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Bioconversion Pathway of CO_2 in the Presence of Ethanol by Methanogenic Enrichments from Production Water of a High-Temperature Petroleum Reservoir

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Abstract: Transformation of CO_2 in both carbon capture and storage (CCS) to biogenic methane in petroleum reservoirs is an attractive and promising strategy for not only mitigating the greenhouse impact but also facilitating energy recovery in order to meet societal needs for energy. Available sources of petroleum in the reservoirs reduction play an essential role in the biotransformation of CO_2 stored in petroleum reservoirs into clean energy methane. Here, the feasibility and potential on the reduction of CO_2 injected into methane as bioenergy by indigenous microorganisms residing in oilfields in the presence of the fermentative metabolite ethanol were assessed in high-temperature petroleum reservoir production water. The bio-methane production from CO_2 was achieved in enrichment with ethanol as the hydrogen source by syntrophic cooperation between the fermentative bacterium Synergistetes and CO_2-reducing *Methanothermobacter* via interspecies hydrogen transfer based upon analyses of molecular microbiology and stable carbon isotope labeling. The thermodynamic analysis shows that CO_2-reducing methanogenesis and the methanogenic metabolism of ethanol are mutually beneficial at a low concentration of injected CO_2 but inhibited by the high partial pressure of CO_2. Our results offer a potentially valuable opportunity for clean bioenergy recovery from CCS in oilfields.

Keywords: methanogenesis; CO_2 bioconversion; ethanol; oil reservoir; energy recovery

1. Introduction

Carbon capture and storage (CCS) in deep subsurface geological formations is widely accepted as a potential and attractive way for the mitigation of greenhouse gas effects of carbon dioxide emission from the fossil fuel combustion [1,2]. Petroleum reservoirs are considered as potential candidates to perform this project and have been successfully tried and applied in CCS in many countries [3,4]. During this process, enhanced oil recovery (EOR) associated with CO_2 injection from CCS project can be a considerable economic incentive and offset the high investment involved in CCS at the same time. Moreover, oil reservoirs are also defined as natural bioreactors, and indigenous microbial communities such as methanogens provide the potential possibility to transform injected CO_2 into methane as clean energy, for energy recovery and the reduction of carbon dioxide [5,6]. From the

above-mentioned perspective, the injected CO_2 can also be regarded as the potential carbon source for clean energy methane production by hydrogenotrophic methanogens, as predominant members inhabiting petroleum reservoirs, commonly with hydrogen and carbon dioxide as substrates, in order to achieve the in situ energy recovery from CO_2 utilization and to promote deployment of CCS [7–11].

Available sources of power reduction such as hydrogen play a significant role in the metabolic activity of CO_2 biotransformation into methane and are often deemed as restricting factors due to the limited amounts commonly present, and amounts of hydrogen are below the detection limits in most of examined petroleum reservoir production waters [12]. Methanogenesis is the terminal process during the entire degradation of organic compounds, and thus the capability of widespread organics biodegradation completed by a wide range of in situ specialized microorganisms in petroleum reservoirs provides the possibility to accomplish energy recovery through injected CO_2 from CCS projects with a dependence on the cooperation of indigenous microbes [7,13,14]. In our previous study, it has been demonstrated that anaerobic biodegradation of long-chain *n*-alkane (C_{16}) could be regarded as the potential electron donor for the bioconversion of injected CO_2 from CCS in the production water of petroleum reservoirs, but the metabolic rate of methane generation was actually low, possibly due to a slow hydrogen generation rate from alkanes [15]. Therefore, our attention is further caught by the syntrophic cooperation between fermentative bacteria and methanogens for methane production with small intermediates, such as the substrate, based upon interspecific hydrogen transfer. Ethanol as the crucial metabolic product from various natural organics is a potentially available electron donor for CO_2 bioreduction by several methanogens containing either a coenzyme F_{420}-dependent or an NADP-dependent alcohol dehydrogenase such as *Methanobacterium palustre*, *Methanogenium organophilum*, and *Methanoculleus thermophilicus* [16,17]. However, the metabolic pathway of methanogenesis from ethanol and its potential as the electron donor for CO_2 biotransformation to methane in petroleum reservoirs have never been assessed.

In this study, the potential of ethanol acting as the electron donor for CO_2 bioconversion and its methanogenic metabolism in high-temperature petroleum reservoir production water are assessed by constructing a series of enrichments amended with ethanol as the substrate and $NaH^{13}CO_3$ as the carbon dioxide source. The abovementioned targets are achieved by combined analyses of carbon stable isotope, molecular biology, and thermodynamics.

2. Materials and Methods

2.1. Enrichment Cultures

The inoculum for anaerobic incubation cultures in this study was collected from the high-temperature petroleum reservoir production water of block Ba-18 at Huabei Oilfield. The physicochemical characteristics of the water-flooded oilfield have been described previously [18], and the temperature was about 55 °C. Two mL of the above inoculum were transferred into a 120 mL autoclaved serum bottle containing 50 mL of a basal medium (g/L): NaCl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.075; NH_4Cl, 0.25; KH_2PO_4, 0.75; $K_2HPO_4 \cdot 3H_2O$, 1.4; KCl, 0.5; $Na_2S \cdot 9H_2O$, 0.50; rezasurin, 0.0001; vitamin, 1 mL/L; and trace element, 1 mL/L. The detailed compositions of vitamin and trace element stock solution have been reported previously [19]. ^{13}C-labeled bicarbonate with final concentrations of 0, 30, 60, and 90 mM and 10 mM of ethanol were also supplemented into different incubation treatments as the source of carbon dioxide and substrate, respectively. Blank controls were conducted with addition of the above-mentioned concentrations of bicarbonate in the absence of ethanol. Different treatments were prepared in triplicates and incubated anaerobically at 55 °C in the dark. Notably, S0, S30, S60, S90, and blank were respectively used as abbreviations for the abovementioned experimental treatments in the following analysis.

2.2. Chemical Analysis

Gas chromatography (GC112A, Shanghai Precision and Scientific Instrument Co., Ltd, China) was used to monitor gas compositions including methane and hydrogen in the headspace of different incubation cultures during the whole incubation period periodically, and the detailed operations and temperature programming conditions have been described previously [20]. The amounts of ^{13}C-labeled methane and carbon dioxide in the headspace after 230 days of anaerobic incubation were detected by analysis of isotopic ratio mass spectrometer (IRMS Delta V PLUS, Thermo Scientific CO., USA) [18]. The apparent fractionation factors (αC) in different enrichments used as the indicator of the dominant methanogenic pathway were calculated according to the following formula: $\alpha C = (\delta^{13}CO_2 + 10^3)/(\delta^{13}CH_4 + 10^3)$ ($\delta^{13}CH_4$, ^{13}C-labeled CH_4; $\delta^{13}CO_2$, ^{13}C-labeled CO_2) [21]. Detections of volatile fatty acids (VFAs, mainly formate and acetate) and residual ethanol at the end of incubation were respectively accomplished by ion chromatography (IC DX-600, Dionex Co., Sunnyvale, CA, USA) and gas chromatography–mass spectrometry (GC-MS, Agilent Technologies, Inc.) according to the previous description [22].

2.3. DNA Extraction and Construction of Bacterial and Archaeal 16S rRNA Gene Clone Libraries

Five mL of the incubation medium from different treatments at the end of anaerobic incubation as well as the inoculum were used for the total genomic DNA extraction, and the centrifuged biomass pellets were conducted according to the method from manufacturer's instructions of the genomic DNA Kit (Axygen Biosciences, Inc, Corning, NY, USA). Bacterial and archaeal partial 16S rRNA genes were respectively amplified by using the universal primer sets of 8F/805R [23] and 340F/1000R [24]. Polymerase chain reaction (PCR) amplification was carried out in a 25 μL reaction volume including: each primer (1 μL), ~50 ng DNA template (2 μL), 2× PCR master mix (12.5 μL, Lifefeng Biotechnology, Shanghai, China), and ddH_2O (8.5 μL). PCR amplification procedures and construction of phylogenetic trees for archaeal and bacterial community compositions followed the previous description [18]. The valid sequencing data used for phylogenetic analysis in this study were deposited to the GenBank database with the accession numbers KR017718 to KR017730, KR049100 to KR049111, KR476582 to KR476604, and KR535989 to KR535991.

2.4. Phylogenetic Analysis

DNA sequences with more than 97% similarity were assembled into the same operational taxonomic units (OTUs), and one representative sequence was chosen from each OTU to compare with sequences in the GenBank Database to identify the most closely related ones. Phylogenetic trees were generated using MEGA5 software. The topology of the trees was obtained by the neighbor-joining method and 1000 bootstrap replicates were applied to estimate the support for the nodes in the tree.

2.5. Thermodynamics Analysis

Calculations of standard Gibbs free energy changes for possible methanogenic pathways from ethanol at 55 °C were performed by using the related thermodynamic data and methods reported elsewhere [25]. The opportunity window of thermodynamics for different metabolic pathways of ethanol possibly involved in the experimental condition was obtained from the modified Gibbs free energy change ($\Delta G^{\circ\prime}55^{\circ}C$) with the amendment of the relevant proton number and neutral conditions (pH = 7.0) according to the calculation methods previously described [12,26].

3. Results and Discussion

3.1. Biotransformation of CO_2 into Methane in the Presence of Ethanol

Gas compositions including methane and hydrogen in the headspace of different treatments in this study during the entire incubation period are shown in Figure 1. The related metabolites in

the cultures after 230 days of anaerobic incubation are summarized in Table 1. Methane production was detected in the treatments amended with ethanol, including S0, S30, S60, and S90, while no methane was detected in the corresponding blank controls without any addition of ethanol (Figure 1A). Methane was generated in all enrichments with the addition of ethanol after an initial lag phase of about 80 days, and 1.25, 327, 308, and 204 μmol of methane were accumulated in the treatments of S0, S30, S60, and S90, respectively, after 230 days of anaerobic incubation. The methane production rate and total amount of methane in the enrichments with the amendment of ethanol and bicarbonate (28.4 μmol CH_4 day^{-1} l^{-1}, 26.8 μmol CH_4 day^{-1} l^{-1}, and 17.7 μmol CH_4 day^{-1} l^{-1} in S30, S60, and S90, respectively) were much higher than those in S0 without bicarbonate, and they were much higher than previously reported biological methane production rates of 1.3–80 nmol CH_4 day^{-1} l^{-1} in subsurface petroleum reservoirs [27]. Maximum methane production occurred in S30, followed by S60, S90, and S0, which showed a similar trend with the amounts of consumed ethanol in the corresponding cultures, indicating that ethanol metabolism was directly or indirectly related to activities of methanogenesis under the experimental conditions. Similarly, hydrogen production was also observed in all treatments with the addition of ethanol, while any hydrogen produced in the blank controls without ethanol addition was below the detection limit. The amount of hydrogen stayed below 0.3 μmol throughout the incubation period in treatments with additions of ethanol and bicarbonate in S30, S60, and S90, while levels of above 0.7 μmol of hydrogen were detected and accumulated in the headspace of S0 without any bicarbonate amendment after 116 days of anaerobic incubation. The amounts of ^{13}C-labeled methane and carbon dioxide and apparent fractionation factors in different treatments after anaerobic incubation are displayed in Figure 2. Carbon isotope ratios of methane and carbon dioxide rose with the increasing concentration of injected bicarbonate from 0 mM to 90 mM. The apparent fractionation factor was 1.008, 1.083, 1.122, and 2.269 in S0, S30, S60, and S90, respectively.

Table 1. Metabolites (μmol/bottle) detected in different treatments after anaerobic incubation for 230 days. *d*, 0—day 0; *d*, 230—day 230.

Samples	Ethanol (*d*, 0)	Ethanol (*d*, 230)	Total CH_4	$^{13}CH_4$	$^{12}CH_4$	CO_2	H_2	Formate	Acetate
S0	500	297	1.25	0	1.25	60.7	0.722	0.81	0.45
S30	500	123	327	201	126	2698	0.216	3.23	6.89
S60	500	138	308	211	97	2515	0.293	3.42	5.77
S90	500	210	204	164	40	2693	0.253	0.05	2.35

Stimulation of methane production was observed in cultures amended with ethanol and bicarbonate (S30, S60, and S90) in comparison with the blank controls with addition of bicarbonate solely, indicating that ethanol was favorable and might act as the potential electron donor for methane production under the experimental conditions. In this study, the methane generation rate with ethanol as the substrate for CO_2 bioconversion was also much higher than that observed in the culture amended with long-chain *n*-alkane (C_{16}) and CO_2 driven by indigenous microorganisms in petroleum reservoirs [15]. In fact, it has been proved that methanogenic archaea containing either a coenzyme F_{420}-dependent or an NADP-dependent alcohol dehydrogenase such as *Methanobacterium palustre*, *Methanogenium organophilum*, and *Methanoculleus thermophilicus* are capable of using ethanol or other alcohols as electron donors for CO_2-reducing methanogens in a previous study [16]. Microorganisms known to have alcohol dehydrogenase are widely distributed in various ecosystems including both natural (hot springs, volcanic marine sediments) and anthropogenic biotopes (petroleum reservoirs) [28]. Moreover, alcohols are not only microbial metabolites from degradation of hydrocarbons but also important substrates for methanogenesis, which are commonly regarded as electron donors in the abovementioned biochemical process [29–31]. Ethanol as the crucially fermentative product could be obtained with variety of organic compounds during the process of microbial metabolism, such as *Thermoanaerobacter subterraneus* isolated from the oilfield [17]. A symbiotic association between ethanol oxidation microorganisms and methanogen was found in 1967 [32]. During the above biological process, ethanol was firstly oxidized into acetate and

hydrogen, and subsequently consumed by methanogenic organisms. None of them could grow well in the pure culture when ethanol was added as the sole substrate. Similarly, a small amount or no methane were detected in the treatment with the addition of only ethanol (S0) and blank controls, respectively, while detectable amounts of methane were produced in the active enrichment simultaneously amended with ethanol and bicarbonate in this study. Thus, it was confirmed that metabolic activity of methanogenesis was very low when either only ethanol or only bicarbonate were added as the substrate. Moreover, the amount of ^{13}C-labeled methane was notably accumulated in S30, S60, and S90, which further indicates that ethanol could be considered as the potential hydrogen source for CO_2-reducing methanogenesis by microbial consortium derived from production water of a high-temperature oil reservoir. In addition, metabolic activity of methane production would also be activated after nutrient injection in petroleum reservoirs as the promising strategy for energy recovery [33,34], and thus it might provide an opportunity for ethanol to be involved to convert CO_2 to methane for clean energy. This could also avoid extra materials addition in comparison with other approaches, such as hydrogen or using electrical energy [35]. Such abovementioned beneficial relationships between fermentative bacteria and methanogens derived from indigenous microorganisms of petroleum reservoirs may stimulate energy recycling of carbon dioxide from CCS.

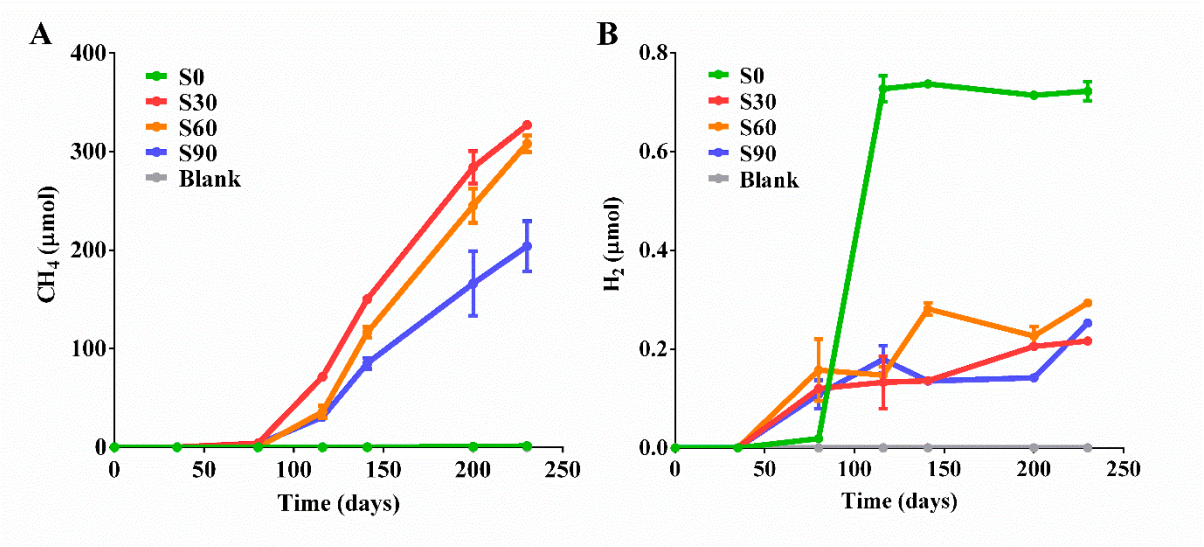

Figure 1. Gas compositions (CH_4 and H_2) in the headspace of enrichment under different treatments during the entire incubation time. (**A**) CH_4 production in serum bottle maintained under anaerobic conditions; (**B**) H_2 production in serum bottle maintained under anaerobic conditions.

Although the addition of bicarbonate lead to the stimulation of methane production (S30, S60, and S90) in comparison with S0, the amount of $^{12}CH_4$ generated from ethanol decreased with an increasing concentration of bicarbonate from 30 mM to 90 mM and showed a positive correlation with totally consumed ethanol, indicating that a higher concentration (above 30 mM in this study) of bicarbonate (carbon dioxide) inhibited the metabolic activity of ethanol methanogenesis. *Methanobacterium* can use ethanol for methanogenesis [32,36]. It seems logical from the perspective of a chemical equilibrium according to the reaction of ethanol methanogenesis in sulfate-free cultures: $2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$ [37]. Different amounts of ^{13}C-labeled methane were observed and the maximum $^{13}CH_4$ production was detected in S60 followed by S30 and S90. The amount of ^{13}C-labeled methane generated from injected bicarbonate via CO_2-reducing methanogenesis with ethanol as the substrate was 201, 211, and 164 μmol in S30, S60, and S90, respectively. $^{13}CH_4$ was derived from added ^{13}C-labeled bicarbonate via CO_2-reducing methanogenesis and this biochemical process was based upon ethanol as the hydrogen source under the experimental conditions. To a great extent, the interaction between metabolic activities of ethanol

degradation and CO_2-reducing methanogenesis depended on the interspecies hydrogen transfer between hydrogen-producing bacteria from ethanol ($CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$) [37,38] and hydrogen-utilizing methanogens ($HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O$). Metabolic activity of CO_2-reducing methanogenesis is facilitated by increasing the concentration of injected bicarbonate when the amount of CO_2/bicarbonate is below the upper limit. With further increased concentration of CO_2/bicarbonate, methanogenesis from carbon dioxide is possibly restricted by the limited hydrogen source due to the low metabolic activity of ethanol degradation (hydrogen production). Therefore, methanogenic metabolism from CO_2 was stimulated and accelerated by the addition of a certain amount of bicarbonate when the amount of ethanol as the sole hydrogen source was fixed according to the abovementioned analysis. In the present study, the maximum metabolic activity of CO_2-reducing methanogenesis from added ^{13}C-labeled bicarbonate was achieved with 60 mM of bicarbonate.

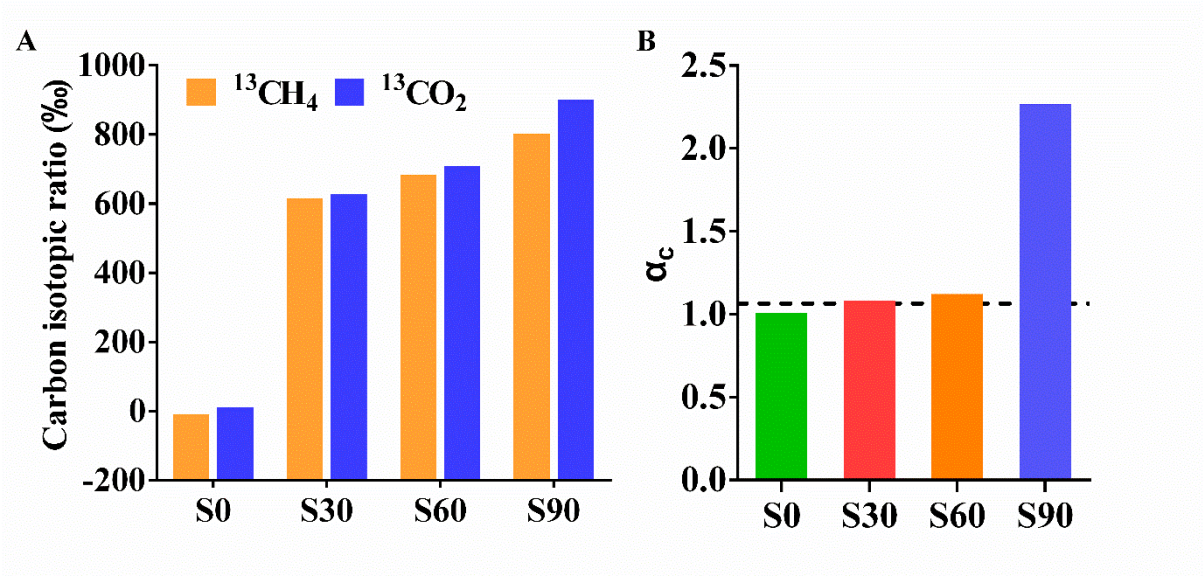

Figure 2. Carbon stable isotope ratios of methane and carbon dioxide and apparent fractionation factors in different enrichments after 230 days of anaerobic incubation. The dashed line represents 1.065. (**A**) Carbon stable isotope ratios of methane and carbon dioxide in different enrichments; (**B**) Apparent fractionation factors in different enrichments.

3.2. Microorganisms Occurring in the Enrichment Cultures

Phylogenetic trees of bacterial and archaeal 16S rRNA genes from different incubation cultures at the end of anaerobic incubation as well as the inoculum are respectively shown in Figures 3 and 4. The dominant bacterial members in both inoculum (52% of occupation) and S0 (92% occupation) were affiliated with *Thermodesulfovibrio* belonging to the phylum Nitrospirae, while in other treatments amended with ethanol and bicarbonate (S30, S60, and S90) *Anaerobaculum* within the phylum *Synergistetes* (92% in S30, 77% in S60, and 72% in S90) prevailed in bacterial communities after the entire incubation period. Other phylum including Actinobacteria, Chloroflexi, Acetothermia, Bacteroidetes, Firmicutes, and Proteobacteria were also detected in this study. Regarding the archaeal communities, the genus *Methanosaeta* within the order Methanosarcinales was encountered as the most prevalent taxon in the inoculum, while in the enrichments (S0, S30, S60, and S90) genus *Methanothermobacter* within the order Methanobacteriales turned out to be the dominant taxon after the whole incubation period.

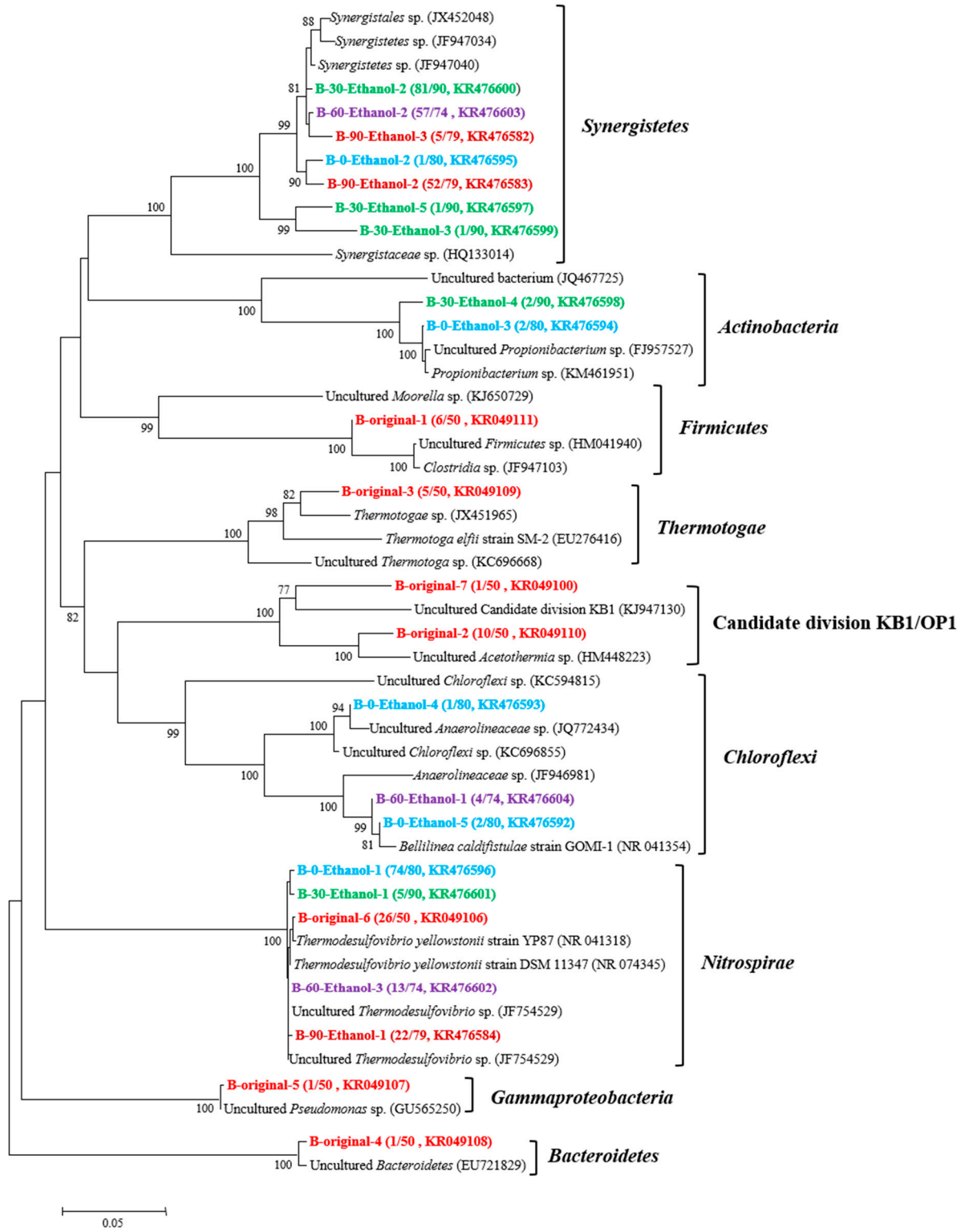

Figure 3. Phylogenetic tree of the 16S rRNA gene sequences of bacteria from different enrichment cultures after anaerobic incubation (indicated by different colors) revealed by the clone libraries. Scale bars show five (0.05) nucleotide substitutions per 100 nucleotide base pairs. The number in parentheses is the number of clones.

Based on an analysis of bacterial community composition, sequences affiliated with Synergistetes dominated in S30, S60, and S90, and their abundance decreased with both the increased concentration of amended bicarbonate and the decreased amount of consumed ethanol, indicating that the dynamic

change of the bacterial community showed a close association with the amount of bicarbonate and ethanol metabolism. Synergistetes characterized as the potential indigenous microorganism in petroleum reservoirs was widely distributed in many methanogenic petroleum hydrocarbon-degrading systems [39–43] and was confirmed to be capable of metabolizing ethanol in cooperation with hydrogenotrophic methanogen [44]. An isolate affiliated with genus *Anaerobaculum*, known as the fermenting bacteria, was obtained from the production water of the oil reservoir and affirmed to have the ability to produce hydrogen from a vast number of amino acids and organic acids [45]. It was reasonably speculated that Synergistetes was directly or indirectly involved and played the crucial role in the degradation of ethanol for hydrogen production in co-culture in cooperation with hydrogenotrophic methanogens under the experimental conditions in this study.

Thermodesulfovibrio as the predominant genus in the inoculum and S0 was abundant in methanogenic alkane-degrading systems and known to be capable of syntrophically degrading organic compounds and producing methane in association with hydrogenotrophic methanogens in the absence of sulfate [15,46–48]. In the current study, acetate oxidation generated from ethanol metabolism is probably performed by *Thermodesulfovibrio* and subsequently consumed for methane production by hydrogenotrophic methanogens, thus *Thermodesulfovibrio* might play a crucial role in methanogenesis. It seems logical that *Thermodesulfovibrio* was distributed in all treatments after 230 days of anaerobic incubation.

The most prevalent methanogen turned out to be a typically hydrogenotrophic methanogen, *Methanothermobacter*, in all enrichment cultures (S0, S30, S60, and S90) from the acetoclastic methanogen *Methanosaeta* in the inoculum, indicating that the dominated methanogenic pathway has been altered in association with ethanol addition but not bicarbonate injection during the incubation period. *Methanothermobacter* as the thermophilic methanogen with H_2+CO_2 as substrates was widely distributed in petroleum reservoirs and responsible for producing methane [48–50]. It was also found that *Methanothermobacter* appeared to be the most frequently encountered taxon in different samples of production water from petroleum reservoirs amended with zero-valent iron as the electron donor and was responsible for producing methane [26]. Moreover, active methanogenic activity conducted by *Methanothermobacter* was confirmed under the CCS conditions in petroleum reservoirs [27]. In the current study, the dominance of *Methanothermobacter* as the sole methanogen detected in all incubation cultures supported its competitive role in methane productionwith ethanol addition. At the same time, bicarbonate injection with different concentrations showed little impact on methanogenic pathways and involved methanogen communities under the experimental conditions according to the little difference in archaeal community compositions among S0, S30, S60, and S90. Thermophilic conditions (55 °C in this study) might also favor the growth of rod-like or coccoid hydrogenotrophic methanogens such as *Methanothermobacter* [31]. *Methanosaeta* affiliated with the order Methanosarcinales was capable of producing methane with acetate as the substrate and was commonly detected in the petroleum reservoirs [9,51]. Acetoclastic methanogenesis mainly conducted by *Methanosaeta* was invoked by high partial pressure of CO_2 in place of hydrogenotrophic methanogenesis in the simulated oil reservoir bioreactor [52]. Based on non-detected *Methanosaeta* after the entire incubation period in this study, it was confirmed that hydrogenotrophic methanogenesis is the dominant or sole methanogenic pathway instead of acetoclastic methanogenesis in the inoculum. The apparent fractionation factor (αC) calculated from isotope data of carbon dioxide and methane further confirmed the abovementioned conclusion because αC as the indicator for the dominant methanogenic pathway was greater than 1.065 in S30, S60, and S90, indicating that hydrogenotrophic methanogenesis was the main methanogenic route in this study [21,53].

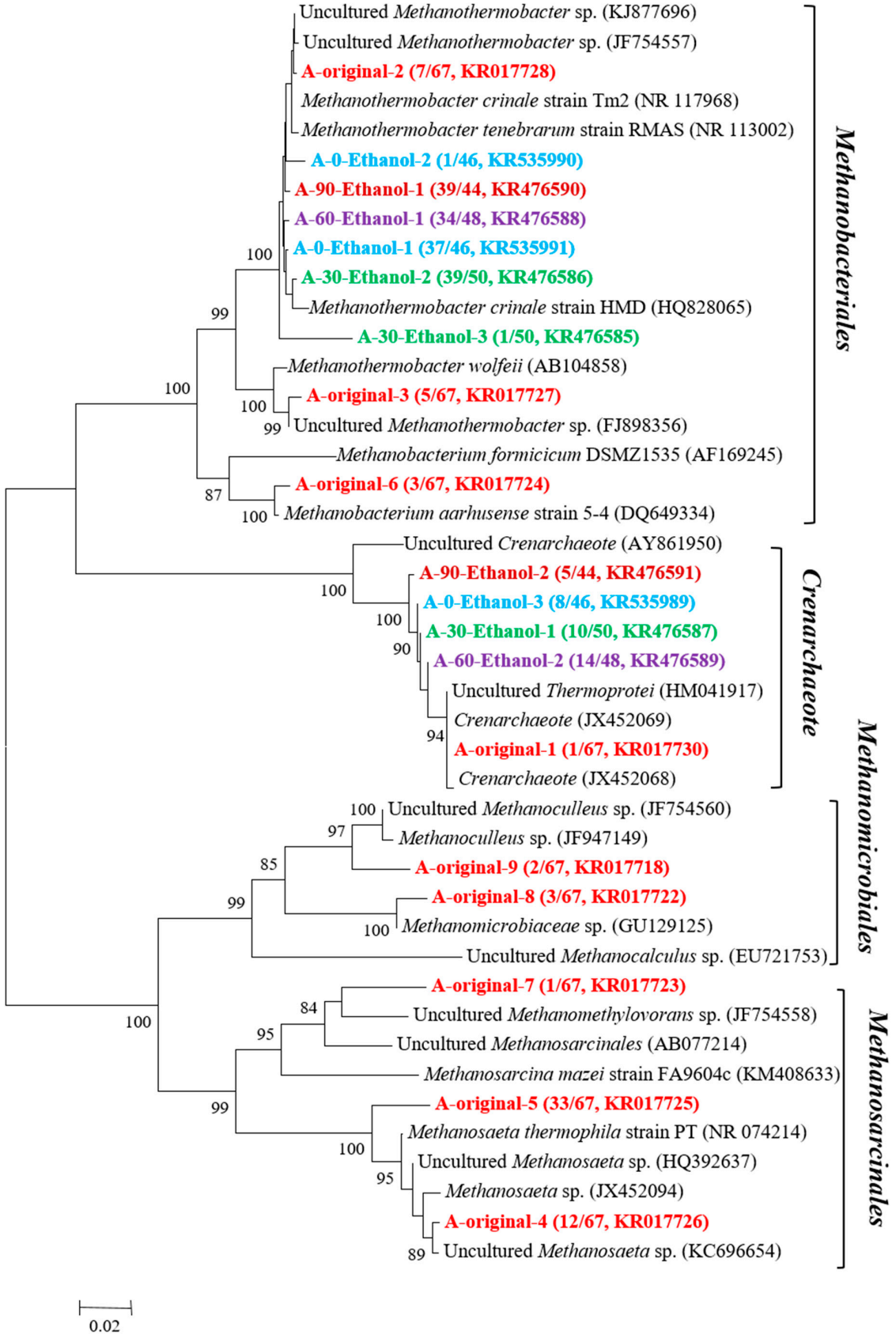

Figure 4. Phylogenic tree of the 16S rRNA gene sequences of *Archaea* revealed in the clone libraries from different enrichment cultures after anaerobic incubation (indicated by different colors). Scale bars show five (0.02) nucleotide substitutions per 100 nucleotide base pairs. The number in parentheses is the number of clones.

3.3. Metabolic Pathways for Methanogenesis from Ethanol

Five possible methanogenic pathways from ethanol are summarized in Table A1 (Appendix A) and the corresponding opportunity window of thermodynamics is shown in Figure 5. Route 1, including reactions 1 and 2, represents that ethanol is firstly converted into acetate and then consumed by acetoclastic methanogen. Route 2, containing reactions 1, 4, and 5, indicates that ethanol is firstly transformed to acetate and subsequently metabolized into hydrogen and carbon dioxide in corporation with hydrogenotrophic methanogens. According to route 3, ethanol is first bioconverted to acetate and hydrogen, and then the produced acetate and hydrogen are further utilized for methane production via acetoclastic and CO_2-reducing methanogenesis (reactions 6, 2, and 5), or, according to route 4, it is transformed to hydrogen and carbon dioxide through syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis (reactions 6, 4, and 5). Route 5 shows that ethanol is directly metabolized to hydrogen and carbon dioxide (reaction 7) and subsequently used for producing methane via CO_2-reducing methanogenesis (reaction 5). Routes 1 and 3 could not happen in our incubation cultures due to the lack of acetoclastic methanogens, as determined by the microbial community analysis. At the same time, route 2 and 5 could not have occurred in this study because not enough hydrogen was generated from the acetate oxidation via reaction 4 in route 2 and ethanol oxidation via reaction 7 in route 5 to transform the injected ^{13}C-labeled bicarbonate based upon significantly detectable ^{13}C-labeled methane in S30, S60, and S90. Consequently, the most favorable methanogenic pathway in our enrichment cultures appears to be bioconversion of ethanol to hydrogen and acetate coupled with syntrophic acetate oxidation and CO_2-reducing methanogenesis (route 4 indicated by orange color in Figure 5).

It was found that syntrophic cooperation between ethanol-oxidizing bacteria and hydrogenotrophic methanogen rather than the pure culture played an important part in metabolizing ethanol and producing methane [32,36,54]. Hydrogen produced by fermenting bacteria (Synergistetes in this study) could have served as the reducing power for injected-CO_2 reduction to methane by hydrogenotrophic methanogens (*Methanothermobacter* in this study), and in reverse, the consumption of hydrogen by partner methanogens would maintain a low partial pressure of hydrogen (<100 Pa) and further keep the biochemical process of endergonic ethanol oxidation proceeding. From the above analysis and thermodynamic results, ethanol oxidation would be stimulated and accelerated by the injection of bicarbonate at low concentrations but inhibited at high concentrations. As a result, methane production in enrichment cultures amended with ethanol and bicarbonate mainly depended on the activity of ethanol metabolism and the concentration of injected bicarbonate, which respectively contributed to the hydrogen and $^{12}CH_4$ production or $^{13}CH_4$ generation. The low hydrogen partial pressure (e.g., $\leq$40 Pa) was also favorable for acetate oxidation conducted by *Thermodesulfovibrio* in this study after ethanol conversion into acetate and hydrogen [31] and subsequent methane production. Furthermore, since interspecies hydrogen transfer played an important role between small organics (ethanol) degradation and CO_2 reduction to methane and was abundant in natural environments such as petroleum reservoirs, we suppose that injection of CO_2 from CCS in oil reservoirs is not only favorable for oil recovery but also possible for the generation of methane from the greenhouse gas carbon dioxide. The methane generation rate was stimulated and accelerated by the degradation of small intermediate (ethanol) in comparison with hardly-decomposed organic compounds [15]. Such syntrophical interspecies relationships may widely exist in petroleum reservoirs and could be deemed as the potential breakthrough for energy recovery from CCS, which showed to be commercially very promising.

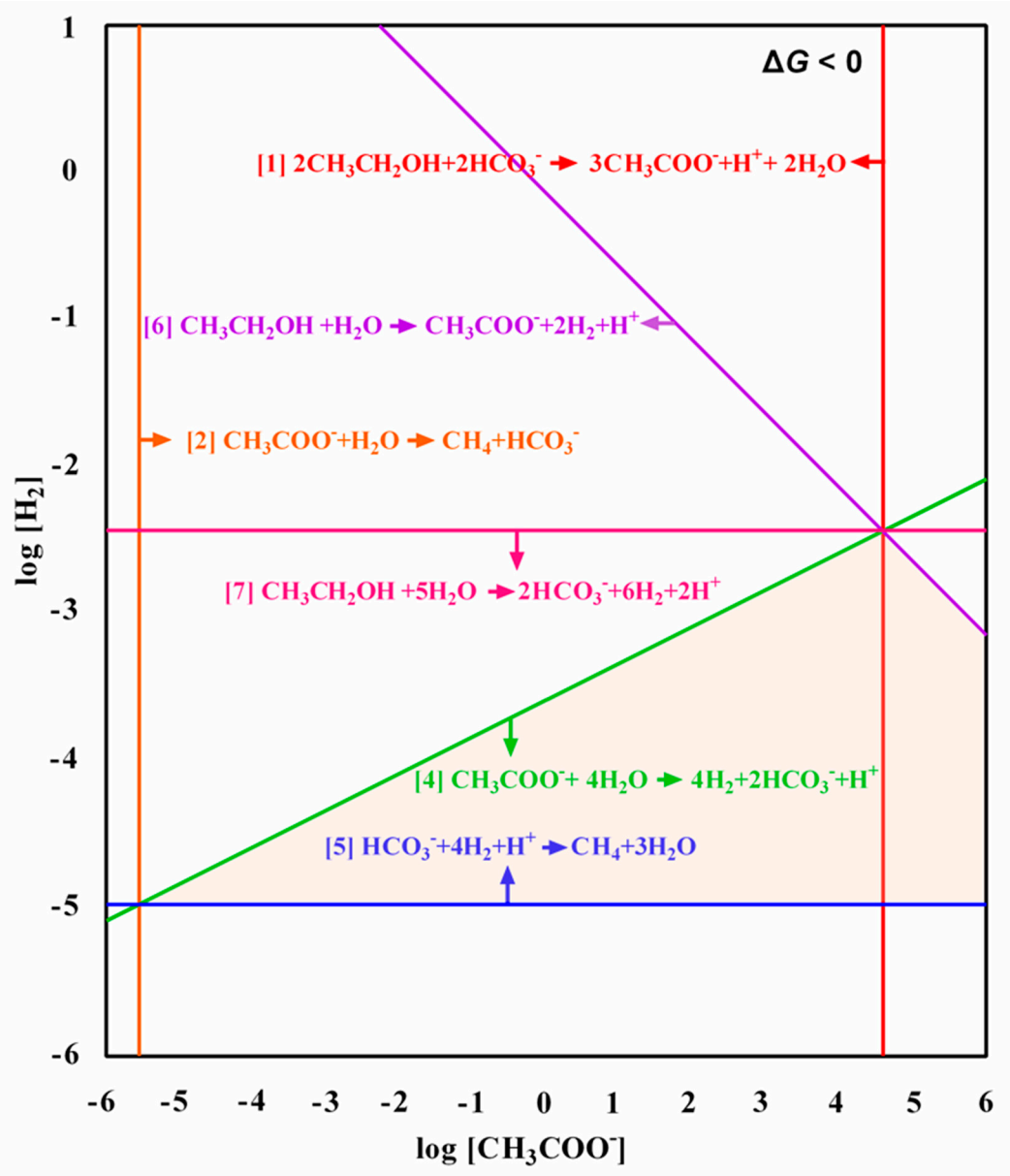

Figure 5. The opportunity window of thermodynamics for different metabolic pathways involved in methane production from ethanol with concentrations of acetate and hydrogen as thermodynamic constraints. The orange area represents route 4, containing reactions 6, 4, and 5, i.e., the bioconversion of ethanol to acetate and hydrogen, linked with syntrophic acetate oxidation and hydrogenotrophic methanogenesis.

4. Conclusions

CO_2 reduction into methane as a clean energy source by indigenous microorganisms of high-temperature petroleum reservoir production water can be effectively stimulated and promoted by using ethanol as a source of reducing power, and, on the other hand, the favorable condition of low hydrogen partial pressure for methanogenic activity of ethanol metabolism is also created by hydrogen consumption during the abovementioned process. The results obtained in this study suggest that biotransformation of CO_2 used for CCS projects might be stimulated by injecting nutrients such as ethanol in petroleum reservoirs, and it also provides the opportunity for a value-added CO_2 management strategy in petroleum reservoirs.

Author Contributions: J.-D.G. and B.-Z.M. designed the experiments, G.-C.Y. did the experiments, G.-C.Y., L.Z. and SM carried out the microbial analysis. J.-D.G. and B.-Z.M. gave the suggestion for the experiments and results analysis. G.-C.Y. prepared the manuscript with contributions from all co-authors.

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Conflicts of Interest: All authors declare that they have no conflict of interest in research reported here.

Appendix A

Table A1. Gibbs free energy changes for the feasible chemical processes at 328.15 K and neutral pH (pH = 7.0).

Pathways	$\Delta G°$25°C (kJ/mol)	$\Delta G°$55°C (kJ/mol)	$\Delta G°'$55°C (kJ/mol)	
Route 1 Bioconversion of ethanol to acetate, linked to acetoclastic methanogenesis				
$2CH_3CH_2OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 2H_2O$	−45.87	−42.58	−86.56	[1]
$3CH_3COO^- + 3H_2O \rightarrow 3CH_4 + 3HCO_3^-$	−93.45	−104.61	−104.61	[2]
SUM: $2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$	−139.32	−147.19	−191.17	[3]
Route 2 Bioconversion of ethanol to acetate, linked to syntrophic acetate oxidation and CO_2-reducing methanogenesis				
$2CH_3CH_2OH + 2HCO_3^- \rightarrow 3CH_3COO^- + H^+ + 2H_2O$	−45.87	−42.58	−86.56	[1]
$3CH_3COO^- + 12H_2O \rightarrow 12H_2 + 6HCO_3^- + 3H^+$	432.51	401.88	269.94	[4]
$3HCO_3^- + 12H_2 + 3H^+ \rightarrow 3CH_4 + 9H_2O$	−525.96	−506.49	−374.55	[5]
SUM: $2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$	−139.32	−147.19	−191.17	[3]
Route 3 Bioconversion of ethanol to hydrogen and acetate, linked to acetoclastic methanogenesis and CO_2-reducing methanogenesis				
$2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2H^+$	98.30	91.38	3.42	[6]
$2CH_3COO^- + 2H_2O \rightarrow 2CH_4 + 2HCO_3^-$	−62.30	−69.74	−69.74	[2]
$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$	−175.32	−168.83	−124.85	[5]
SUM: $2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$	−139.32	−147.19	−191.17	[3]
Route 4 Bioconversion of ethanol to hydrogen and acetate, linked to syntrophic acetate oxidation and CO_2-reducing methanogenesis				
$2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COO^- + 4H_2 + 2H^+$	98.30	91.38	3.421	[6]
$2CH_3COO^- + 8H_2O \rightarrow 8H_2 + 4HCO_3^- + 2H^+$	288.34	267.92	79.96	[4]
$3HCO_3^- + 12H_2 + 3H^+ \rightarrow 3CH_4 + 9H_2O$	−525.96	−506.49	−374.55	[5]
SUM: $2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$	−139.32	−147.19	−191.17	[3]
Route 5 Bioconversion of ethanol to hydrogen and CO_2, linked to CO_2-reducing methanogenesis				
$2CH_3CH_2OH + 10H_2O \rightarrow 4HCO_3^- + 12H_2 + 4H^+$	386.64	359.30	183.36	[7]
$3HCO_3^- + 12H_2 + 3H^+ \rightarrow 3CH_4 + 9H_2O$	−525.96	−506.49	−374.55	[5]
SUM: $2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$	−139.32	−147.19	−191.17	[3]

$\Delta G°' = \Delta G° + m2.303RTlog10^{-7}$(m is the net number of protons formed in the equation). ΔG°T: standard Gibbs free energy at temperature T, reactants and products at 1 M concentration in the aqueous phase, and gases at the partial pressure of 1 atm.

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