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# Repeated batch methanol production from a simulated biogas mixture using immobilized *Methylocystis bryophila*

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ABSTRACT

In this study, biological methanol production under repeated batch conditions by immobilized *Methylocystis bryophila*, using simulated biogas of methane ($CH_4$) and carbon dioxide ($CO_2$) as a feed is demonstrated for the first time. The composition of the simulated gas mixtures significantly influenced methanol production by *M. bryophila,* and in all cases, higher concentrations were achieved than with pure $CH_4$ alone. Under optimum conditions, maximum methanol concentrations of 4.88 mmol $L^{-1}$, 7.47 mmol $L^{-1}$, and 7.02 mmol $L^{-1}$ were achieved using the gas mixtures $CH_4$:$CO_2$ (2:1 ratio), $CH_4$:hydrogen [$H_2$, (4:1 ratio)], and $CH_4$:$CO_2$:$H_2$ (6:3:2 ratio), respectively, as feed, with a fixed $CH_4$ concentration of 30%. Methanol yield was increased to 7.85 mmol $L^{-1}$ using covalently immobilized cells and the simulated gas mixture $CH_4$:$CO_2$:$H_2$ (6:3:2 ratio). Under repeated batch conditions, immobilized cells produced a significantly higher cumulative methanol concentration (25.75 mmol $L^{-1}$) than free cells (15.50 mmol $L^{-1}$), using a simulated biogas mixture of $CH_4$:$CO_2$ (2:1) and eight reuse cycles, suggesting that this mixture can potentially be utilized as a feed for the production of methanol. Furthermore, the effective utilization of low-cost feedstock, derived from natural sources, containing gas mixtures of $CH_4$:$CO_2$, $CH_4$:$H_2$, or $CH_4$:$CO_2$:$H_2$, constitute an economical and environmentally friendly approach to the reduction of greenhouse gases.



## 1. Introduction

The development of renewable energy sources as an alternative to use of fossil fuels, and the consequent wide ranging harmful environmental effects, is a major worldwide concern. Of particular concern is the increased emission of greenhouse gases (GHGs), such as methane ($CH_4$) and carbon dioxide ($CO_2$), due to significant growth in the industrial, agricultural and municipal sectors in both developed and developing countries [1–9]. In recent decades, significant attention has been given to sustainable energy sources, such as biofuels, including hydrogen ($H_2$) and alcohols produced in biological processes [7,10–15]. The global warming potential of $CH_4$ is approximately 25-fold higher than that of $CO_2$, and its utilization in methanol production has been investigated [2,7]. Methanol has 400-fold higher energy density than $CH_4$, as well as lower storage and transportation costs and a better safety profile [2]. In addition, it has broad applications in the synthesis of industrially important chemicals such as formaldehyde and higher alcohols [16].

The feasibility of microbial biotransformation of $CH_4$, $CO_2$ and their mixtures into methanol has been demonstrated using methanotrophs [17–20]. Methanotrophs primarily belong to the Proteobacteria group of prokaryotes, and they are widely distributed throughout diverse environmental habitats, playing a key role in the carbon cycle [21]. Methanotrophs firstly convert $CH_4$ into methanol using methane monooxygenases (MMOs), including the particulate form (pMMO, associated with cellular membranes) and the soluble form (sMMO, found in the cytoplasm). The methanol is

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then converted into $CO_2$ via a complex enzymatic network involving methanol-, formaldehyde-, and formate-dehydrogenases. Methanotrophs are classified into three groups according to the MMO forms expressed: type I (pMMO only), type II (both pMMO and sMMO), and type X (containing properties of both type I and type II organisms) [2,22]. The bioconversion of pure $CH_4$ into methanol has been demonstrated under batch conditions, using differing types of methanotrophs. The type I methanotrophs used include *Methylocaldum* sp., [19], *Methylococcus capsulatus* [23] *Methylomonas* sp., [24], and *Methylomonas methanica* [13], and the type II organisms that have been utilized include *Methylocella silvestris* [13], *Methylocella tundrae* [25], *Methylocystis bryophila* [13], *Methyloferula stellata* [13], *Methylosinus sporium* [16,26,27], and *Methylosinus trichosporium* [10,28–30]. However, the high cost of pure $CH_4$ remains a limitation in methanol production by methanotrophs, and therefore the use of low-cost feedstock such as biogas (a mixture of $CH_4$ and $CO_2$) could be advantageous [27,31,32]. In this study, the methanol production potential of *M. bryophila* was evaluated using different gas mixtures of $CH_4$, $CO_2$, and $H_2$ gases. Furthermore, the use of repeated batch techniques was shown to improve the methanol yield from a simulated biogas containing $CH_4$ and $CO_2$. This is the first report of repeated batch methanol production using covalently immobilized cells and a simulated biogas ($CH_4$:$CO_2$) as a feedstock.

## 2. Materials and methods

### 2.1. Materials

*M. bryophila* strain DSM 21852 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). All chemicals and reagents were of analytical grade and from commercial sources, unless otherwise stated. High purity $CH_4$, $CO_2$, and $H_2$ gases were purchased from NK Co. (Busan, Korea). Amberlite (XAD-2, XAD-4, and XAD-7HP) and Duolite A-7 resins were bought from Sigma-Aldrich (USA). Chitosan (90% deacetylated, from crab shells) was obtained from Bio Basic Inc. (Canada).

### 2.2. Growth conditions

*M. bryophila* was grown on nitrate mineral salt (NMS) medium composed of (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; $Na_2MO_4 \cdot 2H_2O$, 0.026. A trace element solution containing: $ZnSO_4 \cdot 7H_2O$ (0.4 g), $H_3BO_3$ (0.015 g), $CoCl_2 \cdot 6H_2O$ (0.05 g), $Na_2$-EDTA (0.25 g), $MnCl_2 \cdot 4H_2O$ (0.02 g), and $NiCl_2 \cdot 6H_2O$ (0.01 g) was then added at 1 mL per L of NMS medium. The pH of the medium was adjusted to 7.0 using $H_2SO_4$ (1 N) or NaOH (1 N). Millipore water (18 MΩ) was used in the preparation of all reagents. Cells were cultivated in a 1 L flask (Duran-Schott, Germany) as described previously [13], and their growth was monitored by measuring the optical density (OD) at 595 nm with a UV/Vis spectrophotometer (Jenway Scientific, UK) [16]. Once confluent, cells were harvested by centrifugation, and stored at 4 °C until use, as described previously [33].

### 2.3. Methanol production

The production of methanol by free or immobilized *M. bryophila* cells was evaluated under batch conditions, in a 120 mL serum bottle (Sigma-Aldrich, USA). The 20-mL reaction volume contained 20 mM of $MgCl_2$, 100 mM of formate, 10 μM of Fe (II), and 5 μM Cu(II), with a dry cell mass (DCM) of 3 mg $mL^{-1}$ free cells in 100 mM sodium phosphate buffer used as an inoculum [13]. The head-space was saturated with either pure $CH_4$ or the simulated gas mixtures to be used as feedstock, and the cultures were incubated at 30 °C, with shaking at 175 rpm, for up to 120 h [13]. The methanol production profile of free or immobilized *M. bryophila* from pure $CO_2$ (15%, v $v^{-1}$) as feed was assessed up to 60 h of incubation [34]. The influence of feed concentration in the range of 10–40% was also evaluated on methanol production.

### 2.4. Preparation of simulated gas mixture

Various ratios of pure gases were used to produce synthetic gas mixtures, mimicking biogas, for methanol production. Gas mixtures were: (i) $CH_4$ and $CO_2$, (ii) $CH_4$ and $H_2$, and (iii) $CH_4$, $CO_2$ and $H_2$.

### 2.5. Methanol dehydrogenase (MDH) activity measurements

The MDH activity was determined spectrophotometrically by phenazine methosulfate-mediated reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm [16]. In brief, 1 mL reaction in phosphate buffer (pH 7.5) was performed using 10 mM of $CaCl_2$, 0.13 μM of DCPIP, 45 mM of $NH_4Cl$, whole cell supernatant (5 mg of DCM), and phenazine methosulfate (3.3 μM).

### 2.6. Electrochemical measurements

Cyclic voltammetry (CV) analysis was carried out using SP-150 potentiostat (BioLogic, USA), with a 3 electrodes system of glassy carbon, platinum and Ag/AgCl as a working, counter, and reference electrodes in 20-mL reaction cell [35]. Electrochemical impedance spectroscopy (EIS) was analyzed in a potentiostatic mode from 100 KHZ to 10 mHZ, with an amplitude voltage of 10 mV. From nyquist plot, the charge transfer resistance was noted from the low frequency region. Charge transfer resistance was calculated on the basis of circular fit analysis from the instrument [36].

### 2.7. Effect of liquid to headspace volume ratio ($V_{liq}$ $V_{gas}^{-1}$)

The effect of the volume ratio $V_{liq}$ $V_{gas}^{-1}$ on methanol production using the simulated gas mixture $CH_4$:$CO_2$:$H_2$ (30:15:10), was evaluated at the ratios 1:1, 1:3, 1:5, 1:7, and 1:9. These ratios were achieved by varying the reaction volume from 10 to 60 mL, in a 120-mL serum bottle.

### 2.8. Whole cell immobilization

Different solid supports, including Amberlite (XAD-2, XAD-4, and XAD-7HP), Duolite A-7, and Chitosan, were used to immobilize *M. bryophila* via adsorption or covalent immobilization methods. Each support (25 g) was washed with distilled water and functionalized with glutaraldehyde, which adds an aldehyde group to the particles, as described previously [34,35,37]. Functionally activated supports were sedimented by centrifugation at 4000 rpm for 30 min, before being washed three times with distilled water and once with phosphate buffer (20 mM) to remove residual glutaraldehyde. Whole cell immobilization was performed by loading 100 mg of DCM per g of activated support (in 50 mM sodium phosphate buffer, pH 7.0), and shaking at 100 rpm for 24 h at 4 °C. Loosely bound cells were separated by centrifugation, and 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 percentage immobilization yield (IY) was calculated as described previously [16].

### 2.9. Repeated batch methanol production

Both free and covalently immobilized *M. bryophila* were