of *M. sporium* through Na-alginate and silica gel resulted in relatively higher stability of the cells over the pH range 6.4–7.5, with the optimum pH being 7.0. Maximum methanol production of 3.35 and 3.62 mM was observed by the encapsulated cells. Besides the pH, the temperature also influenced the activity of MMOs, thus modulating methanol production by methanotrophs [17, 18, 29]. Methanol production increased with increasing the incubation temperatures from 25°C to 30°C, and a decrease was obtained beyond this temperature (Fig. 4B). Maximum methanol production by both free and encapsulated *M. sporium* cells was obtained at 30°C.

Methanotrophs differ in their CH_4 requirements. Besides this, a variation in agitation rate, which affects the extent of mixing in shake flasks, influences the nutrient availability [17, 29, 31]. The effect of varying the agitation rate on methanol production was investigated (Fig. 4C). Maximum methanol production by free and encapsulated cells through Na-alginate and silica gel methods were found to be 3.84, 3.43, and 3.73 mM, at agitation rates of 150, 175, and 175 rpm, respectively. Higher methanol production by encapsulated *M. sporium* cells at increased agitation rates could be attributed to limited diffusion. A decrease in methanol production by free cells was obtained upon increasing the agitation rate to 225 rpm. Overall, the encapsulated cells exhibited higher stability for the methanol production than the free cells.

Methanol Production Profile and Reusability

Encapsulation of *M. sporium* cells through Na-alginate and silica gel resulted in stable methanol production up to 120 h post incubation, as compared with the free cells (Fig. 5A). Under optimum conditions, methanol production after 120 h of incubation by free and encapsulated cells was 1.78, 2.65, and 2.86 mM, respectively. After six cycles of methanol production under batch culture conditions, the encapsulated *M. sporium* cells retained 61.8% and 51.6% of

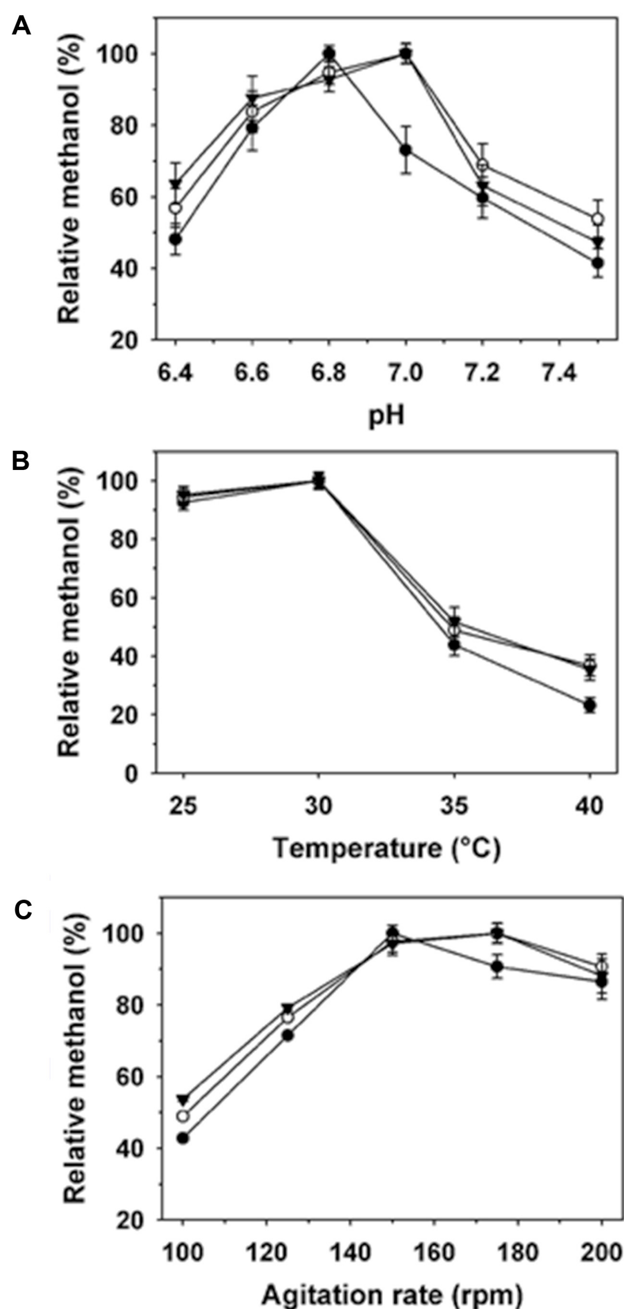

Fig. 4. Effect of the process parameters (**A**) pH, (**B**) temperature, and (**C**) agitation rate on methanol production by encapsulated cells.

Methanol production was evaluated in phosphate buffer (100 mM) at different pH values (6.4–7.5), containing Cu (5 μM), Fe (10 μM), $MgCl_2$ (20 mM), sodium-formate (40 mM) and inoculum (1.5 mg of DCM/ml), followed by incubation for 24 h using 20% CH_4 as a feed. Maximum methanol production by free (●) and encapsulated cells through alginate (○) and silica gel (▼) was taken as 100%. Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

Table 2. Comparison of methanol production by encapsulated *M. sporium*.

Strain	Support	CH_4 (%)	Cells inoculum (mg/ml)	Methanol (mM)	Reference
M. sporium B-2119	Polymer matrix	22	35	1.68	
M. sporium B-2120	Polymer matrix	22	35	1.43	
M. sporium B-2122	Polymer matrix	22	35	1.37	[39]
M. sporium B-2123	Polymer matrix	22	35	1.84	
M. sporium B-2121	Polymer matrix	22	35	2.34	
	Polyvinyl alcohol	22	70	1.94	[37]
M. sporium DSMZ 17706	Na-alginate	20	1.5	3.43	This study
	Silica gel	20	1.5	3.73	

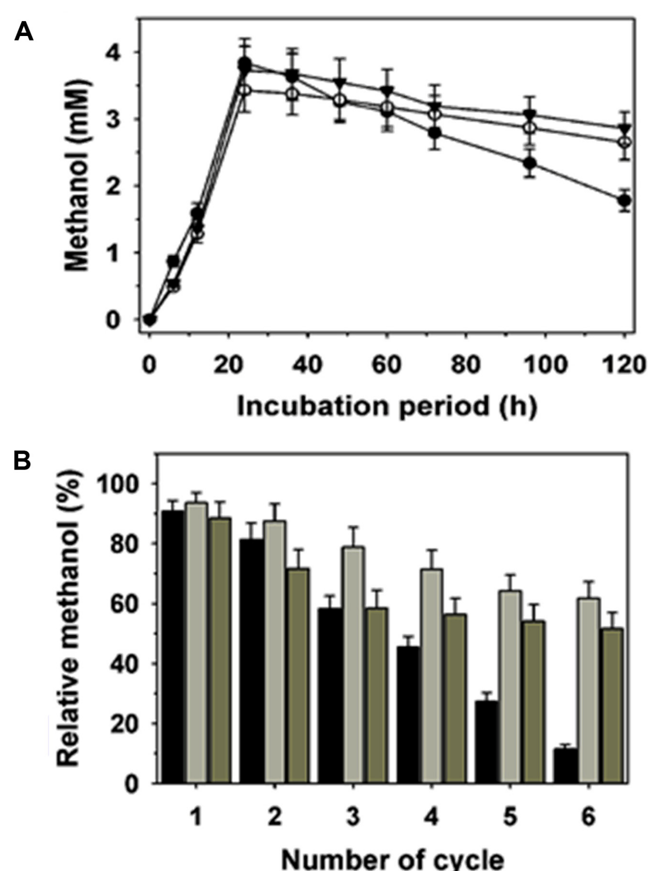

Fig. 5. Methanol production profiles (**A**) and reusability (**B**) of the encapsulated cells.

The methanol production profiles of free cells (●) and cells encapsulated through alginate (○) and silica gel (▼) were evaluated in phosphate buffer (100 mM) containing Cu (5 µM), Fe (10 µM), $MgCl_2$ (20 mM), sodium-formate (40 mM), and inoculum (1.5 mg of DCM/ml) after incubation for up to 120 h using 20% CH_4 as a feed, under optimum conditions. To assess the reusability, the production of methanol by free or encapsulated cells at the initial time was considered as 100% and relative efficiency was calculated after every cycle of reuse. Each value represents the mean of triplicate measurements that varied from the mean by no more than 10%.

their methanol production efficiency (Fig. 5B). However, the methanol production efficiency of free *M. sporium* cells reduced to 11.5%. Polymer matrix-encapsulated *M. sporium* (B2119-B2123) and *M. trichosporium* (B2117 and B2118) strains had significantly reduced (up to 95%) methanol production efficiency after three cycles of reuse [39]. Similarly, Na-alginate-encapsulated *M. tundrae* DSMZ 15673 displayed a lower efficiency of 57.5% after five cycles of reuse under batch conditions [17]. Overall, encapsulation of *M. sporium* cells through Na-alginate and silica gel resulted in 5.4- and 4.5-fold higher methanol production efficiency than the free cells after six cycles of reuse. A comparative analysis of methanol production by different encapsulated *M. sporium* strains is presented in Table 2.

In conclusion, methanotrophic bacteria are known to activate the strong C-H bond of CH_4 under ambient conditions. Therefore, these organisms have been used as eco-friendly alternatives to convert CH_4 to methanol. This study highlighted the effect of cell immobilization approaches on the methanol production stability of methanotrophs. Under optimum conditions, maximum methanol production of 3.43 and 3.73 mM was obtained by Na-alginate- and silica gel encapsulated *M. sporium* cells, respectively, in comparison with 3.84 mM methanol by free cells. After six cycles of reuse, the encapsulated cells retained their methanol production potential and exhibited 5.4- and 4.5-fold higher methanol production efficiency than the free cells. This is the first report on methanol production by silica gel-encapsulated methanotrophic bacteria, which also highlights the potential of metabolic engineering and modeling studies to improve the methanol production potential of methanotrophs.

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