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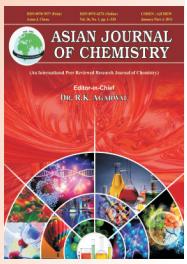

Mechanisms of Generation of Biogenic Methane Influenced by Types of Strain and Disodium EDTA

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In adoption of plate isolation, anaerobic culture and PCR amplification techniques, a methanogens-culture experiment was conducted for investigating the mechanisms of generation of biogenic methane, during which coals of Shaqu colliery were used as substrates and mine water collected from Zhongtai and Shaqu collieries, respectively. The white-rot fungi preserved in laboratory were utilized as strain sources. Methane production was fully examined with the addition of disodium EDTA under various concentration gradients. The results show that there are methanogens present in mine water of Zhongtai, while the growth of methanogens in mine water of Shaqu is possibly discouraged due to the tested existence of denitrifying bacteria and overhigh pH. Hence, there is no methane being produced from coals of Shaqu as compared to the occurrence of gaseous production from that of Zhongtai under the same conditions. Meanwhile, when disodium EDTA is added at 1 g/L can the CH_4 production reach a peak of 10 mL/g after a period of increase and the pH value can also see a notable rise after reaction.

Keywords: Methanogens, Biogenic methane, Disodium EDTA, Gaseous production.

INTRODUCTION

Biogenic methane, produced initially and developed successfully form Powder River Basin and San Juan Basin, USA, has made people gradually aware of the great significance bio-generated methane could play in the increase of coal bed methane (CBM) resources[1]. So far substantial studies have been focused on biogenic methane[2-6] and the microbes that create it, which have in some cases been evidenced by a series of laboratory experiments where coals can be converted into gaseous fuels (mainly methane) through biodegradation[7-10].

Methanogens are considered as the key driver for biological generation of methane[11-14], thus the types and quantities of methanogens needed in bio-generation process are not only regarded as the fundamental research subjects of biogenic methane, but as a great support for the research on the heredity and breeding of methanogens and the corresponding biophysical and biochemical properties of them.

To investigate the impact of methanogens on coal fermentations to evolve biogenic gases and further to study the mechanisms and contributing factors responsible for biologically-generated methane[15], withe-rot fungi and anaerobic microbial consortia originated in mine water were used as strain sources in this experiment and through the enrichment and identification of indigenous microbial of mine water, the traits of its growth and reproduction[16] were studied. Additionally, disodium EDTA was used to treat coal samples with high content of heavy metals[17,18], by which the metabolic activities of microbial in the anaerobic fermentation system were activated, further the function of microbial was evidenced.

EXPERIMENTAL

Coal substrates: Differing from previous work[19], high-grade bituminous coal samples selected, respectively from Shaqu colliery of Huajin Coking Coal Co., Ltd., Shanxi Province and from Zhongtai colliery of Hebi Coal Co., Ltd, Henan Province were applied in the experiments as coal substrates. Coal samples were grounded into powders to the size of 60-80 mesh using a grinder in laboratory prior to being sterilized in an automatic autoclave at 121 ºC for 0.5 h and then being dried and reserved for use.

Strain sources: (1) White-rot fungi were provided by the Biology Technology Laboratory, Henan Polytechnic University, after being inoculated onto the potato-dextrose-agar (PDA) slant medium they were preserved in a refrigerator at 4 ºC; (2)

Microbial consortia for anaerobic fermentation were sampled from the mine water of Shaqu and Zhongtai collieries, respectively, derived from the drainage ditches in the mining working face and packed with axenic plastic buckets. A quick seal should be guaranteed before being brought back to the lab and reserved in a refrigerator at 4 ºC.

Medium: (1) Medium for the enrichment of white-rot fungi; (2) Medium for the enrichment of methanogens: L_1 1.0 g, S_1 0.4 g, L_2 4.0 g, J 1.0 g, L_3 0.1 g, Y 1.0 g, L_4 0.5 g, mine water 1000 mL; (3) Medium for the isolation and enumeration of denitrifying bacteria (BTB medium): KNO_3 1.0 g, CH_3COONa 1.0 g, KH_2PO_4 1.0 g, $FeCl_2$ 0.05 g, $CaCl_2$ 0.2 g, $MgSO_4$ 1.0 g, BTB 1.0 mL (1 %, dissolved in alcohol), agar 20.0 g, ultrapure water 1 L, pH ≈ 7.3.

Experimental equipments: The equipments involved in the experiments include automatic autoclave, multifunctional intelligent anaerobic system, electro-thermal incubator, anaerobic workstation, X-ray diffractometer, gas chromatograph, inverted fluorescence microscope, high-speed tabletop centrifuge, *etc*. The experimental device as shown in Fig. 1 was used to collect the gaseous products.

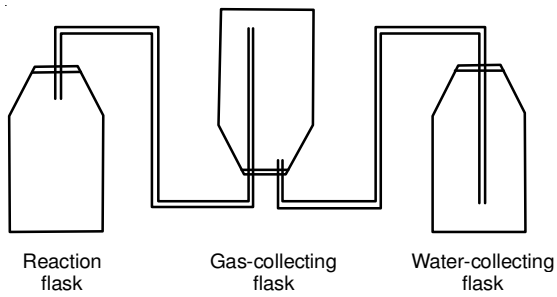


Fig. 1. Experimental device

Experimental methods

Identification of methanogens: Species-specific PCR method was adopted for a quick identification of methanogens in mine water including the following procedures: (1) Culture: Medium was prepared and sterilized in an automatic autoclave at 121 ºC for 20 min first before being divided equally into 3-4 culture dishes; mine water was inoculated onto the medium in an anaerobic environment and spread evenly by a sterilized spreader; N_2 was introduced for the displacement of O_2 within the dishes prior to being placed in an incubator chamber at 35 ºC for 10-20 days of methanogens growth. (2) DNA extraction: Already-formed microbial colonies on the medium were slowly rinsed off the surface with a trace amount of anaerobic ultrapure water to make them into 2 mL centrifuge tubes; OD (optical density) values were determined through a spectrophotometer at the wavelength of 600 nm. To meet the requirements of DNA extraction kits, anaerobic ultrapure water was used to regulate the concentration of OD value at around 1.0×10^9. (3) PCR amplification: a specific primer of 16SrDNA: Met 83F (5V-ACKGCTCAGTAACAC-3V), Met 1340R (5V-CGGTGTGTGCAAGGAG-3V) was used for the amplification of object genes of methanogens[20].

PCR amplification system: 10 × Taq polymerase reaction buffer 2.5 μL; dNTP (20 mmol/L) 2.5 μL; 5'-end primer (25 pmol/μL) 1 μL; 3'-end primer (25 pmol/μL) 1 μL; Mg^{2+} (2.5 mmol/L) 2 μL; DNA template 1 μL; Taq DNA polymerase (5U/μL) 0.2 μL; dd H_2O14.8 μL; total volume 25 μL.

Conditions: Pre-denaturation for 3 min at 94 ºC; denaturation for 30 s at 94 ºC; renaturation for 30 s at 58 ºC; extension for 90 s at 72 ºC; extension for 10 min at 72 ºC after 30 cycles. All these procedures should be conducted within the PCR instrument.

Isolation and enumeration of denitrifying bacteria: Dilution spread plate method was introduced to enumerate denitrifying bacteria in mine water. Already-inoculated medium was placed into an incubator chamber at 35 ºC and some single colonies observed with an appearance of a blue aureola after 3-4 days would be identified as denitrifying bacteria.

Methane-production experiment with strain sources from mine water of Shaqu colliery: The Shaqu coals, mine water of Shaqu and white-rot fungi were used as materials (Table-1). By the addition of disodium EDTA under various concentration gradients into the medium, methane-production laws were further studied.

TABLE-1
MATERIALS APPLIED IN METHANE-PRODUCTION EXPERIMENT OF SHAQU COLLIERY

Number	Sample (g)	Mine water (mL)	White-rot fungi (mL)	Disodium EDTA (g/L)
A_1	20	200	20	0
A_2	20	200	20	1.0
A_3	20	200	20	2.0
A_4	20	200	20	3.0

Methane-production experiment with strain sources from mine water of Zhongtai colliery: The Shaqu coals, mine water of Zhongtai and white-rot fungi were used as materials (Table-2). By the addition of disodium EDTA under various concentration gradients into the medium, methane-production laws were studied.

TABLE-2
MATERIALS APPLIED IN METHANE-PRODUCTION EXPERIMENT OF ZHONGTAI COLLIERY

Number	Sample (g)	Mine water (mL)	White-rot fungi (mL)	Disodium EDTA (g/L)
B_1	20	200	20	0
B_2	20	200	20	1.0
B_3	20	200	20	2.0
B_4	20	200	20	3.0

Analytical items and testing methods: **(1) Identification of methanogens:** The products of PCR amplification and loading buffer were mixed and then subjected to gel electrophoresis in 2 % agarose gel before comparing to a DNA maker. The presence of a bright DNA band at 1200 bp would suggest the existence of methanogens in tested mine water, otherwise no methanogens are there. **(2) Determination of gas production:** The gaseous products derived from the fermentation would be determined by the measurement of amount of water drained off through the experimental device in Fig. 1. **(3) Analysis of gas composition and concentration:** Analysis of composition and concentration of fermentation-produced gas can be based on the interpretation of gas chromatograph equipped with a TCD detector. **(4) Isolation and enumeration**

of denitrifying bacteria: Given the fact that denitrifying bacteria are capable of secreting a large amount of alkaline substances while growing up, BTB medium was used and dilution spread plate method was adopted.

RESULTS AND DISCUSSION

Results of gas production with strain sources from Shaqu colliery: Gas components and concentrations of each samples produced by fermentation were tested after 40 days as tabulated in Table-3.

Table-3 showed that none of samples had the production of CH_4. Further the mine water of Shaqu was identified by means of species-specific PCR method in contrast to the mine water of Zhongtai. Fig. 2 shows the results of DNA amplification through gel electrophoresis.

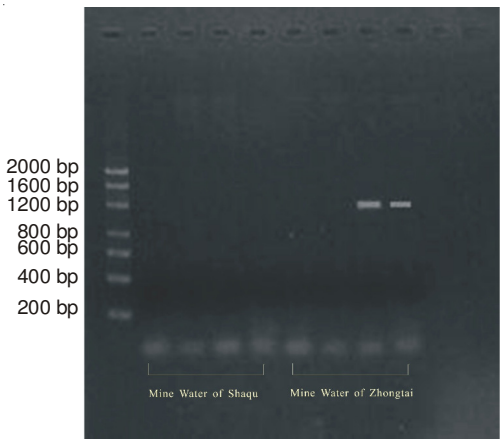
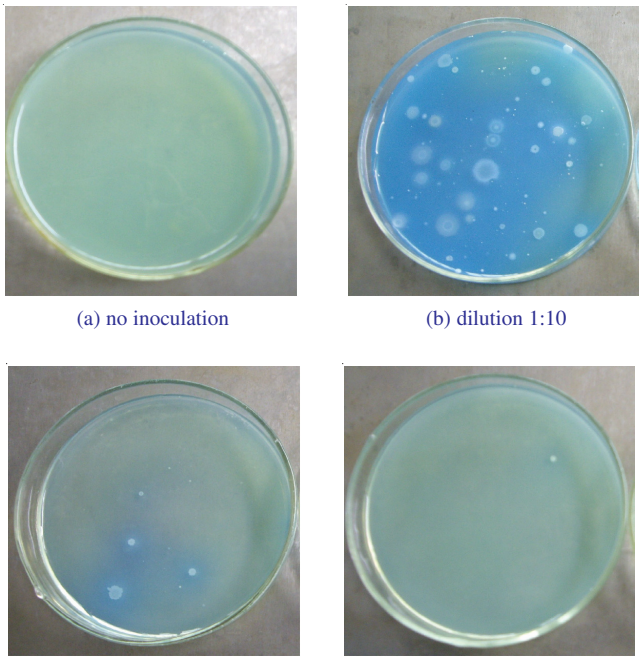


Fig. 2. Result of PCR amplification

As can be seen from Fig. 2, the strain sources originated in mine water of Zhongtai colliery was observed as presenting a specific DNA band at 1200 bp which was indicative of the existence of methanogens after DNA extraction and PCR amplification, while for mine water of Shaqu no such a band was found.

The results indicate the presence of methanogens in mine water of Zhongtai and the absence of methanogens in mine water of Shaqu, so that it is not surprising to note that no methane produced from any of the samples of Shaqu in this experiment.

Table-3 indicates that despite none of samples had the production of methane, yet a large amount of N_2 was produced from all the samples, particularly the highest of 10.5 mL/g produced from sample A_2. This is mainly due to the denitrification of denitrifying bacteria in mine water during anaerobic fermentation, thereby resulting in the significant production of N_2. The denitrifying bacteria in mine water of Shaqu were enumerated at 1.75×10^3. The specific growth conditions of denitrifying bacteria at different concentrations of dilution were also as shown in Fig. 3.



(a) no inoculation (b) dilution 1:10

(c) dilution 1:100 (d) dilution 1:1000

Fig. 3. Growths of denitrifying bacteria under various dilutions

The morphology of denitrifying bacterial colonies under an inverted fluorescence microscope were also observed as pictured in Fig. 4 including spherical and rod-shaped colonies, respectively.

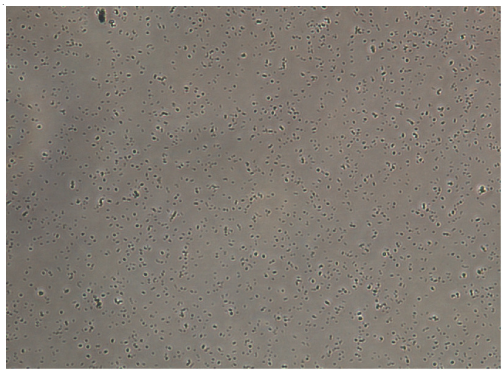


Fig. 4. Morphologies of denitrifying bacteria observed under microscope

Results of gas production with strain sources from Zhongtai colliery: Gas components and concentrations of each samples produced by fermentation were tested after 40 days as given in Table-4.

TABLE-3
RESULTS OF GAS PRODUCTION FOR SHAQU COLLIERY

Sample	Disodium EDTA (g/L)	Results					
		H_2 (%)	CH_4 (%)	CO_2 (%)	N_2 (%)	Total (mL)	CH_4 production (mL/g)
A_1	0.0	0.00	0.0	6.0	94.0	73	3.1
A_2	1.0	0.03	0.0	1.1	98.9	237	10.5
A_3	2.0	0.00	0.0	6.4	93.6	40	1.9
A_4	3.0	0.02	0.0	9.4	91.6	38	1.7

TABLE-4
RESULTS OF GAS PRODUCTION FOR ZHONGTAI

Sample	Disodium EDTA (g/L)	Results					
		H_2 (%)	CH_4 (%)	CO_2 (%)	N_2 (%)	Total (mL)	CH_4 production (mL/g)
B_1	0.0	0.00	31.5	10.4	58.1	166	2.6
B_2	1.0	0.10	70.7	6.5	22.8	284	10.0
B_3	2.0	0.03	56.0	10.7	33.3	278	7.8
B_4	3.0	0.02	49.0	8.9	42.0	260	6.4

Fig. 5 showed the strain sources from mine water of Zhongtai saw a production of CH_4 which increased at the beginning and then decreased with the rising concentrations of EDTA. As shown in Table-4, the production of CH_4 peaked at 10 mL/g when EDTA was 1 g/L, which was nearly 3.8 times greater than that of when EDTA was absent. Presumably, a moderate amount of EDTA added in use can improve the production of CH_4 to a great extent.

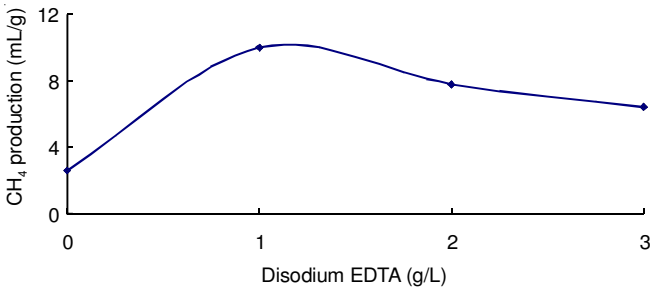


Fig. 5. Relation between disodium EDTA and CH_4 production

Hence, disodium EDTA is able to maintain the equilibrium of heavy-metal ions in solutions, making the concentration of heavy-metal ions neither too low to weaken the metabolic activities of microbial nor too high to exhibit a toxic inhibition to the their growth. In addition, as can be indicated from Table-4, when EDTA was beyond 1.0 g/L, the production of CH_4 experienced a decreasing trend as a sign of negative inhibition exhibited from EDTA resulting from its complexation with heavy-metal ions on the surface of microbial cell membrane and further causing the death of microbial.

As given in Table-4, a relative high proportion of N_2 sustained which was caused by the metabolism of denitrifying bacteria. Dilution spread plate method was adopted to isolate and enumerate the denitrifying bacteria in mine water of Zhongtai and the result was 8.50×10^2, which was far lower than that of in mine water of Shaqu.

Analyses of pH in solutions before and after fermentation: Table-5 displays the changes of pH value of each samples tested before and after fermentation.

TABLE-5
pH VALUES IN SOLUTIONS BEFORE AND AFTER REACTION

Sample	pH before reaction	pH after reaction	Variations of pH
A_1	7.05	8.41	1.36
A_2	7.05	8.44	1.39
A_3	7.00	8.40	1.40
A_4	7.04	8.53	1.49
B_1	6.97	8.01	1.03
B_2	7.00	8.08	1.08
B_3	7.01	8.13	1.12
B_4	6.98	8.16	1.18

Table-5 showed that there was a notable increase of pH values for all the tested samples before and after fermentation[15]. The main causes can be analyzed as follows: First, denitrifying bacteria and methanogens can use such acidic substances as formic acid, acetic acid and propionic acid, *etc.*, which are all derived from the coals degradation as carbon sources by fermentation-hydrolysis bacteria for their metabolism and growth, therefore the degree of acidification in ultimate solutions is declined. Second, methanogens are able to utilize part of CO_2 generated during the fermentation process to produce CH_4 through reduction. Third, denitrifying bacteria will secrete a great deal of alkaline substances further causing the increase of pH in solutions.

Variations of gaseous products during the experiment: The gaseous products from sample A_2 of Shaqu and those from sample B_2 of Zhongtai all experienced a whole-process monitor on the variations of gaseous concentrations as portrayed in Fig. 6.

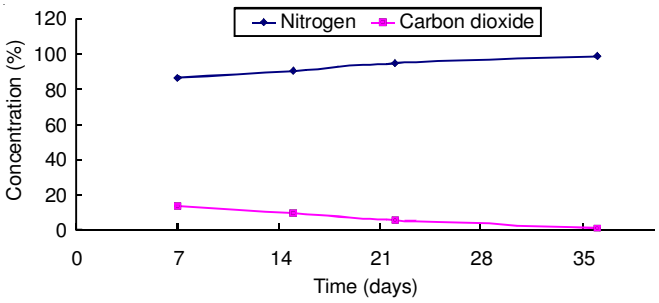


Fig. 6. (a) Concentrations of gaseous products from A_2

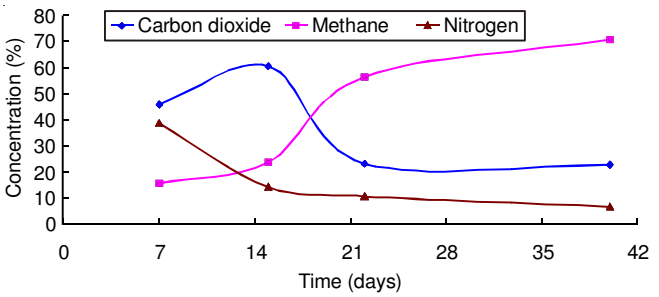


Fig. 6. (b) Concentrations of gaseous products from B_2

Gases produced from A_2 mainly consisted of N_2 and CO_2, of which N_2 was on the rise above 90 % with a dominant advantage over CO_2 at a relative low and stable level, illustrating the whole-process impact of denitrifying bacteria. While B_2 of Zhongtai generally witnessed two gas-production peaks during this period, in which CO_2 gradually reached a peak at 60 % first on the 15th day with a recognizable higher concentration over CH_4 before CH_4 began to rise dramatically from the 18th day, when CH_4 has replaced CO_2 to be the dominant

TABLE-6
DATA OF X-RAY DIFFRACTION OF COAL SAMPLES

No.	$2\theta_{002}$ (°)	$2\theta_{100}$ (°)	$FWHM_{002}$ (°)	$FWHM_{100}$ (°)	d_{002} (Å)	L_a (Å)	L_c (Å)	Nc
1#	25.377	40.35	6.545	6.169	3.507	28.049	12.995	4.705
2#	24.822	43.341	6.983	8.017	3.584	21.800	12.167	4.395

gas since then till the very end. This indicates a fact that the denitrifying bacteria are quite active at the beginning owing to its shorter growth cycle than that of methanogens, which further can be evidenced by a constant decline of N_2.

Analyses of solid products after fermentation: Sample B_2 from Zhongtai was subjected to XRD (Table-6) before and after the fermentation and was numbered as 1# and 2#, respectively.

The contrasts in the crystal parameters used to characterize the aromatic degree of coal samples were shown by a bar chart as portrayed in Fig. 6.

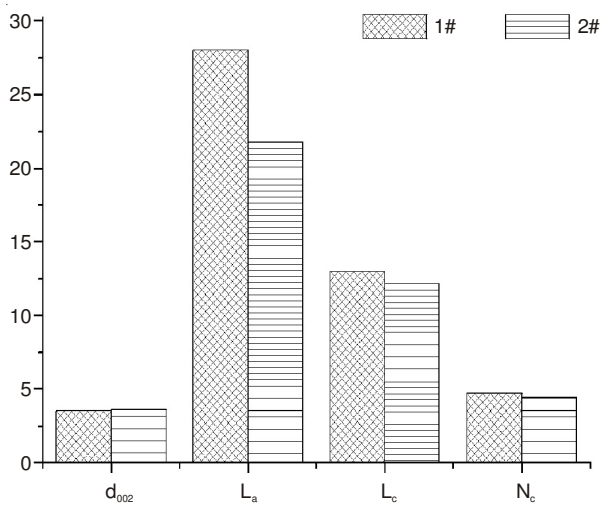


Fig. 7. Variations of crystal parameters

As can be suggested from Fig. 7, the small-molecule structures on the lateral chains of aromatic rings are mainly the targets of degradation rather than the macromolecules, while the degree of aromatization will see a slight drop at the same time, which can be evidenced by the signatures that L_a was becoming smaller and the diffraction peak gradually shifted to a bigger θ side.

Conclusions

Through the experiments, these conclusions can be drawn as follows:

• An overhigh pH value and big amount of denitrifying bacteria in mine water of Shaqu are the major reasons for the absence of methanogens.

• Denitrifying bacteria can produce a big amount of N_2, which results in the decline of the production of CH_4, consumption of acidic metabolic products, secretion of alkaline substances and further the rise of pH values in solutions.

• Disodium EDTA is able to maintain the concentration equilibrium of heavy-metal ions in solutions so that a notable growth of CH_4 can occur. However, when the concentration of disodium EDTA reaches an overhigh (more than 1.0 g/L and over), the production will decrease due to the death of microbial.

ACKNOWLEDGEMENTS

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