

Article

Bio-Methanol Production Using Treated Domestic Wastewater with Mixed Methanotroph Species and Anaerobic Digester Biogas

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Abstract: The development of cost-effective methods, which generate minimal chemical wastewater, for methanol production is an important research goal. In this study, treated wastewater (TWW) was utilized as a culture solution for methanol production by mixed methanotroph species as an alternative to media prepared from commercial or chemical agents, e.g., nitrate mineral salts medium. Furthermore, a realistic alternative for producing methanol in wastewater treatment plants using biogas from anaerobic digestion was proposed. By culturing mixed methanotroph species with nitrate and phosphate-supplemented TWW in municipal wastewater treatment plants, this study demonstrates, for the first time, the application of biogas generated from the sludge digester of municipal wastewater treatment plants. NaCl alone inhibited methanol dehydrogenase and the addition of 40 mM formate as an electron donor increased methanol production to 6.35 mM. These results confirmed that this practical energy production method could enable cost-effective methanol production. As such, methanol produced in wastewater treatment plants can be used as an eco-friendly energy and carbon source for biological denitrification, which can be an alternative to reducing the expenses required for the waste water treatment process.

Keywords: biomethanol; treated wastewater; mixed methanotroph species; biogas; wastewater treatment plant

1. Introduction

A large amount of energy is needed to extract, convey, treat, and deliver potable water. Additionally, energy is required to collect, treat, and dispose of wastewater. In 2010, the U.S. water system consumed over 600 billion kWh, or approximately 12.6% of the nation's energy [1], of which the energy used for sewage treatment was 0.1–0.3% of the total energy consumption in the U.S. [2]. In Korea, which is the subject of this study, 1.02% of total energy was used in the water supply and sewerage sectors in 2014. In Seoul, the water supply accounts for 0.8% of total electric power consumption, and the sewerage system uses 0.9% [3]. In Korea, the Ministry of Environment has established and is implementing the “2010 Basic Energy Independence Plan for Sewage Treatment Plant” for energy independence through renewable energy production in sewage treatment plants [4]. Purification of methane from biogas is a typical application of renewable energy production in a wastewater treatment plant (WWTP). In some cases, renewable energy production involves hydroelectric power generation

using the discharge fall head of treated wastewater, use of inflow sewage heat, and photovoltaics utilizing effective sites. This study attempted to present a novel bio-methanol production method using treated wastewater and biogas, which has not been considered in the existing sewage treatment plant-based renewable energy production.

Studies of methanol production using methanotrophs have focused on single species of type II methanotrophs (e.g., *Methylosinus trichosporium* OB3; OB3b; NCIB) using nitrate mineral salts medium (NMS medium) [5–13]. However, the preparation of synthetic culture medium and the use of commercially available media are costly, and both generate chemical wastewater. Furthermore, to provide conditions under which a particular dominant species can survive, energy consumption requires high quantities of gas, the culture medium injected into the methanol production process is consumed, and further manipulation is required to prevent external pollution. Accordingly, these processes are difficult to commercialize. Moreover, to obtain high-purity methane ($\text{CH}_4 > 97\%$), purification and separation processes are necessary, which are highly expensive [14,15]. Previous studies have only been conducted under controlled conditions, without field applications, owing to the lack of cost-effective alternatives to these high-cost processes [14].

Thus, to ensure the cost-effectiveness of methanol production using methanotrophs, it is necessary (1) to develop an inexpensive and widely available culture solution to replace expensive chemical culture media, such as NMS, which generates chemical wastewater, (2) to develop practical methods to maintain methanotroph species, and (3) to reduce the methane purification cost. To address these challenges, several recent studies have attempted to reduce costs by replacing high-purity methane with biogas. The production of $0.43 \pm 0.00 \text{ g} \cdot \text{L}^{-1}$ methane has been achieved using biogas produced at a commercial anaerobic digestion facility with a high concentration of formate. Furthermore, raw biogas has been used as a carbon source for *Methylosinus sporium* to prepare 0.51–4.62 mM methane [15] and simulated biogas mixtures have been used as carbon sources to co-culture and co-immobilize *Methylomonas methanica* and *Methylocella tundrae*, thereby producing methanol [16].

However, alternatives to artificial culture solutions, such as NMS medium, have not been examined. Furthermore, few studies have evaluated methanol production using mixed methanotroph species collected and cultured in the natural environment.

In this study, a realistic approach was developed using (1) treated wastewater (TWW) from municipal sources as a methanotroph culture solution, (2) mixed methanotroph species collected and cultured in a natural environment, and (3) anaerobic digester biogas from a wastewater treatment plant to produce methanol. This approach has various advantages over previous methods, i.e., the wastewater generated during methanol production can flow into the wastewater treatment process, and the remaining biogas can be circulated into the digester system for biogas collection. Owing to the low water solubility of methane (22 mg/L at 25 °C), which is a strong greenhouse gas, the remaining methane is typically released into the atmosphere. Our approach can resolve this issue. This is the first study to use TWW for methanotroph-mediated methanol production and to simultaneously apply conditions (1), (2), and (3) described above.

2. Materials and Methods

2.1. Cultivation of Methanotrophs

Soil was collected at a depth of 15–20 cm from the upper layer in an area where methane production was predicted at a metropolitan landfill (20,749,874 m², (N37.57, E126.62)) in Incheon, Korea, whose use had been terminated 2 years prior. Soil samples were collected at three separate points, uniformly mixed, filtered through a sieve (No. 50; 300 µm), and stored at 4 °C. To culture methanotrophs from the soil samples, NMS (modified Higgins nitrate mineral salt) medium was used [13,17]. The medium contained the following (per L of distilled water): KNO_3 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ 0.2 g; 3.8% (*w/v*) Fe-EDTA solution 0.1 mL; 0.1% (*w/v*) $\text{NaMo} \cdot 4\text{H}_2\text{O}$ 0.5 mL; KH_2PO_4 26 g; $\text{Na}_2\text{HPO}_4 \cdot 7(\text{H}_2\text{O})$ 62 g. Additionally, 1 mL of trace element solution was added (per L of

distilled solution: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 500 mg; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 400 mg; $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ 20 mg; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 50 mg; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 10 mg; H_3BO_3 (boric acid) 15 mg; EDTA 250 mg). The pH was adjusted to 6.8 using HCl. All chemical reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Next, 200 mL of NMS medium was added to a 350-mL conical flask, and 5 g of the soil sample prepared as described above for microbial inoculation was added. The flask was closed on the open end with a silicon stopper and a gas-tight syringe was used to add 20% (V%) methane to the upper 150 mL of the head space. The flask was then sealed with Parafilm and cultured in a rotary shaker (Lab Champion IS-971R; Champion Laboratories, Albion, IL, USA). Following 24 h of spinner culture at 250 rpm at 30 °C, the solution was left to settle for 10 min, after which 100 mL of the supernatant was removed and added to a fresh 350-mL conical flask containing NMS medium. The procedure was repeated four times, and the resulting solution containing NMS medium was finally separated using by centrifugation (Centrifuge-416; Dongseo Science, Ltd., Dangjin, Korea) at $2700 \times g$. The centrifuged pellet was freeze-dried at -55 °C in a freeze dryer (OPERN FDS-12003; Seoul, Korea) for subsequent use.

In subsequent experiments, the lyophilized cells were inoculated into 50 mL of the cultured solution in a 160-mL serum bottle (Sigma-Aldrich, St. Louis, MO, USA) to adjust the concentration to 520 mg/L. When using purified methane gas (99.99%), the O_2/CH_4 content was adjusted to 18.5 mL/22.0 mL by replacing 22 mL, i.e., 20% of the head space (110 mL), with methane. The sample was then incubated in a shaking incubator at 30 °C and 150 rpm.

2.2. Treated Wastewater in the Wastewater Treatment Plant

TWW and anaerobic digestion biogas were generated at the Ilsan wastewater treatment plant in Goyang-si, South Korea. The Ilsan wastewater treatment plant is a tertiary advanced treatment facility with a treatment capacity of $270,000 \text{ m}^3 \cdot \text{day}^{-1}$. TWW was used, rather than the raw sewage, as a culture solution because raw sewage has a high ammonia content. When ammonia is present during metabolic processes in methanotrophs, it competes with methane as a co-metabolic substrate of methane monooxygenase (MMO), an enzyme that mediates methane oxidation and acts as an inhibitor of methane oxidation by methanotrophs [17–19]. The process of ammonia oxidation by methanotrophs is similar to the ammonia oxidation pathway of ammonia-oxidizing bacteria. Numerous studies have focused on the competitive inhibition of MMO by ammonia [20,21] and the effects of toxic intermediates, i.e., hydroxylamine and nitrite, produced during ammonium oxidation [22].

Unlike type I methanotrophs, a high copper ion concentration is detrimental to growth and soluble methane monooxygenase (sMMO) expression in type II methanotrophs [23]. Thus, the concentration of copper ions is a major determinant of methanotroph growth. The copper ion concentration in the TWW of the target wastewater treatment plant in this study was in the range of 12 μM to 34 μM . The properties of the TWW used to replace NMS medium were as follows: pH 7.0, $10.2 \text{ mg} \cdot \text{L}^{-1}$ nitrate, $3.1 \text{ mg} \cdot \text{L}^{-1}$ ammonium ions, $0.2 \text{ mg} \cdot \text{L}^{-1}$ phosphate ions, 0.001 ($15.7 \mu\text{M}$) $\text{mg} \cdot \text{L}^{-1}$ Cu, $0.0021 \text{ mg} \cdot \text{L}^{-1}$ K, $0.4920 \text{ mg} \cdot \text{L}^{-1}$ Mg, $0.0232 \text{ mg} \cdot \text{L}^{-1}$ Ca, $0.0132 \text{ mg} \cdot \text{L}^{-1}$ Zn, $0.0123 \text{ mg} \cdot \text{L}^{-1}$ Mn, $0.0116 \text{ mg} \cdot \text{L}^{-1}$ Mo, $0.0092 \text{ mg} \cdot \text{L}^{-1}$ Co, $0.045 \text{ mg} \cdot \text{L}^{-1}$ Fe, $0.0024 \text{ mg} \cdot \text{L}^{-1}$ Ni. NaNO_3 and KH_2PO_4 were used to supply nitrogen and phosphorus, which are required for methanotroph growth. The samples were stored in a refrigerator at 4 °C.

2.3. Biogas in the Wastewater Treatment Plant

The final anaerobic sludge digester biogas of the wastewater treatment plant was composed of CH_4 (68.9%), CO_2 (29.3%), N_2 (1.1%), and O_2 (0.7%) and was collected and stored in a Teflon bag. When biogas was used, 25 mL, i.e., 22.7% the 160-mL serum bottle headspace space (22.7%), was substituted with biogas to adjust the $\text{O}_2/\text{CH}_4/\text{CO}_2$ content to 17.8 mL/20.7 mL/7.3 mL.

2.4. Biogas and Metabolite Measurements

For the biogas (CH_4 , CO_2 , N_2 , and O_2) analysis, the methane content in the serum bottle headspace was measured using a gas chromatographer (HP Agilent 6890A; Santa Clara, CA, USA) equipped with a packed column (GS-GASPRO; $30\text{ m} \times 0.32\text{ mm}$) and flame ionization detector (FID). The conditions were an inlet temperature of $100\text{ }^\circ\text{C}$, oven temperature of $50\text{ }^\circ\text{C}$ (3 min), and detector temperature of $200\text{ }^\circ\text{C}$, using He as the carrier gas and setting the flow rate to 1.2 mL min^{-1} with a split ratio of 20:1.

For the methanol analysis, a gas chromatographer (HP Agilent 6890A; head space G1888, Santa Clara, CA, USA) equipped with a packed column (INNOWAX $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$) and an FID was used. The conditions were an oven temperature of $50\text{ }^\circ\text{C}$ (5 min), which was raised to $250\text{ }^\circ\text{C}$ (3 min), and a detector temperature of $250\text{ }^\circ\text{C}$, using N_2 as the carrier gas and setting the flow rate to 1.2 mL min^{-1} with a split ratio of 10:1.

For the formaldehyde analysis, high-performance liquid chromatography (HPLC; Agilent 1100 series, Santa Clara, CA, USA) with a column (Eclipse XDB-C18; $5\text{ }\mu\text{m}$, $4.6 \times 150\text{ mm}$) was used. The mobile phases were mobile A (D.W 100%) and mobile B (ACN 100%), with a mobile flow of 1.5 mL/min , eluent A:B of 50:50, runtime of 38 min, and detection wavelength of 360 nm .

For the formate analysis, HPLC (Agilent 1100 series, Santa Clara, CA, USA) equipped with a column (Agilent Hi-Plex H; $8\text{ }\mu\text{m}$, $7.7 \times 300\text{ mm}$) was used. The injection volume was $20\text{ }\mu\text{L}$ and the mobile phase was 100% $0.01\text{ M H}_2\text{SO}_4$, with a mobile phase flow rate of 0.6 mL min^{-1} , eluent of 100% $0.01\text{ M H}_2\text{SO}_4$, runtime of 60 min, and detection wavelength of 210 nm .

2.5. Enzyme Assays

The naphthalene oxidation assay was conducted to quantify the specific activity of sMMO [24]. This method allows specific activity to be measured under the assumption that only sMMO of methanotrophs can convert naphthalene into 1- or 2-naphthol by oxidation. The 1- or 2-naphthol generated during oxidation can react with tetrazotized *O*-dianisidine to form a purple naphthol diazo compound, whose UV absorbance was measured at 530 nm using a UV-visible spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan) as follows. (1) Cell suspensions were transferred in 1-mL aliquots to 10-mL screwcap test-tubes. (2) Next, 1 mL of saturated naphthalene solution was added to each sample. (3) The reaction mixtures were incubated on a rotary shaker at $30\text{ }^\circ\text{C}$ for 1 h. (4) After incubation, $100\text{ }\mu\text{L}$ of freshly prepared 0.2% (*w/v*) tetrazotized *O*-dianisidine solution was added to the reaction mixture. (5) Samples were immediately monitored spectrophotometrically at 525 nm . (6) sMMO is expressed in nanomoles naphthol formed per milligram of cell protein per hour.

Methanol dehydrogenase (MDH) activity was estimated by measuring changes in the UV absorbance of the phenazine methosulfate compound induced by the reduction of 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm using a UV-visible spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan). The assay solution contained CaCl_2 (10 mM), NH_4Cl (45 mM), phosphate buffer (0.3 M , pH 7.5), the whole cell supernatant (DCM 5 mg), DCPIP ($0.13\text{ }\mu\text{M}$), and phenazine methosulfate ($3.3\text{ }\mu\text{M}$) in a 1-mL reaction solution [11,25]. NaCl, which is the cheapest and most effective MDH inhibitor, was used [5,11,26].

All experimental data are presented as the means and standard deviation of the results of three sets of independent experiments.

2.6. Microbial Community Analysis

For the microbiological analysis, DNA extraction, PCR amplification, and pyrosequencing were performed by ChunLab, Inc. (Seoul, Korea). The 16S rRNA genes for each sample were amplified using barcoded universal primers. PCR was performed under the following cycling conditions: An initial denaturation step at $94\text{ }^\circ\text{C}$ for 300 s, followed by 10 cycles of denaturation at $94\text{ }^\circ\text{C}$ for 30 s, annealing at $60\text{ }^\circ\text{C}$ to $55\text{ }^\circ\text{C}$ ($-0.5\text{ }^\circ\text{C/cycle}$, with a touchdown program) for 45 s, and extension at $72\text{ }^\circ\text{C}$ for 90 s.

This was followed by an additional 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 90 s, and a final elongation step at 72 °C for 5 min.

The composition and ratio of microbial species shared by the three sets of samples were calculated.

2.7. Water Quality Analysis

Analyses of water quality and components of TWW were based on the American Standard Methods for the Examination of Water and Wastewater (23rd ed., American Public Health Association) and EPA Methods (EPA Method 1613). Organic components were analyzed by the colorimetric method, and the atomic analysis was performed by atomic-absorption-spectroscopy.

2.8. Statistical Analysis

Statistical significance was determined by repeated measures ANOVA ($\alpha = 0.05$) using SPSS Statistical Software (IBM SPSS 22.0; Cary, NC, USA). Experimental data are presented as means $\pm$ standard error.

3. Results and Discussion

3.1. Analysis of Cultured Methanotrophs

Figure 1 shows the composition and number of species based on PCR assays of bacteria cultured on the NMS medium. The cultured methanotrophs included *Methylobacter*, *Methylosarcina*, and *Methylobacter*. The dominant species were *Methylobacter*, *Methylosarcina*, *Methylobacter*, and *Methylobacter*. *Methylobacter* is mainly found in sediments of lakes, ponds, or freshwater areas [26]. *Methylobacter* includes typical type I or type X methanotrophs [27]. *Methylobacter* is adapted to a wide range of NaCl conditions (approximately 100 mM) according to Bowman et al. [28], and exhibits optimal growth at 37 °C as well as a stable activity with respect to changes in oxygen [29,30]. Table 1 shows the ICM (intracytoplasmic membrane) type and MMO (sMMO or pMMO) type of the major taxa detected in the analysis. All of the type I methanotrophs, except for *Methylobacter*, expressed sMMO and pMMO simultaneously.

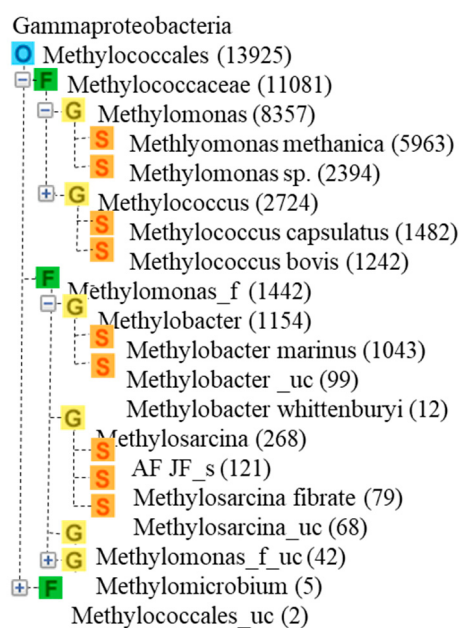


Figure 1. Composition of methanotroph species (output from CL community (ChunLab Inc.) cultured in NMS medium. The numbers in parentheses indicate the number of bacteria belonging to the particular species in the samples.

Table 1. ICM (intracytoplasmic membrane) types and MMO (sMMO, pMMO) types for major species.

Species	MMO Type	ICM Type	Reference
<i>Methylomonas methanica</i>	pMMO, sMMO	Type I	[26]
<i>Methylococcus capsulatus</i>	pMMO, sMMO	Type X	[22]
<i>Methylococcus bovis</i>	pMMO	Type I	[23]
<i>Methylobacter marinus</i>	pMMO, sMMO	Type I	[27]

• sMMO (soluble methane monooxygenase). • pMMO (particulate methane monooxygenase).

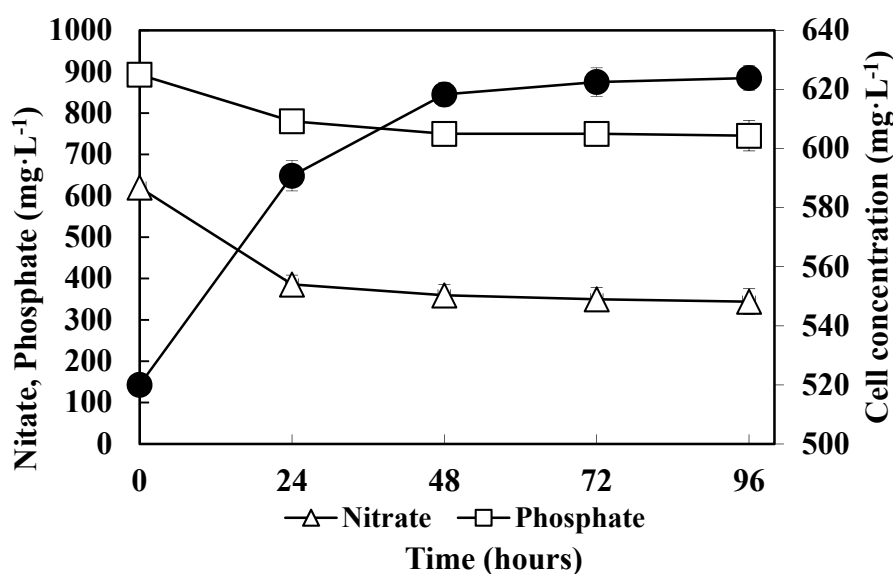
The MMO type and growth rates of methanotrophs depend on the copper (Cu^{2+}) concentration. In the case of *Methylomonas methanica* strain 68-1, MMO has been detected in the form of pMMO, rather than sMMO, for a Cu^{2+} concentration of 1 μM or greater [31]. Previous studies of *Methylococcus capsulatus* Bath have shown increases in cell mass, the abundance of intracytoplasmic membranes, and pMMO in growth medium with a copper concentration of 5–10 μM . *Methylococcus capsulatus* Bath exhibits minimal growth when it is cultured without copper in the growth medium [32,33].

Methylobacter marinus expresses both pMMO and sMMO, as a type I methanotroph [34], and *Methylococcus capsulatus* Bath simultaneously expresses pMMO and sMMO as a type X methanotroph [27]. Moreover, the expression patterns of sMMO and pMMO vary according to the concentration of copper ions [35,36], and the conversion of sMMO into pMMO occurs at a copper ion concentration of 10 mM or greater [37]. The growth rate of *Methylococcus capsulatus* strain M is greater at 40 mM than at lower or higher copper concentrations [38].

3.2. Consumption of Nitrate and Phosphate by Methanotrophs

Methanotrophs require relatively high quantities of nitrogen and phosphorus for growth. Anthony (1982) confirmed that methanotrophs consume 0.25 mol of nitrogen to assimilate 1 mole of carbon from methane [39].

To evaluate nitrate and phosphate consumption in this study, mixed methanotroph species were cultivated in NMS. The consumption levels were 275.0 $\text{mg}\cdot\text{L}^{-1}$ nitrate and 147.7 $\text{mg}\cdot\text{L}^{-1}$ phosphate (Figure 2). Based on these results, NaNO_3 and KH_2PO_4 were added to the TWW to adjust the concentrations of nitrate and phosphate to 300 $\text{mg}\cdot\text{L}^{-1}$ and 160 $\text{mg}\cdot\text{L}^{-1}$, respectively, for use as the methanotroph culture solution in subsequent experiments.

**Figure 2.** Nitrate and phosphate consumption by mixed methanotroph species during growth on NMS medium.

3.3. Comparison of the Methanotroph Growth Rate and Methane Absorption Rate Using Different Culture Solutions

Figure 3 shows a comparison of the methanotroph-specific growth rate (a) and methane uptake rate (b) in NMS medium and TWW supplemented with nitrate and phosphate in TWW. The specific growth rate (Figure 3a) and methane uptake (Figure 3b) of mixed methanotroph species did not differ between the NMS medium and the enriched TWW ($p > 0.05$). These results confirmed that TWW supplemented with nitrogen and phosphate was suitable for use as a methanotroph culture solution.

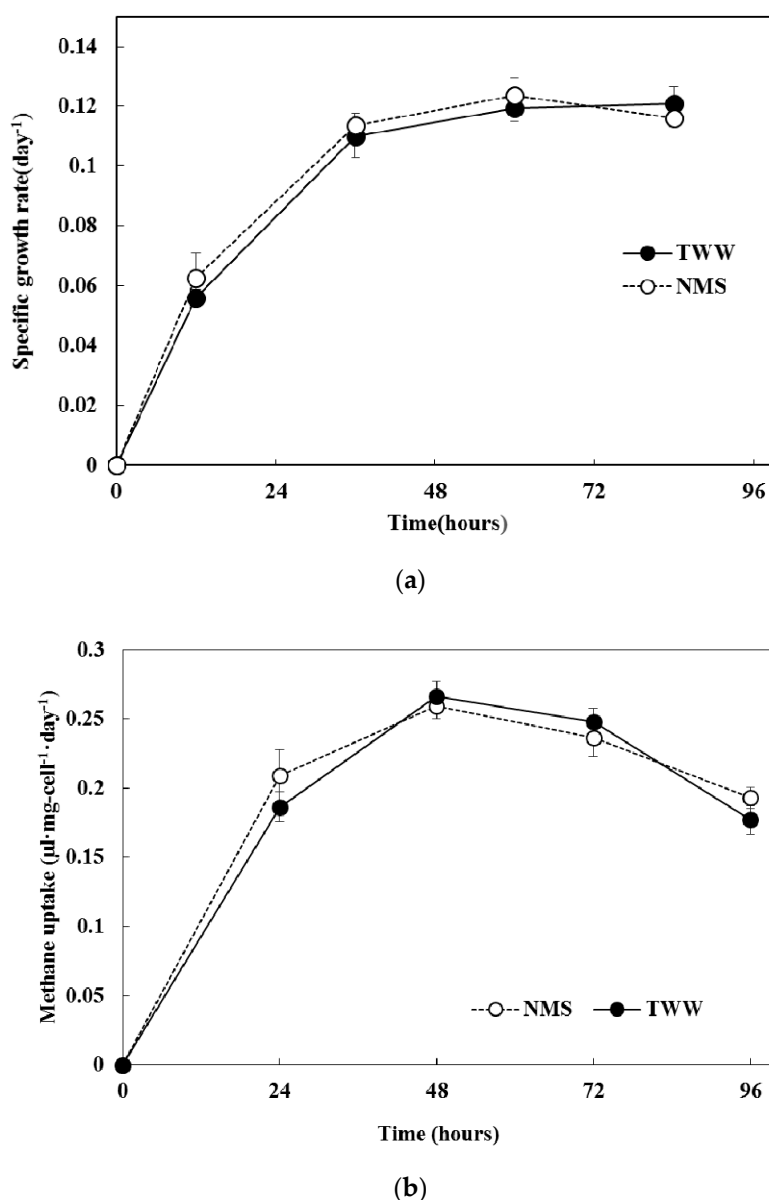


Figure 3. Comparison of the methanotroph-specific growth rate (a) and methane uptake rate (b) in NMS medium and TWW supplemented with nitrate and phosphate.

Methanol production by mixed methanotroph species was similar using NMS medium and pretreated TWW with biogas (Figure 4), e.g., the concentrations were 3.60 ± 0.21 mM using NMS medium and 3.50 ± 0.20 mM using TWW after 48 h. The initially lower methanol production in TWW than in NMS medium could be explained by the partial consumption of MMO by a trace amount of ammonium ($3.1 \text{ mg} \cdot \text{L}^{-1}$) remaining in the TWW.

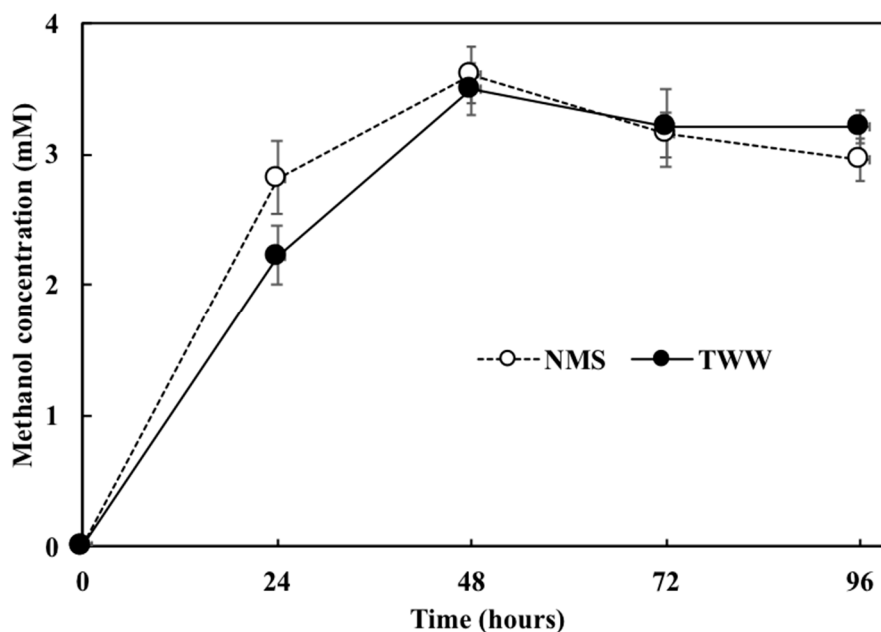


Figure 4. Changes in methanol production in NMS medium and TWW supplemented with nitrate and phosphate.

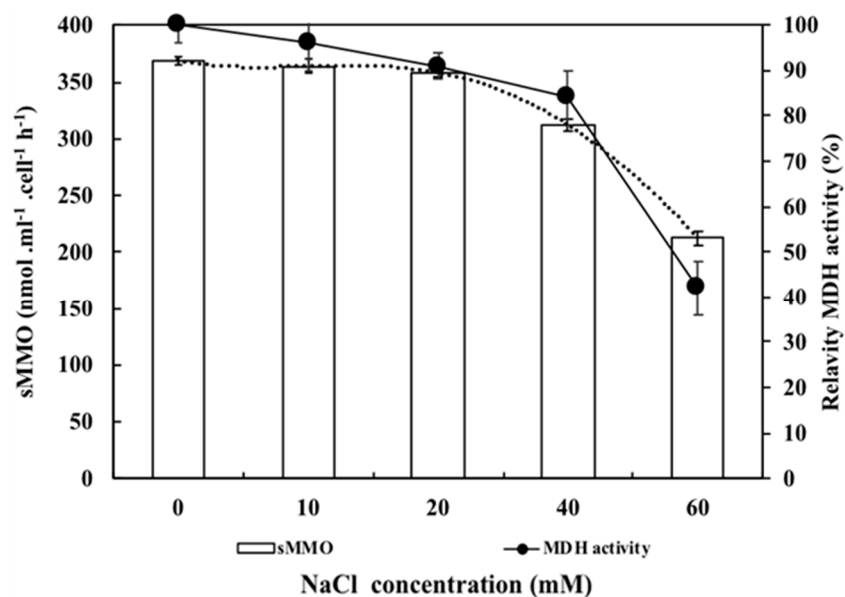
3.4. Effect of NaCl as an MDH Inhibitor, Including Analysis of Methane Uptake and Methanol Production

It is necessary to inhibit MDH activity to prevent the oxidation of the produced methanol. EDTA, cyclopropane, cyclopropanol, dithiothreitol, phenylhydrazine, NaCl, iodoacetate, $MgCl_2$, NH_4Cl , and a high concentration of phosphate have been applied to prevent the conversion of methanol to formaldehyde by inhibiting MDH activity. For example, chelating agents, such as EDTA, significantly inhibit the activity of methanol oxides and MDH [40]. Among these materials, NaCl is the most inexpensive and widely available MDH inhibitor [7–9,11,12,41].

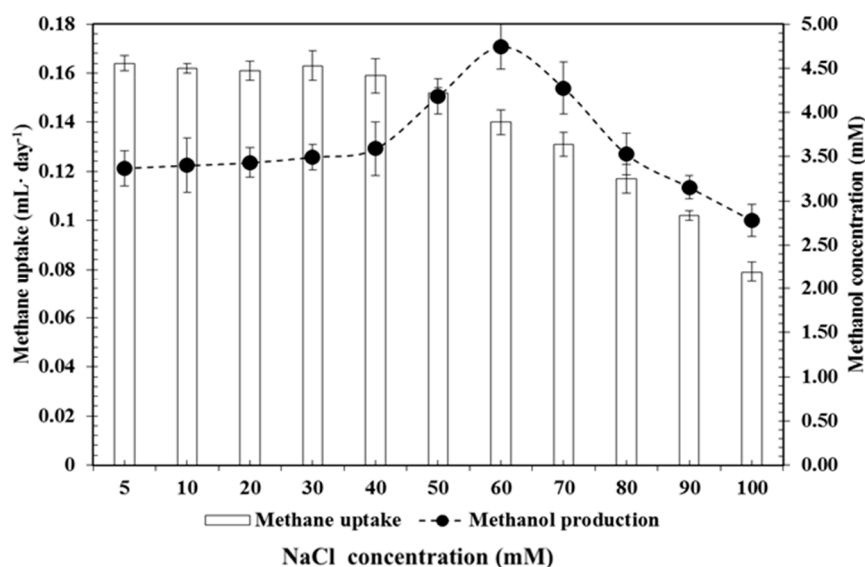
Methylosinus trichosporium OB3b was used in the presence of NaCl 200 mM, 12.9 mM phosphate (pH 7), and 20 mM sodium formate (at 25 °C for 36 h in NMS medium, methane/air (1:4 (v/v))) to produce 7.7 mM methanol [42]. *Methylosinus trichosporium* OB3b in the presence of 100 mM NaCl and 1 mM EDTA as MDH inhibitors and 20 mM sodium formate with 10 mM phosphate buffer (pH 7.0) (at 25 °C for 12 h in NMS medium methane/air (1:1 (v/v), 5 μM $CuSO_4$)) yields 13.2 mM methanol [43].

The inhibitory effects of NaCl alone, without other MDH inhibitors, such as EDTA and a high concentration of phosphate buffer, on MDH were evaluated. In particular, the effects of NaCl concentration on sMMO expression and MDH activity were analyzed (Figure 5). sMMO expression gradually decreased and as the NaCl concentration increased to 40 mM. MDH activity gradually decreased to 84% for NaCl concentrations of up to 40 mM and then sharply decreased to 42% for concentrations of up to 60 mM (Figure 5a).

The MDH inhibitory effect was consistent with previous results indicating that the optimal concentration of NaCl for MDH inhibition using the type II methanotroph *Methylocystis bryophila* is 50 mM [11]. For mixed methanotroph species cultured on enriched TWW medium, changes in methane consumption and methanol production were compared according to the concentration of NaCl as an MDH inhibitor (Figure 5b). The maximum methanol production by mixed methanotroph species was 4.74 mM at an NaCl concentration of 60 mM.



(a)



(b)

Figure 5. Changes in sMMO expression and MDH activity (a) and methane uptake rate and methanol production with respect to NaCl concentration (b) in treated wastewater medium.

3.5. Effect of Formate

In some studies, approximately 2 mM formate, formic acid, ascorbic acid, or citric acid has been used to maintain the high activity of MMO [44], and sodium formate has been used as a reductant to induce sMMO activity [45]. These studies are based on the assumption that reversible enzymatic reactions can occur. In this study, sodium formate was injected to supply formate, which is the final product of methane oxidation, and changes in methanol production were observed. The maximum methanol production of 6.35 mM was detected using 60 mM NaCl and 40 mM formate (Figure 6). In this case, the concentration of sMMO increased from 445 nmol mL⁻¹ cell⁻¹ h⁻¹ to 595 nmol mL⁻¹ cell⁻¹ h⁻¹ by the injection of 40 mM formate.

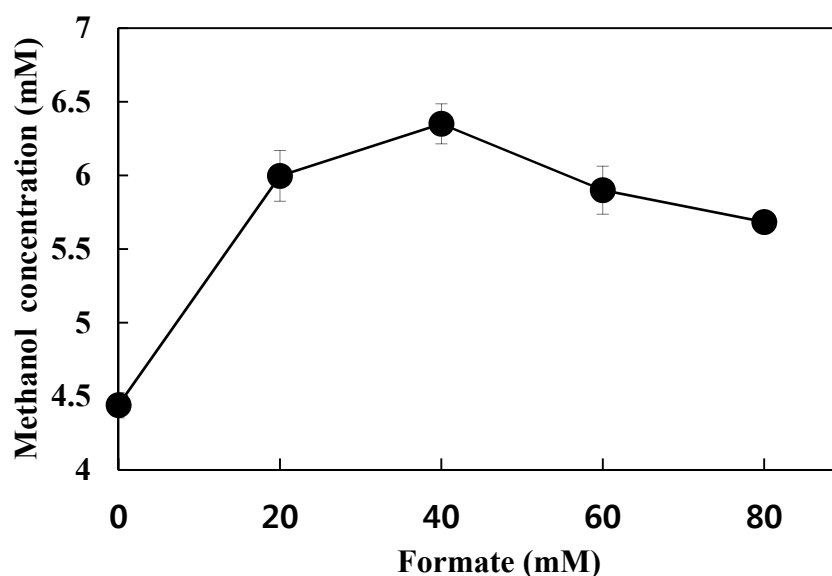


Figure 6. Effect of formate concentration on methanol production from biogas and NaCl (60 mM) in treated wastewater medium.

Changes in metabolites were evaluated with respect to MDH inhibition (Table 2).

Table 2. Concentrations of methanotroph metabolites (methanol, formaldehyde, and formate) in NMS medium according to the NaCl concentration (1) and comparison of methanol production among studies using NaCl as an MDH inhibitor (2).

(1) Metabolite Production				
MDH Control Condition (mM)	Methanol	Formaldehyde	Formate	Total Metabolites
Control	3.46 (± 0.17)	2.04 (± 0.13)	0.80 (± 0.09)	6.30 (± 0.18)
NaCl 60 mM	4.52 (± 0.19)	0.81 (± 0.11)	0.13 (± 0.03)	5.46 (± 0.13)
NaCl 60 mM + sodium formate 40 mM	6.35 (± 0.14)	0.72 (± 0.12)	-	-
(2) Comparison of Methanol Production in Various Studies Using NaCl as an MDH Inhibitor				
MDH Control Condition	Methanol (mM)	Methanotroph Species	Reference	
50 mM MgCl ₂	2.21 mM	<i>Methylocystis bryophila</i>	[11]	
50 mM NaCl + 10 mM NH ₄ Cl	<2.2 mM			
50 mM MgCl ₂ + 100 mM sodium formate	4.15 mM	<i>Methylosinus trichosporium</i> OB3b	[9]	
1 mM EDTA + 100 mM NaCl + 20 mM sodium formate	13.2 mM			
NaCl (100 mM)	0.60 mmol (6.0 mM)	<i>Methylosinus sporium</i>	[41]	
40 mM (NH ₄ Cl)	0.74 mmol (7.4 mM)			
200 mM NaCl + 20 mM sodium formate	7.7 mM	<i>Methylosinus trichosporium</i> OB3b	[42]	

Data represent means from three replicates $\pm$ standard deviations.

The methanol component was 54.9% (3.46 mM) in TWW supplemented with nitrate and phosphate (control), and 82.7% (4.52 mM) using 60 mM NaCl. The total amount of whole metabolites (methanol, formaldehyde, and formate) was 6.3 mM, which was greater than that in the control (without NaCl as an MDH inhibitor). Although the addition of an inhibitor of MDH increases methanol production, it also induces the inhibition of methanotroph activity, and the degree of inhibition can be indirectly determined by total metabolite concentration.

In comparison with methanol production (2.21–13.2 mM) observed using NaCl as an MDH inhibitor in NMS medium for single species using purified methane [9,11,41,42], TTW (tertiary treatment) supplemented with nitrate and phosphate, biogas generated from the digester, and mixed methanotrophs resulted in 6.35 mM methanol. These results confirmed that the use of TWW as a culture solution for methanotrophs could be a cost-effective approach for methanol production.

4. Conclusions

This study is the first to propose a bio-methanol production method as a renewable energy using treated wastewater and biogas generated from a sewage treatment plant. Mixed methanotroph species were cultivated with nitrate and phosphate in a municipal wastewater treatment plant using biogas generated from the sludge digester. Methanol production of 4.52 mM was achieved using only NaCl as an MDH inhibitor. The addition of 40 mM formate increased MMO activity, yielding 6.35 mM methanol. These results confirmed that biogas as well as TWW generated from the wastewater treatment plant can be used for cost-effective methanol production, without additional contamination by wastewater and waste biogas in the wastewater treatment plant.

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