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Methane Generation from Anthracite by Fungi and Methanogen Mixed Flora Enriched from Produced Water Associated with the Qinshui Basin in China

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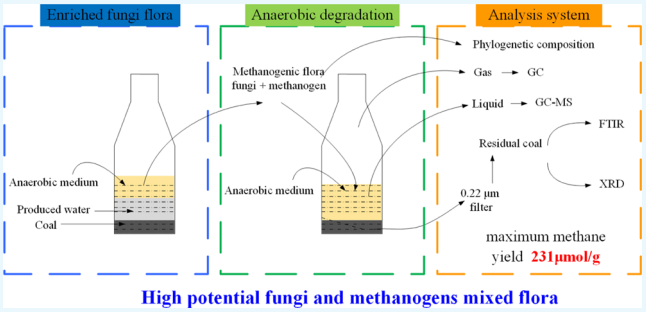
ABSTRACT: Biogenic coalbed methane (CBM) is generally believed to be formed by anaerobic bacteria and methanogens, while a few studies took fungi into account. Here, the microflora consisting of fungi and methanogens was enriched from the produced water associated with the Qinshui Basin using anthracite as the only carbon source. The maximum methane yield of 231 μmol/g coal was obtained after 22 days of cultivation under the optimum temperature of 35 °C, pH of 8, salinity of 0−2%, particle size of 0.075−0.150 mm, and the solid−liquid ratio of 1:30. It could remain active even after exposure to air for 24 h. Miseq results showed that the archaea were mainly composed of *Methanocella*, a hydrogenotrophic methanogen, followed by acetoclastic methanogen *Methanosaeta* and *Methanosarcina*, which could use various methanogenic substrates. The fungal communities mainly included *Amorphotheca*, *Alternaria*, *Aspergillus*, and *Penicilium*, which are all able to degrade complex organics such as aromatics and lignin. After cultivation, the crystal structure of anthracite became looser, as shown by XRD results, which might be due to the swelling effect caused by the destruction of the aromatic ring structure of coal under the function of fungi. The stretching vibration intensity of each functional group in coal decreased with cultivation, as revealed by FTIR. The GC-MS results showed that the concentration of alkanes and alcohols decreased significantly, which are the products of ring-opening of aromatics by fungi. These results suggested that fungi and methanogens in the coalbed also can syntrophically degrade coal effectively, especially for aromatics in coal.



1. INTRODUCTION

In recent years, the exploitation and development of unconventional natural gas has been widely carried out to meet the increasing energy demand.[1] As an important unconventional natural gas, CBM refers to the methane adsorbed in coal seam,[2] which is being vigorously developed in the major coal-producing countries of the world. Biogenic CBM is an important part, which accounts for more than 20% of CBM worldwide and an additional 10% may also be of microbial origin.[3,4] Most of the biogenic CBM retained in coalbed nowadays is secondary, which is generated after coal formation by the anaerobic microorganisms in coalbed under suitable environmental conditions.[5,6] Based on the formation of biogenic CBM, the technology of microbially enhanced CBM (MECBM) was proposed to convert coal into CH_4 via specific microorganisms. It can not only biomine raw coal and residual coal but also increase the porosity of coal and decrease the affinity of coal for CH_4 to improve the development of CBM.[7] In addition, other organic liquid products could also be generated from coal during MECBM, which further added to its value.[8,9]

As coal is a complex organic compound, which is composed of aromatic hydrocarbons, aliphatic hydrocarbons, and heterocyclic compounds, various microorganisms with diverse metabolic characteristics are required to degrade coal to produce methane.[10] Only one paper reported methane production from coal by a single methanogen.[11] It is generally believed that microorganisms mainly break the coal structure and metabolize the intermediates such as fatty acids, organic acids, and alcohols to generate substrates for methanogens.[12] Most of the previous studies analyzed the methanogens and bacteria that functioned in coal biodegradation. Various methanogens have been detected in the produced water and the culture solution in the laboratory, such as acetoclastic

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Methanoseta, hydrogenotrophic *Methanobacterium*, *Methanocella*, and methylotrophic *Methanolubus*. *Proteobacteria*, *Firmicutes*, *Clostridiales*, *Actinobacteria*, and *Bacteroidetes* are found to be the dominant bacteria in CBM fields and the main participant in coal degradation.[3,13−17] Several microflora consisting of bacteria and methanogens have also been enriched from the produced water.[18−20] Jones et al. enriched a methanogenic mixed culture from wetland named WBC-2 with the ability to degrade coal, which was mainly composed of *Clostridium* sp., *Bacteroides* spp., *Acetobacterium* sp., and acetoclastic methanogen *Methanomicrobia*.[21]

A great number of evidence have shown that fungi are good degrader for macromolecular compounds such as lignin, lignocellulose, lignin-derived compounds, synthetic dyes, and polycyclic aromatics (PAHs).[22,23] Some fungi have also been employed to ferment coal. Fungi flora AD-1, isolated from the low rank coal, was found to have the abilities of decarboxylation and deamination, as well as breaking the side chains of the aromatic rings.[24] *Trichoderma atroviride* was used to biosolubilize coal in a stirred tank reactor.[25] *Polyporus versicolor* and *Poria monticola*, two species of basidiomycete fungi, were reported to attach to the coal surface and liquefy and biodegrade lignite.[26] Haider et al. isolated fungi MW1 to pretreat the low rank coal to improve the production of humic acids.[27] Actually, the syntrophic relationship between anaerobic fungi and methanogens has been detected in methane production in bovine rumen, and the microflora consisting of anaerobic fungi and methanogens has also been isolated with the high fiber degradation ability.[28,29] However, only a few studies reported the fungi communities related to the formation of biogenic CBM.[30,31] A surprising diversity of the fungal community was found in the produced water from the Qinshui Basin. The main fungi included *Rhodotorula*, *Mortierella*, *Acremonium*, *Fusarium*, *Trichoderma*, *Aspergillus*, and *Schizophyllum*, which could generate a significant methane yield from coal in collaboration with methanogens.[30] In the laboratory, fungi were also found to play a role in coal degradation together with bacteria and methanogens.[31] Thus, the fungi community are the non-negligible part in coal biodegradation to produce methane.

In this study, a microflora with fungi and methanogens to degrade anthracite and produce methane was enriched from the produced water obtained from the Qinshui Basin. The fungal and archaeal communities in the microflora were evaluated using Miseq. The growth conditions of microflora were optimized. The success of the coal structure and intermediates during methane production was analyzed by Fourier transform infrared spectrometry (FTIR), X-ray diffraction (XRD), and gas chromatography-mass spectrometry (GC-MS) to discuss the mechanism of coal biodegradation by the microflora.

2. RESULTS AND DISCUSSION

2.1. Physicochemical Properties of Coal and Formation Water. The proximate analysis and ultimate analysis of coal samples showed that M_{ad}, A_{ad}, and V_{daf} were 1.90, 10.41, and 8.82%, respectively, and C_d, H_d, O_d, N_d, and S_{td} were 83.01, 3.24, 1.76, 1.27, and 0.31%, respectively, which belonged to the range of anthracite.[32] The concentrations of major cations, anions, and ammonium in the formation water are shown in Table 1, which are similar with the previous research in the same site; both detected high salinity with high concentration of Na^+.[33]

Table 1. Analysis Result of Formation Water Samples

parameter	units	values
K^+	mmol/L	0.02
Na^+	mmol/L	18.01
Ca^{2+}	mmol/L	0.12
Mg^{2+}	mmol/L	0.01
Cl^-	mmol/L	1.36
SO_4^{2-}	mmol/L	0.32
NO_3^-	$\times10^{-3}$ mmol/L	0.48
NO_2^-	$\times10^{-3}$ mmol/L	0.22
TOC	mmol/L	0.14

2.2. High Methane Yielded by the Microflora with Fungi and Methanogens. The methanogenic flora with fungi and methanogen was successfully enriched from the produced water with the ability to degrade coal and produce methane. Figure 1 shows the methane productions in the 1st,

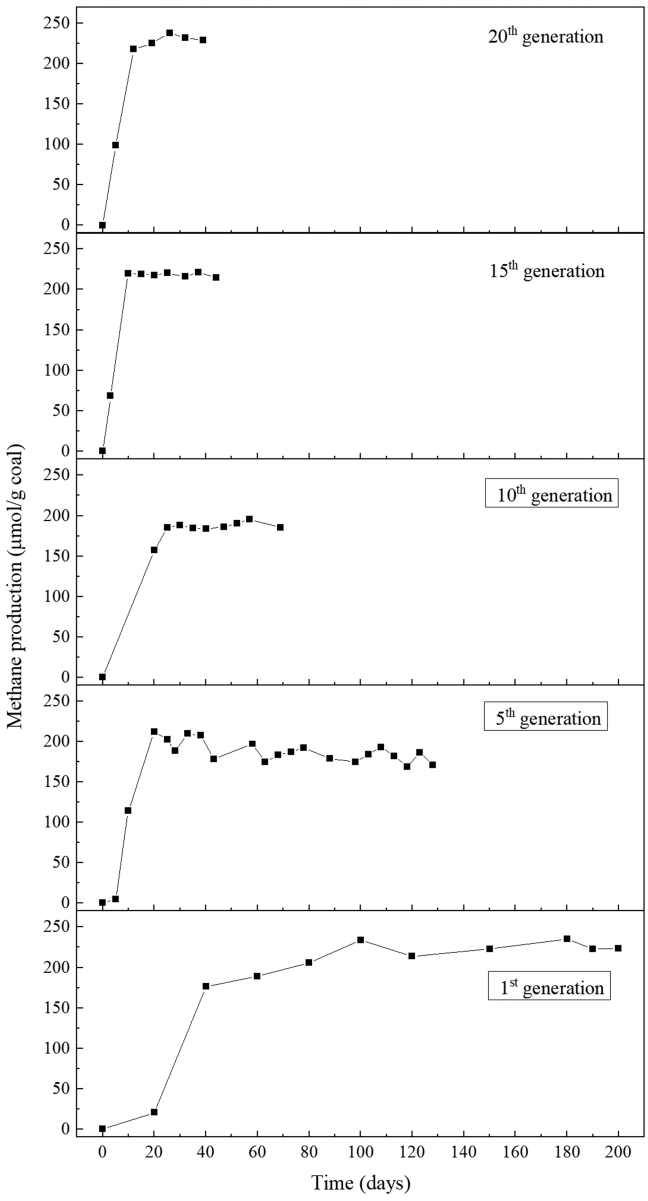

Figure 1. Methane productions in 1st, 5th, 10th, 15th, and 20th generations.

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Table 2. Summary of Archaeal and Fungal MiSeq Reads, OTUs, and Diversity Estimators of Enriched Microflora at 97% Similarity

reads	OTUs	Ace	Chao1	Shannon	Simpson	coverage
fungi						
31 887	33	33.00 (33.00, 33.00)	33.00 (33.00, 33.00)	1.84 (1.83, 1.85)	0.25 (0.25, 0.25)	0.9999
archaea						
39 169	15	16.00 (15.13, 22.46)	15.00 (15.00, 0.00)	0.76 (0.75, 0.77)	0.68 (0.67, 0.68)	0.9999

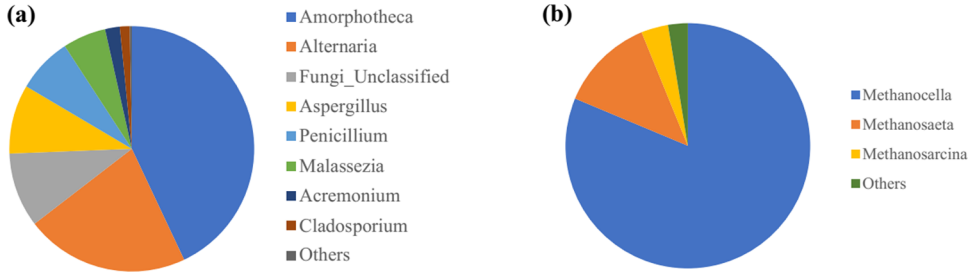

Figure 2. Phylogenetic compositions of (a) fungal and (b) archaeal communities at the genetic level in the enriched microflora based on the MiSeq data. The genera that contained <1% of the sequence reads were grouped into "Others".

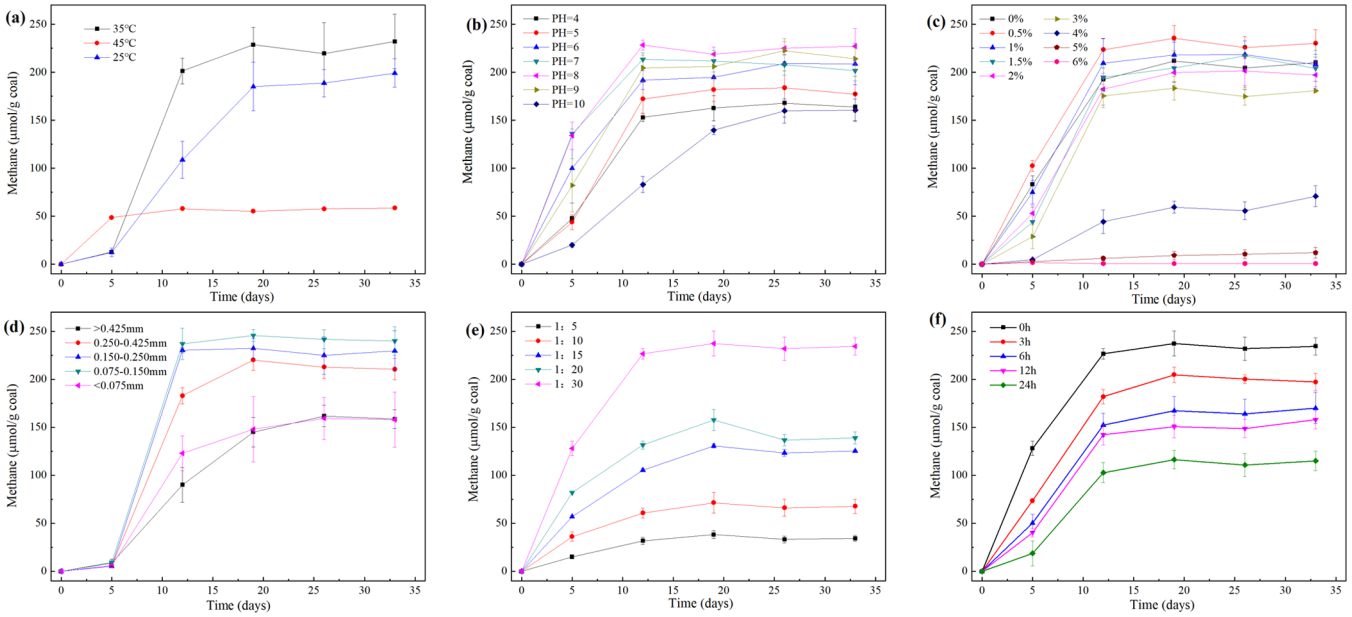

Figure 3. Methane produced by the microflora under different culture conditions, including (a) temperature, (b) pH, (c) salinity, (d) coal particle size, (e) solid−liquid ratio, and (f) oxygen content.

5th, 10th, 15th, and 20th generations of enrichment. With transferring, the time for the microflora to reach the maximum methane yield decreased and the methane production became constant. The maximum methane yield of about 220 μmol/g coal was reached on day 20 incubation after enrichment, suggesting that fungi in produced water could degrade the high-rank coal such as anthracite to supply substrates for methanogens to produce methane.

2.3. Phylogenetic Composition of Microbial Communities in Microflora. The summary of fungal and archaeal MiSeq reads, OTUs, and diversity estimators of enriched microflora at 97% similarity are shown in Table 2. A total of 32 018 fungal sequence reads and 39 691 archaeal sequence reads were generated by MiSeq, and 33 fungal OTUs and 15 archaeal OTUs were obtained. The coverages were both above 0.999. The Shannon and Simpson estimators showed that the fungal diversity was more than two times higher than archaeal diversity.

The phylogenic composition of communities is shown in Figure 2. There are five main fungal genera with more than 5% of sequence reads including *Amorphotheca* (42.95%), *Alternaria* (21.62%), *Aspergillus* (9.13%), *Penicilium* (7.25%), and *Malassezia* (5.7%). They all had potential to degrade aromatic and lignin-derived compounds in coal.[34] *Amorphotheca* is known to utilize different kinds of organic substances such as alkanes, acetic acid, and lignocellulose biomass.[35] It has been confirmed that *Amorphotheca* can degrade various inhibitor compounds such as a high level of acetic acid from pretreated lignocellulose feedstock, and it has been applied for producing ethanol, lactic acid, gluconic acid, and microbial lipid with a high product yield and zero wastewater generation.[36−38] *Alternaria* is one of only a few of fungi reported so far to be capable of degrading heavy crude oil, which is composed of

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PAHs and has a higher metabolization of the aromatic fraction.[39] *Aspergillus* was reported to use polysaccharide wastes as substrates to produce VFAs, acetate, and butyrate, and promote H_2 generation.[40,41] The growth of *Aspergillus* was also found on the surface of aromatic polyesters,[42] demonstrating its remarkable ability to degrade complex aromatics, especially the high-molecular-weight PAHs.[43] Previous studies have also shown that *Aspergillus* can degrade carbohydrates in plants and rice straw.[44] *Penicillium* is a typical fermenter that can degrade cellulose and lignin effectively,[45] and it was also be found to efficiently degrade PAHs such as phenanthrene,[46] fluorene,[47] and pyrene.[48]

The archaea mainly included *Methanocella* (81.29%), *Methanosaeta* (12.54%), and *Methanosarcina* (3.55%). It suggested that the main metabolic type of the enriched consortia was hydrogenotrophic methanogenesis, as *Methanocella* is a thermophilic hydrogenotrophic methanogen.[49] Methane could also be generated by other methanogenic pathways considering that the metabolic type of *Methanosaeta* is acetoclastic, and *Methanosarcina* can utilize different substrates, such as CO_2, H_2, acetate, and methylamine.[20,31] Thus, the enriched microflora can produce methane through diverse metabolic pathways, while hydrogenotrophic methanogenesis is the main pathway.

2.4. Effects of Culture Conditions on Methane Production. The most critical step in the technology of MECBM was to obtain the high-efficiency methanogenic microflora. The culture conditions were one of the main factors influencing the efficiency of coal biodegradation by microflora. Thus, five critical factors were evaluated here, including temperature, salinity, particle size, pH, and the solid−liquid ratio. At the same time, the effect of the oxygen content was also evaluated.

Methane production was significantly affected by the culture temperature. As shown in Figure 3a, the highest methane yield was 231 μmol/g coal at 35 °C, while methane yields were 199 μmol/g coal at 25 °C and 58.47 μmol/g coal at 45 °C. However, at the initial stage, especially the first 5 days, the methane generating rate at 25 and 35 °C (both about 12 μmol/g coal per day) was lower than that at 45 °C (48 μmol/g coal per day). It showed that the higher temperature could enhance the production of methane in the early time.[50] Simultaneously, the higher temperature also shortened the reaction period, which was about 33 days at 25 °C, 19 days at 35 °C, and 12 days at 45 °C.

The cultivations can be divided into two groups according to methane yields when pH changed from 4 to 10 (Figure 3b). When pH = 6−9, methane yields did not change significantly, and 209.08−226.95 μmol/g coal methane was observed when the maximum methane yield was obtained at pH = 8. When pH = 4, 5, and 10, methane yields were only 160, 177, and 160 μmol/g coal, respectively. The activity of microorganisms was inhibited under higher acidity or alkalinity.[51] The microflora was more suitable for the weak alkalinity condition.

The salinity tolerance of the microflora was as high as 4% (Figure 3c). The optimum salinity range for coal degradation was 0−3%, and the highest methane yield of 230 μmol/g coal was observed at a salinity of 0.5%. With the increase in salinity, the maximum methane production decreased, which was consistent with the previous study.[52] The methane production decreased significantly at a 4% salinity with only 50 μmol/g coal, and even no methane was detected at 5−6% salinity. The good salinity tolerance of the microflora is related to its origin habitat where a high concentration of Na^+ was detected (Table 2).

There was also an optimal coal particle size for methane production. A maximum methane yield of about 231 μmol/g coal was obtained when the coal particle size was in the range of 0.075−0.150 mm (100−200 mesh) (Figure 3d). When the coal particle size was >0.425 and <0.075 mm, methane yields were both lower with only 159 μmol/g coal. It did not agree with the previous study, which showed that the smaller the coal particle size, the higher the methane production.[50] It was believed that the surface area of coal increased with the decrease in coal particle size, leading to the increase of contact between organisms and coal. However, too small coal particles (<0.075 mm) would result in the reintegration of coal particles, leading to a decrease in the contact area and low methane production.

Methane production was negatively correlated with the solid−liquid ratio (Figure 3e). The maximum methane production of 231 μmol/g coal was observed at a solid−liquid ratio of 1:30. When the solid−liquid ratio was 1:5 (6 g coal), the methane production was only 38 μmol/g coal. The higher coal loading capacity would bring more toxic substances into the culture, which inhibited the microbial activities, leading to a decrease in methane production.[53]

It is hard to keep the microflora away from air all the time when injecting it into coal seam. Thus, oxygen resistance is essential for the application of microflora. As shown in Figure 3f, methane production decreased with the prolongation of exposure time in the air. In the anaerobic environment, the microflora could show the best biodegradation ability, and the highest methane yield was 237 μmol/g coal. When the exposure time was 3 h, the methane yield was about 200 μmol/g coal. After exposure for 6 h, it decreased to 170 μmol/g coal and continued to decrease to 150 μmol/g coal after 12 h and 115 μmol/g coal after 24 h. It is expected that coal biodegradation could be performed aerobically as many fungi detected here are facultatively anaerobic. At present, most methanogens are generally believed to be strictly anaerobic,[54,55] while some methanogens are reported to survive in the oxygen environment for several hours and days with very low methane generating rates.[56−58] Here, the microflora were active even after 24 h of exposure to air, although the methane production was half of the maximum. The methane generating trend did not change under different oxygen exposure times. The only difference was the amount of methane produced, suggesting that the microbial activities decreased after oxygen exposure. Thus, it can be believed that methane production would be recovered after several transfers and the microflora is adaptable in the field. These results supported the feasibility of injecting microflora into the coal seam.

2.5. Dynamic Changes in the Coal Crystal Structure during Anaerobic Biodegradation. The XRD spectra of raw coal and residual coal after 15 and 27 days of cultivation are shown in Figure 4, and the corresponding structural parameters are shown in Table 3. In general, the change in the coal structure mainly occurred before 15 days, and significant changes were not detected between 15 and 27 days, which was consistent with methane production, which was almost completed at 15 days. The stacking height (L_c), aromatic layers (N), and L_a/L_c increased with culture time. It can be inferred that the interaction of coal and microflora caused the swelling effect on the coal crystallite structure. A_{26} and A_{20} are believed to be equal to the number of aromatic carbon (C_{ar})

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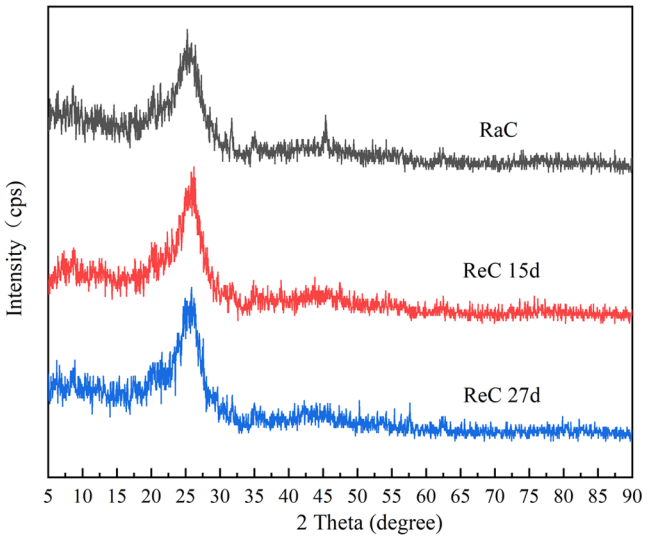

Figure 4. XRD curves of raw coal (RaC) and residual coal after 15 days (ReC15d) and 27 days (ReC27d) of cultivation.

atoms and aliphatic carbon (C_{al}) atoms.[59] The intensity ratio (A_{26}/A_{20}) of the two peaks can reflect the aromaticity of coal.[39] During the reaction, A_{26} and A_{20} decreased from 11 706 to 8547 and 1269 to 1185, respectively, suggesting that the number of aromatic and aliphatic carbon atoms decreased by 26.98 and 6.6%. The ratio of A_{26}/A_{20} decreased from 9.22 to 7.21, indicating that the aromaticity and the ordered degree of the crystallite structure decreased. It can be inferred that the fungal flora acted on aromatic parts of coal, breaking the connection between aromatic rings and degrading the aromatic compounds effectively.

2.6. Changes of Functional Groups in Coal during Anaerobic Biodegradation. The FTIR spectra of coal samples are shown in Figure 5a. Similar to the results of XRD, the FTIR spectra of coal samples changed significantly between 0 and 15 days, which showed that almost all of the bands decreased sharply, while they barely changed between 15 and 27 days. The wavelengths between 3000 and 700 cm^{-1} were divided into three regions according to the previous studies.[60,61] The wavelengths between 900 and 700 cm^{-1} are attributed to aromatic groups, those between 1800 and 1000 cm^{-1} are attributed to oxygen-containing groups, and those between 3000 and 2700 cm^{-1} are attributed to aliphatic hydrocarbon groups. Their curve-fitting FTIR spectra are shown in Figure 5b–d. The details for each adsorption band in all three groups, including the center position, height, area, area %, and the assignment, are listed in Tables S1–S3.

There were primarily six absorption bands detected in the infrared spectrum of aromatic structures (Figure 5b). The most apparent stretching vibration intensity of aromatic functional groups in raw coal (RaC) is the stretching vibration of aromatics with two substitutions, which accounted for 27.43% of the aromatics. After 27 days of biodegradation, the stretching vibration intensity of each characteristic peak in the aromatic functional group weakened obviously when the range of peak intensity decreased from 4.17–11.94 to 1.3–6.11, which showed a correlation with the decrease of A_{26} in XRD that the aromatic carbon decreased. It suggested that the fungi in microflora could destroy the complex aromatic macromolecules in coal and utilize the simple aromatic substances in coal to generate the substrate of methane production,[62,63] leading to the depolymerization of coal and conducive to the follow-up biological reaction.

Eight absorption bands were found in the region of oxygen-containing function groups (Figure 5c). The stretching vibration intensity of each functional group decreased obviously during biodegradation, especially for aliphatic with an oxygen functional group, with the peak intensity of C–O alcohols and C–O ethers decreased by 81.17 and 86.38%. It is expected because oxygen-containing groups in coal are believed to be the acting site for microorganisms.[64] These results suggested that the oxygen-containing groups were also the favorite parts for fungi in microflora, which was similar to bacteria.[63]

The curve-fitting infrared spectra of aliphatic functional groups in the raw coal and degraded coal are shown in Figure 5d, which were mainly divided into 10 adsorption bands. The most obvious changes in this region were the sym. R_2CH_2, which decreased from 22.75 to 6.51, and asym. RCH_3, which increased from 12.28 to 18.17. The CH_2/CH_3 (2920/2950 cm^{-1}) ratios are often used to estimate the length and degree of branching of aliphatic side chains.[65] During the biodegradation, the stretching vibration of $-CH_3$ was weaker than $-CH_2$, leading to the decrease in the ratio of CH_2/CH_3 from 2.95 to 2.64, which suggested that the aliphatic chains in coal were degraded by the microorganism, resulting in the aliphatic chains becoming shorter or less branched.

2.7. Evolution of Intermediate Metabolites during Microbial Degradation of Coal. Figure S1 shows the GC-MS chromatogram of organics on 0, 15, and 27 days of cultivation. There were 20 prominent peaks divided into seven categories: aliphatic acids, aromatic acids, heterocyclic compounds, aliphatic alcohol, aliphatic ester, alkanes, and aromatic compounds (Figure 6). The area of each peak was analyzed by NIST. The percentage of different kinds of organic compounds in 20 peaks was calculated. The mechanism of fungi biodegradation of coal was further explored by analyzing the percentage changes of different organic components. The aromatics, aliphatics, and alkanes were the main parts of the organic matter. It was found that the alkanes in coal continuously degraded to produce methane, decreasing from 35.45 to 8.11%. This change was consistent with the decreasing trend of alkanes side rings [$(CH)_n$, $n > 4$] in FTIR and bigger than that caused by bacterial degradation,[63] indicating that fungi have a better ability to degrade alkanes than bacteria. The aromatic compounds in the culture only changed from 15.96 to 18.71%. It seems that the release of aromatics from coal was kept in balance with the transformation of aromatics in culture by the dominant fungi in the

Table 3. Microcrystalline Structure Parameters of Coal during Degradation

sample	d_{002}/nm	L_c/nm	L_a/nm	N	L_a/L_c	A_{26}	A_{20}	A_{26}/A_{20}
RaC	0.35	1.63	10.8	4.65	6.62	11 706	1269	9.22
ReC15d	0.34	2.02	9.75	5.94	4.83	11 575	1241	8.52
ReC27d	0.34	2.18	10.48	6.3	4.78	8547	1185	7.21

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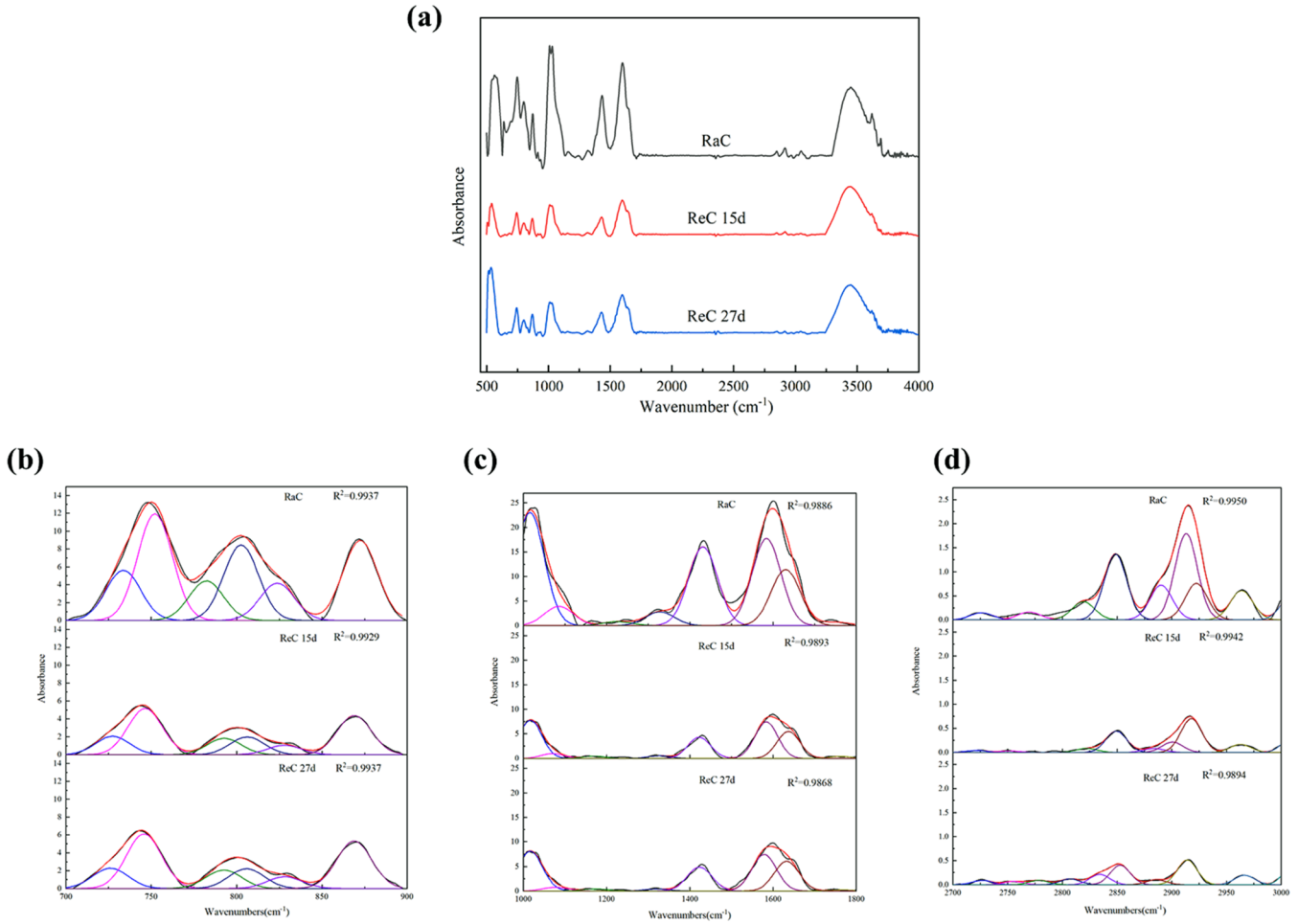

Figure 5. FTIR spectra and curve-fitting FTIR spectra of coal samples including raw coal (RaC) and residual coal after 15 days (ReC15d) and 27 days (ReC27d) of cultivation. (a) FTIR spectra. (b) Aromatic functional groups. (c) Oxygen-containing functional groups. (d) Aliphatic functional groups.

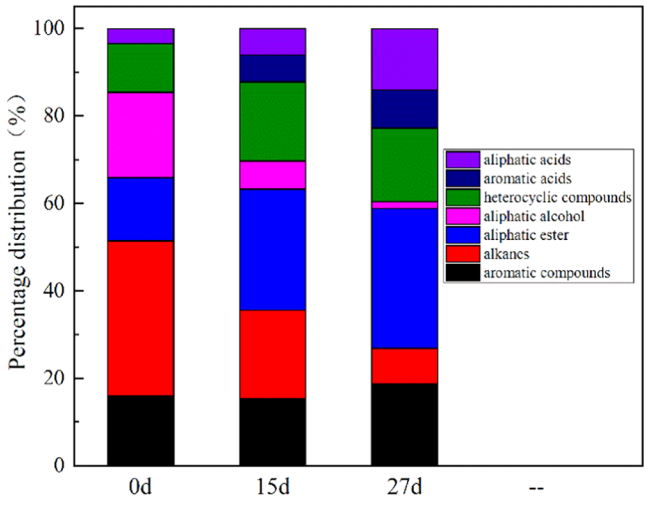

Figure 6. Classification of organic compounds and their distributions in culture on 0, 15, and 27 days of coal biodegradation.

microflora, *Alternaria*, *Penicillium*, and *Aspergillus*, which all have good ability to degrade aromatic compounds. In addition, these fungi would also degrade aromatic compounds by the ring-opening way,[66] resulting in the generation of alkanes, heterocyclic compounds, aliphatic alcohols, and aliphatic ester. This might be the reason for the reduction of aliphatic alcohols from 19.56 to 1.6% during methane production.

It is believed that methane production is restricted by the toxic matter released from coal at a later stage of coal biodegradation.[53] However, fungi would still act on the coal matrix in this process because they are more tolerant of harsh environments, leading to the accumulation of some intermediates during methane production. The GC-MS results showed that aromatic acids, aliphatic acids, and aliphatic esters were accumulated at the later stage from 15 to 27 days when methane production was completed. The proportion of aromatic acids, aliphatic acids, and aliphatic esters increased from 0, 3.39, and 14.4% in the initial stage to 8.74, 14.03, and 31.95% at the end of cultivation. These results suggested that aromatic acids, aliphatic acids, and aliphatic esters were more likely to be the key intermediates in coal biodegradation by fungi. This is consistent with the enriched fungal properties that *Amorphotheca* can degrade lignocellulose and other components to produce acidic substances and ester substances, and *Aspergillus* can degrade aromatic substances to produce acid substances.

3. CONCLUSIONS

The microflora with fungi and methanogens was enriched from produced water from the Qinshui Basin in this study. The

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microflora could degrade anthracite to generate methane with the maximum methane yield of 231 μmol/g coal after 22 days of cultivation under the optimum conditions; the temperature, pH, salinity, particle size, and the ratio of solid−liquid were 35 °C, 6−9, 0−2%, 0.075−0.150 mm (100−200 mesh), and 1:30, respectively. It can maintain methane generation activity even after exposure to air for 24 h. According to the results of Miseq, the archaea were mainly composed of *Methanocella*, followed by *Methanosaeta* and *Methanosarcina*, showing that the methanogenic pathways were mainly hydrogenotrophic methanogenesis. *Amorphotheca*, *Alternaria*, *Aspergillus*, and *Penicilium* were dominant fungi, which were able to degrade aromatic and lignin-derived compounds in coal. XRD analysis showed that the aromatic compounds were degraded effectively by fungi in the microflora, which would cause a swelling effect, making the crystal structure of anthracite looser. After biodegradation, the stretching vibration intensity of each functional group in coal decreased; GC-MS results showed that the concentration of alkanes and alcohols in the culture decreased significantly during methane production, which are the products of aromatic biodegradation by fungi. These results suggested that the microflora with fungi and methanogens enriched from the produced water could degrade anthracite and generate methane, especially ferment aromatic compounds in coal effectively. They also provided a new way to better understand the mechanism of biogenic CBM formation and bioconversion of high-rank coal.

4. MATERIALS AND METHODS

4.1. Sample Collection. The anthracite sample was obtained from no. 3 coal seam in the Sihe coal mine in the Qinshui, Basin which is located in the southeast of Shanxi Province, China,[33] and pulverized to pass through a 120 mesh. The produced water samples were collected from active CBM wells near the Sihe coal mine in sterile 2 L bottles with 20 mL of sterilized water containing 0.1% resazurin, 1.25% cysteine, and 1.25% Na_2S. After sampling, the bottles were tightly sealed and transported to the laboratory on ice as soon as possible. The concentrations of major cations in the produced water were measured utilizing an inductively coupled plasma optical emission spectrometer (ICPOES, Spectro Analytical Instruments, Kleve, Germany). Major anions were analyzed using an ion chromatograph (Metrohm Ltd., Herisau, Switzerland). The concentration of ammonium was analyzed utilizing a colorimetric method with mercuric iodide and potassium iodide according to the Chinese standard methods (GB 5749-2006).

4.2. Microflora Enrichment. The anaerobic cultivation was performed according to Guo et al.[63] Briefly, the anaerobic medium included (1 L) 100 mL of a basic medium, 30 mL of a trace metal solution, 30 mL of a vitamin solution, 10 mL of $Fe(NH_4)_2(SO_4)_2$ (6%), 10 mL of cysteine (15%)−Na_2S (15%), and 1 mL of resazurin (1%). Overall, 1 g of pulverized coal was placed in preautoclaved 100 mL serum bottles with produced water (30 mL). The headspace was filled with N_2 gas at 1 atm. Bottles were incubated without shaking at 35 °C. To inhibit the effect of bacteria, ampicillin and streptomycin were added to the bottle as the antibiotic with final concentrations of 0.1 and 0.2 mM. The concentration of biomethane in the headspace was measured at regular intervals. Every 2−3 weeks, when the gas production was stable, 10% of the enrichment was transferred to the new medium with 1 g of coal until the fluctuation of the highest production of methane was no more than 5%. All of the experiments were carried out in triplicate.

4.3. Optimization of Growth Conditions of the Microflora. To determine the effects of culture conditions on methane production, six factors were evaluated including temperature, pH, salinity, particle size of coal, the ratio of coal to inoculum, and aerotolerant. The culture system and methane determination were the same as that used in the enrichment. Overall, 3 mL of enriched fungi microflora was added to each serum bottle with an anaerobic medium (27 mL). Specific parameters were set as follows: (1) temperature—25, 35, and 45 °C; (2) pH—4, 5, 6, 7, 8, 9, and 10; (3) salinity—0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0%; (4) coal particle size—>0.425, 0.425−0.85, 0.25−0.425, 0.15−0.25, 0.075−0.15, and <0.075 mm; (5) the ratio of coal and inoculum volume (g/mL)—1:5, 1:10, 1:15, 1:20, and 1:30; and (6) aerotolerant—0, 3, 6, 12, and 24 h. Under the optimal conditions, the coal sample and the culture medium were obtained on days 0, 15, and 27 for the determination of microbial communities, coal structure, and intermediates.

4.4. DNA Extraction and Miseq. The DNA extraction and Miseq were performed according to Guo et al.[63] Briefly, the microorganisms in cultivation were collected with a 0.22 μm membrane filter (Millipore). DNA was extracted using the UltraClean Soil DNA Isolation Kit (Mobio). The DNA was extracted and quantified. The archaea-specific primer pairs Arch344F/Arch915R[30,67,68] and fungi-specific primer pairs ITS1f/ITS2R[35] were used to amplify the archaeal 16S rRNA gene and the fungal ITS gene, respectively. The PCR was performed as follows: 95 °C for 5 min, 27 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and finally 72 °C for 10 min. Sequencing was performed using an Illumina Miseq platform.

All of the sequence reads were quantified and primer dimers were removed.[69] Operational taxonomic units (OTUs) were assigned at 97% similarity using Usearch v 7.1. The 16S rRNA gene sequences were compared against the Silva database,[70] and the fungal gene sequence was compared against the Unite fungal database.[71] Diversity and richness estimators were calculated by Mothur v.1.30.1.[72] The sequences derived from Miseq have been deposited in the NCBI Sequence Read Archive with accession number PRJNA657731.

4.5. FTIR Analysis. FTIR technology was used to analyze the effect of anaerobic biodegradation on the coal structure and determine the changes of different functional groups following cultivation. The coal samples of raw coal and the dried residual coal on the 15th and 27th day of cultivation were analyzed. A glass fiber filter (0.7 μm, GF/F, Whatman) was used to separate the residual coal and cultivation solution. The pulverized coal on the filter was removed and dried at 60 °C to obtain a constant weight for FTIR and XRD analysis and the filtrate was collected for further GC-MS analysis. The FTIR spectra of raw coal and residual coal after incubations were determined using a Bruker Tensor 27 FTIR spectrometer, as described by Liu et al.[73] Coal powder and KBr were pelleted at a ratio of 1:100. The scanning range was 4000−400 cm^{-1} with a resolution of 2 cm^{-1}. PeakFit 4.12 was used to further analyze the FTIR data.[74]

4.6. XRD Analysis. The samples determined by XRD were the same as the FTIR. XRD was accomplished with a TD-3500 X-ray diffractometer with Cu Kα radiation at 30 kV and 20 mA. The coal samples were continuously scanned from 5 to 90° with a scanning rate of 6°/min, 0.05°/step. Jade software

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was used for fitting the diffractograms between 16−34 and 39−49°. In this region, the diffractograms were fitted to two Gaussian peaks around 26 and 43°, representing the 002 peak and the 100 peak, respectively. Then, the position, intensity, width, and area of each diffractogram were obtained. The crystallite diameter (L_a) and the stacking height (L_c) of coal were calculated by Scherrer equations[74,75]

$$L_a = 1.84\lambda/(\beta_a \cos \varphi_a)$$

$$L_c = 0.94\lambda/(\beta_c \cos \varphi_c)$$

where λ is the wavelength of X-ray used, β_a and β_c are the half-widths of the 100 peak and the 002 peak, respectively, and φ_a and φ_c are the corresponding scattering angles. The stacking layer number (N) of aromatic carbon corresponds to L_c/d_{002} and $d_{002} = \lambda/2 \sin \varphi_c$.

4.7. GC-MS Analysis. The culture solution was filtered through a 0.7 μm filter membrane. Sodium chloride was added to the filtrate for saturation. The solution was extracted with sequentially with 30 mL of dichloromethane three times under acid, alkali, and neutral conditions, respectively. The pH of each condition was adjusted using NaOH and HCl. After extraction, 270 mL of the extracted liquid product was obtained and dried by sodium sulfate. Then, it was evaporated with a temperature of 40 °C and concentrated to 0.5−1 mL by blowing N_2.

The organics in solution were analyzed by GC-MS (Agilent 7890B-5977B). A gas chromatography column, HP-INNOWax (30 m × 0.25 mm × 0.25 μm), was used. The carrier gas was He (99.999%), and the velocity was 30 mL/min. The specific heating procedures were as follows: the temperature of the column box was kept at 60 °C for 3 min, then increased to 150 °C at 20 °C/min, 230 °C at 5 °C/min, and finally for 5 min.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04705.

Curve-fitting parameters, GC-MS chromatogram, and organic compounds (PDF)

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Notes

The authors declare no competing financial interest.

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