Methanol production from simulated biogas mixtures by co-immobilized Methylomonas methanica and Methylocella tundrae

by Ahmad Saiful Haqqi

Submission date: 07-Apr-2022 06:43PM (UTC-0600)
Submission ID: 1804769088
File name: BioTech2018_co-immobilization.pdf (1.88M)
Word count: 7652
Character count: 40684

Bioresource Technology 263 (2018) 25–32

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Methanol production from simulated biogas mixtures by co-immobilized *Methylomonas methanica* and *Methylocella tundrae*

Sanjay K.S. Patel, Virendra Kumar, Primata Mardina, Jinglin Li, Rowina Lestari, Vipin C. Kalia, Jung-Kul Lee*

Department of Chemical Engineering, Konkuk University, Seoul 05029, Republic of Korea

ARTICLE INFO

Keywords:
Co-culture
Immobilization
Methanol
Methylocella tundrae
Methylomonas methanica

ABSTRACT

In the present study, co-cultures of the methanotrophs *Methylocella tundrae*, *Methyloferula stellata*, and *Methylomonas methanica* were evaluated for improving methanol production with their application. Among the different combinations, the co-culture of *M. tundrae* and *M. methanica* increased methanol production to 4.87 mM using methane (CH_4) as feed. When simulated biogas mixtures were used as feed, the maximum methanol production was improved to 8.66, 8.45, and 9.65 mM by free and encapsulated co-cultures in 2% alginate and silica-gel, respectively. Under repeated batch conditions, free and immobilized co-cultures using alginate and silica-gel resulted in high cumulative production, up to 24.43, 35.95, and 47.35 mM, using simulated biohythane (CH_4 and hydrogen), respectively. This is the first report of methanol production from defined free and immobilized co-cultures using simulated biogas mixtures as feed.

1. Introduction

The greenhouse gas (GHG) nature of methane (CH_4), and its continuous increasing global emissions (774 Tg $year^{-1}$) through anthropogenic as well as natural processes, has had a great negative influence on the environment (Strong et al., 2015). Therefore, the utilization of CH_4 as a promising feedstock to produce value-added products may reduce these effects. CH_4 (113 trillion m^3) reserves as natural fuel sources and it has an energy potential of 2.0×10^{15} kWh (Ge et al., 2014). Additionally, generation of CH_4 through anaerobic digestion (AD) of lignocellulosic biomass has been demonstrated (Liu et al., 2016). The global warming potential of CH_4 is very high and approximately 25-fold greater than that of carbon dioxide (CO_2). Thus, utilization CH_4 has been recommended to reduce its negative environmental effects. Methanotrophs can biotransform CH_4 into value-added bioproducts such as biopolymers, methanol, and lipids (Fei et al., 2014; Ishikawa et al., 2017; Strong et al., 2016; Su et al., 2017). Recent studies suggested that the conversion of GHGs into liquid fuels such as methanol by methanotrophic strains is a more effective than chemical methods for their reduction, because of the environmental friendly nature, high conversion rates, selectivity, and low capital/energy costs of this method (Barzgar et al., 2017; Hur et al., 2017; Patel et al., 2018a; Strong et al., 2015). Additionally, GHG conversion can be broadly applied to synthesize industrially important chemicals such as formaldehyde and higher alcohols (Ge et al., 2014; Lee et al., 2016; Whitaker et al., 2015). Methane monooxygenase (MMO) enzymes [particulate (pMMO) and soluble (sMMO) forms] are involved in the oxidation of CH_4 to methanol by methanotrophs. Subsequently, methanol is oxidized to formaldehyde and then to formate, via methanol dehydrogenase (MDH) and formaldehyde dehydrogenase, respectively. Finally, CO_2 is produced through the oxidation of formate by formate dehydrogenase (Lawton and Rosenzweig, 2016; Li et al., 2018). sMMO requires the cofactor nicotinamide adenine dinucleotide (NADH) to oxidize CH_4 into methanol, whereas pMMO catalyzes NADH-independent oxidation of CH_4. Generally, lower methanol accumulation has been observed in methanotrophs because of its further oxidation by MDH (Han et al., 2013; Yoo et al., 2015). Therefore, to enhance methanol production, various MDH inhibitors including ammonium chloride, cyclopropanol, ethylenediaminetetraacetic acid, magnesium chloride ($MgCl_2$), phosphate buffer, and sodium chloride have been used (Ge et al., 2014; Han et al., 2013; Sheets et al., 2016). Because the production of methanol by sMMO is highly dependent on the effective

Abbreviations: AD, anaerobic digestion; CH_4, methane; CO_2, carbon dioxide; DCM, dry cell mass; DCPIP, 2,6-dichlorophenol-indophenol; FTIR, Fourier transform infrared; GC, gas chromatography; GHG, greenhouse gas; FE-SEM, field emission scanning electron microscopy; MDH, methanol dehydrogenase; NADH, nicotinamide adenine dinucleotide; NMS, nitrate mineral salt; pMMO, particulate methane monooxygenase; rpm, revolution per minute; sMMO, soluble methane monooxygenase; µM, micromolar; mg, milligram; mL, milliliter; mM, millimolar

* Corresponding author at: Department of Chemical Engineering, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 05029, Republic of Korea.
E-mail address: jkrhee@konkuk.ac.kr (J.-K. Lee).

https://doi.org/10.1016/j.biortech.2018.04.096
Received 10 March 2018; Received in revised form 21 April 2018; Accepted 23 April 2018
Available online 25 April 2018
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regeneration of NADH, partial inhibition of NADH and supplementation of formate has been suggested to increase methanol production (Ge et al., 2014).

Previously, the conversion of CH_4 into methanol using a methanotrophic consortium, including *Methylosinus sporium* NCIMB 11126, *Methylosinus trichosporium* OB3b, and *Methylococcus capsulatus* Bath, as a mixed culture inoculum developed by enriching landfill cover soil samples, was adopted to improve methanol production (Han et al., 2013). Similarly, a thermotolerant methanotrophic consortium of mixed culture was developed for methanol production through enrichment of the digestate in the AD system (Su et al., 2017). Here, the syntrophic behavior of strains resulted in high methanol production. Additionally, the use of pure culture methanotrophs is vulnerable to contamination by other organisms, has narrow ranges of physical stability, or is prone to inefficient utilization of raw feed as biogas mixtures contain inhibitory gases, which may lead to process failure during large-scale production. Therefore, the use of a defined methanotroph consortium, selective methanotroph co-culture, or association with another type of organism as an inoculum may improve process efficiency through better utilization of biogas, increase production, and reduce process variability, compared with the results achieved using an undefined methanotrophic consortium (Han et al., 2013; Hill et al., 2017; Su et al., 2017). The immobilizations strategies have been well demonstrated to improve the properties of biocatalysts (Jiang et al., 2016; Ling et al., 2016; Zhuang et al., 2017). The use of immobilized cells has also been suggested as an effective approach for enhancing the biotransformation efficiency because of their higher stability than free cells, including methanotrophs (Mehta et al., 1991; Patel et al., 2015; Senko et al., 2007; Sheets et al., 2017; Sun et al., 2018). However, no studies have examined methanol production from GHGs using immobilized, defined mixed culture or co-culture. In this study, the enhancement of methanol production using co-cultures of the methanotrophic strains *Methylocella tundrae*, *Methyloferula stellata*, and *Methylomonas methanica* was evaluated. Immobilization of co-culture by encapsulation using two different polymeric matrixes of alginate and silica-gel improved methanol production stability using simulated biogas (CH_4 and CO_2) and biohythane [CH_4 and hydrogen (H_2)] mixtures as a feed. Further, effective methanol production under repeated batch conditions was verified. The results showed that co-culture of *M. methanica* and *M. tundrae* produced more methanol than pure cultures and other combinations, i.e., co-cultures of two and three strains. These results suggest that immobilization of co-culture is a valuable approach for improving methanol production from simulated biogas and biohythane under repeated batch conditions. This is the first report of using immobilized, defined co-cultures of methanotrophs for methanol production using simulated biogas and biohythane as a feed.

2. Materials and methods

2.1. Materials

Methanotrophic strains *M. tundrae* (DSMZ 15673), *M. stellata* (DSM 22108), and *M. methanica* (DSM 25384) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Pure CH_4, CO_2, and H_2 were purchased from NK Co. (Busan, Republic of Korea). Municipal waste treatment anaerobic digester (Seoul, South Korea) raw biogas procured from Phygen Co. Ltd. Glycerol, pluronic (P-123) tri-block polymer [poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)], poly-ethyleneglycol, sodium-alginate, and tetraethylorthosilicate (TEOS) were procured from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Culture conditions and preparation of co-cultures

Strains were cultured in nitrate mineral salts medium, as reported previously (Patel et al., 2016a,b,c). These strains were grown in 1-L Erlenmeyer flasks (200 mL working volume) containing 20% of CH_4 feed and incubated for 5 days under shaking (150 rpm) at 30 °C. Cell growth was monitored, and cells were harvested by centrifugation as described previously (Mardina et al., 2016). The co-cultures of two (three sets) and three (one set) strain combinations were prepared by mixing individual strains in equal proportions, obtaining a final dry cell mass (DCM) concentration of 3.0 mg mL^{-1} reaction mixture.

2.3. Methanol production

Initially, the methanol production conditions under batch conditions were optimized for *M. stellata* and *M. methanica* using different concentrations of phosphate (20–120 mM, pH 7.0), $MgCl_2$ (5–60 mM), and formate (20–120 mM) with Fe^{2+} (10 μM), Cu^{2+} (5 μM), and 3.0 mg DCM mL^{-1} of cells as the inoculum at 30 °C and under 150 rpm shaking (Mardina et al., 2016; Patel et al., 2016d). The final reaction volume of 20 mL was prepared using pure or co-culture in serum bottles (120 mL) and CH_4 (30%) was used as feed, with replacement of an equal volume of headspace air as described previously (Patel et al., 2016d).

2.3.1. Effect of inoculum and feed concentration

The influence of the ratio of the strains (*M. tundrae*: *M. methanica*) at 1:3, 1:2, 1:1, 2:1, and 3:1 in the co-culture as inoculums with a fixed final inoculum of 3.0 mg DCM mL^{-1}, on methanol production using 30% CH_4 as feed, was examined after incubation for 24 h. Further, the effects of CH_4 concentration (10–50%) on methanol production during co-culture of *M. tundrae* and *M. methanica* incubated for up to 96 h were evaluated.

2.4. MDH and MMO activity

MDH activity was measured by phenazine methosulfate-mediated reduction of 2,6-dichlorophenol-indophenol (DCPIP) at a wavelength of 600 nm, as described previously (Patel et al., 2016d). Briefly, the 1mL reaction assay was evaluated using $CaCl_2$ (10 mM), NH_4Cl (45 mM), phosphate buffer (0.3 M, pH 7.5), cell supernatant (5.0 mg DCM), DCPIP (0.13 μM), and phenazine methosulfate (3.3 μM). Similarly, naphthalene oxidation was performed to evaluate sMMO activity using a 2mL reaction mixture containing 0.9 mL of naphthalene saturated solution, 1 mL of cell suspension (5.0 mg DCM), and 0.1 mL of 0.2% (w v^{-1}) of tetrazotized *o*-dianisidine at 530 nm, as described previously (Han et al., 2013).

2.5. Whole cell encapsulation

Co-immobilization of *M. methanica* and *M. tundrae* was performed by encapsulation of different sodium-alginate concentrations (1.0–3.0%) in cells loaded with 1.0 and 2.0 mg DCM mL^{-1} mixture, respectively, as reported previously (Mardina et al., 2016). Further, loosely bound cells from the Na-alginate beads were removed by washing twice with saline solution. The encapsulation of co-cultures through silica gel was accomplished using 20 mL of precursor solution (mixture of TEOS/P-123/H_2O/ethanol/HCl/glycerol in a molar ratio of 1.0:0.015:5.3:18.1:0.3:1.13, pH 5.0) and 40 mL of cells (3.0 mg DCM mL^{-1}, 40 mL), as described previously (Niu et al., 2013). Thereafter, loosely bound cells were separated by washing twice with distilled water followed by washing with buffer solution. These immobilized cells were stored at 4 °C.

2.6. Methanol production by immobilized co-cultures

The methanol production profile of free and immobilized co-cultures was assessed using 30% CH_4 with cell inoculums of 3.0 mg DCM mL^{-1} for 96 h with shaking at 150 rpm.

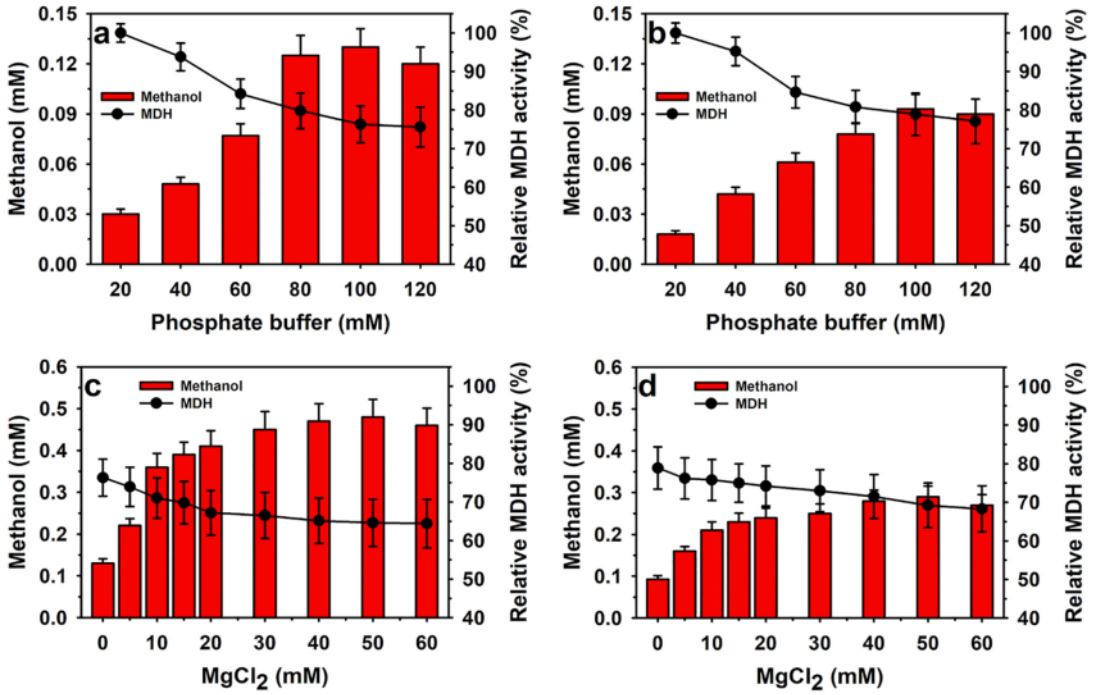

Fig. 1. Effect of MDH inhibitors [phosphate buffer (a, b), and $MgCl_2$ in 100 mM phosphate buffer (c, d)] concentrations on methanol production by *M. methanica* (a and c) and *M. stellata* (b and d) using CH_4 (30%) as feed.

2.6.1. Effect of feed composition on methanol production

To evaluate the effect feed composition on methanol production, different gas mixtures were prepared as a simulated biogas [CH_4 (30%) and CO_2 (15%)] in a ratio of 2:1 (v v^{-1}), and biohythane [CH_4 (30%) and H_2 (7.5%)] in a ratio of 4:1 (v v^{-1}) and used as feed for cell inoculums of 3.0 mg DCM mL^{-1}, which were incubated for 96 h with shaking at 150 rpm. The conversion yield (%) of gas mixtures to methanol was calculated by dividing the moles of methanol produced by the moles of CH_4 consumed in the feed.

2.6.2. Scanning electron microscopy (SEM) analysis

The immobilized co-culture cells through silica-gel and alginate beads were dried at 25 °C for 24 h. The analysis was performed by field emission SEM (FE-SEM, JEOL, Tokyo, Japan) after coating with platinum (Patel et al., 2016d).

2.6.3. Fourier transform infrared (FTIR) analysis

Infrared spectra of the free cells, silica-gel, and encapsulated cells dried samples were analyzed using FTIR (JASCO, FTIR 300E spectrometer, Japan) with a scan range of 2000–600 cm^{-1} (Patel et al., 2016e, 2017a).

2.7. Methanol production under repeated batch conditions

Repeated batch culture methanol production by free and immobilized co-cultures with an inoculum of 3.0 mg DCM mL^{-1} was assessed using simulated biogas [CH_4 (30%) and CO_2 (15%)] in a 2:1 (v v^{-1}) ratio and simulated biohythane [CH_4 (30%) and H_2 (7.5%)] in a 4:1 (v v^{-1}) ratio for eight cycles of reuse (Patel et al., 2017b). After each cycle (24 h), free and immobilized cells were collected by centrifugation and further used as an inoculum for the subsequent cycle (Patel et al., 2016d).

2.8. Analytical methods

The gas (CH_4, CO_2, and H_2) composition was analyzed by gas chromatography (GC, Agilent 7890A; Agilent Technologies, Santa Clara, CA, USA) equipped with a Carboxen 1010 fused silica capillary column (Supelco, Bellefonte, PA, USA) and thermal conductivity detector, as reported previously (Patel et al., 2016b, 2018a). The methanol concentration was investigated with a GC system equipped with an HP-5 column (Agilent 19091J-413) and flame ionization detector as described previously. Statistical significance was analyzed by analysis of variance ($\alpha = 0.05$) using GraphPad Prism 5 software (GraphPad, Inc., Inc., La Jolla, CA, USA) (Patel et al., 2018b). All the methanol synthesis reactions were performed in serum bottles (120 mL) with the working volume of 20 mL, using free or immobilized cells at 30 °C mechanically agitated at 150 rpm. All experiments were performed in triplicate and data are presented as the mean values ± standard deviations.

3. Results and discussion

3.1. Co-culture and methanol production

Pure cultures of methanotrophs, *M. sporium*, *M. trichosporium*, *M. tundrae*, *Methylomonas* sp., and *Methylocaldum* sp., have been widely studied for methanol production (Hur et al., 2017; Mardina et al., 2016; Senko et al., 2007; Sheets et al., 2017). The use of microbial co-culture or consortia has been adopted to improve the effectiveness of biotransformation processes, including biofuel production, because of its high productivity and stability (Hill et al., 2017, Patel et al., 2014; Su et al., 2017). A few reports are available on a landfill soil-enriched consortium (including, *M. sporium* NCIMB 11126, *M. trichosporium* OB3b, and *M. capsulatus* Bath) and thermotolerant methanotrophs consortia enriched from digestate in an AD system as a mixed culture to

produce methanol (Han et al., 2013; Su et al., 2017). These studies suggested that the use of undefined methanotrophic mixed culture has a variable influence on methanol production because of variations in their microbial composition and inoculum source. However, the preparation of selective methanotroph-defined mixed culture may be effective for use in methanol production to overcome the lower production potential and operational stability of individual strains. Additionally, the addition of MDH inhibitors and formate has been suggested to be essential for methanol accumulation by methanotrophs to reduce MDH activity and as an alternative to generating the reducing equivalent (NADH), respectively (AlSayad et al., 2018; Han et al., 2013). To prepare effective co-cultures of *M. methanica* and *M. stellata* also containing *M. tundrae*, methanol production conditions were optimized using different concentrations of phosphate, $MgCl_2$ in 100 mM phosphate buffer, and formate at pH 7.0 (Fig. 1). Phosphate buffer as an individual MDH inhibitor at an optimum concentration of 100 mM resulted in methanol production of 0.13 and 0.09 mM by *M. methanica* and *M. stellata*, respectively. Remarkably, the combined influence of phosphate buffer (100 mM) and $MgCl_2$ (50 mM) as MDH inhibitors showed significantly improved methanol production, of 0.48 and 0.29 mM, respectively. The higher methanol production was associated with higher MDH activity inhibition, of 35.4% and 30.8%, compared with that achieved by individual phosphate buffer with values of 23.7% and 21.1%, respectively (Duan et al., 2011). Formate supplementation (100 mM) enhanced methanol production by 8.0- and 9.1-fold, to 3.86 and 2.64 mM for *M. methanica* and *M. stellata*, respectively. Overall, the optimum concentrations of phosphate, $MgCl_2$, and formate were 100, 50, and 100 mM to achieve the maximum methanol production by *M. methanica* and *M. stellata*. The time profile of methanol production over incubation for up to 96 h of *M. methanica*, *M. stellata*, and *M. tundrae* is presented in Fig. 2. Initially, methanol production increased for up to 24 h of incubation with the maximum values of 3.86, 2.65, and 3.57 mM, for *M. methanica*, *M. stellata*, and *M. tundrae*, respectively. Longer incubation for up to 96 h resulted in lower methanol production of 3.09, 1.87, and 2.79 mM, for *M. methanica*, *M. stellata*, and *M. tundrae*, respectively.

Co-cultures were prepared by mixing pure cultures in an equal ratio with a final concentration of 3.0 mg DCM mL^{-1}. The combinations of two and three strains in the co-culture resulted in 3.21–4.69 mM methanol production (Supplementary information). Among these combinations, the co-cultures of *M. methanica* and *M. tundrae* exhibited the maximum methanol production, of 4.69 mM, which was 21.5% and 31.4% higher than that obtained from their pure cultures. In contrast, the *M. stellata* and *M. tundrae* co-culture showed the lowest methanol production, of 3.21 mM. These results suggest that synchronization of

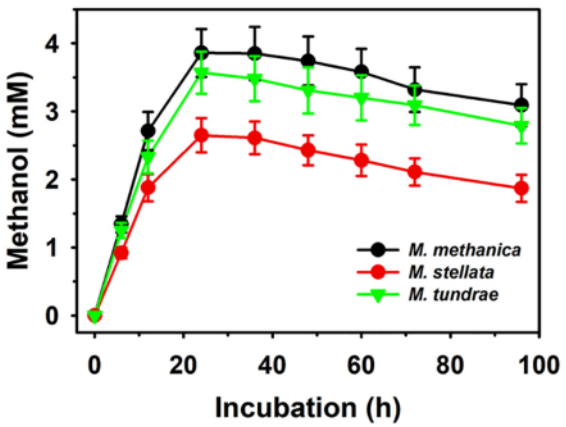

Fig. 2. Pure cultures methanol production profile using CH_4 (30%) as feed.

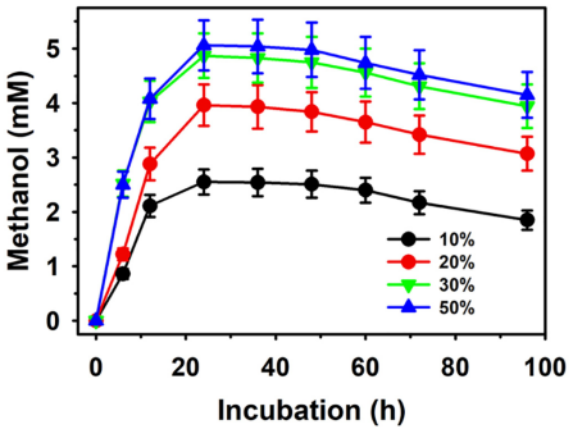

Fig. 3. Methanol production profile of the 2:1 co-culture ratio of *M. methanica* and *M.* using different CH_4 concentrations as feed.

M. methanica with *M. tundrae* is more productive than their individual performance and the other combinations with *M. stellata*.

To determine a suitable ratio of *M. methanica* and *M. tundrae* as an inoculum of co-culture for effective methanol production, the ratios 1:3, 1:2, 1:1, 2:1, and 3:1 were evaluated (Supplementary information). These ratios resulted in methanol production of 3.78–4.87 mM. The maximum methanol production of 4.87 mM was observed at a ratio of 2:1 (*M. methanica*: *M. tundrae*). In contrast, at a higher ratio of 3:1 (*M. methanica*: *M. tundrae*), lower methanol production was observed. These results suggest better compatibility between *M. methanica* and *M. tundrae* at a ratio of 2:1 for improved methanol production. Further, the influence of various feed contents of CH_4 (10–50%) on methanol production during co-culture was evaluated (Fig. 3). Methanol production significantly increased from 2.55 to 4.87 mM as CH_4 content increased from 10% to 30%. Further, an increased CH_4 content to 50% slightly increased methanol production to 5.06 mM. In contrast, *M. sporium* KCTC 22312 was reported to produce only 0.72 mM methanol (Yoo et al., 2015). Low production may be associated with either further methanol utilization in subsequent metabolic pathways because of less MDH inhibition or a high feed concentration (Fei et al., 2014; Yoo et al., 2015).

3.2. Encapsulation of co-culture

Immobilized methanotrophs have been widely studied for methanol production because of their higher stability than the free cells (Mehta et al., 1991, Patel et al., 2016d; Yu et al., 1998). However, no studies have examined immobilized co-cultures for methanol production from GHGs. Previously, covalent and encapsulation methods were used to improve methanol production from CH_4 using pure cultures (Senko et al., 2007; Mehta et al., 1991). In this study, immobilization of co-cultures was evaluated by entrapping cells in alginate beads and silica-gel. Initially, different concentrations of alginate (1–3%, w v^{-1}) were used containing total cells loaded with 3.0 mg DCM mL^{-1} in a *M. methanica* and *M. tundrae* ratio of 2:1 (Supplementary information). The methanol production efficiency of encapsulated co-cultures was in the range of 68.0–86.7%. The optimum alginate concentration of 2% (w v^{-1}) showed the maximum methanol production 4.22 mM. However, further increasing the alginate concentration to 3% resulted in lower methanol production 3.31 mM. This lower methanol production may be associated with mass-transfer limitations or the high rigidity of alginate beads (Mardina et al., 2016). Photographs of immobilized co-culture with alginate are presented in the Supplementary information. Immobilization was further confirmed by cross-sectional FE-SEM

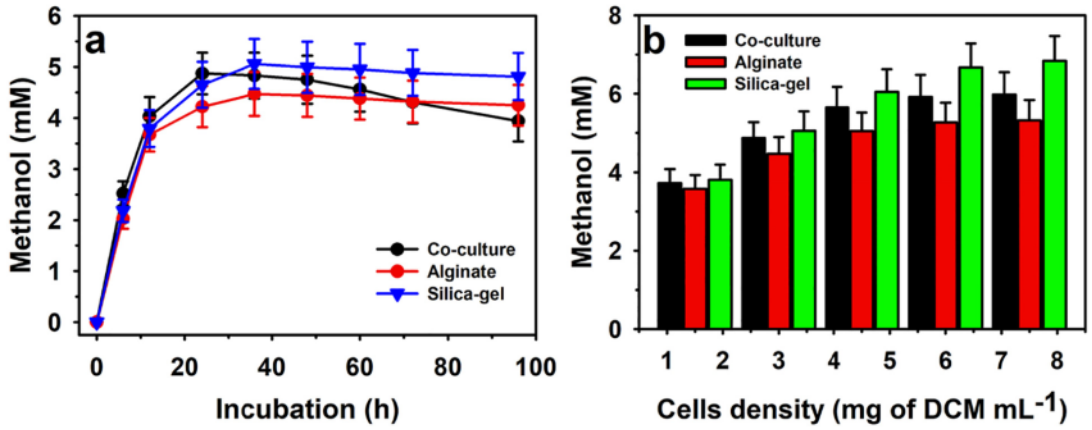

Fig. 4. Methanol production profile of free and encapsulated co-cultures (a), and effect of cell density (b) using CH_4 (30%) as feed.

analysis of the alginate beads (Supplementary information). In contrast, silica-gel based encapsulated co-culture showed higher methanol production efficiency, of 95.4%, with methanol production of 4.65 mM, compared with the alginate-encapsulated cell methanol production of 4.22 mM (86.7%) (Supplementary information). The encapsulated co-cultures with alginate and silica-gel exhibited residual MMO activities of 88.5 and 98.2%, respectively (Supplementary information). Here, the better MMO activity retention within silica-gel suggested that silica-gel is more biocompatible than alginate. The immobilization of co-culture through silica-gel was confirmed by FE-SEM analysis (Supplementary information). Further, the FTIR peaks of Si–O–Si stretching at 1095, 950, and 800 cm^{-1} for pure silica-gel and strong peaks for amide I (1650 cm^{-1}) and amide II (1530 cm^{-1}) bands associated with the secondary structures of intracellular proteins confirmed the immobilization of co-culture within the silica-gel (Niu et al., 2013).

The methanol production profiles of encapsulated cells through alginate and silica gel are presented in Fig. 4a. After incubation for 36 h, encapsulation of co-cultured cells resulted in higher maximum methanol production compared with the production from free co-culture cells. Initially, both co-cultured cells encapsulated by alginate and silica gel showed similar trends of increased methanol production for up to 36 h of incubation, with the maximum productions of 4.47 and 5.06 mM, respectively. Further, increasing the incubation time to 96 h resulted in slightly lower methanol production, of 4.25 and 4.81 mM, respectively. Both encapsulated co-culture cells exhibited higher methanol production stability than the free cells. Here, methanol production by alginate and silica gel immobilized co-cultures was significantly higher than those (1.37–1.97 mM) of previously reported immobilized pure cultures of *M. sporium* and *M. trichosporium* strains encapsulated in a polymeric matrix (Senko et al., 2007). Similarly, 1.94 mM of methanol production was observed with polyvinyl alcohol-encapsulated *M. sporium* B2121 (Razumovsky et al., 2008). In contrast, alginate-encapsulated pure cultures of *M. tundrae* and *M. sporium* resulted in methanol production of 3.75 and 3.17 mM, respectively (Mardina et al., 2016; Patel et al., 2016a).

3.2.1. Effect of co-culture cells density

During biotransformation of CH_4 to methanol, the cell density of methanotrophs as an inoculum had a variable influence on methanol production (Duan et al., 2011; Senko et al., 2007). Therefore, the potential of free and encapsulated co-cultures in alginate and silica-gel on methanol production was evaluated using CH_4 (30%) as feed at optimum incubation times of 24, 36, and 36 h, respectively (Fig. 4b). Initially, the co-culture exhibited increased methanol production, from 3.72 to 5.92 mM, with an increase in cell density from 0.75 to 6.0 mg of DCM mL^{-1}. Thereafter, production of methanol was stabilized at a higher cell density of 7.5 mg of DCM mL^{-1} with a maximum production of 5.98 mM. A similar trend was observed for methanol production by encapsulated co-cultures through alginate and silica-gel, with a maximum methanol production of 5.32 and 6.84 mM, respectively, and cell density of 7.5 mg of DCM mL^{-1}. Interestingly, silica-gel encapsulated co-culture exhibited 28.6% higher methanol production than immobilized co-culture through alginate under similar conditions. This higher methanol production may be associated with better compatibility or operational stability of the co-culture within silica-gel over alginate (Niu et al., 2013). Overall, these encapsulated co-culture cells resulted in significantly higher methanol production than that previously reported for pure cultures of *M. sporium* and *M. trichosporium* strains encapsulated in a polymeric matrix in the range of 1.37–1.97 mM, with up to 9.3-fold higher cell density (70 mg DCM mL^{-1}) (Senko et al., 2007). Similarly, polyvinyl alcohol-encapsulated *M. sporium* B2121 showed the maximum methanol production of 1.94 mM at a cell density of 105 mg DCM mL^{-1} (Razumovsky et al., 2008).

3.2.2. Methanol production from simulated biogas mixture

Previous studies suggested that simulated biogas (CH_4 and CO_2) is a more effective feed than CH_4 for methanol production by pure cultures methanotrophs (Patel et al., 2016b; Yoo et al., 2015). However, methanol production from simulated biogas using co-culture has not been demonstrated. Therefore, in this study, the methanol production potential of free and immobilized co-cultures from simulated biogas was evaluated. The methanol production profile of free and encapsulated co-cultures through alginate and silica-gel using a simulated biogas as a feed and inoculum of 3.0 mg of DCM mL^{-1} is presented over an incubation period of 96 h in Fig. 5a. Initially, simulated biogas containing CH_4 and CO_2 gas mixture at a ratio of 2:1 (v v^{-1}) was prepared at a fixed concentration of CH_4 (30%) and used for methanol production. Free and encapsulated cells through alginate and silica-gel showed maximum methanol production of 5.20, 5.16, and 6.04 mM after incubation for 48 h, respectively. These results suggest that simulated biogas is an effective feed because co-culture cells produce higher quantities of methanol when fed this biogas mixture, compared with when they were fed pure CH_4, which resulted in lower methanol production of 4.87, 4.47, and 5.06 mM using free, alginate encapsulated, and silica-gel encapsulated cells, respectively. Here, free and encapsulated co-cultures, those immobilized within alginate, and those immobilized within silica-gel exhibited maximum methanol productivity of 0.52, 0.37, and 0.43 mmol L^{-1} h^{-1}, while those obtained using pure CH_4 were 0.42, 0.34, and 0.36 mmol L^{-1} h^{-1},

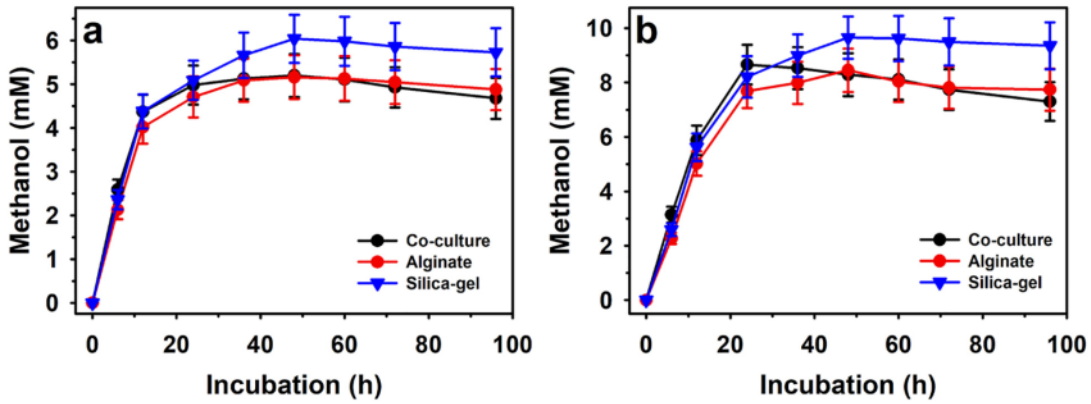

Fig. 5. Methanol production profile of free and encapsulated co-cultures of *M. methanica* and *M. tundrae* using simulated biogas [CH_4 and CO_2, 2:1 (v v^{-1})] (a) and simulated biohythane [CH_4 and H_2, 4:1 (v v^{-1})] (b) as feed.

respectively. Enhanced (6.8–19.4%) methanol production was observed using simulated biogas as a feed as opposed to using pure CH_4. The conversion yields of simulated biogas to methanol by free and encapsulated co-cultures, those immobilized within alginate and those immobilized within silica-gel were 56.8, 59.7, and 62.4%, while those obtained using pure CH_4 were 50.2, 52.6, and 54.8%, respectively. The high conversion yield of simulated gas to methanol may be associated with differential responses to the feed by free and encapsulated co-cultures or higher stability of methanol production in the presence of CO_2, as described previously (Patel et al., 2018a; Xin et al., 2004). In contrast, *M. trichosporium* IMV 3011 showed very low methanol production, of 0.02 mM, cultured using a biogas mixture of CO_2 (40%) and CH_4 (30%) (Xin et al., 2004). Similarly, a pure culture of *M. sporium* KCTC 22312 showed methanol production of 0.71 mM, upon being cultured using a synthetic biogas mixture of CH_4 and CO_2 at a ratio of 1:1 (v v^{-1}) (Yoo et al., 2015).

3.2.3. Methanol production from biohythane

Biogas (~$2.6 per 1000 ft^3) appears to be a more viable feed for effective methanol production than the costly pure CH_4 (~$300 per 1000 ft^3) (Zhang et al., 2016). Biohythane is a biogas that is produced mostly by mixed cultures from lingo-cellulosic biowaste through the AD process, and contains CH_4 and H_2 in a range of 60–70% and 10–15% (v/v) of the total evolved gases, respectively, thereby promising to be a more viable alternative feed than pure CH_4 for producing methanol (Mountfort et al., 1990; Patel et al., 2017a). Therefore, a similar composition of simulated biohythane containing CH_4 and H_2 in a ratio of 4:1 (v v^{-1}) was prepared and used as a feed for methanol production by free and immobilized co-cultures. The methanol production profile of free and encapsulated co-cultures through alginate and silica-gel using a simulated biohythane as a feed is presented over an incubation period of 96 h in Fig. 5b. H_2 showed positive effects on methanol production by free and encapsulated co-cultures through alginate and silica-gel. Maximum methanol production levels of 8.66, 8.45, and 9.65 mM were observed after incubation for 24, 48, and 48 h, respectively. Here, free and encapsulated co-cultures, those immobilized within alginate, and those immobilized within silica-gel exhibited maximum methanol productivity of 0.43, 0.35, and 0.39 mmol L^{-1} h^{-1}, respectively. Enhancement of 77.8%, 81.9%, and 90.7% in methanol production was observed using simulated biohythane as feed compared with that obtained using pure CH_4 (which showed lower methanol production, of 4.87, 4.47, and 5.06 mM, respectively). The conversion yields of simulated biohythane to methanol by free and encapsulated co-cultures, those immobilized within alginate, and those immobilized within silica-gel were 61.6, 62.5, and 66.1%, respectively. Higher methanol production using simulated biohythane may be associated with the positive role of H_2 as an electron source for a pyridine nucleotide-linked hydrogenase reaction (Mountfort et al., 1990). Overall, methanol production by co-culture was higher than those previously reported using pure methanotrophs, including *M. trichosporium* OB3b and *M. tundrae* DSM 21852 from simulated biogas (Mountfort et al., 1990; Patel et al., 2017a).

3.2.4. Methanol production from raw biogas

Biogas (CH_4 and CO_2) produced through the AD process contains hydrogen sulfide (H_2S), which negatively affects CH_4 oxidation or methanol production (Patel et al., 2016b; Zhang et al., 2016). Therefore, raw biogas comprising CH_4 (63.4%), CO_2 (35.6%), and H_2S (0.13%) was used as a raw material for methanol production, using a free and immobilized co-culture. A free and immobilized co-culture, co-cultures immobilized within alginate, and those immobilized within silica-gel resulted in the production of 4.56, 4.73, and 5.68 mM methanol, respectively. Here, inhibition of methanol production by 14.0, 9.1, and 6.3% was observed compared with the maximum methanol production of 5.20, 5.16, and 6.04 mM, respectively, using simulated biogas without H_2S. In contrast, an H_2S concentration of 0.05% yielded significantly greater inhibition of up to 34.0% in methanol production, using a pure culture of *Methylocaldum* sp. SAD2 (Zhang et al., 2016).

3.3. Repeated batch methanol production

To demonstrate effective methanol production using simulated biogas (CH_4:CO_2, 2:1) and biohythane (CH_4:H_2, 4:1), repeated batch production was evaluated for up to eight cycles of reuse. The cumulative methanol production profile of free and immobilized co-cultures is presented in Fig. 6. After eight cycles of reuse, free co-culture exhibited residual methanol efficiencies of 8.2% and 2.1%, with cumulative methanol production of 19.47 and 24.43 mM using simulated biogas and biohythane as a feed, respectively. Under similar conditions, encapsulated co-culture through alginate retained significantly higher residual methanol production efficiency values of 31.8% and 18.2%, with the cumulative production of 25.72 and 35.95 mM, respectively. Similarly, encapsulated co-culture through silica-gel exhibited maximum cumulative methanol production of 32.04 and 47.35 mM from simulated biogas and biohythane, respectively. Silica-gel immobilized co-culture was found to be more suitable for methanol production than the free and alginate immobilized forms, possibly because of its higher production stability after cell immobilization. Using simulated biogas as a feed, silica-gel immobilized co-culture exhibited 6.6- and 1.7-fold higher stability in methanol production, with a residual efficiency of

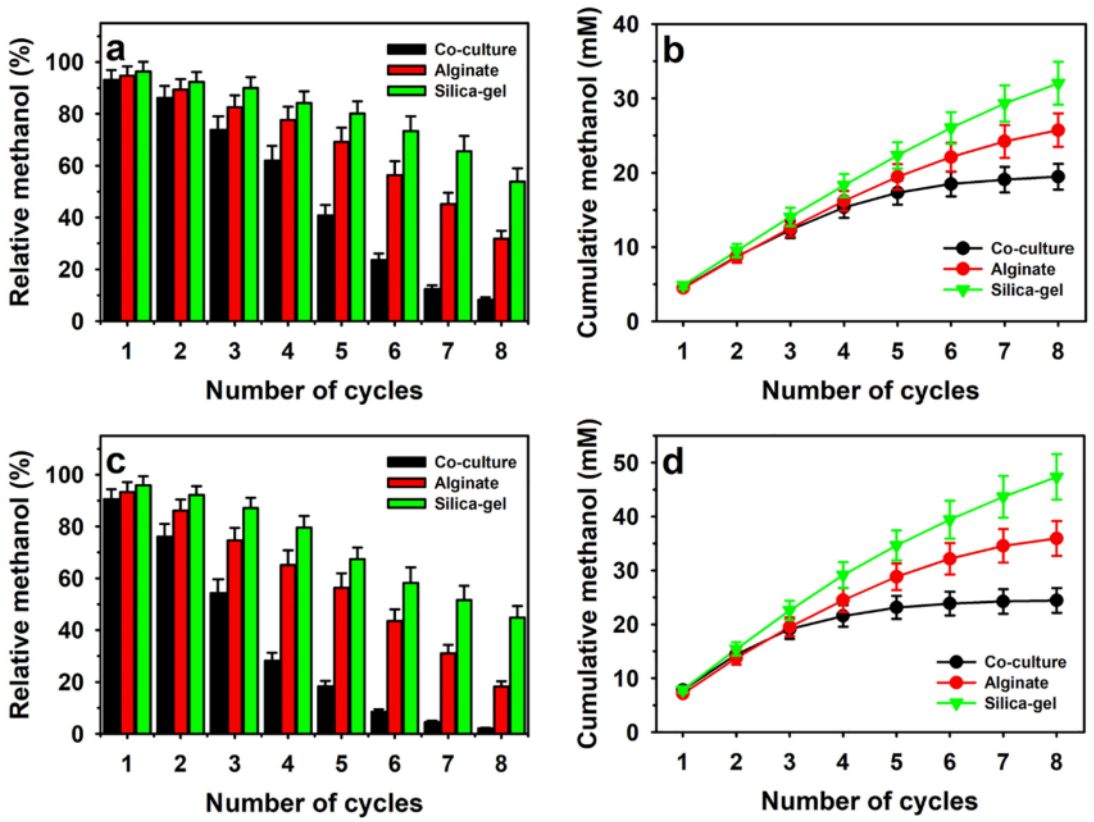

Fig. 6. Repeated batch methanol production by free and encapsulated co-cultures of *M. methanica* and *M. tundrae* using simulated biogas [CH_4 and CO_2, 2:1 (v v^{-1})] (a and b) and simulated biohythane [CH_4 and H_2, 4:1 (v v^{-1})] (c and d) as feed.

53.8% compared with that of the free and alginate encapsulated co-cultures. When simulated biohythane was used as a feed, silica-gel immobilized co-culture showed a residual efficiency of 44.8%, with 21.3- and 2.5-fold improvement, respectively, over free and alginate encapsulated co-cultures. Previously, *M. capsulatus* (Bath) membrane-bound pMMO embedded in polyethylene glycol diacrylate hydrogel resulted in only 20 μM methanol production from CH_4, even with the use of costly NADH as a co-factor (Blanchette et al., 2016). Similarly, repeated batch methanol production using pure cultures of *M. sporium* (B2119-B2123) and *M. trichosporium* (B2117 and B2118) encapsulated in the polymeric matrix resulted in significant reductions in residual efficiency by up to 90% within three cycles of reuse (Senko et al., 2007). Overall, these results suggest that the silica-gel based encapsulated system is an effective approach for higher cumulative methanol production. This is the first study to examine methanol production by immobilized co-culture methanotrophs using simulated biogas or biohythane as a feed.

4. Conclusions

The utilization of biogas as a feed for methanol production by methanotrophs may be a more effective approach than using costly pure CH_4. In this study, methanol production by immobilized co-cultures of defined methanotrophic strains was reported. Co-culture was more effective, up to 21.5%, than pure culture for methanol production. Co-culture immobilized within silica-gel yielded greater methanol productivity of 32.04 and 47.36 mM from simulated biogas and biohythane, under repeated batch conditions than free co-culture (19.47 and 24.43 mM), respectively. Further, the use of raw biogas as a feed produced through AD revealed a promising approach for methanol production.

Acknowledgements

This research was supported by Basic Science Research Program (2017R1A2B3011676, 2017R1A4A1014806) and by the Intelligent Synthetic Biology Center of Global Frontier Project (2013M3A6A8073184) through the National Research Foundation of Korea, South Korea (NRF) funded by the Ministry of Science, ICT & Future Planning,South Korea. This work was supported by Brain Pool grant (NRF-2018H[illegible]A2001746) by NRF, South Korea to work at Konkuk University. This work was supported by the Energy Efficiency & Resources Core Technology Program of the Korea Institute of Energy Technology Evaluation and Planning (KETEP, South Korea), granted financial resource from the Ministry of Trade, Industry & Energy, South Korea (20153030091450). This work was supported by KU Research Professor program of Konkuk University, South Korea.

Conflict of interest

The authors declare that they have no conflicting interests associated with this publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the

online version, at https://doi.org/10.1016/j.biortech.2018.04.096.

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