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How Low Can You Go: Methane Production of *Methanobacterium congolense* at Low CO_2 Concentrations

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Autotrophic hydrogenotrophic methanogens use H_2/CO_2 as sole carbon and energy source. In contrast to H_2, CO_2 is present in high concentrations in environments dominated by methanogens e.g., anaerobic digesters (AD), and is therefore rarely considered to be a limiting factor. Nonetheless, potential CO_2 limitation can be relevant in the process of biomethanation, a power-to-gas technology, where biogas is upgraded by the addition of H_2 and ideally reduce the CO_2 concentration in the produced biogas to 0–6%. H_2 is effectively utilized by methanogens even at very low concentrations, but little is known about the impact of low CO_2 concentrations on methanogenic activity. In this study, CO_2 consumption and CH_4 production kinetics under low CO_2 concentrations were studied, using a hydrogenotrophic methanogen, *Methanobacterium congolense*, as model organism. We found that both cellular growth and methane production were limited at low CO_2 concentrations (here expressed as Dissolved Inorganic Carbon, DIC). Maximum rates (V_{max}) were reached at [DIC] of 100 mM (extrapolated), with a CO_2 consumption rate of 69.2 *f*mol cell^{-1} d^{-1} and a CH_4 production rate of 48.8 *f*mol cell^{-1} d^{-1}. In our experimental setup, 80% of V_{max} was achieved at [DIC] >9 mM. DIC half-saturation concentrations (K_m) was about 2.5 mM for CO_2 consumption and 2.2 mM for CH_4 production. No CH_4 production could be detected below 44.4 μM [DIC]. These data revealed that the limiting concentration of DIC may be much higher than that of H_2 for a hydrogenotrophic methanogen. However, DIC is not a limiting factor in ADs running under standard operating conditions. For biomethanation, the results are applicable for both *in situ* and *ex situ* biomethanation reactors and show that biogas can be upgraded to concentrations of 2% CO_2 (98% CH_4) while still retaining 80% V_{max} at pH 7.5 evaluated from *M. congolense*. Since DIC concentration can vary significantly with pH and pCO_2 during biomethanation, monitoring DIC concentration through pH and pCO_2 is therefore important for keeping optimal operational conditions for the biomethanation process.

Keywords: methanogenesis, hydrogen, carbon dioxide, biomethanation, CO_2 threshold, CO_2 kinetics, biogas upgrading

INTRODUCTION

Methanogenic archaea play a key role in the production of biogas from anaerobic digesters (AD), yielding a product gas with 50–75% CH_4 and 25–50% CO_2 (Plugge, 2017). Methanogenic archaea here produce CH_4 from either H_2/CO_2 (hydrogenotrophic methanogensis, $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$) or acetate (acetoclastic methanogensis, $CH_3COOH \rightarrow CH_4 + CO_2$). Hydrogenotrophic methanogens are ubiquitous in natural anaerobic environments other than engineered AD systems, e.g., the gastrointestinal tracts, flooded soils, and anoxic lake and marine sediments (Whitman et al., 2014). In anaerobic environments, H_2 is an intermediate produced by fermentative and syntrophic bacteria, where it undergoes rapid turnover and its concentration is extremely low (Lin et al., 2012). Use of H_2 as an electron donor is however not restricted to hydrogenotrophic methanogens, but other anaerobic microorganisms, e.g., sulfate reducers and acetogens compete for available H_2 with methanogens in anoxic environments (Robinson and Tiedje, 1984; Cordruwisch et al., 1988; Kotsyurbenko et al., 2001). Therefore, many studies have been committed to the understanding of H_2 uptake kinetics of hydrogenotrophic methanogens through either pure cultures or the whole microbial community in environmental samples (e.g., Conrad, 1999; Kotsyurbenko et al., 2001; Eecke et al., 2012, 2013).

In an AD, dissolved H_2 concentration is usually low [0.5–3 μM, (Frigon and Guiot, 1995)]. Low H_2 concentration limits methane production through hydrogenotrophic methanogenesis, which has been verified by many studies devoted to biogas upgrading by injecting H_2 directly into the AD (Luo and Angelidaki, 2012; Agneessens et al., 2017). Above studies showed that the addition of H_2 to ADs greatly increases methane concentration in the biogas, while decreasing CO_2 concentration—a process known as biomethanation.

Through biomethanation the CH_4 concentration is increased to as high as natural gas quality (>95%), and thus this process dramatically alters the standard operational conditions in AD because CO_2 concentration is correspondingly reduced to lower than 5%. Such low CO_2 concentration is rarely seen in natural anaerobic environments where methanogens are present, so it is not clear whether such low CO_2 concentration affects the activities of hydrogenotrophic methanogens. However, CO_2 is known to be an important substrate for hydrogenotrophic methanogens, as it serves as both electron acceptor for energy production and (sole) carbon source for biosynthesis through the Wood-Ljungdahl pathway (Berg, 2011; Borrel et al., 2016). To the best of our knowledge, there is limited knowledge about CO_2 uptake kinetics of methanogens in literature. Nevertheless, understanding CO_2 uptake kinetics of methanogens could consequently be crucial when dealing with the concept of biomethanation, which aims at upgrading the CH_4 concentrations in biogas to >95% by consuming CO_2 to as low concentration as possible.

Previous work gives some insights about limitation of CO_2 consumption rate and methanogenic rate at low CO_2 concentrations during biomethanation (Luo et al., 2012; Garcia-Robledo et al., 2016; Agneessens et al., 2017). Here it was shown that H_2 consumptions rate decreased when headspace CO_2 concentrations was lower than 12% during H_2 pulse injection batch experiments in bioreactors (Agneessens et al., 2017), while an inhibition of H_2 consumption rate was found when CO_2 concentration was below 6% in a methanogenic manure samples (Garcia-Robledo et al., 2016). These results thus indicate that CO_2 uptake rate and methanogenic rate is limited at low CO_2 concentrations with great implications for the limits of the biomethanation technology. However, the exact impact of CO_2 on the methanogenic activity was not clearly depicted by those studies, as they were conducted on complex microbial communities that include both CO_2 consumers and producers—including homoacetogens that compete with methanogens for H_2 and CO_2. Therefore, a thorough understanding of methanogenic reaction kinetics at low CO_2 concentrations seems necessary in order to find out under which conditions hydrogenotrophic methanogenic rates will be reduced or even inhibited during biomethanation. This will enable us to optimize the efficiency of biomethanation.

In this study, we present the first trial to examine CO_2 uptake kinetics of a hydrogenotrophic methanogen, *Methanobacterium congolense*, by studying its CH_4 production and CO_2 consumption rates at low CO_2 concentrations with surplus of H_2. We chose to study a model organism from the genus *Methanobacterium* because methanogens of this genus were found to increase substantially after pulse H_2 injections in reactors with mesophilic sludge (Agneessens et al., 2017). *M. congolense* was used as test strain because it is a mesophilic methanogen originally isolated from a mesophilic anaerobic digester, and also because it solely utilizes H_2 and CO_2 as substrates for growth and methane production (Cuzin et al., 2001).

MATERIALS AND METHODS

Strains and Culture Medium

Type strain *Methanobacterium congolense* (DSM7095) was purchased from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The methanogen was cultivated with a H_2:CO_2 (4:1) gas mixture at 37°C in versatile medium for methanogenic archaea, based on the medium developed by Khelaifia et al. (2013), but adding only acetate, formate and $NaHCO_3$ as carbon source.

For the kinetic studies of CO_2 uptake by *M. congolense*, the versatile medium was prepared (pH 7) without any carbon sources, by omitting the addition of acetate, formate, and $NaHCO_3$ (hereinafter referred to as mineral medium). CO_2 gas injected into the headspace at the beginning of incubation was thus the sole carbon source and electron acceptor for *M. congolense* during all incubations. To minimize pH effects by high amount of CO_2, a version of mineral medium with higher buffering capacity, 10 times higher K_2HPO_4 (5.0 g/L) and 5 times higher KH_2PO_4 (2.5 g/L) than that of the mineral medium, was employed for cultures with high CO_2 concentration (HBC mineral medium, pH 7). *M. congolense* performed normal

growth in either medium or HBC mineral medium as in the versatile medium.

Experimental Setup

Two series of batch culture experiments were carried out for determination of CO_2 uptake kinetics of *M. congolense*: long-term batch culture lasted for about 1 week and short-term batch culture lasted for around 3 h (see details below). For both batch cultures, *M. congolense* was cultivated in 330 mL serum bottles filled with 150 mL sterile medium and sealed with butyl rubber stoppers. Cultures were transferred several times in either mineral medium or HBC mineral medium before batch culture experiments, in order to make certain that they were adapted to the medium.

Long-Term Batch Culture

A series of 330 mL serum bottles filled with 150 mL mineral medium were prepared aseptically for the long-term batch culture. Firstly, the bottles were flushed thoroughly with sterile H_2 gas (0.22 μm filtered) through butyl rubber septa and headspace pressure was kept at around 2 atmospheres eventually. Different volumes of sterile CO_2 (0.22 μm-filter filtered), 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 mL, were subsequently injected into the headspace to reach different CO_2 partial pressure in the bottles. The mole fraction of H_2:CO_2 was higher than 4:1 in all bottles, so H_2 was in excess. The bottles were incubated overnight at 37°C with continuous shaking (90 rpm) to allow the partitioning of CO_2 between the gas and liquid phases and the dissolution of CO_2 in water to be at equilibrium before the experiment started. Following overnight incubation, headspace pressure in each bottle was measured and gas composition was determined by gas chromatography (GC). Based on pressure and gas composition, CO_2 partial pressure in the headspace (pCO_2) were calculated and used to make a series of standard curves for describing the carbonate system in the bottles with sterile medium (details in Standard Curves).

Each of the long-term bottles was inoculated with 8 mL inoculum from an exponential growth phase culture of *M. congolense*. The bottles were incubated at 37°C and shaken at 90 rpm for about 1 week. During incubation, headspace pressure was monitored and 1 mL headspace was taken for gas composition analysis at regular intervals. 2 mL of medium was sampled aseptically once a day for optical density at 600 nm (OD600) and pH measurement.

Short-Term Batch Culture Experiment

M. congolense was incubated in either mineral medium or HBC medium with 4:1 H_2:CO_2 gases in the headspace. When the methanogens reached late exponential growth phase, as indicated by OD600, the short-term batch cultures were flushed thoroughly with sterile H_2 gas (0.22 μm filtered) through a needle submerged in the liquid phase. The final pressure of H_2 in the headspace was kept at around 2 atmospheres to ensure that H_2 was in excess. Different volumes of sterile CO_2 gas was subsequently injected into the headspace: 2, 4, 8, 12, 16, 20, 24, 32, and 40 mL for bottles with mineral medium and 4, 12, 14, 20, 28, 32, 40, 60, 80, and 90 mL for bottles with HBC mineral medium. Some of the above setup was repeated to check data reproducibility.

Following CO_2 gas injection, the bottles were incubated in a rotary incubator (37°C, 90 rpm) for about 1 h to allow the partitioning of CO_2 between the gas and mineral medium and the dissolution of CO_2 in the medium to reach equilibrium. The headspace pressure and gas composition (CO_2 and CH_4) were monitored with an interval of 15–25 min for 1.5–2 h. At the beginning and end of the experiment, 2 mL liquid were removed aseptically from the bottle for OD600 and pH measurement and 2 mL for determination of cell abundance using quantitative PCR (qPCR). OD600 stayed nearly constant during the experiment. pH at the beginning of the experiment varied with CO_2 amount injected to the bottle. The maximum pH variation was observed with 40 mL CO_2 injected into bottles with mineral medium, where pH dropped to 6.44, compared to pH 7 before adding any CO_2. pH dropped to 6.63 with 90 mL CO_2 injected into HBC mineral medium bottles.

Analytical Measurements

The pressure of headspace was monitored by gas pressure sensor during the incubation. Headspace gas composition (CO_2 and CH_4) was determined immediately on a gas chromatograph equipped with a thermal conductivity detector (Shimadzu-2014) and a stainless steel column packed with Poropaq Q column. The carrier gas was helium. OD600 was measured on a Genesys 10 UV-VIS spectrophotometer (ThermoFisher, USA). pH was measured using a pH meter B-71X (Horiba, Kyoto, Japan).

Cell Abundance Estimation

Liquid samples for cell abundance were flash frozen with liquid nitrogen and stored at −20°C until analysis. DNA was extracted by using FastDNA kit (MP Biomedicals, LLC) and quantitative PCR was executed for quantification of cell abundance by using archaeal 16S rRNA primer pair–arc806F and arc915r-mod (Chen et al., 2017). Cell abundances of *M. congolense* were estimated by dividing the 16S gene copies with a factor of 3, since *M. congolense's* genome harbors three 16S rRNA gene copies (Tejerizo et al., 2017).

Carbonate System Calculation

Standard Curves

Following the measurements in Long-Term Batch Culture, standard curves were generated with sterile medium for carbonate system calculation. Under gas-liquid equilibrium conditions, CO_2 gas injected into the 330 mL bottles with 150 mL sterile mineral medium at the starting time ultimately split into two fractions, CO_2 gas in the headspace [$CO_{2(g)}$] and dissolved inorganic carbon (DIC) in the medium. The latter was composed of three species (dissolved CO_2 gas–$CO_{2(aq)}$, HCO_3^- and CO_3^{2-}).

To generate standard curves, pCO_2 values at equilibrium [$pCO_{2(eq)}$] was firstly measured in each bottle receiving different amounts of CO_2 ($\sum CO_2$). Then $pCO_{2(eq)}$ was plotted against $\sum CO_2$, and the resulting curve was used for the calculation of total CO_2 in each bottle during the experiments. Furthermore, [DIC] at equilibrium [$[DIC]_{(eq)}$] were calculated by subtracting

$CO_{2(g)}$ from $\sum CO_2$, and both $pCO_{2(eq)}$ and $\sum CO_2$ were plotted with $[DIC]_{(eq)}$ to generate standard curves for deduction of [DIC] during experiments.

Standard curves for carbonate system in the HBC mineral medium were generated in a similar fashion.

Carbonate System Calculation During Incubation

During the microbial growth, we assumed that the buffering capacity in the medium changed very little because the uptake of phosphate was minor compared to the amount of phosphate present in the medium. Thus, the carbonate system in bottles with methanogens behaved similarly as in sterile medium without microbial activity. This was supported by the fact that, when CO_2 was nearly completely consumed by methanogens at the end of long-term batch culture experiments, medium pH returned to nearly 7, which was the initial sterile medium pH before CO_2 injection.

Based on the above assumption, $\sum CO_2$ in the bottle and [DIC] in the medium during the incubation were estimated from measured pCO_2 by using the standard curves generated in Standard Curves. Concentrations of three inorganic carbon species in the medium ($CO_{2(aq)}$, HCO_3^- and CO_3^{2-}) were estimated by software CO_2SYS (Pierrot et al., 2006) by providing pCO_2 and [DIC]. In this study, carbonate is negligible because the pH range was about 6.44–7, so $[DIC] \approx [CO_{2(aq)}] + [HCO_3^-]$. Both $CO_{2(aq)}$ and HCO_3^- are bioavailable carbonate species, and they are shown to be utilized by methanogens in different steps in methanogenesis and carbon fixation (Ferry, 2013). Moreover, the enzyme carbonate anhydrase, which can actively transform HCO_3^- to CO_2 gas or vice versa, is found to be ubiquitous in the culturable methanogens isolated so far, including *M. congolense*. Therefore, DIC is used as the main parameter for studying the CO_2 uptake kinetics here.

Calculation of Kinetic Parameters

Short-Term Batch Culture Experiment

$\sum CO_2$ consumption and CH_4 production kinetics were estimated from specific $\sum CO_2$ consumption rates ($V_{\sum CO_2}$) and specific CH_4 production rates (V_{CH_4}) in a range of [DIC] in the medium.

[DIC] values were estimated from the CO_2 gas volumes injected in the bottles by fitting them to the standard curve of [DIC] and initial CO_2 amount injected as mentioned in Standard Curves.

In each incubation, $V_{\sum CO_2}$ was calculated by linear fitting of the $\sum CO_2$ concentration as a function of time. The $\sum CO_2$ concentrations in the incubations were estimated as indicated in Standard Curves. Similarly, V_{CH_4} was estimated by linear fitting of the methane concentration in the headspace as function of time in each incubation. We assumed here that the dissolved CH_4 was negligible due to its low solubility in water.

Long-Term Batch Culture

During the long-term incubation, specific $\sum CO_2$ consumption rates ($V_{\sum CO_2}$) were calculated by dividing $\sum CO_2$ consumed between two sampling points with time and normalized with cell abundance. Specific CH_4 production rates (V_{CH_4}) were calculated in a same manner. Since $V_{\sum CO_2}$ and V_{CH_4} changed with time during incubation, only the maximum $V_{\sum CO_2}$ ($r_{\sum CO_2}$) and V_{CH_4} (r_{CH_4}) from each bottle were taken for kinetic analysis.

The growth yields ($Y_{biomass}$) were calculated at the end of incubation when CO_2 was nearly consumed. We assumed that the CO_2 fraction, which was not converted to CH_4, was assimilated into cell biomass. Thus,

$$Y_{biomass} = 1 - [CH_{4(end)}] / \left([\sum CO_{2(initial)}] - [\sum CO_{2(end)}] \right)$$

Here, $CH_{4(end)}$ refers to total CH_4 amount produced by the end of incubation; $[\sum CO_{2(initial)}]$ refers to the $\sum CO_2$ amount injected at the beginning of the experiment; $[\sum CO_{2(end)}]$ refers to the $\sum CO_2$ amount left in the bottle at the end of the incubation.

During the exponential growth phase, cell numbers were plotted against the volume of $\sum CO_2$ consumed and CH_4 produced, respectively. The best-fit linear slopes were taken as the growth yields with respect to CO_2 consumed and CH_4 produced (Y_{CO_2} and Y_{CH_4}).

Modeling of Kinetics

In summary, we estimated $V_{\sum CO_2}$ and V_{CH_4} from three different experiment setups: (1) short-term incubation with versatile mineral medium; (2) short-term incubation with HBC mineral medium; (3) long-term incubation with versatile mineral medium ($r_{\sum CO_2}$ and r_{CH_4}).

We used the Michaelis-Menten equation to represent the effect of [DIC] on the performance of methanogen's $\sum CO_2$ consumption rate and CH_4 production rate, with the consideration of [DIC] threshold [DIC]*:

$$V_{\sum CO_2} = V_{\max-CO_2} \times ([DIC] - [DIC]^*) / (K_{m-CO_2} + [DIC] - [DIC]^*)$$

$$V_{CH_4} = V_{\max-CH_4} \times ([DIC] - [DIC]^*) / (K_{m-CH_4} + [DIC] - [DIC]^*)$$

Where: $V_{\max-CO_2}$ and $V_{\max-CH_4}$ are the maximum specific $\sum CO_2$ consumption rate and CH_4 production rate, respectively; K_{m-CO_2} and K_{m-CH_4} are the [DIC] giving one-half the maximum specific $\sum CO_2$ consumption rate and CH_4 production rate respectively.

By the end of the batch culture experiments, headspace CO_2 was depleted to a nearly constant partial pressure without further consumption, even though H_2 was still in surplus. This constant CO_2 partial pressure remained unchanged over a period even longer than the period of active CO_2 uptake. This indicates that there is a CO_2 and/or [DIC] threshold for *M. congolense*. The [DIC]* concentration at which no active CO_2 uptake activity could be detected was found to be $44.4 \pm 0.4\,\mu M$ of DIC ($N = 7$) in this study.

TABLE 1 | Growth parameters of *M. congolense* under different initial CO_2 amounts injected into the bottles.

Initial CO_2 amount (mL)	[DIC] (mM) at equilibrium	pCO_2 at equilibrium (matm)	Growth rate (d^{-1})	Maxmium CH_4 production rate, r_{CH_4} ($f$mol CH_4 cell^{-1} d^{-1})	Maxmium CO_2 consumption rate, $r_{\sum CO_2}$ ($f$mol CO_2 cell^{-1} d^{-1})	Growth yield		
						$Y_{biomass}$ (mol C per mol CO_2)	Y_{CO_2} (log number of cells per mol CO_2)	Y_{CH_4} (log number of cells per mol CH_4)
2	0.44	2.9	<0.02	0.26	0.21	21%	N.D.	N.D.
4	0.85	6.6	0.06	3.23	2.61	16%	N.D.	N.D.
8	1.62	14.6	0.06	6.65	5.04	22%	N.D.	N.D.
12	2.33	24	0.33	20.37	18.99	17%	14.25	14.22
16	2.96	34.9	0.28	34.42	32.87	11%	13.96	13.94
20	3.55	46.9	0.56	30.72	31.09	15%	13.96	13.96
24	4.13	59	0.47	43.94	47.6	21%	13.92	13.96
28	4.68	71.8	0.84	41.03	40.33	15%	13.95	13.93
32	5.13	87.5	1.12	47.76	57.12	26%	14.09	14.19
36	5.58	102.3	0.64	108.99	69.62	24%	14.07	14.11
40	6.16	114.7	0.94	66.83	80.9	22%	13.89	13.93

RESULTS

Growth of *M. congolense* in Long-Term Batch Cultures

Table 1 summarizes cell growth rates, maximum $\sum CO_2$ consumption ($r_{\sum CO_2}$) and CH_4 (r_{CH_4}) production rates and growth yield of *M. congolense* incubated under different initial $\sum CO_2$ amount and surplus H_2. Growth rates, r_{CH_4} and $r_{\sum CO_2}$ increased linearly with added $\sum CO_2$. Growth rate increased from <0.02 d^{-1} with 2 mL CO_2 (∼0.09 mmol, [DIC] = 0.44 mM) injected to about 1.12 d^{-1} with 32 mL CO_2 (∼1.43 mmol, [DIC] = 5.13 mM) injected. Lowest r_{CH_4} and $r_{\sum CO_2}$ (0.26 and 0.21 $f$mol cell^{-1} d^{-1}, respectively) were found in the bottle receiving least amount of CO_2 (2 mL). The bottle receiving 36 mL CO_2 showed the highest r_{CH_4} of about 108.99 $f$mol cell^{-1} d^{-1}, while the bottle receiving 40 mL CO_2 showed the highest $r_{\sum CO_2}$ of about 80.90 $f$mol^{-1} cell d^{-1}. The growth yield, $Y_{biomass}$-carbon assimilated for anabolism with respect to total CO_2 assimilated, was in the range of 11–26%, but it did not show a clear trend with $\sum CO_2$ amount injected. Cell specific growth yields with respect to $\sum CO_2$ consumption (Y_{CO_2}) and CH_4 production (Y_{CH_4}), which were estimated from data acquired during the exponential growth phase, were nearly equivalent in all conditions. Y_{CO_2} and Y_{CH_4} were consistent at around 0.8–1.8 × 10^{14} cells per mole gas consumed or produced, except for the low CO_2 conditions (2–8 mL) where cell growth was not observed during incubation. Y_{CO_2} and Y_{CH_4} estimated in the bottles receiving <8 mL CO_2 can be biased due to slow growth, therefore the yields were not used here.

In this long-term batch culture experiment, both r_{CH_4} and $r_{\sum CO_2}$ were of first order kinetics and showed a linear correlation with [DIC] instead of showing Michaelis-Menten kinetics. This implies that the saturation concentration of [DIC] for *M. congolense* is higher than 6 mM, which was the highest concentration tested in this study (**Table 1**). Unfortunately, attempts to further increase [DIC] by injecting more CO_2 gas (60 mL) with the same setup led to a pH drop from 7 to <6.4 and substantial reduction of methanogenic activity (data not shown).

Kinetics of $\sum CO_2$ Consumption and CH_4 Production

Figure 1 gives an example of how $V_{\sum CO_2}$ and V_{CH_4} were estimated in short-term batch culture experiments. The CO_2 gas in the headspace started to dissolve in the medium right after its injection, and cells started to utilize CO_2 and produce CH_4. CH_4 production rates were low but kept increasing in the first 1–1.5 h during CO_2 gas dissolution until CO_2 equilibrium between gas and liquid phase reached. After the CO_2 gas-liquid equilibrium, CH_4 production rate was stable for a few hours (data not shown). To minimize the influence of increasing cell abundance due to growth during experiment, specific CH_4 production rates were retrieved within 1–1.5 h after reaching CO_2 gas-liquid equilibrium. In CO_2-depleted medium *M. congolense* produced CH_4 at extremely low rates in the presence of H_2 (data not shown).

Figures 2A,B show the estimation of $V_{\sum CO_2}$ and V_{CH_4} from three independent experimental setups, respectively: (1) short-term incubation with versatile mineral medium; (2) short-term incubation with HBC mineral medium; (3) long-term incubation with versatile mineral medium. $V_{\sum CO_2}$ and V_{CH_4} were both dependent on available [DIC] and followed Michaelis-Menten kinetics. Maximum specific CO_2 consumption rate (V_{max-CO_2}) was estimated to be about 69.2 f mol CO_2 cell^{-1} d^{-1} and half-saturation concentration of DIC (K_{m-CO_2}) was about 2.5 mM. Maximum specific CH_4 production rate of *M. congolense*, V_{max-CH_4}, was about 48.8 f mol CH_4 cell^{-1} d^{-1} and half-saturation concentration of DIC (K_{m-CH_4}) was estimated to be about 2.2 mM. Reaction speeds of 80% V_{max-CH_4} could be reached at 9 mM [DIC]. Extrapolation of the fitted curve in **Figure 2B** shows that DIC concentrations needed for 90% V_{max-CH_4} and 100% V_{max-CH_4} would be 22 mM and 100 mM. As these values are outside the range of [DIC] that could be tested in the present setup without inferring changes in media pH, these

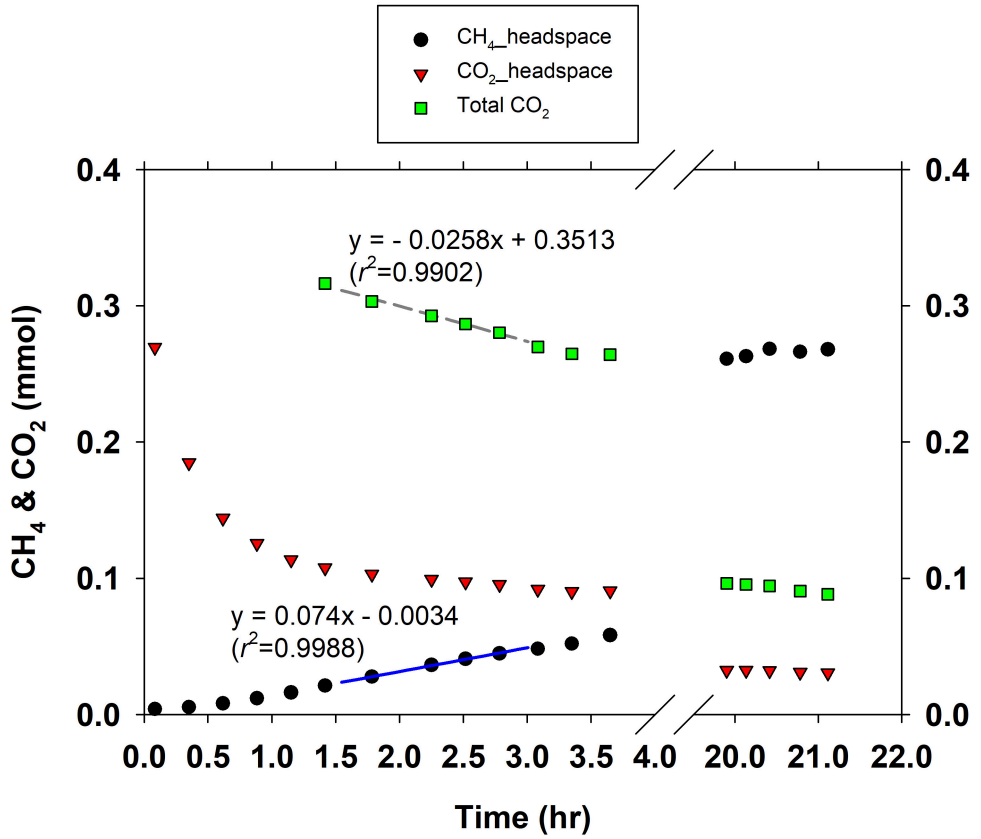

FIGURE 1 | An example for determination of methane production rate and DIC consumption rate in a short-term batch culture.

concentrations could not be verified experimentally and should thus be interpreted with great care.

Figure 2C shows that there was good correlation between $V_{\sum CO_2}$ and V_{CH_4} for cells in late exponential growth phase in the short-term batch culture, where $V_{\sum CO_2}$ was about 1.3 times higher than V_{CH_4}.

Estimation of Methanogenic Activities Under Various CO_2 Headspace Concentrations

Biomethanation aims at decreasing pCO_2 in the off-gas, which will directly influence [DIC] in the liquid (slurry) in AD. The [DIC] is furthermore dependent on slurry pH as this determines the CO_2/HCO_3^- partitioning. **Figure 3A** shows the profile of [DIC] with pH in liquid (salinity = 0) under headspace CO_2 concentration of 2, 5, 25, and 50%, assuming that the headspace pressure was 1 atmosphere and in equilibrium with slurry and using the growth pH range for *M. congolense* (5.9–8.2) (Cuzin et al., 2001) for calculation. It can be seen that increased pCO_2 in headspace refers to higher [DIC] in the slurry and [DIC] further increases with pH. It can be calculated that [DIC] is tens of mM in the slurry in a normal mesophilic AD, where CO_2 gas composes 25–50% of the biogas (Plugge, 2017). The lowest [DIC] is found at the lowest pH tested (5.9), about 9 mM, which can support the methanogenic rate of 80% of V_{max-CH_4}.

For biomethanation, it is critical to know how much pCO_2 can be lowered through upgrading CO_2 to CH_4 but without affecting methanogenic activity. In **Figures 3B,C**, we model the lowest pCO_2 required to maintain 2.2 and 9 mM [DIC] at different pH so as to maintain 50 and 80% of V_{max-CH_4} according to the kinetic modeling. It is found that CO_2 can be lowered to 5% and 2% at pH >6.2 and pH >6.8, respectively, with a methanogenic rate of 50% V_{max-CH_4} (**Figure 3B**). Correspondingly, with a methanogenic rate of 80% V_{max-CH_4}, CO_2 can be lowered to 5% and 2%, respectively, at pH >7.1 and pH >7.5 (**Figure 3C**).

DISCUSSION

Using batch-culture experiments, we provided the first estimation of CO_2/DIC uptake kinetics of an autotrophic hydrogenotrophic methanogen, *M. congolense*. We found that the affinity for DIC was dramatically lower than that of H_2, the other reactant involved in hydrogenotrophic methanogenesis. With a K_m of 2.2–2.5 mM, the affinity for DIC was shown to be a few tens to thousands times lower than the K_m of H_2, 0.44–66 μM, as previously reported for other hydrogenotrophic methanogens (Kotsyurbenko et al., 2001; Karadagli and

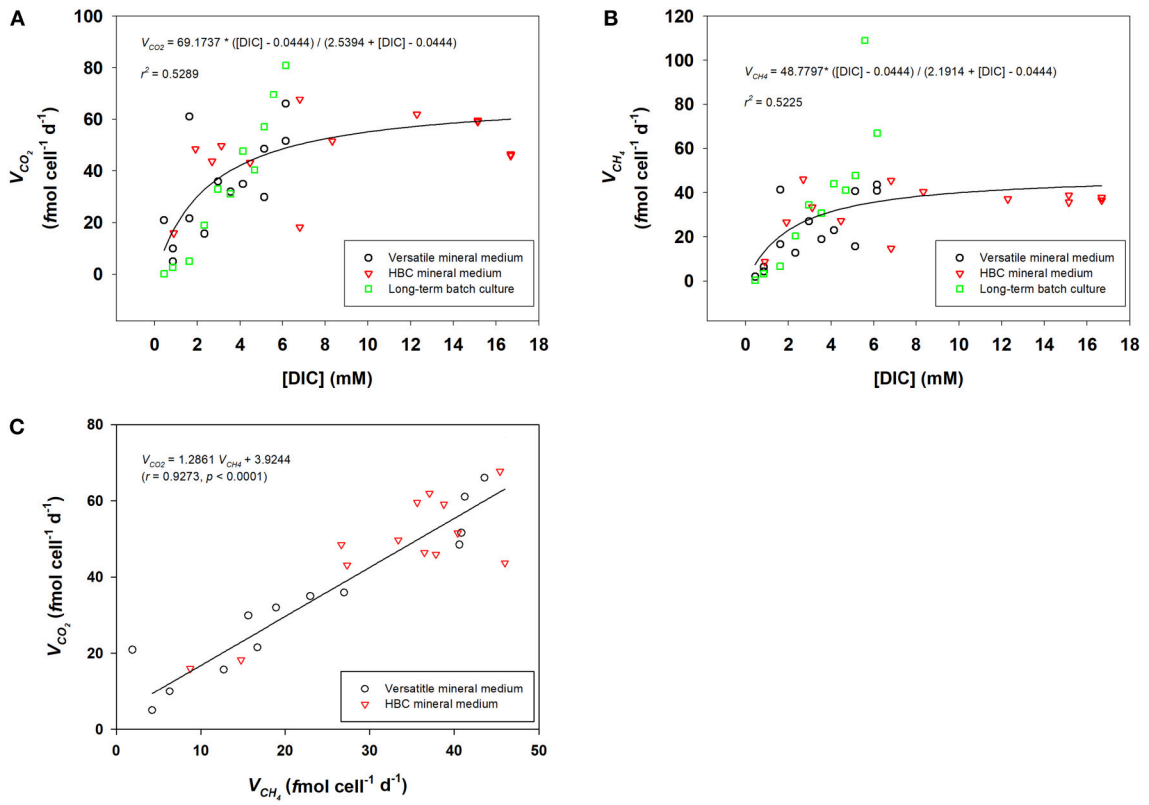

FIGURE 2 | K_m and V_m of DIC consumption **(A)** and methanogenic activity **(B)** for *M. congolense*, determined from both short-term and long-term batch cultures by Michaelis-Menten fitting. **(C)**, Correlation between CO_2 consumption rate (V_{CO_2}) and methane production rate (V_{CH_4}) in short-term batch cultures with both versatile mineral medium and HBC mineral medium ($N = 25$).

Rittmann, 2007). Likewise, DIC threshold of 44.4 μM, at which concentration the methanogenic activity could no longer be detected for *M. congolense*, was also hundreds to thousands times higher than reported H_2 thresholds of 6–70 nM, observed for methanogens (Lin et al., 2012). Such high K_m and threshold of DIC might be related to the CO_2 fixation pathway used by methanogens. *M. congolense* utilizes the Wood-Ljungdahl pathway for CO_2 fixation, which has previously been shown to have the highest K_m of DIC among the six autotrophic inorganic carbon assimilation pathways (Raven et al., 2012). From an evolutionary perspective, poor affinity for DIC is in accordance with the ubiquitous distribution of methanogens in habitats with high CO_2 concentrations, such as anaerobic digesters, animal guts and sediments. Maximum CH_4 production rate (V_{max-CH_4}, 48.8 *f*mol CH_4 cell^{-1} d^{-1}) of *M. congolense,* estimated from the kinetic model here is comparable to methanogenic rates found for methanogens from other complex environments incubated at a comparable temperature (35°C): 108-135 *f*mol CH_4 cell^{-1} d^{-1} in anaerobic reactors (Li and Noike, 1992) and 31.5 *f*mol CH_4 cell^{-1} d^{-1} in lake sediments (Lay et al., 1996).

Addition of different amounts of CO_2 gas at the beginning of the batch culture experiments caused the decrease of media pH due to the dissolution of CO_2 gas into the medium, where pH decreased more in bottles receiving higher amounts of CO_2. A further challenge was that the CO_2 concentration changed constantly during the incubation, due to continuous CO_2 consumption. Therefore, we controlled the amount of CO_2 added to keep pH within an optimal range for *M. congolense* (pH 5.9–8.2) (Cuzin et al., 2001), so that growth and methanogenic activity of *M. congolense* were not affected during our trials. A previous study on an obligate hydrogenotrophic autotrophic methanogen, *Methanocaldococcus* strain JH146, showed that pH did not affect methanogenic activity when it was within the range for optimal growth (Eecke et al., 2013). In our experiments, addition of 2–40 mL CO_2 lowered pH from 7 to 6.44–6.94 in the mineral medium, which has lower buffering capacity but methanogenic rates kept increasing with CO_2 amount and reached a rate of ∼44 *f*mol CH_4 cell^{-1} d^{-1} with 40 mL CO_2 (pH = 6.44). Moreover, specific $\sum CO_2$ consumption rates and CH_4 production rates determined from all three different experimental setups here were in good agreement with respect to [DIC], regardless of buffering capacity or incubation time (**Figure 2**). Thus, pH seems to have little impact on the rates within the experimental range. However, addition of excessive CO_2 beyond the buffering capacity was shown to greatly inhibit methanogenic activity: addition of 60 mL CO_2 into the mineral medium decreased pH to 6.01 and resulted in a dramatic reduction of methanogenic activity to ∼12 *f*mol CH_4 cell^{-1} d^{-1}.

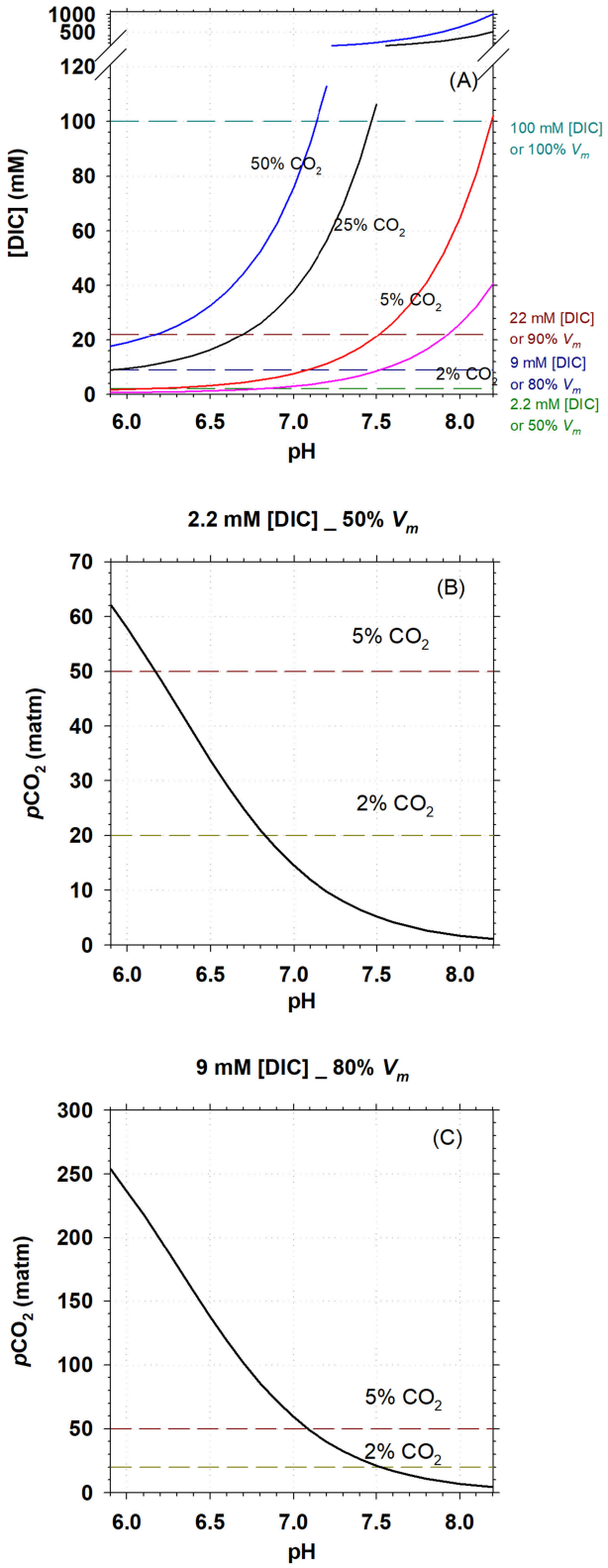

FIGURE 3 | Carbonate systems, methanogenic activity, and pH in mesophilic AD. **(A)**, Calculated DIC concentration in sludge in a mesophilic AD with headspace CO_2 concentration of 2, 5, 25, and 50%; pH was set to be within *(Continued)*

FIGURE 3 | the growth range for *M. congolense*. **(B,C)**, pCO_2 at pH range of 5.9–8.2 in an mesophilic AD, when [DIC] = 2.2 and 6 mM, respectively. Cell specific methanogenic rate for *M. congolense* is estimated to be 50 and 80% of V_{m-CH_4} at [DIC] of 2.2 and 9 mM, respectively, following the kinetic modeling from **Figure 2B**.

Our study also shows that DIC concentration influences the microbial growth rate. This is revealed by a clear reduction of growth rate from 1.12 d^{-1} at 5.13 mM [DIC] to <0.02 d^{-1} at the lowest DIC concentrations tested (0.44 mM) in the long-term batch culture experiment (**Table 1**). A previous study showed that the mixotrophic methanogen, *Methanosarcina barkeri*, has very slow growth and low methanogenic rate when incubated with only H_2 but lacking CO_2 (Weimer and Zeikus, 1978).

Here we showed that growth of *M. congolense* was limited when [DIC] was lower than 1.6 mM, although methanogenesis still continued at low rates. Methanogens fix CO_2 autotrophically into biomass through the Wood-Ljungdahl pathway, with which its methanogenesis pathway is associated (Berg, 2011). Therefore, whether the lowered growth rate at low [DIC] was due to reduced assimilation of carbon for biomass formation, or due to a reduced energy generation from methanogenesis remains unknown. Nonetheless, cell growth yield at the end of long-term incubation seems to be consistent for all DIC concentration tested: the methanogen converts approximately four moles of CO_2 into CH_4 for each mole of CO_2 incorporated into biomass (**Table 1**). Similar fraction of CO_2 was assimilated into biomass by late exponential growth phase cells during our short-term batch incubation, as $\sum CO_2$ consumption rate was about 1.3 times higher than methane production rate for all bottles (**Figure 2C**). The ratio of CO_2 used in dissimilatory and assimilatory metabolisms were thus independent of the [DIC] concentration.

As pCO_2 concentrations in anaerobic digesters is often high (25–50%), our data showed that inorganic carbon availability might not limit methanogenic activity of *M. congolense* under standard operating conditions of an anaerobic digester. Fermentation processes in the sludge will furthermore supply CO_2 to the hydrogenotrophic methanogens and hereby decrease the likelihood of CO_2 limitation under standard conditions. This study is of primary importance for biomethanation, a power-to-gas technology used for biogas upgrading to increase CH_4 concentration in the produced biogas through reduction of the CO_2 concentration. Knowledge on methanogen's CO_2/DIC kinetics is relevant as low CO_2 concentrations (<2%) are required to fulfill criteria for injection of upgraded biogas to the natural gas grid. If *M. congolense* is treated as a representative of hydrogenotrophic methanogens, the biogas can be upgraded to >98% CH_4 (<2% CO_2) at 80% V_{max-CH_4} when slurry pH in the reactor is >7.5 (**Figure 3C**) and 50% V_{max-CH_4} at pH >6.8 (**Figure 3B**). Our result suggests that the availability of bioavailable inorganic carbon under low CO_2 concentration might not greatly decrease methanogenic activity during biogas upgrading, but other factors, such as pH, might have greater impact on methanogenic rates.

A decrease in methanogenic activities to 50% of the maximum was shown in previous studies at different CO_2 concentrations: 2.9% CO_2 (59 mM [DIC] in methanogenic manure samples, Garcia-Robledo et al., 2016) and 10% CO_2 (257 mM [DIC] in anaerobic digestate, Agneessens et al., 2017). These [DIC] concentrations were higher than the K_m values reported here for *M. congolense*, which would indicate that the organisms in these studies either had lower affinities for [DIC]/CO_2 than *M. congolense*, or that their methanogenic activities were inhibited by other factors like NH_3 or pH. The pH levels of 8.2 (Garcia-Robledo et al., 2016) and 8.3 (Agneessens et al., 2017), were close to the value of 8.5 reported to be inhibitory to the biomethanation process (Angelidaki et al., 2018). As bicarbonate is the dominant buffering system in anaerobic slurries, it is often difficult to separate effects by high pH from effects by low CO_2 concentrations here, as these are inversely related. Through the pure culture study on *M. congolense* reported here, it was possible to separate the direct pH effect from low concentrations of CO_2 and hereby elucidate microbial physiological limitations to the process of biomethanation of a methanogenic type strain. The results are applicable to both *in situ* methanation, where H_2 and CO_2 are converted by methanogens in the main reactor, and separate *ex situ* reactor harboring specialized methanogenic communities.

CONCLUSIONS

Although CO_2 affinity of *M. congolense* is many times higher than H_2 affinity, CO_2 concentrations will only become severely limiting for biomethanation at very low [DIC] concentrations. Experiments were only carried out on a single methanogenic strain here and further testing of other methanogens will reveal if they elicit a similar affinity for CO_2.

AUTHOR CONTRIBUTIONS

XC performed the experiments and drafted the manuscript. All authors designed the study, interpreted the data and wrote the manuscript.

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