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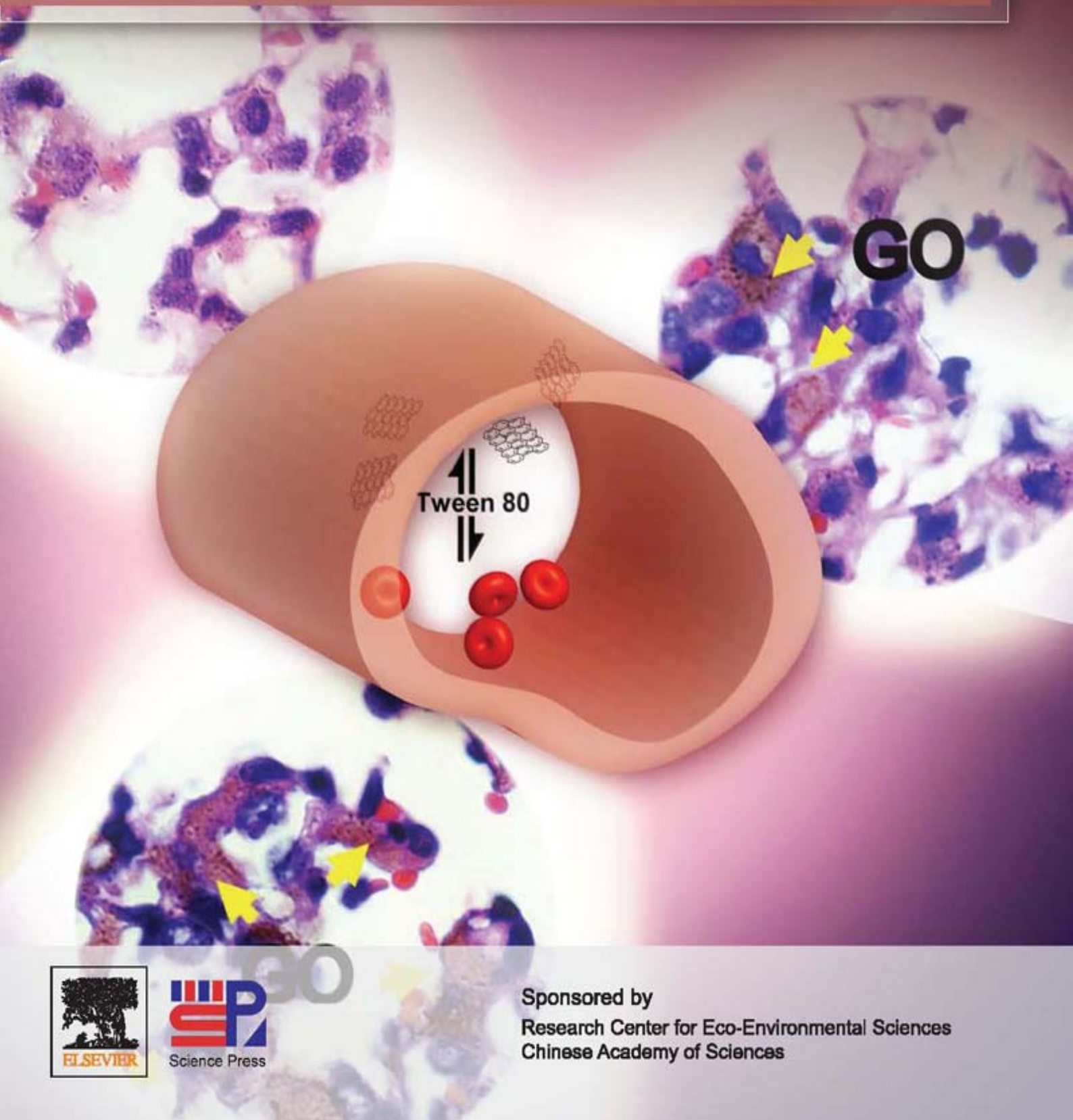

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Response of anaerobes to methyl fluoride, 2-bromoethanesulfonate and hydrogen during acetate degradation

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Abstract

To use the selective inhibition method for quantitative analysis of acetate metabolism in methanogenic systems, the responses of microbial communities and metabolic activities, which were involved in anaerobic degradation of acetate, to the addition of methyl fluoride (CH_3F), 2-bromoethanesulfonate (BES) and hydrogen were investigated in a thermophilic batch experiment. Both the methanogenic inhibitors, i.e., CH_3F and BES, showed their effectiveness on inhibiting CH_4 production, whereas acetate metabolism other than acetoclastic methanogenesis was stimulated by BES, as reflected by the fluctuated acetate concentration. Syntrophic acetate oxidation was thermodynamically blocked by hydrogen (H_2), while H_2-utilizing reactions as hydrogenotrophic methanogenesis and homoacetogenesis were correspondingly promoted. Results of PCR-DGGE fingerprinting showed that, CH_3F did not influence the microbial populations significantly. However, the BES and hydrogen notably altered the bacterial community structures and increased the diversity. BES gradually changed the methanogenic community structure by affecting the existence of different populations to different levels, whilst H_2 greatly changed the abundance of different methanogenic populations, and induced growth of new species.

Key words: methanogenic inhibitor; hydrogen; thermophilic anaerobic digestion; microbial diversity; denaturing gradient gel electrophoresis (DGGE)

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Introduction

Anaerobic digestion of biomass for methane production is attracting increasing attention owing to its potential as a substitute for fossil fuels and to reduce carbon dioxide emissions. A better understanding of the mechanisms responsible for methane production will help to improve operational strategies and to solve the existing technical problems such as the accumulation of volatile fatty acids (VFAs) and the following inhibition of methanogenesis. Externally added selective inhibitors, therefore, present potentially powerful tools in the study of microbial processes.

In general, there are specific and nonspecific inhibitors. For example, chloroform ($CHCl_3$), which was firstly found to inhibit methanogenesis by Bauchop (1967), is known to block the function of corrinoid enzymes and to inhibit methyl-coenzyme M reductase of methanogens (Gunsalus and Wolfe, 1978). As a classic nonspecific inhibitor for methanogens, it can also inhibit the activity of homoacetogens and acetate-consuming sulfate-reducing bacteria (Scholten et al., 2000). By contrast, 2-bromoethanesulfonate (BES), as a structural analogue of coenzyme M, was used to block methane formation by methanogens (Zinder et al., 1984). A further inhibitor, methyl fluoride (CH_3F), has been widely used to specifically inhibit acetoclastic methanogenesis (AM) with hydrogenotrophic methanogenesis (HM) unaffected, if appropriate dose was applied (Janssen and Frenzel, 1997). Vinyl acetate also showed the potential as an AM inhibitor, which was recently reported to irreversibly inhibit AM (Durán et al., 2011). However, its inhibitory effect on various anaerobic metabolisms needs to be further confirmed. Thus, to separately investigate different methanogenic pathways, without significantly disturbing other non-methanogenic metabolisms, combined application of CH_3F and BES is the best choice. Quantitative information on the contribution of different metabolic pathways to methane generation and waste reduction is expected to be obtained by this method. In fact, these two classic inhibitors have been widely used as metabolic probes for quantitative analysis of the carbon and electron flow in natural and engineered methanogenic systems (Xu

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et al., 2010).

Although the inhibition of methanogenesis by CH_3F and BES in anaerobic environments has been well established, most studies only focused on the practical applications. Little is known about their effects on the *in situ* diversity of microbial community structures. Recently, BES has been reported to inhibit or promote the growth of certain bacteria (Chiu and Lee, 2001). Due to the potential nonspecific or unintended effects of the methanogenic inhibitors, further study is necessary to fully understand their influence on the ecology of microbial community in the target system.

Metabolism of acetate is a key link in the degradation of accumulated VFAs and initiation of methanogenesis. Two pathways could be responsible for the methanogenic conversion of acetate: AM and the syntrophic acetate oxidation (SAO) coupled with HM. Thermodynamically, SAO is inferior to AM and can only proceed if H_2 partial pressure (pH_2) is kept at a low level (**Table 1**). On the contrary, acetate production via homoacetogenesis (HA) is favorable at high pH_2. Although AM was generally considered as the main mechanism for methane production, the potential significance of SAO was gradually recognized recently (Hao et al., 2011). It has been found in various anaerobic environments (Zinder and Koch, 1984; Li et al., 2009; Liu and Conrad, 2010), and to be predominant under thermophilic or other stressed conditions (Schnürer et al., 1999; Karakashev et al., 2006; Qu et al., 2009). To study the metabolism involved in methanogenic degradation of acetate at high concentrations, the application of inhibitors to separate different pathways is a considerable choice.

In the present work, CH_3F and BES were used to inhibit AM pathway and total methanogenesis respectively. Since there are not specific inhibitors for SAO, we created conditions with high pH_2 to thermodynamically block SAO. Their effects on the microbial diversity and metabolic activities of the involved reactions were studied during the incubation with high concentration of acetate, by using denaturing gradient gel electrophoresis (DGGE) fingerprinting of the bacterial and archaeal communities, and by monitoring the carbon flow and thermodynamic analysis.

1 Materials and methods

1.1 Experimental set-up

All experiments were performed with freshly collected methanogenic granular sludge cultivated at 55°C in the dark. Twenty milliliters of sludge mixture was transferred into each 1.2-L reactor with 450 mL basal medium. Acetate was added as the substrate to reach a final concentration of 100 mmol/L to simulate the VFAs accumulation. Detailed information on the seed sludge, added solution and gas phase control was described by Hao et al. (2011). The pH range was 6.8–7.8, regulated by using 5 mol/L H_3PO_4 solution.

Total methanogenesis was inhibited by addition of 2-bromoethanesulfonate (BES) (98.5%, Aladdin, China) to final concentration of 50 mmol/L. For specific inhibition of AM, CH_3F (99%, Shanghai Chunyu Special Gas Corporation Limited, China) was injected to reach a content of 2.5% (V/V) in the headspace after equilibrium between the gaseous and liquid phases. To provide high pH_2

Table 1 Reactions involved in methanogenic conversion of acetate

Pathway	Reaction	$\Delta G^{0\prime}$ (kJ/mol)
(1) Acetoclastic methanogenesis (AM)	$CH_3COOH \rightarrow CH_4 + CO_2$	–31.0
(2) Syntrophic acetate oxidation (SAO)	$CH_3COOH + 2H_2O \rightarrow 2CO_2 + 4H_2$	+104.6
(3) Hydrogenotrophic methanogenesis (HM)	$4H_2 + CO_2 \rightarrow CH_4 + H_2O$	–135.6
(4) Homoacetogenesis (HA)	$4H_2+2CO_2 \rightarrow CH_3COOH + 2H_2O$	–104.6
(5) Sum (2) + (3) (SAO-HM)	$CH_3COOH \rightarrow CH_4 + CO_2$	–31.0

The standard Gibbs free energy change ($\Delta G^{0\prime}$) values were calculated from Thauer et al. (1977).

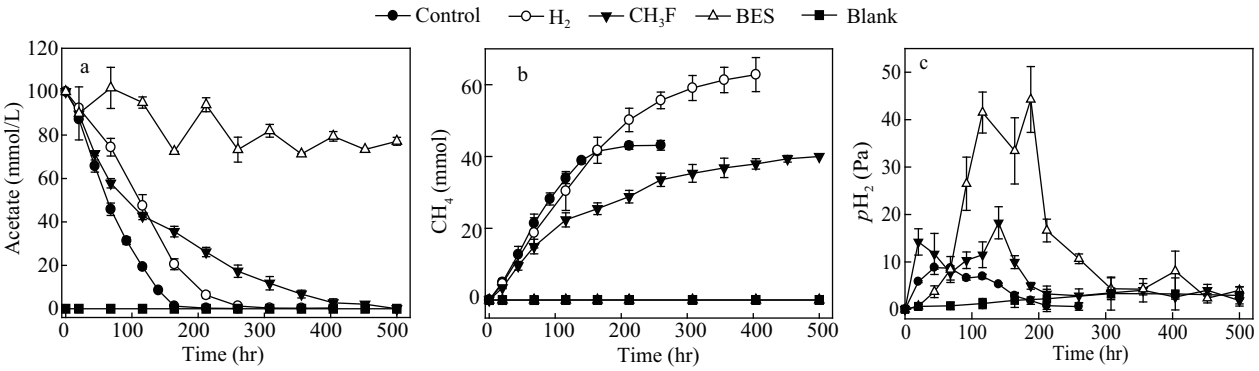

Fig. 1 Temporal change in acetate concentration (a), accumulated methane (b) and hydrogen partial pressure (pH_2) (c). Gas sampling was stopped three days after the acetate could not be detected.

condition, pure H_2 (99.999%, Shanghai Chunyu Special Gas Corporation Limited, China) was used instead of N_2 to refill the headspace of the H_2-amended reactors. Both CH_3F and BES doses were selected based on literature values (Janssen and Frenzel, 1997; Zinder et al., 1984) and preliminary experiments conducted in our laboratory. Endogenous methanogenesis was studied as the blank without acetate and inhibitor addition. Treatments, blanks and controls without inhibitor were incubated statically in duplicate at 55°C in the dark. Liquid and gas samples were taken periodically for analysis until the added acetate was exhausted, and the gaseous phase was exchanged with N_2 or H_2 after each sampling event.

1.2 Analysis of the samples

Gas composition (CH_4, CO_2 and H_2), volume and pressure were periodically monitored. The liquid samples were analyzed for pH, total organic carbon, total inorganic carbon and VFAs. The analytical methods were described elsewhere (Hao et al., 2011).

1.3 DNA extraction and PCR-DGGE

Sludge samples were taken from each reactor after the added acetate was exhausted, while at 500 hr for the blank. To capture the hypothesized gradual change of the microbial community structure in the BES-treated sets, sludge was sampled at 116, 212, 356 and 500 hr. DNA was extracted from the seed and incubated sludge. The strategy for DNA extraction and PCR-DGGE was described in our previous work (Hao et al., 2011).

1.4 Data processing

The thermodynamic analysis referred to the study of Lee and Zinder (1988). Based on the DGGE profiles, the structural diversity of the microbial community was estimated by the Shannon index of general diversity (H) as described by Tiwar and Mishra (1993). The evenness of microbial structure was evaluated by the indice of evenness (E) (Pielou, 1966) and Pareto-Lorenz (PL) curves (Wittebolle et al., 2008). For the latter, the value of cumulative proportion of intensities corresponding with the 20% level on the proportion of band numbers (20%PL evenness) was used to characterize the functional organization of the populations.

2 Results

2.1 Methanogenic conversion of acetate

Figure 1a shows that methanogenesis started up immediately and acetate was completely utilized in 212 hr in the control. While, CH_3F prolonged the incubation time to nearly 500 hr for depletion of the added acetate, obviously due to the inhibition of AM. Addition of H_2 slowed down the consumption of acetate compared with the control, especially in the initial period. It seemed that the suppression of SAO by quite high pH_2 contribute to the decreased acetate-utilizing rate. Interestingly, acetate concentration slowly decreased in the BES-treated sets, with substantial fluctuation during the long-term incubation. Considering methanogenesis was already completely inhibited, it indicated that metabolism of acetate by other microbes was stimulated by BES.

CH_4 produced in the H_2-amended set was nearly 34%–37% higher than that in the control and CH_3F-treated one (**Fig. 1b**). The increase of CH_4 production was most probably due to the active HM reaction under high pH_2.

Figure 1c indicates that, in the initial period of incubation, fast conversion of acetate was accompanied by accumulation of H_2, implying SAO was involved in the process. Nevertheless, pH_2 suddenly decreased and remained at quite low levels in the later stage, which might result from the change of SAO and HM activities or the participation of other H_2-utilizing bacteria.

2.2 Thermodynamic analysis

ΔG_{SAO} in the control, CH_3F- and BES-treated sets were quite negative in the beginning and then increased with incubation time, and finally reached about –20 kJ/mol, while that in the H_2-treated one was always highly positive (**Fig. 2a**), demonstrating that SAO was thermodynamically unfeasible. ΔG_{HM} and ΔG_{HA} maintained at quite low levels after addition of H_2, which indicated the potential utilization of H_2 via HM and HA, with the former reaction being more favorable. ΔG_{HM} in the control and CH_3F-treated sets was around the thermodynamic threshold of –20 kJ/mol throughout the incubation, which was likely to result from the low pH_2 induced by the gas exchange or a close coupling of SAO and HM. Similar phenomena showing that microbial metabolism can proceed close to thermodynamic limits have also been discovered in other anaerobic environments (Jackson and Mclnerney, 2002). AM, on the other hand, was always thermodynamically feasible during the incubation.

2.3 Microbial community structure

Figure 3A illustrates the DGGE profiles of the PCR-amplified 16S rRNA gene fragments of bacteria from the seed sludge and different cultures after incubation for 500 hr. It seems that, CH_3F-treatment did not affect the bacterial structure compared with the control, with H for CH_3F-treatment and control being 1.79 and 1.74 respectively (**Table 2**). On the contrary, the addition of BES greatly changed the structure of bacterial community and improved the diversity (H = 1.91). For the culture receiving BES, 3 bands essentially disappeared, and 6 new bands came out. As indicted by the presence of several new bands, H_2 addition obviously increased the diversity of bacterial community structure (H = 2.54), with the possible enrichment of more H_2-utilizing bacteria.

It was observed that the acetate concentration period-

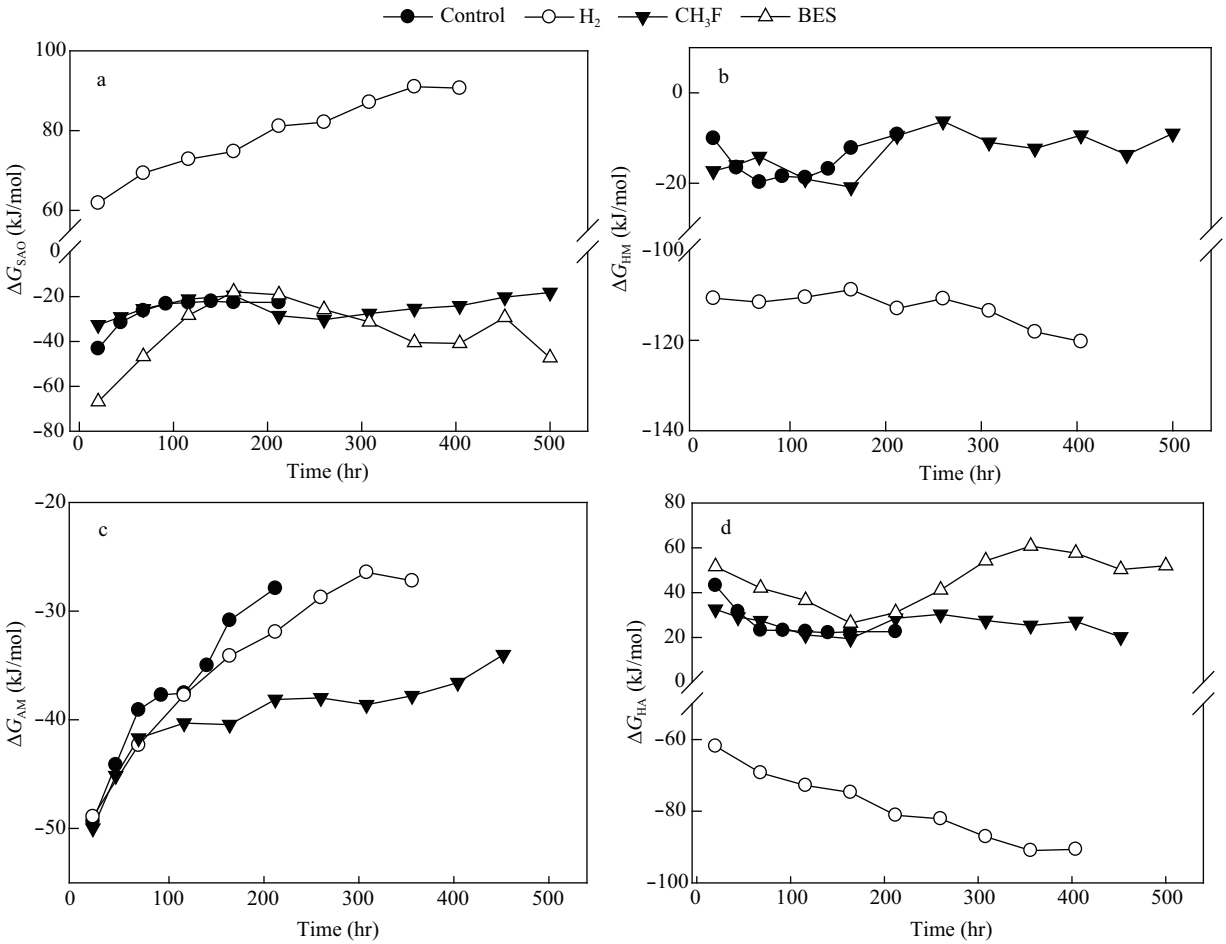

Fig. 2 Temporal change of Gibbs free energy (ΔG) for syntrophic acetate oxidation (a), hydrogenotrophic methanogenesis (b), acetoclastic methanogenesis (c) and homoacetogenesis (d).

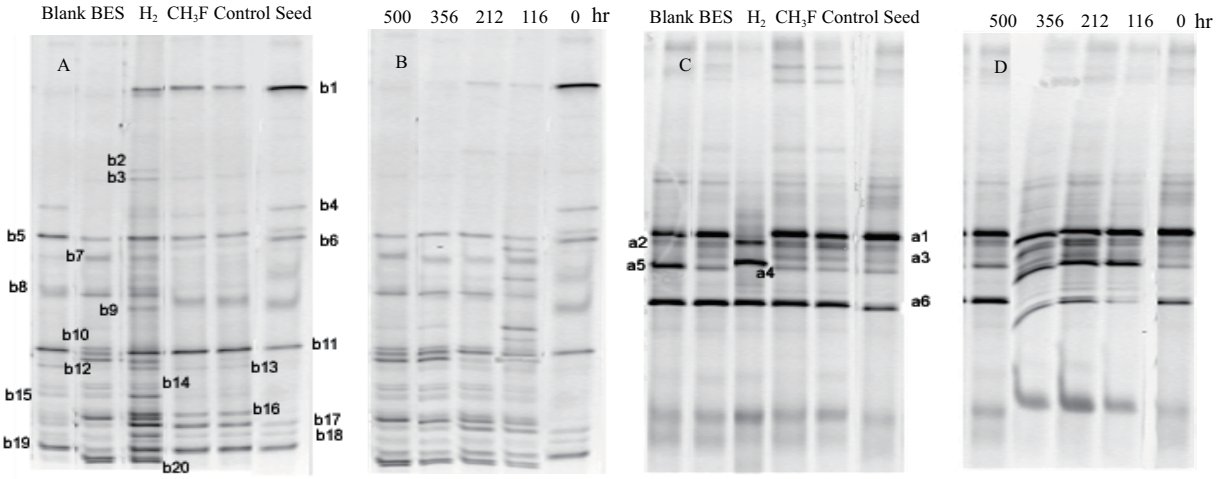

Fig. 3 DGGE fingerprints of the bacterial community in the inoculum, blank and cultures after incubation with 100 mmol/L acetate (A) and in the BES culture after incubation for 0, 116, 212, 356 and 500 hr (B); the archaeal community in the inoculum, blank and cultures after incubation with 100 mmol/L acetate (C) and in the BES culture after incubation for 0, 116, 212, 356 and 500 hr (D).

ically fluctuated, which we hypothesized to be induced by the shift of the functions or structure of the microbial populations. To validate this, we monitored the evolution of the microbial community structure in the BES-treated cultures. As shown in **Fig. 3B**, after 116-hr exposure to BES, H quickly increased from the initial 1.53 to 2.16,

Table 2 Summary information for the DGGE profiles in the present study

	Bacteria				Archaea			
	H[a]	E[b]	20%PL evenness[c]	S[d]	H[a]	E[b]	20%PL evenness[c]	S[d]
Seed	1.53	0.6	66%	9	1.35	0.65	56%	13
Control	1.74	0.68	53%	15	1.38	0.67	51%	11
CH_3F	1.79	0.7	53%	22	1.39	0.67	51%	17
H_2	2.54	0.78	40%	26	1.38	0.66	50%	12
BES	1.91	0.71	56%	15	1.36	0.65	53%	16
Blank	1.51	0.63	60%	11	1.32	0.64	51%	8
0 hr	1.53	0.6	66%	9	1.35	0.65	53%	13
116 hr	2.16	0.76	41%	17	1.34	0.65	54%	5
212 hr	1.97	0.75	42%	14	1.51	0.73	43%	14
356 hr	1.99	0.72	51%	16	1.52	0.73	43%	11
500 hr	1.91	0.69	56%	15	1.36	0.65	54%	16

[a] Shannon index of general diversity (H) as described by Tiwar et al. (1993); [b] indice (E) to evaluate the evenness of microbial structure as calculated by Pielou (1966); [c] based on the Pareto principle, the cumulative y axis value (in this case the proportion of intensities) corresponding with the 20% level on the x axis (in this case the cumulative proportion of band numbers) (20%PL evenness) used to evaluate the Lorenz curves by Wittebolle et al. (2008); [d] richness (S) of the bacterial and archaeal communities (Chau et al., 2011), which is the number unique taxa present in the sample (here represented by the number of bands in the DGGE lanes).

with 9 new bands emerged although most of them disappeared later. To note that, H_2 and acetate both accumulated during this stage. At 212 hr, several bands disappeared with H decreased slightly (H = 1.97), accompanied with the sudden decrease of pH_2. The stage after 356 hr was characterized by low pH_2 and fluctuant acetate concentration. Just at this stage, the DGGE band patterns began stabilized, with the calculation of H around 1.9, which indicated the formation of a stable bacterial community structure. The newly appeared bands such as b11, b12, b16 and b20 in the lane BES were also present in the lane H_2, implying their appearance was closely related with the presence of abundant H_2.

As shown in **Fig. 3C**, mainly 5 bands were detected in the seed sludge with a1 and a6 predominating in the archaeal DGGE lanes. The lane patterns did not change much after a long-term incubation in control, blank, CH_3F- and BES-treated sets with H around 1.36. By contrast, the methanogenic community structure was greatly altered by the supply of H_2. In the lane H_2, a1 was eliminated, and a2 and a6 were promoted with the appearance of a new band a4.

Sequencing analysis (**Table 3**) showed that, the sequence of a1 was related to an acetotrophic mathanogen *Methanosarcina thermophila* (97% similarity in 16S rRNA gene sequence), while sequences of a2 and a3 were similar to hydrogenotrophic *Methanobacterium* species (96% and 98% similarity) with a5 being assigned to *Methanothermobacter* species (97% similarity). During the 500 hr incubation in the presence of BES, bands a2 and a5 became quite intensive while a1 and a6 got relatively fainter and fainter, indicating the methanogenic community structure gradually changed.

Figure 4 illustrates the PL curve distribution patterns of the DGGE profiles. For the bacterial community in different treatments, it was observed that all the PL curves

Table 3 Phylogenetic sequence affiliation of amplified 16S rRNA gene sequences excised from DGGE gels

Band ID	Phylogenetically most closely related organism (accession no.)	Similarity
Amplified 16S rRNA gene sequences (around 100 bp) excised from archaeal DGGE gels		
a1-1	*Methanosarcina thermophila* (M59140)	97%
a1-2	Uncultured archaeon (EF512456)	97%
a2	Uncultured *Methanobacterium* sp. (AB479402)	96%
a3	*Methanobacterium* sp.169 (AB368917)	98%
a5	*Methanothermobacter marburgensis* (EU807735)	97%
a6	Uncultured archaeon (AB539930)	97%
Amplified 16S rRNA gene sequences (150–170 bp) excised from eubacterial DGGE gels		
b1	Bacteroidales bacterium (GU129118)	98%
b2	Uncultured bacterium clone QEEA1CG01 (CU918505)	94%
b4	Uncultured bacterium clone 822_12_pH7_1 (GQ453634)	98%
b5	Uncultured Firmicutes bacterium (AB451804)	96%
b6	*Bacillus* sp. (EU938359)	95%
b11	Uncultured bacterium (FN436125)	98%
b13	Uncultured bacterium clone NGA11 (EF613928)	98%
b16	Uncultured manure pit bacterium (AF261818)	94%
b17	Uncultured bacterium (FJ227290)	99%
b19	Uncultured Thermotogae bacterium (CU918794)	97%

Multi-fragments named a1-1 and a1-2 were found in the single band a1.

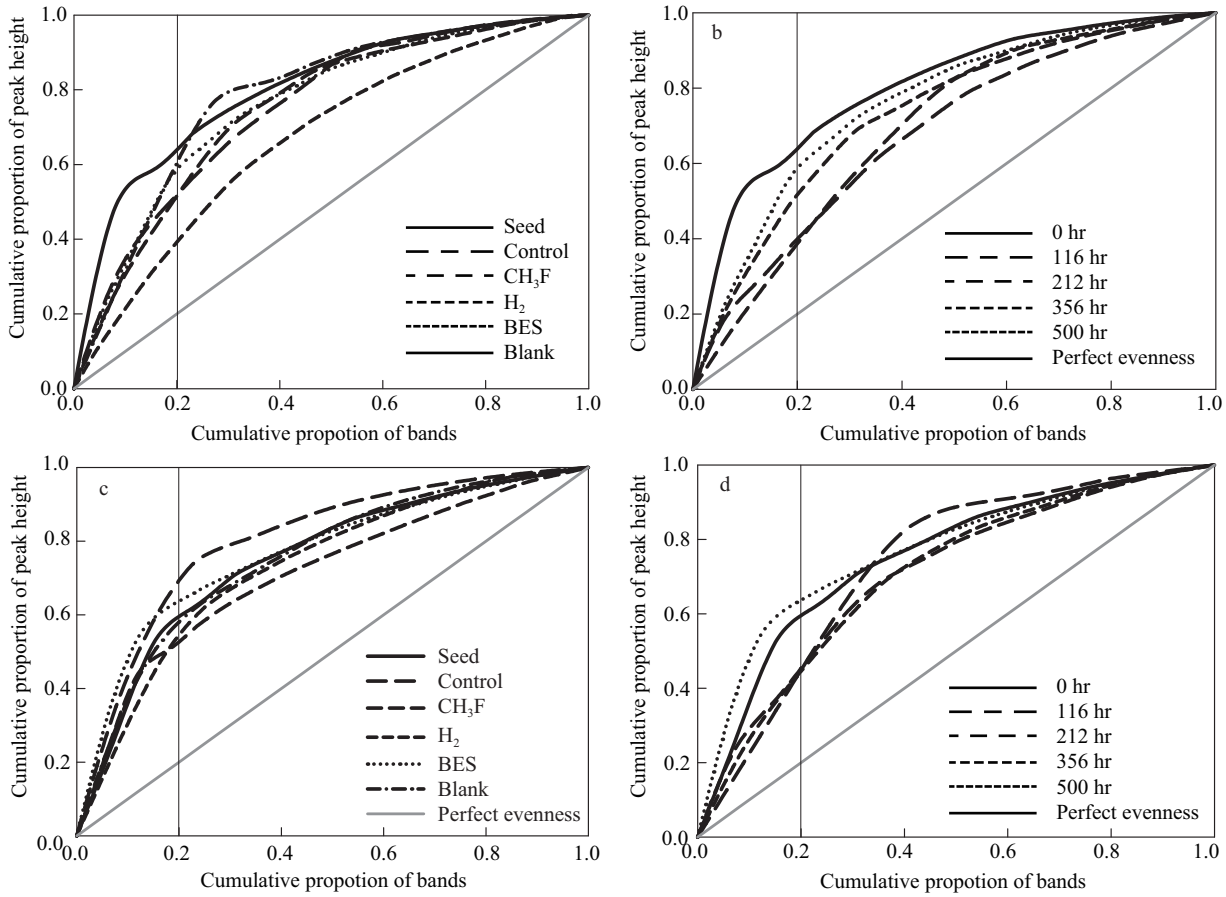

Fig. 4 Pareto-Lorenz distribution curves based on PCR-DGGE analysis for (a) the bacterial community structure in the inoculum, blank and cultures after incubation with 100 mmol/L acetate (a) and in the BES culture after incubation for 0, 116, 212, 356 and 500 hr (b); the archaeal community structure in the inoculum, blank and cultures after incubation with 100 mmol/L acetate (c) and in the BES culture after incubation for 0, 116, 212, 356 and 500 hr (d).

were similar except that of the H_2-incubated one (**Fig. 4a**). In these sets, 20%PL evenness was 53%–66% with E ranged from 0.60 to 0.71, indicating only a small group of bacterial species were numerically dominant (**Table 2**). However, the H_2-amended PL curve represented bacterial community with much higher evenness (E = 0.78) in which 20%PL evenness was 40%. Sufficient substrates of H_2 and acetate in the H_2-amended set reduced the environmental stress and the competition among microbial species, thus led to higher diversity and evenness.

For the archaeal community (**Fig. 4c**), the similar PL curves, in which 20%PL evenness was 50%–56%, indicated that there was no significant difference in the evenness of microbial species (E = 0.64–0.67). While, for the BES-treated set, both the bacterial and archaeal communities experienced a first increase and a following decrease in evenness (**Fig. 4b, d**). It is likely that some bacteria could use BES as a substrate, and that BES may also selectively exclude some bacteria, and thus change the microbial structure and diversity. Evolution of the functional populations occurred during this process, as implied by the fluctuation in the microbial structure diversity and evenness.

3 Discussion

3.1 Effect of methanogenic inhibitors

The analysis based on the PCR-DGGE fingerprinting showed, application of CH_3F did not have much influence on the microbial diversity, while long-term exposure to BES altered both the archaeal and bacterial community structures. Similar results were reported in the previous studies. For example, Penning and Conrad (2006) found that CH_3F could change the population size of the methanogens due to the specific inhibition on AM, while did not affect the diversity of the methanogeinc community. Nevertheless, there is still not much detailed information on the effect of CH_3F on bacterial populations. BES has been shown to directly or indirectly inhibit certain non-methanogenic microbes such as some dechlorinating organisms, gram-negative bacteria and syntrophic acetogenic bacteria (Chidthaisong and Conrad, 2000; Chiu and Lee, 2001; Scholten et al., 2000). It was speculated that BES affected some of these bacteria directly by acting as a competing substrate or through other mechanisms, since the sulfonate moiety of BES can serve as an alternative electron acceptor for sulfate-reducing bacteria (Chiu and

Lee, 2001), and that might be responsible for the sudden decrease of pH_2 and the fluctuation of acetate levels in this study. Alternatively, bacteria which consume and/or produce substrates for methanogens, such as H_2 and acetate, would be affected indirectly by BES through the inhibition of methanogens. For instance, Xu et al. (2010) observed that BES stimulated the homoacetogenesis from H_2/CO_2 under suppressed methanogenesis. Treatment by BES also altered the methanogenic community structure, with different changing behaviour of the bands representing different methanogenic species. It was reported that the concentration of BES used for inhibiting AM (1.0 mmol/L) was much higher than that for inhibiting HM (50 mmol/L) (Zinder et al., 1984). Belay and Daniels (1987) also found that BES could completely inhibit two *Methanococcus* species, while only partially inhibited two *Methanobacterium* strains. Hence, the change of the DGGE band patterns might be due to the different sensitivities of the methanogenic populations to BES. In consideration of these unspecific effects, one should carefully determine the incubation time and exercise great caution in interpreting the data when using BES to specifically inhibit methanogenesis.

3.2 Effect of high hydrogen partial pressure

As mentioned above, the reduction of acetate-consumption rate may result from the suppression of SAO by quite high pH_2, however, the possible participation of homoacetogens may produce acetate from H_2 and CO_2, which was a potential explanation as well (Nozhevnikova et al., 2007). Addition of H_2 could greatly change the microbial structure as promoting the growth of H_2-utilizing microbes, especially those with low hydrogen affinity. pH_2 in the H_2-incubated sets was always above 4 kPa (data not shown), while that without H_2 addition kept below 50 Pa throughout the incubation. Thus, under low pH_2 conditions, the H_2-consuming bacteria with low H_2 affinity like the homoacetogens could not compete with those with high affinity such as the sulfate reducers and hydrogenotrophic methanogens. For instance, the species of homoacetogenic *Acetobacterium* have much higher threshold concentration of H_2 at 52–95 Pa (Cord-Ruwisch et al., 1988). These low-H_2-affinity microbes might only proliferate with external H_2 addition. Besides, the mixtrophic *Methanosarcina* could alter its methanogenic pathway from acetoclastic to hydrogenotrophic under specific conditions (Qu et al., 2009), that could also be an alternative reason at such high pH_2.

Therefore, H_2 is not proper to be considered as a metabolic blocker in the methanogenic systems, since other related reactions or the microbial behavior were quickly influenced due to the H_2 addition.

It is worth noting that, the shortcomings of DGGE should not be ignored. For example, not all the visible bands can produce sequences that could be used satisfactorily in the BLAST analysis, since the sequences retrieved from them are not long enough. The PCR bias, presence of more than one band due to heterogeneous rRNA operons in the same organism, one band representing different organisms due to identical partial sequences of the 16S rRNA gene, etc., all contribute to difficulties in the precise determination of community complexity. Meanwhile, there was no metabolite flux analyzed by isotopic tracers, which limits the identification of functionally active microbes. Therefore, it is suggested to use more powerful techniques such as stable isotope probing combined with metagenomics to identify microorganisms involved in specific metabolic processes.

4 Conclusions

The process performance and DGGE profiles under different incubation conditions were compared. The results demonstrated, during the thermophilic anaerobic degradation of acetate at high concentration, CH_3F effectively inhibited acetoclastic methanogenesis, while the microbial community structure kept similar without significant change in the microbial diversity; BES completely inhibited methane production while stimulated other acetate metabolism, and the bacterial and archaeal community structures were gradually changed with the microbial diversity increased; H_2 addition thermodynamically blocked syntrophic acetate oxidation, yet promoted H_2-utilizing reactions as hydrogenotrophic methanogenesis and homoacetogenesis, and therefore, significantly altered the bacterial and archaeal community structures and increased the microbial diversity.

Acknowledgments

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Supporting materials

Supplementary data associated with this article can be found in the online version.

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Supporting materials

Thermodynamic calculation

Gibbs free energy values of acetate oxidation (ΔG_{SAO}), homoacetogenesis (ΔG_{HA}) and CH_4 production directly from acetate (ΔG_{AM}) and H_2/CO_2 (ΔG_{HM}) were calculated from the actual concentrations of reactants and products using Nernst's equation as follows:

$$\Delta G_{SAO} = \Delta G^{0'}_{SAO} + RT\frac{[HCO_3^-]^2(pH_2)^4}{[CH_3COO^-]} \quad (1)$$

$$\Delta G_{AM} = \Delta G^{0'}_{AM} + RT\frac{pCH_4[HCO_3^-]}{[CH_3COO^-]} \quad (2)$$

$$\Delta G_{HM} = \Delta G^{0'}_{HM} + RT\frac{pCH_4}{(pH_2)^4[HCO_3^-]} \quad (3)$$

$$\Delta G_{HA} = \Delta G^{0'}_{HA} + RT\frac{[CH_3COO^-]}{(pH_2)^4[HCO_3^-]^2} \quad (4)$$

where, $\Delta G^{0'}_{SAO}$, $\Delta G^{0'}_{HA}$, $\Delta G^{0'}_{AM}$ and $\Delta G^{0'}_{HM}$ represent the Gibbs free energy values of acetate oxidation, homoacetogenesis and CH_4 production directly from acetate and H_2/CO_2 at standard state conditions, in which the partial pressures of any gases involved in the reaction is 10^5 Pa, the concentrations of all aqueous solutions are 1 mol/L, and the temperature for reaction is 55°C (328 K), as corrected by Lee and Zinder (1988); R is the ideal gas constant of 8.314 J/(mol·K); T represents the temperature which is 328 K in this study; pH_2 and pCH_4 are the contents of H_2 and CH_4 in the gaseous phase; CH_3COO^- (mol/L) and HCO_3^- (mol/L) represent the concentration of these anions in the liquid phase.

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