Research Article

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Methanol excretion by *Methylomonas methanica* is induced by the supernatant of a methanotrophic consortium

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Abstract

BACKGROUND: Methanotrophs play an important role in mitigating methane (CH_4) emissions in ecosystems. They closely interact with other microorganisms forming communities where the cross-feeding of metabolites, presumably methanol (MeOH), is essential for the growth and activity of non-methanotrophs. The experiments in this study were focused on investigating the effect of adding the supernatant from a methanotrophic consortium to pure cultures of *Methylomonas methanica*.

RESULTS: Methanol dehydrogenase inhibition caused the accumulation of MeOH, which resulted in a significant production after 3 h, with 1.99 mmol L^{-1} CH_3OH. The addition of the supernatant was associated with the excretion of MeOH by *M. methanica* and enhancement of the CH_4 consumption rate, despite a reduction in growth. The maximum MeOH concentrations were between 0.7 and 1.5 mmol L^{-1} CH_3OH.

CONCLUSION: These findings indicate that MeOH excretion may indeed be linked to a metabolic imbalance, which could potentially be compensated through the cross-feeding of metabolites within the methanotrophic community.

Supporting information may be found in the online version of this article.

Keywords: biofiltration of waste gases; bioconversion; biomethanol; environmental biotechnology

INTRODUCTION

The emission of greenhouse gases (GHG) is the leading cause of global warming and climate change. Methane (CH_4) is a powerful GHG, with a global warming potential 28–34-fold higher than CO_2.[1] Approximately 63% of CH_4 emissions are mainly of anthropogenic origin, from activities such as rice cultivation, cattle ranching, sanitary landfills, wastewater treatment, gas refining and coal mining.[1]

Aerobic methanotrophs use CH_4 as their carbon (C) and energy source, which is essential in the global C cycle as the ecosystem's primary biological sink for CH_4. They consume ≈90% of the CH_4 that is naturally produced, in addition to anthropogenic CH_4.[2,3] Thus, they are considered valuable agents in developing strategies for CH_4 emissions mitigation. Furthermore, the abundance of natural CH_4 sources, makes it a potential alternative feedstock to produce proteins, polymers and valuable chemicals with methanotrophic cultures.[4,5]

Methanotrophs are distributed within the phyla Proteobacteria (Alphaproteobacteria and Gammaproteobacteria), Verrucomicrobia, and the candidate division NC10.[6] Many of these microorganisms have been identified in CH_4-rich ecosystems, usually forming communities, and establishing close interactions with other non-methanotrophic microorganisms.[7] Stable isotope labeling studies (SIP) have shown that non-methanotrophs can assimilate the C derived from CH_4.[8,9] This has suggested that methanotrophs may support the development of other microorganisms by providing an accessible C source of CH_4-derived products through community cross-feeding.[8,10] These interactions in methanotrophic communities could stimulate the activity and growth of methanotrophs,[9] improving robustness and adaptability, thereby providing economic and practical incentives for industrial applications.[11]

The pathways by which methanotrophs oxidize CH_4 to CO_2 have methanol (CH_3OH or MeOH), formaldehyde (CH_2O) and formate (CHOOH) as intermediates.[6] Methanol synthesis is the first step of CH_4 metabolism and is catalyzed by the methane monooxygenase (MMO) enzyme. Two forms of MMOs are found in

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methanotrophic bacteria; the sMMO (soluble) located in the cytoplasm, and the pMMO (particulate), located on the cell membrane.[12] The more common is the membrane-bound form[13] that synthesizes MeOH in the periplasm, which may facilitate its diffusion out of the methanotrophic cell. As the next step for incorporating carbon into cells is MeOH oxidation by methanol dehydrogenase (MDH) to formaldehyde, MeOH consumption can be inhibited by using MDH inhibitors. The use of different inhibitors of MDH activity has been reported, such as phosphate buffer, NaCl, cyclopropanol, EDTA, $MgCl_2$ and NH_4Cl.[14-16]

Methanol has been proposed to be one of the leading intermediaries of cross-feeding in these methanotrophic communities. Krause[17] observed that MeOH excretion by methanotrophs can be stimulated when cultivated with methylotrophic microorganisms. However, other studies have proposed that methanotrophs can also excrete multi-C compounds, such as acetate, formate, lactate or succinate, via methanotrophic fermentation,[18-20] suggesting that cross-feeding might not be solely related to MeOH production. Therefore, other interactions may be involved, expanding the compounds and phenomena implicated in the cross-feeding of methanotrophs with non-methanotrophs.

This study reports the effect of the organic compounds produced by a methanotrophic consortium on the excretion of MeOH by a pure culture of the methanotroph *Methylomonas methanica*. The results of the addition of the supernatant from the growth and stationary phases were compared with the MeOH produced with MeOH dehydrogenase inhibitors. For this, MeOH concentration was measured and related with CH_4 and O_2 consumption and CO_2 production rates and yields. Results in this study aimed to a better understanding of the interactions between methanotrophs and other non-methanotrophic microorganisms, as these communities represent an attractive option to produce valuable metabolites, such as MeOH, together with CH_4 emissions mitigation.

MATERIALS AND METHODS

CIR culture and growth conditions

Methanotrophic enrichment experiment

The methanotrophic consortium (CIR) was enriched from sediment samples of a pond (1858.6076 N, 09905.7232 W). It was cultivated with nitrate mineral salts medium (NMS) composed of (g L^{-1}): 0.2 $MgSO_4{\cdot}7H_2O$, 0.067 $CaCl_2{\cdot}2H_2O$, 1.0 KNO_3, 0.348 KH_2PO_4, 0.242 $Na_2HPO_4{\cdot}12H_2O$, 0.005 $FeSO_4{\cdot}7H_2O$, and 0.1% (v/v) of a trace elements solution containing (g L^{-1}): 0.22 $CuSO_4{\cdot}5H_2O$, 0.44 $ZnSO_4{\cdot}7H_2O$, 0.15 $MnSO_4{\cdot}H_2O$, 0.1 H_3BO_3, 0.18 $CaCl_2{\cdot}2H_2O$, 0.06 $Na_2MoO_4{\cdot}2H_2O$. The medium pH was adjusted to 7.0. Three replicates of 25 mL of the sample were incubated in 125 mL serological bottles with butyl septa at 28 ± 1 °C and 150 rpm. The cultures were fed with 10% CH_4 (99% v/v; Praxair, México) in air, the gas composition was monitored periodically. After exhausting the CH_4, 5 mL each replicate were mixed to be used as inoculum for cultures in serial batches in which the dilution rate was gradually reduced to 0.003, 0.006, 0.008, 0.013, 0.039 and 0.078 h^{-1}. Each transfer was performed in three replicates and at the end 5 mL was mixed to use as inoculum for the next culture. The last enrichment was named CIR and was used in subsequent experiments.

CIR consortium culture

The CIR consortium was grown in a 1.23-L culture bottle with 230 mL NMS medium and a port for gas injection and sampling.

Table 1. Experimental set-up of *M. methanica* cultivation with CIR consortium supernatants

Experiment	Volume of supernatant added (%)	CIR culture stage
MM	Without inhibitors	
MI	With $MgCl_2$ and phosphate as inhibitors	
1 (5G)	5	Growth
2 (25G)	25	Growth
3 (5S)	5	Stationary
4 (25S)	25	Stationary

Two replicates were made for each treatment.

Cultivation was performed at 28 ± 1 °C and 150 rpm in an orbital incubator. The reactors were replenished with 10% CH_4 in the headspace every 24 h. The initial and final gas compositions were measured daily by gas chromatography (GC-TCD). The optical density (OD_{600}) was measured with a UV–visible spectrophotometer (Genesys30; Thermo Fisher Scientific, Waltham, MA, USA) at 600 nm with the proper dilutions. Liquid samples (20 mL) were used for the biomass concentration, pH, total nitrogen (TN), total organic C (TOC) and inorganic C (IC). Dry cell weight (DCW) was determined by vacuum-filtering liquid samples of culture broth through a pre-weighted 0.2-µm-pore-size cellulose acetate membrane (Sartorius) followed by drying at 60 °C for 24 h.

MDH inhibition of *Methylomonas methanica*

The methanotroph *M. methanica* (ATCC 51626) was propagated at 28 ± 1 °C in a culture bottle as previously described for the CIR consortium. DCW was determined periodically. The MDH inhibition experiments with cultures of *M. methanica* were done in 125-mL serological bottles with butyl rubber stoppers with 25 mL culture volume and 10% CH_4 in the headspace incubated at 28 ± 1 °C and 150 rpm. The inhibiting solution contained 50 mmol L^{-1} $MgCl_2$ and 100 mmol L^{-1} phosphate at pH 7.0 as suggested by Hur[14] and Patel.[16] Cultivation was initiated from actively growing *M. methanica* with an initial biomass concentration of 0.7–0.8 g_{DCW} L^{-1}. Cell growth was followed by optical density (OD_{600}). Additionally, the initial and final biomass concentration (DCW) was determined, and liquid samples were taken for MeOH quantification (see Analytical Procedures section).

Methylomonas methanica cultivation with CIR consortium supernatants

Pure cultures of *M. methanica*, were grown with the supernatant of the CIR consortium. Samples of 50 mL from the CIR culture bottle were centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was first vacuum-filtered through 0.22-µm cellulose acetate membrane (47 mm; Sartorius AG, Goettingen, Germany), and then filtered under sterile conditions with a 0.22-µm membrane. The supernatants were maintained at 4 °C until further use.

Two concentrations, (5 or 25% v/v), and supernatants from two stages of the growth of the CIR consortium [growth at Day (D)6 or stationary at D25] were evaluated as depicted in Table 1. Cultures were initiated from growing *M. methanica* cultures with an initial biomass concentration of 0.7–0.8 g_{DCW} L^{-1} in 125-mL serological bottles with butyl rubber stoppers with 25 mL culture volume. Methane consumption, cell growth and MeOH concentration

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were analyzed to compare the effect of the supernatant addition with experiments of MDH inhibition as positive control.

Analytical procedures

CIR consortium bacterial diversity

DNA extraction and Illumina sequencing of 16S rRNA genes were performed to determine the bacterial composition of the CIR methanotrophic consortium. For DNA extraction, 50 mL culture broth were centrifuged at 10,000 rpm and washed twice with PBS 1X. A DNeasy PowerSoil DNA Isolation Kit (Qiagen Sciences, Germantown, MD, USA) was used following the manufacturer's instructions. DNA samples were submitted to RTL Genomics (Lubbock, TX, USA) for sequencing of the V4 region using primers 515F and 806bR[21] through the Illumina platform. Raw sequences were processed using QIIME2 v2023.5.[22] After a graphical quality analysis, the forward and reverse sequences were truncated at position 260. The DADA2 plug-in with standard parameters was used for denoising and chimera filtering.[23] The resulting amplicon sequence variants (ASVs) were taxonomically classified using the Alignment, Classification, and Tree Service (ACT) from the SILVA database v138.1.[24,25] Raw Illumina reads were submitted to the NCBI database under Bioproject PRJNA1008177 deposited with accession no. SRR25729140.

Methanol quantification

Liquid samples (500 µL) were drawn from the cultures of the experiments of the MDH inhibition or CIR supernatant experiments. Then, 1 µL HCl 10 mol L^{-1} was added and centrifuged at 10,000 rpm recovering the supernatant liquid after filtering with a syringe filter with a 0.22-µm pore. The samples were stored at 4 °C until further analysis. Methanol concentration was analyzed using an Agilent 7890B gas chromatograph equipped with an HP-5 column (19 091J-413E; Agilent Technologies, Santa Clara, CA, USA) and a flame ionization detector (FID). Nitrogen was used as carrier gas at 0.4 mL min^{-1} and the temperatures of the injector, oven and detector were 100 °C, 100 °C and 250 °C, respectively. Liquid samples (10 µL) were collected and placed into 10-mL vials with Mininert valves. Vials were heated at 65 °C for 10 min, and then 500 µL gas phase were taken with a syringe and injected into the chromatograph. The standard curve was made by preparing standard solutions of MeOH in water at concentrations ranging from 0.1 to 10 mmol L^{-1}.

Gas concentrations

Headspace composition was analyzed from 200-µL gas samples drawn with an airtight syringe. The analysis was conducted in a Gow-Mac Series 580 gas chromatograph with a CTR1 column (Alltech, Nicholasville, KT, USA) equipped with a thermal conductivity detector (TCD). Helium was used as carrier gas at 100 mL min^{-1}. The temperature settings were set to 50 °C in the injector, 40 °C in the column and 115 °C in the TCD detector.

Soluble carbon

Liquid samples of co-culture were filtered through a 0.2-µm-pore-size membrane and the supernatant assayed for TC and IC with a TOC-L CSH analyzer (Shimadzu, Kyoto, Japan) equipped with an infrared detection system (NDIR) via the oxidative catalytic combustion method. Acidification was done with 0.1 mol L^{-1} HCl, and the combustion temperature was 680 °C. Total organic carbon was calculated as the difference between the measured TC and IC. Total nitrogen was determined with the TNM-L chemiluminescence module in the same equipment.

Statistical analysis and calculations

Experiments were carried out with two replicates of each treatment. Results are presented as the average ± standard deviation (SD) ($n = 2$). Analysis of variance (ANOVA; EXCEL 2021) was used to compare the means of gases consumption or production rate between treatments. A significance level of 0.05 was used to calculate the *P*-value. ANOVA results are reported in Supporting Information, Tables S1–S3. To determine the difference between means, Fisher's least significant difference (LSD) *post hoc* test was used.

For the calculation of consumption and production rates, the Gompertz model was used, with the following formula:

$$C(t) = a\exp(-\exp(b-ct))$$

where $C(t)$ is the concentration at time t, a represent the highest value of one asymptote, b is a relative value of the other asymptote and c is the growth rate (in t^{-1}). The maximum rate parameter was used to describe the kinetics, which is obtained as $q_{max} = a{\cdot}c/\exp(1)$. The time at which the maximum rate is obtained is calculated as $t_{q_{max}} = b/c$. The estimation of the parameters and characteristic values was made with the *Excel Solver* function in EXCEL 2021with a bootstrap ($n = 30$) using experimental data from the two replicates of each treatment to determine the parameters and their statistics.

RESULTS AND DISCUSSION

Characterization of CIR methanotrophic consortium

Analysis of sequencing results from the CIR consortium, in Fig. S1, revealed the presence of two bacterial phyla: Bacteroidetes and Proteobacteria. Although the sequencing results identified the presence of bacterial clades frequently enriched along methanotrophs (e.g. Burkholderiales, Rhizobiales, Sphingomonadales, Bacteroidetes),[26-28] it was unexpected to find that the only methanotroph detected, an Alphaproteobacteria belonging to the genus *Methylocystis*, represented only 1% of the total community composition. Instead, ≈89% of the consortium was composed of methylotrophs from the family Methylophilaceae. These methylotrophs systematically co-occur with Gammaproteobacteria methanotrophs of the family Methylococcaceae, suggesting that the pairing of these two families occurs nonrandomly, providing ecological advantages over other co-occurrence partners.[26,29] The long-standing hypothesis for the co-occurrence of these families is that it is a result of MeOH cross-feeding subjected to abiotic factors, such as O_2 levels[30] or N sources;[31] or biotic factors, such as changes in expression patterns of MDHs;[17] however, the exact mechanism behind this cross-feeding interaction has not yet been elucidated. Although infrequent, the co-occurrence of Methylophilaceae methylotrophs with Alphaproteobacteria methanotrophs is not unique to this study,[32] suggesting that the phenomenon leading to MeOH cross-feeding is not specific to the pairing of Methylophilaceae methylotrophs with Methylococcaceae methanotrophs.

CIR consortium cultivation

During growth kinetics, the accumulated consumed CH_4 and O_2 were 67 mmol CH_4 (1.07 g CH_4) and 54 mmol O_2 (1.73 g O_2), while the accumulated produced CO_2 was 27 mmol (0.75 g CO_2) (Fig. S2). The calculated rates referred to the medium volume were 38.4, 13.4 and 28.7 mmol L^{-1} day^{-1} for CH_4, O_2 and CO_2, respectively ($R^2 = 0.99$, 0.95 and 0.97). The CO_2 production rate

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was similar to the values reported by López[33] for an enriched methanotrophic culture (≈23–32 mmol L^{-1} CO_2 day^{-1}). The low presence of methanotrophs in CIR suggests that the non-methanotrophic microorganisms, such as *Methylophilus*, may play an important role in enhancing the activity of the methanotroph *Methylocystis*.[34] Figure 1(a) shows that from D0 to D20, CIR biomass increased linearly at a growth rate of 0.21 g_{DCW} L^{-1}-day^{-1}. Simultaneously, the N concentration decreased from 145.2 to 3.0 mg N L^{-1} in the first 9 days with a consumption rate of 15.6 mg N L^{-1}-day^{-1}. Despite N exhaustion at D9, DO_{600} continued incrementing until D21, and from D21 to D25 the growth rate decreased to 0.06 g_{DCW} L^{-1}-day^{-1}.

The carbon analysis of the supernatant [Fig. 1(b)] showed that between D0 and D9 the IC concentration increased from 4.3 to 105.7 mg IC L^{-1}. During the same period, the largest increase in pH, from 7.3 to 9.3, was also recorded. The increment of pH in the supernatant may be related, on the one hand, to the consumption of protons during NO_3^- assimilation by the nitrate reductase.[35,36] On the other hand, periodic aeration every 24 h facilitates the removal of the produced CO_2, hence avoiding medium acidification, which is a common phenomenon in methanotrophic cultures. The increase of IC may be related to the equilibrium of CO_2 due to the formation of carbonates in alkaline pH. From D9 to D12, the IC accumulation ceased and reached 110.1 mg IC L^{-1}, whereas the pH continued between 9.3 and 9.4. Finally, the TOC remained in the range 11.6–15.3 mg TOC L^{-1} until D9, rising to 51.4 mg TOC L^{-1} on D12 after N depletion.

Based on these results, growth of the CIR consortium can be divided into three stages: (i) growth stage, from D0 to D9; (ii) transition stage, from D9 to D20; and (iii) stationary stage, from D20 to D25. The supernatants for the experiments were obtained from the growth (D6) and the stationary (D25) stages, with initial MeOH concentrations of 0.95 ± 0.21 and 0.55 ± 0.12 mmol L^{-1}, respectively. The final concentration of biomass was 7.11 g_{DCW} L^{-1}. The increment in biomass after the N source was exhausted, can be associated to the accumulation of reserve molecules, because it is known that under different limiting conditions, such as N deprivation, some methanotrophic bacteria can store C substances such as polyhydroxyalkanoates (PHAs) or carbohydrates.[37,38]

Methanol production by *Methylomonas methanica*

Methanol production in *M. methanica* was tested with the concurrent effect of $MgCl_2$ and phosphate. The maximum MeOH concentration found in the supernatant of the experiment with inhibitors (MI) was close to 2 mmol L^{-1} (1.99 ± 0.05) after 3 h, with a yield of 0.31 ± 0.07 mol CH_3OH/mol CH_4 consumed. The maximum rate of CH_4 consumption (1.90 ± 0.17 mmol CH_4 g_{DCW}^{-1}-h^{-1}) occurred at 2.8 ± 0.4 h, concurrent to the maximum MeOH accumulation [Fig. 2(b)]. The excretion and accumulation of MeOH was favored by the effect of high concentrations of salts, that inhibits the electron transport thus blocking the interaction between the MDH enzyme and cytochromes,[39] and in turn limiting further oxidation of MeOH to formaldehyde. After 3 h, the MeOH concentration decreased to 0.4 mmol L^{-1} despite the presence of the MDH inhibitors. The decrease in MeOH concentration is consistent with other observations in extended cultivation that have shown lower MeOH production.[14,40] This phenomenon may be possibly to partial inhibition of the MDH. By contrast, MeOH remained below detectable limits (0.1 mmol L^{-1}) during the entire experiment without inhibitors (MM), suggesting that the produced MeOH was further metabolized by *M. methanica* [Fig. 2(a)].

The CH_4 consumption rate in the MI experiment was lower compared to 2.55 mmol CH_4 g_{DCW}^{-1}-h^{-1} obtained in the control without inhibitors (MM). As observed in other studies, MDH inhibitors may also affect other enzymes involved in the supply of reducing power.[41] Furthermore, reduction in CH_4 consumption rate in inhibited experiments could be a result of a reduction in the regeneration of reducing power necessary to sustain the activity of MMO. Patel[16] reported that *M. methanica* attained a MeOH concentration of 0.48 mmol L^{-1} with phosphate buffer (100 mmol L^{-1}) and $MgCl_2$ (50 mmol L^{-1}) and that the addition of formate (a source of reducing power) enhanced the MeOH production to 3.86 mmol L^{-1}. This difference in MeOH production highlights the importance of reducing power to sustain CH_4 oxidation to MeOH, whereas in our study an external source of reducing power was not used. The headspace CO_2 production rate in the MI experiment was ≈2.4-fold higher than in the MM experiment (2.75 compared to 1.16 mmol CO_2 g_{DCW}^{-1}-h^{-1}). The global CO_2/CH_4 yield in MM was 0.39 ± 0.02 whereas the MI experiment presented a higher yield, 0.71 ± 0.06 mol CO_2/mol CH_4 (see

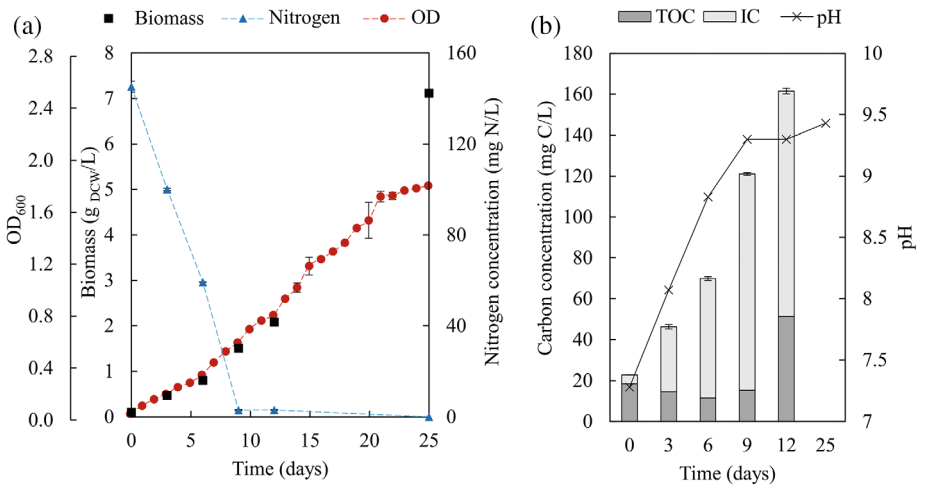

Figure 1. CIR consortium culture: (a) growth in DCW and OD_{600}, and N concentration during cultivation time; (b) soluble C composition (TOC and IC) and pH.

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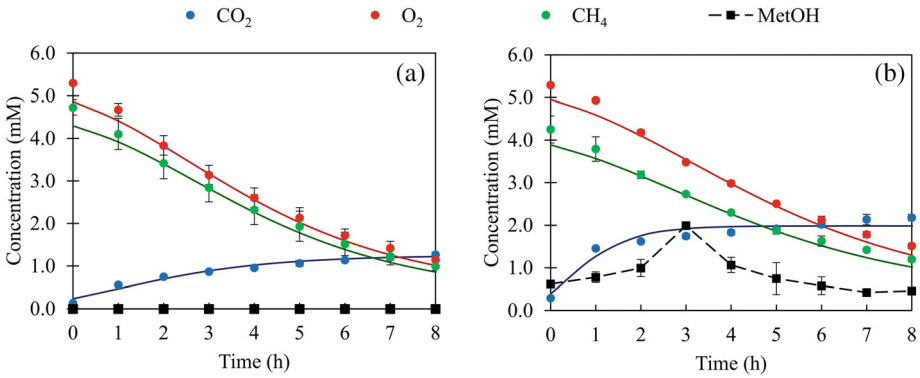

Figure 2. MDH inhibition kinetic experiments in pure cultures of *M. methanica*: (a) culture without inhibitors (MM) and (b) culture with inhibitors (MI), 100 mmol L^{-1} phosphate and 50 mmol L^{-1} $MgCl_2$. Means and errors bars of concentrations were calculated from two replicates.

Table S4). An increase in CO_2 production rate and yields suggest that, under MDH inhibition, a higher fraction of formaldehyde is oxidized to CO_2 to supply redox equivalents required for MMO activity. Therefore, it resulted in the decrease of the CH_4-C incorporated into biomass.[39] Another aspect that needs to be considered when analyzing the headspace CO_2 is that the final pH for MI was 7.85 whereas for MM it was 8.66, suggesting that a higher fraction of the produced CO_2 in the MM may have been retained as carbonates.

Effect of the CIR consortium supernatants on pure cultures of *Methylomonas methanica*

Methanol accumulation by *M. methanica* with supernatant of the CIR consortium showed maximum MeOH concentrations of 1.53, 0.70. 1.18, and 1.15 mmol L^{-1} CH_3OH for 25G, 5G, 25S and 5S, respectively [Fig. 3(a)–(d)], which were lower than MI. The measured MeOH concentrations indicate that it was produced during the CH_4 consumption. This MeOH produced is then partially excreted into the medium, where it accumulates, and it can be further consumed. The coexistence of methylotrophs with methanotrophs is usually attributed because are fed with MeOH that is excreted from methanotrophic cells owing to metabolic overflow.[8,29] Alternatively, a study by Krause[17] suggested that the excretion of MeOH is related to a change in the expression patterns of alternative MDHs: calcium-dependent MDH (MxaF) or the lanthanide-dependent MDH (XoxF). However, in this study the medium used does not contain lanthanides, and metabolic overflow could be discarded because the experiment MM did not show significant MeOH excretion.

The maximum specific rates for CO_2 production and O_2 and CH_4 consumption in these experiments are depicted in Fig. 3(e)–(g). The CH_4 consumption rate between experiments decreased in the following order 25G > 5G > 5S > 25S, with 2.21, 1.76, 1.50 and 1.39 mmol CH_4 g_{DCW}^{-1}-h^{-1}, respectively [see Fig. 3(g)], although the rates using the supernatants in the stationary stage did not show statistically significant difference ($P = 0.86$). The addition of supernatant in the stationary stage decreased the CH_4 consumption by about two-fold in both levels. During N limitation in the stationary stage of CIR consortium, the accumulation of organic compounds in the supernatant could be a cause of the inhibitory effect observed.

As in the experiment with inhibitors, an increase in the global CO_2/CH_4 yield was observed with the addition of 5% of the supernatant, with values of 0.61 ± 0.12 and 0.64 ± 0.05 mol CO_2/mol CH_4 for growth and stationary stages, respectively. By contrast, by increasing the concentration of supernatant, the yields of CO_2/CH_4 were closer to the experiment without inhibition, 0.41 and 0.42 mol CO_2/mol CH_4 for growth and stationary stages, respectively (see Table S4).

The biomass concentration increased to 10.4 mg_{DCW} L^{-1} in MM in 24 h (not measured in MI). With the addition of the supernatants, the highest growth was 18.8 mg_{DCW} L^{-1} in 5G (Fig. 4), whereas the growth in the other experiments was less than that in MM (7.8, 5.56 and 1.3 mg_{DCW} L^{-1} for 25G, 5S and 25S, respectively). No growth or relevant activity (CO_2 produced or O_2 consumed) was observed in CH_4 free headspace (see Fig. S3). Therefore, the effect depended only on the presence of CH_4, indicating that the organic compounds found in the supernatant cannot be used alone as a source of C or energy. The final pH values in the experiments were 8.04, 8.35, 8.52 and 8.7 for 25G, 5G, 25S and 5S, respectively. It was observed that the pH in cultures with 5% v/v of supernatant was higher compared to those with 25% v/v of supernatant. This difference could be attributed to experiments with lower concentration of supernatant experiencing more growth of biomass. This increased growth could be associated with a higher consumption of nitrates, leading to the alkalinization of the medium.

The results suggest that the methanotrophic activity increases with further addition of supernatant in the growth stage, despite the diminishing growth. In this sense, a higher concentration of supernatant in the growth stage increases the excreted MeOH. Observed effects could be related to the energy imbalance generated for MeOH accumulation. Whereas both the CH_4 to MeOH reaction and cell growth depends on energy production during CH_4 metabolism, it is proposed that the organic compounds in the supernatant induce the MeOH excretion which, in turn, is related to methanotrophic activity by improving the MMO activity and thus the CH_4 consumption rate. Concurrently, the MeOH excretion and accumulation can be related to a decrease in metabolic efficiency of MeOH consumption, that would, eventually, result in a reduction of biomass production. Therefore, the methanotroph in the methanotrophic consortium may benefit from organic compounds to increase the CH_4 consumption while providing MeOH to sustain the growth of methylotrophic microorganisms in consortium.

CONCLUSIONS

The effect of the organic compounds of methanotrophic consortium supernatant on the excretion of MeOH by the methanotroph

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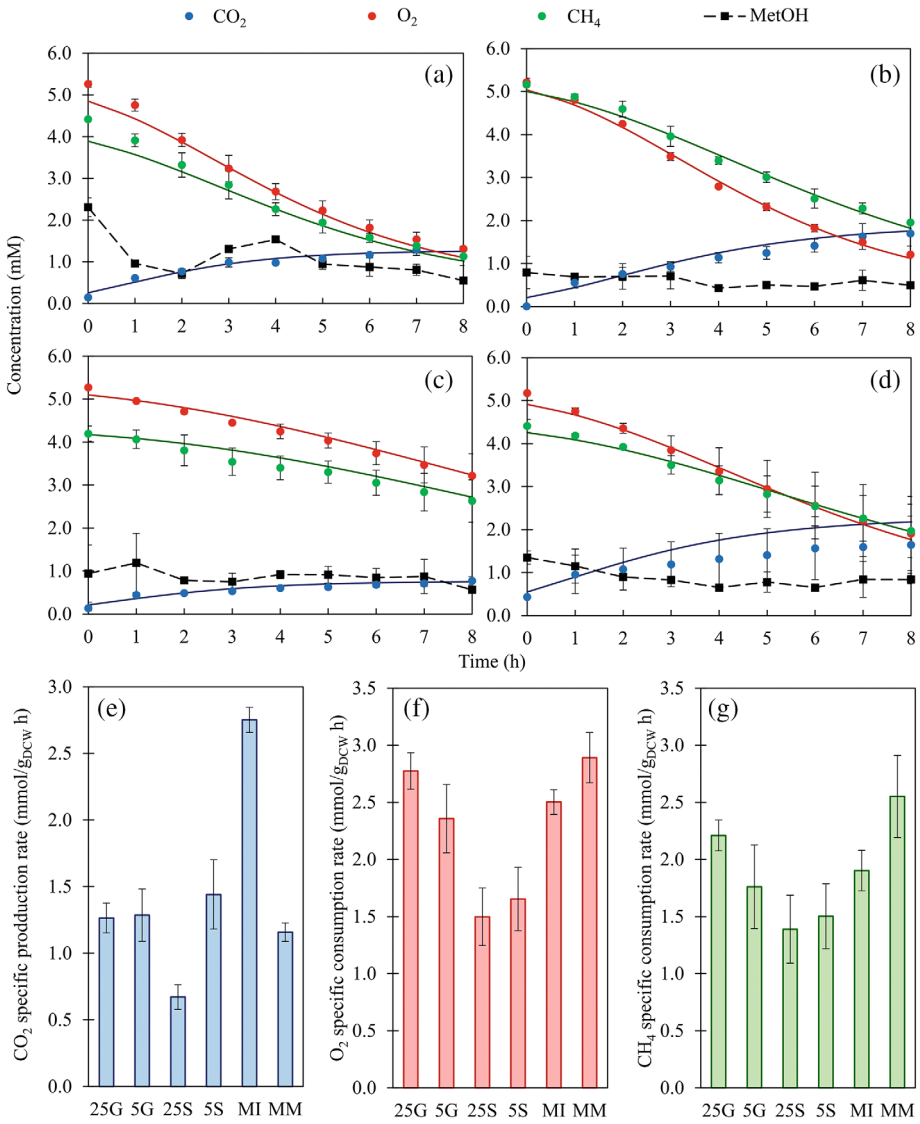

Figure 3. Kinetics of CH_4 and O_2 consumption, CO_2 production and MeOH accumulation for (a) 25G (b) 5G (c) 25S and (d) 5S experiments with ClR supernatants ($n = 2$) and maximum specific rates obtained from (e) CO_2, (f) O_2 and (g) CH_4 data Gompertz fitting from bootstrap ($n = 30$) dataset (see values in Table S5).

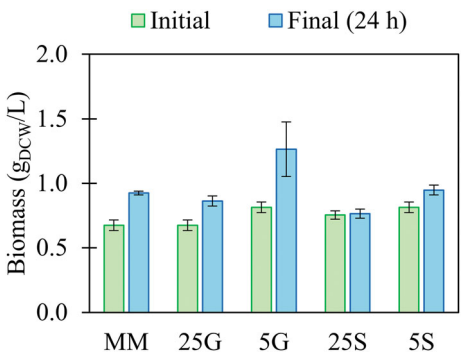

Figure 4. Biomass initial and final of *M. methanica* experiments with the supernatant and control without inhibitors (MM). Means and errors bars of concentrations were calculated from two replicates.

was confirmed in experiments with *M. methanica* as a model methanotroph. This work demonstrated that interactions within the methanotrophic consortium can be carried out through the exchange of compounds in the supernatant between the methanotroph and methylotroph microorganisms. However, the cross-feeding of compounds was observed to require a more intimate methanotroph–methylotroph relationship, rather than being the result of metabolic overflow of *M. methanica*. Methanol excretion by *M. methanica* increased in response to a higher concentration of supernatant, even at stationary stage, suggesting that the methanotroph–methylotroph interaction remains stable within the consortium. The enhancement of the CH_4 consumption rate appears to be linked to the fact that the energy cost of MeOH excretion is offset in methanotrophs by the improvement in MMO activity, yet the growth of methanotrophs is reduced. Therefore, the results obtained help to understand the interactions between methanotrophs and methylotrophs in methanotrophic communities, although further studies are required to identify the compounds responsible for the excretion of MeOH by the methanotroph. Furthermore, conducting studies of activity and expression of key enzymes involved in CH_4 metabolism can aid in comprehending the impact of interactions within this type of methanotroph–methylotroph community.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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