Biological conversion of biogas to methanol using methanotrophs isolated from solid-state anaerobic digestate

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Abstract

The aim of this work was to isolate methanotrophs (methane oxidizing bacteria) that can directly convert biogas produced at a commercial anaerobic digestion (AD) facility to methanol. A methanotrophic bacterium was isolated from solid-state AD (SS-AD). The isolate had characteristics comparable to obligate methanotrophs from the genus *Methylocaldum*. This newly isolated methanotroph grew on biogas or purified CH₄ and successfully converted biogas from AD to methanol. Methanol production was achieved using several methanol dehydrogenase (MDH) inhibitors and formate as an electron donor. The isolate also produced methanol using phosphate with no electron donor or using formate with no MDH inhibitor. The maximum methanol concentration (0.43±0.00 g L⁻¹) and 48-h CH₄ to methanol conversion (25.5±1.8 %) were achieved using biogas as substrate and a growth medium containing 50 mM phosphate and 80 mM formate.

Keywords: anaerobic digestion, biogas, biological conversion, methanol, methanotrophs

1. Introduction

Biogas produced during anaerobic biodegradation of organic wastes contains methane (CH_4) as the major component (30-70%) and it is an emerging renewable energy source. In the United States alone, it is estimated that over 650 billion ft^3 of biogas could be captured yearly from landfills and anaerobic digestion (AD) systems (USDA et al., 2014). This large source of energy could be used to power more than 3 million homes or generate enough compressed natural gas (CNG) to replace 2.5 billion gallons of gasoline (USDA et al., 2014, USDOE, 2014). However, biogas is difficult to store and transport because it is a gas at ambient conditions (Ge et al., 2014). This issue could be addressed by converting biogas to methanol, a valuable liquid chemical that can be used directly as fuel or further converted to other products such as olefins and gasoline. CH_4 can be thermo-chemically converted to methanol, but the process uses expensive metal catalysts and operates at high temperatures (200-900°C) and pressures (5-20 MPa)(Riaz et al., 2013). Besides, biogas contains carbon dioxide (CO_2 , 30-70%) and trace impurities such as hydrogen sulfide (H_2S , 0-2000 ppm) (Yang et al., 2014), while thermochemical technologies require a CH_4 source that is free of impurities (Riaz et al., 2013). Although biogas can be cleaned and upgraded to purified CH_4 (>97%), the purification processes are usually expensive (Yang et al., 2014). Biological conversion of biogas to methanol is an emerging, attractive approach as it may not require biogas purification and uses ambient conditions, reducing operational requirements and energy demands (Ge et al., 2014).

Methanotrophs are aerobic bacteria that can convert CH_4 to methanol using the enzyme methane monooxygenase (MMO) (Kalyuzhnaya et al., 2015). Under normal conditions, methanotrophs further oxidize methanol to CO_2 via methanol dehydrogenase (MDH) and other enzymes in a sequential manner, generating both ATP and reducing power that can be used for

other metabolic reactions (Kalyuzhnaya et al., 2015). While MDH inhibitors, such as phosphate and NaCl, have been used to increase methanol production by methanotrophs (Duan et al., 2011, Mehta et al., 1991), inhibition of methanol oxidation by MDH inhibitors decreases production of ATP and reducing power; thus, another organic substrate, such as formate, is needed to maintain the metabolic activity of methanotrophs. Recently, electrochemical production of formic acid from CO₂ and H₂O has been achieved, which provides an opportunity to effectively reduce the cost of formate as an electron donor (Li et al., 2012, Reda et al., 2008). However, improvements in several aspects are still needed for biological conversion of biogas to methanol to reach industrial scale. Currently, pure CH₄ is routinely used for growing methanotrophs. No studies have used methanotrophs to convert biogas from a commercial AD facility to methanol.

Isolation of robust methanotrophs that can directly use biogas will improve the feasibility of biological conversion of CH₄ to methanol. Methanotrophs have been isolated from a variety of sources such as soil, natural gas fields, and waste treatment facilities (Ge et al., 2014), and a few studies have isolated methanotrophs to produce methanol (Han et al., 2013). Methanotrophs have also been detected and isolated from commercial liquid AD (L-AD) systems that operate at total solids (TS) contents less than 15% (Corder et al., 1986, Rastogi et al., 2009). This indicates that methanotrophs can survive long-term anoxia and may use biogas as their sole source of carbon and energy (Ge et al., 2014). Compared to L-AD, solid-state AD (AD) operates at TS contents greater than 15% (Sheets et al., 2015), and has higher porosity than L-AD, which may provide higher O₂ levels that support the growth of methanotrophs (Ahn et al., 2010). Potentially, methanotrophs isolated from SS-AD could be used to directly convert biogas to methanol. To date, no methanotrophs have been isolated from SS-AD or used to convert biogas from a commercial AD facility to methanol. Additionally, no studies have examined the effects of MDH

inhibitors and formate on the biological conversion of biogas to methanol by methanotrophs. To address these research gaps, this study aimed to: 1) isolate and characterize methanotrophs from an SS-AD system; 2) compare biogas and purified CH₄ as substrates for a methanotrophic isolate; and 3) determine the effects of MDH inhibitors and formate on biological conversion of biogas to methanol.

2. Materials and methods

2.1. Isolation of methanotrophs

Eight digestate samples (5 g each) were collected from SS-AD reactors described in Sheets et al. (2015). The 1-L SS-AD reactors were fed switchgrass (Feedstock (F)) and inoculated with digested wastewater sludge (Inoculum (I)) at an F/I ratio of 3. The SS-AD reactors were controlled at either 20% or 30% TS and incubated under mesophilic (36±1°C) conditions for 70 days. The 70-day CH₄ yield of the SS-AD reactors varied from 88 L kg⁻¹ volatile solids (VS_{added}) (30% TS) to 113 L kg VS_{added}⁻¹ (20% TS) (Sheets et al., 2015). The isolation procedure was a modified version of the protocols described in Bowman (2006) and Dedysh and Dunfield (2011). The SS-AD samples were inoculated into a nitrate mineral salts (NMS) medium (Bowman, 2006) in 250-mL flasks (45 ml NMS per flask). The NMS medium contained MgSO₄·7H₂O (1.0 g L⁻¹), KNO₃ (1.0 g L⁻¹), KH₂PO₄ (0.272 g L⁻¹), Na₂HPO₄ (0.284 g L⁻¹), CaCl₂·2H₂O (0.134 g L⁻¹), chelated Fe solution (0.2% (v/v)), and a trace element solution (0.05% (v/v)). The chelated Fe solution contained ferric (III) ammonium citrate (1.0 g L⁻¹), EDTA (2.0 g L⁻¹), and concentrated HCl (0.3% (v/v)) in deionized (DI) water. The trace element solution contained EDTA (500 mg L⁻¹), FeSO₄·7H₂O (200 mg L⁻¹), ZnSO₄·7H₂O (10 mg L⁻¹), MnCl₂·4H₂O (3.0 mg L⁻¹), H₃BO₃ (30 mg L⁻¹), CoCl₂·6H₂O (20 mg L⁻¹), CaCl₂·2H₂O (1.0 mg L⁻¹), NiCl₂·6H₂O (2.0 mg L⁻¹), and Na₂MoO₄·2H₂O (3.0 mg L⁻¹) in DI water. The initial pH of the

NMS medium was 6.6-6.8. Each of the flasks was sealed with a rubber stopper and its headspace was filled with a filtered (0.2 μ m) mixture of purified CH₄ (99% purity) and air at a CH₄: air ratio of 1:4 (v/v). The flasks were incubated at 37°C with continuous shaking at 200 rpm. After three days, 5-10 ml sample of each enriched culture was transferred into a new flask that contained 40-45 mL of fresh NMS medium and a similar mixture of CH₄ and air. This process was repeated every 3 days. After 25 days, the enriched culture from each flask was spread on an NMS agar plate. The plates were then incubated at 37°C in an anaerobic jar, with a headspace of CH₄ and air at a ratio of 1:4 (v/v), for one to two weeks. Individual colonies were picked and streaked on fresh NMS agar plates until a single colony morphology (based on color, size, and shape) was observed. Fifteen isolates were obtained and each was transferred to 1 mL of NMS medium in a 15 mL sealed vial with a headspace containing filtered (0.2 μ m) CH₄ and air at a ratio of 1:4. The vials were incubated at 37°C with continuous shaking at 300 rpm until there was an observable increase in culture turbidity. Cell morphology was examined by light microscopy. Cultures were considered to be pure if they did not grow in NMS amended with 0.05% glucose (Dedysh and Dunfield, 2011).

For each methanotrophic isolate, the full-length 16S rRNA gene was amplified and sequenced at the Plant-Microbe Genomics Facility at The Ohio State University, Columbus, Ohio, USA. The 16S rRNA gene sequences were analyzed and taxonomically classified using RDP Classifier (Wang et al., 2007) and BLAST against the NCBI RefSeq database. The 16S rRNA gene sequence of all 15 isolates shared >97% sequence similarity with strains of *Methylocaldum*, a genus containing obligate methanotrophs. One of the fastest growing isolates, henceforth referred to as *Methylocaldum* sp. 14B, was selected for further experiments. Strain 14B was isolated from the digestate of a mesophilic (37°C) SS-AD reactor that was operated at

30% TS content and produced biogas composed of 50-60% (v/v) CH₄ and 1-3% (v/v) O₂ (Sheets et al., 2015).

2.2. Cultivation with different chemical and physical inputs

To test the ability of strain 14B to utilize various carbon sources, it was aerobically grown in 1 mL of liquid NMS medium supplemented with 0.1% (w/v) of methanol, formate, acetate, xylose, glucose, or citrate. Cultivation was conducted in 15 mL vials at 37°C and 200 rpm. Growth was determined by the change in optical density (OD) at 600 nm and cell morphology was examined by light microscopy.

The effects of copper (Cu²⁺), NaCl, nitrogen source, pH, and temperature on cell growth using CH₄ as the sole carbon source were evaluated by culturing strain 14B in 20 mL of NMS medium in 125 mL flasks with CH₄ and air (at 1:4 ratio, v/v) in the headspace. Copper was tested because it was shown to influence cell growth, especially for those methanotrophs that possess the particulate methane monooxygenase (pMMO) (Kalyuzhnaya et al., 2015). The Cu²⁺ concentrations assessed were 0, 1.0, 5.0, and 10 µM of CuCl₂ supplemented in NMS. Liquid NMS medium containing 1 µM CuCl₂ was amended with 0, 2, 5, 10, or 20 g L⁻¹ NaCl to determine the effects of different salt concentrations. Growth on ammonium was examined by replacing the KNO₃ in NMS medium (containing 1 µM CuCl₂) with 1.0 g L⁻¹ ammonium sulfate ((NH₄)₂SO₄). The optimal growth pH was evaluated using NMS medium containing 1 µM CuCl₂ at pH 5.0-7.6. For tests to evaluate Cu²⁺, NaCl, nitrogen source, and pH, the cultures were incubated at 37°C with continuous shaking at 200 rpm in a walk-in incubator. To evaluate the effect of temperature, strain 14B was cultured in NMS medium (NO₃⁻ as nitrogen source, 1 µM CuCl₂, no NaCl, initial pH of 6.6-6.8) and incubated at 37, 42 or 47°C with continuous shaking at 200 rpm. Cell growth was determined by measurement of the optical density (OD_{600nm}), and

growth rate was calculated using the OD values. For each of the above tests, at least two replicates were performed. Gases were filtered (0.2 μ m) prior to use.

2.3. Cultivation on different methane sources

Biogas was collected from quasar energy group's commercial scale digester (Wooster, OH, USA) that treats food waste. According to gas chromatography (GC) analysis, the biogas was composed of 69.8% CH₄, 28.8% CO₂, 1.0% N₂, and 0.4% O₂. The biogas had less than 50 ppm of H₂S as measured using the Dräger Short Term Detector Tubes (Fisher Scientific, Hampton, NH, USA), since the AD facility implements control technologies to reduce H₂S content in the biogas (quasar energy group, 2015). Purified CH₄ (99% purity) was purchased from Praxair[®] (Danbury, CT, USA). Kinetic parameters for substrate consumption and cell growth were determined by cultivating strain 14B with either purified CH₄ (99% purity) or the biogas as the carbon source. Strain 14B was cultured at 37°C and 200 rpm in 50 mL of NMS medium (w/ 1 μ M CuCl₂) in 250-mL flasks with a 500-mL Tedlar gas bag connected to each flask. The headspace and Tedlar gas bag contained CH₄ and air at a 1:4 ratio (v/v) or biogas and air at a 1:2.5 ratio (v/v). These two gas ratios provided a comparable initial headspace concentration of ~20% CH₄ (v/v) and 16-18% O₂ (v/v) for aerobic cultivation (Bowman, 2006, Dedysh and Dunfield, 2011). Cell growth (OD_{600nm}) and gas composition (CH₄, O₂, N₂, CO₂) were measured (via GC) periodically until the stationary phase of growth was reached. Three replicates were performed.

2.4. Methanol formation

To screen for effective MDH inhibitors, strain 14B cells grown to the exponential/early stationary phase were harvested by centrifugation (10,000 rpm, 15 min) and then resuspended in

4.1 mL of NMS medium (w/ 1 μ M CuCl_2) containing either 0 mM or 80 mM sodium formate and varied concentrations of MDH inhibitors. The initial $\text{OD}_{600\text{nm}}$ of the cell suspension was 0.89 ± 0.02 . EDTA (0.5, 5.0 mM), phosphate buffer (100, 200 mM as $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$), NaCl (100, 200 mM), and MgCl_2 (20, 40 mM) were tested as potential MDH inhibitors (Ge et al., 2014). Before incubation, a 1-mL sample of the cell suspension was removed and filtered (0.2 μ m) and the filtrate was analyzed by GC to test for the initial presence of methanol. The remaining 3.1 mL of the cell suspension was transferred to 40-mL glass vials, which were each sealed with a rubber stopper. Six mL of purified CH_4 (99% purity) was added to each vial to reach a CH_4 : air ratio of 1:5.15 in the headspace. All the vials were then incubated at 37°C with continuous shaking at 200 rpm. After 6 hours, the cell suspension was filtered and the filtrate was subjected to GC analysis for methanol concentration. Two replicates were performed for each vial. The volume of CH_4 (6 mL) was selected to provide similar quantities of CH_4 (0.25 mmol) and O_2 (0.27 mmol) to the reactor headspace, while 80 mM formate was used to provide an essential amount of formate (0.25 mmol) corresponding to the CH_4 and O_2 levels in the headspace.

The effects of formate and phosphate on biogas to methanol conversion were evaluated using a 4 x 4 factorial design to test four levels of phosphate (0, 50, 100, and 200 mM as $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) and four levels of sodium formate (0, 40, 80, and 120 mM). The reactors were set up as described above (3.1 mL cell suspension and 40 mL reactor volume) except biogas was the source of CH_4 (9 mL, biogas: air ratio of 1:3.1 in the headspace) and the initial $\text{OD}_{600\text{nm}}$ was 0.67 ± 0.01 . At least three replicates were performed for each condition except for those with 0 mM phosphate, which had two replications. Low formate concentrations (10-20

mM) were not used because a preliminary experiment showed that methanol production was inconsistent at these levels (data not shown).

The time course of methanol production was examined by cultivating strain 14B at 37°C and 200 rpm in 50 mL of NMS medium (containing 1 μ M CuCl₂ and 50 mM phosphate) with different sodium formate levels (40, 80, or 120 mM) in 250-mL flasks with a 500-mL Tedlar gas bag connected to each flask. Biogas was the CH₄ source and the initial OD_{600nm} of the cell suspension was 0.75±0.02. The headspace and Tedlar gas bag contained biogas and air at a 1:2.5 ratio (v/v). Cell density (OD_{600nm}), gas composition (CH₄, O₂, N₂, CO₂), and methanol content were measured periodically. Three replicate reactors were set up for each condition. The gas composition data from one reactor (one replicate at 80 mM formate) were not included because of gas measurement error, and all the data from another reactor (one replicate at 120 mM formate) were not used due to culture seal failure.

2.5. Analytical methods

Samples of the cell suspension (1 mL) were collected for OD analysis. Optical density of the cell suspension was measured at 600 nm with an Eon[®] microplate spectrophotometer (Biotek, Winooski, VT, USA). Each sample (200 μ L) was measured at least three times. The specific growth rate (μ , h⁻¹) was determined by

$$\mu = \left[\ln\left(\frac{OD_t}{OD_0}\right) \right] / t \quad (1)$$

where OD₀ is the OD_{600nm} at the beginning of the exponential growth phase, OD_t is the OD_{600nm} at the end of the exponential growth phase, and t is the time period of the exponential growth phase (h). The pH of cultures was measured using a pH probe.

A linear relationship was determined between the OD at 600 nm and bacterial biomass. The dry weight of cell suspension was determined using an adapted version of the methods described in Cai et al. (2013). Briefly, 50 mL samples of cell suspension of known OD_{600nm} were centrifuged at 10,000 rpm for 15 min. The supernatant was discarded and the cell pellet was resuspended in 25 mL of 0.5 M NH₄HCO₃ to remove residual salts. The samples were centrifuged again at 10,000 rpm for 15 min. After the supernatant was discarded, the cell pellet was resuspended in 3 mL of 0.5 M NH₄HCO₃ and transferred to a pre-ignited (550°C) porcelain crucible and dried in a Thelco Model 18 oven (Precision Scientific, Chennai, India) at 105°C for 12 h. Ash weight was determined by heating the samples in an Isotemp muffle furnace (Fisher Scientific, IA, USA) at 550°C for four hours. The cell yield (Y_{x/CH_4}) for batch cultivations was determined by dividing the ash free dry weight (AFDW) at the stationary phase by the amount of CH₄ consumed during cultivation.

The composition of headspace gas (CO₂, CH₄, N₂, and O₂) was analyzed by GC according to methods described in Sheets et al. (2015). The methanol content of filtrate samples was analyzed using a GC (Shimadzu, 2010PLUS, Columbia, MD, USA) equipped with a Stabilwax polar phase column (30 m × 0.32 mm × 0.5 µm) and flame ionization detector. The temperatures of the injector and detector were both set at 250°C. The oven temperature was set at 50°C and gradually increased to 80°C at a rate of 5.0°C min⁻¹. Helium was used as the carrier gas with a total flow rate of 24.8 mL min⁻¹ and split ratio of 15. The concentration of methanol (g L⁻¹) was determined using an external standard curve prepared using pure methanol. Methanol productivity (g L⁻¹ d⁻¹) was determined by dividing the methanol content (g L⁻¹) by the cultivation time. CH₄ to methanol conversion (%) was determined by dividing the moles of methanol produced by the moles of CH₄ consumed.

2.6. Statistical analysis

Statistical significance was determined with analysis of variance (ANOVA, $\alpha=0.05$) or Tukey's Honestly Significant Difference (HSD) test ($\alpha=0.05$) using JMP Statistical Software from SAS Institute Inc. (Version 10.0.2, Cary, NC, USA). Experimental data are presented as average values $\pm$ standard error.

3. Results and discussion

3.1. Isolation and characterization of a methanotrophic strain from digestate

When the enrichment cultures were grown on agar plates with CH₄ as the sole substrate, shiny, smooth, butyrous/cartilaginous light brown or brown colonies formed. Strain 14B was one of the fastest growing isolates, and it was further characterized. An increase in culture turbidity was observed within 3-5 days after a colony of strain 14B was inoculated into liquid NMS medium with 20% CH₄ in the headspace. Slow growth was observed on 0.1% methanol, but no growth was detected on 0.1% (w/v) formate, acetate, xylose, glucose, or citrate. These results indicated that strain 14B was an obligate methanotroph. The V1-V3 region of its 16S rRNA gene shared 99%, 98%, and 98% sequence similarity with that of *Methylocaldum tepdium*, *Methylocaldum gracile*, and *Methylocaldum szegediense*, respectively, all of which are obligate methanotrophs (Bodrossy et al., 1997). Strain 14B also had morphological and physiological characteristics comparable to those of *Methylocaldum* species, especially *M. gracile* (Table 1) (Bodrossy et al., 1997, Eshinimaev et al., 2004). The type strain of *M. gracile* was isolated from activated sludge (Bodrossy et al., 1997, Eshinimaev et al., 2004, Ge et al., 2014), and strain 14B might also come from the inoculum of the SS-AD reactors. Additionally, strain 14B had a similar growth rate when either NO₃⁻ (0.048 h⁻¹) or NH₄⁺ (0.045 h⁻¹) was used as the nitrogen

source. Because NH_4^+ is the primary component of total nitrogen in AD, strain 14B may have used NH_4^+ as a nitrogen source in the SS-AD reactors (Shi et al., 2014).

The pH range for growth was 6.0 to 7.6 (no clear optimum), which was slightly lower than the pH of digestate samples (pH=8.0) (Sheets et al., 2015). Strain 14B had stable growth within the mesophilic temperature range of 37-42°C, but did not grow at or above 47°C. NaCl concentration above 2 g L⁻¹ significantly reduced the specific growth rate ($p < 0.05$) and no growth was observed above 10 g L⁻¹ NaCl, which was comparable to the *Methylocaldum* strains isolated by Eshinimaev et al. (2004). Similar to the *Methylocaldum* strains isolated by Bodrossy et al. (1997), strain 14B grew slowly in NMS medium without Cu^{2+} supplement, but grew well at 1.0-10.0 μM CuCl_2 . As minimizing input costs is critical for scale-up of the process, 1.0 μM CuCl_2 was used in remaining experiments. To the authors' knowledge, this is the first methanotrophic strain isolated from SS-AD, which confirms that AD systems can be a source to isolate methanotrophic bacteria (Corder et al., 1986, Ge et al., 2014).

3.2. Comparison of growth on different methane sources

Strain 14B was able to grow using either biogas or purified CH_4 as the primary carbon source (Figure 1), and no significant difference (Tukey HSD, $p > 0.05$) between biogas and purified CH_4 was noted for growth rate, CH_4 consumption, or biomass yield of strain 14B (Table 2). Additionally, strain 14B had a growth rate ($\mu = 0.06 \text{ h}^{-1}$) and CH_4 conversion rate (0.68-0.71 mmol L⁻¹ h⁻¹) comparable to those of *Methylosinus trichosporium* OB3b ($\mu = 0.024$ -1.0 h⁻¹, 0.8-0.9 mmol L⁻¹ h⁻¹), the most commonly studied methanotroph for CH_4 to methanol conversion (Duan et al., 2011, Ge et al., 2014, Mehta et al., 1991, Rostkowski et al., 2013). Lower cell yield (0.2 g cells g⁻¹ CH_4) compared to *M. trichosporium* OB3b (0.5-0.7 g cells g⁻¹ CH_4) indicated

strain 14B oxidized CH₄ primarily for energy generation rather than for biomass production (Kalyuzhnaya et al., 2015, Rostkowski et al., 2013).

The main difference between biogas and purified CH₄ is that biogas contains CO₂ and H₂S. CO₂ can lower culture pH and has also been shown to inhibit MDH activity (Xin et al., 2004). However, the pH of the cultures grown on biogas was close to neutral (6.0-6.8) because of sufficient buffering in the NMS medium. Lack of methanol accumulation during all growth experiments also suggested that MDH activity was not inhibited by the CO₂ present in the biogas. Additionally, the H₂S content of the biogas was less than 50 ppm, which was much lower than the levels inhibitory to methanotrophs (>100 ppm) (Cáceres et al., 2014). Overall, biogas from a commercial scale AD system is a suitable growth substrate for strain 14B. This indicates that the costly purification steps used in conventional biogas upgrading are not needed for a biological upgrading process that uses this methanotroph and biogas with low H₂S content (Ge et al., 2014, Yang et al., 2014). Biogas produced at other AD facilities may have higher H₂S content and could negatively impact the growth of methanotrophs. Further studies could be conducted to determine the inhibitory levels of H₂S on biological conversion of biogas to methanol (Cáceres et al., 2014).

3.3. Screening of methanol dehydrogenase inhibitors for methanol production

All the tested MDH inhibitors caused strain 14B to accumulate methanol (Table 3), indicating several different chemicals can be used for this purpose (Hwang et al., 2015). Surprisingly, methanol was accumulated even without the addition of MDH inhibitors (i.e. 80 mM formate). In some cases, a small amount of methanol was also produced when only MDH inhibitors were added, for example 200 mM phosphate (Table 3), suggesting not all of the

methanol produced was further oxidized. Methanol production at different levels of formate and phosphate was further investigated with a full factorial experiment (Section 3.4).

3.4. Effects of formate and phosphate concentrations on methanol production

In the absence of formate, methanol production increased linearly ($R^2=0.94$) with increasing phosphate concentration (Figure 2), indicating higher levels of phosphate caused a decrease in MDH activity (Duan et al., 2011). However, the maximum methanol content in those cultures was low ($0.04\text{--}0.05\text{ g L}^{-1}$ at 200 mM phosphate). This is likely attributed to the limited supply of reducing equivalents for CH_4 to methanol conversion by MMO (Ge et al., 2014). When 40 mM formate was added, methanol productivity increased by 4- to 22-fold (Figure 2), because formate dehydrogenase (FDH) oxidized the exogenous formate to produce electrons for methanol production (Mehta et al., 1991, Takeguchi et al., 1997).

Interestingly, high methanol productivities ($0.5\text{--}1.0\text{ g L}^{-1}\text{ d}^{-1}$) were obtained in reactors supplied with formate but not phosphate (Figure 2). This result indicated that electron generation by FDH was the limiting factor for methanol production by strain 14B and may have been caused by an already low MDH activity under those cultivation conditions. Previously, Eshinimaev et al. (2004) showed that FDH in *M. szegedienze* had much higher activity ($85\text{--}211\text{ nmol min}^{-1}\text{ mg}^{-1}\text{ protein}$) than MDH ($2\text{--}3\text{ nmol min}^{-1}\text{ mg}^{-1}\text{ protein}$) at 37°C . Therefore, strain 14B may have rapidly generated electrons that were used to oxidize CH_4 to methanol faster than MDH could convert methanol to formaldehyde. Additionally, Leak and Dalton (1983) showed that MMO activity in *Methylococcus capsulatus* (Bath) was stimulated by formate (5 mM). Higher activities of MMO and FDH and low activity of MDH might have contributed to methanol accumulation. The results from this study suggest that high concentrations of formate and small amounts of MDH inhibitors can be used to promote methanol production by strain

14B. After plating the seed culture that was used for one replicate of the full factorial experiment on NMS solid medium with 0.05% glucose, a non-methanotrophic bacterial isolate was observed. However, methanol production was consistent among all replicates (Figure 2), indicating the process was resistant to contamination. No other non-methanotrophic bacteria were observed during any other experiments.

The maximum methanol productivity ($1.09 \text{ g L}^{-1} \text{ d}^{-1}$) was obtained in reactors supplied with 50 mM phosphate (Figure 2). Currently, this is the third highest reported methanol productivity, behind that reported by Mehta et al. (1991) ($4.1 \text{ g L}^{-1} \text{ d}^{-1}$) and Hwang et al. (2015) ($1.18 \text{ g L}^{-1} \text{ d}^{-1}$) and is supported by a recent review by Ge et al. (2014). A kinetic study was conducted to further elucidate the effects of formate on biogas to methanol conversion (Section 3.5).

3.5. Effects of formate concentration on biogas to methanol conversion

Methanol production increased linearly (from 0 g L^{-1} to $0.23\text{-}0.27 \text{ g L}^{-1}$) in the first 12 hours at all formate concentrations (Figure 3). However, faster CH_4 consumption and higher CO_2 production were observed in reactors with low formate concentration (40 mM). In those reactors, methanol production also ceased after 12 hours, and CO_2 and cell biomass increased rapidly (Figure 3a). This suggests that reducing power had been depleted in these reactors and strain 14B had to use methanol as a source of energy and reducing power (Ge et al., 2014). Consequently, the 48-h CH_4 to methanol conversion ratio was $0.0\pm0.0\%$ in reactors with 40 mM formate.

More than 40 mM formate was needed to maintain methanol production. For example, reactors with 80 mM and 120 mM formate produced methanol at a peak level ($0.34\text{-}0.43 \text{ g L}^{-1}$) in 48-50 hours, and then declined after 50 hours (data not shown). This is a commonly observed phenomenon that has been attributed to mass transfer limitations and/or cell inhibition caused by

methanol accumulation (Kim et al., 2010, Pen et al., 2014). As an example, Furuto et al. (1999) showed that methanol concentrations above 0.32 g L^{-1} could inhibit pMMO activity in *M. trichosporium* OB3b. Decreases in pMMO activity would explain the lower CH_4 oxidation rates (Figure 3b-c) and the decline in cell density after methanol accumulated in reactors at 80 mM and 120 mM formate (Figure 3b-c). Reduced methanol production at 120 mM formate was likely because the higher formate concentrations can inhibit the activity of the FDH enzyme (Yoch et al., 1990). Decreased FDH activity would reduce the amount of electrons available for MMO, thereby lowering methanol production (Yoch et al., 1990). The 48-h CH_4 to methanol conversion ratio was $23.3 \pm 0.7\%$ in reactors with 120 mM formate.

Overall, maximum 48-h CH_4 to methanol conversion ratio ($25.5 \pm 1.8\%$) and methanol concentration ($0.43 \pm 0.00 \text{ g L}^{-1}$) were obtained in reactors with 80 mM formate (Figure 3b). In general, these results are comparable to others (conversion=27-80%, methanol concentration= $0.44\text{-}1.12 \text{ g L}^{-1}$) (Corder et al., 1986, Duan et al., 2011, Ge et al., 2014, Kim et al., 2010). Although Duan et al. (2011) achieved a methanol content of 1.12 g L^{-1} , they used 5% paraffin to increase gas-liquid mass transfer of gaseous substrates (CH_4 , O_2) and to maintain a very high cell density ($>1.0 \text{ g L}^{-1}$) (Duan et al., 2011). It is reasonable to envisage that comparable or higher methanol productivities could be obtained with strain 14B using reactors that are designed to overcome mass transfer limitations to provide high cell densities (Pen et al., 2014). Currently, the high price of formate limits its use as an electron donor for biological conversion of biogas to methanol. Economic feasibility could be improved by genetically modifying methanotrophs to use carbon sources in wastewater (i.e. acetate) as electron donors. Furthermore, isolation of methanotrophs that can use renewable electricity as an electron source is an attractive area of future research (Ge et al., 2014).

4. Conclusion

A methanotrophic bacterial isolate with similar characteristics to *Methylocaldum* species was isolated from solid-state anaerobic digestate. The isolate successfully grew on biogas and purified CH₄ as the carbon source. The methanotrophic isolate also converted biogas to methanol. Several methanol dehydrogenase (MDH) inhibitors increased methanol accumulation from biogas. Methanol production was also achieved by adding only phosphate to the cultivation medium, but was improved when formate was included. High levels of methanol could also be attained using formate and no MDH inhibitor.

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487 **Figure Captions**

488 **Tables**

489 Table 1: Comparison of growth characteristics of strain 14B to those of *Methylocaldum* species

490 Table 2: Effect of CH₄ source on growth of strain 14B

491 Table 3: Effects of MDH inhibitors on methanol production

492 **Figures**

493 Figure 1: Growth, gas consumption, and gas production using purified CH₄ (a) and biogas (b) a
494 substrate

495 Figure 2: Effects of phosphate and formate concentrations on methanol production from biogas.
496 *=no methanol detected.

497 Figure 3: Time course of methanol production at 40 mM formate (a), 80 mM formate (b), and
498 120 mM formate (c).

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507 Table 1: Comparison of growth characteristics of strain 14B to those of *Methylocaldum* species

	Strain 14B	<i>M. gracile</i>	<i>M. tepidium</i>	<i>M. szegidienze</i>
Cell morphology	Coccus/pleomorphic	Thin rod/coccus	Rod/pleomorphic	Rod/Pleomorphic
Motility	+	+	+	+
Colony description	Irregular, shiny, smooth, butyrous/cartilaginous, light brown to brown	Irregular, shiny, smooth, pasty/cartilaginous, brown to dark brown	Irregular, shiny, smooth, butyrous/cartilaginous, light brown	Irregular, shiny, smooth, mucoid/cartilaginous, brown to dark brown
Color in liquid	Light brown to brown	Brown	Brown	Brown
Growth on C2-C6 compounds	-	-	-	-
Growth on other C1 compounds	+ ^a	ND	-	-
Cu ²⁺ range	1.0-10 µM ^b	ND	1 µM ^c	1 µM ^c
Temperature range (°C)	37-42	20-47	30-47	37-62
pH range	6.0-7.6 ^d	ND	ND	ND
Nitrogen sources	NO ₃ ⁻ , NH ₄ ⁺	NO ₃ ⁻ , ^e	NO ₃ ⁻ , ^e	NO ₃ ⁻ , ^e
NaCl range	0-2 g L ⁻¹	ND	ND	ND
Isolation source	Solid-state anaerobic digestate	Activated sludge	Agricultural soil	Anaerobic effluent of hot spring
Reference	This study	(Bodrossy et al., 1997)	(Bodrossy et al., 1997)	(Bodrossy et al., 1997)

508 ^a. slight increase in turbidity in 0.1% methanol

509 ^b. no significant difference (Tukey HSD, p>0.05) in growth rate at each level. 1.0 µM selected as
510 preferred level

511 ^c. only concentration reported

512 ^{d.} no growth below pH 5.5
513 ^{e.} only nitrogen source reported
514 ND=not determined

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Table 2: Effect of CH₄ source on growth of strain 14B

CH ₄ source	Growth rate (h ⁻¹)	Yield (g cells g ⁻¹ CH ₄)	CH ₄ consumption (mmol L ⁻¹ h ⁻¹)
Pure CH ₄	0.062±0.00	0.19±0.02	0.68±0.03
Biogas	0.056±0.00	0.20±0.04	0.72±0.05

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Table 3: Effects of MDH inhibitors on methanol production

MDH inhibitor	MDH inhibitor concentration (mM)	Formate (mM)	Methanol productivity (g/L/d)
None	0	80	0.98±0.05
	0	0	ND
EDTA	0.5	80	1.12±0.03
	5.0	80	1.04±0.14
	5.0	0	ND
Phosphate	100	80	1.17±0.04
	200	80	0.95±0.13
	200	0	0.28±0.00
NaCl	100	80	1.00±0.04
	200	80	0.85±0.09
	200	0	0.08 ^a
MgCl ₂	20	80	0.98±0.13
	40	80	1.04±0.01
	40	0	ND

^a.Detectable in only one samplePurified CH₄ used as substrate

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(a)

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Figure 1: Growth, gas consumption and gas production using purified CH₄ (a) and biogas (b) as substrate

Figure 2: Effects of phosphate and formate concentrations on methanol production from biogas.
 *=no methanol detected.

(a)

(b)

(c)

Figure 3: Time course of methanol production at 40 mM formate (a), 80 mM formate (b), and 120 mM formate (c).