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Degradation of Ethanol and Ethanethiol By Methanosarcina Horinobensis

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ABSTRACT

In this study, *Methanosarcina horinobensis* was used as the object to explore the degradation mechanism of sulfur-containing small molecular organics (ethanethiol, ethanol, etc.) and the formation process of hydrogen sulfide (H_2S) and methane (CH_4) under strict anaerobic conditions. Through anaerobic fermentation experiments, combined with gas chromatograph and ion chromatograph analysis of gas and liquid phase composition changes, it was found that when ethanol was used as the substrate, the methanogenic capacity of the flora reached a peak at 14~26 days, and the cumulative gas production was 21.317 mL/g, and the concentration of CO_2 fluctuated. When ethanethiol was used as the substrate, the production of H 2 S reached the peak (6.78 mL/g) in the first 4 days of fermentation, and the production of CH was weak (0.00116 mL/g), and the peak period of the two was consistent. The liquid phase analysis showed that the concentration change of sulfur-containing inorganic ions (such as SO_4^{2-} and $S_2O_3^{2-}$) was not directly related to the formation of H_2S, suggesting that the degradation of ethanethiol directly generated H_2S and CH_4. The study revealed that the strain catalyzed the decomposition of methyl compounds by methyltransferase, and used HS-CoM as an electron donor to convert methyl groups into CH_4, accompanied by the formation of H_2S. The conclusion shows that methanogens can directly use sulfur-containing methyl compounds, which provides a theoretical basis for revealing the biological origin and control technology of H_2S in coal seams.

KEYWORDS

Methanosarcina horinobensis; Ethanol; 2-Ethylthiol; H_2S; CH_4.

1. INTRODUCTION

Many coal mines have been threatened by hydrogen sulfide (H_2S) to varying degrees, especially the high-sulfur coal seams of the late Permian Longtan Formation in the south and the early Permian Taiyuan Formation in the north[1-5]. With the gradual increase of mining depth, the abnormal emission of hydrogen sulfide in coal seam is becoming more and more frequent, which seriously threatens the safe and efficient mining of coal mine and the physical and mental health of personnel. Therefore, it is more and more important to find out the way of hydrogen sulfide production in order to find a way to inhibit its production [6]. At present, the causes of H_2S in natural gas (coalbed methane) are generally considered to have two kinds of biological and thermal causes. Biogenesis includes biodegradation dominated by the decomposition of sulfur-containing organic matter and microbial sulfate reduction. Thermogenic H_2S is divided into thermochemical decomposition and sulfate thermochemical reduction. Thermochemical decomposition is that sulfur-containing organic compounds are broken and decomposed to produce a large amount of H_2S under the action of heat[7].

However, with the deepening of research, the causes of H_2S are far more than these two. Some denitrifying bacteria use plant remains to degrade to produce H_2S, and some microorganisms degrade organic sulfides to produce H_2S. The main degradation method of organic sulfides is biological method. The biological method [8-10] has attracted much attention due to its mild reaction conditions, low energy consumption, low operating costs, and small secondary pollution. The premise of the biological method is to screen one or more single strains that can efficiently tolerate and degrade sulfides. At the same time, it is also hoped that the reasons for the efficient degradation of strains can be understood and explained from the perspective of mechanism and molecular biology [11], so as to grasp the key to the degradation of sulfides by degrading bacteria, improve the efficiency of degrading strains for organic sulfides from the source, and reduce the rate-limiting link in the degradation process.

At present, there are few studies on biological hydrogen sulfide in coal seams at home and abroad, and there is little detailed description of the degradation mechanism of organic sulfide. Studies have shown that under anaerobic conditions, the possible degradation pathway of methyl mercaptan (MT) is that MT is converted into DMDS, DMS, DMSO and $DMSO_2$ in turn, and finally SO_4^{2-}can be generated by methanesulfonic acid (MSA)[12, 13]. The methanogenic transformation of dimethyl sulfide (DMS) can be explained in lake sediment slurry, in which 14C-DMS is converted into ^{14}C-CH_4 and ^{14}C-CO_2 [14, 15]. Through the study of inhibitors in marine and estuarine sediments, it is concluded that methanogens and sulfate-reducing bacteria compete for DMS when the concentration is lower than 10μM, while methanogens dominate DMS degradation at higher concentrations [16]. Methanogens reduce DMS to methane and methyl mercaptan (MT/MeSH), which is then disproportionated to CH_4, CO_2 and H_2S.

DMSO metabolism is mediated by DMSO reductase, and NADH is the preferred electron donor [17, 18] to promote its reduction to DMS and H_2O. The stoichiometry of DMS formation is that 2 mol DMSO will be reduced by adding 1 mol formaldehyde, which is consistent with the role of NAD^+-dependent formaldehyde and formate dehydrogenase in the oxidation of formaldehyde to CO_2. A number of studies have shown that the degradation of soluble organic sulfur mainly has the following steps: sulfur-containing proteins are decomposed into SAAs (mainly Cys and Met) by proteases and peptidases secreted by anaerobic bacteria, and then Cys is mainly degraded into HS^-, $S^{2-}$and H_2S through cysteine lyase, which is considered to be an organic precursor of H_2S. The conversion of Met during anaerobic fermentation can eventually be degraded into various forms such as DMS, dimethyl disulfide (DMDS) and H_2S through different pathways.

Specifically, Met is first decomposed into MM by methionine lyase, and then further converted into DMDS by chemical oxidation or DMS by methylation. It was observed that the methylation of MM was closely related to the activity of methanogens. When the methanogens in the anaerobic fermentation system are inhibited, the concentration of MT will accumulate to a higher level. In addition, organic sulfur compounds can be converted into CS_2 by fermentation. CS_2, MT, DMS and DMDS can be further degraded. MT, DMS and DMDS can be degraded into H_2S by methanogens or SRM.

However, studies have shown that methylated sulfur compounds can be used as sulfur sources for several methanogens, but these methanogens cannot form methane from these compounds. [19] Under anaerobic conditions, it is not clear whether methyl-type methanogenic bacteria can produce methane by degrading methylated sulfur compounds. The metabolic pathway of sulfur-containing small molecule organic compounds corresponding to the occurrence state of organic sulfur in coal is metabolized to produce hydrogen sulfide during fermentation is not clear. In this process, it is urgent to answer whether sulfate substances are generated as intermediate products to participate in the cyclic metabolism of sulfur and the types of bacteria involved in metabolism. Therefore, in this paper, methyl sulfide, dimethyl sulfoxide, ethyl mercaptan, cyclohexane, n-16 alkanes and methyl cyclohexane were used as sulfur sources, and pure methanogens (*Methanosarcina horinobensis*) were used to identify the production process of H_2S through strict anaerobic fermentation experiments.

Combined with the changes of H_2S concentration and other gas components in the process of gas production, the metabolic pathways of small molecule sulfides were systematically discussed, and the intermediate products and metabolic processes of methyl sulfides degraded by pure methyl methanogens were identified.

2. EXPERIMENTAL MATERIALS AND TEST METHODS

Methanosarcina horinobensis was anaerobically fermented with different methyl compounds as substrates to explore the metabolism of methanogens.

2.1. Experimental materials

Two 3-litre glass bottles, two 1-litre glass bottles, room temperature incubator, ultrasonic cleaner, gas chromatograph, electronic balance, H2S detector, ethanethiol, ethanol, *Methanosarcina horinobensis*, syringe, gas collection bag, sealing film, tin paper.

2.2. Experimental steps

Blank group: only *Methanosarcina horinobensis*;

Experimental group: On the basis of the blank group, ethanethiol and ethanol were added respectively.

Phase I: *Methanosarcina horinobensis* for domestication experiments

(1) Prepare 3L and 1L glass bottles and put them into the sterilization pot for sterilization.

(2) After sterilization, the prepared culture medium was added, the air was removed by argon gas, and then put into the sterilization pot again for sterilization. At the same time, ultraviolet sterilization was performed on the sterile table for 30 min.

(3) After sterilization and cooling, the culture solution was put into the sterile room together with the syringe for ultraviolet sterilization for 30 min.

(4) The bacterial liquid was injected into the sterile table according to the bacterial liquid ratio of 1: 10, and the culture was carried out in the incubator at 35℃.

(5) The second phase of the experiment was started after 5 days of culture.

The second stage: To investigate the metabolism of Methanosarcina horinobensis on different substrates.

(1) Prepare 2 glass bottles of 3 L and 1 L, and put them into the sterilization pot for sterilization (3 L bottle to take liquid, 1 L bottle to take gas).

(2) The preparation work before injection was the same as the first stage. The bacterial liquid cultured in the first stage was injected into the sterile table and cultured in the incubator at 35℃ for 5 days.

(3) Two 3 L and two 1 L glass bottles were injected with ethanethiol and ethanol at a ratio of 1: 100 on a sterile bench, respectively, and cultured in an incubator at 35℃.

(4) The liquid was taken every 2 days, and the gas was measured every 1 day. At the same time, the liquid phase and solid phase were collected and cryopreserved.

(5) After the end of the reaction, the samples collected at all stages were analyzed and tested to explore the metabolic mechanism.

2.3. Test method

(1) Gas phase

The gas components produced in anaerobic fermentation were measured every 2 days. The gas components of H_2, N_2, CH_4 and CO_2 in the measured gas were obtained by gas chromatograph GC-4000 A. The total gas in the gas collection bag was obtained by syringe extraction, and the gas production was calculated.

(2) Liquid phase

Inorganic ions: HCO_3^-/CO_3^{2-}, sulfur-containing inorganic ions (focusing on HS^-. SO_4^{2-}.SO_3^{2-}. S^{2-}. $S_2O_3^{2-}$) in the fermentation broth were tested by ion chromatograph and national standard test methods;

3. EXPERIMENTAL RESULTS

3.1. Gas production characteristics

In the anaerobic fermentation system with ethanol as the substrate, the methanogenic capacity of the bacteria was weak before 14 days, and the strongest at 14-26 days. The methane production reached the peak at 20 days, which was 4.436 mL/g. After 24 days, the gas production capacity decreased significantly, and the cumulative gas production at 32 days reached 21.317 mL/g (Fig.1). In the anaerobic fermentation system with ethanethiol as the substrate, the production time of H_2S was mainly concentrated in the early stage of fermentation (0~16 d), and the peak of H2S production was reached at 4 d, and the yield was 6.78 mL/g at this stage. The cumulative production of H2S during the whole fermentation process reached 29.545 mL/g; the methane production capacity of this fermentation system was relatively weak and concentrated in 0 ~ 6 d. The peak gas production of CH_4 was 0.00116 mL/g in 4 d, and the cumulative production was 0.00116 mL/g, which was consistent with the concentrated production time of H_2S (Fig.2).

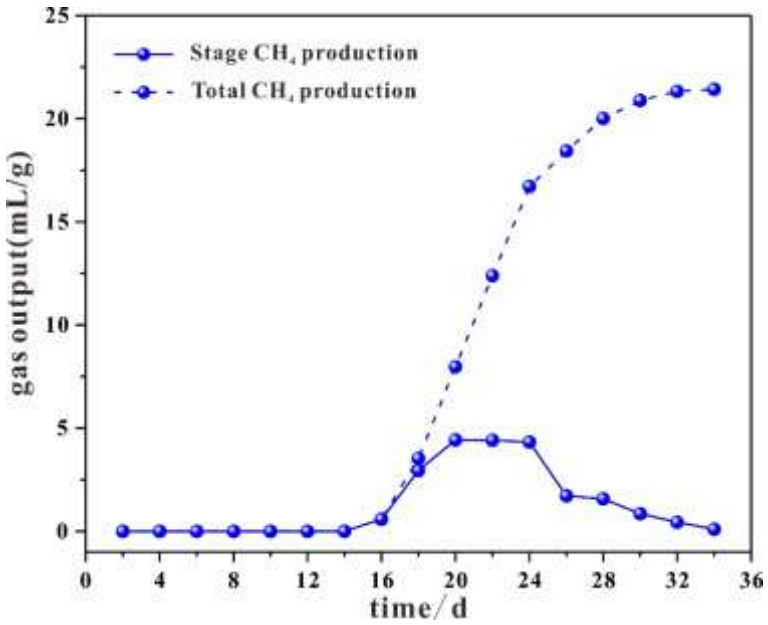

Figure 1. Stage gas production and cumulative gas production of ethanol substrate CH_4 during anaerobic fermentation

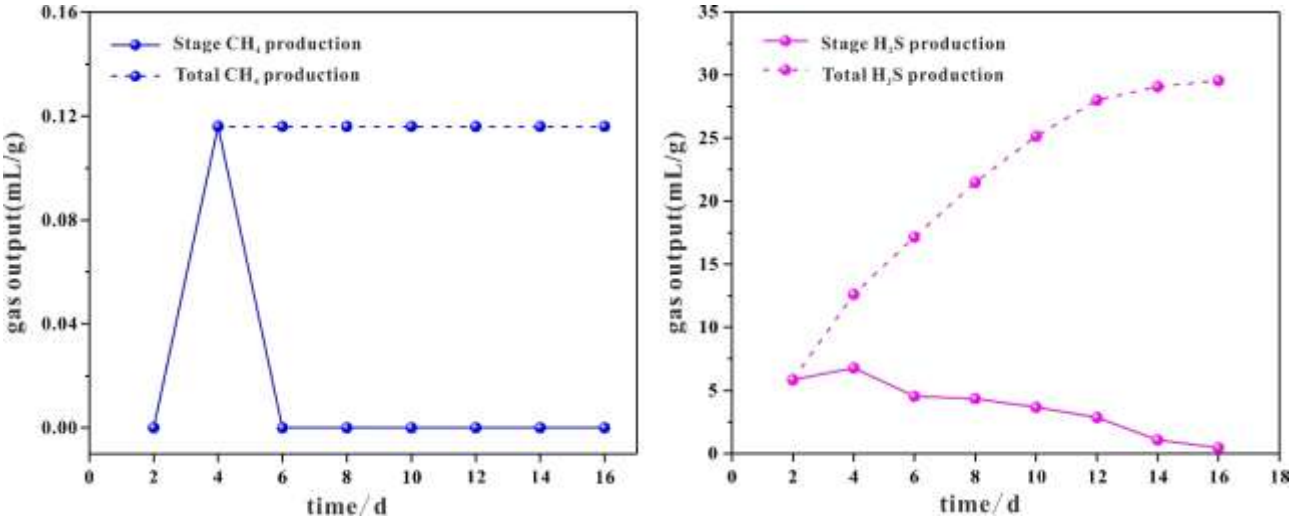

Figure 2 Stage gas production and cumulative gas production of CH_4 and H_2S during anaerobic fermentation of ethanethiol substrate

3.2. Variation characteristics of inorganic ions in anaerobic fermentation system

(1) Ethanol as substrate

First of all, the concentration of $HCO_3^-$and CO_2 ions showed a fluctuating upward trend. The ion concentration of HCO_3^-increased dynamically from 85.65 mg/L to 106.17 mg/L on the 8th day, then decreased sharply to 73.22 mg/L on the 10th day, then increased to 152.55 mg/L on the 16th day, and finally stabilized at 147.67 mg/L. The overall CO_2 increase in 10~24 days may be caused by the use of ethanol by methanogenic bacteria (Fig.3).

The pH continued to decrease from 7.32 on the 2nd day to 6.89 on the 8th day, and finally gradually increased to 7.22 on the 16th day, then decreased to 6.85 on the 24th day, and fluctuated to 6.99 in the subsequent gas production cycle (Fig 3).

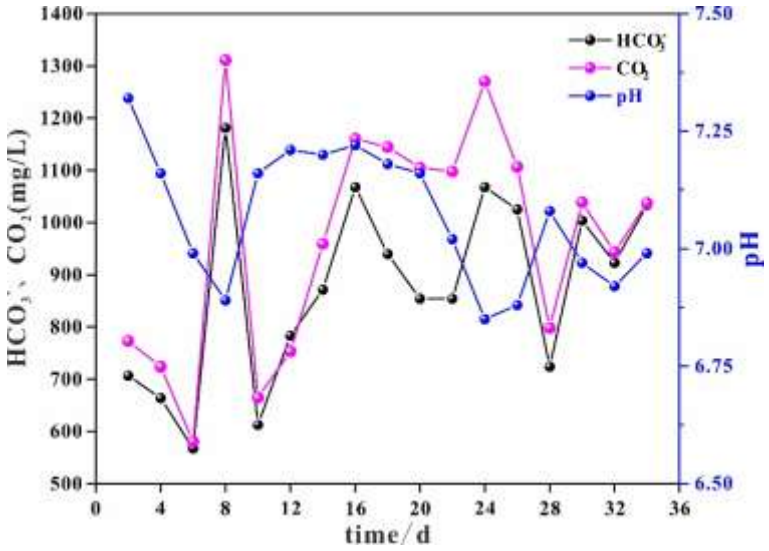

Figure 3 Changes of ion concentration and pH value of ethanol substrate

(2) Using ethanethiol as substrate

First, the ion concentration of SO_4^{2-}reached a peak of 1152 mg/L on the 4th day, and then decreased to 38.4 mg/L from 4 to 16 days. The ion concentration of SO_3^{2-}reached a peak of 2200 mg/L on the 12 th day, and fluctuated greatly within 0~16 days. The final ion concentration reached 840 mg/L; the ion concentration of $S_2O_3^{2-}$ reached a peak of 1640 mg/L on the 10 th day, and increased to 1444

mg/L from the 2 nd to 16 th day. From the linear fitting coefficient R2 of 0.1, it can be seen that the formation of H_2S has no obvious linear relationship with SO_4^{2-}, $S_2O_3^{2-}$and SO_3^{2-}, and sulfur-containing ions do not participate in the degradation reaction (Figure 5).

Secondly, the concentration of $HCO_3^-$and CO_2 ions increased first, then decreased and then increased. The ion concentration of HCO_3^-dynamically increased from 68.34 mg/L at the beginning to 106.17 mg/L on the 10 th day, then increased to 82.38 mg/L on the 14 th day, and increased to 98.85 mg/L on the 16 th day. The overall increase of CO_2 in 0~10 d may be caused by the use of ethanethiol by methanogens (Figure 5).

In addition, pH continued to decrease from 7.32 on the 2nd day to 6.89 on the 8th day, and finally gradually increased to 7.22 on the 16th day, then decreased to 6.85 on the 24th day, and fluctuated to 6.99 in the subsequent gas production cycle (Figure 5).

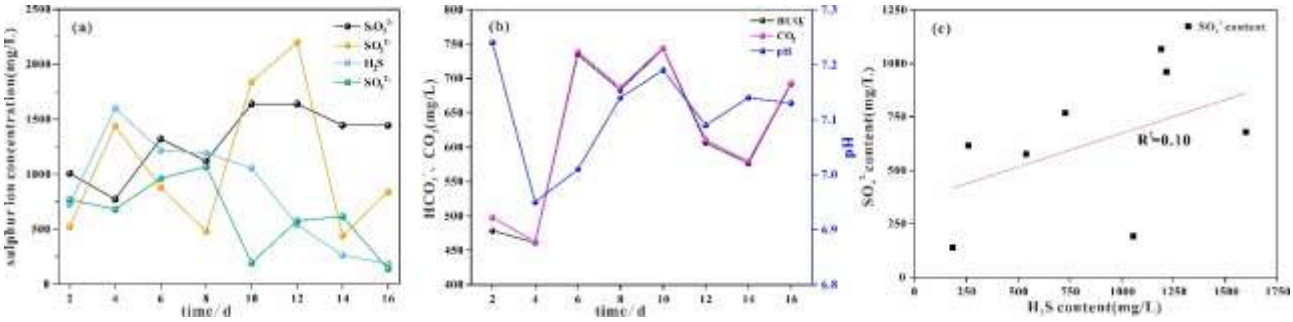

Figure 4 The concentration of sulfide ion (a), pH (b), the linear relationship between sulfide ion and H_2S (c).

4. ANALYSIS AND DISCUSSION

4.1. The formation mechanism of methane and hydrogen sulfide in anaerobic fermentation system

Biogas (CH_4, CO_2, H_2S) was generated in the fermentation system with ethanethiol as the substrate, and CH_4, CO_2, etc.were generated in the fermentation system with ethanol as the substrate, which proved that both ethanethiol and ethanol in the system can be effectively utilized by *Methanosarcina horinobensis* (Fig. 2, Fig. 3). The experimental flora *Methanosarcina horinobensis* belongs to *Methanosarcinales*. It is a methylotrophic methanogen that can ferment simple methyl compounds (formic acid, methanol, methylamines, etc.) and produce CH_4, CO_2, H_2S and NH_3.

In the anaerobic fermentation system with ethanol as substrate, biological CH_4 began to be produced after 14 days, and the gas production cycle was 34 days. At this time, the flora was *Methanosarcina horinobensis*, indicating that the biological methane in the fermentation system was mainly decomposed by ethanol. At 14-26 days of anaerobic fermentation of ethanol substrate, it was in the peak period of methane production. The flora used the hydrogen ions in the nutrient solution as the electron donor to reduce the methyl group in the methyl compound to produce methane, and produced part of CO_2, which was also the reason for the fluctuation of CO_2 in the fermentation system. At this time, the pH also changes from neutral to acidic. It has been reported that methanogens such as *Methanococcus* use simple methyl compounds as substrates to reduce the methyl groups in the methyl compounds with the reduction equivalent generated by the external supply or the oxidation of the methyl compounds themselves as electron donors to generate methane. Also explains the fluctuation of HCO_3^-concentration during this period. In the late stage of anaerobic fermentation, the substances that can be used by microorganisms in the system are exhausted, the microbial activity is inhibited, and the methane production stops.

In the anaerobic fermentation system with ethanethiol as the substrate, biological CH_4 was generated on the 4th day, and the yield of H_2S also reached the peak at this time, indicating that *Methanosarcina horinobensis* produced methane while using ethanethiol to produce H_2S.As the accumulation of H2S produced, it had a certain toxic effect on the growth of the flora, which inhibited the gas production cycle of the flora after 16 days. It is worth noting that there is no linear relationship between the change of sulfur ion concentration and the formation of H_2S by detecting the change of sulfur ion concentration, indicating that sulfur ion does not participate in the degradation reaction. At the same time, the concentration of CO_2 increases in a fluctuating manner during this process, which further confirms that the degradation of ethanethiol directly produces methane and CO_2. In this process, the formation rate of $HCO_3^-$is greater than the consumption rate. The concentration of HCO_3^-in this period shows an overall upward trend. The sudden decrease of pH in the first 4 days is due to the dissolution of some CO_2 in the fermentation system to generate carbonic acid. In the later stage, the pH of unstable decomposition of carbonic acid is stable at about 7.1.

4.2. Pathway analysis of methane and hydrogen sulfide production

Methanosarcina horinobensis uses HS-CoM as an electron donor and ethanol, ethanethiol and methyl-containing sulfides as electron acceptors. Under the catalysis of coenzyme M methyltransferase (Mt), the methyl group on the electron acceptor is transferred to the sulfhydryl group of the electron donor to form CH_3-CoM, and then hydrogen is used as an electron donor to produce methane [20].In fact, when ethanol is the substrate of methyl compounds, three methyl compounds are activated by Mtr, and one of them is oxidized to CO_2 through the reverse H_2/CO_2 reduction pathway. The remaining two were reduced to CH_4 and H_2O. When ethanethiol was used as the substrate of methyl compounds, two methyl compounds were activated by Mtr, one of which was oxidized to CO_2 and H_2S through the reverse H_2/CO_2 reduction pathway, and the remaining one was reduced to CH_4 and H_2S.

5. CONCLUSION

During the anaerobic fermentation process, ethanol and ethanethiol were used as carbon and sulfur sources, respectively, in the role of *Methanosarcina horinobensis* to degrade CH_4, H_2S, etc., indicating that this flora can degrade simple methyl compounds. The methyl group on it promotes the production of CH_4. When the substrate is a sulfur-containing methyl compound, H_2S is also produced. It can also be concluded that sulfur-containing organic matter can be degraded and utilized by methanogens. The metabolism of methane is mainly derived from the degradation of methyl compounds.

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