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ORIGINAL PAPER

Bioconversion of H_2/CO_2 by acetogen enriched cultures for acetate and ethanol production: the impact of pH

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Abstract Syngas fermentation into ethanol and other bioproducts by mixed cultures is considered a promising biotechnology. Effects of pH on product generation and microbial community during H_2/CO_2 utilization by acetogen enrichment cultures were investigated in this work. The maximum acetate concentration reached 95.41 mmol L^{-1} at pH 7, which was 71.7, 21.8 and 50.9 % higher than at pH 5, 9 and 11, respectively. The maximum ethanol concentration at pH 7 was 45.7, 50, 72 % higher than that at pH 5, 9 and 11, respectively. The CO dehydrogenase (CODH) gene copy number was highest at pH 7, indicating that metabolically active acetogens reached their highest level at pH 7. The CODH gene copy number at pH 9 was lower than at pH 7, but higher than at pH 5 and 11. Correspondingly, the enrichment cultures at pH 7 had the highest species richness and diversity, while those at pH 9 had the second highest diversity, and those at pH 5 and 11 had the lowest diversity. The shift in microbial community structure and the different active acetogen contents resulting from different pHs were responsible for the differences in acetate and ethanol production.

Keywords pH · H_2/CO_2 · Mixed culture · Acetogen · Ethanol

Introduction

The production of biofuels from syngas using a variety of feedstocks has received much attention in recent years. Acetogens possess the ability to use various gaseous substrates (CO/CO_2 and H_2) to produce chemicals and/or biofuels such as acetate and ethanol (Fast et al. 2015). Acetogens cannot be classified phylogenetically, but can be considered a type of organism with the same metabolic pathway. Though pure culture based biotechnology has been explored for the production of acetate and ethanol, it requires harsh conditions for the good growth of acetogens, including a strict anaerobic environment, appropriate pH, elimination of contaminating bacteria, and so on. Mixed culture biotechnology could become a more attractive addition or alternative to the traditional pure culture based approach (Kleerebezem and van Loosdrecht 2007; Cata Saady 2013). The advantages of mixed culture biotechnology include no sterilization requirements, more adaptive capacity in various conditions due to microbial diversity, and the possibility of a continuous process (Kleerebezem and van Loosdrecht 2007).

Production of ethanol from biogases is an important method to obtain renewable energy and conducive to sustainable economic development. As ethanol is not the main product of acetogens, some studies have been carried out to adapt cultures from acidogenesis to solventogenesis, thus enhancing ethanol yields (Mohammadi et al. 2012). Fermentation pH plays an important role in regulating the metabolic pathway of acetogens and thus affects the metabolic products and their yields (Mohammadi et al.

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2011). As a regulator, external pH can lead to production of solvents such as ethanol to balance low intracellular pH (Kundiyana et al. 2010). Sakai et al. (2004) reported that ethanol production by *Moorella* sp. HUC22-1 from H_2 and CO_2 in a fermenter was 3.7-fold higher after 220 h when the culture pH was controlled at 5 than that in a pH-uncontrolled culture. Cotter et al. (2009) reported that the initial medium pH significantly affected the metabolism of growing cultures of *Clostridium ljungdahlii*. More acidic pH levels led to slower growth rates and lower ethanol yields.

It remains unclear how the medium pH affects product formation in mixed culture conditions. The medium pH may affect the performance of enzymes and shift the microbial community in a mixed culture, so it is necessary to identify which factors are critical for acetate and ethanol production. In order to investigate pH regulation of metabolic pathways during the bioconversion of gas substrates, acetogen enriched mixed cultures were applied as inocula in different pH conditions. The gene quantities of key enzymes and the constituents of the microbial communities were detected to show the response of microorganisms to pH change. The results of this study provide a novel insight into the mechanism of pH regulation of gas bioconversion by mixed cultures.

Materials and methods

Inocula and media

Manure samples were collected from a cattle farm (WuXi, China). The basic characteristics of the cow manure are shown in Table 1.

The enrichment medium contained (per liter, pH 6.8): sodium formate (3.25 g), NH_4Cl (0.5 g), KH_2PO_4 (0.25 g), $K_2HPO_4{\cdot}3H_2O$ (0.25 g), $MgCl_2{\cdot}6H_2O$ (0.3 g), $FeCl_3{\cdot}6H_2O$ (25 mg), $NiSO_4{\cdot}6H_2O$ (16 mg), $CaCl_2$ (25 mg), $ZnCl_2$ (11.5 mg), $CoCl{\cdot}6H_2O$ (10.5 mg), $CuCl_2{\cdot}2H_2O$ (5 mg), and $MnCl_2{\cdot}4H_2O$ (15 mg). 2-Bromoethanesulfonate was added into the enrichment medium to 50 mM to inhibit methanogenesis during the experiment (Xu et al. 2010). Resazurin stock solution (0.2 %, 0.2 mL L^{-1}) was used as an indicator of oxidation–reduction potential. The components in the medium used for bioconversion of CO_2/H_2 were the same as in the enrichment medium except sodium formate was omitted.

Table 1 Basic characteristics of cow manure samples

Basic characteristics	Parameter value
Total solids (TS, g L^{-1})	79.58 ± 0.16
Volatile solids (VS, g L^{-1})	58.47 ± 0.13
Chemical oxygen demand (COD, mg L^{-1})	15840 ± 5.64
Water content (%)	92.04 ± 0.02
Protein (mg L^{-1})	284.92 ± 4.01
Polysaccharides (mg L^{-1})	369.21 ± 2.22

Experimental setup and operation

Two hundred grams of cow manure (Table 1) were incubated in 1000 mL serum bottles containing 500 mL enrichment medium. The liquid medium was boiled before incubation and flushed with N_2 (99.9 %) for 20 min. The cultures were incubated at 37 °C for 1 month.

The fermentation experiments were conducted in 250 mL fermentation reactors (Fig. 1) containing 100 mL liquid medium. An artificial mixed gas substrate containing CO_2/H_2 (80/20, v/v) was daily flushed into the reactor through an inlet steel needle to 1 standard atmosphere pressure. Oxygen was eliminated through an outlet steel needle. Fermentation experiments were carried out at 37 °C. NaOH (5 mol L^{-1}) and H_3PO_4 (3 mol L^{-1}) were used daily to adjust the pH to 5, 7, 9 or 11 as appropriate, in order to maintain the pH values stably during the whole experimental period. The control group was treated the same as the fermentation experiments, but flushed daily with N_2 instead of CO_2/H_2 and without pH regulation.

Chemical analysis methods

The medium pH was monitored with a pH meter (Mettler-Toledo GmbH, Greifensee, Switzerland). CO_2 and H_2 in the headspace gases were measured every 24 h by GC-2010 gas chromatography (Shimadzu Corporation, Tokyo, Japan) with a gas-tight pressure lock syringe (Shimadzu Corporation, Tokyo, Japan). Liquid samples were collected

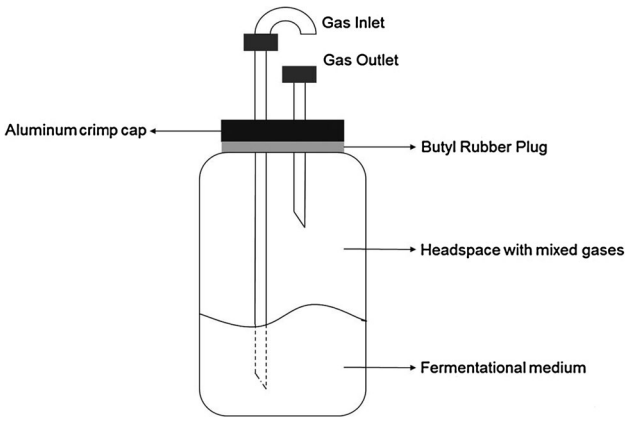

Fig. 1 Experimental setup for conversation of mixed gases (CO_2/H_2) into acetate and ethanol

every 24 h for volatile fatty acids (VFAs) measurement by a gas chromatograph equipped with an AOC-20i auto injector (Shimadzu Corporation, Tokyo, Japan) using a fused-silica capillary column (PEG-20 M, 30 m × 0.32 mm × 0.5 μm, China) (Xu et al. 2009). Before measuring the VFA content, liquid samples were centrifuged at 10,000 rpm for 10 min and filtered through a 0.45 μm membrane.

DNA extraction and real-time quantitative PCR (qPCR)

Samples from the fermenters (0.25 g) were collected every 5 days. Total genomic DNA was extracted with a MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA).

Forward primer 27f and reverse primer 1401r were used to amplify bacterial 16S rRNA genes to quantity the total numbers of bacteria. Each 25 μL PCR mixture contained 12.5 μL 2× QuantiFast SYBR Green PCR Master Mix, 0.25 μL of each primer (10 μM), 2 μL of template DNA and RNase free dH_2O to complete the final volume. The 16S rRNA gene PCR protocol was: initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 50 s, and elongation at 72 °C for 1 min. This was followed by a final extension at 72 °C for 10 min.

The copy number of gene *fhs*, encoding 10-formyltetrahydrofolate synthetase (FTHFS), a functional gene of acetogens, can be used to identify acetogens (Ryan et al. 2008). Primers used in PCR amplification and the reaction conditions were as described by Xu et al. (2009).

Reverse transcription quantitative PCR (RT-qPCR)

Gene expression of the catalytic subunit of carbon monoxide dehydrogenase (CODH, KF224973) was monitored by RT-qPCR. Table 2 shows the concentrations and sequences of the primers used.

RNA was extracted from 2.5 g Samples from the fermenters using a RNA PowerSoil® Total RNA Isolation Kit according to the manufacturer's protocol. Gene expression of CODH was measured by using a One Step SYBR PrimeScript™ PT-PCR Kit (TaKaRa, Japan). Each 25 μL RT-qPCR amplification mixture contained 12.5 μL of 2× One Step SYBR RT-PCR Buffer, 2.5 U of TaKaRa Ex *Taq* HS, 0.5 μL of PrimeScript RT Enzyme Mix, 0.25 μL of each primer (10 μM), 2 μL RNA templates and RNase free dH_2O to complete the final volume. The previously described RT-qPCR program (Kundiyana et al. 2010) was modified as follows: holding at 42 °C for 30 min and predenaturation at 94 °C for 5 min to complete reverse transcription; then, 40 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s, and elongation at 72 °C for 45 s; followed by a final extension for 10 min at 72 °C. Neither nonspecific bands nor primer-dimers could be visualized on agarose gels after this RT-qPCR procedure.

Clone library of 16S rRNA genes

Six clone libraries were generated using samples of raw material, and the incubations from four sets of pH conditions and from the control group at the end of anaerobic fermentation. 16S rRNA genes were amplified using universal primers 27f/1401r in the thermal conditions as described in the method of qPCR. PCR products were clearly visualized by agarose gel electrophoresis without special bands and were ligated into the pMD®18-T Vector system (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China,). This ligation mixture was used to transform *Escherichia coli* JM109 using standard methods. One-hundred-eighty clones from six libraries in total from six libraries were picked for phylogenetic analysis by Sangon Co. (Shanghai, China). 16S rRNA gene sequences were compared online using GenBank's Basic Local Alignment Search Tool (BLAST).

Terminal restriction fragment length polymorphism (T-RFLP) analysis

PCR was carried out using universal primer set 27f and 1401r in the thermal conditions used to prepare the clone

Table 2 Primers used for PCR amplifications in this study

Primer	Sequence (5′–3′)	Conc. (μM)	Amplicon (bp)	References
27f	AGTTTGATCATGGCTCAG	10	18	Universal primer
1401r	CGGTGTGTACAAGACCC	10	17	Universal primer
FTHFS-f	GTWTGGGAAAAGGYGGMGAAGG	10	22	Xu et al. (2009)
FTHFS-r	GTATTGDGTYTTRGCCATACA	10	21	Leaphart and Lovell (2001)
CODH-f	TGGTTGGATCAGAGGCT	10	23	Ukpong et al. (2012)
CODH-r	GTCTTAAGTCCAGGACCAGCGAGT	10	24	Ukpong et al. (2012)

library. The 5′ end of the 27f primer was labeled with 6-carboxyfluorescein (FAM). PCR products were purified using a MiniBEST Agarose Gel DNA Extraction Kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) and digested for 3 h at 37 °C with restriction endonucleases *Hha*I and *Hae*III (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China), respectively, which were then inactivated at 65 °C for 10 min. Digested products were analyzed by Shanghai GeneCore BioTechnologies Co., Ltd. The relative abundance of each T-RF was determined by the percentage peak area relative to the total area of all peaks, and T-RFs with percentage peak areas relative to the total area of all peaks <1 % were excluded from the analysis.

Nucleotide sequence accession numbers

Sequences obtained in this study were deposited in Genbank with accession numbers KF224973 and KP134302 to KP134335 (Supplementary Table S1).

Results

Gas substrate consumption

Figure 2 shows the daily utilization of CO_2 and H_2. The utilizations of CO_2 at pH 5 and pH 9 were much lower than those that at pH 7 and pH 11 (Fig. 2a). The average daily consumption of CO_2 was 0.03 and 0.05 mmol at pH 5 and pH 9, respectively. At pH 7, about 0.12 mmol CO_2 were consumed every day during the initial 16 days, and the level then decreased to about 0.06 mmol day^{-1} until the end of the experiment (25 days). At pH 11, the daily CO_2 consumption gradually increased and reached 0.30 mmol at day 13, and then decreased to 0.22 mmol day^{-1} by the end of the fermentation. The average CO_2 consumption at pH 11 was 87.8, 56.1 and 79.3 % higher than those at pH 5, pH 7 and pH 9, respectively. The average daily H_2 use at pH 5, 7, 9 and 11 was 0.18, 0.44, 0.28 and 0.28 mmol, respectively (Fig. 2b), indicating that the H_2 consumption was highest at pH 7.

In alkaline conditions the CO_2 was not only consumed by acetogens, but also in neutralization reactions with alkali. Thus, CO_2 consumption at pH 11 might mainly be by neutralization, instead of in acetogen metabolism. Given that acetogens use CO_2 and H_2 simultaneously to synthesize metabolites, we conclude that neutral pH was more favorable for CO_2 and H_2 use by acetogens in this study.

Acetic acid and ethanol production

In control samples, the pH fluctuated between 6.0 and 7.0 during the fermentation (Fig. S1). The pH decreased from 6.8 to 6.0 during the initial 6 days and acetate accumulated from 2.5 to 26.1 mmol L^{-1}, and then the pH increased to 6.5 and maintained this level until the end of the experiment. Meanwhile, the acetate concentration in the control samples decreased to 15.0 mmol L^{-1} and then remained stable. Thus, the organic matter in the controls became limited from day 7.

Figure 3 shows the accumulation of acetic acid and ethanol at pH 5, 7, 9 and 11. Much more acetic acid and ethanol accumulated at pH 7 than in the other pH conditions. The acetic acid concentration at pH 7 increased gradually during the initial 8 days and was then maintained stably at 95.41 mmol L^{-1} during the next 8 days; finally, it decreased to 68.90 mmol L^{-1} on day 21. The trends in acetic acid accumulation at pH 5, 9 and 11 were similar to those at pH 7, but the final acetic acid concentrations were

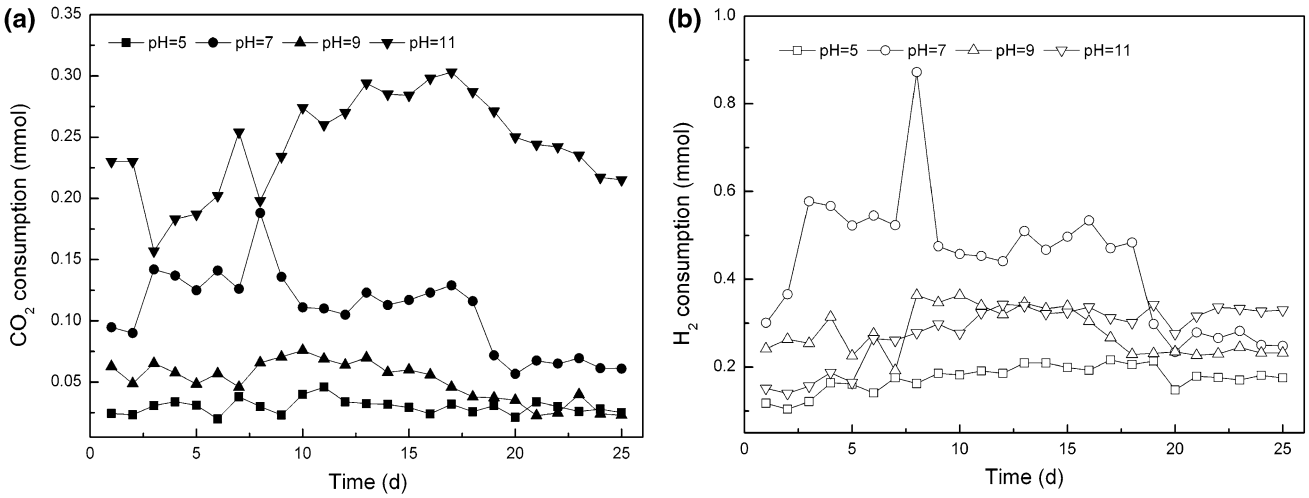

Fig. 2 CO_2 (**a**) and H_2 (**b**) consumption during the process of fermentation in different pH conditions

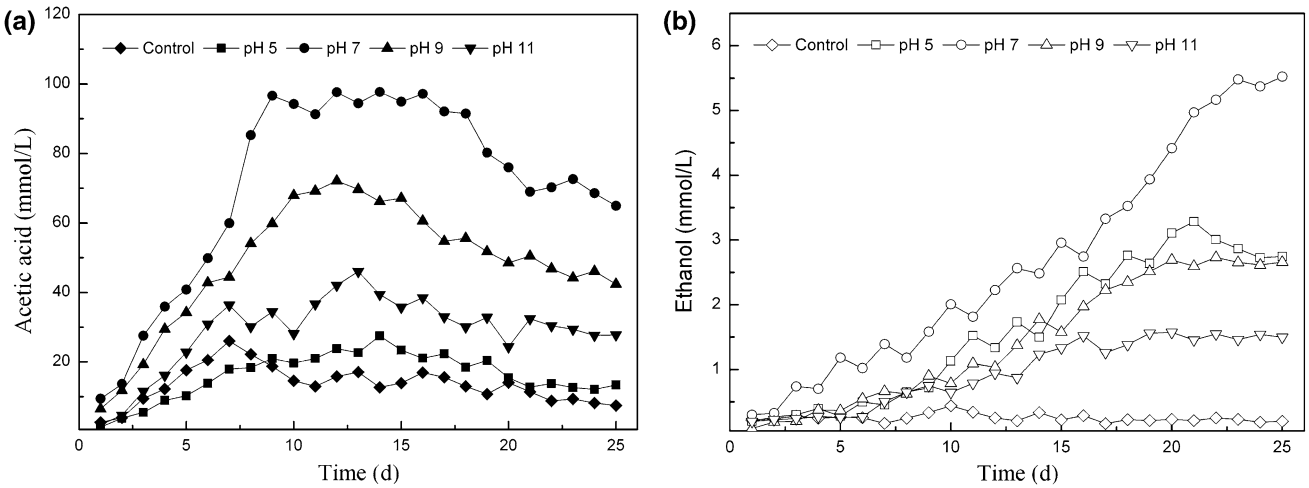

Fig. 3 Production of acetic acid (**a**) and ethanol (**b**) during the process of fermentation

much lower, being 13.42, 42.40 and 27.81 mmol L^{-1} at pH 5, 9 and 11, respectively (Fig. 3a). For all the pHs, the ethanol concentration increased gradually. The final concentrations of ethanol were 2.71, 5.49, 2.72 and 1.51 mmol L^{-1} at pH 5, 7, 9 and 11, respectively (Fig. 3b).

The maximum molar ratios of ethanol to acetic acid at pH 7 and pH 9 were 0.09 and 0.06, respectively, which were significantly lower than that at pH 5 (ratio 0.26). We suggest that a lower pH favors a higher maximum molar ratio of ethanol to acetic acid, which is similar to previous results using pure cultures (Phillips et al. 1993). In the present study, acetogens seem to switch from acetogenesis to solventogenesis in order to save themselves from damage due to lower external pH (Kundiyana et al. 2011; Barik et al. 1988). This product shift, induced by pH regulation, is important for the potential industrial applications of syngas fermentation by mixed cultures. It is possible to obtain large amounts of ethanol from syngas fermentation at neutral pH. If the goal is to obtain a larger proportion of ethanol, it is necessary to reduce the pH value. It is promising to realize directional ethanol production at acidic pH.

FTHFS and CODH gene quantification

As a key enzyme in the acetyl-CoA pathway, FTHFS is conserved both structurally and functionally (Lovell and Leaphart 2005). Thus the FTHFS gene (*fhs*) has been used as a functional marker for acetogens (Henderson et al. 2010). CODH is the condensing enzyme that forms acetyl-CoA from CO_2 and H_2 (Schnürer et al. 1997; Henstra et al. 2007; Can et al. 2014) and plays an important role in both acetate synthesis and acetate oxidation (Lee and Zinder 1988).

The gene copy numbers of *fhs*, CODH and the percentage of *fhs* containing bacteria to total bacteria at days 15 and 25 of the fermentations are shown in Table 3. The *fhs* gene copy number was of the same magnitude during the anaerobic fermentation process at pH 5, 7 and 9. However, the copy number at pH 11 was 1–3 orders of magnitude lower than at the other pHs (Table 3). Correspondingly, the percentage of *fhs* to total bacteria was the lowest at pH 11 and reduced dramatically from 16.2 to 3.0 % during the final 10 days of fermentation at this pH. During the same time period, the percentage of *fhs* to total bacteria decreased from 70.1 to 51.0 % at pH 7 (Table 3). At pHs 5 and 9, the percentages remained relatively stable. As a result of being flushed with N_2 instead of CO_2/H_2 as substrate, the percentage of *fhs* to total bacteria in the control group decreased significantly from 39.2 to 7.1 % from day 15 to day 25. Obviously, the majority of microbes contained in the mixed cultures cannot sustain themselves in strongly alkaline conditions.

The CODH gene copy number was highest at neutral pH, and was one and two orders of magnitude higher than at pH 9 and 11, respectively. The copy number at pH 7 was also two orders of magnitude higher than that at pH 5 at day 15. As acetogens prefer neutral pH conditions, it is much easier for them to sustain high bacterial cell viability around pH 7, which resulted in the high copy number of key enzyme genes (represented by CODH). The highest CODH gene copy number at pH 7 was generally in accordance with the largest utilization of gas substrates and the highest accumulation of acetic acid.

Bacterial community structures

Sequences obtained from 16S rRNA gene libraries were used to analyze the diversity of bacterial communities associated with mixed cultures exposed to pH 5, 7, 9 and 11 (Fig. 4 and Supplementary Fig. S2 showing phylogenetic trees of the clones based on partial 16S rRNA sequences).

Table 3 Gene copy numbers of *fhs* and CODH, and the percentages of *fhs*-containing bacteria to total bacteria

	fhs (copies g^{-1} $soil^{-1}$)	CODH (RNA copies μL^{-1})	*fhs*/total bacteria (%)
Day 15			
Control group	$10^{6.44}$	$10^{5.03}$	39
pH 5	$10^{7.32}$	$10^{5.17}$	66
pH 7	$10^{7.68}$	$10^{7.49}$	70
pH 9	$10^{7.47}$	$10^{6.62}$	50
pH 11	$10^{6.38}$	$10^{5.98}$	16
Day 25			
Control group	$10^{5.31}$	$10^{3.5}$	7
pH 5	$10^{7.15}$	$10^{5.03}$	63
pH 7	$10^{7.31}$	$10^{5.47}$	51
pH 9	$10^{7.17}$	$10^{4.79}$	45
pH 11	$10^{4.57}$	$10^{3.7}$	3

Ten operational taxonomic units (OTUs) were detected in the raw samples. *Advenella faeciporci*, *Turicibacter sanguinis*, *Defluviitoga tunisiensis* and *Terrisporobacter glycolicus* represented 3.3, 6.2, 6.2 and 10.1 % of the total clone sequences, respectively, with a similarity of 99.0 %. Meanwhile, 20.8 % and 17.2 % of the total clone sequences were assigned to *Romboutsia lituseburensis* and *Clostridium sordellii*, respectively, with a similarity of 98.0 %. Two clone sequences (3.3 % of the total clones) were 97.2 % similar to *Bacillus benzoevorans* and *Tepidiphilus margaritifer*, respectively. *Lachnoclostridium phytofermentans* were the closest bacteria to 29.6 % of the total clone sequences (93.0 % similarity). In addition, one clone sequence (3.3 % of the total clones) was closely related to *Papillibacter cinnamivorans* with a similarity of 88.0 %.

In the clone library from the control group, five OTUs were detected, including species affiliated with *R. lituseburensis*, *C. sordellii*, *T. sanguinis*, *D. tunisiensis* and *Sedimentibacter hongkongensis*. 23.3 and 10.2 % of the total clone sequences could be recognized as *T. sanguinis* and *D. tunisiensis*, with similarities of 99.0 %. 10.1 % of clone sequences were 98.0 % similar to *R. lituseburensis* and 10.1 % of clone sequences were 98.0 % similar to *C. sordellii*. 20.2 % of the clone sequences showed 97.0 % similarity to *S. hongkongensis*. 10.0, 10.0, 3.3 and 3.3 % of the total clones were closely related to *L. phytofermentans*, *Natronoflexus pectinivorans*, *Clostridium mangenoti* and *Sporobacterium olearium*, with respective similarities of more than 90.0 %.

Thirty clone sequences were assigned to two OTUs in the bacterial library at pH 5. Twenty-one clone sequences (70.0 % of the total clones) belonged to *R. lituseburensis* with a similarity of 98.0 % and three sequences (10.0 % of total clones) were affiliated to *C. sordellii* (98.0 % similarity). Four (13.3 % of clones), one and one clone sequences were closely related to *L. phytofermentans*, *Cellulosilyticum ruminicola* and *Thermoanaerobacterium thermosaccharolyticum*, with similarities of 93.0, 96.0 and 94.0 %, respectively.

In the bacterial library obtained from samples fermented at pH 7, thirty clone sequences were assigned to three OTUs. One sequence (3.3 % of total clones) could be recognized as *D. tunisiensis* with a similarity of 99.0 %. Six (20.0 % of clones) and six (20.0 % of clones) clone sequences were affiliated to *R. lituseburensis* and *C. sordellii*, with similarities of 98.0 and 97.0 %, respectively. In addition, four clone sequences each belonged to the two groups that were closest to *S. hongkongensis* and *Anaerovorax odorimutans*, with similarities of 95.0 and 93.0 %, respectively. Four clone sequences (13.3 % of the total clones) were closely related to *L. phytofermentans*, and the others were distributed in *Clostridium ultunense* (3.3 % of clones), *Pelotomaculum schinkii* (3.3 % of clones), *Proteiniphilum acetatigenes* (3.3 % of clones) and *Acetivibrio cellulolyticus* (3.3 % of clones), with similarities >90.0 %.

Thirty clone sequences were assigned to four OTUs in the bacterial library obtained at pH 9. Nineteen (63.3 % of the total clones) and one (3.3 %) sequences were assigned to *A. faeciporci* and *Tissierella praeacuta* with similarities of 99.0 and 98.0 %, respectively. Another four sequences were evenly assigned to OTUs that were close to *S. hongkongensis* and *C. formicaceticum* with similarities higher than 97.0 %. Four sequences were recovered for which *L. phytofermentans* were the closest bacteria to the clones (>90.0 % similarity). The other bacteria found were closely related to *Clostridium acidurici* and *Desulfotomaculum alkaliphilum*, respectively.

Twelve clone sequences from the bacterial library at pH 11 were affiliated to *A. faeciporci* with similarities of 99.0 %. Nine sequences (30.0 % of clones) were recovered with *D. alkaliphilum* being the closest bacteria to the clones (93.0 % similarity). The remaining sequences were assigned to groups that were closely related to *S. hongkongensis*, *P. acetatigenes* and *A. cellulolyticus*, with similarities more than 90.0 %.

In acetogen mixed cultures, the diversity of the bacterial community is influenced by both the richness and abundance of species in the community. Our results showed that the

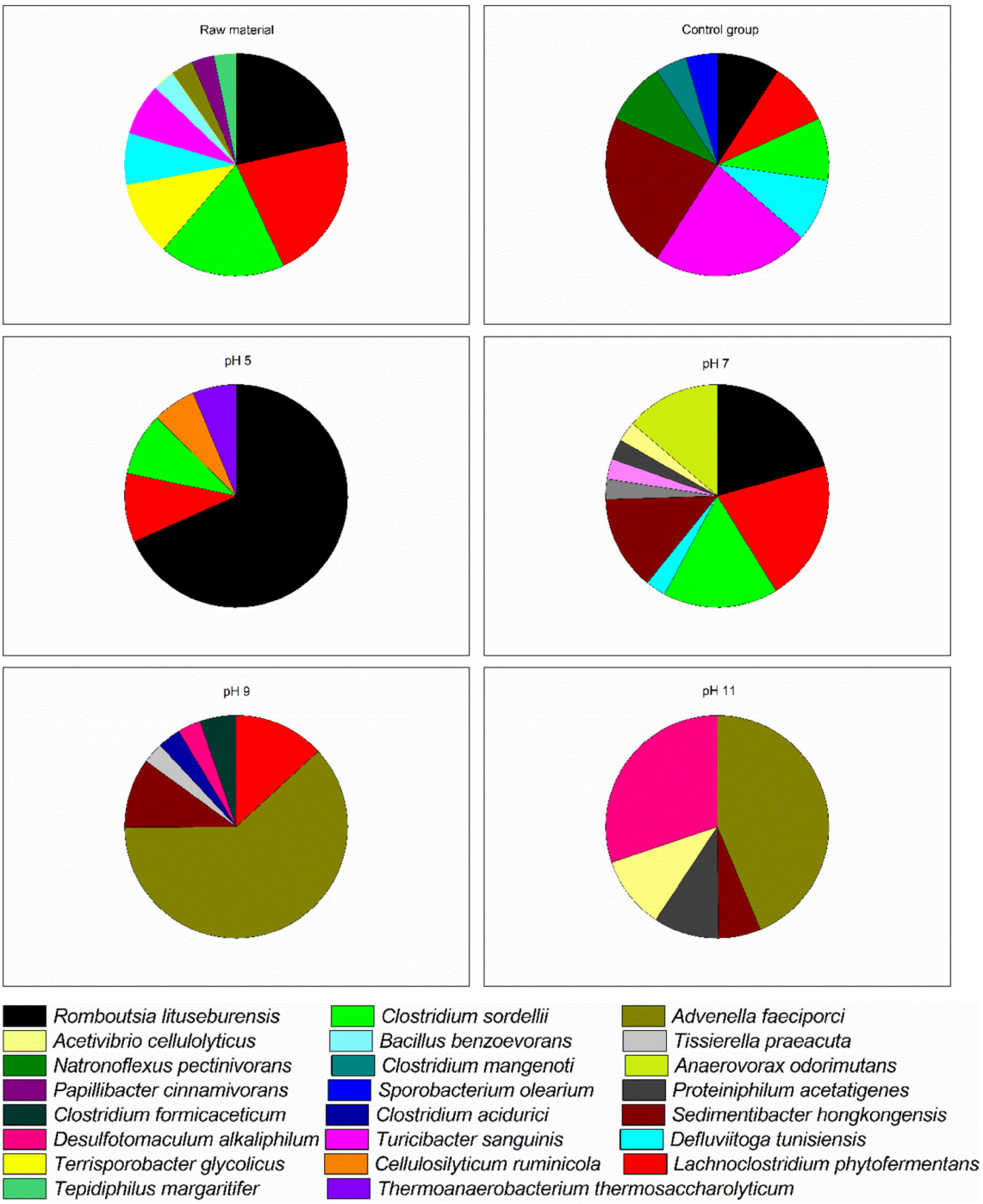

Fig. 4 Distribution of 16S rRNA clones detected in the raw samples, the control samples, and the bacterial communities at pH 5, 7, 9 and 11

bacterial community had the highest species richness at pH 7; the richness decreased at pH 9 and was lowest at pHs 5 and 11. Evenness is also a key factor in preserving biodiversity and could be important for bacterial function in mixed cultures. The bacterial community at pH 7 had the highest species evenness, and that at pH 11 had the lowest. The calculation of the Shannon-Wiener index (H) and Evenness (E) from the number of T-RFs detected by 16S rRNA T-RFLP, indicting species diversity and evenness, showed similar differences between the bacterial communities (Fig. S3).

Discussion

Syngas fermentation into ethanol and other bioproducts by mixed cultures is considered to be an attractive biotechnology. In this study, different acetate and ethanol

production from H_2/CO_2 substrates by acetogen enrichment cultures were induced by pH regulation. The maximum acetate concentration (97.7 mmol L^{-1}) was achieved at pH 7, and decreased in the following order: pH 9 (72.1 mmol L^{-1}), pH 11 (46.0 mmol L^{-1}) and pH 5 (27.5 mmol L^{-1}). The maximum ethanol concentration at pH 7 was 5.5 mmol L^{-1}, which decreased to 3.3, 2.7 and 1.5 mmol L^{-1} at pH 5, 9 and 11, respectively. The results showed an optimum pH of 7 for maximum acetate and ethanol production in the bioconversion of H_2/CO_2 by acetogen enrichment cultures. However, the highest concentration ratio of ethanol to acetate was obtained at pH 5. Thus, higher production of acetate and ethanol was achieved under neutral and weakly alkaline pH conditions, while weakly acidic conditions were good for enhancing the proportion of ethanol in the products. Researchers have reported that some *Clostridium* species switch from acetogenesis (acetic acid production) to solventogenesis (ethanol production) at pH below 5 (Ahmed et al. 2006; Kundiyana et al. 2011).

Fermentation pH can alter the growth and activity of acetogens. pH values above 9 were outside the optimal range for growth and metabolism of the acetogens. Fermentation at pH 5–9 did not reduce the content of acetogens but influenced the number of active acetogens (Table 3). These data indicate that the metabolism of the acetogens shifted in response to the pH, which was associated with different acetate and ethanol concentrations. Firstly, fermentation at pH below 5 and above 9 might cause an unfavorable environment for acetogen growth and adaptation, leading to lower active acetogen concentrations. Secondly, fermentation pH at 5 induced a physiological stress on the acetogenic bacteria and the acetogens regulated the pH gradient between the medium and the intercellular environment by producing solvents such as ethanol (Ahmed et al. 2006). It has been reported that unionized weak organic acids diffuse through cell membranes and result in a lower intercellular pH (Taherzadeh et al. 1997).

pH change from 5 to 11 affected the microbial community strongly. *R. lituseburensis* is the dominant bacterium in the bacterial library at pH 5. Its disappearance from the bacterial libraries obtained at pH 9 and 11 indicated that the clones affiliated with *R. lituseburensis* prefer acidic conditions but can also grow well at neutral pH. Given that the percentage of *R. lituseburensis* at pH 5 was over 68.0 %, but the accumulations of acetate and ethanol at pH 5 were the lowest observed among the four pH conditions, one might suggest that *R. lituseburensis* is not a major acetate and ethanol producer. Similar results were observed for clones affiliated with *L. phytofermentans*. The percentages of clones showing higher than 90.0 % similarity to the 16S rRNA of *L. phytofermentans* in the clone libraries of raw samples, the control group, and pH 7 and 9 samples were 10.0, 10.0, 13.3, and 3.3 %, respectively. However, these bacteria disappeared from the clone libraries at pH 11. We suggest that the clones related to *L. phytofermentans* grew at pH 7–9, which is generally in accordance with the reports of Yutin and Galperin (2013). Zuroff et al. (2013) reported that ethanol was produced from α-cellulose using a consortium of *Clostridium phytofermentans* (latterly *L. phytofermentans*) and yeast. In the present study, lignocellulose was replaced by CO_2/H_2 and the largest amount of ethanol accumulated at pH 7, which is the optimal pH condition for *L. phytofermentans*. Thus there may be a positive correlation between ethanol accumulation and the growth of *L. phytofermentans*.

Our results showed that the percentages of *S. hongkongensis* at pH 7, 9 and 11 were 18.5, 10.0 and 6.1 %, respectively. According to Zhang et al. (1994), *S. hongkongensis* is a Gram-positive, spore-forming, anaerobic rod that utilizes amino acids rather than sugars. Its optimal temperature and optimal pH for growth are 33–34 °C and 7–8, respectively, and acetic acid can stimulate its growth. The highest percentage of *S. hongkongensis* observed, at pH 7, may relate to the highest acetate accumulation, which was also observed at neutral pH.

Clostridium sordellii and *Clostridium difficile* are closely related anaerobic, Gram-positive, spore-forming human pathogens (Liggins et al. 2011). It is feasible to draw analogies between *C. sordellii* and *C. difficile* since most articles about *C. sordellii* are focused on its clinical features. *C. difficile* is able to grow on CO_2/H_2 as sole carbon and energy source to produce acetate as the main product (Köpke et al. 2013). It is reasonable to assume that *C. sordellii* may be also capable of using CO_2/H_2 to produce acetate. The community composition at pH 7 showed that the percentage of *C. sordellii* was 21.1 %, which was 56.9 % higher than at pH 5, corresponding to the acetate accumulation patterns in these pH conditions. In addition, the percentages of clones showing higher than 97.0 % similarity to the 16S rRNA of *C. sordellii* in the clone libraries of raw samples, the control group, and pH 5 and 7 samples were 17.2, 10.0, 10.0 and 20.0 %, respectively. These clones were eliminated in the clone libraries at pH 9 and pH 11, suggesting that bacteria closely related to *C. sordellii* could grow at pH 5–7.

Clones that were close relatives of *A. faeciporci* accounted for 63.3 and 40.0 % of the total clones at pH 9 and 11, but were only detected in those bacterial libraries, suggesting that clones related to *A. faeciporci* grew well in alkaline pH conditions. *A. faeciporci* is a nitrite-denitrifying bacterium (Matsuoka et al. 2012), meaning that it is not a typical acetate or ethanol producer. *D. alkaliphilum* is the major bacterium in the microbiological community at

pH 11. It is a moderately thermophilic, alkaliphilic, sulfate-reducing, chemolithoheterotrophic bacterium, capable of using H_2, acetate, formate, ethanol, lactate and pyruvate as electron donors and sulfate, sulfite and thiosulfate as electron acceptors (Pikuta et al. 2000). Therefore, *D. alkaliphilum* prefers to decompose acetate and ethanol rather than produce them. It is therefore reasonable that we obtained lower acetate and ethanol yields at pH 11 when a consumer of acetate and ethanol was a major constituent of the systems.

The effects of pH on the acetogenic quantity and microbial community were investigated to provide a novel insight into the mechanism of pH impact on gas bioconversion by acetogen enrichment cultures. The maximum acetate and ethanol concentration was achieved at pH 7, and those at pH 9 were in second place. The CODH gene copy number was highest at pH 7, indicating that active acetogens reached their highest level at pH 7 in this study. The CODH gene copy numbers at pH 9 were lower than that at pH 7 but higher than at pH 5 and 11. Correspondingly, the enrichment cultures at pH 7 had the highest species richness and diversity, while those at pH 9 had the second highest diversity and those at pH 5 and 11 had the lowest diversity. The majority of the bacterial populations may be restricted by acidic and strong alkaline conditions, which could explain the lower species diversity and acetate production at pH 5 and 11. In this study, the pH was responsible for the dominance of the microbial communities and the active acetogen contents in gas bioconversion by acetogen enrichment cultures, thus influencing the acetate and ethanol production.

Conclusions

During CO_2/H_2 fermentation by acetogen enriched mixtures, pH change resulted in different bacterial community structure and acetogen contents, thus affecting acetate and ethanol production. The maximum acetate and ethanol concentration was achieved at pH 7, and that at pH 9 took second place. RT-qPCR results show that the CODH gene copy number was highest at pH 7 and second highest at pH 9. Correspondingly, the enrichment cultures at pH 7 had the highest species richness and diversity, while those at pH 9 had the second highest diversity and those at pH 5 and 11 had the lowest diversity.

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