微生物学通报
Microbiology China
tongbao@im.ac.cn

Jul. 20, 2017, 44(7): 1563−1578
http://journals.im.ac.cn/wswxtbcn
DOI: 10.13344/j.microbiol.china.170084

研究报告

Acetate production from H_2/CO_2 by mixed cultures from diverse ecosystems and their application for syngas fermentation

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Abstract: [Objective] We studied acetate production from H_2/CO_2 by mixed cultures from diverse ecosystems and applied the system for syngas fermentation. **[Methods]** Undefined mixed cultures from waste activate sludge, freshwater sediment, anaerobic methanogenic sludge and cow manure were used for the bioconversion of H_2/CO_2 and enriched H_2/CO_2-converting cultures from cow manure were applied as inoculums for syngas fermentation. Gas and volatile fatty acids contents were measured by a gas chromatograph. We used 454 pyrosequencing and qPCR assay to reveal the community structure during the bioconversion. **[Results]** Acetate, ethanol and butyrate accumulated in cow manure and waste activated sludge during the incubation under H_2/CO_2 headspace with final acetate concentrations of approximately 63 mmol/L and 40 mmol/L, respectively, significantly higher than that of 3 mmol/L and 16 mmol/L in freshwater sediment and anaerobic methanogenic sludge. Diverse types of acetogens were found in waste activated sludge and cow manure. The major putative acetogens belonged to the species *Clostridium* spp., *Sporomusa malonica* and *Acetoanaerobium noterae* in waste activated sludge, and to *Clostridium* spp., *Treponema azotonutricium* and *Oxobacter pfennigii* in cow manure. **[Conclusion]** Both the richness and the number of acetogens were important factors for the bioconversion of H_2/CO_2 by mixed cultures. Enriched H_2/CO_2-converting cultures can use syngas as carbon source to produce acetate and ethanol.

Keywords: Actate, Acetogen, H_2/CO_2, Microbial community, Syngas fermentation

Foundation item: National Natural Science Foundation of China (No. 21206056); Natural Science Foundation of Jiangsu Province (No. BK2012121, BK20141112); CAS Key Laboratory of Bio-Based Materials (No. KLBM2016007)

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Received: January 31, 2017; **Accepted:** April 07, 2017; **Published online** (www.cnki.net)**:** April 20, 2017

基金项目：国家自然科学基金项目(No. 21206056)；江苏省自然科学基金项目(No. BK2012121，BK20141112)；中国科学院生物基材料重点实验室开放基金项目(No. KLBM2016007)

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收稿日期：2017-01-31；接受日期：2017-04-07；优先数字出版日期(www.cnki.net)：2017-04-20

不同生境微生物转化 H_2/CO_2 产乙酸及其在合成气发酵中应用

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摘 要:【目的】合成气发酵对大力开发可再生资源和促进国家可持续发展具有重要意义，研究旨在探究不同生境微生物转化 H_2/CO_2 产乙酸及其合成气发酵的潜力。【方法】采集剩余污泥、牛粪、产甲烷污泥和河道底物样品在中温(37 °C)条件下生物转化 H_2/CO_2 气体，将来源于牛粪样品的 H_2/CO_2 转化富集物用于合成气发酵，通过 454 高通量技术和定量 PCR 技术分析复杂微生物群落的组成，GC 气相色谱法检测气体转化产生的挥发性脂肪酸(VFAs)浓度。【结果】牛粪和剩余污泥微生物利用 H_2/CO_2 气体生成乙酸、乙醇和丁酸等，最高乙酸浓度分别为 63 mmol/L 和 40 mmol/L，明显高于河道底物和产甲烷污泥样品的最高乙酸浓度 3 mmol/L 和 16 mmol/L。牛粪和剩余污泥微生物中含有种类多样化的同型产乙酸菌，剩余污泥中同型产乙酸菌主要为 *Clostridium* spp.、*Sporomusa malonica* 和 *Acetoanaerobium noterae*，牛粪中则为 *Clostridium* spp.、*Treponema azotonutricium* 和 *Oxobacter pfennigii*。【结论】同型产乙酸菌的丰富度和数量两个因素都对复杂微生物群落转化 H_2/CO_2 产乙酸效率至关重要；转化 H_2/CO_2 得到的富集物可用于合成气发酵产乙酸和乙醇，这为基于混合培养技术的合成气发酵提供了依据。

关键词: 乙酸，同型产乙酸菌，H_2/CO_2，微生物群落，合成气发酵

1 Introduction

Syn(thesis) gas (primarily contains CO, H_2 and CO_2) has been used as a major feedstock in production of fuels and chemicals. Several laboratory-scale studies focused on acetate or ethanol production from syngas by pure cultures were done[1-2]. Mixed cultures biotechnology could become a more attractive addition or alternative to the traditional pure culture based approach[3] due to its several advantages as no sterilization requirements, more adaptive capacity under various conditions due to microbial diversity, and the possibility of a continuous process implementation[4]. Undefined mixed cultures from raw sludge and anaerobic granular sludge were used for fermentative CO conversion[5]. Singla et al.[6] enriched anaerobic mixed cultures for conversion of syngas to ethanol. H_2/CO_2 are essential syngas components and are also the products of fermentative CO conversion, however, little attention was paid on the conversion of H_2/CO_2 by mixed cultures approaches.

H_2/CO_2 can be biologically converted into chemicals and/or bioenergy such as acetate and ethanol by acetogens[7]. Ryan et al. enriched acetogens by supplying a variety of acetogenic growth substrates to two laboratory-scale high rate upflow anaerobic methanogenic sludge bed reactors operated at 37 °C and 55 °C[8]. Alves et al. reported homoacetogenic communities enriched with a syngas mixture from thermophilic anaerobic sludge[9]. Our previous studies have shown different abundances of acetogens in different natural and engineered environmental samples[10]. However, in these studies, the potential of the undefined mixed cultures from diverse inoculums in bioconversion of H_2/CO_2 and its application in syngas fermentation was not studied. Furthermore, little information was given about the microbial composition of undefined mixed cultures from diverse inoculum types during the bioconversion of H_2/CO_2.

In the present study, the undefined mixed cultures from waste activated sludge, freshwater sediment, anaerobic methanogenic sludge and cow manure were used for H_2/CO_2 bioconversion. Formyltetrahydrofolate synthetase gene (*fhs*)-specific Real-time quantification PCR (qPCR) assay and Terminal restriction fragment length polymorphism (T-RFLP) analysis, 16S rRNA gene based 454 high-throughput pyrosequencing were also introduced to determine the diversity of the bacterial and acetogenic community during the incubation under H_2/CO_2. Anaerobic mixed culture obtained from cow manure were applied as inoculum

for syngas fermentation.

2 Materials and Methods

2.1 Original samples

Waste activated sludge, freshwater sediment, anaerobic methanogenic sludge and cow manure samples were collected from dewatered sludge of a municipal wastewater treatment plant (Wuxi, China), Lihu Lake (Wuxi, China), a kitchen waste anaerobic digestion reactor (Suzhou, China) and a cattle farm (Wuxi, China), respectively. Basic characteristics of original samples were summarized in Table 1.

2.2 Experimental setup and operation

H_2/CO_2-incubation experiments were conducted in 1 000 mL serum bottles containing 500 mL culture medium[11] and 200 g of original samples. The culture medium consisted of the following basal salts (mmol/L): NH_4Cl 9.35, KH_2PO_4 1.84, $K_2HPO_4{\cdot}3H_2O$ 1.10, $MgCl_2{\cdot}6H_2O$ 1.48, $FeCl_3{\cdot}6H_2O$ 0.09, $NiSO_4{\cdot}6H_2O$ 0.06, $CaCl_2$ 0.23, $ZnCl_2$ 0.08, $CoCl{\cdot}6H_2O$ 0.04, $CuCl_2{\cdot}2H_2O$ 0.03, and $MnCl_2{\cdot}4H_2O$ 0.08. Oxygen was removed by purging the bottles with nitrogen gas for 5 minutes and 2-bromoethanesulfonate (50 mmol/L) was added to inhibit methanogenesis during incubation. H_2/CO_2 gas (4:1, *V/V*) (18.0 mmol/L H_2 and 4.4 mmol/L CO_2) was purged into headspace every day for substrates. The cultures were incubated at 37±1 °C for 30 days. All experiments were carried out in duplicate.

The optimization of trace metal ions and cysteine sulfide for enhanced ethanol production from H_2/CO_2 was conducted in 100 mL fermentation reactors with 50 mL liquid medium. The gas substrate containing CO_2/H_2 (80/20, *V/V*) was flushed daily into the reactor through an inlet steel needle to one standard atmosphere and oxygen was eliminated through an outlet steel needle. The experiments were carried out at 37 °C. The concentration of metal ions were 0, 20.4, 204 μmol/L Fe^{2+} and 0, 6.96, 69.60 μmol/L Zn^{2+}, respectively, while the concentration of cysteine sulfide were 0, 0.5, 1.0, 2.0 g/L. The control group was flushed with N_2 to one standard atmosphere.

The syngas fermentation experiment was conducted in 250 mL fermentation reactors with 100 mL liquid medium. The gas substrate containing CO_2/H_2 (80/20, *V/V*) or $CO/CO_2/H_2$ (40/30/30, *V/V/V*) was flushed daily into the reactor through an inlet steel needle to one standard atmosphere. Oxygen was eliminated through an outlet steel needle. Syngas fermentation experiment was carried out at 37 °C. 5 mol/L NaOH and 3 mol/L H_3PO_4 were used to adjust pH at 7.0. The control group was flushed with N_2 to one standard atmosphere.

2.3 Chemical analysis methods

pH values were measured by a pH meter (METTLER FE 20, Shanghai), chemical oxygen demand (COD), total solids (TS) and volatile solids (VS) were determined according to standard methods described by APHA[12]. Polysaccharides were quantified by phenol-sulfuric acid method using glucose as standard[13] and volatile fatty acids (VFAs) contents were measured every 3 days by a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with an auto injector (AOC-20i, Shimadzu) using a fused-silica capillary column (PEG-20M, 30 m × 0.32 mm×0.5 μm, China) as described by Liu et al.[14]. The VFA samples were filtrated through a 0.45 μm membrane before measurement. CO_2 and H_2 gas samples (0.05 mL) were taken with a gas-tight pressure lock syringe (Shimadzu, Japan) and quantified by gas chromatography (GC-2010, Shimadzu, Japan) equipped with a packed column Porapak Q (50/80 mesh) and a thermal conductivity detector (Shimadzu, Japan)[15].

Table 1 Basic characteristics of original seed used in the experiment
表 1 初始接种物的基本性质

Origin sources 初始样品	pH	TS (%)	VS (%)	COD (mg/L)	Protein (mg/L)	Polysaccharide (mg/L)
Waste activated sludge 剩余活性污泥	7.7±0.0	21.0±0.8	53.0±0.4	1 977.0±5.0	740.0±3.70	453.0±2.3
Freshwater sediment 淡水河道底泥	6.2±0.0	60.0±1.0	5.0±0.1	108.0±3.5	17.0±0.90	35.0±0.2
Anaerobic methanogenic sludge 厌氧产甲烷污泥	7.2±0.0	6.0±0.4	42.0±0.6	5 229.0±6.4	374.0±1.92	72.0±1.4
Cow manure 牛粪	7.6±0.0	18.0±0.6	81.0±0.8	3 469.0±5.7	760.0±3.80	308.0±1.5

2.4 DNA extraction

2 mL of culture samples were collected every 6 days and centrifuged at 10 000×*g* for 20 min at 4 °C. Total DNA was then extracted by MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions. DNA samples were assayed spectrophotometrically by a NanoDrop-2000 system (Thermo Scientific, USA). DNA samples were stored at −20 °C prior to use.

2.5 qPCR assay

Formyltetrahydrofolate synthetase *fhs* gene was chosen as an indicator for the abundance of acetogens during incubation. Copy numbers of *fhs* gene were measured by qPCR assay according to the description by Xu et al.[10].

Bacteria 16S rRNA genes were amplified by qPCR using forward primer 519F and reverse primer 907R for quantification of total bacteria. Each 25 μL reaction mixture contained 12.5 μL QuantiFast SYBR Green PCR Master Mix (2×), 0.25 μL of each primer (10 μmol/L), 2 μL of DNA sample and 10 μL purified water to complete the final volume. Amplification was carried out with a program consisting of an initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and a final elongation cycle at 72 °C for 5 min.

qPCR assay was performed using a Rotor-Gene Q system (QIAGEN, Germany). All the PCR assays were carried out using the QuantiFast SYBR Green PCR Kit (QIAGEN, Germany) and reactions were modified based on the instructions of the manufacturer.

2.6 Terminal restriction fragment length polymorphism (T-RFLP) profiling

Amplification of the 16S rRNA gene and selection of enzymes for the T-RFLP were performed in accordance with Xu et al.[15]. Bacteria 16S rRNA genes were amplified by qPCR using FAM labeled primer 27F and 1401R. Purified PCR products (10 μL) were digested with 1 μL of restriction enzyme *Msp* I (TaKaRa, Japan) in a total volume of 20 μL for 3 h at 37 °C followed by 10 min at 65 °C. The digested products were analyzed by Shanghai GeneCore BioTechnologies Co., Ltd. T-RFLP profiles were analyzed on the basis of the peak size and area. Terminal restriction fragments (T-RFs) that differed by ±1 bp in different profiles were considered as identical. Relative abundance of each T-RF was the proportion of that single T-RF's peak area of all T-RFs' total peak area within one sample and the mean of the relative abundances of T-RFs are the results presented.

The Shannon-Wiener index (H') of TRFLP-based species diversity was calculated by the following equation:

$$H' = -\sum_{i=1}^{s} P_i \ln P_i$$

Where P_i represents the proportional area of T-RF i in the sum of peak areas in a given T-RFLP and i is the number of T-RFs of each T-RFLP pattern and s is the total number of T-RFs. The evenness (E) of TRFLP-based species diversity was calculated by the following equation:

$$E=H'/H'_{max},\ H'_{max}=\ln S$$

t test was performed using Microsoft Excel 2007, and the significance level (P) was determined.

2.7 16S rRNA gene based-454 pyrosequencing

The primers 515F (5′-GTGCCAGCMGCCGCG GTAA-3′) and 926R (5′-CCGTCAATTYYTTTRAG TTT-3′) were used to amplify approximately 412 bp of variable regions V4 to V5 of bacterial 16S rRNA genes. 454 pyrosequencing was accomplished with a Genome Sequencer FLX system (Shanghai Hanyu Bio-Tech Co., Ltd.). All 16S rRNA genes pyrosequencing reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) according to protocols described in 454 overview tutorial: de novo OTU picking and diversity analyses using 454 data (http://qiime.org/tutorials/tutorial.html#picking–operational-taxonomic-units-otus-through-making–out-table)[16]. Barcode 16S rRNA gene sequences and information of primers were imported to filter different reads according to Phred quality scores. A minimum sequence length of 300 bp (no "N" was included in a sequence) and a minimum average quality score of 20 were selected as quality criteria. Sequence reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UCLUST and longest sequence in a cluster was used as the representative sequence for that OTU. Then the RDP classifier was applied to assign taxonomic data to each representative sequence[17]. Bacterial taxonomic classifications of our sequences were obtained by BLAST searching against the SILVA database[18]. Sequences were aligned against the Greengenes

database[19] using Python Nearest Alignment Space Termination (PyNAST) tool[20].

For all bacterial OTUs at the family level, sequences belonged to the same family were considered as a group, and the sum of percentage of these sequences were considered to represent the relative abundance of that unique group. The 16S rRNA gene sequence analysis was carried out using the BLAST search program[21] against the GenBank and RDPII database[22]. The 16S rRNA gene sequences of OTU#174, OTU#235, OTU#279, OTU#832, OTU#1179, OTU#2347, OTU#2439 and OTU#2848 were submitted to the GenBank and the accession numbers are from KJ125514 to KJ125521. The phylogenetic analysis was conducted using MEGA version 5.0[23].

3 Results

3.1 VFAs production during H_2/CO_2-incubation

Acetate, ethanol and butyrate are the main VFAs produced, with the four inocula showing different trends of acetate accumulation during the incubation (Figure 1A). Acetate accumulated in cow manure and waste activated sludge with final concentrations of approximately 63 mmol/L and 40 mmol/L, respectively, which were significantly higher than that of 3 mmol/L and 16 mmol/L in freshwater sediment and anaerobic methanogenic sludge. Acetogenic populations in freshwater sediment and anaerobic methanogenic sludge seemed to poorly develop using H_2/CO_2 as substrates.

Ethanol productions from cow manure and waste activated sludge samples reached the highest level (around 8 mmol/L) before Day 3, while those from the other two groups were below detection limit (Figure 1B, E). Butyrate accumulations in waste activated sludge samples were concomitant with acetate accumulations. Butyrate concentration reached the highest level of 13 mmol/L on Day 9 and remained constant until Day 21, decreasing to about 8.6 mmol/L afterwards. In the meantime, acetate concentrations increased slightly (Figure 1A, C). In addition, butyrate accumulation or decline was not observed in cow manure samples grown on H_2/CO_2. The butyrate concentration in cow manure samples maintained at 6−8 mmol/L after 3 days of rapid accumulation (Figure 1C, F).

Substrate consumption during incubation was also monitored. As shown in Figure 1, hydrogen and CO_2 consumption (Figure 1D, F) were in accordance with the VFA accumulation profile (Figure 1A−C). Substrates consumption by waste activated sludge and cow manure inocula were much greater than that of the other two groups. Substrates utilization rate increased initially but decreased significantly after 6 days of incubation. Freshwater sediment samples showed faster hydrogen consumption than anaerobic methanogenic sludge, and their CO_2 consumption trends were similar. The trends of substrates consumption were highly alike with the products accumulation.

3.2 Abundances of acetogenic *fhs* gene during H_2/CO_2-incubation

The copy numbers of *fhs* genes and 16S rRNA genes were quantified by qPCR approach to measure the abundance of acetogenic bacteria and total bacteria. The initial copy numbers of acetogenic *fhs* gene in the original samples were $(7.39\pm1.81)\times10^5$, $(1.28\pm1.04)\times10^5$, $(6.44\pm0.72)\times10^6$ and $(3.14\pm0.48)\times10^5$ copies/g dry weight (DW) for waste activated sludge, freshwater sediment, anaerobic methanogenic sludge and cow manure, respectively. The quantities of *fhs* genes in these samples were similar to our previous study[10]. The quantities of *fhs* gene copy numbers in freshwater sediment and cow manure samples increased about 17 and 29 times at the end of incubation (Figure 2A), while the acetogenic proportions in total bacteria of these two groups reached 5.47%±0.12% and 4.69%±0.32%, which were approximately 5 and 10 times higher than that of the original samples (Figure 2B). The highest copy number of *fhs* genes occurred in anaerobic methanogenic sludge with $(1.95\pm0.02)\times10^7$ copies/g DW (Figure 2A), but only about 3-fold increase of acetogens. The final copy numbers of *fhs* gene in the waste activated sludge was $(5.10\pm0.58)\times10^6$ copies/g DW (Figure 2A). Furthermore, the acetogenic proportions in total bacteria in waste activated sludge and anaerobic methanogenic sludge incubations increased only around 3.2 fold after 30 days (Figure 2B). Considering that acetogenic proportion in total bacteria are about 0.6% to 0.9% in most natural anaerobic environments, this abundance was the upper detection limit of acetogenic detection using Most Probable Numeration as indicated by Harriott and Frazer[24].

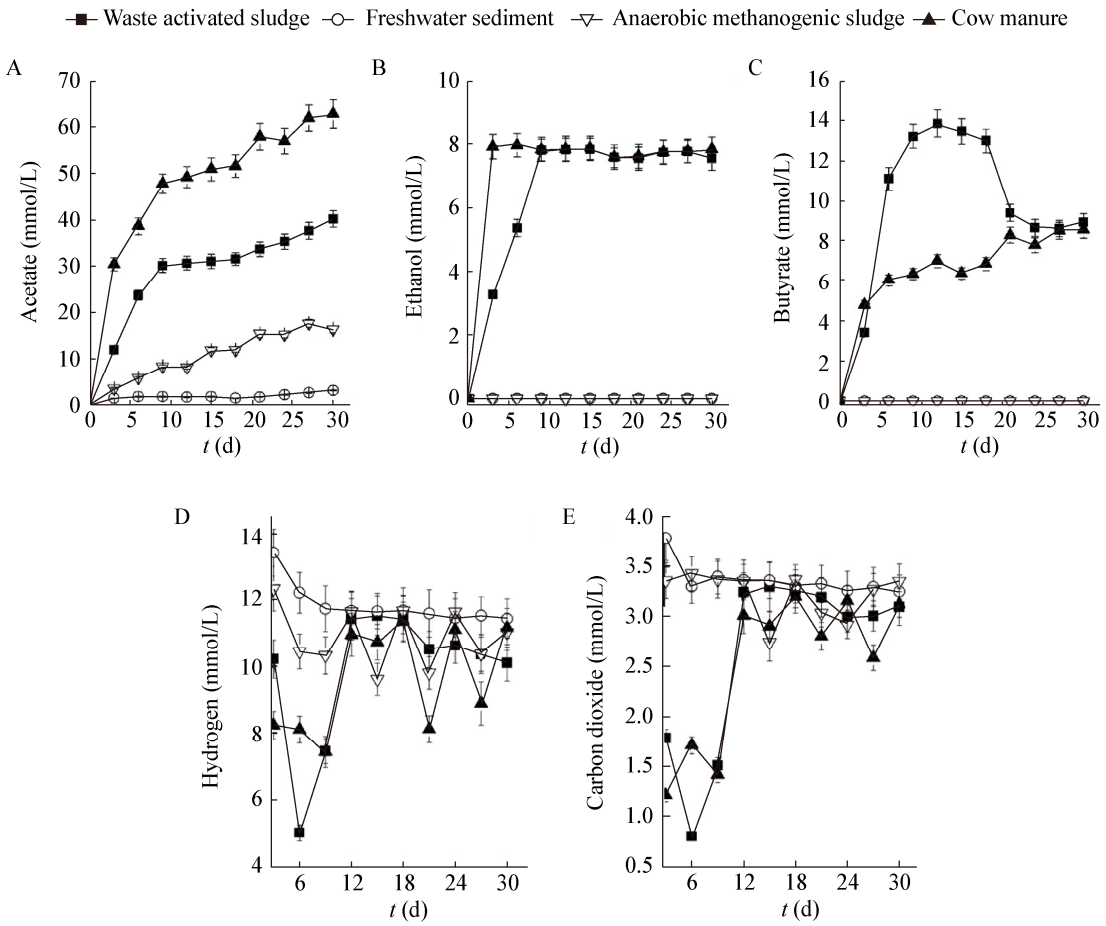

Figure 1 Acetate (A), ethanol (B), butyrate (C) production and H_2 (D), CO_2 (E) consumption during incubation under H_2/CO_2

图 1 H_2/CO_2 培养过程中乙酸(A)、乙醇(B)和丁酸(C)生成及底物 H_2 (D)和 CO_2 (E)消耗

Note: Initial concentrations of H_2, CO_2 were 18.0 and 4.4 mmol/L.

注：H_2 和 CO_2 的初始浓度分别为 18.0 mmol/L 和 4.4 mmol/L.

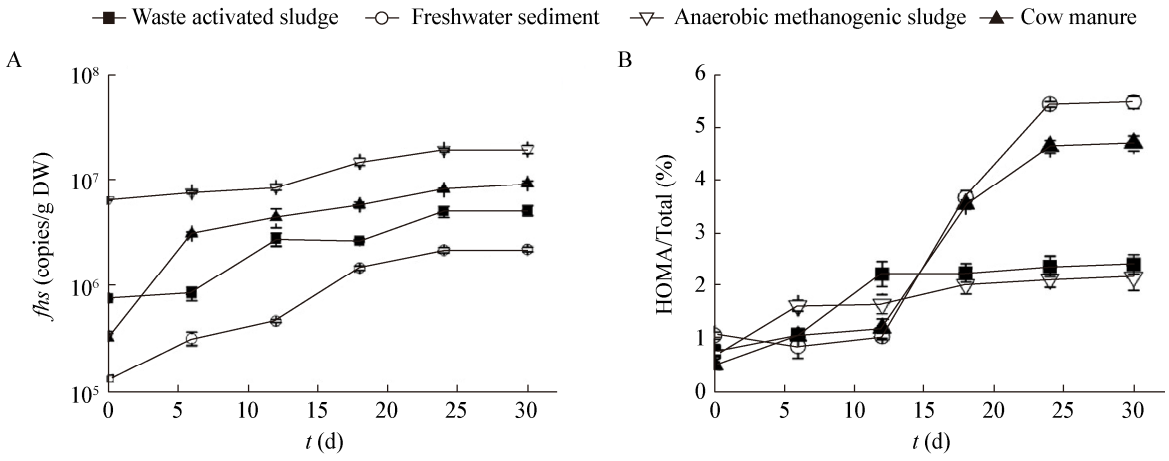

Figure 2 Copy numbers of *fhs* gene and its proportion in total bacteria of cultures using H_2/CO_2

图 2 H_2/CO_2 培养物中 *fhs* 基因拷贝数及其占总细菌的比例

3.3 Bacterial community structures during H_2/CO_2-incubation

The most abundant sequences in the four samples belonged to the following phyla: Firmicutes, Spirochaetes, Proteobacteria, and Bacteroidetes, accounting for 62.1%–99.1% of the total reads, and other phyla represented smaller fractions (0.9%–37.9%) (Figure 3). The dominant family of Firmicutes shifted from Carnobacteriacea (55% of total Firmicutes) to Clostridiaceae (increase from 32% to 52% of Firmicutes) in waste activated sludge samples (Figure 4A). Similar patterns of community shift were also observed in freshwater sediment. The family of Carnobacteriacea accounted for 48% of total Firmicutes in the initial period, but Clostridiaceae became the dominant family at the end, increasing from 31% to 50% (Figure 4A). For anaerobic methanogenic sludge and cow manure, Clostridiaceae dominated in family Firmicutes throughout the incubation process, but Carnobacteriacea disappeared at the final period. Ruminococcaceae remained stable at about 28% of total Firmicutes in cow manure samples, while Clostridiaceae increased from 42.07% to 59.64% of total Firmicutes (Figure 4A). Bacterial community shift in the family Firmicutes suggested that the cultures were dominated by Clostridiaceae.

Among the families belonged to Spirochaetes phylum, the Spirochaetaceae was dominant in the original waste activated sludge, fresh sediments samples and cow manure with proportions ranging from 67% to 98% of total Spirochaetes (Figure 4B). However, the ratio of this family decreased during the incubation process. The share of the family Sphaerochaetaceae significantly increased from 31.34% to 85.80% in the four cultures. The family of Protebacteria phylum was much richer than the other three phyla. Among the Family composition of Proteobacteria was quite different among the four origins (Figure 4C). Sulfur metabolism-associated OTUs belonging to Betaproteobacteria, Deltaproteobacteria, or Epsilonproteobacteria have been identified from all the samples. Considerable attention should be given to sulfate reducing bacteria populations belonging to Desulfovibrionaceae, which appeared in all the four samples.

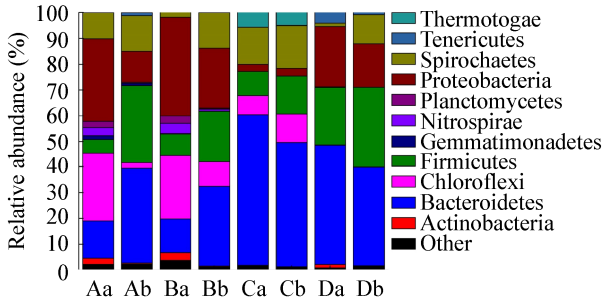

Figure 3 Relative abundances of bacterial OTUs based on 454 sequencing of the V4−V5 regions of 16S rRNA genes at phylum level

图 3 基于 16S rRNA 基因 V4−V5 区 454 高通量测序的微生物 OTUs 门水平的相对丰度

Note: DNA samples were obtained from a: Initial inocula; b: Final enrichment vials containing H_2/CO_2; A: Waste activated sludge; B: Freshwater sediment; C: Anaerobic methanogenic sludge; D: Cow manure.

注：a：初始样品；b：H_2/CO_2 培养结束时的样品；A：剩余活性污泥；B：淡水河道底泥；C：厌氧产甲烷污泥；D：牛粪.

75 acetogen-related OTUs were identified to represent significant increases of acetogenic species with the addition of H_2/CO_2 as substrate. Based on the acetogenic physiology, these OTUs were classified in three physiological groups: H_2/CO_2 utilizing acetogen, heterotrophic fermentative (often proteolytic) acetogen and autotrophic sulfate reducing acetogen. These physiological acetogenic groups, which are widely distributed at different levels of Firmicutes and Spriochaetes, showed clearly origin-related patterns.

OTU#514 shares 95% identity with heterotropic acetogenic, facultatively sulfur-reducing *Sporanaerobacter acetigenes* Lup 33[23], which abundance increased in waste activated sludge; while another OTU#666 (94% identical to *Caloramator proteoclasticus* DSM 12679)[25] showed a relatively higher abundance than OTU#514. OTU#5218 was much less abundant than OTU#514 and shares 92% identity with a fermentative acetogen *Sporanaerobacter acetigenes* Lup33 (DSM 13106)[26].

In the mixed cultures of anaerobic methanogenic sludge, OTU#5142, 5573, and 216 were 96%−99% identical to *Atopobium parvulum* DSM 20469[27], which dominated in the acetogenic community at the level of Clostridia class; while they were not detected in other samples. *Atopobium parvulum* has been reported as a proteolytic, fermentative bacterium associated with sulfur metabolism[27]. The genus *Sphaerochaeta* in the family Sphaerochaetaceae and the genus *Treponema* in the family Spirochaetaceae

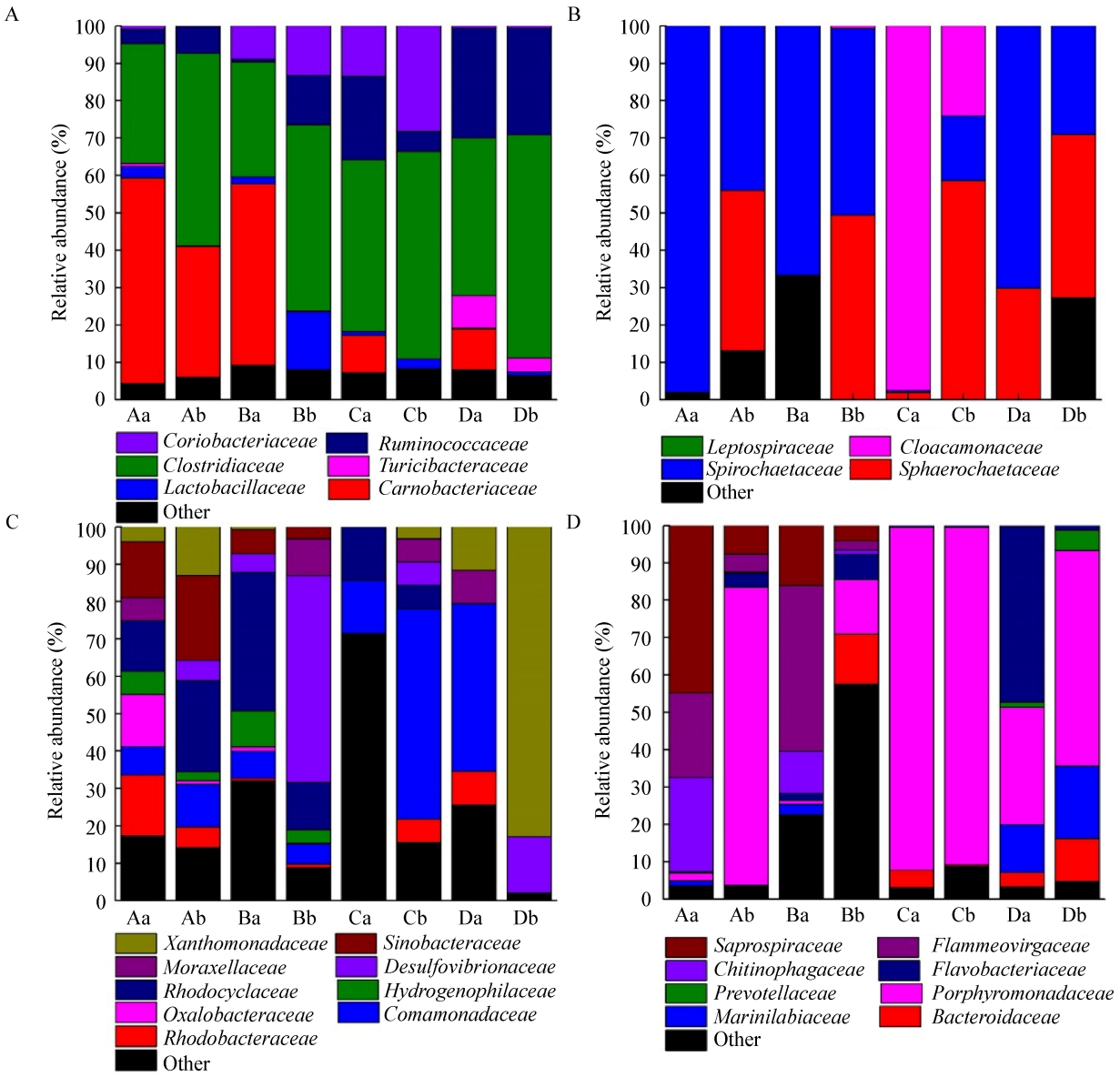

Figure 4 Abundances of the phyla Firmicutes (A), Spirochaetes (B), Proteobacteria (C) and Bacteroidetes (D) 16S rRNA genes at the family level

图 4 厚壁菌门(A)、螺旋体门(B)、变形菌门(C)和拟杆菌门(D) 16S rRNA 基因科水平的丰度

Note: DNA samples were obtained from a: Initial inocula; b: Final cultures vials containing H_2/CO_2; A: Waste activated sludge; B: Freshwater sediment; C: Anaerobic methanogenic sludge; D: Cow manure.

注：a：初始 DNA 样品；b：H_2/CO_2 培养结束时 DNA 样品；A：活性污泥；B：淡水河道底泥；C：厌氧产甲烷污泥；D：牛粪.

were two dominant populations in the pylum Spirochaetes in three samples, except for anaerobic methanogenic sludge. Instead of using H_2/CO_2, both *Sphaerochaeta* and *Treponema* are sugar utilizing fermentative acetogens.

OTU#5774 from freshwater sediment sample represented the most dominant species. It was probably belonged to *Sulfuricurvum kujiense* with 98% identity, a facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacterium[28]. These results suggested that sulfur metabolism had been highly activated at the end of incubation. There are two possible explanations for sulfate reducing bacterial growth: (1) Some autotrophic sulfate reducing bacteria could use H_2/CO_2 as electron donor and carbon source, and use remaining sulfate as

electron acceptor for their growth; (2) The addition of 50 mmol/L 2-bromoethanesulfonate inhibits methanogens[15]; However, this significant amount of 2-bromoethanesulfonate also could possibly be used as carbon source/electron donor and electron acceptor to support sulfate reducing bacteria growth.

More importantly, OTU#2347 (84% identical to *Acetoanaerobium noterae* ATCC 35199)[29] represented common bacterial acetogens in waste activated sludge (Figure 5). In addition, three OTUs were only detected in waste activated sludge. They are OTU#174 and OUT#829, which shared 89% and 92% identity with

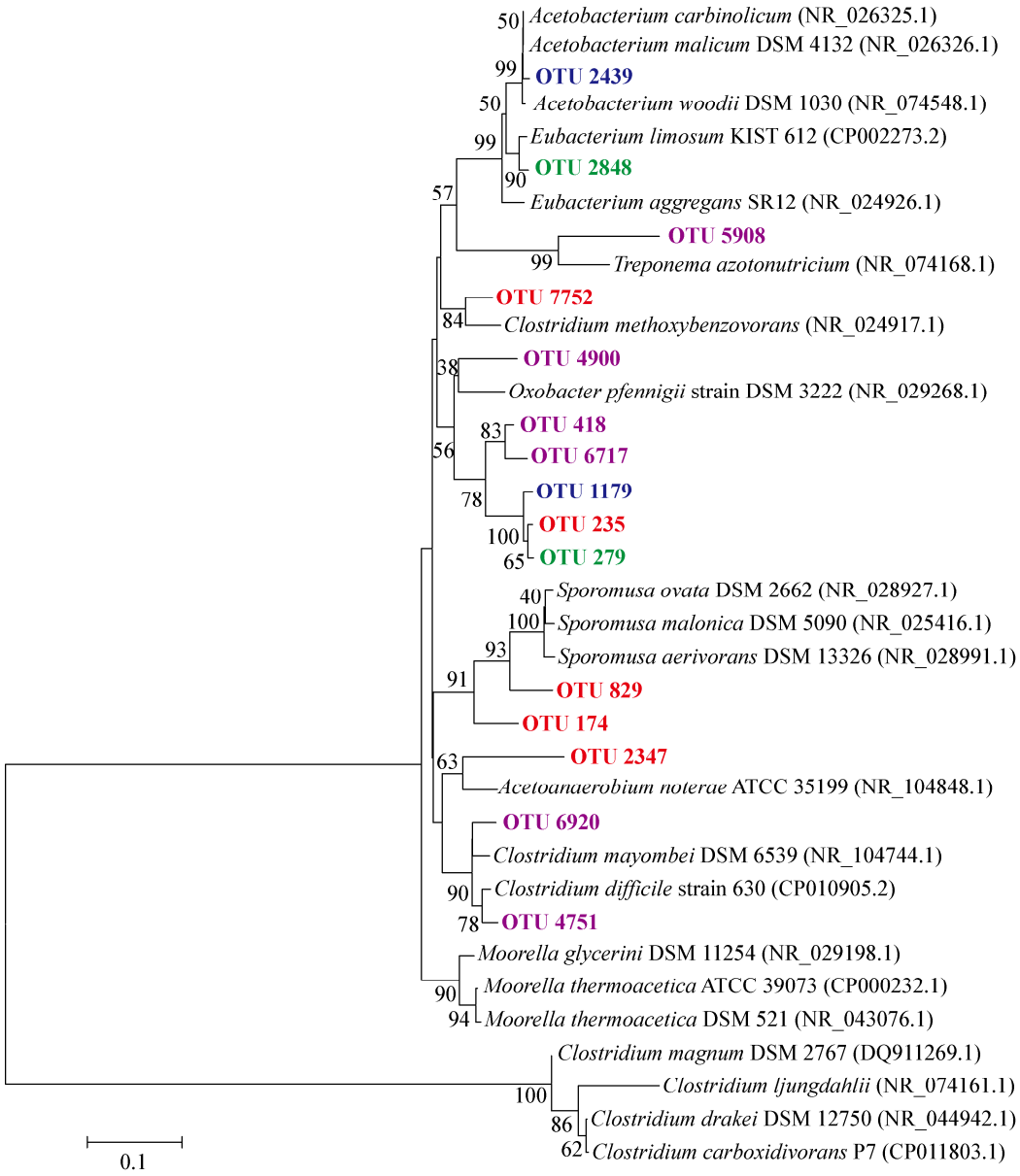

Figure 5 Maximum-likelihood phylogenetic tree of 16S rRNA gene sequences from putative acetogens grown on H_2/CO_2 or formate from waste activated sludge (red), freshwater sediment (blue), anaerobic methanogenic sludge (green) and cow manure (purple) and their closely related species

图 5 不同生境样品中产乙酸菌基于 16S rRNA 基因序列的系统发生树

Note: Red: Waste activated sludge; Blue: Freshwater sediment; Green: Anaerobic methanogenic sludge; Purple: Cow manure; The GenBank accession numbers appear in brackets; Numbers at internal nodes are the percentage of 100 bootstrap samples; Bar=0.1 represent distance scale.

注：红色：活性污泥；蓝色：淡水河道底泥；绿色：厌氧产甲烷污泥；紫色：牛粪；括号中为菌株序列号；每个节点的数字代表自举值；标尺 0.1 为距离标尺.

Sporomusa malonica DSM 5090[30] and *Sporomusa ovata* strain H1[31], respectively, and represented the highly abundant taxa; and OTU#235, 94% identical with *Clostridium carboxidivorans* P7[32], which were much less abundant. Besides, OTU#7752 was representative in H_2/CO_2-utilized waste activated sludge, which was 93% identical with *Clostridium methoxybenzovorans* SR3[33]. Two acetogenic OTUs (#2848 and #279) were detected from anaerobic methanogenic sludge samples. OTU#2848 was closely related to *Eubacterium limosum* KIST612[34] with 16S rRNA gene sequence identity of 98%, while OTU#279 was 97% identical to *Clostridium magnum*. *Clostridium magnum* related OTU#1179 also was detected in H_2/CO_2 when freshwater sediment samples were utilized. Besides, OTU#2439, which was 99% identical to *Acetobacterium carbinolicum* strain VNs25[35], was the representative sequence in H_2/CO_2 utilized freshwater sediment. It was not detected from the other three kinds of samples. The OTU#6717, #4751 and #6920 were found with highest relative abundances in all the cow manure samples, which had sequence similarities of 90%, 96% and 95% to *Clostridium ljungdahlii* DSM 13528[36], *Clostridium difficile* 630[37] and *Clostridium mayombei* strain SFC-5[38], respectively. OTU#418 with sequence similarity of 92% to *Clostridium drakei* strain SL1[4] was present in original and H_2/CO_2-incubated cow manure, and its relative abundance increased. Specially, OTU#5908 and #4900 were close relatives of *Treponema azotonutricium* ZAS-9[39] and *Oxobacter pfennigii* strain V5-2[40] with 16S rRNA sequence identity of 89% and 90%, respectively.

3.4 Effect of trace metal ions and cysteine sulfide on H_2/CO_2 conversion

Enzymes have an important effect on the metabolism of acetogens, and metal enzymes involves in acetyl-CoA such as carbon monoxide dehydrogenase (CODH) and hydrogenase all contain iron-sulfur protein[41]. Aldehyde:ferredoxin oxidoreductases (AORs) also contain ferredoxin. In addition, acetyl-CoA is catalyzed by alcohol dehydrogenase (ADH) to be reduced to ethanol in solventogenic *Clostridiums*, and ADH in *Clostridiums* also contains iron ion or zinc ion. In order to study the effect of trace metal ions on H_2/CO_2 conversion by mixed culture, different concentration of Fe^{2+} and Zn^{2+} were added to test their effects on acetate and ethanol production from H_2/CO_2 by mixed cultures from cow manure.

Addition of Zn^{2+} and Fe^{2+} promoted the utilization of gas substrate, as well as acetate and ethanol production from the conversion of H_2/CO_2 (Figure 6), and the increase of acetate yield and ethanol increased with the rise in Zn^{2+} and Fe^{2+} concentration. The maximum concentration of acetate and ethanol were 12.83 mmol/L and 7.70 mmol/L, respectively, when the Zn^{2+} concentration was 6.96 μmol/L. When the concentration of cysteine sulfide increased to 1.0 g/L and 2.0 g/L, the ethanol production increased significantly and the maximum yields were 6.09 mmol/L and 10.65 mmol/L, respectively (Figure 7A, B).

3.5 Syngas fermentation by H_2/CO_2-converting cultures from cow manure

3.5.1 Acetate and ethanol production from syngas fermentation: The aim of the syngas fermentation was to examine the selected mixed culture for acetate and ethanol production by utilizing syngas as carbon source. The mixed culture from cow mature produced maximum ethanol and acetic acid during the incubation utilizing H_2/CO_2 as substrate (Figure 1), and was selected for syngas fermentation. The acetic acid and ethanol accumulations are shown in Figure 8A, B. The acetate and ethanol concentration increased gradually and then remained relatively stable. The final concentrations of acetate were 32.1, 97.1 and 55.4 mmol/L for control, H_2/CO_2 and syngas fermentation, respectively (Figure 8A), and those of ethanol were 1.78, 8.90 and 11.8 mmol/L respectively (Figure 8B). The average daily consumption of CO_2 was 0.12 and 0.14 mmol for H_2/CO_2 and syngas fermentation, respectively (Figure 8C, D). During the H_2/CO_2-incubation, the H_2 consumption gradually increased and reached 0.41 mmol at Day 7, and then decreased to 0.31 mmol at the end of the fermentation. For syngas fermentation, about 0.35 mmol H_2 was consumed every day during the first 16 days, and then decreased to about 0.22 mmol until the end. The CO consumption gradually increased and reached 0.28 mmol at Day 11, and then decreased to 0.12 mmol at the end. The maximum ethanol production during syngas fermentation was 27.3% higher than the one from CO_2/H_2-incubation. It is suggested that the presence of CO contributes with ethanol production, which is in agreement with the previous results using pure culture as inoculum[42].

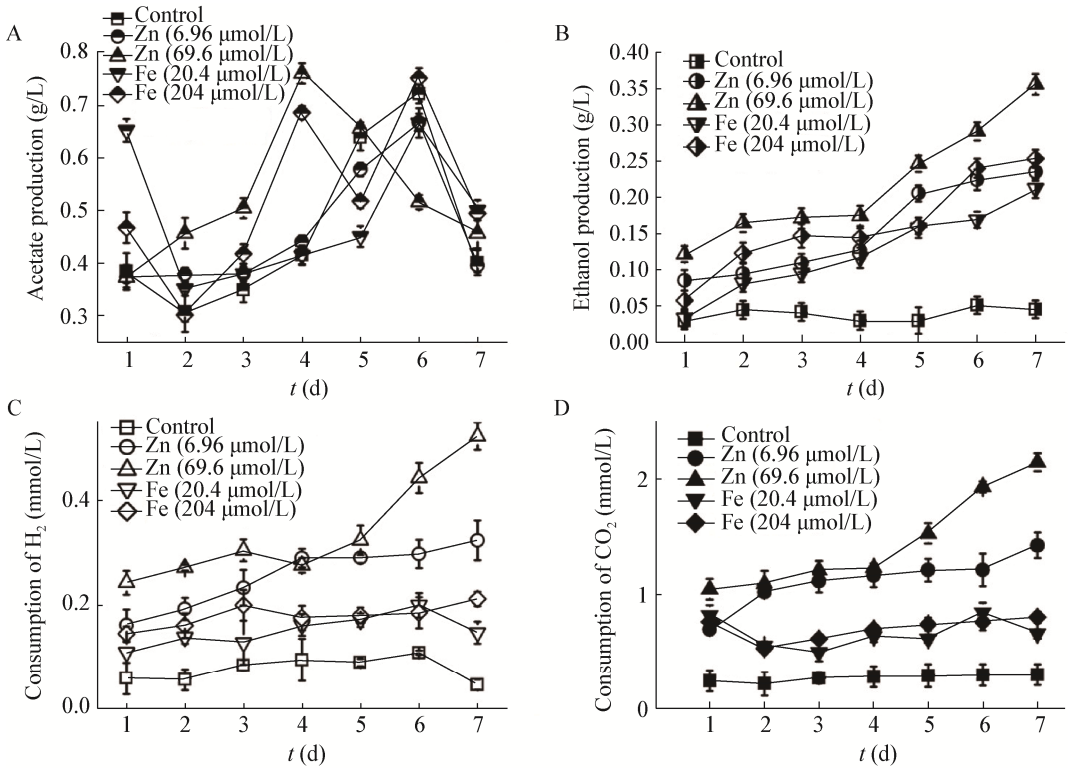

Figure 6 Acetate (A), ethanol (B) accumulation and H_2 (C), CO_2 (D) consumption under different trace metal ion concentration

图 6 不同痕量金属浓度下乙酸(A)和乙醇(B)生成以及 H_2 (C)和 CO_2 (D)消耗

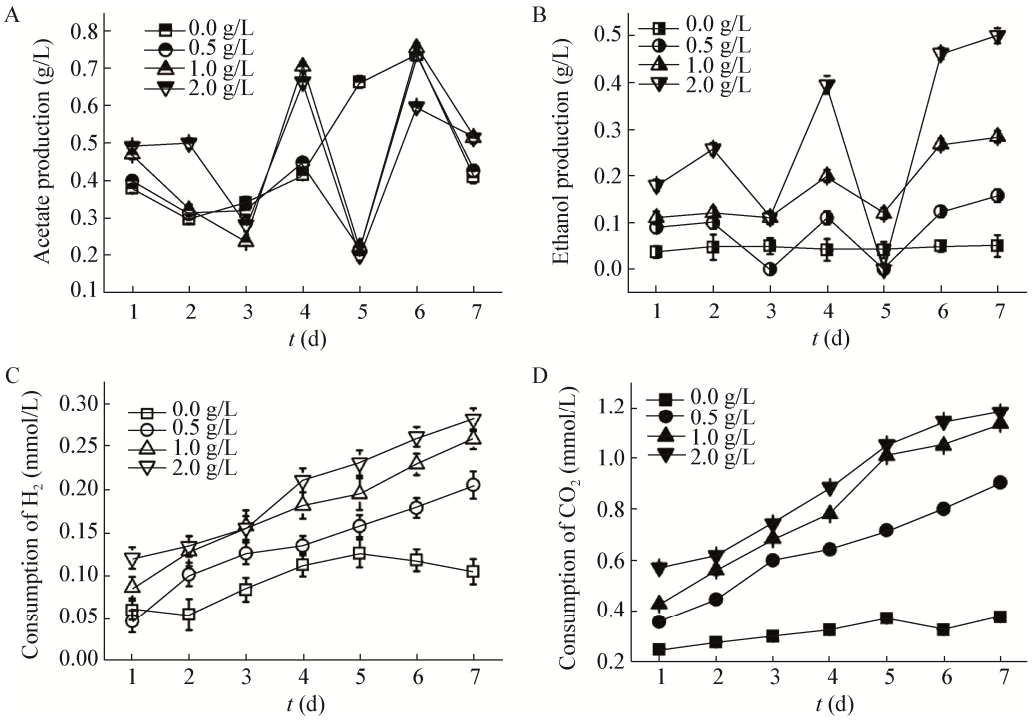

Figure 7 Acetate (A), ethanol (B) accumulation and H_2 (C), CO_2 (D) consumption under cysteine sulfide concentration

图 7 不同半胱氨酸硫化物浓度下乙酸(A)和乙醇(B)生成以及 H_2 (C)和 CO_2 (D)消耗

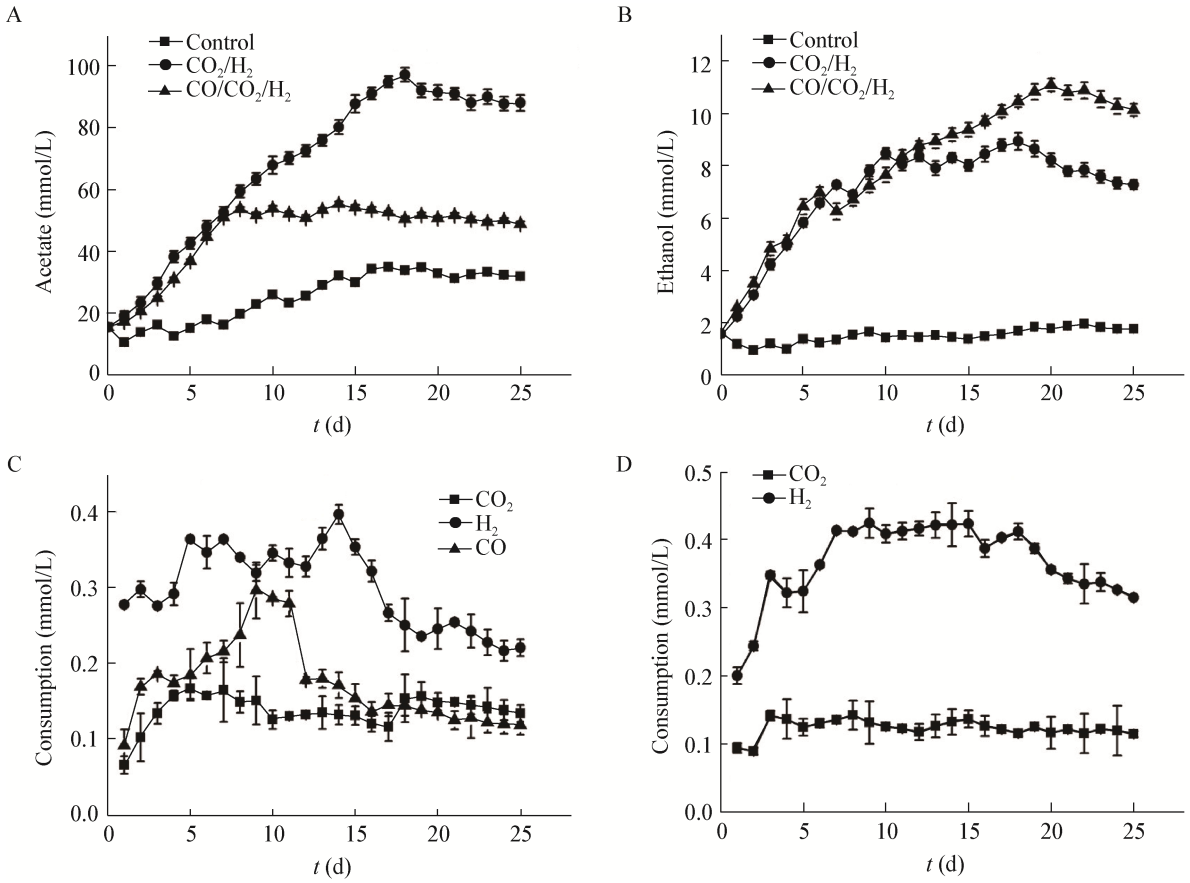

Figure 8 Acetate (A) and ethanol (B) accumulation and gas substrates consumption of syngas-incubation (C) and CO_2/H_2-incubation (D) during syngas fermentation

图 8 合成气发酵过程中乙酸(A)和乙醇(B)的积累以及合成气(C)和 H_2/CO_2 (D)的消耗

3.5.2 Bacterial community shift during syngas fermentation: In mixed culture from cow manure, the diversity indexes *H′* for the bacterial communities in the initial samples was 2.98, which decreased to 2.73 and 2.59 after H_2/CO_2 and syngas fermentation, respectively, showing the reduction in diversity of bacterial community species after the H_2/CO_2 and syngas fermentation. Bacterial communities involved in syngas fermentation had lower species diversity than H_2/CO_2 incubation. The Shannon-Wiener index is influenced by both the richness and abundance of species in the community. T-RFLP-based *H′* index increases with increasing number of T-RFs or even distribution of individual T-RFs. Most numbers of T-RFs were detected in the initial samples (14 T-RFs) with maximum species evenness (*E* values of 0.85). 9 T-RFs were detected in the samples of syngas fermentation with *E* values of 0.80. The samples of H_2/CO_2 incubation had higher species richness (10 T-RFs) and higher species evenness (*E* values of 0.83), which resulted in higher species diversity than that of syngas fermentation.

The response of bacterial community in cow manure samples to H_2/CO_2 and syngas were different. 27, 183 and 190 bp T-RFs accounted for abundance proportions of 22.4%, 17.1% and 27.8% in the populations of initial samples. The three T-RFs were also the main bacterial composition in samples of H_2/CO_2 incubation with abundance proportions of 25.5%, 15.3% and 28.7% (Figure 9). For cow manure using syngas as substrates, the proportion of 60 bp increased to about 15.1% comparing to 1.9% in the initial samples (Figure 9). Interestingly, T-RFs of 30 bp and 244 bp disappeared throughout the process of syngas fermentation (Figure 9).

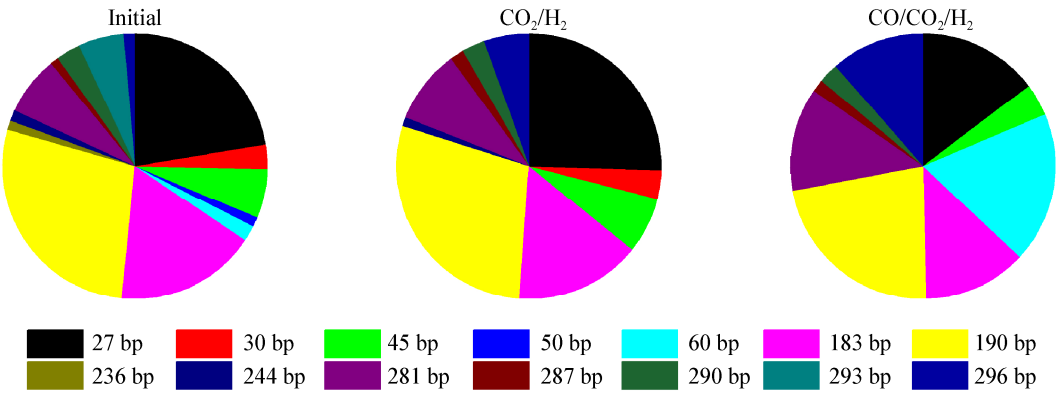

Figure 9 Microbial community dynamics as determined by the DNA-based T-RFLP in enrichment of cow manure during syngas fermentation

图 9 牛粪富集物合成气发酵过程中基于 T-RFLP 技术的微生物群落动态变化

4 Discussion

Acetate was the main product of the H_2/CO_2 bioconversion for each inoculum (Figure 1A−C), which was in accordance with the increase in the number of acetogenic bacteria during H_2/CO_2-incubation experiments. The copy numbers of *fhs* genes and the acetogenic proportions in total bacteria increased after incubation under H_2/CO_2 headspace for each inoculum (Figure 2), indicating the increase of acetogenic number. Acetogens used CO_2 as carbon source and H_2 as electron donor to synthesize acetyl coenzyme A (acetyl-CoA) through acetyl-CoA pathway and then acetyl-CoA can be converted into acetate or ethanol[43]. 0.3 ATP was generated in the whole process of metabolism when acetyl-CoA was converted into acetate, and thus the energy was positive in this process. 0.1 ATP was needed when acetyl-CoA was converted into ethanol, being the energy balance of this metabolic pathway negative[44]. As a result, acetate production from H_2/CO_2 was more favorable than ethanol production, and the main product in the present study was acetate. However, ethanol can also be produced by reducing acetate through Aldehyde:ferredoxin oxidoreductases (AOR). Many acetogens such as *Clostridium ljungdahlii* and *Eubacterium limosum* KIST612 have been reported to have AORs[44], this maybe the reason for a small amount of ethanol formed in cow manure and waste activated sludge samples.

Relied on high throughput 16S rRNA genes pyrosequencing, the signature OTUs representing acetogenic bacteria from different samples were identified. Different acetogens have been obtained from four different ecosystems after H_2/CO_2 incubation. Diverse acetogenic bacteria were found in waste activated sludge and cow manure. The major putative acetogenic species belonged to *Clostridium* spp., *Sporomusa malonica* and *Acetoanaerobium noterae* in waste activated sludge, and those were *Clostridium* spp., *Treponema azotonutricium* and *Oxobacter pfennigii* in cow manure. Predominant putative acetogens in methanogenic sludge samples were the species *Eubacterium limosum* and *Clostridium magnum*. Acetogenic bacteria were composed also of two species *Acetobacterium carbinolicum* and *Clostridium magnum*. The above OTUs sequences provide molecular bioindicators for monitoring key species development and bacterial community dynamics during incubation. The results indicated that the acetogens composition in the studied cultures was decided by the sources of natural inocula under similar incubation condition.

When treated with H_2/CO_2, anaerobic methanogenic sludge inoculated group possessed the highest number of acetogens, while freshwater sediment inoculated group had the lowest number of that. Besides, cow manure and freshwater sediment inoculated groups achieved the biggest extent of growth (16.8 times and 29.3 times higher). Accordingly, highest proportions of acetogens in total bacteria were detected in these two groups (5.47% and 4.69%). VFAs concentrations, especially acetate, could be indicators to evaluate the capacity of

acetogenic bacteria during incubation. Among the four cultures, highest yields of acetate, ethanol, and butyrate were produced in cow manure group after 30 days of incubation. The following was waste activated sludge group. The acetate production in anaerobic methanogenic sludge and freshwater sediment group was much less than in the former two groups. The results showed that the biggest amount of acetogens in mixed culture did not correspond to largest acetate accumulation. Intragenomic heterogeneity of 16S rRNA gene was reported to cause the overestimation of microbial diversity when using 16S rRNA gene-based methods, and overestimation was the least for the V4−V5 region under the dissimilarity level of 3%[45]. In the present study, variable regions V4−V5 of bacterial 16S rRNA genes were amplified through 454 pyrosequencing and sequence reads were clustered into operational taxonomic units (OTUs) at 97% sequence similarity. Although the application of 16S rRNA gene-based methods caused inevitable overestimation of bacterial diversity, this overestimation was minimized in this study, as suggested. Comparatively, acetogenic population of higher diversity with relatively higher content led to higher acetate production from H_2/CO_2 under the studied situation. Both the richness and number of acetogens in the mixed culture were key factors that regulate the capacity of acetogens to produce acetate.

As ADH contained iron or zinc, the increase of Zn^{2+} concentration might stimulate the activity of ADH, which catalyzes acetyl-CoA to produce ethanol in solventogenic *Clostridiums*. Similarly, the addition of Fe^{2+} also enhanced the yield of acetate and ethanol (Figure 7). The addition of Zn^{2+} promoted the growth of acetogens. Compared with the control, there was an increase of two orders of magnitude for the Zn^{2+} concentration of 69.6 μmol/L (Table 2). The existence of cysteine sulfide benefited ethanol and acetate production significantly. Cysteine sulfide reduced the redox potential as reductant[46] and it also provided electrons for acetogens, making the electron flow towards the direction of ethanol production[47]. In addition, cysteine sulfide stimulated the activity of some key enzymes such as CODH involved in acetyl-CoA pathway containing iron-sulfur protein[48]. Cysteine sulfide had no significant effect on the growth of acetogens (Table 2).

Table 2 Numbers of *fhs* under different trace metal ion or cysteine sulfide concentrations
表 2 不同痕量金属及半胱氨酸硫化物浓度下 *fhs* 基因拷贝数

Trace metal ion/ Cysteine sulfide 痕量金属/半胱氨酸硫化物		*fhs* gene copies numbers (copies/g) *fhs* 基因拷贝数(copies/g)
Trace metal ion	痕量金属	
	Control	$10^{8.03}$
Zn^{2+}	6.96 μmol/L	$10^{10.43}$
	69.6 μmol/L	$10^{8.23}$
Fe^{2+}	20.4 μmol/L	$10^{8.43}$
	204 μmol/L	$10^{7.18}$
Cysteine sulfide	半胱氨酸硫化物	
	Control	$10^{8.41}$
	0.5 g/L	$10^{8.43}$
	1.0 g/L	$10^{8.59}$
	2.0 g/L	$10^{8.83}$

The presence of CO is favorable to higher ethanol production and this may be related to the energetics of acetogens in acetyl-CoA pathway. CO can act as a carbon source as well as electron donor to provide reducing equivalents during the syngas fermentation[44]. When using H_2/CO_2 as energy and carbon source, the reaction is more favorable for the acetate production. 1.5 ATP was generated during converting acetyl-CoA into acetate using CO as electron donor, while 1.7 ATP was generated during converting acetyl-CoA into ethanol. In addition, reducing acetate to form ethanol through AOR generated 2.1 ATP[44].

5 Conclusion

The studied cow manure and waste activated sludge possess an ability to convert H_2/CO_2 to acetate, ethanol and butyrate. The major putative acetogens belonged to *Clostridium* spp., *Sporomusa malonica* and *Acetoanaerobium noterae* in waste activated sludge, and those were *Clostridium* spp., *Treponema azotonutricium* and *Oxobacter pfennigii* in cow manure. Diverse acetogens were found in waste activated sludge and cow manure. Both richness and number of acetogens in mixed cultures are important for regulating acetogenesis during the bioconversion of H_2/CO_2. The addition of trace metal ions (Fe^{2+} and Zn^{2+}) and cysteine sulfide effectively improved ethanol production during the conversion

of H_2/CO_2 by mixed culture from cow manure. Mixed culture obtained from cow manure had potential for acetate and ethanol production from syngas fermentation.

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