RESEARCH ARTICLE

The diversity of hydrogen-producing microorganisms in a high temperature oil reservoir and its potential role in promoting the *in situ* bioprocess

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Abstract: Hydrogen-producing microorganisms are believed to play an important role in energy metabolism of microorganisms in anaerobic environments and hence are one of the crucial factors for influencing the activity and development of these microorganisms. Consequently, they provide the biological foundation for the biotechnology such as MEOR (Microbial Enhanced Oil Recovery) and microbial fixation of CO_2 and conversion of it into CH_4 and *etc*. However, knowledge on the community of hydrogen-producing microorganisms and their potential in subsurface formations are still limited. In this study, hydrogen-producing microorganisms in the production water from an oilfield as well as enrichment cultures were analyzed with clone library analysis of *[FeFe]*-hydrogenase encoding genes. The results show that *[FeFe]*-hydrogenase genes in production water are diverse and related to *Bacteroidetes*, *Firmicutes*, *Spirochaetes* and uncultured. Anaerobic incubations established within the oil reservoir production water and generating 202 mmol H_2/mol glucose during 7-day incubation at 55°C indicate a high frequency of members of the *Firmicutes*. This study implies that hydrogen-producing microorganisms in oil reservoir may play a positive role in promoting the *in situ* bioprocess via hydrogen production once common nutrients are available. These data are helpful for evaluating, developing, and utilizing hydrogen-producing microorganisms in oil reservoirs for biological fixation and conversion of CO_2 into CH_4 as well as MEOR.

Keywords: microbial community, hydrogen-producing microorganisms, functional gene biomarker, enrichment culture, oil reservoir

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1. Introduction

Hydrogen plays a crucial role in affecting subsurface microbial community structure due to the fact that hydrogen occupies a central place in the energy metabolism of anaerobic microorganisms. Indeed, it is well accepted that hydrogen generated in subsurface environments could be of diverse origins including biogenic, thermogenic, and others. Biogenic formation of hydrogen in such deep environments is of paramount interest and a good knowledge of functional microorganisms responsible and/or involved in this for-

mation process is appealing. Petroleum reservoirs are typical subsurface environments, in which, hydrogen may also be produced by physiological distinct microorganisms dominated by anaerobes (including bacteria and maybe also archaea). Hydrogen produced by microorganisms *in situ* is believed to be the trophic and energy links between H_2-producers and consumers such as methanogens, sulfate- and nitrate-reducers as well as metals reducers, *etc*[1–4]. Study of these microorganisms of oil reservoirs may be useful in the development of practical technologies such as MEOR[5,6], microbially influenced corrosion control[7,8] as well as the most promising technology such as CO_2 fixation and bioconversion into methane[9,10].

The production and consumption of hydrogen are believed to be catalyzed by hydrogenases. Till now, hydrogenases are classified into three groups: *[NiFe]-*, *[FeFe]-* and *[Fe]*-hydrogenases[11]. *[NiFe]*-hydrogenases are considered to be mainly involved in H_2 consumption, *[FeFe]*-hydrogenases mainly in H_2 production and *[Fe]*-hydrogenases in H_2-forming. The latter are only found in some methanogens. For H_2 production, only *[FeFe]*-hydrogenases are of scientific and technological interest[12,13] and they have been used as specific biomarkers for investigation of H_2-producing microbial communities[9,14–17]. Recently, three sets of primers, i.e., hydF1/hydR1, 272F/FeFe-427R, and HydH1f/HydH3r, have been developed and used to analyze H_2-producing communities inhabited in anaerobic environments[14,16–19]. The obtained results have provided novel knowledge on the structures of H_2-producing communities in these various environments. However, till now, there are limited reports on the microbial communities that may be responsible and/or involved in the hydrogen formation process in oil reservoirs.

The present study was motivated by practical MEOR field applications, in which, carbohydrates (mainly glucose and/or sucrose) are commonly used as specific nutrients to stimulate the microbial community *in situ* for enhancement of oil recovery purposes. Injected nutrients such as glucose and/or sucrose may undergo *in situ* fermentation process with hydrogen, organic acids, CO_2, and methane as intermediate metabolites and end products. Here, we used primer sets specific to *[FeFe]*- hydrogenase genes to elucidate the diversity of functional microorganisms that may be implicated in hydrogen production in oil reservoir fluids. Our research revealed the high diversities of *[FeFe]*-hydrogenases in the oil reservoir, and the possibility of hydrogen producing microorganisms to be activated by nutrient addition to produce hydrogen which may play a positive role in the biotechnology such as MEOR as well as CO_2 bio- fixation and its conversion into CH_4 in oil reservoirs.

2. Materials and Methods

2.1 Sampling

Samples were collected from four producing wells in Huabei Oilfield (55°C). Each production water sample (about 5 L) was collected from the valve at the production well head and completely filled into a sterilized bottle. The bottles were tightly sealed and immediately transported to the laboratory. The physicochemical properties of these water samples are presented in Table 1.

Table 1. The physicochemical properties of water samples

Parameter	Huabei oilfield
Depth (m)	1450.0
Temperature (°C)	57.0
pH	7.2
Salinity (mg L^{-1})	5745.0
Water type	$NaHCO_3$
Formate (mg L^{-1})	ND
Acetate (mg L^{-1})	58.8
Propionate (mg L^{-1})	ND
Butyrate (mg L^{-1})	3.1
Iso-butyrate (mg L^{-1})	ND

ND: Not Detectable

2.2 DNA Extraction

Microbial cells in the water samples or enrichment culture was collected by filtration with a 0.2 μm membrane filters as described by Wang *et al*.[20] Cells obtained from 2.0 L of production water sample were used for the extraction of total genomic DNA using AxyPrep™ Bacterial Genomic DNA Miniprep Kit according to the protocol provided by manufacturer. The afterward purification of genomic DNAs were performed with a DNA purification kit following the manufacturer's instructions. The purified DNAs were stored at −20°C before the PCR amplification of functional genes.

2.3 PCR Amplification

The amplification of a fragment of a *[FeFe]*-hydrogenase-encoding gene was conducted with the primer set of 272F (5′-GCHGAYMTBACHATWATGGARGA-3′) and 427R (5′-GCNGCYTCCATDACDCCDCCNGT-3′) under the conditions described by Schmidt *et al*.[16]. Five parallel PCR reactions were performed for functional gene fragments in a Peltier thermal cycler (Bio-Rad, USA), and the obtained PCR products were subsequently pooled for cloning exercises and construction of the respective gene libraries.

2.4 Construction of Functional Gene Clone Libraries

The PCR products were first purified with the Gel Ex-

traction Kit (U-gene, China), and then it was cloned into *Escherichia coli* using a pMD19®-T simple vector kit (Takara, Japan) according to the manufacturer's instructions. For each gene clone library, white colonies were picked randomly and inoculated to Luria broth (LB) medium supplemented with ampicillin (50 mg L^{-1}) before incubation for overnight at 37°C. The inserted DNAs were verified by amplification with primers of M13-47 and RV-M and following agarose gel electrophoresis with ethidium bromide staining[21].

2.5 Sequencing and Phylogenetic Analyses

An ABI 377 automated sequencer was used for sequencing. After removing the vector, sequences were checked with Bellerophon for identification of putative chimera[22] before the assemblage of OTUs (operational taxonomic units) at similarity of 97% using FastGroupII[23]. From each OTU, one representative sequence was chosen to compare with sequences in the BLAST network service[24]. Phylogenetic trees were generated using MEGA5 software[25]. The phylogenetic tree was generated by neighbor-joining and the Poisson correction method[26]. The nodes in the tree were estimated by 1000 bootstrap replicates.

2.6 Incubation

Enrichment cultures were conducted with 120 mL serum bottles containing 60 mL of basal medium, 6 mL of the production water, 1.0 mL of trace element, and 1.0 mL of vitamin stock solution. The basal medium contained (g L^{-1}): Glucose 10.0, KH_2PO_4 1.6, K_2HPO_4 1.0, NH_4Cl 0.5, $MgCl_2 \cdot 6H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.02, $NaHCO_3$ 1.0, NaCl 0.5, Na_2S 0.6, Yeast extract 2.0 and Peptone 1.0. Trace element and vitamin stocks solutions are the same as descripted by Wang *et al*[27]. The incubation was performed in triplicate at 55°C. The produced gas was analyzed and the average of the test triplicates was obtained as the final results.

Headspace gas in serum bottles was analyzed by a gas chromatograph equipped with a 1.5 m column filled with 5 Å carbon molecular sieves. The temperature of the injector and detector were set at 200°C, and that of the column was program-controlled as: 60°C for 12 min, increment of temperature from 60 to 200°C at a rate of 15°C/min and finally kept at 200°C for 24 min.

2.7 Nucleotide Sequence Accession Numbers

The data of gene sequences for *[FeFe]*-hydrogenase are available in GenBank sequence database with the accession numbers KT955908~KT955977 for production water and KT964905~KT964998 for enrichment culture.

3. Results

3.1 The Diversity of Hydrogenase Encoding Genes

The *[FeFe]*-hydrogenase encoding genes were analyzed for the original production water samples. The phylogenetic affiliation based on the protein sequences derived from these genes is presented in Table 2 and the corresponding

Table 2. Phylogenic affiliation of hydrogen producing bacteria based on the protein sequence derived from *[FeFe]*-hydrogenase encoding gene

OTU	Accession No	Most closely related sequence (NCBI Blast)	Accession no (NCBI Blast)	Similarity (%)
HB-56	KT955963	Yellowstone geothermal spring clone	ADC53605	100
HB-47	KT955954	*Bacteroidales_bacterium*_enrichment	AHX56817	77
HB-48	KT955955	*Bacteroidales_bacterium*	WP_025310303	81
HB-70	KT955977	*Bacteroidales_bacterium*	WP_025310303	76
HB-49	KT955956	Subsurface aquifer sediment	EKD30691	87
HB-43	KT955950	*Bacteroidales_bacterium*	GAP42690	90
HB-41	KT955948	Saline pond clone	ACM67619	91
HB-27	KT955934	*Bacteroidales_bacterium*	GAP70735	96
HB-40	KT955947	Phreatic limestone sinkholes clone	ACQ94954	90
HB-12	KT955919	*Proteiniphilum_acetatigenes*	WP_019541397	98
HB-1	KT955908	*Proteiniphilum_acetatigenes*	WP_019541397	91
HB-16	KT955923	*Alistipes*_sp.	CCY35044	83
HB-68	KT955975	Oil reservoir clone	AHL26948	94
HB-22	KT955929	*Desulfotomaculum_gibsoniae*	AGL03894	74
HB-23	KT955930	*Proteiniclasticum ruminis*	WP_031576066	86
HB-51	KT955958	Oil reservoir clone	AHL26938	98
HB-54	KT955961	Oil reservoir clone	AHL26938	97
HB-55	KT955962	*Holdemania_filiformis*	EEF67834	70

NCBI blast was accessed on Oct. 2015

phylogenetic tree constructed at the protein level is shown in Figure 1.

From the sequence of 70 clones retrieved from the production water sample, 18 OTUs were identified using FastGroupII. The OTU HB-56 shares similarities with sequence from *Treponema caldarium* within *Spirochaetes*. OTU HB-47, HB-48, HB-70, HB-49, HB-43, and HB-27 were similar to sequence from members of the order of *Bacteroidetes*. The OTU HB-12 and HB-1 share high similarity with *Proteiniphilum acetatigenes*, and HB-16 with high similarity to *Alistipes sp*, all of which are affiliated to the *Bacteroidales*. The OTU HB-22, HB-23, and HB-55 share high similarity with *Desulfotomaculum gibsoniae*, *Proteiniclasticum ruminis*, and *Clostridium spiroforme*, respectively, are all affiliated to the *Firmicutes*. The dominant OTUs, HB-1 (20%) and HB-12 (10%), are affiliated to *Proteiniphilum acetatigenes* (with similarity values of 91% and 98%, respectively) within the *Bacteroidetes*. The latter was originally isolated from waste water of beer production with a characteristical production of acetate from protein, amino acids, and pyruvate[28]. The OTU HB-27 (20%) shares high similarity with genes extracted from microorganisms in rice soil[19]. Obviously, these sequences are affiliated mainly to three different phyla of *Spirochaetes*, *Bacteroidetes*, and *Firmicutes*, which showed great diversity of hydrogen-producing microorganisms. Our results are in agreement with those obtained by Grabowski, who found that the main hydrogen-producing microorganisms in the enrichment cultures of samples

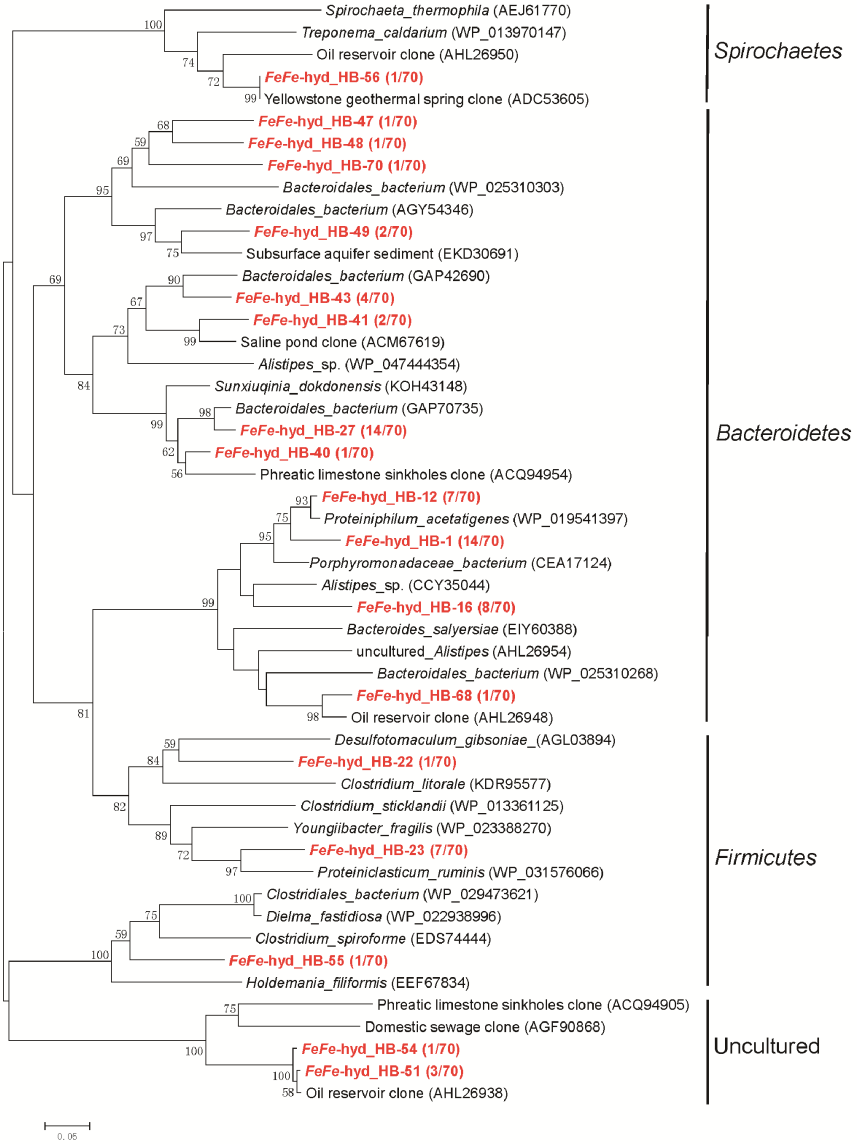

Figure 1. Neighbor-joining tree of *[FeFe]-Hydrogenase* gene amplified from DNA extracted from oil reservoir samples (red) and related gene sequences from GenBank. Bootstraps below 50% are not shown. 5% amino acid substitution is shown on the scale bar.

from low temperature oil reservoirs are affiliated with *Spirochaetes*, *Bacteroidetes*, and *Firmicutes*[29].

3.2 Biohydrogen Production

The microorganisms inhabiting in the production water from Huabei oil reservoir were incubated anaerobically with basal medium at 55°C and hydrogen production at different incubation periods was monitored. The results are shown in Figure 2. The yield of hydrogen reached its maximum value (about 11 mmol H_2/L medium or 202 mmol H_2/mol glucose) at the 7th incubation day and then decreased. On the other hand, methane was produced from the 7th day and increased with incubation time.

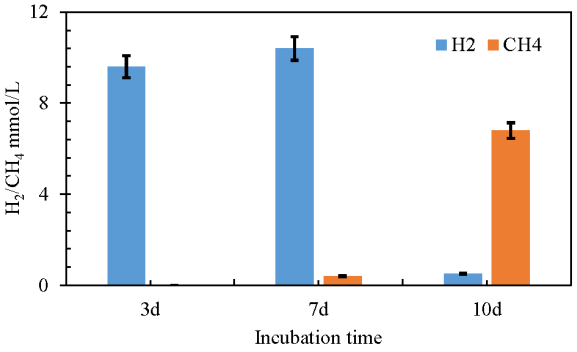

Figure 2. The hydrogen and methane production by enrichment culture with production water sample at 55°C.

3.3 Hydrogen-producing Bacterial Community from Enrichment Culture

The *[FeFe]*-hydrogenase encoding genes were analyzed for the enrichment sample incubated at 55°C and the phylogenetic affiliation of the hydrogen-producing bacteria is listed in Table 3. The corresponding phylogenetic tree was constructed at the protein level and is shown in Figure 3.

Table 3. Phylogenic affiliation of hydrogen producing bacteria based on the protein sequence derived from *[FeFe]*-hydrogenase encoding gene

OTU	Accession no	Most closely related sequence (NCBI Blast)	Accession no (NCBI Blast)	Similarity (%)
Enrichment-94	KT964998	*Proteiniphilum_acetatigenes*	WP_019541397	87
Enrichment-01	KT964905	*Clostridium*_sp.	AIC77297	97
Enrichment-48	KT964952	*Clostridium_sticklandii*	WP_013361125	99
Enrichment-59	KT964963	*Proteiniclasticum_ruminis*	WP_031576066	88
Enrichment-76	KT964980	*Clostridium_aceticum*	WP_044826304	76
Enrichment-91	KT964995	*Dielma_fastidiosa*	WP_022938996	77

NCBI blast was accessed on Oct. 2015

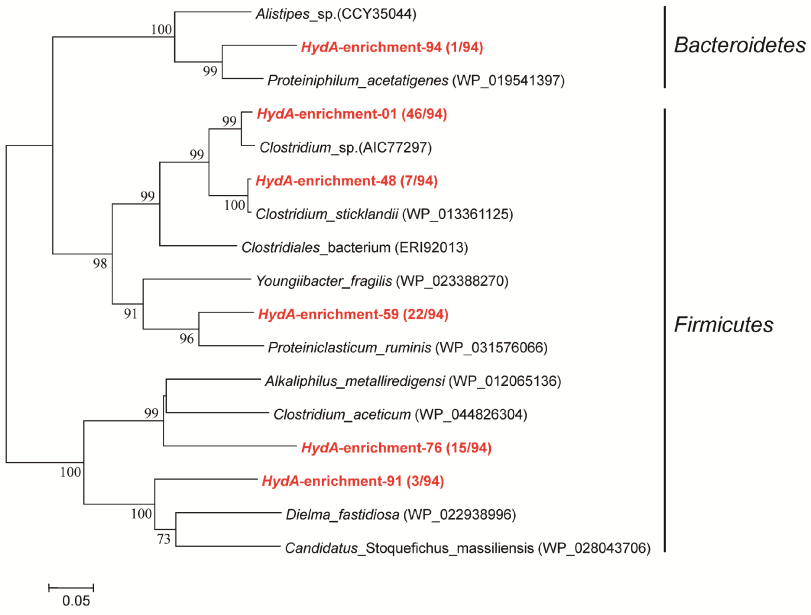

Figure 3. Neighbor-joining tree of *[FeFe]-Hydrogenase* gene amplified from DNA extracted from the enrichment culture at 55°C (red) and related gene sequences from GenBank. Bootstraps below 50% are not shown. 10% amino acid substitution is shown on the scale bar.

We randomly selected 94 clones and grouped into 6 OTUs after sequencing. Clones represented by enrichment-01 (46 clones), enrichment-48 (7 clones), enrichment-59 (22 clones), enrichment-76 (15 clones) and enrichment-91 (3 clones) share high similarity with *Clostridium sp.*, *Clostridium sticklandii*, *Proteiniphilum acetatigenes*, *Alkaliphilus_metalliredigensi* and *Dielma_fastidiosa*, respectively, which are all within phylum *Firmicutes*. Only one OTU, enrichment-94 (1 clone), was classified as *Proteiniphilum acetatigenes* within the *Bacteroidetes*.

4. Discussion

4.1 The Diversity of Hydrogen-producing Bacteria in Oil Reservoir

The hydrogen-producing microorganisms in original production water samples were diverse and included *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, and uncultured microorganisms. The enrichment culture was less diverse and composed only by microorganisms affiliated with the *Firmicutes* and *Bacteroidetes*. Members of the *Bacteroidetes* are frequently found among hydrogen producing microorganisms[30,31]. A novel member of the family *Porphyromonadaceae*, designated as strain ING2-E5B, was found by complete genome sequencing to possess a *[FeFe]*-hydrogenase encoding gene. This indicates that this strain may be involved in hydrogen production[32]. In our study, *Clostridium* was the most encountered microorganism in the enrichment culture and thus, might play a considerable role in H_2 production even though it was negligible in the original production water sample. A recent study showed that *Clostridium* inhabited in production water of Daqing oilfield subjected to CO_2- and water-flooding with a frequency of 38% and 6% respectively[9]. Also, members of *Clostridia* showed the highest abundant microorganisms in microbial mat, H_2-producing sludge, and marine geothermal environment[14,15,33,34]. Our results are in good agreement with those of previous studies conducted in oil reservoirs or similar subsurface environments.

The present study shows that the diversity of *[FeFe]*-hydrogenase encoding genes extracted from the enrichment cultures is quite different from those of the original production water samples. Members of the *Firmicutes*, mainly *Clostridia*, were predominant in the enrichment cultures with a relative proportion of 72% whereas *Proteiniphilum* majored in original production waters with relative proportion of 30%. The same phenomenon was also found under both thermophilic and mesophilic culture conditions[30,35,36]. The significant shift in the microbial community structure from *Bacteroidetes* dominant in original production water samples to *Firmicutes* dominant in enrichment culture may be caused by the difference in carbon source available as it is hydrocarbon or its intermediary metabolites in oil reservoir rather than glucose or its intermediary metabolites in the enrichment culture.

Petroleum reservoirs, with unique physico-chemical conditions, are inhabited by taxonomically, physiologically, and phylogenetically unusual microorganisms[9,21,27,37,38]. As for hydrogen production, different members within mainly four phyla have been intensively investigated, such as *Oceanotoga*[39], *Petrotoga*[40–43], *Thermotoga*[44–46] within *Thermotogae*; *Thermoanaerobacter*[47–49] within *Firmicute*; *Spirochaeta*[50] within *Spirochaeta*; and *Anaerobaculum*[51] within *Synergistetes*. Till now, the most frequently encountered hydrogen-producing bacteria in oil reservoirs are members within *Thermotoga* and *Firmicute*. The present study shows that sequences obtained in clone libraries constructed for *[FeFe]*–hydrogenase encoding gene are of high similarities with those from *Firmicutes* and *Bacteroidetes*. Our results are consistent with those mentioned above.

4.2 Investigation of Hydrogen-producing Bacterial Community

Among the three major groups of hydrogenases: *[NiFe]*-, *[FeFe]*-, and *[Fe]*-[52], *[FeFe]*-hydrogenase are often found to be involved in hydrogen production[11] and the genes encoding *[FeFe]*-hydrogenase have been used as specific biomarkers for hydrogen-producing bacteria[9,14–17,33]. In addition, only *[FeFe]*-hydrogenase is believed to be scientifically and technologically important for H_2 production[12,13]. This is the first time to use specific primer sets to investigate hydrogen-producing microbial community in production water sample from oil reservoir. Our results imply that this primer is suitable and effective for investigating hydrogen-producing microorganisms in oil reservoirs.

It is important to point out that the relative proportion of hydrogen-producing microorganisms obtained from the clone libraries of hydrogenase genes is only of referential value, because multiple hydrogenases may be present in given microbial genomes[11].

4.3 The Potential Role of Hydrogen-producing Microorganisms in Oil Reservoirs

As for the hydrogen energy production, the present research shows that hydrogen-producing microorganisms occur in oil reservoirs with great diversity. Also, in our enrichment cultures, *Clostridia* were identified to be the dominating H_2-producing bacteria. The *Clostridia* are the most widely studied microorganisms for hydrogen production because of their high H_2 yield[53,54] and high rate of H_2 production[55]. The potential of *Clostridia* for hydrogen production have been widely and systematically

studied[56]. Members of the *Firmicutes* and the *Bacteroidetes* are of high potential once they are activated with proper nutrients to produce hydrogen in oil reservoirs.

Moreover, our results indicate that methane was produced in case the hydrogen content reached a certain concentration. This implies that methanogens inhabiting the production water samples were activated. Microbial conversion of CO_2 into methane in oil reservoirs is believed to be one of the most promising solutions for a mitigation of CO_2 emissions. Positive results have been obtained demonstrating that hydrogen-producing bacteria and hydrogenotrophic methanogenic archaea were of great significance with respect to microbial conversion of CO_2 into methane in oil reservoirs[57]. Also, this bioprocess using enrichment cultures has been studied[58]. The present data implies that the hydrogen producing microorganisms may play a positive role in promoting methanogenesis process such as microbial conversion of CO_2 into methane.

In case of MEOR, hydrogen-producing microorganisms may play a significant role in stimulating the functional microorganisms. Successful field pilot tests have been conducted by injection of nutrients containing glucose[59], molasses[60,61] and sucrose[62] which can be metabolized by fermentative bacteria for hydrogen production.

5. Conclusion

Hydrogen-producing bacterial community in the production water from an oil reservoir as well as its enrichment cultures were analyzed by means of functional gene approach. The results showed that *[FeFe]*-hydrogenase genes were of great diversity which mainly related to members of the *Bacteroidetes*, the *Firmicutes*, and the *Spirochaetes* in production water but was dominated only by the *Firmicute* in anaerobic enrichment culture. Our results revealed the members of hydrogen- producing bacteria and its positive role in promoting bioprocess in the oil reservoir such as MEOR and biogenic methane production which are helpful in evaluating, developing, and utilizing hydrogen-producing microorganisms in oil reservoirs.

Author Contributions

Liu J-F, Gu J-D, and Mu B-Z designed the full experiments. Ke W-J and Mbadinga S M conducted the microbial and chemical analysis. Liu J-F prepared the manuscript with the help of all co-authors.

Conflict of Interest and Funding

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