Methane and ethane production rates by methyl-coenzyme M reductase in cell extracts from different methanogens

V. P. Thinh Nguyen[†], Sebastian Keller & Silvan Scheller*

Department of Bioproducts and Biosystems, School of Chemical Engineering, Aalto University, FI-02150, Espoo, Finland

*Corresponding author: silvan.scheller@aalto.fi

[†]Current address: Canadian Glycomics Network, E5-33 Gunning/Lemieux Chemistry Centre, T6G 2E2, Edmonton, Alberta, Canada

ORCID

Silvan Scheller 0000-0002-0667-9224

V. P. Thinh Nguyen 0000-0002-2499-2900

Sebastian Keller 0000-0001-7477-1201

Abstract

Methyl-Coenzyme M reductase (Mcr) plays an important role in the regulation of the global carbon cycle. The enzyme catalyzes the reversible conversion of methyl-coenzyme M and coenzyme B to methane and the corresponding heterodisulfide CoM-S-S-CoB, which constitutes the first step of the anaerobic oxidation of methane. Mcr homologs of varies methanogenic archaea also catalyze the anaerobic oxidation of extended alkanes. Fully active, purified enzyme is exclusively achievable *via* strictly anaerobic purification from *Methanothermobacter marburgensis.* This microbe expresses two isoenzymes and most studies were performed with isoenzyme I that exhibits a limited substrate-promiscuity (c.a. 0.5%) towards the homologous substrate ethyl-coenzyme M. Here, cell-free lysates from the different species such as *Methanosarcina mazei*, *Methanococcus maripaludis*, *Methanothermococcus okinawansis,* and *Mt. marburgensis* were screened for Mcr activity and substrate promiscuity. An assay relying on titanium (III) citrate and cobalamin to regenerate coenzyme B and coenzyme M was used to test the ability of different cell extracts for the catalytic activity towards the C2-substrate ethyl-coenzyme M. Cell extracts from *M. mazei* showed a ratio of ethane- to methane- production of ca. 8% at 37 °C, and about 14% at 49 °C. The level of substrate-promiscuity towards ethyl-coenzyme M for *M. marburgensis* cell extracts under our assay conditions were much higher than previously reported for purified isoenzyme I, indicating that isoenzyme II is much more promiscuous for ethane formation than isoenzyme I. Our experiments demonstrate that Mcr activity can be quickly and conveniently studied *via* cell-free lysates, and that substrate promiscuity towards ethane formation is generally larger than anticipated.

Keywords

Enzyme catalysis, methanogenic archaea, methyl-coenzyme M reductase, biological methane formation, C1-metabolism, substrate promiscuity, enzyme assays, response surface methodology

Introduction

Methyl-Coenzyme M reductase (Mcr) catalyzes the reversible last step of methanogenesis, where it catalyzes the conversion of methyl-Coenzyme M (CoM) and the thiol coenzyme B (CoB-SH) to methane and the corresponding heterodisulfide coenzyme B–coenzyme M (CoB-S-S-CoM) in all methanogenic archaea [1] (**Figure 1**). Mcr also catalyzes the first step of anaerobic oxidation of methane, and even more extended alkanes in alkanotrophic archaea [2–4]. This archaeal reductase plays an important role in the global carbon cycle by catalyzing the last step in archaeal methane formation with an estimated global flux of c.a. 1 Gt per year of methane [5]. Investigating the anaerobic oxidation of ethane to corresponding ethyl-CoM mediated by *Methanothermobacter marburgensis's* Mcr has shown the promiscuous conversion of ethyl-CoM to the corresponding ethane [6]. Although an early study has reported an accumulation of ethane from ethyl-CoM carried out by cell-free extracts from *Methanosarcina barkeri* under hydrogen supply [7], the Mcr enzyme activity for alkyl-CoM of *M. barkeri* and other methanogens, besides *Mt. marburgensis*, remains limited.

As mentioned previously, most of the kinetic information was collected from *Mt. marburgensis* Mcr's studies [8,9], whereas insights into the substrate specificity and enzyme activity of other methanogens remain limited up-to-date. It is reported that Mcr isolated from *Mt. marburgensis* possesses two different isoenzymes. The Mcr activity of cell lysates harvested in the late log phase is mainly attributed to the type I isozyme [10]. This later constitutes 0.14% of the Mcr remaining in the harvested cell [9,11], while the isoenzyme II was exclusively expressed in the early log phase. The specific enzyme activity of isoenzyme II (11.7 U/mg) was reported to be significantly higher than that of isoenzyme I (4.5 u/mg) [11]. It is noteworthy that the specific activity of Mcr also varies dependently on the amount of the MCR-red1 form residing in the harvested cell. Specific activities for the methyl-CoM reduction of purified *Mt. marburgensis* Mcr were shown to be elevated over 20 U/mg when Ti(III) citrate and cobalamin were added for the reactivation of the NI(I) state of cofactor F_{430} [11,12], with maximal 100 U/mg upon fully activation of the enzyme [11].

Methyl-CoM reductase requires the nickel-porphinoid [13] cofactor F_{430} in its Ni(I) oxidation state to be active [11,12]. In the *in vitro* assay, Mcr owes the efficient reduction of CoM-S-S-CoB heterodisulfide to the presence of Co(I) or Co(II) of Ti(III)citrate-reduced cyanocobalamin. The hypothesis is this reduction system can be implemented to other Mcr homologues from other methanogenic and alkanotrophic species to maintain or reactivate the enzyme activity for methyl-CoM and even for more extended alkyl-CoM derivatives (e.g., ethyl-CoM). However, the purification of active Mcr from other methanogens appears to be challenging due to the high O_2-sensitivity, as no such successful procedure has been communicated in the literature. This inactivation of Mcr is prompted by the presence of oxidized form of Ni-porphinoid to (II) and (III) oxidation states [13]. Nevertheless, Mcr-Ni(III) can be further

reactivated by using strong electron-donors such as Ti(III) citrate or DTT under either H_2 or CO atmosphere. A lack of critical post-translational modifications furthermore impactfully contribute to the inactivity of Mcr [14,15]. In spite of the aforementioned limitations, Mcr-Ni(III) can be further reactivated by using strong electron-donors such as Ti(III) citrate or DTT under either H_2 or CO atmosphere. Taken together, the functional heterologous expression of Mcr remains challenging [15] hampering the study of this of interest enzyme. Moreover, the utilization of the Ti(III) citrate/cobalamin reduction system to rescue the Mcr activity is exclusively limited to *Mt. marburgensis* purified Mcr [6,9,11].

Cell-free lysate can be used instead of purified Mcr [7]. This approach will allow one to address the limit of arduous purification or reactivation processes of Mcr. Employing cell-free extract is thereby an excellent strategy for rapid assay of Mcr enzyme activity for alkyl-CoM to form corresponding alkanes. In this context, we aim to provide herein the optimal conditions of Mcr enzyme activity for ethane formation from corresponding ethyl-CoM basing on the study conducted with cell-free lysate isolated from *M. acetivorans* (strain WWM73) grown on methanol (MeOH), as well as to investigate the substrate promiscuity of Mcr from different methanogen cell-free extracts.

Results

In this study, we have adapted the previously published enzyme activity assay of Mcr purified from *M. marburgensis* [9] to cell-free lysates isolated from the other cultivable methanogens such as *Methanosarcina* and *Methan*ococcaceae. We aimed to compare the enzyme activities towards C1- and C2-alkyl donor (methyl- and ethyl-CoM, respectively). The promiscuity of cell-free lysates, defined by the ratio between ethane and methane formation from respective ethyl- and methyl-CoM, were also studied. The optimization of the Mcr assay was conducted using cell-free lysates from *M. acetivorans* (strain WWM73) regarding its fast growth pace on methanol (MeOH) [16]. The culture was supplemented with 100 mM MeOH and 2.5% Na_2S as sulfur source under strict anaerobic conditions. Once the desired growth phase reached, the cell-free lysate was then prepared from the active culture and will be used to catalyze the conversion of methyl- and ethyl-CoM to corresponding methane and ethane, respectively. The standard Mcr assay contained 0.5 mM alkyl-CoM, 0.25 mM CoB, 0.2 mM vitamin B_{12}, 20 mM of Ti(III) citrate, and 100 µg of cell-free lysate at pH 7 and 25 °C, while the enzyme activity was determined by methane and ethane level detection via GC-FID.

An experimental design approach employing response surface methodology was performed (see **Supporting Information)** to provide further insights concerning the effects of predefined parameters onto enzyme activity for ethyl-CoM alongside with the ratio between enzyme activity for ethyl-CoM and that for methyl-CoM (henceforth referred as the promiscuity). The outcomes of this experimental design afforded optimal operating conditions

which would be next implemented to assay enzyme activity for ethyl-CoM and the promiscuity of different methanogens. We would expect to identify the most promiscuous methanogen-Mcr among tested methanogens.

Determination of parameters having effects onto Mcr activities

It appeared that selected parameters such as the growth phase at harvesting, the incubation temperature and the B_{12} concentration involving in the CoB-S-S-CoM heterodisulfide reduction have significant effects on Mcr enzyme activity for methyl-CoM. In order to study these effects, we devised a response surface design involving 3 factorial variables (OD_{600} at harvesting, incubation temperature and vitamin B_{12} concentration) that included, not only the enzyme activity for methyl-CoM as a response variable, but also comprised the enzyme activity for ethyl-CoM, and the promiscuity (ratio between ethane and methane formed from ethyl-CoM and methyl-CoM, respectively) with regards to the main goal of this study is to assay the ethane formation ability of studying methanogens. Thus, the optimal operating conditions were implemented to explore the methane and ethane formation ability, as well as the promiscuity of cell-free lysates from different methanogens.

Growth phase

Cell lysates of different growth phases, with OD600 at harvesting were categorized as early (low OD, value between 0.1-0.3), middle (moderate OD, value between 0.5-0.7) and late growth phase (high OD, value between 0.9-1.2). Mcr assays employing these cell lysates were conducted to determine the effects of growth phase on methanogenic activities. Methyl-CoM was used as an alkyl donor, and methane was quantified by GC-FID using methane as an external standard curve. Our results indicated that *M. acetivorans* Mcr was most active at mid growth phase (OD600 value between 0.5-0.8), whereas harvesting at early growth phase or late growing time resulted in lower enzyme activity as depicted in **Figure 2**.

Incubation temperature and vitamin B_{12} concentration

We investigated the effects of incubation temperature during the assay onto the enzyme activity by conducting Mcr assay at different temperatures (25 and 37 °C) employing different cell-free lysates prepared at different growth phases (low and high OD levels). Our results presented in **Figure 3** shows that low OD culture exhibited higher enzyme activity at 37 °C. On the other hand, the high OD cell-free lysate did not show any improvement in enzyme activity for methyl-CoM at higher temperature.

Ti(III) citrate is used along with the addition of cobalamin to reduce the generated CoB-S-S-CoM heterodisulfide complex in the Mcr activity assay [11,12]. The concentration of Ti(III) must be in excess, whereas the cobalamin concentration was reported to be between 0.2-2 mM to ensure the highest Mcr activity [12]. Taken together, we hypothesized that cobalamin concentration might have an indirect effect on Mcr assay. Therefore, the concentration of

vitamin B_{12}–the source of cobalamin in our assay–was also included as a factorial variable for our design of experiment. We investigated the concentration range of vitamin B_{12} between 0.2 to 1.1 mM, as higher concentration might not be directly beneficial to our assay. Our results depicted in **Figure 3** showed that low OD and low cobalamin concentration had significant positive effect onto enzyme activity for methyl-CoM, whereas increasing cobalamin concentration only had a slight declination of the enzyme activity for high OD cell-free lysate. These results confirmed our aforementioned hypothesis concerning the indirect effect of cobalamin onto Mcr enzyme activity.

Based on our previous results, we proceeded with the optimization of the Mcr enzyme activity with a non-C1 substrate, ethyl-CoM, in parallel in order to determine the enzyme activity of Mcr in cell-free lysates towards more extended substrate (C2 in this study), as well as the promiscuity of Mcr towards these substrates. For the latter purpose, we determined the ratio between ethane and methane formed by separately providing methyl-CoM and ethyl-CoM as alkyl donor was therefore determined. Response surface methodology is a powerful statistical technique that has been successfully employed to optimize the methanogenic activity of methanogens [17]. Thereby, we believed that employing this statistical approach would afford an efficient and highly reproducible procedure that enables alkane formation from corresponding alkyl-CoM. The outcomes of this optimization process would allow us to investigate the enzyme activity for ethyl-CoM and promiscuity of cell-free lysate from *M. acetivorans*. The model fitting and further optimization of enzyme activity are presented in **the Supporting Information**. Confirmatory experiments were conducted in duplicate according to the operating conditions afford *via* the prediction model to confirm the estimating values. The outcomes were reported in **Table 1**, with the enzyme activity for methyl-CoM appeared to be higher than the predicted value. Nevertheless, the value of this latter remained satisfactory in the context of our study. Thus, we proceeded to the implementation of our optimized assay conditions onto well-known cultivable methanogens. The results of this process are described in the following section.

Implementation of optimal Mcr assay to investigate the enzyme activity and promiscuity of different cell-free lysate harvested from available methanogens

Enzyme activity towards ethyl-CoM and the promiscuity of cell-free lysates from different cultivable methanogens such as *M. mazei*, M. *M. maripaludis*, *Mt. okinawansis* and *Mt. marburgensis* were studied under previously optimized operating conditions (OD_{600} at harvesting at moderate/low, 49 °C and 1.1 mM B_{12}). In addition, experiments under conventional assay conditions (moderate OD600, incubation temperature 37 °C and vitamin B_{12} concentration 0.2 mM) were also conducted, as we were concerned that the high incubation temperature and vitamin B_{12} concentration might significantly affect onto the Mcr activity of non-thermal methanogens, originally living in cold conditions [17]. For *M. mazei,*

low protein concentration in lysate prepared from cell harvested at OD600 between 0.4-0.6 occurred. As a result, the cell was instead harvested at OD600 value between 1.4-1.6 to afford the sufficient amount of protein for the Mcr assay. In contrast to other methanogens, *Mt. okinawansis* cell-free lysates appeared inactive under both conventional and optimal conditions. The results are reported in **Figure 4**.

Dependence of substrate promiscuity of *Methanothermobacter marburgensis* on different growth conditions

We investigated the high promiscuity and ethane formation from ethyl-CoM of *Mt. marburgensis* by conducting the Mcr assays with different cell-free lysates obtained *via* different growth methods: batch growth in serum vials with frequently supplement of H_2/CO_2, and biofermentation in bioreactor with a continuous flow of H_2/CO_2 [6,9]. OD_{600} value at harvesting of the former growth method was reported to be between 0.5-0.7, whereas the latter one was harvested during the plateau growth phase (OD_{600} between 5-6). In both cases, the cell suspension appeared to be slightly fluorescent green due to enrichment of flavin-based cofactor F_{420}. Upon lysis, the cell-free extracts were retrieved and stored at - 80 ºC under H_2 until further uses.

Upon obtaining the cell-free lysates, we conducted assays of Mcr activity using either methyl- or ethyl-CoM alone, or mixing methyl- and ethyl-CoM at different ratios to study Mcr activities of lysates. The conventional assay conditions (0.2 mM vitamin B_{12} concentration, 20 mM Ti(III) citrate) and high incubation temperature (65 °C) were implemented to compare the enzyme activity for methyl- & ethyl-CoM. Our results in **Figure 5** indicated that cell-free lysate of *M. marburgensis* grown in serum vial and low-OD600 value at harvesting exhibited better enzyme activity for ethyl-CoM, with the enzyme activity for methyl-CoM is c.a. 19 times higher than that for ethyl-CoM. In contrast, the lysate growth in bioreactor showed lower activity for ethyl-CoM, with the enzyme activity ratio between for methyl-CoM and for ethyl-CoM was determined to be c.a. 230 times, which is in agreement with our previously reported data [6].

We observed that the presence of ethyl-CoM in high concentration drastically inhibited the conversion of methyl-CoM to corresponding methane in cell-free lysates of *M. marburgensis* grown in bioreactor while varying the concentration of methyl- and ethyl-CoM in the alkyl-CoM mixture (**Figure 5**). In contrast, *M. marburgensis* cell-free lysate harvested at early growth phase showed higher tolerance towards the presence of ethyl-CoM in the alkyl-CoM mixture. Ethane formation was observed in both cases where only ethyl-CoM was supplemented.

In order to rationalize our observation, Mcr composition was analyzed via SDS-PAGE following the protocol described by Rospert et al. [18]. Thus, lysates of *Mt. marburgensis*, collected either at a low (vial), or high OD (bioreactor) were step-wise enriched with ammonium sulfate to saturated level for a partial purification of the Mcr isoenzymes (**Figure 6**). The bioreactor sample from a late growth phase showed basically three main bands corresponding well to Mcr isoform I [10,18]. Both Mcr isoforms can be distinguished basing the size difference of the gamma subunit *via* SDS-PAGE analysis. The respective sizes of small γ subunits are 38 kDa for isozyme I and 33 kDa for isozyme II [10]. In the case of the vial sample that originated from an earlier growth phase, Mcr isoform I appeared to dominate the total Mcr fraction. However, a faint band occurred at the corresponding size of the Mcr II gamma subunit (33 kDa), which was absent in the bioreactor sample. We also observed a ratio of ca. 3:1 for Mcr I to Mcr II based on the intensity of the two gamma subunit bands in the vial sample.

Discussion

Optimal Methyl-Coenzyme M reductase assay enhancing enzyme activity and the promiscuity for ethyl-CoM

In this study, we have optimized the operating conditions for the Mcr methane formation assay, that so far exclusively employs purified Mcr from *M. marburgensis,* onto other methanogens such as *M. acetivorans*, *M. mazei*, *M. maripaludis,* and *M. okinawansis*. The optimal conditions of 3 factorial variables such as OD_{600} at harvesting, incubation temperature, and B_{12} concentration were afforded *via* an experimental design using the response surface methodology with Box-Behnken design. Our prediction model suggested that increasing the incubation temperature at high B_{12} concentration efficiently enhance the formation of ethane from ethyl-CoM along with the promiscuity towards more extended alkane from corresponding alkyl-CoM. Experiments conducted with cell-free lysate of *M. acetivorans* harvested at low/moderate OD_{600} (OD_{600} value at harvesting 0.3-0.5) at high B_{12} concentration (1.1 mM) and temperature (49 °C) afforded higher enzyme activity for methyl- and ethyl-CoM. These outcomes suggested the thermostability of Mcr from *M. acetivorans*. This can involve the different composition of the whole Mcr complex between these species [19]. Nevertheless, the improvement of the conversion of methyl- and ethyl-CoM to corresponding alkane prompt by increasing incubation temperature and B_{12}-concentration deserves further investigation, with high regards to limited respective information relating to Mcr thermostability and enzyme reactivation mechanism.

Our results depicted in **Figure 5** showed that cell-free lysate from different methanogens exhibiting the activity for conversion of methyl- and ethyl-CoM to corresponding alkanes under both conventional conditions. Despite the high OD600 value at harvesting, the cell-

free lysate from *M. mazei* also showed the highest Mcr activity for both provided alkyl-CoM substrates as well as the promiscuity. These values even exceeded that of thermo-methanogens such as *Mt. marburgensis* and *Mt. okinawansis*. In contrast, cell-free lysate *M. maripaludis* was determined to be the less efficient material to carry out Mcr activity for both methyl- and ethyl-CoM. Overall, the Mcr activity appeared to improve at increasing vitamin B_{12} concentration and incubation temperature. Regardless the low activity, this is the very first time Mcr activity of two different cultivable members of *Methanococcacaea* (*M. maripaludis* and *Mt. okinawansis*) has been reported according to our best knowledge.

We have successfully implemented the optimal Mcr conditions for ethane formation onto different methanogenic archaea and have identified *M. mazei* to be the most promiscuous Mcr for ethane formation as well as the promiscuity for ethyl-CoM as substrate.

Cell-free lysate as materials to assay Mcr activity towards non-C_1 alkyl-Coenzyme M

As previously mentioned, data relating to Mcr enzyme activity is mainly reported by studying pure Mcr isoenzyme I isolated from *Mt. marburgensis* [6,9,15]. In this study, we have demonstrated that cell-free lysate can also be used to quantify, in a preliminary manner, the Mcr activity for methyl-CoM and ethyl-CoM. Optimized Mcr assay employing Ti(III) citrate/B_{12} heterodisulfide reducing system furthermore resulted in our findings about the enhancement of activity and promiscuity with increasing assay temperature and B_{12}-concentration for extended alkyl-CoM of multiple methanogen Mcr such as *M. mazei*, *M. acetivorans*, *Mt. marburgensis* (**Figure 6**). In contrast, Mcr from *Methanococcacaea* appears to be thermos- and B_{12}-sensitive. Further investigation is needed to fully understand the behavior of these latter.

The cell-free lysate approach employed in this study enables a quick analytical method to assay the promiscuity of Mcr towards non-C1 alkyl-CoM. Our results furthermore confirmed that this assay is applicable on a broad range of cultivable methanogens. We also showed that *Methanosarcina* is a potential role model for overexpression of yet-to-be alkyl-Coenzyme M reductase, with high regards to its promiscuity towards non-C1 substrates. Moreover, our method emphasized the H_2 availability- and/or the abundance of Mcr isoenzyme-dependence of substrate promiscuity of *Mt. marburgensis*.

Optimal Mcr assay allowing further understanding onto Mcr activity of *Methanothermobacter marburgensis*

Our results showed that cell-extracts of *Mt. marburgensis* grown under non-hydrogen limitation, in which Mcr-isoenzyme II is stronger expressed, exhibited an ethane-formation rate of ca. 7.7 %. This later ethane formation level showed that the well-studied enzyme variant Mcr-I from *M. marburgensis* has a low substrate promiscuity towards C2, and substrate promiscuity is generally higher than anticipated. Mixtures of methyl- and ethyl-

coenzyme M showed a substantial inhibitory effect of ethyl-coenzyme M for methane formation, in particular for Mcr-I, which is in line with the previous finding that ethyl-coenzyme M is readily processed by Mcr, but ethane formation is blocked in this enzyme variant.

We have attempted without success the conversion of propyl-CoM employing different promiscuous cell-free lysates such as *M. mazei*, *M. acetivorans*, and *Mt. marburgensis* under different assay conditions (data not shown). Nevertheless, multiple genetic modification tools for methanogens have been developed [20,21] along with expression of uncultured archaea Mcr [22] which may allow better conversion of longer alkyl-CoM (C3 and above) in the near future. To conclude, employing our cell-free lysate Mcr assay approach to investigate this enzyme family is a rapid and efficient assay to test the enzyme activity of these recombinant enzymes in order to provide a first milestone for further engineering of artificial cultivable alkanogens.

Materials and Methods

Chemical and Materials

Methyl-CoM, ethyl-CoM, and coenzyme B were synthesized by following the reported protocol [19]. Gas formation was recorded on an Agilent 6890 GC-FID system (Agilent Technologies, CA, USA) and analyzed using OpenLab CDS software (Agile Technologies, CA, USA). OD600 value and Bradford assay were recorded on BioPhotometer Plus 6132 spectrophotometers (Eppendorf, Germany) at 595 nm wavelength. All chemicals were purchased from Merck. Titanium (III) citrate were prepared following the description elsewhere [23]. MilliQ water was produced by Milli-Q Direct (Merck Millipore, MA, USA). Universal Hood II-Gel Doc system (Bio-Rad) was used to display the SDS-PAGE gel.

Strains, cultivation, and cell-free lysate preparation

M. barkeri (strain Fusaro), *M. acetivorans* (strain WWM73) [16], and *M. mazei* (strain Gö I) [21], *M. maripaludis* (strain JJ:*Δupt*) [24], *Mt. okinawansis* [25] and *M. marburgensis* [9], were used for this study. For *M. acetivorans,* cell pellets were obtained at different OD600 values. For the other strains, cell pellets were obtained at OD600 value between 0.3-0.5.

For *M. acetivorans*, cultures were first grown statically in High-Salt (HS) medium supplemented with 150 mM MeOH as substrate and 1 mM Na_2S as sulfur source at 37 °C for 1 day [26]. Typical OD_{600} reaches 0.18-0.20. For low OD_{600} experiments, cultures were then harvested by centrifugation, resuspended in HS medium to yield the cell pellet. For moderate and high OD_{600} experiments, cultures were grown at 25 °C (for moderate OD_{600}) or 37 °C (for high OD_{600}) until OD_{600} reaching the desired value range. The cell pellet was then obtained by centrifugation.

For *M. barkeri,* the desired OD_{600} value (0.3-0.5) can be reached within 3-4 days by statically cultivating at 37 °C in HS medium supplemented with H_2 and 1% of H_2S as growth substrate and sulfur source, respectively. For *M. marburgensis*, the cell pellet was obtained using reported cultivation method [27] under H_2/CO_2 with H_2S as growth substrate and sulfur source, respectively. For *M. mazei*, the HS medium was used as growth liquid supplemented with 125 mM MeOH as growth substrate and 1% H_2S as sulfur source [28]. For *M. maripaludis and Mt. okinawansis*, the liquid McFc medium was used for cultivation [29].

Cell-free lysate was prepared by sonication (Bandelin Sonopuls, Germany) at 30% amplitude, 30 s pulse and 30 s rest time for 10 min. The cell-lysate fraction was then retrieved by centrifugation and the protein concentration was determined by Bradford assay [30]. Cell-free lysates were then aliquoted in 10 mL serum vials, of which the headspace was filled with 100% H_2 and then stored at -80 °C until further uses.

Enzyme activity assay employing cell-free lysate

The enzyme assay was performed following previously published protocol [31]. In brief, the assay solution was prepared in 10 mL serum vial containing 0.5 mM alkyl-CoM, 0.25 mM CoB, 0.2-1.1 mM of vitamin B_{12}, 20 mM of Ti(III) citrate, and 100 µg of cell-free lysate (gradually warmed at RT for 30 min. prior to the assay). Tris-HCl (50 mM, pH 7) was then added to make up to 2 mL total volume. The solution was then mixed by pipetting and then closed with a rubber stopper. The head space was flushed with 100% H_2 and then immediately transferred into heat block at different temperature (25, 37, and 49 °C) for incubation. The gas formation was monitored by injecting 100 µL of the head space into the GC-FID.

For the assay employing mixture of methyl- and ethyl-CoM, the previously described protocol was employed with slight modification. The assay solution was prepared in 10 mL serum vial containing methyl- and ethyl-CoM at different concentration were used as following; for only methyl-CoM–5mM of methyl-CoM; for 9 equivalence (Eq) methyl-CoM:Eq ethyl-CoM–4.5 mM of methyl-CoM and 0.5 mM of ethyl-CoM; for 1 Eq methyl-CoM:1 Eq ethyl-CoM–2.5 mM of methyl-CoM and 2.5 mM of ethyl-CoM; for 1 Eq methyl-CoM:9 Eq ethyl-CoM–0.5 mM of methyl CoM and 4.5 mM of ethyl-CoM; for only ethyl-CoM: 5 mM of ethyl-CoM. 0.25 mM CoB, 0.2 mM of vitamin B_{12}, 20 mM of Ti(III) citrate, and 100 µg of cell-free lysate (gradually warmed at RT for 30 min. prior to the assay) were then sequentially added. Tris-HCl (50 mM, pH 7) was then added to make up to 2 mL total volume. The solution was then mixed by pipetting and then closed with a rubber stopper. The head space was flushed with 100% H_2 and then immediately transferred into heat block at 65 °C) for incubation. The gas formation was monitored by injecting 100 µL of the head space into the GC-FID.

SDS-PAGE analysis of cell-free lysates of *Mt. marburgensis* harvested under different growth conditions

A fractionated ammonium sulfate precipitation of the *Mt. marburgensis* lysates was performed as described elsewhere [18]. The cell extracts were first well mixed with ammonium sulfate reaching a 70 % salt concentration and kept on ice for 2 h. After centrifugation (14,000 rpm, 5 min, 4 °C) the supernatants were further enriched with ammonium sulfate until the salt concentration reached about 100 %. After a second centrifugation step the pellets were resuspended in 10 µl sterile water. The protein concentration was estimated by the NanoDropTM Lite Spectrophotometer (Thermo Fisher). The samples were subsequently mixed with loading dye and incubated for 5 min at 95 °C.

For SDS PAGE a 10 % polyacrylamide gel was loaded with 2 µg protein/lane for both samples. Precision Plus Protein Dual Color Standard (#1610374, Bio-Rad) was applied as a size marker. The electrophoresis was performed at 120 V for 80 min before the gel was processed by Coomassie staining.

Statistical analysis

The results were expressed as the mean ± standard deviation. Replication was conducted in duplication. The significance of each coefficient was determined by using the Student *t-test* and p-value. The ANOVA analysis was conducted at 95% significance level. The analysis software used for this study was Minitab® 21 (version 3.1) (Minitab Inc, State College, Palo Alto, CA, USA.).

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

T.N. and S.S: conceptualization and data curation, T.N. & S.K: conducting experiments, T.N., S.K., & S.S. drafting the manuscript, S.S.: supervising, project administration and funding acquisition. All authors commented and approved the final version of the manuscript.

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Figures

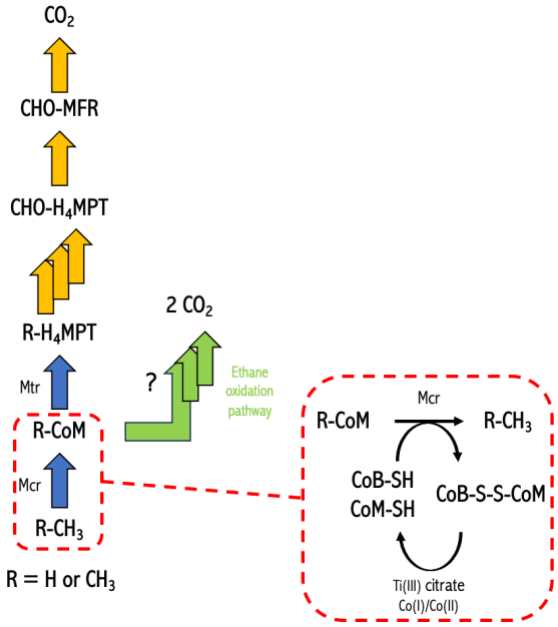


Figure 1. Anaerobic oxidation of alkane pathway occurred in archaea. The discontinued box highlights the conversion of alkyl-Coenzyme M to corresponding alkane catalyzed by Mcr including the heterodisulfide reducing system involving the utilization of Ti(III) citrate and Co(II)/Co(I).

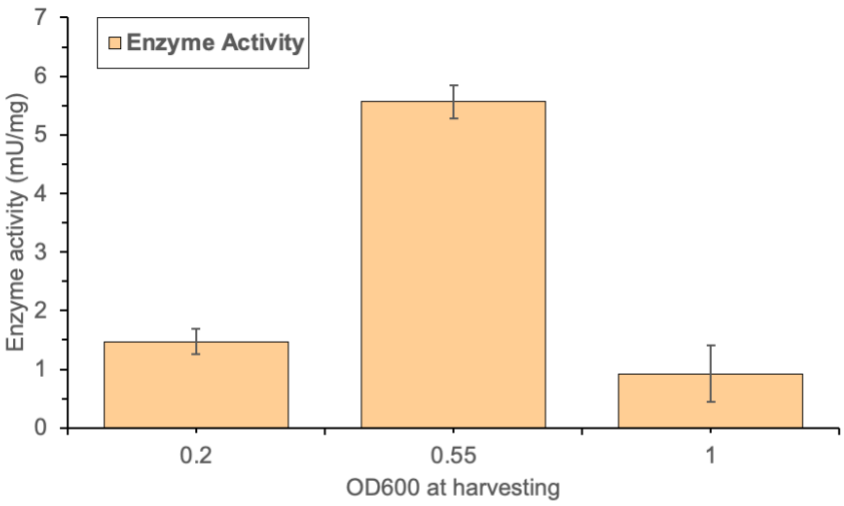


Figure 2. Influence of the growth phase onto Mcr methane formation activity from methyl-CoM and Coenzyme B in cell-free lysates prepared from M. acetivorans supplemented with MeOH.

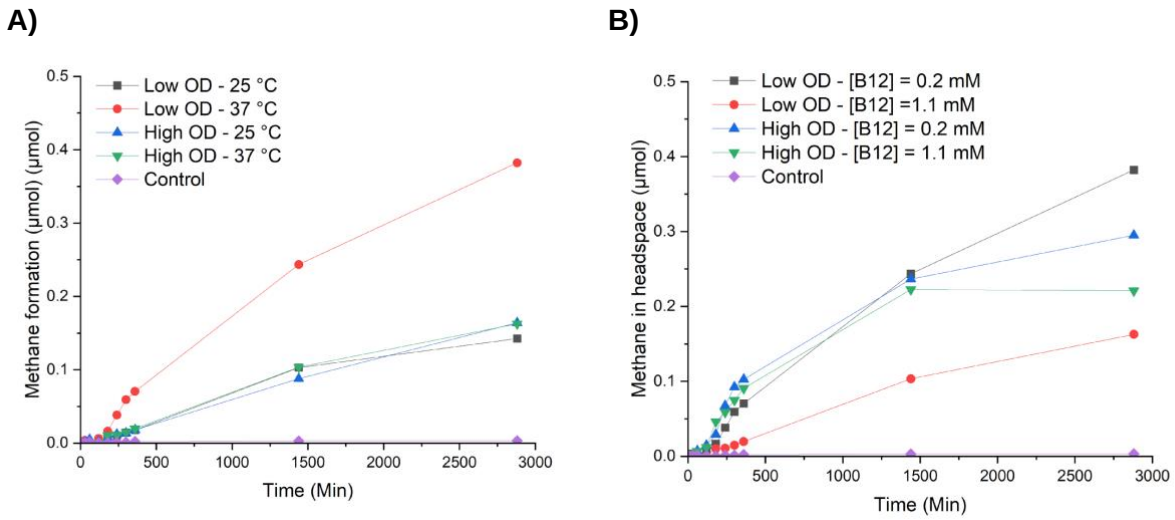
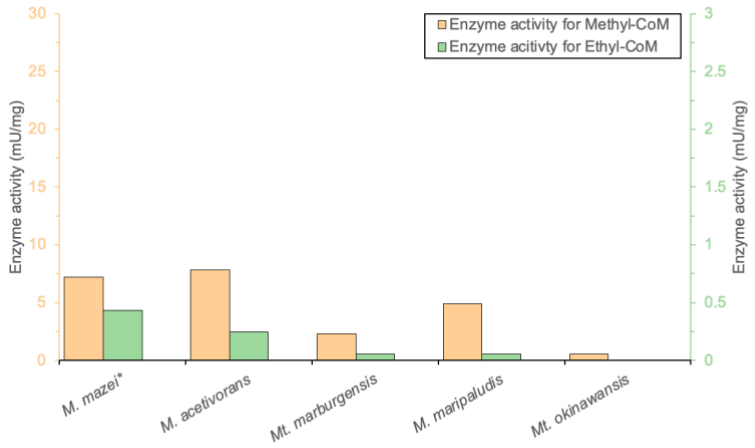


Figure 3. Comparison of M. acetivorans cell-free lysates enzyme activity under (A) different incubation temperatures (25 and 37 °C) of different harvested at different growth phase (low and high OD_{600}); and (B) different vitamin B_{12} concentrations (0.2 mM, 1.1 mM) at different growth phase (low and high OD_{600}. Assays were conducted in duplicate under conventional assay conditions (100 µg of protein in cell lysate, 0.2 mM vitamin B12, 0.2 mM B_{12}, 37 °C).

A)



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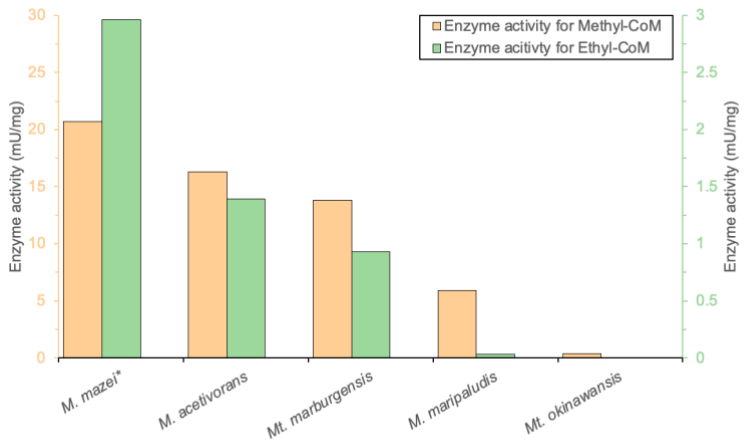
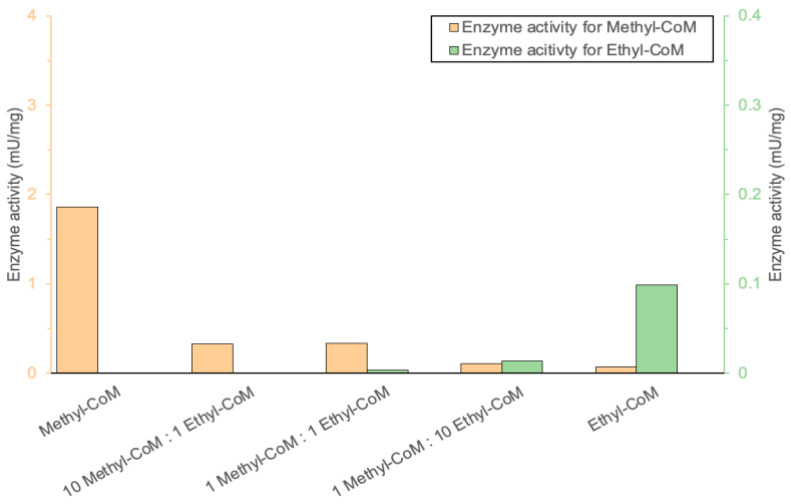


Figure 4. Promiscuity and enzyme activity for ethyl-CoM and promiscuity of cell-free lysates from different methanogens at different conditions: **(A)** conventional conditions: 37 °C, 0.2 mM B12, 20 mM Ti (III) citrate, and OD600 at harvesting between 0.3-0.5; **(B)** optimal conditions: 49 °C, 1.1 mM B12, 20 mM Ti(III) citrate, and OD600 at harvesting between 0.3-0.5. *For M. mazei, due to low protein concentration in cell lysate, cell was harvested at OD600 between 1.4-1.6.

A)



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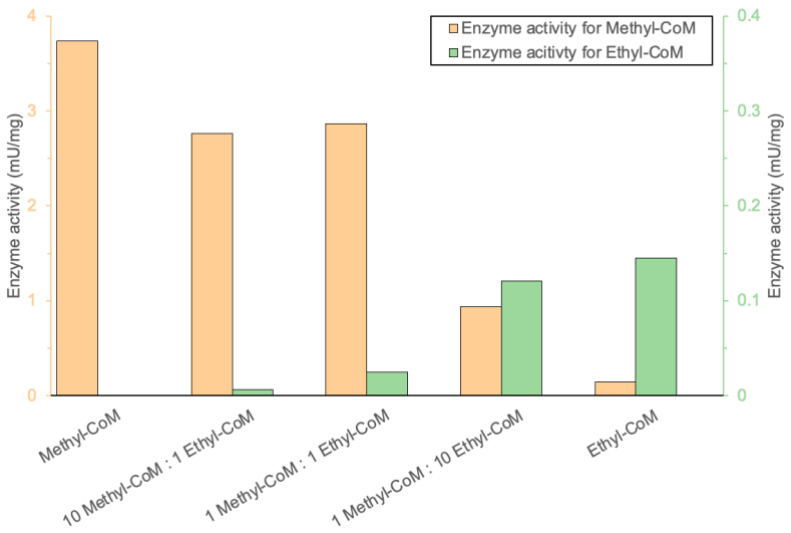

Figure 5. Comparison of enzyme activity for methyl- and ethyl-Coenzyme at different mixture concentrations employing different cell-free lysate from Mt. marburgensis grown either **(A)** in bioreactor with a continuous flow of growth substrate or **(B)** in serum vial with a periodic fill of growth substrate under high pressure. The experiments were carried out under conventional conditions: 37 °C, 0.2 mM B12, 20 mM Ti (III) citrate, and OD600 at harvesting between 0.3-0.5.

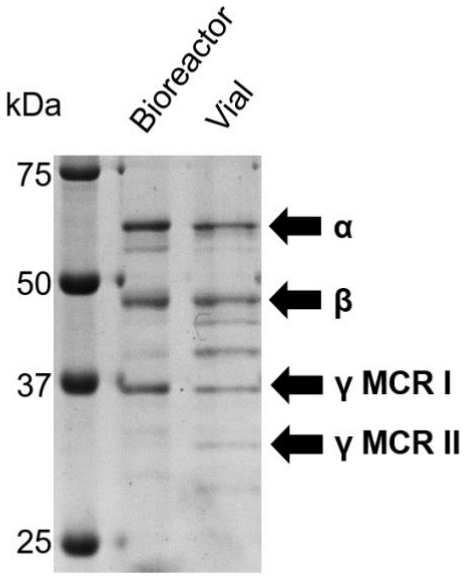

Figure 6. SDS-PAGE of ammonium sulfate-precipitated Mt. marburgensis Mcr extracts collected from two different growth conditions. The bioreactor and the vial sample samples were harvested at high- and low-optical density, respectively. The sizes of the subunits of both Mcr isoforms are: 65 kDa (alpha), 49 kDa (beta), 38 kDa (gamma of Mcr I), and 33 kDa (gamma of Mcr II).

Table 1. Enzyme activity for methyl- and ethyl-CoM, and the promiscuity of cell-free lysate under optimal conditions generated by the prediction models.

	Activity methyl-CoM (mU/mg)	**Activity ethyl-CoM (mU/mg)**	**Promiscuity (%)**
Prediction value	8.4	1.5	10.9
Replicate 1	16.3	1.37	8.5
Replicate 2	16.7	1.43	8.9
Mean ± S.D.	16.5 ± 0.21	1.43 ± 0.06	8.7 ± 0.2