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RESEARCH ARTICLE

Characteristics of microbial communities in water from CBM wells and biogas production potential in eastern Yunnan and western Guizhou, China

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Abstract The study of microbial communities in the produced water of coalbed methane (CBM) wells is an important aspect of microbial-enhanced methane production. Water produced from 15 CBM wells in four synclines in eastern Yunnan and western Guizhou was collected. Through the use of 16S ribosomal RNA (16S rRNA) amplicon sequencing and realtime fluorescence quantitative polymerase chain reaction (PCR), the characteristics of bacterial and archaeal communities before and after enrichment culture were studied. The methanogenic pathways of secondary biogas were discussed, and potential microbial-enhanced methane production was preliminarily evaluated. The results showed that the bacterial DNA content in uncultured produced water was low, so it is difficult to detect. After enrichment, the dominant bacteria phyla were *Proteobacteria, Bacteroidetes,* and *Firmicutes*. A total of seven phyla were detected in the uncultured produced water, and the dominant archaeal phylum was *Euyarchaeota*. Methanogens were the main component of archaea. The dominant archaeal genera were *Methanobacterium, Methanoculleus* and *Methanobrevibacter*. The community structure of the archaea changed noticeably after four days of enrichment culture. The relative abundance of *Euryarchaeota* increased to 99% in most samples after enrichment culture. It was found that there was a transition from *Methanoregula* to *Methanobacterium* within genera. The relative abundance of *Methanobacterium* increased, which can produce hydrogenotrophic methane. Combined with the isotopic composition of the produced water and gas, it is considered that the CBM in the Tucheng and Enhong synlines consists of a mixture of thermogenic gas and biogas. The proportion of secondary biogas in the Tucheng and Enhong synlines are estimated to range from 10.89% to 49.62%. There are mainly hydrogentrophic methanogens in the study area, and CO_2 reduction is the main way of microbial gas production. After enrichment culture of produced water in the study area, the hydrogenotrophic methanogens were enriched. These two areas have strong potential for microbial-enhanced methane production.

Keywords eastern Yunnan and western Guizhou, produced water form CBM wells, 16S amplicon sequencing, secondary biogas, microbial-enhanced methane production

1 Introduction

Coalbed methane (CBM) is a kind of clean unconventional natural gas energy. Under the international background of “carbon neutral”, “stabilizing oil and increasing gas” is China’s long-term energy strategy, and vigorously developing shale gas and CBM is an urgent need to realize this strategy. However, the permeability of the CBM reservoir in China is low, so it is generally necessary to reconstruct the reservoir to obtain industrial gas flow. The development of reservoir reconstruction technology is the core demand for improving the production of CBM. In recent years, reservoir modification and engineering experiments of microbial-enhanced methane production have been widely carried out in China and abroad (Ritter et al., 2015; Sun et al., 2018). According to

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the mechanism of biogas generation, the methanogenic bacteria or the nutrient solution used to cultivate the methanogenic bacteria are artificially injected into the coal seam with suitable conditions, so that the coal seam can produce gas again. In this way, the production of CBM wells can be increased, so as to enhance the recovery factor (Su et al., 2020).

The basis of these studies is to understand the characteristics of microbial community in coal reservoirs. The previous research mainly used 16S rDNA sequencing technology to study the microbial community structure by collecting underground water or coal samples. Yubai (Shimizu et al., 2007) in Japan, Surat Basin, Sydney Basin and Port Phillip Basin (Li et al., 2008; Vick et al., 2018) in Australia, Illinois Basin (Strąpoć et al., 2008; Zhang et al., 2015) and Powder River Basin (Klein et al., 2008) in America, Waikato coalfields (Fry et al., 2009) in New Zealand, Alberta Basin (Penner et al., 2010) in Canada, Ordos Basin (Guo et al., 2012) and Huaibei Mine (Liu et al., 2019) in China have all done sequencing research, and different bacteria and archaea have been found. *Methanobacterium, Methanolobus* and *Methanoculleus* were the most common archaea. *Firmicutes, Proteobacteria* and *Bacteroidetes* were the most common bacteria. Generally speaking, the diversity of bacteria was higher than that of archaea (Parkes et al., 2000; Su et al., 2018). To reduce the reservoir pressure, it is necessary to discharge the water from the coal seam during the exploitation of CBM well. During the stable production period, the groundwater discharged is generally the water from the original coal seam. The produced water contains abundant microbial information, which can reflect the microbial community structure of the underground coal seam to a certain extent (Beckmann et al., 2019), which provides the possibility for more convenient and rapid study of microbial community structure in coal reservoirs.

At the same time, microbial enhanced methane production also needs to pay attention to the type of CBM in the original coal seam, which can be thermogenic gas, protobiogas, and secondary biogas or a mixture of the three (Glasby, 2006; Wang et al., 2018). The reservoir with secondary biogas is more suitable for microbial enhanced methane production.

Eastern Yunnan and western Guizhouis is an important coal and CBM resources area in southern China, with the CBM geological resources of the Upper Permian accounting for approximately 10% of China's coal resources. This area has the geological characteristics of multiple and thin coal seams, a wide range of coal ranks, high *in situ* stress, low water saturation, and complex coal structure (Gao et al., 2009; Yang et al., 2019, 2020). In recent years, the exploration and development of CBM has made important breakthroughs, and the maximum daily production of some vertical wells has exceeded 6000 m^3. However, due to the complex coal structure, the effect of coal reservoir transformation is limited to a certain extent, and the gas production effect of some wells is poor, so it is important to explore new reservoir reconstruction technology. Therefore, water samples from 15 CBM wells in four synclines in the study area were collected for anaerobic fermentation and cultivation in the laboratory. By using high-throughput sequencing technology and geochemical analysis methods combined with the environmental characteristics of the formation water in different synclines, the characteristics of bacterial and archaeal community changes, methanogenic pathways and gas production potential were studied and evaluated. This study provides a theoretical basis for microbial-enhanced methane production from the coal seams of the Longtan Formation in eastern Yunnan and western Guizhou.

2 Geological settings

The coal-bearing strata in eastern Yunnan and western Guizhou are the Late Permian Longtan Formation/Xuanwei Formation, with multiple coal seams and many synclines as the main coal-accumulating units. Long-flame coal, gas coal, fat coal, coking coal, thin coal, meager coal, and anthracite all occur. Development test wells in western Guizhou are mainly distributed in Songhe (SH), Zhijin (ZJ), Faer (FE) and northern Guizhou blocks (Fig. 1). The Songhe and Faer CBM production wells were selected as the research objects, and the two regions are close. The Songhe block currently has eight CBM production wells, which are a clustered well group in the Tucheng (TC) syncline. Well GP1 and GP2 were put into production in January 2014, and wells GP3 to GP8 were put into production in January 2015. The commingled gas production is typically from 6 to 9 layers. The depth of the bottom coal seam is about 564.5–977.08 m (Table 1). By October 2020, the maximum daily CBM production was approximately 3000 m^3/d, and the stable production was approximately 300 m^3/d. Each single well had a cumulative water production of approximately 1400–3300 m^3. After January 2018, the GP1 well was subjected to secondary fracturing and was replaced by single-layer drainage of the 1 + 3 coal seam, which is one of the numbers of the main coal seams in Songhe block.

There are two CBM wells in the Faer (FE) syncline, wells YC1 and QC1. Both wells are multi-layer commingled production wells, and the combined production layer is 3 layers. The depth of the bottom coal seam is 659–739 m. Well YC1 was put into operation in January 2017, with a maximum gas production of 5400 m^3/d. At present, the YC1 well has a stable production of 1400 m^3/d and a cumulative water production of approximately 1100 m^3. Well QC1 was put into operation in September 2017, with a maximum gas production of 1000 m^3/d. At present, the QC1 well is stable at 420 m^3/d, and the

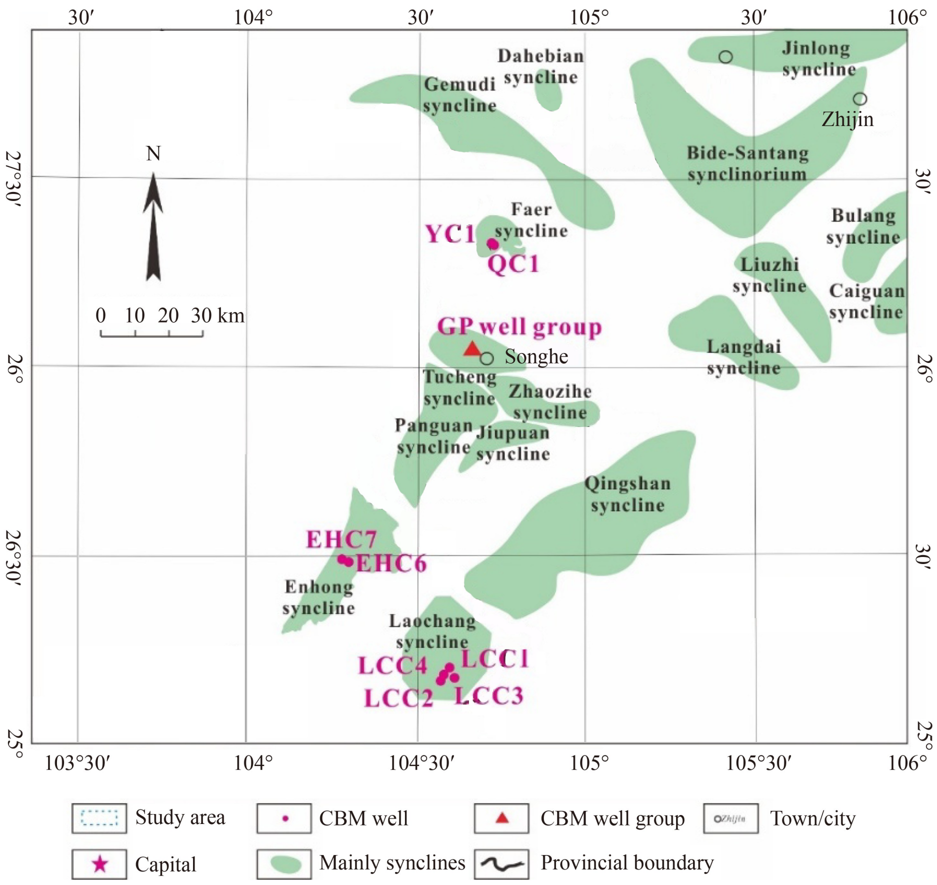

Fig. 1 Distribution of CBM wells in the study area.

cumulative water production is approximately 800 m^3 (Table 1).

There are eight CBM wells in eastern Yunnan, mainly located in the Enhong (EH) and Laochang (LC) syncline (Fig. 1). The EHC6 and EHC7 wells are located in the EH syncline and are multi-layer combined mining wells with 3 to 4 layers of production. The depth of the bottom coal seam is 1036–1182 m. The two wells were put into production in January 2018. By October 2020, the maximum gas production reached 300 m^3/d, and the cumulative water production of each individual well ranged from 600 to 800 m^3 (Table 1).

LCC1, LCC2, LCC3, LCC4, LCS1, and LCS2 wells are located in the Laochang (LC) syncline, in which LCC4, LCS1, and LCS2 are a well group (Fig. 1). All wells adopt multi-layer combined production, and the production layers are generally 3 to 4 layers. The depth of the bottom coal seam is 712.58–832 m. Most of them started drainage in April 2018. By October 2020, the maximum gas production reached 800 m^3/d, and the cumulative water production of each individual well ranged from 450 to 5000 m^3 (Table 1).

Among the four synclines, the Enhong and Tucheng synclines mainly develop coking coal, the Faer syncline mainly develops lean coal, while the Laochang (LC) syncline mainly develops anthracite (Table 1). The four synclines are adjacent to each other.

3 Experiment

3.1 Sample collection

Fifteen samples were collected from five wells in the GP well group of in the Tucheng (TC) syncline, two wells in the Faer (FE) syncline, six wells in the Laochang (LC) syncline in Yunnan and two wells in the Enhong (EH) syncline in late October 2019. Plastic bottles were used to collect 500 mL of produced water directly from the outfall of each of the CBM wells and were then sealed. Three water samples were collected from each well, and one sample was sent directly to the State Key Laboratory of Environmental Geochemistry, Guiyang Institute of Geochemistry, Chinese Academy of Sciences for geochemical testing. The other two samples were transported at low temperature, one of which was enriched in the laboratory. Samples before and after culture were sent to the Sangon (Shanghai) CO., Ltd for 16S rRNA amplicon sequencing. Gas samples were collected by the drainage and gas gathering method and were then sent to the Key Laboratory of Oil and Gas Resources Research, Chinese Academy of Sciences, for testing.

Table 1 Basic information of CBM wells in the study area

Syncline	Well name	Development layer	$R_{\text{o,max}}$/%	Depth/m	Temperature/°C	Start time of drainage
Tucheng	GP1	6/9/12/13/15/16/29	1.40–1.69	847.00	31.00	2014.1
	GP2	1 + 3/5/9/10/11/13/15/16	1.40–1.69	764.00	34.00	2014.1
	GP3	6/9/12/13/15/29	1.40–1.69	610.00	37.00	2015.1
	GP6	1/3/4/5/6/9/15/16/26/27/29	1.40–1.69	617.00	35.14	2015.1
	GP7	1 + 3/4/5/12/15/27/29	1.40–1.69	902.00	48.00	2015.1
Faer	YC1	5/7/13	1.75	659.00	29.00	2017.1
	QC1	13/21	1.80	739.00	30.00	2017.9
Enhong	EHC6	7 + 8/9/16/21	1.21	1182.00	25.00	2018.2
	EHC7	16/17 + 18	1.20	1036.00	24.00	2018.1
Laochang	LCC1	7 + 8/13	3.00	778.30	26.00	2018.4
	LCC2	16/18/19	3.02	735.00	30.00	2018.4
	LCC3	13/14/19	3.40	832.00	36.00	2018.5
	LCC4	13/16/18/19	3.38	712.58	32.00	2018.5
	LCS1	13/19	3.38	750.00	42.85	2018.6
	LCS2	14/16/18	3.38	687.00	41.13	2018.5

3.2 Geochemical test of produced gas and produced water

The test contents of water samples were hydrogen and oxygen isotopes, dissolved inorganic carbon isotopes and trace elements of water. Hydrogen and oxygen isotopes were measured by laser liquid isotope mass spectrometer (MAT253, USA) and dissolved inorganic carbon isotopes were measured by gas isotope mass spectrometer (MART252, USA). The trace element detection instrument was inductively coupled plasma mass spectrometer (NexION300X ICP-MS). Test procedures were strictly in accordance with national norms. Gas sample testing included carbon and hydrogen isotope tests of alkanes. The carbon and hydrogen isotopes of alkane gas were tested by a Delta V Advantage isotope mass spectrometer. The detection was based on the general rule of mass spectrometry analysis method GB/T 6041–2002. Test results wre shown in Table 2.

3.3 Enrichment culturing of microorganisms and 16S rRNA amplicon sequencing

3.3.1 Enrichment culturing of microorganisms

One of the water samples collected from CBM wells was sealed and transported to the laboratory at low temperature. After sterilization, the added nutrients were weighed in a triangular flask, and the water samples were transferred to a triangular flask and placed in a constant temperature incubator at 35°C for 4 days. The specific components of nutrient solution are as follows: 1000 mL of distilled water was added to 1.5 g of triglycolamic acid, 0.5 g of $MnSO_4 \cdot 2H_2O$, 3.0 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of NaCl, 0.1 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.05 g of H_3BO_3, 0.01 g of $AlK(SO_4)_2$, 0.02 g of $NiCl_2 \cdot 6H_2O$, and 0.05 g of Na_2MoO_4. Thirty milliliters of the cultured sample was poured into a centrifuge tube, subjected to deoxidation sealing treatment and then sent to be tested after culturing.

3.3.2 16S rRNA amplicon sequencing

16S amplicon sequencing analysis of the water samples from six CBM wells of the GP well group was carried out. The sequencing was completed at Sangon Biotech (Shanghai) Co., Ltd. DNA extraction was achieved using a kit (E.Z.N.ATM Mag-Bind Soil DNA Kit). Archaea and bacteria detection was conducted by polymerase chain reaction (PCR) with three rounds of amplification. In the first round, the M-340F and GU1ST-1000R primers were used for amplification, and in the second round, the first round PCR primers were used for amplification. The primers used were fused with the V3–V4 universal primers of the Miseq sequencing platform, including the primers 341F: CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG and primers 805R: GACTGGAG TTCCTTGGC ACCCGAG AATTCCA GACTACHVGGGTATCTAATCC. A 30 μL of reaction mixture for PCR amplification contained 15 μL of Taq master Mix, 1 μL of Bar-PCR primers F (10 μMol/L), 1 μL of primers R (10 μMol/L), 10–20 ng of bulk DNA solution, and add water to 30 μL. The PCR conditions were initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 30 s, denaturation at 45°C for 20 s, denaturation at 65°C for 30 s and extension at 72°C for 5 min. The PCR conditions were initial denaturation at 94°C for 3 min, followed by denaturation

Table 2 Development information and produced water geochemical test data of CBM wells

Syncline	Well name	TDS/(mg·L^{-1})	pH	Fe/(μg·L^{-1})	Ni/(μg·L^{-1})	δ^{13}C$_{DIC}$ /‰	δD /‰	δ^{18}O /‰	δ^{13}C(CH$_4$) /‰	δD(CH$_4$) /‰
Tucheng	GP1	2715	7.9	212.92	0.53	1.440	−24.99	−6.15	−41.30	−172.70
	GP2	3305	7.8	34.05	0.09	14.990	−26.31	−6.03	−42.30	−188.60
	GP3	3815	7.8	38.01	3.90	8.799	−35.04	−6.86	−41.20	−171.20
	GP6	4220	7.6	91.83	0.27	8.629	−29.94	−6.51	-	-
	GP7	4020	7.8	61.18	0.30	2.639	−31.92	−6.15	−41.00	−169.60
Faer	YC1	2949	7.9	19.91	0.00	−0.319	−30.32	−6.01	−36.00	−160.70
	QC1	3949	7.7	41.34	0.06	−0.389	−39.07	−6.73	−35.90	−157.10
Laochang	LCC1	2670	8.3	13.38	0.14	−4.450	−73.71	−11.30	−33.70	−143.00
	LCC2	2910	8.3	22.63	0.08	−8.650	−84.71	−12.39	−36.40	−150.00
	LCC3	1024	8.4	828.05	0.49	−8.900	−88.79	−13.00	−35.60	−144.30
	LCC4	3355	7.9	28.63	0.00	−4.030	−80.52	−12.15	−34.80	−145.30
	LCS1	2408	8.3	16.42	0.01	−5.490	−86.28	−12.79	−35.50	−145.40
	LCS2	2922	8.3	17.92	0.00	−4.430	−83.28	−12.45	−35.30	−145.80
Enhong	EHC6	2237	8.3	997.76	8.49	12.730	−72.54	−11.56	−45.40	−214.20
	EHC7	1594	8.6	13.49	0.00	20.020	−58.15	−10.28	−50.70	−230.60

Notes: a) TDS: Total dissolved solids; b) “-” indicates no detection.

at 94°C for 30 s, denaturation at 45°C for 20 s, denaturation at 65°C for 30 s and extension at 72°C for 5 min.

In the third round, Illumina bridge PCR-compatible primers were introduced. A 30 μL of reaction mixture for PCR amplification contained 15 μL of Taq master Mix, 1 μL of Bar-PCR primers F(10 μMol/L), 1 μL of primers R(10 μMol/L), the second round PCR primers, and add water to 30 μL. The PCR conditions were initial denaturation at 94°C for 3 min, denaturation at 94°C for 20 s, denaturation at 55°C for 20 s, denaturation at 72°C for 30 s, and extension at 72°C for 5 min.

3.3.3 Sequencing data analysis

The PCR products were checked using electrophoresis in 1% (w/v) agarose gels. Total DNA products recovery was performed using a magnetic bead nucleic acid purification kit. The concentration of DNA was measured using a Qubit 2.0 (life, USA). Sequencing was performed using the Illumia Miseq system (Illumia Miseq, USA).

After all samples were sequenced, effective sequences were obtained for subsequent analysis after quality control. The sequences were clustered into operational taxonomic units (OTUs). On the basis of OTU clustering results, the most abundant sequence was selected as the representative sequence of OTU, and various analyses were carried out. Chao index and ACE index were used to evaluate the richness of the microbial community. The larger values of Chao 1 index and ACE index represent more microbial biomass. Shannon index and Simpson index were used to evaluate the diversity of microbial community. The larger values of Shannon index represent richer community diversity, while Simoson index is the opposite. The test results are shown in Table 3.

3.4 Realtime fluorescence quantification PCR

To detect the changes of biomass before and after culture, realtime fluorescence quantitative PCR was carried out. The primers of archaea used in this experiment are 340F: CCCTACGGGGYGCASCAG and 519R: TTACCGCGG CKGCTG. The primers were designed and synthesized by Sangon (Shanghai) CO., Ltd using primer premier 5.0 software. The quantitative PCR reagent used was 2xsg fast qPCR Master Mix (b639271, BBI, Roche Roche), and the quantitative PCR instrument was lightcycler480 II fluorescent quantitative PCR (Roche, rotkreuz, Switzerland). The DNA sample was diluted 10 times as a template for detection. PCR conditions consisted of an initial denaturation step of 95°C for 3 min, and followed by 95°C for 5 s, 60°C for 30 s. The final samples were detected by realtime fluorescence quantitative PCR.

The realtime fluorescence quantitative PCR test of archaea in the produced water of 15 CBM wells collected in October 2019 was carried out, including a total of 30 samples of before and after enrichment culture in the laboratory. The test results are shown in Table 3.

4 Results

4.1 Community structure of archaea in uncultured water

In the water produced from the CBM wells, a total of seven archaeal phyla were detected, among which

Table 3 The information of Seq-Number and OTUs and the results of realtime fluorescence quantitative PCR

Sample		Effective sequence number	OTUs	Shannon	Chao	Ace	Simpson	Average copies /(copies·μL^{-1})
Archaea (before culture)	GP1	79318	109	2.676914	140.66670	192.01900	0.127298	619.6386
	GP2	74689	238	3.669296	249.66670	260.65180	0.075506	424.4985
	GP3	95335	208	2.709824	232.00000	239.11940	0.223403	536.9054
	GP6	114520	93	0.564902	120.14290	161.13970	0.830923	609.2864
	GP7	108355	105	0.640935	144.42860	129.18810	0.760809	650.2727
	YC1	91201	77	1.347247	90.20000	88.51642	0.388553	675.6968
	QC1	40063	111	3.418395	132.00000	170.16690	0.075087	635.7875
	LCC1	84060	207	2.938889	208.11110	208.71740	0.128711	586.9863
	LCC2	87998	155	2.689045	190.00000	201.58050	0.256630	661.4936
	LCC3	98142	275	2.222372	285.11110	282.13140	0.311218	517.3710
	LCC4	82165	226	2.110939	232.56250	231.83340	0.284999	467.0808
	LCS1	77015	70	2.851636	91.00000	89.95899	0.100348	475.1358
	LCS2	82030	67	2.012512	72.00000	75.29911	0.231284	613.3209
	EHC6	91389	143	2.431259	155.00000	150.19760	0.175003	637.0635
	EHC7	89749	39	0.889882	44.00000	49.58599	0.585256	518.2393
Archaea (after culture)	GP1	29408	60	2.750319	82.50000	70.19988	0.140437	1665.4060
	GP2	90209	86	1.518124	99.57140	102.90910	0.288684	153453.8000
	GP3	84524	62	1.351330	74.21430	86.06072	0.348370	42400.4600
	GP6	81894	78	1.724473	124.75000	181.83750	0.235420	567935.6000
	GP7	96916	26	1.043127	31.00000	31.90723	0.390325	112317.3000
	YC1	107199	103	0.314287	105.14290	105.99700	0.922528	64917.6700
	QC1	81294	74	0.540438	87.00000	81.87146	0.734516	311554.4000
	LCC1	96374	80	0.06289	84.12500	85.72118	0.987094	162915.3000
	LCC2	112159	31	1.129833	46.16667	89.91944	0.401601	233650.4000
	LCC3	97771	114	0.074028	167.00000	233.59930	0.983106	587858.8000
	LCC4	100742	31	1.036109	64.00000	70.73032	0.422829	14565.3000
	LCS1	95249	37	0.347614	48.14286	48.26662	0.839801	56138.2600
	LCS2	101257	45	0.460523	95.60000	129.10200	0.777521	37225.3900
	EHC6	83518	65	1.529978	102.50000	140.84310	0.309206	169197.9000
	EHC7	89358	52	0.814535	91.42860	136.23830	0.577505	165155.7000
Bacteria (after culture)	GP1	53616	25	1.615902	25.60000	27.18625	0.271231	-
	GP2	53451	45	2.290351	45.75000	46.19055	0.139808	-
	GP3	52746	74	2.061296	87.00000	87.48082	0.233719	-
	GP6	54107	30	1.745363	37.20000	42.92479	0.228786	-
	GP7	60243	18	1.085732	19.20000	21.54145	0.392638	-
	YC1	54483	40	1.952130	42.00000	42.17778	0.203598	-
	QC1	55247	58	2.103187	59.20000	60.49471	0.198752	-
	LCC1	44199	53	2.111865	58.25000	59.51334	0.184093	-
	LCC2	49117	71	2.470686	77.42857	90.34014	0.136720	-
	LCC3	57114	58	1.526263	69.00000	68.31053	0.354134	-
	LCC4	54984	62	2.064624	64.50000	65.22739	0.214903	-
	LCS1	52060	79	2.338743	96.14286	108.01760	0.143459	-
	LCS2	62536	94	2.752088	114.00000	110.43200	0.126117	-
	EHC6	52808	50	2.472064	50.60000	51.60826	0.117960	-
	EHC7	46782	52	2.385944	59.00000	57.28552	0.145706	-

Note: a) “-” means not detected.

Euryarchaeota was the dominant archaeal phylum with an average relative abundance of 90.56%, and methanogens belong to *Euryarchaeota*. In addition, *Thaumarchaeota*, *Crenarchaeota*, and *Woeserchaeota* were detected in all samples, while *Pacearchaeota*, *Nanohalorchaeota*, and *Dipherotrites* were only detected in some samples. The relative abundances of *Thaumarchaeota* and *Crenarchaeota* in the water produced from well GP2 in western Guizhou Province were relatively high, and the relative abundances of *Euryarchaeota* in the water produced from the other wells were above 85% (Fig. 2).

Methanogens can be divided into six orders, namely, *Methanobacteriales* (36.42%), *Methanomicrobiales* (29.99%), *Methanosarcinales* (13.885%), *Methanomassiliicoccales* (7.69%), *Methanococcales* (0.03%), and *Methanocellales* (0.03%). The average relative abundances of *Methanobactales*, *Metonomicrobiales*, *Metonosarcinales*, and *Methanogasiliccacales* were high, and they were detected in all samples (Fig. 3). *Methanococcales* and *Methanocellales* were detected in some samples. Some archaea belonging to other phyla were also determined to have high relative abundances. For example, *Nitrososphaerales*, which belong to *Thaumarchaeota*, had a relative abundance of 34%–35% in the water samples from GP2 well (Fig. 3). *Nitrososphaerales* are important ammonia oxidizing archaea and play an important role in nitrogen and carbon circulation (Zhang and He, 2012). *Halobacteria*, which belong to *Euryarchaeota*, had an average relative abundance of 1.72%, are extremely halophilic, and their optimum NaCl concentration is 3–4 mol/L. *Halobacteria* grow in salt lakes, salt fields, and salted fish. High concentrations of electrolytes are used to maintain the structural integrity of the plasma membrane and ribosomes of *Halobacteria*. The relative abundance of *Halocharacteria* in the GP well group is higher than that in other areas, which reflected the water from GP well group has higher total dissolved solids (TDS).

Various methanogens constituted the main archaea in each sample within genera. The dominant archaeal genera in the produced water from different wells in the study area were mainly *Methanobacterium*, *Methanoculleus*, *Methanobrevibacter*, *Methanothrix*, and *Methanoregula*. *Methanobacterium* has been detected as a dominant archaea in the produced water of many CBM wells and can utilize both H_2 and methyl compounds for metabolism. *Methanobacterium* is a hydrogenotrophic and methyltrophic methanogen. *Methanoculleus*, *Methanobrevibacter*, and *Methanoregula* are hydrogenotrophic methanogens, while *Methanothrix* is an acetoclastic methanogen. These four dominant archaeal genera were detected to different degrees in all the produced water samples, which indicates that there are abundant methanogenic pathways in the coal seams of eastern Yunnan and western Guizhou. In addition, *Methanosarcina* was also detected in all the wells containing produced water and has three methane synthesis pathways and can utilize at least nine different substrates. And many types of methanogens were found in anthracite.

4.2 Community structure of archaea in cultured water

To verify the experimental anaerobic culture results, the changes in the microbial community structure before and after cultivation were compared. After four days of enrichment, the water samples were sequenced.

After enrichment culture, *Euryarchaeota* was enriched to a certain extent within phyla. *Crenarchaeota* and *Thaumarchaeota* were only found in the GP1, GP6, and

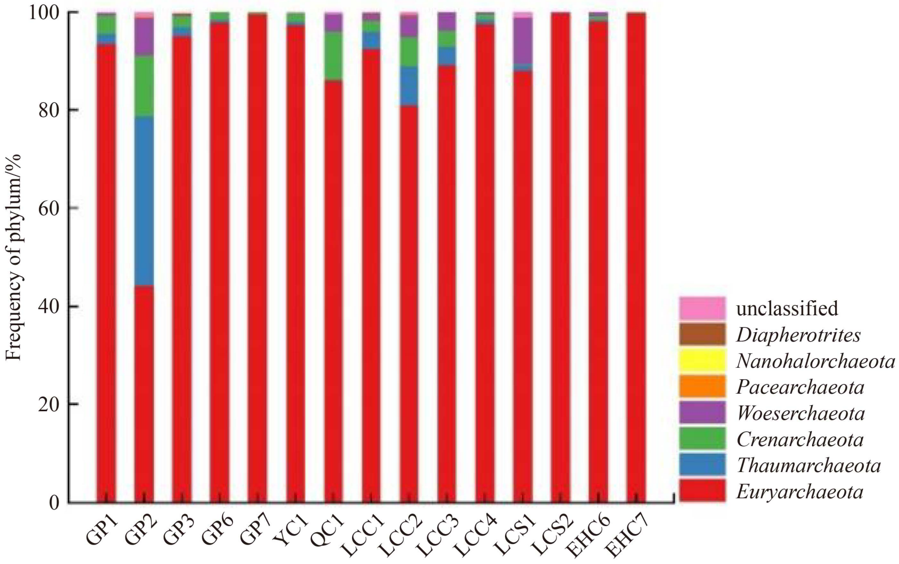

Fig. 2 Relative abundances of archaea in uncultured water within phyla.

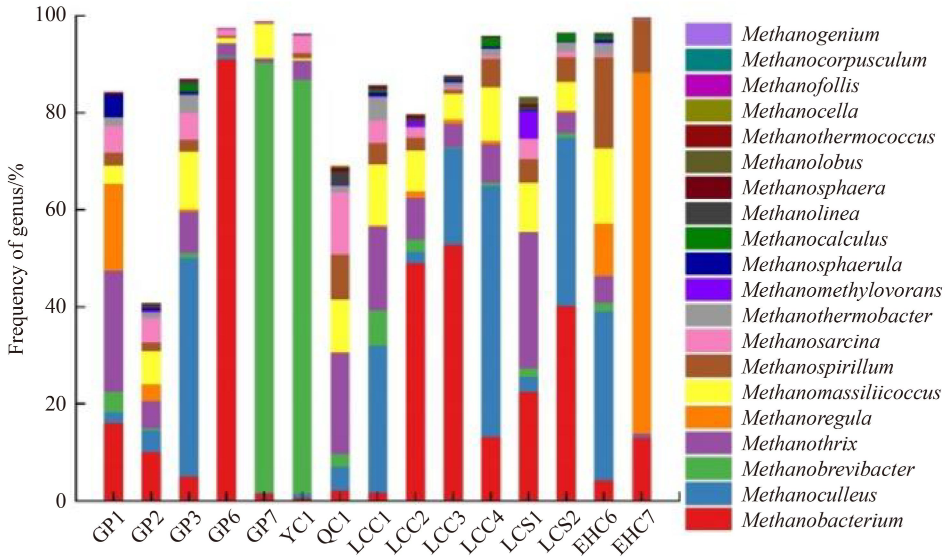

Fig. 3 Relative abundances of archaea in uncultured water within genera.

GP7 well samples. *Euryarchaeota* accounted for the majority of in water samples from other wells, with relative abundances above 99% (Fig. 4).

The average relative abundances of *Methanomicrobiales* and *Methanobacteriales* were the highest within orders, and the other methanogens were mainly distributed among *Methanosarcinales*, *Methanogasiliccales*, and *Methanocellales*. Other archaea detected in the produced water, including *Thermoproteales*, *Nitrososphaerales*, *Halobacteria*, *Desulfurococcales*, and *Haloferacals*, were also detected in the cultured water. The relative abundances of *Methanobacteriales* and *Methanomicrobiales* increased to some extent. The dominant archaeal genera were *Methanobacterium*, *Methanoregula*, *Methanobrevibacter*, and *Methanospirillum*. Among them, the dominant archaea in the YC1 well and six wells in the LC area were *Methanobacterium*, and their relative abundances were high (Fig. 5).

4.3 Community structure of bacteria in cultured water

The 16S rRNA gene of bacteria in the water produced from CBM wells was not amplified, which indicated that the bacteria in the produced water were lower than the detection level. After completion of collection, the produced water samples were sent to the laboratory for enrichment culture without contacting other bacterial sources. Therefore, the bacteria in the water samples after enrichment culture can also reflect bacterial communities in the coal-seam water to a certain extent, even if the microbial community structures change during the enrichment and cultivation processes. Because of the

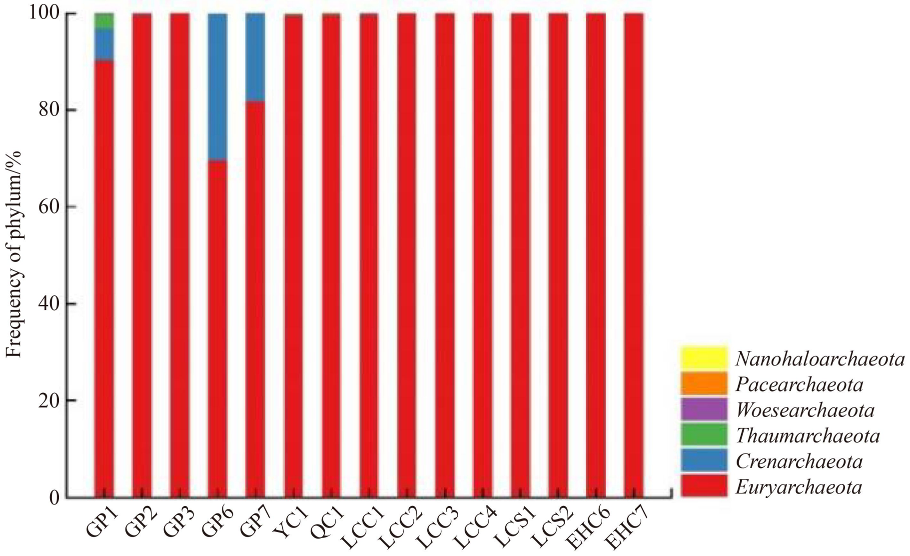

Fig. 4 Relative abundances of archaea in cultured water within phyla.

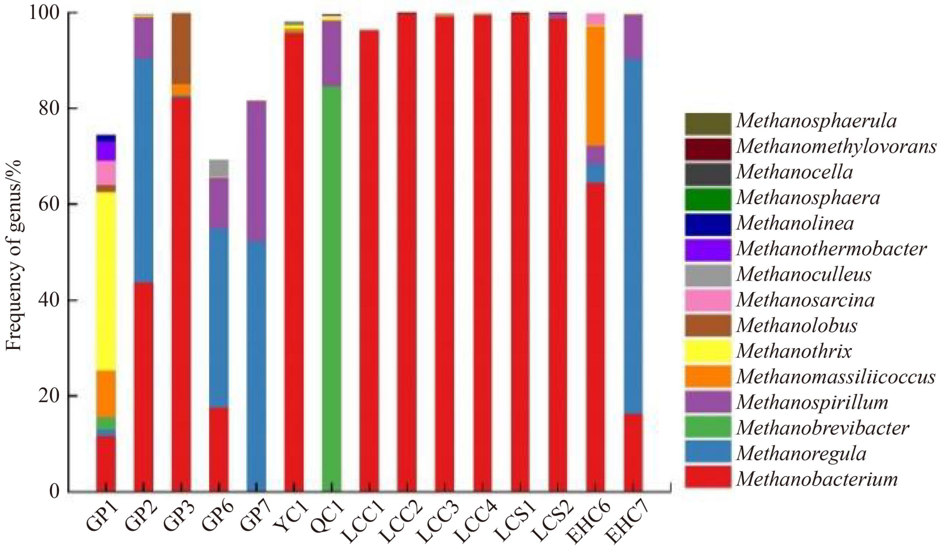

Fig. 5 Relative abundances of archaea in cultured water within genera.

complexity of the bacterial community structures in the cultured water, a heatmap was used to represent this complexity, which is shown in Fig. 6. Most of the bacteria were distributed among *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, and other bacteria with low relative abundances were detected, such as *Synergistetes*, *Spirochaetes*, *Fusobacteria*, and *Acinobacteria*. These bacteria are commonly associated bacteria in coal seams or coalbed water, and their common characteristics are diverse metabolic activities and hydrocarbon degradation abilities.

Bacteroidetes are common in sediments and are chemoautotrophic microorganisms. *Bacteroidetes* found in coalbed water are mainly involved in the degradation of macromolecular substances, such as proteins, sugars and cellulose, which are fermented into formic acid, hydrogen, and carbon dioxide (Guo et al., 2012). *Macellibacteroides*, which belongs to *Bacteroidetes*, was the dominant genus in most samples. The relative abundance of *Dysgonomonas* was also high. *Macellibacteroides* are acetogenic bacteria and are the main bacteria in the hydrogen and acetic acid synthesis stages. The function of *Macellibacteroides* is to ferment macromolecular soluble organic matter, such as short chain cellulose to produce pyruvic acid and fumaric acid to produce small-molecule acetic acid, propionic acid, butyric acid and other fatty acids (Jabari et al., 2012).

Proteobacteria are closely associated with methanogens (Guo et al., 2012), which are abundant bacteria. The *Proteobacteria* detected in this study were mainly *Epsiloproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria*. *Deltaproteobacteria* contain a variety of

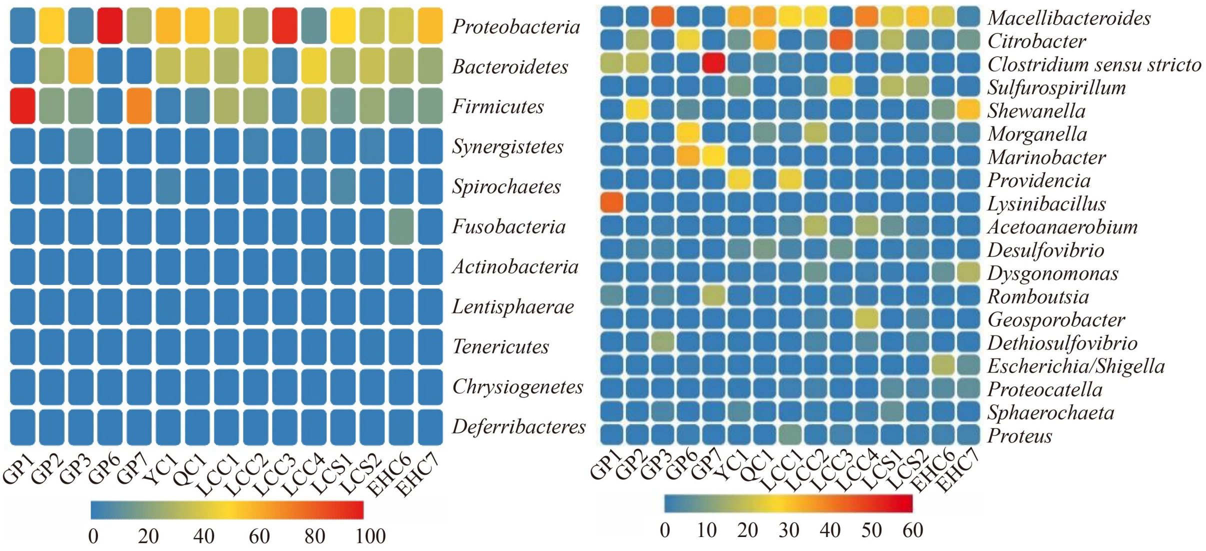

Fig. 6 Heatmap of the bacterial communities in cultured water (relative abundance > 1%; left: phylum level; right: genus level).

sulfate reduction microorganisms that can also degrade naphthalene or other aromatic hydrocarbons. *Desulfocibrio,* which belong to *Deltaproteobacteria,* were detected in all samples. *Desulfovibrio* have the potential to metabolize heterotrophic energy under sulfate reduction conditions. *Desulfovibrio* cannot only metabolize complex carbohydrates but can also be cocultured with methanogenic archaea to metabolize simple compounds, such as lactic acid and ethanol, to produce methane (Green et al., 2008).

Firmicutes are important bacteria involved in biogenic methane. The bacterial genus in *Synergistetes* is mainly *Dethiosulfovibrio*, which is an important amino acid-degrading and acetogenic bacterium in anaerobic systems (Wang, 2009). Hydrogen-producing acetic acid-producing bacteria can further degrade propionic acid, butyric acid and other volatile organic acids (VFAs) as well as ethanol produced in the previous stage into acetic acid, carbon dioxide and hydrogen, which provide direct substrates for methanogens in the last stage (Strąpoć et al., 2011).

The main bacteria in *Spirochaetes* were *Sphaerochaeta*. It has been reported that *Spirochaeta* can degrade carbohydrates and produce ethanol, acetic acid, lactic acid, hydrogen and carbon dioxide (Li et al., 2019).

Compared with the bacterial communities detected by many other scholars in coal or coal seam water, the detection results lack many sulfate reducing and denitrifying bacteria. Only part of *Desulfovibrio* was detected in the sequencing results. The sulfate reducing bacteria detected in coal seam water mainly include *Desulfaculales*, *Desulfobacteria*, *Desulfovibrionales*, and *Desulfurmonadales* (Penner et al., 2010). Denitrifying bacteria detected in coal seam water mainly include *Pseudomonas*, *Thauera*, *Janthinobacterium*, and *Mesorhizobium*. The low abundance of these two kinds of bacteria reflects the low efficiency of sulfur and nitrogen cycle in the production horizon in the study area. It can also be seen from the geochemical test of produced water that the concentrations of SO_4^{2-} and NO_3^- in almost all wells are low or even undetectable. It has been found that sulfate reduction and denitrification process can significantly inhibit the activity of methanogens (Vizza et al., 2017). However, they are also important microorganisms in the gas production stage, so the effect of these microorganisms on the methane production efficiency needs to be further studied.

In all the samples, the three bacteria with the highest relative abundances after anaerobic enrichment culture were *Macrobacteria*, *Citrobacter*, and *Clostridium sense stricto*, which belong to *Bacteroidetes, Proteobacteris*, *Firmicutes.* Due to the lack of genetic information on microbial communities in *in situ* coalbed water, it was impossible to compare the effects of enrichment culture on the bacterial community structure in the laboratory. Penner et al. (2010) compared microbial communities before and after enrichment culture of coal samples and found that after anaerobic enrichment culture in the laboratory, the bacterial microbial population changed from oligotrophic and chemotrophic *Proteobacteria* to heterotrophic fermentation *Firmicutes* and *Bacteroidetes*. Therefore, it can be inferred that *Macellibacteroides,* with higher abundances in the sequencing results, were strengthened by the nutrient solution.

5 Discussion

5.1 Changes in the archaeal community before and after culturing

Shannon index and Simpson index are two commonly used Alpha diversity assessment indices to estimate microbial diversity in samples. They generally do not focus on comparison, but only evaluate the degree of diversity in the environment. The Shannon index is used to describe the disorder and uncertainty of individual species. The higher the uncertainty, the higher the diversity, that is, the greater the Shannon index, indicating the higher the community diversity. The Simpson index describes the probability that the number of individuals obtained from two consecutive samplings of a community species belongs to the same type. The larger the Simpson index value is, the lower the community diversity is (Simpson, 1949; Chao and Shen, 2003).

Comparing the changes of the Shannon index and the Simpson index before and after enrichment culture, it was found that the diversity of archaea community in most samples decreased after anaerobic enrichment culture (Fig. 7).

To characterize the abundance of Archaea before and after anaerobic culture more precisely, the biomass was measured by realtime fluorescence quantitative PCR. It is found that the biomass of Archaea community in the produced water of different CBM wells has little difference, and the distribution range is 424.50–675.70 copies/μg, and the average value is 25 copies/μg. The low value of biomass appeared in GP2 well and Laochang area. After 4 days of enrichment culture, the biomass increased significantly, which was between 1665.41–587858.80 copies/μg, and the average value is 11 copies/μg. The biomass of nine wells exceeded 10000 copies/μg (Fig. 8).

The reason is that in the process of enrichment culture, a large number of ions or elements that were missing or less in the original coal seam water were added according to the best living conditions of methanogens. The sensitivity of archaea to different ions or elements and culture temperature in anaerobic incubator was different. The optimal enrichment culture conditions of different archaea are not consistent, which leads to the enhancement of some archaea and the disappearance or decrease of the relative

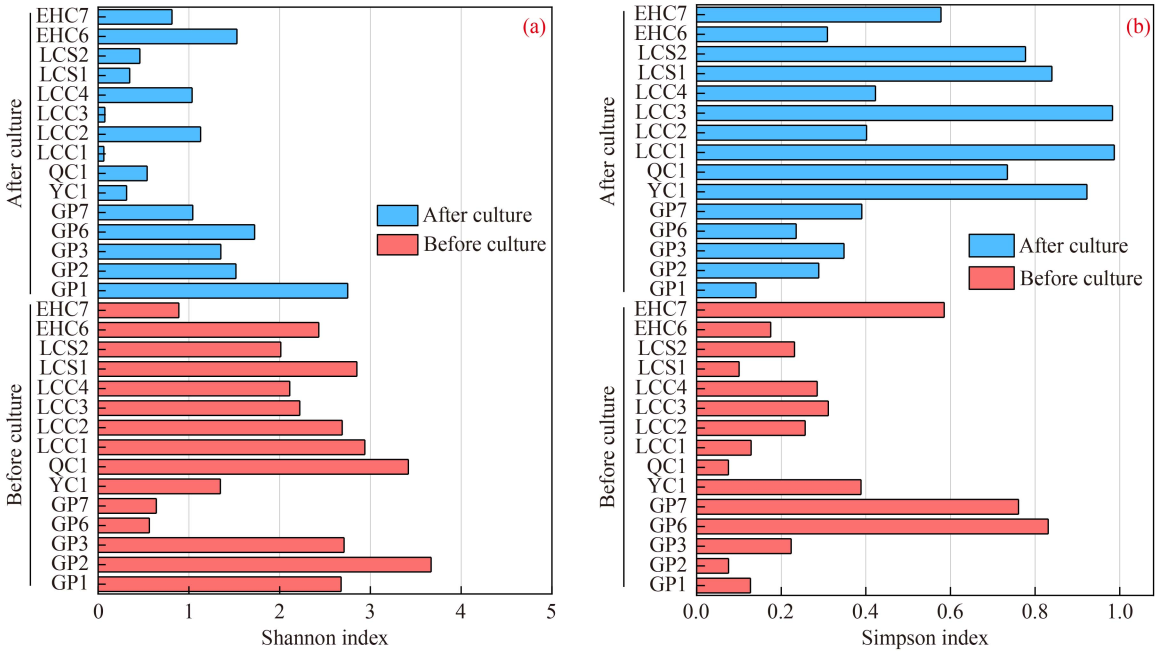

Fig. 7 Changes of archaea community diversity before and after enrichment culture in produced water of CBM well.

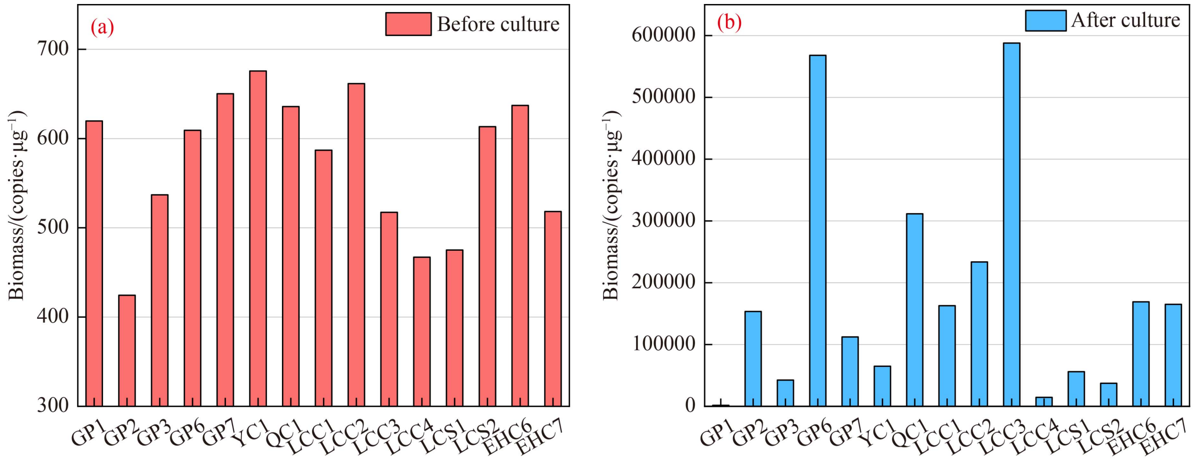

Fig. 8 Biomass changes of archaea community before and after enrichment culture in produced water of CBM wells.

abundance of other archaea, that is, the decline of Archaea community diversity. On the whole, nutrient solution and culture environment were suitable for the growth and reproduction of archaea, and the abundance of microbial community increased rapidly in a short time.

Lefse and Welch’s *t*-test analysis were used to evaluate the significance of laboratory anaerobic culture on the changes in archaeal community structures (Figs. 9 and 10). The results showed that the transformations were mainly from *Methanoregula* to *Methanobacterium* within genera and from *Methanomicrobia* to *Methanobacteria* within orders.

Four methanogens had high relative abundances: *Methanobacterium*, *Methanoregula*, *Methanospirillum*, and *Methanobrevibater*. Among them, only the relative abundance of *Methanobacteria* increased significantly, while the relative abundances of *Methanobacteria, Methanopirillum,* and *Methanobrevibater* decreased significantly. All of these results indicated that after anaerobic culture, archaeal diversity decreased, the relative abundances of many methanogens decreased, and the relative abundances of *Methanobacteria* with two metabolic pathways, hydrogen and methyl, increased.

5.2 Evaluation of biogenic enhancement of methane production potential

5.2.1 Methanogenic pathway of secondary biogas

Methanogens can survive in most natural environments and even in some extreme environments. In recent years, there have been an increasing number of reports on the

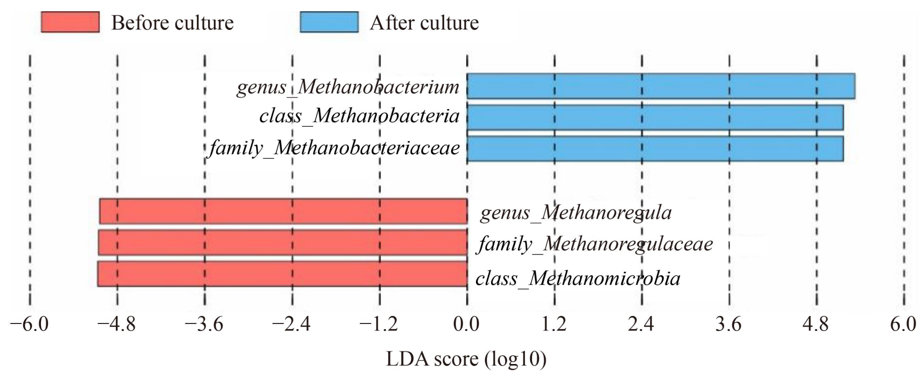

Fig. 9 LEfSe analysis of archaeal communities before and after culture.

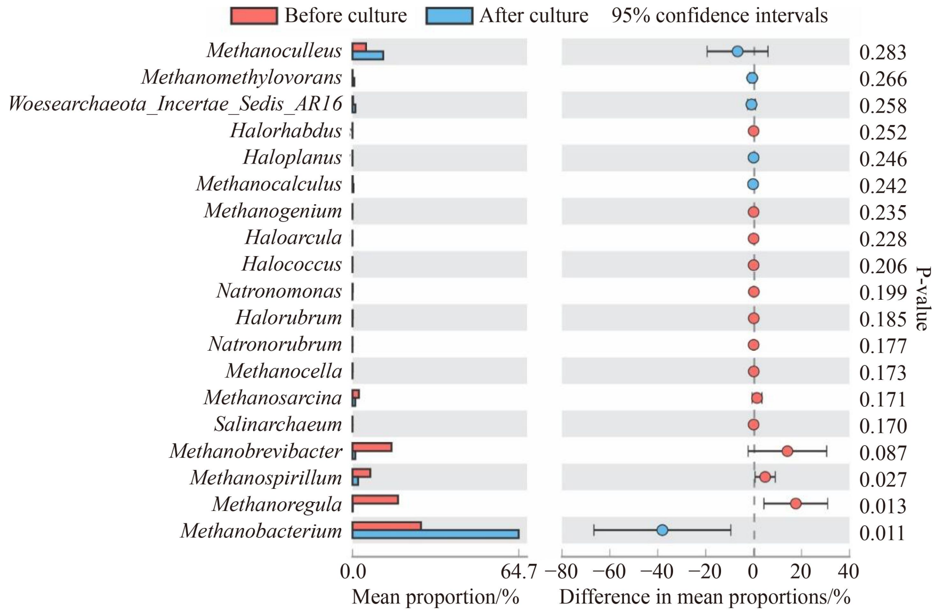

Fig. 10 Welch's *t*-test within genera before and after culture.

diversity of methanogens in coal seams or coalbed water, and high-abundance methanogens have been found in some high-rank bituminous coal and anthracite mining areas (McInerney et al., 1981; Xiao et al., 2013; Su et al., 2018; Nie et al., 2019). A large number of methanogens, such as LC syncline CBM development wells, were detected in the anthracite area of eastern Yunnan and western Guizhou for the first time. However, whether there is a large amount of secondary biogas produced by methanogens in the study area is the basic condition for microbial-enhanced methane production. Therefore, it is necessary to discuss the genetic types of CBM in the study area.

The carbon and hydrogen isotopic characteristics of methane in CBM can be used to identify the genetic types. According to the template of Whiticar et al. (1986), the gas produced in CBM wells of the Tucheng, Faer, Enhong, and Laochang areas was identified. It can be seen from Fig. 11 that most of the gas in the study area is thermogenic gas, some wells in the Enhong area are located in the range of mixed gas, and wells in Tucheng area are close to the range of mixed gas. However, the use of methane carbon and hydrogen isotopes to directly distinguish thermogenic gas and biogenic gas has certain limitations. The secondary biogenic gas is that during the burial process of coal seam, due to the tectonic uplift, it receives surface water supply, so that microorganisms can enter the coal seam, and under specific reservoir conditions, methane is generated through carbon dioxide reduction or acetic acid fermentation. If the generated secondary biogenic gas is mixed with the previously formed thermogenic gas, the content of $\delta^{13}C$ will be reduced, and then the gas genetic identification will be biased.

Some scholars have proposed a method to identify biogenic gas based on the content of $\delta^{13}C_{DIC}$ in produced water (Golding et al., 2013; Yang et al., 2019). In the process of microbial fermentation to produce biogenic gas, methanogens could preferentially use ^{12}C, making ^{13}C gradually enriched in water, resulting in a higher

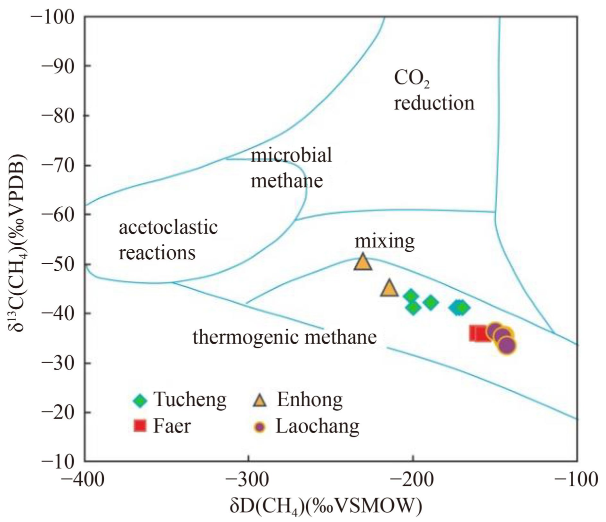

Fig. 11 Origin identification of CBM (Whiticar plate).

positive value of $\delta^{13}C_{DIC}$. Studies have shown that the presence of biogenic gas can be indicated when the $\delta^{13}C_{DIC}$ value is higher than 10‰. (Yang et al., 2019, 2020). It can be seen from Fig. 12 that the $\delta^{13}C_{DIC}$ in Enhong area is greater than 10‰, and the average value of $\delta^{13}C_{DIC}$ in Tucheng synclines is 7.29 ‰, with the maximum value of 14.99‰. Therefore, it can be considered that there is biogenic gas in Tucheng and Enhong synclines. The $\delta^{13}C_{DIC}$ values of Faer and Laochang areas are negative, indicating that they did not have a lot of biogenic gas formation or preservation in their geological history.

Based on the mixed model proposed by Tao et al. in 2007, the proportions of biogas and thermogenic gas in the coal seams in the TC and EH areas were quantitatively estimated. The mixing formula is as follows (Eq. (1))

$$Ax + B(1 - x) = C, \tag{1}$$

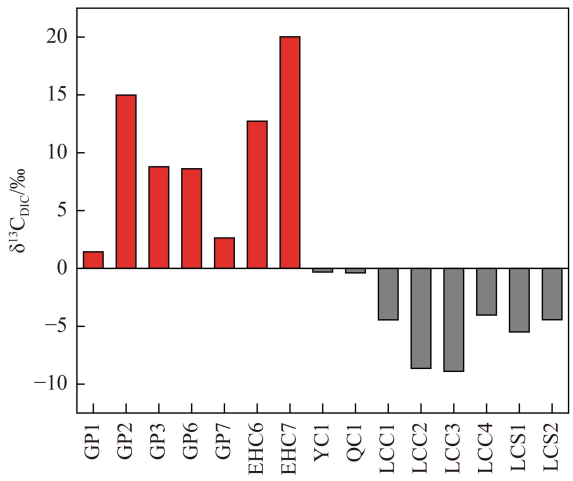

Fig. 12 $\delta^{13}C_{DIC}$ distribution of produced water from CBM wells in the study area.

$$\delta\,^{13}C(CH_4) = 22.42 \lg R_0 - 34.8. \tag{2}$$

In Eq. (1), A is the end member of the $\delta^{13}C$-CH_4 value of biogas, which is −70‰. B is the end member of the $\delta^{13}C$-CH_4 value of thermogenic gas, which is calculated by the regression formula for coal rock thermal simulation of primary CBM, without an obvious secondary transformation effect, in Eq. (2) (Liu and Xu, 1999). The C value represents the $\delta^{13}C$-CH_4 value of CBM that was obtained from experimental tests, and the calculated x value is the proportion of biogas in the CBM. The results show that the proportion of biogas in the TC area is 16.83%–30.52% and that in the EH area is 27.95%–48.46%. The proportion of secondary biogas in Enhong area is higher than that in Songhe area, which is related to the low rank of coal in Enhong area.

Further analysis of the secondary biogenic methane production pathway is the basis for evaluating the microbial-enhanced methane production. In the final stage of gas production, hydrogenotrophic methanogens can use H_2 and formic acid as electron donors to reduce CO_2 to produce methane; acetoclastic methanogens decompose acetic acid, oxidize hydroxyl groups in acetic acid to CO_2, and reduce methyl groups to methane; and methylotrophic methanogens produce methane by H_2 reduction of methyl compounds or by disproportionation of methyl compounds. In many basins around in the world, CO_2 reduction is more extensive than the acetic acid/methyl fermentation pathway for forming biogas, such as the Bowen Basin (Kinnon et al., 2010; Golding et al., 2013), Sydney Basin (Faiz and Hendry, 2006), and Power River Basin (Flores et al., 2008; Rice et al., 2008; Bates et al., 2011).

Among the top five methanogens with high relative abundances in the produced water from the CBM wells in the Tucheng and Enhong areas, only *Methanthrix* is an acetoclastic methanogen. Most of the other methanogens are hydrogenotrophic and use CO_2 to reduce H_2 to produce methane. The hydrogen atoms in CH_4 that are produced by hydrogenotrophic methanogens all come from a symbiotic water medium, and the relationship follows Eq. (3) (Whiticar, 1999; Golding et al., 2013):

$$\delta D(CH_4) = \delta D(H_2O) - 160‰ \pm 10‰. \tag{3}$$

Combined the sampling results of the research group at other times in 2019 (Fig. 13 and Fig. 14), it was determined that these areas are located near the hydrogentrophic methane, which do not completely fall into this range. The reason is that gas produced by the CBM wells in these two areas is a mixed genetic gas, and the isotopic composition of different time periods is also affected by precipitation.

Based on the results of 16S rRNA amplicon sequencing and the geochemical characteristics of the produced gas and water, it is concluded that the two areas are dominated by hydrogenotrophic methanogens. CO_2 reduction

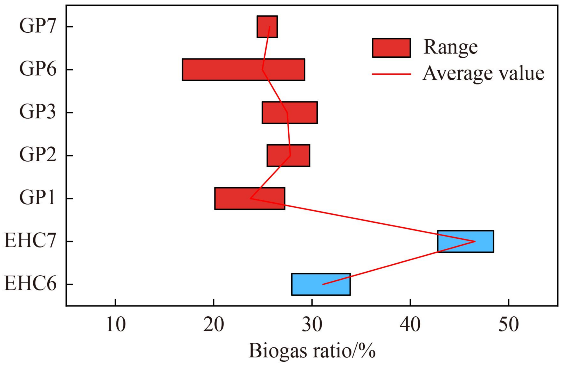

Fig. 13 Biogas ratios of seven CBM wells in the TC and EH areas.

is the main method of biogas production, and there is also thermogenic gas, which is consistent with the conclusion that most biogas is produced by hydrogenotrophic methanogens (Kirk et al., 2012).

5.2.2 Feasibility of microbial-enhanced methane production

In thermokinetics, Gibbs free energy is used to determine whether a chemical reaction will occur spontaneously. At a constant temperature, when the Gibbs free energy of a reaction is negative, the reaction can proceed spontaneously. Methanogens act on the last stage of microbial degradation of organic matter in coal. Although there are large differences in the phylogeny of different methanogens, most of their final products are CH_4 and CO_2, and few substrates can be utilized. The Gibbs free energy levels are different in the processes of methanogens that use different substrates (Table 4). Gibbs free energy can reflect the difficulty of a reaction. The lower the Gibbs energy is, the easier the reaction is. The gas production reaction of hydrogentrophic methanogens was the most likely to occur, followed by methyltrophic methanogens and acetoclastic methanogens.

In the study area, there were mainly hydrogenotrophic methanogens in the produced water from the CBM wells, and there were also methanogens with acetic acid and methyl nutrition. The metabolic pathways of methane production are diversified. After anaerobic culture, *Methanobacterium* was highly enriched, and the other methanogens exhibited higher relative abundances, such as *Methanoregula*, *Methanobrevibacter* and *Methanospirillum*, which are hydrogenotrophic methanogens. After anaerobic enrichment culture, although the diversity of the methanogenic pathways decreased, the number of hydrogentrophic methanogens increased. Compared with the methyltrophic and acetoclastic methanogens, these methanogens can produce higher energy in the methanogenesis process, and the reaction is easier, which indicates that adding nutrient solutions could improve the methane production potential of microorganisms.

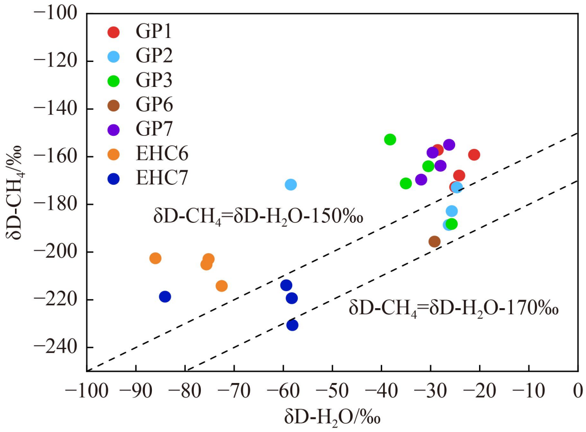

Fig. 14 Relationships between δD-CH_4 and δD-H_2O in the produced water of CBM wells in the TC and EH areas.

Most methanogens are extremely anaerobic microorganisms that are sensitive to the environment and can survive only in specific reservoir environments. First, coal rank affects the methanogen community structure. Low-rank coal contains more hydrogen, oxygen and nitrogen. With increasing coal rank, the numbers of hydrogen and oxygen side chains in coal decrease, and the available nutrients of microorganisms are reduced. Scott et al. (1994) found that the $R_{o,\max}$ of coal rock generated by secondary biogas was 0.3%–1.5%. Compared with domestic data analysis, Li Guihong and Zhang Hong (2013) found that the coal rock $R_{o,\max}$ generated by secondary biogas was 0.29%–2.01%. The $R_{o,\max}$ distribution range of five wells in the Tucheng area was 1.4%–1.69% and that of two wells in the Enhong area was approximately 1.21%, which are suitable for

Table 4 Gibbs free energy changes of different methanogenic bacteria during biogas production (Cheng et al., 2016)

Substrate	Product	$\Delta G^{0\prime}$/(kJ·mol^{-1} CH_4)	Methanogens
$4HCO_3^- + H^+ + H_2O$	$CH_4 + 3HCO_3^-$	−145	Hydrogentrophic
$4H_2 + HCO_3^- + H^+$	$CH_4 + 3H_2O$	−135	Hydrogentrophic
$HCOO^- + H^+ + 3H_2$	$CH_4 + H_2O$	−134	Hydrogentrophic
$CH_3COO^- + H_2O$	$CH_4 + HCO_3^-$	−31	Acetoclastic
CH_3OH	$3CH_4 + HCO_3^- + H^+ + H_2O$	−105	Methylotrophic
$4(CH_3)_3$–$NH^+ + 9H_2O$	$9CH_4 + 3HCO_3^-\ 4NH_4^+ + 3H^+$	−76	Methylotrophic

secondary biogas generation.

The survival of methanogens also requires suitable temperatures, TDS, trace elements, and other environmental conditions. When the reservoir temperature is between 35°C and 42°C, methanogens are most likely to survive. Tang et al. (2012) selected 35°C as the optimal temperature for anaerobic fermentation of methanogens. A higher TDS can inhibit the growth of microorganisms. With increasing TDS, methane production gradually decreases. Some trace elements are indispensable elements in the process of microbial growth. Su et al. (2018) found that Fe and Ni are involved in the synthesis of several key enzymes in the metabolism of microorganisms. The producing layers of CBM wells in the study area are generally less than 1000 m deep. The average temperature range of the production layers that span the TC area is 30.87°C–35.9°C, and that in the EH area is 23.6°C–27.43°C. The TDS concentrations are generally 4500 mg/L in the Tucheng area and are lower in the Enhong area, with values of 1594 mg/L and 2237 mg/L in the two wells, respectively. High Fe and Ni contents were detected in the produced water. The geological conditions in the producing layers in TC and EH are suitable for survival of the methanogen community, so engineering tests of enhanced methane production by nutrient injection can be carried out.

6 Conclusions

In this study, produced water from 15 CBM wells of four synclines in eastern Yunnan and western Guizhou was taken as the research object. Through laboratory cultivation of the produced water, the bacterial and archaeal community structures in the water before and after cultivation were tested by 16S rRNA amplicon sequencing and realtime fluorescence quantitative PCR. Combined with the geochemical characteristics of the produced water and gas, the potential and feasibility of microbial-enhanced methane production were discussed. The following conclusions are drawn.

1) The bacterial DNA content in uncultured produced water was lower than the detection level, so it is difficult to detect. After enrichment, the dominant bacteria phyla were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and other low abundance bacteria were also detected.

2) A total of seven phyla were detected in the uncultured produced water of CBM wells, and the dominant archaeal phylum was *Euyarchaeota*, with an average relative abundance of 90.56%. Methanogens were the main component of archaea in each sample. The dominant archaeal genera were *Methanobacterium*, *Methanoculleus*, and *Methanobrevibacter*. The community structure of the archaea changed noticeably after enrichment culture. The relative abundance of *Euryarchaeota* increased to 99% in most samples after enrichment culture. Meanwhile, archaea grew rapidly, but the species diversity decreased. Using LefSe and Welch's *t*-test analysis, it was found that there was a transition from *Methanoregula* to *Methanobacterium* within genera. The relative abundances of many methanogens decreased, but the relative abundance of *Methanobacterium* increased, which can produce hydrogenotrophic methane.

3) Combined with the isotopic composition of the produced water and gas, it is considered that the CBM in the Tucheng and Enhong synlines consists of a mixture of thermogenic gas and biogas. The proportion of secondary biogas in the Tucheng area is estimated to range from 10.89% to 35.35%, and that in the Enhong area is estimated to range from 29.58% to 49.62%. There are mainly hydrogentrophic methanogens in the study area, and CO_2 reduction is the main method of microbial gas production. This provides a basis for microbial-enhanced methane production.

4) The CBM wells in the Tucheng and Enhong areas are classified as coking coal, with suitable reservoir temperatures, low TDS and rich trace element contents, which can provide a suitable living environment for methanogen growth. After enrichment culture of produced water in the study area, the hydrogenotrophic methanogens were enriched. These two areas have strong potential for microbial-enhanced methane production.

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