

Stepwise utilization of H₂ between *Geobacter sulfurreducens* and *Methanosarcina barkeri* promoted CO₂-to-CH₄ conversion

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Abstract

A syntrophic relationship was established between *Methanosarcina barkeri* (*M. barkeri*) and *Geobacter sulfurreducens* (*G. sulfurreducens*) to promote CO₂-to-CH₄ conversion using H₂. The purpose was to improve the H₂ utilization and CH₄ production rate in the H₂/CO₂ biomethanation systems due to gas-liquid mass transfer limitations. Proteomics analysis revealed that membrane-bound [NiFe] hydrogenase Hyb in *G. sulfurreducens* accelerated H₂ consumption during methanogenesis, while methyl-coenzyme M reductase, ferredoxin, flavodoxin, membrane-associated ATP synthase, and the membrane-associated proton pump were significantly up-regulated. Consequently, carbon fixation, energy conversion, and electron transfer in the metabolic pathways of *M. barkeri* cells were all improved. Interspecific electron transport mediated by fulvic acid-like substances occurred between *G. sulfurreducens* and *M. barkeri*, promoting CO₂-to-CH₄ conversion. The maximum CH₄ production rate (0.158 mL-CH₄/ mL-feedstock H₂/ day) in the *G. sulfurreducens* and *M. barkeri* co-culture was 3.1-fold higher than that in the *M. barkeri* pure culture. The lag and peak rate times were shortened by 24.6 and 45.7%, respectively. Therefore, The *M. barkeri* and *G. sulfurreducens* co-culture provides a sustainable and promising method of promoting CH₄ production using the H₂ and CO₂ feedstock gases.

1. Introduction

Renewable gas has been proposed to decarbonize industrial processes, especially high heat demand. Therefore, some industries are formulating initiatives to substitute a renewable gas for fossil natural gas [1]. Biomethane is a clean, renewable energy, considered an important alternative to fossil fuels. Microbial technologies offer a sustainable and cost-effective way to produce biomethane, and the released CO₂ can be converted using H₂ as an electron donor that is generated by surplus electricity from renewable wind or solar energy [2, 3]. Alternatively, hydrogenotrophic methanogens can reduce the CO₂ by-product to CH₄ using H₂ as the reducing agent [4, 5]. This H₂/ CO₂ biomethanation process reduces CO₂ emission, increases yield, and upgrades the purity of CH₄.

Moreover, the produced biogas has a lower calorific value (20–25 MJ/m³) than CH₄ (35.9 MJ/m³) based on CH₄ content and is considered among the most important renewable energy carriers [2, 6]. The CH₄ content in biogas must be upgraded to at least 95% to meet natural gas specifications, at which point it is referred to as biomethane or bionatural gas [7, 8]. Thus, increasing the CH₄ content by CO₂ removal or conversion into CH₄ is an essential step in biogas upgrading [9]. After biogas upgrading, the products can be directly injected into the natural gas grid, thereby increasing the application prospect of biogas [10].

Although biological CH₄ production from H₂ and CO₂ has many advantages, there are still several technical and economic barriers [11]. The low H₂ solubility reduces the gas-to-liquid mass transfer rate. Dissolved H₂ is key because methanogens use it for CO₂ conversion [11, 12]. Henry's law shows the gas-to-liquid mass transfer rate of H₂ is positively correlated with the gas transfer coefficient. Therefore, increasing the gas transfer coefficient is a direct and convenient method to speed up the mass transfer rate. Intense stirring [13], minimization of gas bubble size by the addition of packing materials [14], biogas recirculation [15] and

an increase in the reactor pressure [16] increase the gas transfer coefficient. Several novel methods, including membrane bioreactors and biological electrochemical systems, have also been developed to overcome this limitation. For instance, a hollow-fiber membrane increased the H₂ gas transfer coefficient from 30 to 430 h⁻¹, upgrading the final CH₄ concentration in the biogas to 95% [17]. However, additional research is required on the new material selection and material or surface modification because of membrane fouling and short service life. Direct electron uptake or *in-situ* H₂ generation in the microbial electrolysis cell is an efficient solution to the problem [18]. However, the electron transfer mechanisms are unclear, and the low cathode potential and energy efficiency limit its applications. Other potential solutions for the problem of low gas-to-liquid phase transfer rate include optimizing the reactor design and improving the H₂ capture capability.

A novel bioaugmentation method involves the application of *G. sulfurreducens* PCA to accelerate methanogenesis [19]. The accelerated methanogenesis observed after *G. sulfurreducens* addition, resulted from a syntrophic association between *G. sulfurreducens* and the methanogens affiliated with *Methanosaetaceae* and *Methanobacteriaceae* [19]. In this hypothetical syntrophic relationship, *Methanosaeta* obtains electrons for the reduction of CO₂ to CH₄ through direct interspecific electron transfer from the co-cultivated *Geobacter* species [20]. Studies concerning syntrophic interactions between *G. sulfurreducens* and methanogens with H₂ and CO₂ as the sole feedstocks are limited to date. While *G. sulfurreducens* are capable of hydrogen oxidation, *Geobacter* species, such as *G. metallireducens*, are incapable of this process [21, 22]. *G. sulfurreducens* reportedly has only one uptake hydrogenase, i.e., the membrane-bound [NiFe] hydrogenase Hyb [23, 24]. While this research has exciting prospects, the extent to which *G. sulfurreducens* co-cultivation with methanogens improves H₂/CO₂ biomethanation is unknown. Hence, additional investigation and the syntrophic interactions between these species must be elucidated.

In the present study, the stepwise utilization of H₂ between *G. sulfurreducens* PCA and *M. barkeri* MS to promote CO₂-to-CH₄ conversion was explored, and the working mechanism was different from previous studies that focused on anaerobic fermentation of organic substrates, such as glucose, ethanol and acetate. Scanning electron microscopy (SEM), three-dimensional fluorescence spectra (3-DEEM), and proteomic analysis were used to reveal the metabolic pathway of CH₄ production with H₂ and CO₂ as the feedstock gases, and the promotion mechanism of the syntrophic relation between *G. sulfurreducens* PCA and *M. barkeri* MS. The aims and objectives of this study were as follows: (1) To obtain a *G. sulfurreducens* and *M. barkeri* co-culture; (2) To observe the cell surface morphology and interaction degree of the co-culture; (3) To analyze the extracellular polymer substances (EPS) in the co-culture; (4) To reveal the syntrophic interaction in the co-culture using H₂ and CO₂ as the sole substrate at a protein level.

2. Materials and methods

2.1 Bacterial and archaeal cultivation

G. sulfurreducens PCA (DSM 12127) and *M. barkeri* MS (DSM 800) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). A culture medium of *G. sulfurreducens* was prepared using the medium 826 provided by DSMZ (Braunschweig, Germany), with 10

mM Na-acetate as the sole electron donor and 50 mM Na₂-fumarate as the electron acceptor. The cultivation process was as follows: NH₄Cl, Na₂HPO₄, and KCl were dissolved, and the medium was purged with an 80% N₂/ 20% CO₂ gas mixture for 30 min to facilitate anoxic conditions. The medium was dispensed under the same gas atmosphere into anoxic serum vials and sterilized using an autoclave (121°C, 20 min); after autoclaving, the remaining ingredients were added to the medium from anoxic stock solutions prepared under a 100% N₂ gas atmosphere and sterilized by 0.22 µm membrane filtration. During membrane filtration, appropriate volumes of the stock solutions were injected into the sterile medium using a disposable syringe. Finally, the strain was cultured in an anaerobic incubator (DG250, Don Whitley Scientific, UK) at 37°C to the logarithmic growth before formally starting the experiment.

The culture medium of *M. barkeri* MS was optimized using medium 120a provided by DSMZ (Braunschweig, Germany), with 30 mM methanol as the sole electron donor (no FeSO₄ was added). The cultivation process was as follows: K₂HPO₄, KH₂PO₄, NH₄Cl, MgSO₄·7H₂O, CaCl₂·2H₂O, and NaCl were dissolved, and the medium was purged with an 80% N₂/ 20% CO₂ gas mixture for 30 min to facilitate anoxic conditions. The medium was then dispensed under the same gas atmosphere into anoxic serum vials (100 mL) to 30% of their volume and sterilized using an autoclave (121°C, 20 min). After autoclaving, the remaining ingredients were added from anoxic stock solutions prepared under a 100% N₂ gas atmosphere and sterilized by 0.22 µm membrane filtration. During membrane filtration, appropriate volumes of the stock solutions were injected into the sterile medium using a disposable syringe.

2.2 Co-cultivation experiment setup

Before the co-cultivation experiment, *M. barkeri* and *G. sulfurreducens* (50 mL each) cultures were prepared using the cultivation methods described above. The two cultures were then condensed by centrifugation (2300 r/min, 4°C) to 2 mL. Two 50 mL anoxic serum vials (with working volume of 20 mL each) were prepared under a pure N₂ gas atmosphere and sealed with butyl rubber and aluminum foil, containing the co-cultivation medium and the condensed strains. The co-cultivation medium (1000 mL) contained 0.35 g K₂HPO₄, 0.23 g KH₂PO₄, 0.50 g NH₄Cl, 0.50 gM gSO₄·7 H₂O, 0.25 g CaCl₂·2 H₂O, 2.25 g NaCl, 1.00 mL trace element solution (see Medium 320 by DMSZ (Braunschweig, Germany)), 0.85 g NaHCO₃, 10.00 mL vitamin solution (see Medium 141 by DMSZ (Braunschweig, Germany)), 0.30 g L-Cysteine-HCl·H₂O, 0.30 g Na₂S·9 H₂O. The serum vials were sterilized using an autoclave (121°C, 20 min), and the co-cultivation medium was sterilized by 0.22 µm membrane filtration.

The co-cultivation experiment was set up in three groups (experimental, control, and blank groups). The experimental group contained condensed *M. barkeri* and *G. sulfurreducens* inoculated into anoxic serum vials using a disposable syringe at a 1:1 ratio. The control group contained only condensed *M. barkeri*, and the blank group contained no substrates. The experimental and control groups were purged with an 80% H₂/ 20% CO₂ gas mixture for 30 min. Next, 20 mL of the 80% H₂/ 20% CO₂ gas mixture was added into the serum vial headspace using a gas-tight syringe (SEG syringe, Australia) at 1 bar overpressure. The 80% H₂/ 20% CO₂ gas mixture comprised feedstock gases (no organic substrates were added). The blank group was prepared using the same process, except that serum vials were purged with pure N₂ for 30 min and contained no substrates. All three groups were purged with a gas mixture for four cycles (5 days per cycle) and placed in a

constant-temperature oscillating incubator at 37°C. The results from the fourth cycle were used for experimental analysis. No other nutrients or nutrient solution components were added during the reaction period.

2.3 Analytical methods

2.3.1 Gas chromatography and scanning electron microscopy analysis

The H₂, CO₂, and CH₄ concentrations were determined by gas chromatography (Agilent 7820A, USA). The gas chromatograph contained a thermal conductivity detector, a 5A sieve column, and a HaySep Q column [25]. A field emission scanning electron microscope (FESEM; Zeiss Gemini 300, Germany) was used to observe the interaction of co-culture after the completion of the experiment [26].

CH₄ production data obtained from gas chromatography was fitted to the modified Gompertz (Eq. 1) using Origin 9.0 software.

$$H = H_m \exp \left\{ \exp \left[\frac{R_m e}{H_m} (\lambda - t) + 1 \right] \right\}$$

1

where H is the cumulative CH₄ yield (mL of CH₄/ mL of feedstock H₂); H_m is the maximum CH₄ yield potential (mL of CH₄/ mL of feedstock H₂); R_m is the peak rate (mL of CH₄/ mL of feedstock H₂/ day); λ is lag time (d); t is reaction time (d); and e is 2.718.

The peak time T_m (d) was calculated using Eq. 2:

$$T_m = \frac{H_m}{R_m e} + \lambda$$

2

2.3.2 Extracellular polymer substances (EPS) extraction and analysis

At the end of the experiment, three EPS layers: tightly bound (TB-EPS); loosely bound (LB-EPS); and soluble (S-EPS) EPS layers, were extracted by thermal extraction [27, 28]. Three-dimensional excitation emission matrix (3-DEEM) fluorescence spectra of these layers were imaged using a fluorescence spectrometer (Aquag, Horiba, France). The emission wavelength (Em) scan was 245–822 nm (increment, 4.48 nm), and the excitation wavelength (Ex) scan was 200–550 nm (increment, 5 nm). An EEM spectrum can be divided into five regions following the Ex/Em wavelength [29–32].

2.3.3 BCA protein test and electron transport system (ETS) activity analysis

The weight of the protein in the microbial cells is approximately 60% of the total cell dry weight [33]. The concentrations of protein were measured by a BCA Protein Detection Kit (Beijing ComWin Biotech Co., Ltd., China) and calculated according to the absorbance value at 562 nm, following the manufacturer's manual (<https://www.cwbiotech.com/goods/index/id/10069>). Meanwhile, *M. barkeri* and *G. sulfurreducens* protein concentrations were tested before the experiment. Thus, the protein concentrations in the pure and co-culture samples were calculated as 0.265 ± 0.010 and 0.487 ± 0.015 mg/mL, respectively. After the experiment, protein concentrations in the pure culture and co-culture samples were 0.294 ± 0.010 mg/mL and 0.362 ± 0.012 mg/mL, respectively.

Then, a 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride-electron transport system (INT-ETS) was used to investigate the ETS activities of *M. barkeri* and *G. sulfurreducens* as earlier detailed [30, 34, 35]. Finally, INT-ETS activity was calculated as $\text{ETS activity} = \text{OD}_{485} (\text{Experimental}) / \text{OD}_{485} (\text{Control})$, where OD_{485} is the absorbance of the supernatant at 485 nm.

2.3.4 Quantitative proteomics analysis

Label-free quantitative proteomics was used to compare the protein levels between samples quantitatively. Label-free quantitative proteomics using the DIA (Data Independent Acquisition) is an important method in proteomics research. The technique has good reproducibility and selectivity, high accuracy, and easy data traceability [36]. Before protein extraction, the cells were condensed by centrifugation (2300 r/min, 4°C) and washed thrice using phosphate buffer to remove impurities. The washed cells were stored at -80°C for subsequent proteomics analysis following a previously discussed method [37, 38]. The quantitative information used to identify the extracted proteins was obtained from the Uniprot database for *M. barkeri* and *G. sulfurreducens*. Functional annotations of the proteins were conducted using the Diamond program of eggNOG-mapper against the non-redundant protein database. The COG (<http://www.ncbi.nlm.nih.gov/COG/>) and the KEGG (<http://www.genome.jp/kegg/>) databases were used to annotate and group the identified proteins. Statistical analysis via one-way ANOVA was performed using the Origin 9.0 software to evaluate the co-cultivation results at $p < 0.05$.

3. Results and discussion

3.1 Promoting methanogenesis by *M. barkeri* and *G. sulfurreducens* co-cultivation

The contents of H_2 and CH_4 in the headspace of the fourth cycle were obtained by gas chromatography (Fig. 1). The kinetic process for CH_4 production was elevated in the experimental group. The H_2 consumption was highest rate (Fig. 1a) when equal stoichiometric volumes of H_2 and CO_2 feedstock were injected. In contrast, the H_2 feedstocks in the experimental group (*M. barkeri* and *G. sulfurreducens*) and control (pure *M. barkeri*) group were completely consumed within four and eight days, respectively. Thus, *G. sulfurreducens* also consumed the H_2 and shortened the reaction time in the experimental group by 50%.

The maximum CH_4 yield of the experimental group was 0.213 mL of CH_4 / mL of feedstock H_2 on the fourth day, while that of the control group was 0.193 mL of CH_4 / mL of feedstock H_2 on the eighth day (Fig. 1b).

These results demonstrate that co-cultivation of *M. barkeri* and *G. sulfurreducens* improved CH₄ production. According to the modified Gompertz fitting of the CH₄ production data (Table 1), the CH₄ yields of the two groups were similar by the end of the experiment because the groups received the same volumes of the sole substrates, H₂ and CO₂. The peak rate of CH₄ production in the experimental group was 0.158 mL of CH₄/ mL of feedstock H₂/ day, which was three-fold higher than the control group (0.051 mL of CH₄/ mL of feedstock H₂/ day). Additionally, the lag and the peak rate times of the reaction were reduced by 24.6 and 45.7%, respectively. These results demonstrate that *M. barkeri* and *G. sulfurreducens* co-cultivation significantly improved the reaction kinetics, the H₂ consumption rate, and the CH₄ production rate. Moreover, co-cultivation partially resolved the H₂ gas-liquid mass transfer limitation.

3.2 SEM images of the co-cultivated *M. barkeri* and *G. sulfurreducens*

After completion of the methanogenesis experiment, the growth and distribution of *M. barkeri* and *G. sulfurreducens* were observed using SEM (Fig. 2). The experimental group had spherical and rod-shaped cells, fitting the morphology characteristics of *M. barkeri* and *G. sulfurreducens*, respectively (Fig. 2b). However, the rod-shaped cells were less abundant than the spherical cells, mainly because *G. sulfurreducens* could not use CO₂ as a carbon source for growth (unlike *M. barkeri*). In the control group, all the cells in the visual field were spherical, consistent with the morphology characteristics of *M. barkeri* (Fig. 2a). The SEM results reveal the concentrated co-distribution of *M. barkeri* and *G. sulfurreducens* and their close association. These SEM and CH₄ production data (Fig. 1) confirm that *M. barkeri* and *G. sulfurreducens* can form a syntrophic relationship.

3.3 EPS and INT-ETS analysis with co-cultivated *M. barkeri* and *G. sulfurreducens*

Geobacter and methanogens are reported to be syntrophic under anaerobic conditions, and the electrons produced by *Geobacter* being transported to methanogens for CO₂-to-CH₄ conversion [20]. However, the syntrophic relationship between *M. barkeri* and *G. sulfurreducens* in the H₂/ CO₂ biomethanation system remains unclear. The 3-DEEM fluorescence spectra of EPS extracted from *M. barkeri* and *G. sulfurreducens* co-culture were divided into five regions, and the relative proportions of each region were evaluated (Fig. 3). In S-EPS, Regions I, II, and IV of the experimental group accounted for 39.1, 37.9, and 8.5%, respectively. The three regions accounted for 85.5% overall. Likewise, Regions I, II, and IV of the control group accounted for 36.6, 36.7, and 6.9%, respectively. The overall proportion of these three regions in the experimental group was significantly higher than in the control group. However, the substances from these three regions are mainly involved in cell movement and metabolic activity. Therefore, whole-cell activity or metabolic ability was stronger in the experimental than in the control group.

In LB-EPS, Regions III and V of the experimental and control groups accounted for 20.6 and 3.3% and 17.5 and 3.1%, respectively. In TB-EPS, Region III and V of the experimental and control group accounted for 19.7 and 2.2% and 16.6 and 3.59%, respectively. Region III and V reported the redox substances that act as a medium for extracellular interspecific electron transfer. Humus substances, including humic acid like and

fulvic acid-like substances, are rich in redox-active groups, such as quinone/hydroquinone and phenolic hydroxyl group, which can exchange electrons between EPS layers and facilitate interspecific electron transfer [39, 40]. Previous studies indicated that electron accepting/donating capacity positively correlates with the content of humus substances because the quinones in humus substances have redox-active groups [41]. Additionally, humus substances such as fulvic acid can facilitate interspecific electron transfer between two *Geobacter* species [42]. The relative proportion of Region III in the experimental group was higher than that in the control group, indicating that extracellular interspecific electron transfer through fulvic acid-like substances was enhanced in the experimental group. Therefore, the syntrophic relationship between *M. barkeri* and *G. sulfurreducens* involved an electron transfer process. Furthermore, the INT-ETS activity analysis showed that the electron transport system activity of *M. barkeri* and *G. sulfurreducens* co-culture was 22% higher than for the pure *M. barkeri*.

3.4 Label-free quantitative proteomics analysis of *M. barkeri* and *G. sulfurreducens*

The proteins of *M. barkeri* and *G. sulfurreducens* were analyzed by label-free quantitative proteomics. In total, 1836 proteins were identified in both *M. barkeri* and *G. sulfurreducens*. In those identified proteins, the most did not change compared with *M. barkeri* only, and a few were up or down-regulated (Fig. S3). Specifically, the *M. barkeri* and *G. sulfurreducens* co-culture had 130 and 93 significantly up-regulated (fold-change ≥ 1.50 and $p \leq 0.05$) and down-regulated (fold-change ≤ 0.67 and $p \leq 0.05$) proteins, respectively, compared to *M. barkeri* only (Fig. 4). Network analysis revealed that 223 of the significantly up-regulated/ down-regulated proteins highly interacted with one another (Fig. S4), suggesting that *G. sulfurreducens* induced systematic changes in the physiological status of *M. barkeri*. These systematic changes included changes in electron transfer and energy conservation.

Functional annotation of the significantly up-regulated/ down-regulated proteins using the clusters of orthologous groups database revealed several differentially expressed proteins (Fig. 4c). The significant, differential expressed proteins contained the C, E, F, G, H, I, P and Q clusters of orthologous groups were classified as the class of metabolism, accounting for 50.0%. Information storage and processing (J and K) and cellular processes and signaling (D, M, O, T, U and V) classes accounted for 10.1 and 15.4%, respectively. Thus, the differentially expressed proteins corresponded to KEGG annotation and enrichment analysis (Fig. S3) and mainly involved the metabolism in the co-culture of *M. barkeri* and *G. sulfurreducens*, directly related to CH₄ production. The differentially expressed proteins in the D, E, F, G, H, I, J, K, L, M, O, T and U orthologous clusters were partly up-regulated alongside inorganic ion transport and metabolism (P) and energy production and conversion (C).

3.4.1 Proteome of *G. sulfurreducens* in co-culture: H₂ utilization and electron transfer

To better understand the H₂ utilization and electron transfer processes in *G. sulfurreducens*, proteins involved in these processes were further analyzed (Table S1). The membrane-bound [NiFe] hydrogenase Hyb (HybL, HybS, and HybB) enzymes correspond to proteins Q74F24, Q74F27, and Q74F25 in the proteomics data.

Membrane-bound [NiFe] hydrogenase Hyb is an uptake H_2 hydrogenase in *G. sulfurreducens* [23, 24]. Hydrogenase Hyb transforms H_2 into protons and electrons. Protein carriers transport the electrons to extracellular electron acceptors, involving proteins Q74GH2, Q747K6, and Q749U9 (Table S2) and three cytochrome b-type or cytochrome c-type proteins. Menaquinone (MQ) and the three cytochrome b-type or cytochrome c-type proteins signal the electron transfer process in the inner membrane of *G. sulfurreducens*. Afterward, the electrons are transported to the periplasmic c-type cytochrome (Ppc) in the periplasm, passed to the outer membrane cytochromes, including OmcB, OmcC, OmcS, and OmcZ, and finally to the extracellular acceptor [43]. Previous research showed that the type IV pili proteins (microbial nanowires) in the *G. sulfurreducens* co-culture proteom can act as a direct interspecific electron transport medium [43, 44]. However, no type IV pili proteins were detected in the experimental group. Therefore, the fulvic acid-like substances mainly mediated the interspecific electron transfer between *G. sulfurreducens* and *M. barkeri* (Fig. 3).

3.4.2 Proteome of *M. barkeri* in co-culture: faster production rate and new pathway

To better understand the changes that occur in the H_2 / CO_2 biomethanation pathway during co-cultivation of *G. sulfurreducens* and *M. barkeri*, proteins involved in carbon fixation, energy conversion, and electron transfer in *M. barkeri* were further analyzed (Fig. 5). Different enzymes catalyzed carbon fixation during H_2 / CO_2 biomethanation (Fig. 6). *M. barkeri* encodes three distinct types of hydrogenase that transform H_2 into protons and electrons, including the F_{420} -dependent (Frh), methanophenazine-dependent (Vht), and ferredoxin-dependent (Ech) hydrogenases [45, 46]. CO_2 reduction and energy conservation in *M. barkeri* involve many complicated reactions in which methanofuran (MFR), tetrahydrosarcinapterin (H_4 MPT) and coenzyme M (HS-CoM) are C1-unit carriers and ferredoxin (Fd), coenzyme F_{420} , coenzyme B (HS-CoB) and methanophenazine (MP) are electron carriers [47]. Moreover, The label-free proteomics data revealed that methyl-coenzyme M (CH_3 -S-CoM) reductase (Mcr) was significantly up-regulated ($p < 0.05$) during co-cultivation of *G. sulfurreducens* and *M. barkeri* (compared with the *M. barkeri* monoculture). However, membrane-associated energy-converting [NiFe]-hydrogenase (Ech, $H_2 + Fd_{ox} \rightarrow Fd_{red}^{2-} + 2H^+$), membrane-associated methanophenazine (MP)-reducing [NiFe]-Hydrogenase (Vht, $H_2 + MP \rightarrow MPH_2$), and cytoplasmic coenzyme F_{420} -reducing [NiFe]-hydrogenases (Frh, $H_2 + F_{420} \rightarrow F_{420}H_2$) were down-regulated in the co-culture. These results indicate that direct conversion of H_2 molecules ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$) was lower in *M. barkeri*, and an alternative pathway ($CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$) facilitated the CO_2 -to- CH_4 conversion. At the same time, ferredoxin (Fd), iron-sulfur flavoprotein (Mbar_A0252), and flavodoxin (Mbar_A2179) were significantly up-regulated, indicating that intracellular redox activity was enhanced and electron transfer was accelerated. Furthermore, membrane-bound $F_{420}H_2$ dehydrogenase (Fpo, $MPH_2 + F_{420} \rightarrow MPH_2 + F_{420}$) was up-regulated, indicating that membrane-bound electron transport was enhanced. Membrane-bound electron transport is also known to play an important role in methanogenesis in *M. barkeri* [47, 48]. Additionally, the up-regulations ($p < 0.05$) of membrane-associated ATP synthase (ATPase) and membrane-associated proton pump (hpaA) in *M. barkeri* probably stimulated its growth and metabolism.

3.5 Stepwise H₂ utilization in *M. barkeri* and *G. sulfurreducens* co-culture promotes CO₂-to-CH₄ conversion

Based on the above analysis, a synergetic metabolic pathway for H₂ and CO₂ in *M. barkeri* and *G. sulfurreducens* co-culture was proposed (Fig. 6). This synergetic metabolic pathway increased H₂ consumption and the CH₄ production rates. In addition to the direct utilization of H₂ and CO₂ through *M. barkeri*, H₂ was transformed into electrons through *G. sulfurreducens*, then *M. barkeri* accepted electrons and reduced CO₂ to produce CH₄. Fulvic acid-like substances predominantly mediated the electron transport between *G. sulfurreducens* and *M. barkeri*. However, because no outer-surface c-type cytochromes or e-pili were identified in *M. barkeri*, its membrane-bound electron acceptor protein remains unknown, and further research is required. The *M. barkeri* and *G. sulfurreducens* co-cultivation provides an alternative pathway for CO₂-to-CH₄ conversion, partially alleviating the slow H₂ utilization. This alternative pathway improved the kinetics of methanogenesis.

4. Conclusion

A syntrophic co-culture of *M. barkeri* and *G. sulfurreducens* was established using an H₂ and CO₂ feedstock, considering the characteristics of *G. sulfurreducens*. Proteomics analysis revealed interspecific electron transfer between *M. barkeri* and *G. sulfurreducens*. Methanogenic proteins, such as Mcr, Fd, flavodoxin and membrane-associated ATP synthase, were up-regulated in the co-culture, indicating that electron transfer and energy conversion were enhanced. The experimental results reveal that the maximum CH₄ production rate (three-fold increment) increased and the lag and peak times (24.6 and 45.7%, respectively) were shortened. In general, extremely low H₂ solubility limits the CH₄ production rate during H₂/CO₂ biomethanation. The biological co-culture method from this provides a green and sustainable method to improve the H₂ utilization and the CH₄ production rates. This method may have wide applicability and requires further study.

Declarations

Author Contribution

Zhengnan Zhong: Writing - original draft, Validation, Formal analysis. Haiquan Dong: Methodology, Investigation. Rongxin Xia: Project administration; Resources. Junhu Zhou: Writing - review & editing. Jun Cheng: Resources; Data curation.

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Table

Table 1. Kinetic parameters of CH₄ production from H₂ and CO₂ in *M. barkeri* and *G. sulfurreducens* co-culture

Experimental Groups	CH ₄ content (mL of CH ₄ /mL of feedstock H ₂)	Dynamic parameters				
		H _m (mL of CH ₄ /mL of feedstock H ₂)	R _m (mL of CH ₄ /mL of feedstock H ₂ / day)	I (d)	T _m (d)	R ²
<i>M. barkeri</i>	0.193±0.004	0.203±0.009	0.051±0.003	1.454±0.155	2.933±0.155	0.993
<i>M. barkeri</i> and <i>G. sulfurreducens</i>	0.213±0.003	0.213±0.001	0.158±0.001	1.097±0.001	1.594±0.001	1

Figures

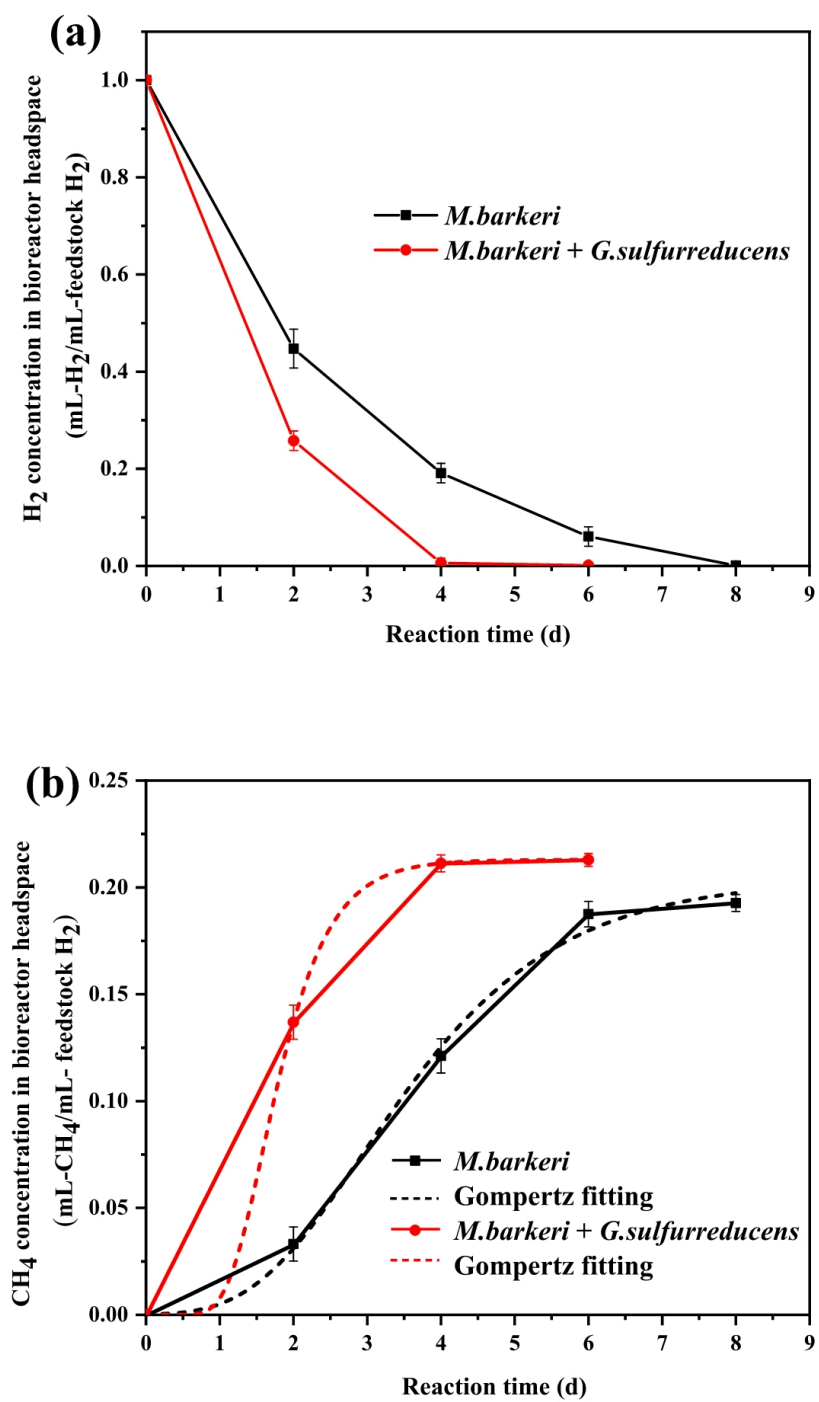


Figure 1

Dynamic concentrations of (a) H₂ feedstock and (b) CH₄ product in the bioreactor headspace.

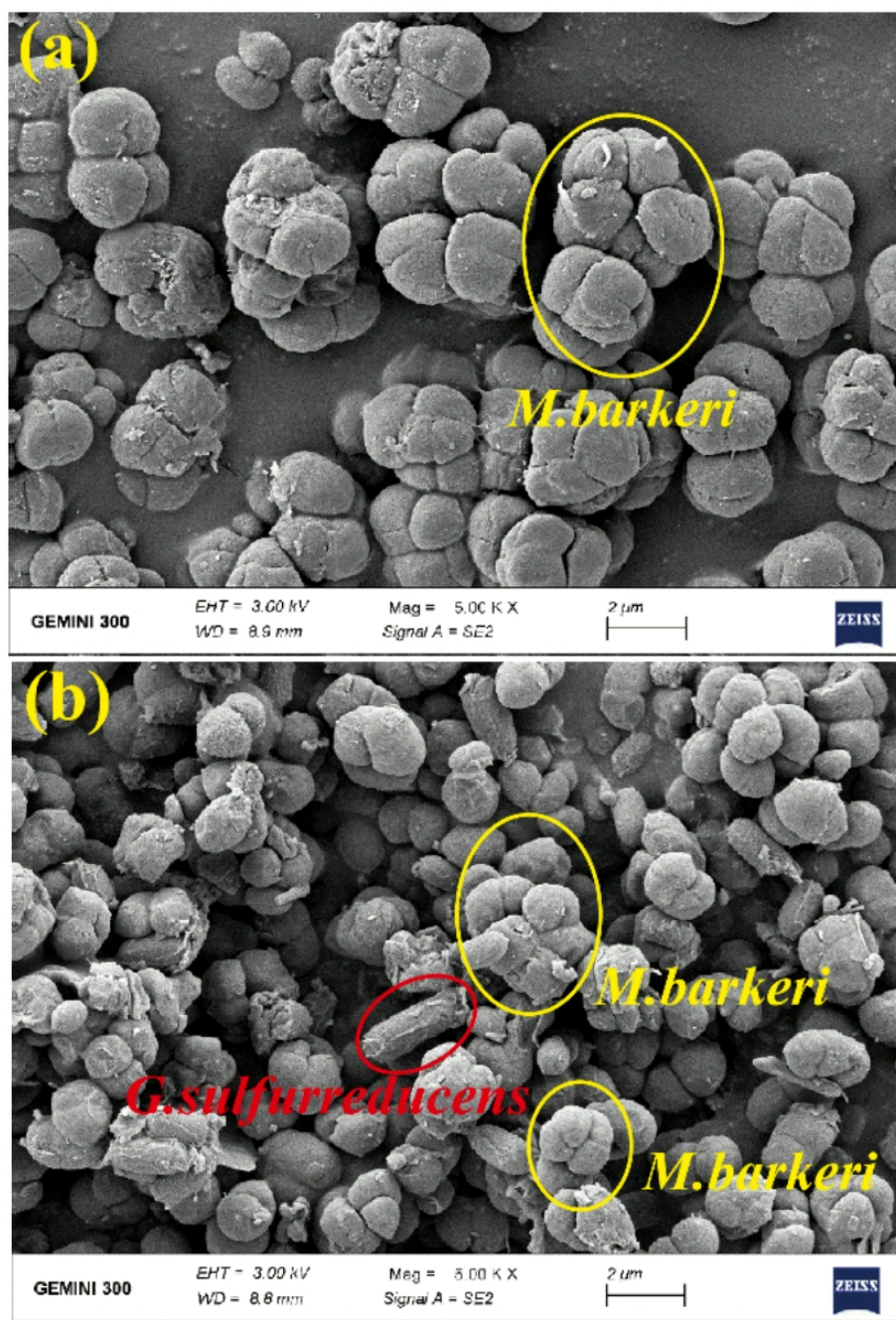
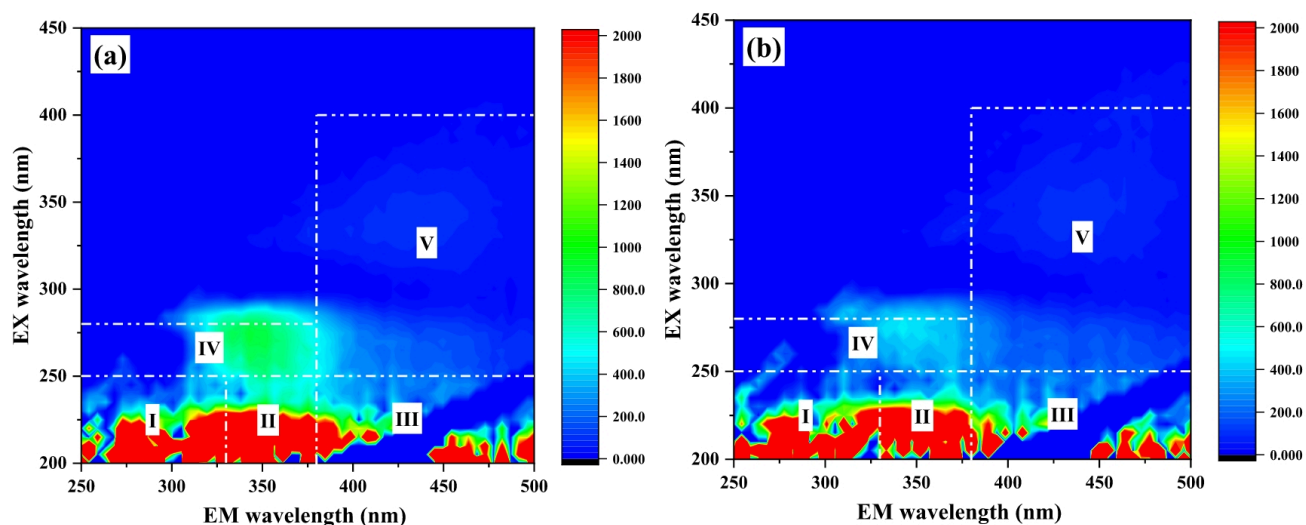


Figure 2

SEM images of *M. barkeri* and *G. sulfurreducens*. (a) Pure culture of *M. barkeri*; (b) Co-culture of *M. barkeri* and *G. sulfurreducens*.



Region	EX/EM wavelengths (nm)	Percent fluorescence response (P_i ; %) over the five regions					
		<i>M.barkeri</i>			<i>M.barkeri</i> + <i>G.sulfurreducens</i>		
		S-EPS	LB-EPS	TB-EPS	S-EPS	LB-EPS	TB-EPS
I	200-250/250-330	36.62	55.16	54.78	39.09	45.34	51.12
II	200-250/330-380	36.70	22.96	23.67	37.95	26.85	24.34
III	200-250/380-500	13.64	17.50	16.64	10.02	20.56	19.65
IV	250-280/250-380	6.91	1.07	1.32	8.48	4.14	2.64
V	250-400/380-500	6.13	3.32	3.59	4.47	3.11	2.24

Figure 3

Three-dimensional excitation emission matrix (3-DEEM) spectra analysis:

M.barkeri group (a) LB-EPS; *M.barkeri* and *G.sulfurreducens* group (b) LB-EPS. (S-EPS, the soluble extracellular polymeric substances; LB-EPS, the loosely bound EPS; TB-EPS, the tightly bound EPS.) Note: Region I were simple aromatic proteins, such as tyrosine; Region II were tryptophan-like substances; Region III were fulvic acid-like substances; Region IV were soluble microbial by-products; Region V were humic acid-like substances.

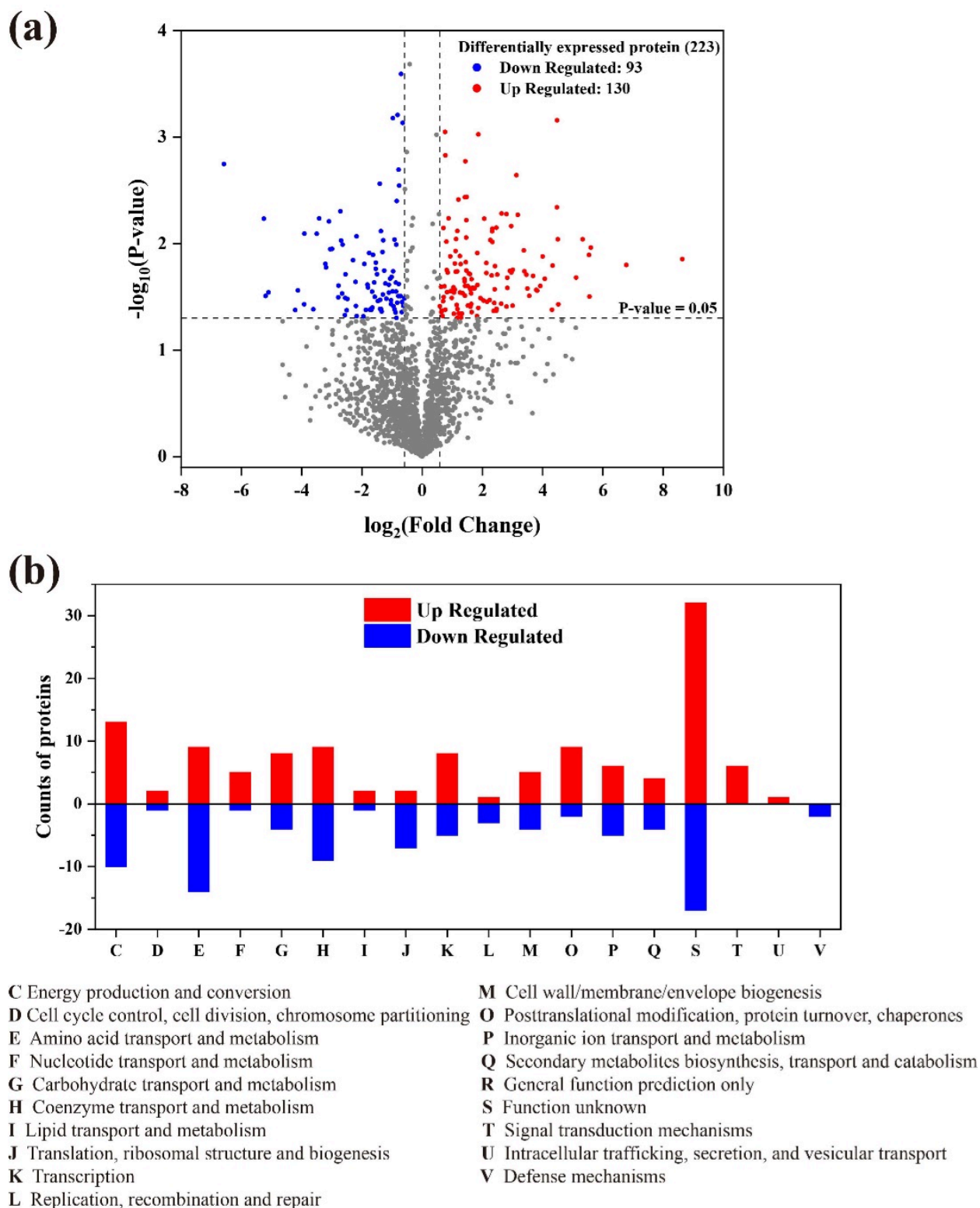


Figure 4

Label-free quantitative proteomics of *M. barkeri* and *G. sulfurreducens*. (a) Volcano plots of identified proteins; (b) COG functional annotation for the 223 proteins with significant variations.

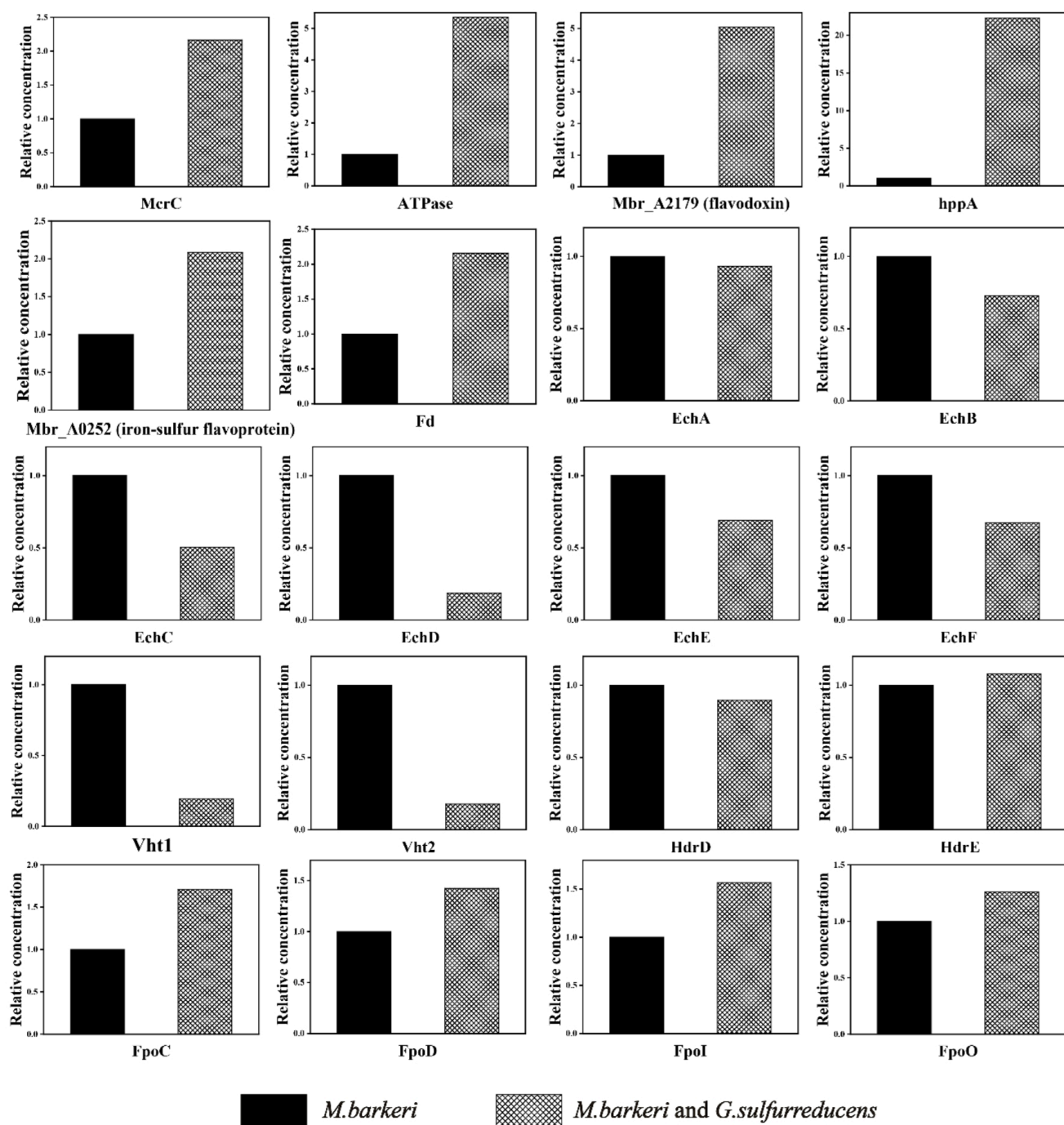


Figure 5

Relative concentrations of differentially expressed proteins during H_2 and CO_2 biomethanation in co-culture (*M. barkeri* and *G. sulfurreducens*) compared with that of pure culture (*M. barkeri*).

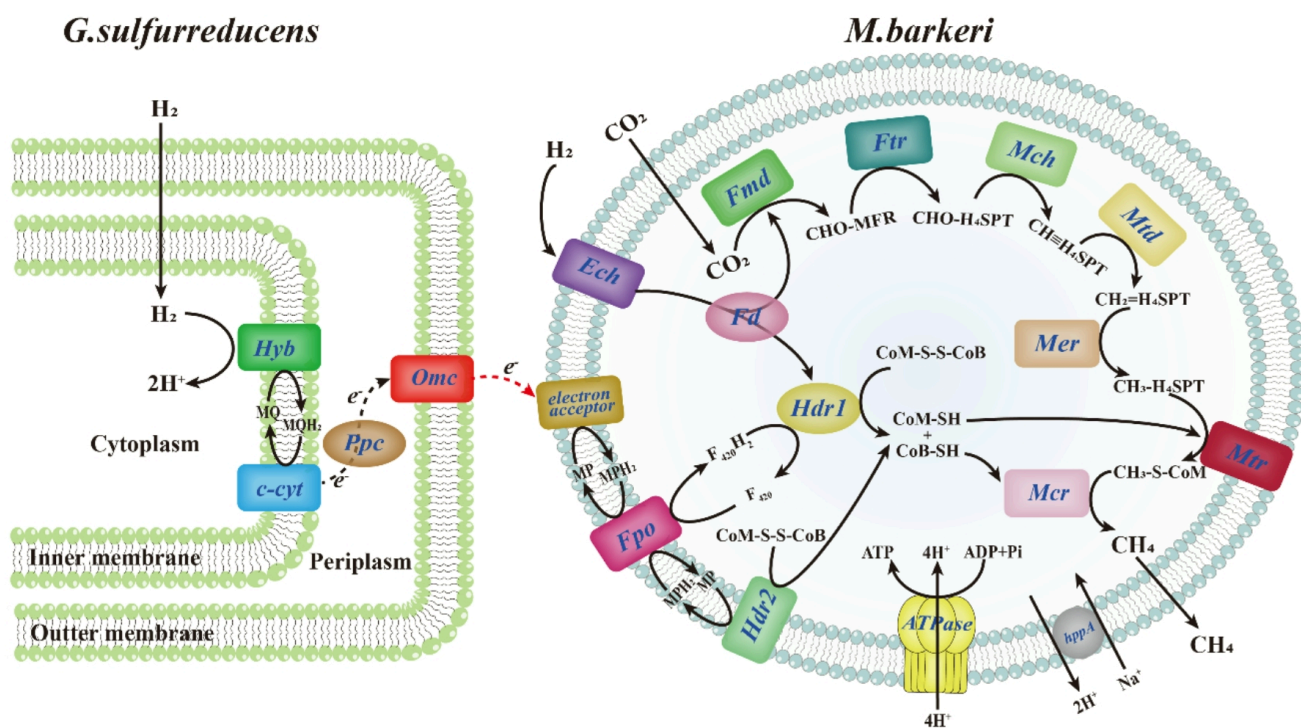


Figure 6

The stepwise utilization of H₂ in *M. barkeri* and *G. sulfurreducens* co-culture promoted CO₂-to-CH₄ conversion.

Supplementary Files

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