Influence of Organic Matrix and Cations on Bio-Methane Yield with Anthracite Methanogenic Consortium

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Abstract

Organic compounds fermentation of coal has been used to generate secondary biogenic gas and enhance gas reservoirs in coalbed. To enhance the bio-degradation process, culture nutrition plays an important role in remediating the nutritional deficiency of the coal seam. The influence of bio-methane yield with organic inputs and cations concentrations was examined. Research of organic matrix influence revealed that the traditional organic material except yeast extract should forbid, and the input of yeast extract should limit at 1.00g/L also. Further, the study demonstrated that the ion concentration of

sodium, potassium, magnesium, calcium and ammonia nitrogen also influenced methane and carbon dioxide yields. And the optimize concentrations for Ca^{2+}, K^{+}, Na^{+}, Mg^{2+} were 5.1, 1.7, 23 and 1.3 mmol/L. The Mg^{2+} was particularly sensitive in inhibiting CH_4 metabolism processes largely for gas-coal methanogenic consortium.

Keywords: Anaerobic bacteria; Culture media; Organic matrix; Coal; Bio-methane.

1. INTRODUCTION

The demand for gas has seen a tremendous increase in the last 10 years [1]. In particular, coal-bed methane enhance with biotechnology represents an ideal method to increase methane productivity from oil deposits and coal seams using underground anaerobic digestion processing [2,3]. With the addition of adequate nutrient supplements, methanogenic consortium has the potential to degrade organic compounds in coal into methane. In this way, gas reserves and productivity could be enhanced along with secondary biogenic gas generation [4–6].

Previous studies involved with the methanogenic microbial traits and fermentation characteristics for bio-methane generation have focused on the processes of bio-degradation by microbial communities in coal seam [2,7,8] Anion research identified that methane biosynthesis process could active where coalbed waters exhibit relatively low salinities (<2 mol/L Cl^{-}) and low SO_4^{2-} concentrations (<10 mmol/L) [9]. Nutrients supply by meteoric water plays an important role in methanogenic microbial metabolism

[10,11]. However, nutritional deficiency exists in most coal seam. Most organic compounds in coal cannot be readily used as nutrients for methanogenic microbes. In addition to the inherent elements present in coal beds, supplemental nutrients are important to remediate the nutritional deficiency and promote microbial growth [12,13]. If concentrations of supplemental nutrients are excessive, the methanogenic consortium will be adversely influenced [14]. The goal of this report is to identify the optimal nutrient dose for microbial community culture. Methane and carbon dioxide yield rate were the indicators used to assess the results of experiments.

2. Materials and methods

2.1 Materials

The coal and coalbed water samples used in this study were collected from Laohutai Fm 103$^{\#}$ coalbed located in Shenyang province of China (GPS coordinates 41.830256, 123.957639). The depth of the Fm 103$^{\#}$ gas-coal sample was 550 meters and the thickness of the coal seam was 6-18 meters. Laohutai Mine, in the east part of Fushun near Shenyang, Liaoning province, opened in 1901. The mine has 55.6 million tons of recoverable coal reserves until 2004[15]. The samples were collected by the State Key Laboratory of Coal Resources and Safety Mining. The field studies did not involve endangered or protected species, and only small sample quantities were used. And no specific permissions were required for these locations sample collection and research.

The coal samples were obtained from a newly exposed coal seam in the heading face by the channel sampling method following ASTM D 4596-86 with nitrogen protection. Two sample points were selected from the Fm 103# coal seam. These samples were sealed in gas-tight steel canisters and set with gas-tight valves (manufactured by J&D Technologies) which enabled inert gas to flush the canister and protected with nitrogen immediately. The coal to be used for the microbe community source was crushed into small pieces (10-15 mm in diameter) at an aseptic bench (manufactured by JingXue Technologies). Nitrogen protection was used to maintain the coal in a continuous anaerobic environment. The sample was sealed in a sterilized gas desorption canister (manufactured by J&D Technologies) to desorb the absorbed gas until no gas desorbed in atmospheric pressure at 25 ℃[16].

The formation of water samples from the Fm 103# coalbed were collected in the same coal-bed in sterile glass bottles (manufactured by Fisher Scientific) which were filled to overflow to prevent oxygen ingress. Coalbed water (≤ 24 hours after collection) was autoclaved at 121 ℃ for 45 minutes and sealed in sterile glass bottles. Argon was infused to seal the top space of the bottles. The coalbed water samples were stored at 4 ℃ for no more than 3 days.

2.2 Culture

Five different nutrition culture media were tested in these experiments: MAC-1,

MAC-2, MAC-3, MAC-4, MAC-5 [4,17] (MAC is the abbreviation of "the influence test of organic matrix and cations"). The final concentrations of the compounds (g/L) were as list in table 1, and 0.1 mL/L of resazurin was added as an oxygen indicator. Yeast extract was produced by Fisher BioReagents, and other chemicals were supplied by Acros Organics.

Table 1. The MAC1-MAC5 nutrition culture media concentrations (g/L)

Culture Media	Sodium Acetate	Glucose	Beef Extract	Yeast Extract	Common Concentrations
MAC-1	2.00	3.00	3.00	2.00	
MAC-2	2.00	1.00	1.50	1.00	NH_4Cl, 1.00; $MgCl•7H_2O$, 1.00;
MAC-3	0.00	1.00	1.50	1.00	KH_2PO_4, 0.40; KCl, 0.50;
MAC-4	0.00	0.00	0.00	1.00	$NaHCO_3$, 1.00; L-Cysteine Hydrochloride Monohydrate, 0.45
MAC-5	0.00	0.00	0.00	0.00	

The distilled water was autoclaved at 121 ℃ for 45 minutes with dissolved oxygen removed. Nutrition medium was prepared in a 500 mL flask with distilled water. The medium was mixed using a magnetic stirrer for 2 hours at 60 ℃ at an aseptic bench and then combined with an equal volume of coalbed water (volume ratio 1:1) for another hour at room temperature. Nitrogen protection was used to maintain nutrition in a continuous anaerobic environment throughout the entire experiment. The final pH was maintained at 6.0 for all nutrition cultures. Control samples of 100 ml MAC-1, MAC-2, MAC-3, MAC-

4, and MAC-5 were sealed in separate sterile glass bottles and stored at -40 ℃. Argon was infused to seal the top space of bottles.

Anaerobic conditions were ensured in flasks using a gas-replacement method. This gas replacing process was monitored in real time with a carbon dioxide monitor system (manufactured by E2V). For each experiment 50.00±1 g of the coal sample and 500.00 mL of medium were used. Nitrogen was used to seal the upper space of the flask at the beginning of the experiment. The flasks were placed in an incubation shaker at 35 ℃ and were agitated at 80 rpm to maximize the coal-liquid mass transfer rates. Twelve parallel tests were designed for each nutrition group which were cultured for 40 days under identical conditions.

The control experiments were performed without coal sample, which named MAC-1*, MAC-2*, MAC-3*, MAC-4*, MAC-5*, to identify whether exogenous organic has the possibility to supply extra carbon to enhance the bio-methane yield. For each control experiment of each nutrition group 100 mL of cultured medium and 400 mL new medium was used. And cultured for 40 days in the same condition as above.

2.3 The cation orthogonal analysis

The cation orthogonal analysis was based on consumption of cation elements in bio-degradation processes, according to the $L_{16}(4^5)$ orthogonal table which includes Na^+, K^+, Ca^{2+}, Mg^{2+}. The lowest ion level was based on the MAC-4 medium while the highest ion

level was based upon that of the East China Sea ion concentration. A quantity of 50.00±1 g of Fm 103# gas-coal sample was used in each experiment. The upper space of the flasks was sealed with nitrogen. The flasks were placed on an incubation shaker at 35 ℃ and agitated at 80 rpm to maximize coal-liquid mass transfer rates. Cation orthogonal experiments were of 40 days of culture.

2.4 Gas analysis

Gas samples were obtained with a 50-μL gas syringe. Methane and carbon dioxide analyses were performed using an Agilent 7890A gas chromatograph (manufactured by Agilent). The nitrogen (carrier gas) flow rate was set at 1.00 mL/min. The injection port was maintained at 150℃ with the oven temperature set at 25℃and the Thermal Conductivity Detector (TCD) at 200℃. Retention times for methane were 3.76 minutes and 5.0 minutes for carbon dioxide. Calibration standards consisted of 40% methane, 20% carbon dioxide, 10% hydrogen and 30% nitrogen, which were injected at atmospheric pressure to generate the calibration plot.

2.5 Nutrient Metabolism Analysis

Nutrient ion concentrations were analyzed using an HC-800 ionization analyzer (manufactured by Histrong Technologies). The main indicators included fluoride, chloride, nitrate and nitrite nitrogen, phosphate, sulfate, carbonate, bicarbonate, ammonia nitrogen, sodium, potassium, magnesium, calcium, pH, water hardness and total

alkalinity.

3. Results

3.1 The nutrient abundance influence for bio-degradation of coal

Five different organic compound nutrient concentrations designated as MAC-1, MAC-2, MAC-3, MAC-4, and MAC-5 were assessed in this study. In these medium, Sodium Acetate is a collective medium, as carbon resource, for *methanogenic* normally [18]. Glucose is the medium for *hydrogen-producing acetogens*. Beef Extract is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals, and some vitamins. It is often used to supply carbon and nitrogen sources. Yeast extract contains a mixture of amino acids, peptides, water soluble vitamins, and carbohydrates. And it is often used in culture media [19]. The rank order of organic concentrations were MAC-1 > MAC-2 > MAC-3 > MAC-4 > MAC-5.

Coalbed methanogenic groups are comprised of a variety of microbial types. With regard to *methanogens*, only a limited number of simple carbon compounds such as CO_2 or acetate can serve as substrates. For the conversion of complex organic compounds to methane, fermentative and *acetogenic bacteria* are required. Thus they group an interactive methanogenic consortium [20]. In the process of gas-coal bio-degradation, the capacity for fermentative bacteria to hydrolyze and ferment the organic compounds of coal plays an important role, and CO_2 is the main compound of gas productions. Beef

146 extract in medium supplied the extra carbon and nitrogen for bacteria. For the *acetogenic*
147 *bacteria* fermentation process, the long chain fatty acids and sugar degrade to form
148 acetate, CO_2 and H_2 [18]. Glucose is the medium to enhance the *acetogenic bacteria*
149 fermentation. And *methanogens* yield CH_4 with CO_2 and H_2 or acetate. Sodium Acetate
150 was introduced to raise the acetate supply for *methanogens* [21].

151 If consortia in a good banlance, most CO_2 and H_2 will be used to format CH_4. And
152 the concentration of CH_4 should be high, meanwhile, the concentration of CO_2 and H_2
153 should keep in low. So the yield gas concentration with microbial fermentation process
154 could reflect the bacteria balance conditions. The presence of sufficient organic nutrients
155 in the medium could promote fermentative and *acetogenic bacteria* flourish and improve
156 methanogen nutrient generation. However, if excessive amounts of organic nutrients are
157 contained within the medium, which like beef extract and glucose, the high propagation
158 of fermentative or *acetogenic bacteria* would break the microbial balance, and carbon
159 dioxide yield rate could be enhanced, meanwhile, methanogenesis could be inhibited.
160 This phenomenon showed in MAC-1, MAC-2 and MAC-3 culture experiments (Figure
161 1).

162 The results confirmed that MAC-4 was the most effective medium for enhancing the
163 bio-methane generation rate. 1 g/L yeast extract provided the best concentration of
164 organics amino acids, peptides, and water-soluble vitamins to optimize the microbial
165 fermentation. The maximal methane concentrations of the MAC-4 culture group achieved

166 23.62% (Figure 1). It was 4 times higher than that of the MAC-2, MAC-3, MAC-5
167 groups on average. The MAC-4* control experiment and MAC-5 group verified that the
168 anthracite is important to provide carbon for bio-methane yield (Figure 1).

169 **Figure 1 here**

170 The high carbon dioxide and low methane concentration data obtained from MAC-1
171 and MAC-2 media demonstrate that hydrolytic bacterial had enhanced with an abundance
172 of organic substrate supplement, however, methane biosynthesis tended to inhibit in
173 culture even with additional sodium acetate. In MAC-1 and MAC-2 experiments, the
174 *acetogenic bacteria* inhibited also with sodium acetate influence in the medium.

175 The MAC-3 medium, which follows the same medium concentration of MAC-2
176 except sodium acetate, enhanced *acetogenic bacteria*, and inhibited the *methanogens*.
177 After 40 days of culture, the average H_2 volume concentration for the MAC-3 and MAC-
178 3* group achieved 67.2% and 59.15% as indicated by gas analysis. This amount was 33-
179 40 times greater than that of all other experimental groups. The exogenous organic
180 material played an important role in carbon supply.

181 However, organic nutrition plays an important to supply the vitamin and other
182 microelements besides organic nutrients. Without the vitamin and microelements besides
183 organic nutrients supply, the consortia would grow at a slower rate, such as that observed
184 for MAC-5.

3.2 The nutritional metabolism analysis of methanogenic consortium

Changes in MAC-4 ion concentrations were analyzed in the initial and final media. The data from this analysis revealed that the concentrations of sodium, potassium, ammonia nitrogen and magnesium were consumed microbially. In particular, 85.78% of the sodium was utilized with microbial fermentation. The medium pH changed from 6.65 to 7.32 over this period. In contrast, the concentrations of sulfate and bicarbonate increased 1.5-7 fold (Table 2). These data indicated that sodium, nitrogen, potassium, and magnesium are the key elements for methanogenic consortium fermentation. However, the sulfate should be maintained at a low level in the medium.

Table 2. The MAC-4 medium ion concentration changes with methanogenic consortium bio-degradation.

	pH	Fluoride	Chloride	Nitrate Nitrogen	Nitrite Nitrogen	Phosphate	Sulfate	Carbonate
Initial	6.65	3.70	81.58	-	-	44.67	195.80	-
final	7.32	5.18	95.90	-	-	38.60	478.40	-
	Bicarbonate	Sodium	Ammonia Nitrogen	Potassium	Magnesium	Calcium	Water Hardness	Total Aalkalinity
Initial	40.66	811.90	193.60	159.00	38.89	21.06	212.56	40.66
final	328.77	115.40	39.23	35.30	14.30	22.76	115.62	328.77

3.3 The cations orthogonal analysis

From the analysis of nutrient element concentrations, it is clear that sodium, ammonia, nitrogen, potassium, and magnesium play important roles in the methanogenic

199 microbial metabolism. The orthogonal analysis experiment was designed to identify the
200 influence of cations in bio-methane yield. The orthogonal module introduced 4 cations,
201 (Na^+, K^+, Ca^{2+}, Mg^{2+}) as tested with 4 different concentrations. The lowest ion
202 concentration was set by MAC-4 and the highest reference level for cation analysis was
203 that of the East China Sea ion concentration. The $L_{16}(4^5)$ orthogonal table was used as an
204 orthogonal analysis module (Table 3).

205 **Table 3.** The cation orthogonal analysis table for CH_4 and CO_2. The results represent
206 volume percent units for both gases. The table shows the analysis of CH_4 and CO_2
207 ranges.

Serial Number		Factors（mmol/L）				Results	
		Na	Mg	Ca	K	CH_4	CO_2
1		23	1.3	0.5	10.7	6.65	4.71
2		23	6.7	1.7	1.7	3.92	4.27
3		23	13.0	3.4	3.4	0.92	4.03
4		23	20.0	5.1	5.1	0.46	3.23
5		130	1.3	1.7	3.4	6.59	4.84
6		130	6.7	0.5	5.1	3.85	3.56
7		130	13.0	5.1	10.7	0.99	3.31
8		130	20.0	3.4	1.7	0.38	2.94
9		260	1.3	3.4	5.1	6.49	4.47
10		260	6.7	5.1	3.4	3.76	4.73
11		260	13.0	0.5	1.7	0.57	2.47
12		260	20.0	1.7	10.7	0.34	2.84
13		390	1.3	5.1	1.7	7.01	4.86
14		390	6.7	3.4	10.7	0.30	3.90
15		390	13.0	1.7	5.1	0.47	2.28
16		390	20.0	0.5	3.4	0.30	2.67
	k_{1j}	2.99	6.69	2.84	2.97		
CH_4	k_{2j}	2.95	2.96	2.83	2.89		
	k_{3j}	2.79	0.74	2.02	2.82		

	k_{4j}	2.02	0.37	3.06	2.07
	R_j	0.97	6.32	1.03	0.90
	k_{1j}	4.06	4.72	3.35	3.64
	k_{2j}	3.66	4.12	3.56	4.07
CO_2	k_{3j}	3.63	3.02	3.84	3.39
	k_{4j}	3.43	2.92	4.03	3.69
	R_j	0.63	1.80	0.68	0.68

Range analysis was used to indicate the affected order among factors. Range R_j was calculated with module 1.

$$R_j = Max_{k_{jm}} - Min_{k_{jm}} \quad (1)$$

Where k_{jm} is the average result of the m level j factor (module 2), (j, m =1, 2, 3, 4).

$$k_{jm} = \sum_{i=1}^{4} y_{jmi} \ /4 \quad (2)$$

Where y_{jmi} is the result of the m level j factor number i data, (j, m, i =1, 2, 3, 4).

Range analysis confirmed that for methanogenic activity, the best ion concentrations for enhancement CH_4 yield were Na_1, Mg_1, Ca_4, and K_1, with the rank order of element effectiveness being Mg > Ca > Na > K. The most effective ion concentration for control CO_2 generation were Na_4, Mg_4, Ca_1, and K_3, with its ran order being Mg > Ca = K > Na.

Notable differences exist regarding the influence of CH_4 and CO_2 on yield rates as a function of cation concentrations. The CH_4 biosynthesis is substantially more sensitive to element content. Maximal Mg^{2+} concentrations to enhance methanogen metabolism processes were 1.3 mmol/L, while maximal cation concentrations for Ca^{2+}, K^+, Na^+ were 5.1, 1.7 and 23 mmol/L, respectively.

223 Variance analysis has been used to analyze the Factor Significance (F) in this

224 system. F was calculated with module 3.

$$F_j = \frac{4\sum_{i=1}^{4} K_{ij}^2 - \left(\sum_{i=1}^{16} x_i\right)^2}{16\sum_{i=1}^{16} x_i^2 - \left(\sum_{i=1}^{16} x_i\right)^2} \times 5 \quad (3)$$

225

$$K_{ij} = \sum_{i=1}^{4} y_{jmi} \quad (4)$$

226

227 Where x_i is the experiment results for every factor and level.

228 **Table 4.** The variance analysis table for 4 factors with CH_4 and CO_2 data.

	Factor	Square of Deviance	Degree of Freedom	F Value	Significant
CH_4	Na^+	2.465	3	0.892	
	Mg^{2+}	100.905	3	36.533	*
	Ca^{2+}	2.486	3	0.900	
	K^+	2.080	3	0.753	
	Error	2.762	3		
CO_2	Na^+	0.842	3	8.592	
	Mg^{2+}	9.120	3	93.061	**
	Ca^{2+}	1.079	3	11.010	*
	K^+	0.954	3	9.735	
	Error	0.098	3		

Based upon the F calculation, Mg^{2+} was the significant cation factor for CH_4 and CO_2 (Table 4). This ion is particularly sensitive in inhibiting CH_4 metabolism processes largely for gas-coal methanogenic consortium.

4. Discussion

Microbial bio-degradation of some organic compounds of coal represents a current technology available to enhance coal-bed methane. Microbial cooperation via anaerobic digestion processes results in the implementation of secondary biogenic methane generation and gas reservoirs enhancement in mining. Based on the experiments performed in this report, the following conclusions can be garnered: (1) The organic nutrient dose should be adjusted to methanogenic consortium requirements. Excessive or deficient organic nutrient support doses adversely affecting biomethane yield; (2) MAC-4 was the most effective medium in enhancing anaerobic digestion in Fushun gas-coal seam; (3) The sodium, nitrogen, potassium, and magnesium are the key elements for methanogenic consortium fermentation; (4) The cation rank order of influence Fushun methanogenic consortium metabolism was Mg > Ca > Na > K, and maximal cation concentrations of Mg^{2+}, Ca^{2+}, K^{+}, Na^{+} were 1.3, 5.1, 1.7, and 23 mmol/L, respectively; (5) Mg^{2+} was a particularly sensitive factor, which could inhibit methanogenic bacteria.

5. Conclusion

5.1 The nutrient concentration for gas-coal methanogenic consortium culture

248 The biomethane generation from coal involves a complex interaction between
249 environmental factors and biotic communities. Hydrolytic fermentative bacteria,
250 syntrophic *acetogenic bacteria*, methanogenic bacteria, and many other bacteria comprise
251 the biotic community. The environment includes not only the physical factors but also the
252 coal, coalbed water, coal-bed gas and other complex formations which we call the
253 environment.

254 The medium injected into coal seam enriching nutrients of the coal seam. The series
255 of experiments of this report confirm that nutrient media require a strict concentration of
256 control to be effective, especially for those involving organic materials. If the organic
257 compounds are too rich, like that modeled with our formulations of MAC-1, MAC-2, and
258 MAC-3, the excessive levels of nutrients will adversely affect the microbial community
259 structure. High rates growth of hydrolytic fermentative bacteria or *acetogenic bacteria*
260 could inhibit methane biosynthesis. In contrast, if lack organic nutrition, such as that
261 modeled in MAC-5, the potential for enhancing methane biosynthesis is low and the yield
262 proceeds at a slow rate. Concentrations of nutrition required should differ as a function
263 of: (1) the methane biosynthesis type, such as carbon dioxide reduction or acetate
264 fermentation; (2) coal maturity grade, such as gas-coal, flame coal, bituminous coal or
265 lignite. The exact organic nutrition required for different coal ranks needs to be identified
266 to maximize bio-methane yield rates.

267 **5.2 Ion concentration for bio-methane yield enhancement**

Organic biological degradation to methane is a microbial cooperation process. Fermentative bacteria initially hydrolyze complex organic compounds to acetate, longer chained fatty acids, carbon dioxide, hydrogen, NH_4^+, and HS^-. Syntrophic hydrogen-producing (proton-reducing) *acetogenic bacteria* reduce intermediary metabolites to acetate, carbon dioxide, and hydrogen. Hydrogen-utilizing *acetogenic bacteria* demethoxylate low molecular weight ligneous compounds and ferment some hydroxylated aromatic compounds. Carbon dioxide reduction methanogenic bacteria are dependent on hydrogen, produced by other bacteria, to reduce carbon dioxide or bicarbonate to methane. And acetate fermentation methanogen yields methane via acetate bio-degradation.

Different nutrients are required for different microbial activity in the community. Results from the cation orthogonal experiments revealed that cation concentrations critically influence the metabolism process of the microbial. Except for the examination of sodium, potassium, magnesium, calcium which analyzed in the cations orthogonal analysis, the nitrogen, yeast extract, salinity and pH could have the potential to influence the biomethane synthesis. More researches to identify the effects of ions upon the coal seam biotic community are needed to reveal the microbial activity in gas-coal beds.

6. Competing interests

The authors declare that they have no competing financial interests.

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8. References

1. Lv Y, Tang D, Xu H, Luo H. Production characteristics and the key factors in high-rank coalbed methane fields: A case study on the Fanzhuang Block, Southern Qinshui Basin, China. Int J Coal Geol. 2012; doi:10.1016/j.coal.2012.03.009

2. Park SY, Liang Y. Biogenic methane production from coal: A review on recent research and development on microbially enhanced coalbed methane (MECBM). Fuel. 2016. doi:10.1016/j.fuel.2015.10.121

3. Scott AR. Improving Coal Gas Recovery with Microbially Enhanced Coalbed Methane. Coalbed Methane: Scientific, Environmental and Economic Evaluation. 1999. doi:10.1007/978-94-017-1062-6_7

4. Green MS, Flanegan KC, Gilcrease PC. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, U.S.A. Int J Coal Geol. 2008; doi:10.1016/j.coal.2008.05.001

5. Penner TJ, Foght JM, Budwill K. Microbial diversity of western Canadian subsurface coal

beds and methanogenic coal enrichment cultures. Int J Coal Geol. 2010; doi:10.1016/j.coal.2010.02.002

6. Strąpoć D, Picardal FW, Turich C, Schaperdoth I, Macalady JL, Lipp JS, et al. Methane-producing microbial community in a coal bed of the Illinois Basin. Appl Environ Microbiol. 2008;74: 2424–2432. doi:10.1128/AEM.02341-07

7. Li D, Hendry P, Faiz M. A survey of the microbial populations in some Australian coalbed methane reservoirs. Int J Coal Geol. 2008; doi:10.1016/j.coal.2008.04.007

8. McIntosh J, Martini A, Petsch S, Huang R, Nüsslein K. Biogeochemistry of the Forest City Basin coalbed methane play. Int J Coal Geol. 2008; doi:10.1016/j.coal.2008.03.004

9. Hall RO, Tank JL, Baker MA, Rosi-Marshall EJ, Hotchkiss ER. Metabolism, Gas Exchange, and Carbon Spiraling in Rivers. Ecosystems. 2016; doi:10.1007/s10021-015-9918-1

10. Beckmann S, Luk AWS, Gutierrez-Zamora ML, Chong NHH, Thomas T, Lee M, et al. Long-term succession in a coal seam microbiome during in situ biostimulation of coalbed-methane generation. ISME Journal. 2018. doi:10.1038/s41396-018-0296-5

11. Chen T, Zheng H, Hamilton S, Rodrigues S, Golding SD, Rudolph V. Characterisation of bioavailability of Surat Basin Walloon coals for biogenic methane production using environmental microbial consortia. Int J Coal Geol. 2017; doi:10.1016/j.coal.2017.05.017

12. Xiao D, Wang E-Y, Peng S-P, Wu J-Y. Responses of coal anaerobic fermentation fractures development. Meitan Xuebao/Journal China Coal Soc. 2017;42. doi:10.13225/j.cnki.jccs.2016.1109

13. Meng J, Nie B, Zhao B, Ma Y. Study on law of raw coal seepage during loading process at different gas pressures. Int J Min Sci Technol. 2015; doi:10.1016/j.ijmst.2014.12.005

14. Galagan JE, Nusbaum C, Roy A, Endrizzi MG, Macdonald P, Fitzhugh W, et al. The

genome of M. acetivorans reveals extensive metabolic and physiological diversity. Genome Res. 2002; doi:10.1101/gr.223902

15. Wang E, Chen P, Li Z, Shen R, Xu J, Zhu Y. Resistivity response in complete stress-strain process of loaded coal. Meitan Xuebao/Journal China Coal Soc. 2014;39. doi:10.13225/j.cnki.jccs.2013.1070

16. Ali Shah F, Mahmood Q, Maroof Shah M, Pervez A, Ahmad Asad S. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. The Scientific World Journal. 2014. doi:10.1155/2014/183752

17. Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil P-A, et al. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat Biotechnol. 2018; doi:10.1038/nbt.4229

18. Zinder SH. Physiological Ecology of Methanogens. Methanogenesis. 1993. doi:10.1007/978-1-4615-2391-8_4

19. Bellou S, Baeshen MN, Elazzazy AM, Aggeli D, Sayegh F, Aggelis G. Microalgal lipids biochemistry and biotechnological perspectives. Biotechnology Advances. 2014. doi:10.1016/j.biotechadv.2014.10.003

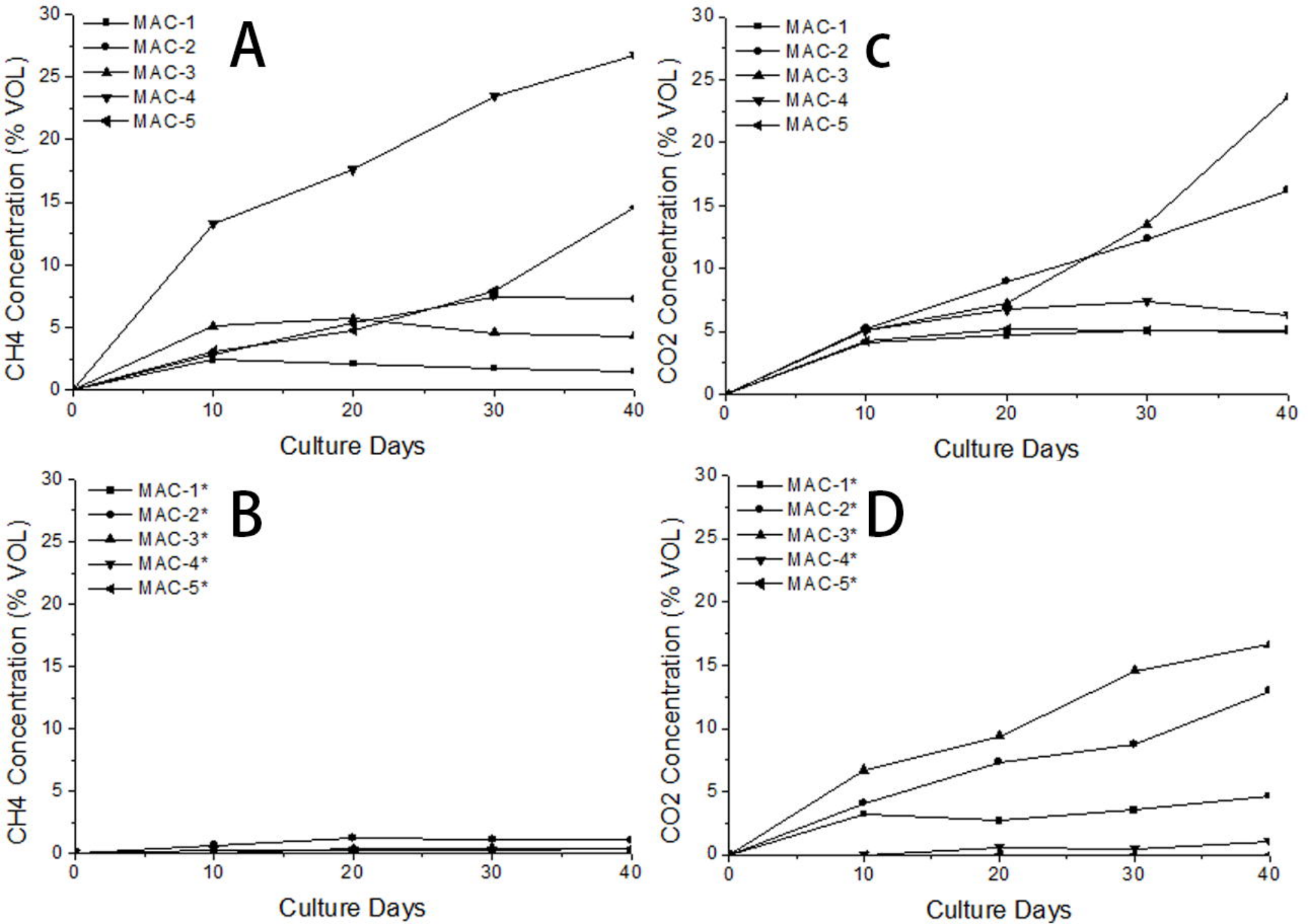
20. Gerritsen J, Fuentes S, Grievink W, van Niftrik L, Tindall BJ, Timmerman HM, et al. Characterization of Romboutsia ilealis gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus Clostridium into the genera Romboutsia gen. nov., Intestinib. Int J Syst Evol Microbiol. 2014; doi:10.1099/ijs.0.059543-0

21. Kwietniewska E, Tys J. Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation. Renew Sustain Energy Rev. 2014; doi:10.1016/j.rser.2014.03.041

356

Figure Legends

Figure 1. Changes in methane and carbon dioxide production as a function of media nutrition and time in culture. Figure A is the methane concentration changes with culture days. And figure B is the control experiment without coal sample supply in experiment. Figure C is the carbon dioxide concentration changes with culture days. And figure D is the control experiment without coal sample supply in experiment. Methane is the main factor to identify the *methanogens* activity. And carbon dioxide is an important factor to identify the fermentative and *acetogenic bacteria* activity. The methane yield rate would high, only if the fermentative and *acetogenic bacteria* activity in a limited condition. Organic material could enhance the fermentative and *acetogenic bacteria*. However, if the activity of fermentative and *acetogenic bacteria* is too high, it would inhibit the *methanogens*. The microbial group will in a good balance when achieved the highest methane yield and the best ratio of methane and carbon dioxide. Thus the MAC-4 medium culture group fit for the requirements.


A
MAC-1
MAC-2
MAC-3
MAC-4
MAC-5
CH4 Concentration (% VOL)
Culture Days
B
MAC-1*
MAC-2*
MAC-3*
MAC-4*
MAC-5*
CH4 Concentration (% VOL)
Culture Days
C
MAC-1
MAC-2
MAC-3
MAC-4
MAC-5
CO2 Concentration (% VOL)
Culture Days
D
MAC-1*
MAC-2*
MAC-3*
MAC-4*
MAC-5*
CO2 Concentration (% VOL)
Culture Days