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Improved Formation of Biomethane by Enriched Microorganisms from Different Rank Coal Seams

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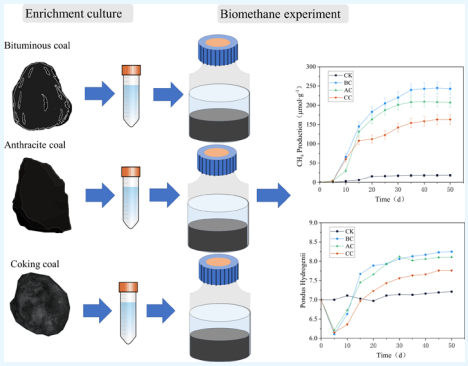
ABSTRACT: The influence of enrichment of culturable microorganisms in in situ coal seams on biomethane production potential of other coal seams has been rarely studied. In this study, we enriched culturable microorganisms from three in situ coal seams with three coal ranks and conducted indoor anaerobic biomethane production experiments. Microbial community composition, gene functions, and metabolites in different culture units by 16S rRNA high-throughput sequencing combined with liquid chromatography-mass spectrometry-time-of-flight (LC-MS-TOF). The results showed that biomethane production in the bituminous coal group (BC)cc resulted in the highest methane yield of 243.3 μmol/g, which was 12.3 times higher than that in the control group (CK). Meanwhile, Methanosarcina was the dominant archaeal genus in the three experimental groups (37.42 ± 11.16−52.62 ± 2.10%), while its share in the CK was only 2.91 ± 0.48%. Based on the functional annotation, the relative abundance of functional genes in the three experimental groups was mainly related to the metabolism of nitrogen-containing heterocyclic compounds such as purines and pyrimidines. Metabolite analysis showed that enriched microorganisms promoted the degradation of a total of 778 organic substances in bituminous coal, including 55 significantly different metabolites (e.g., purines and pyrimidines). Based on genomic and metabolomic analyses, this paper reconstructed the heterocyclic compounds degradation coupled methane metabolism pathway and thereby preliminarily elucidated that enriched culturable bacteria from different coal-rank seams could promote the degradation of bituminous coal and intensify biogenic methane yields.



1. INTRODUCTION

The global energy revolution is accelerating, traditional oil and gas resources are close to depletion,[1,2] and unconventional natural gas, represented by coalbed methane (CBM), has become an important energy source for energy transformation due to its low pollution to the environment.[3,4] According to the gas components, the genesis of CBM can be categorized into biogenic and thermal genesis,[5] and according to the different formation times of CBM, the biomethane can be categorized into primary biogenic gas and secondary biogenic gas.[6,7] Secondary biogenic gas is a mixed gas produced by microorganisms, accounting for nearly 20% of coalbed methane.[8]

The application of microorganisms to enhance biomethane production is a hot topic in the field of coalbed methane bioengineering, and systematic and in-depth research is being carried out.[9] Secondary biogas production largely depends on the cometabolism between microbial communities.[10,11] Specifically, it is difficult or impossible for methanogen to directly utilize the macromolecular organic matter in the coal in the polymerized state,[12] and it is necessary to rely on the bacterial community with degradation function to degrade the macromolecular organic matter and generate the substrate (CO_2, hydrogen, acetate, etc.) that can be directly utilized by methanogenic archaea and finally complete the process of biomethane generation.[13,14]

At present, researchers have explored various methods to stimulate the potential of mine microbes to generate methane. Wang et al.[15] found that enrichment of in situ methanogen strains and addition of exogenous microorganisms can promote in situ biogenic methane formation in coal seams. Li et al.[16] found that altering actinomycete activity can directly affect the negative cohesion of microbial communities and, thus, the potential for methane production. Liu et al.[17] determined the feasibility that enrichment and cultivation of methanogenic bacteria from bituminous coal could be used to improve the production of biological CBM. Guo et al.[18] found that the addition of exogenous carbon could improve the low bacterial activity in lignite and promote its biomethane

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potential. Davis et al.[19] suggested that activation of native microorganisms in coal seams by the addition of nutrients such as algae amendments could promote biomethane production. These studies have shown that the activation of in situ microorganisms in coal seams is the key to biogenic methane production. However, the environments of coal seams in different mining areas and at different depths are intricate, and the methanogenic potential of in situ microorganisms in coal seams of different coal qualities needs to be systematically analyzed.

Coal is a condensed aromatic system composed of aliphatic compounds, aromatic hydrocarbons, and heteroatom compounds.[20] During the anaerobic degradation of coal, the more bioavailable aliphatic compounds are degraded first to methanogenic substrates, followed by water-soluble heterocyclic compounds.[21] However, aromatic compounds are difficult to biodegrade due to the presence of benzene rings. Shi et al.[22] found that microbial reactors degrade difficult-to-degrade heterocyclic aromatic compounds in coal gasification wastewater and generate methanogenesis-related substances such as acetic acid and CO_2. Fu et al.[23] observed the complete degradation of indole to acetate under sulfate-reducing conditions, and the degradation pathway was more similar to the methanogenic metabolic process. All previous studies have shown that degradation of organic matter by functional bacteria is an important part of biomethane production. However, there has been a lack of studies exploring the degradation processes of these organics and related studies to identify, annotate, and categorize specific products.[24]

Based on previous studies, in situ microorganisms from three different coal qualities were selected in this study for enrichment and added to high-volatile bituminous coals for simulated methane production experiments. First, this study compared the methanogenic potential of different enriched microorganisms, then determined their community structure, gene function, and metabolites, and finally constructed the coupled relationship between degradation metabolism and methanogenic metabolism of bacteria through genomics and metabolomics to elucidate the key process of bituminous coal biomethane formation under the action of enriched microorganisms. The results of the study help to reveal the metabolic mechanism of biomethane formation by enriched microorganisms and contribute to the exploration of biomethane formation in coal seams −and its exploitation and utilization.

2. MATERIALS AND METHODS

2.1. Collection of Coal Samples. Coal samples were collected from 1621(1) coal mining face of Panji No. 3 Coal Mine, Anhui province (116°55′E, 32°46′N). The proximate analysis was performed according to ISO 17246−2010, and the coal quality was high-volatile bituminous coal: air-dry moisture 1.23%, air-dry ash (Aad) 13.67%, air-dry volatile (Vad) 33.36%. Through the core drilling rig (ZYW3000, Φ100), 20−30m coal samples were taken from the coal wall of the working face and placed in vacuumable sterile bags, which were stored in ice bags at 4 °C and transported to the laboratory. About 2 cm of outer coal was removed with sterile blades, and the inner coal samples were crushed to 60 mesh with sterile mortar. All operations were carried out in an anaerobic glovebox.

The enriched microorganisms come from different rank coal seams, which are low-volatile anthracite (Sihe No. 2 Coal Mine), high-volatile bituminous coal (Panji No. 3 Coal Mine), and medium-volatile coking coal (Pinggou Coal Mine), respectively. Separately, they were added to 200 mL of culture solution and placed in a constant temperature incubator at 35 °C for enrichment culture for 30 days. At the end of culture, the culture solution was centrifuged at 4000 r/min for 15 min, the supernatant was discarded, and the bacterial body was washed with sterile saline to obtain the enriched bacterial solution, which was vacuum freeze-dried and preserved at 80 °C. The enriched culture medium components were: NH_4Cl 0.8 g/L, NaCl 0.5 g/L, $MgCl_2{\cdot}6H_2O$ 0.5 g/L, $CaCl_2{\cdot}2H_2O$ 0.1 g/L, KCl 0.5 g/L, KH_2PO_4 0.2 g/L, K_2HPO_4 0.4 g/L, peptone 10g/L, and cysteine 0.5 g/L. The pH of the culture solution was adjusted to 7 with NaOH and HCl using a pH meter in each period of cultivation system pH changes.

2.2. Methanogenic Potential Analysis. In this study, four culture systems were designed, including three experimental groups divided into BC (strains enriched in bituminous coal), CC (strains enriched in coking coal), and AC (strains enriched in anthracite coal), as well as a control group, CK (no enriched microorganisms added). Three sets of replicates were set up for each culture system. A 500 mL anaerobic flask with a rubber stopper was used as an incubation vessel configured with 20 g of high-volatile bituminous coal and 100 mL of sterile enrichment culture solution. The headspace gas in the bottle was replaced with nitrogen to simulate an anaerobic environment, and finally, 10 mL of enriched bacterial solution was added. Anaerobic cultivation was conducted in a 30 °C constant temperature incubator for 50 days, and the headspace gas content and solution pH were measured every 5 days. All operations were performed in a sterile environment.

The CH_4 concentrations[4] in the headspace were measured with a gas chromatograph equipped with a thermal conductivity detector (TCD) and a TDX-01 packed column using N_2 as a carrier gas. The temperatures of the detector, injector, and oven were 110, 130, and 90 °C. The formula for methane production (MP) is as follows (CW means coal weight).

$$\mathrm{MP}\ (\mu\mathrm{mol/g}) = \frac{0.45\ \mathrm{L} \times \mathrm{MC}(\%)}{\mathrm{CW}\ (\mathrm{g}) \times 22.4\ \mathrm{L/mol}} \times 10^6$$

2.3. Microbial Composition and Functional Gene Analysis. All culture samples were extracted on the 35th day of the experiment and stored in an ultralow temperature freezer at −80 °C until testing. Total DNA from each cultured sample and bacterial solution was extracted by a FastDNA SPIN Kit for Soil (MP Biomedicals, Cleveland, OH) according to the manufacturer's instructions. The bacterial communities in the bacterial solution were analyzed by high-throughput sequencing of the 16S rRNA genes. The 515*F*/907R (515F: 5′-GTG CCAGCMGCCGCGG-3′; 907R: 5′-CCGTCAATTCMTTTRAGTTT-3′) primer was used to amplify the V4−V5 region of the bacterial 16S rRNA gene, and 1106*F*/1378R (1106F: 5′-TTWAGTCAGCAACGAGC-3′; 1378R: 5′-TGTGCAAGGAGCAGGGAC-3′) in the V8−V9 region for the archaea.

16S rRNA profiling of bacterial solutions was conducted by a HiSeq4000 platform and Illumina MiSeq, respectively, from Hangzhou Lianchuan Biotechnology Co., Ltd. (Hangzhou, China).

For 16S rRNA profiling, the QIIME pipeline was used to quality-filter the raw sequences. Operational taxonomic units

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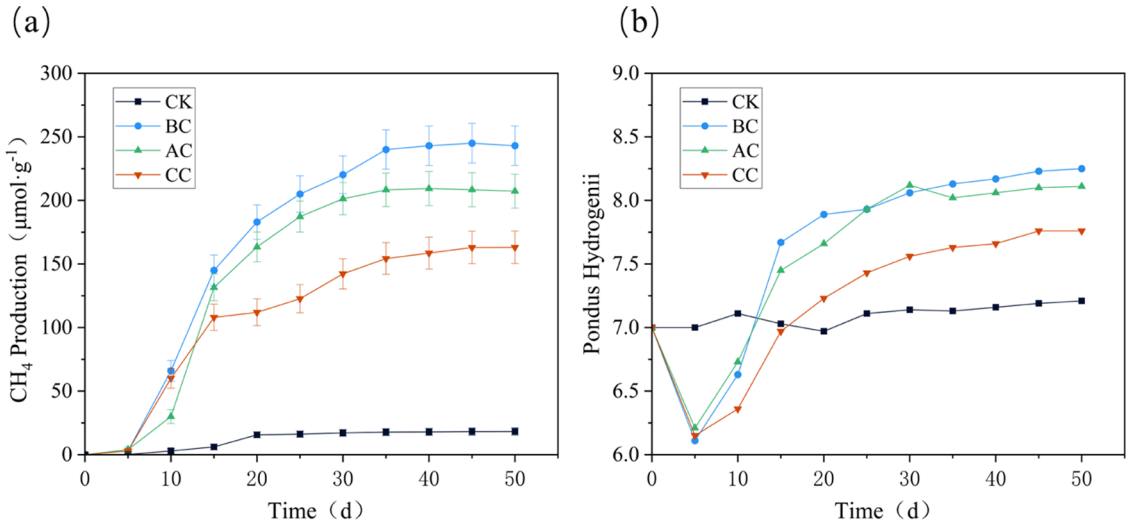

Figure 1. Changes in the biological methanogenic potential and pH among the groups. (a) Biomethane yields. (b) Change in pH.

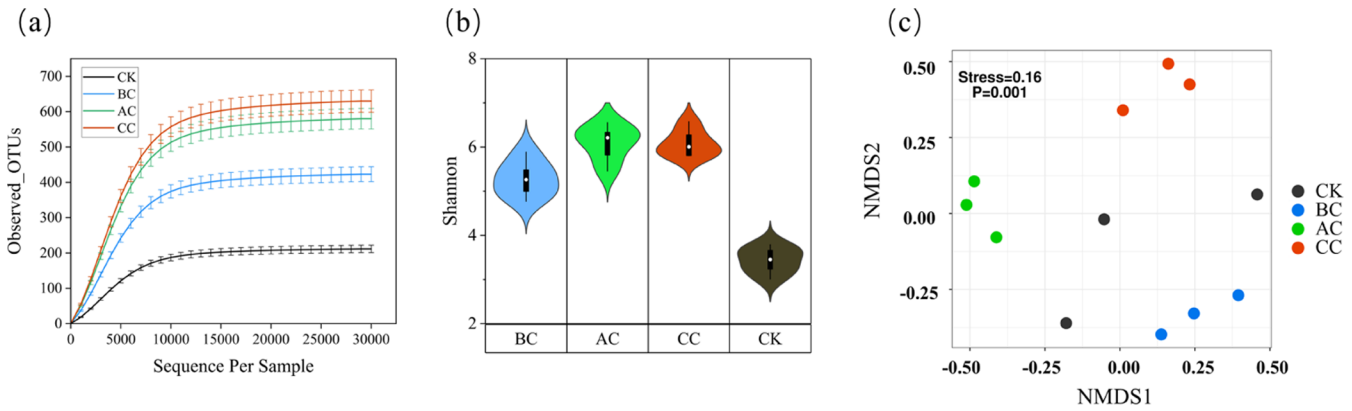

Figure 2. Differences in the diversity of microbial community among the samples. (a) Observed OTUs; (b) Shannon diversity; (c) ordering of the composition of microbial communities by nonmetric multidimensional scaling (NMDS) using the Bray−Curtis dissimilarity index.

(OTUs) were clustered at 97% similarity, and OTU selection and taxonomic assignment were performed according to the SILVA reference data (version 128). The reads that did not align to the anticipated region of the reference alignment and that were classified as "chloroplast," "mitochondria," or "unknown" were removed.

2.4. Untargeted Metabolomics Analysis. The experiments relied on a liquid chromatograph mass spectrometer (Agilent G-6530) for metabolite identification. The instrument is equipped with a C18 chromatographic column (100 mm × 2.7 μm × 3.0 mm). The column oven was maintained at 35 °C. The flow rate was 0.4 mL/min, and the mobile phase consisted of solvent A (water, 0.1% formic acid) and solvent B (Acetonitrile, 0.1% formic acid). Gradient elution conditions were set as follows: 0−0.5 min, 5% B; 0.5−7 min, 5 to 100% B; 7−8 min, 100% B; 8−8.1 min, 100 to 5% B; 8.1−10 min, 5%B.

Rely on Agilent MassHunter Qualitative Analysis 10.0 for peak extraction and filtering, sample retention time alignment, addition ion merging, and missing value filling of raw data. Metabolite annotation was done by comparison to the METLIN database.

Metabolites with a frequency of occurrence greater than 50% in all samples and greater than 66.6% in the same samples were labeled as significant metabolites. Further, univariate analysis of variance multiplicity (fold change) and T.TEST algorithm statistical tests were used, BH correction was performed to obtain *q*-value, and metabolites with *p*-value less than 0.05 and log 2 (fold change) greater than 2 were labeled as significantly differentiated metabolites.

3. RESULTS

3.1. Methanogenic Potential of Enriched Microorganisms. The methane production experiments lasted for 50 days, with gas collected every 5 days and methane concentration measured by gas chromatography. The methane yields of the four culture systems are shown in Figure 1a. The methane production was low in the CK throughout the incubation cycle, with a cumulative methane production of 18.3 μmol/g in 50 days. BC, AC, and CC treatment groups reached the peak of gas production on days 35 to 40, with a cumulative gas production of 243.3, 207.3, and 163.1 μmol/g, respectively. In conclusion, the enrichment of microorganisms in the coal beds significantly improved the biomethane production of bituminous coal, with BC methane production being the highest, which was 12.3 times higher than that of the CK, while AC and CC were also enhanced by 10.3 and 7.9 times.

The pH in the culture solution was tested at the same time as each determination of methane concentration. The pH is an important index to respond to the efficiency of anaerobic

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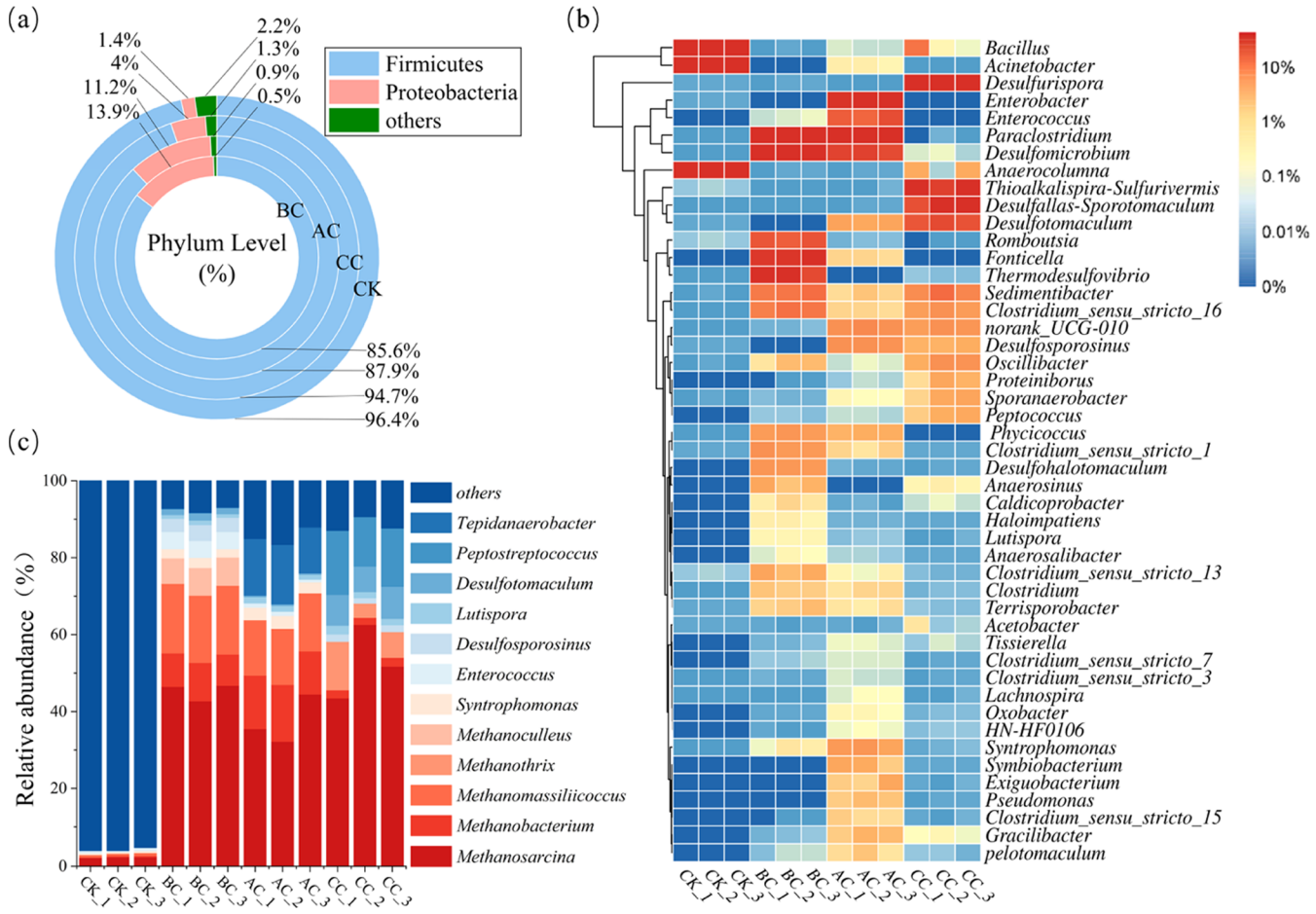

Figure 3. Heatmap for the relative abundance of the main microbial phylum and genera within groups. (a) Bacterial communities at the phylum level. (b) Bacterial communities at the genus level. (c) Archaeal communities at the genus level.

degradation of bituminous coal. The decrease of pH in the culture solution represents the effective hydrolysis of bituminous coal by microorganisms, and the increase of pH represents the beginning of the production of methane by methanogen using the hydrolysis products.[25] The dynamics of pH in the three experimental groups BC, AC, and CC were relatively similar, all showing a "V" shape (Figure 1b). The pH in the solution decreased rapidly to 6.11 ± 0.1 from the beginning of the experiment from day 1 to day 5, then showed an increasing trend with the production of methane from day 5 to day 40, and no longer showed significant changes after 40 days. The final pH in the three experimental groups of BC, AC, and CC was 8.25 ± 0.2, 8.11 ± 0.3, and 7.76 ± 0.2, respectively, while there was no significant change in the pH in the CK, which was maintained at 7 ± 0.5 throughout the incubation period. The pH was maintained at 7 ± 0.5 throughout the incubation cycle.

3.2. Microbial Composition and Diversity. The community structure of microorganisms in the four culture systems was determined based on 16S rRNA sequencing analysis. All operational taxonomic units (OTUs) were clustered at a set cutoff of 0.03 (97% similarity), yielding a total of 1865 OTUs corresponding to bacteria (Figure 2a). In descending order, CC (630 OTUs) > AC (580 OTUs) > BC (423 OTUs) > CK (232 OTUs). The Shannon index was used to characterize the metabolic diversity of the microbial community in the experimental group; the higher the Shannon index, the higher the biodiversity. The results showed (Figure 2b) that CK (3.01 ± 0.78) had a significantly lower Shannon index than BC (5.59 ± 0.5), AC (6.00 ± 0.57), and CC (5.82 ± 0.51). Nonmetric multidimensional scales (NMDS) were used to display the degree of segregation of bacterial community structure. The results showed (Figure 2c) that there were significant differences in bacterial community structure at the genus level among the four culture systems.

The differences in bacterial composition at the Phylum level among the four culture systems were not significant (Figure 3a). Firmicutes (90.96 ± 5.4%) and Proteobacteria (7.65 ± 6.25%) were the dominant phyla common to all four culture systems. Further, the four culture systems showed significant differences in bacterial composition at the genus level (Figure 3b). The dominant genera in the BC treatment group were *Paraclostridium* (17.775.44 ± 6.34%), *Desulfomicrobium* (11.35 ± 0.79%), *Fonticella* (9.3 ± 0.63%), *Thermodesulfovibrio* (13.32 ± 5.37%), and *Romboutsia* (9.82 ± 3.58%); the dominant genera in the AC-treated group were *Paraclostridium* (13.53 ± 2.79%), *Desulfomicrobium* (7.79 ± 0.89%), *Enterobacter* (9.70 ± 1.49%), and *Enterococcus* (5.60 ± 2.01%); the dominant genera in the CC treatment group were *Desulfurispora* (23.75 ± 3.56%), *Thioalkalispira-Sulfurivermis* (12.8 ± 4.03%), *Desulfallas- Sporotomaculum* (9.68 ± 2.23%), and *Desulfotomaculum* (6.88 ± 1.02%); while the dominant genera in the CK were *Acinetobacter* (43.67 ± 0.03%), *Bacillus* (36.14 ± 0.69%), and *Anaerocolumna* (14.84 ± 0.92%). The bacteria in

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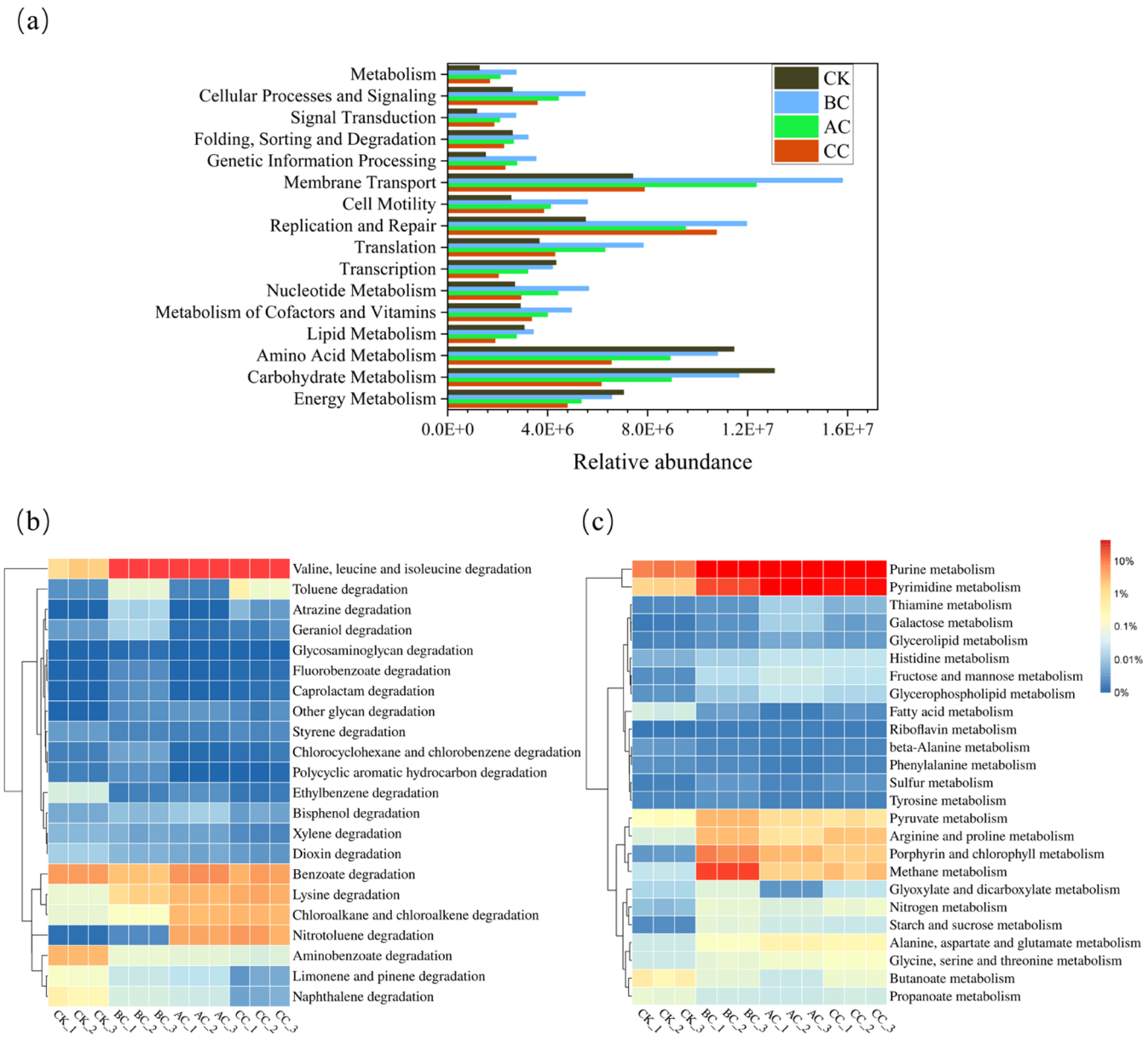

Figure 4. Functional gene difference among samples. (a) Relative abundance of the functional gene at level 2. (b) Heatmap for the relative abundance of degradation at level 3. (c) Heatmap for the relative abundance of metabolism at level 3.

CK mainly act on the hydrolysis stage of coal degradation, including *Acinetobacter* and *Bacillus*. The enrichment culture treatment increased the relative abundance of hydrolysis bacteria (*Sedimentibacter*, *Sporanaerobacter*), acidification bacteria (*Clostridium_sensu_stricto*, *Terrisporobacter*), hydrogen- and acetogen-producing bacteria (*Proteiniborus*, *Enterobacter*, *Clostridium*).

The methanogenic archaeal communities in the three experimental groups were similarly structured at the genus level (Figure 3c). The main groups included *Methanosarcina*, *Methanobacterium*, *Methanomassiliicoccus*, *Methanothrix*, and *Methanoculleus*. Among them, *Methanosarcina* was the main dominant species with a relative abundance of 38.91 ± 13.72%. Methanogen had the highest relative abundance share in the archaeal community at the genus level in the BC experimental group (77.13 ± 3.03%), followed by AC (60.8 ± 9.21%), and CC, the lowest (58.2 ± 9.92%). The control and experimental groups differed significantly at the genus level, with a very low percentage of relative abundance of methanogenic archaea in the CK group (2.91 ± 0.48%).

3.3. Metabolic Pathways Based on Functional Genes.

Functional annotation of genes in the four sets of experimental samples based on the KEGG_LEVEL2 database screened a total of 16 gene functions with relative abundance higher than 100,000 (Figure 4a). The results showed that enrichment of coal microorganisms significantly increased the relative abundance of two metabolic pathways, metabolism of cofactors and vitamins and nucleotide metabolism, and resulted in the maintenance of functional expression of genes encoding ribosomal proteins, membrane transport, energy metabolism, and replication and repair, and other genes encoding ribosomal proteins.

Organic degradation of bituminous coal by bacteria produces aromatic, aliphatic, alkane, and other compounds, and further metabolism and degradation of these compounds provide methanogen bacteria with the necessary metabolic

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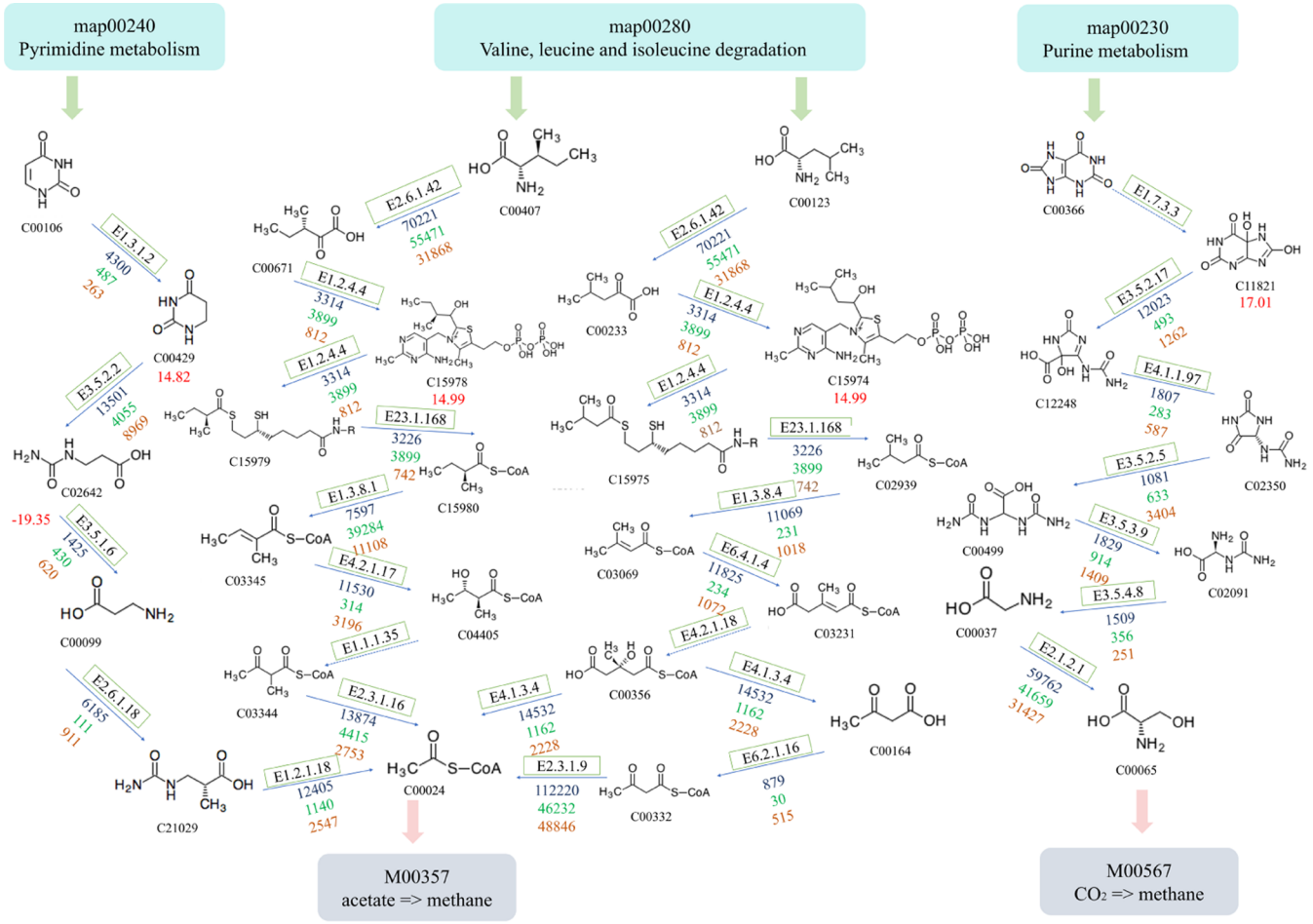

Figure 5. Overview of the anaerobic metabolic pathway of the degradation of nitrogen-containing heterocyclic compounds in three experimental groups. The numbers below the functional enzyme (E-.-.-.-) indicate the relative abundance of genes, and the numbers in red indicate the changes (log 2 FC) of compounds.

substrates.[26] Studies on the organic degradation of bituminous coal can shed more light on the methanogenic potential of enriched microorganisms. Therefore, genes related to organic degradation in bituminous coal were functionally annotated based on the KEGG_LEVEL3 database and categorized into degradation and metabolism categories (Figure 4b,c). Enrichment of coal seam microorganisms resulted in significant enrichment of three metabolic pathways: valine, leucine, and isoleucine degradation (map00280), purine metabolism (map00230), and pyrimidine metabolism (map00240). Significant changes in the relative abundance of six metabolic pathways, pyruvate metabolism, arginine and proline metabolism, porphyrin and chlorophyll metabolism, lysine degradation, chlorane and chlorophyll degradation, and nitrotoluene degradation, were induced.

Functional annotation of genes from enriched microorganisms based on the KEGG KO database reconstructed the metabolic pathways for the degradation of heterocyclic compounds in coal (Figure 5). In the three experimental groups, four classes of enzymes were detected, including oxidoreductase (E1.2.4.4, E1.3.8.1, E1.3.1.2, E1.7.3.3, etc.), transferase (E2.3.1.16, E2.6.1.42, E2.6.1.18, etc.), and oxidoreductase (E1.2.4.4, E1.3.8.1, E1.3.1.2, E1.7.3.3, etc.), hydrolases (E3.5.2.2, E3.5.1.6, E3.5.2.5, E3.5.3.9, etc.), and lyases (E4.1.3.4, E4.1.3.4, etc.). The final products include acetyl-CoA and lysine, which are important metabolic substrates for the acetate ≥ methane (M00357) and CO_2 ≥ methane (M00567) methanogenic pathways.

3.4. Analysis of liquid Metabolites. A total of 1538 soluble low-molecular-weight compounds were found in the four culture systems (Table S1). Enrichment of coalbed microorganisms significantly enhanced compound degradation. (Figure 6). Coal seam native flora in CK promoted the degradation of 127 compounds in bituminous coal and produced 64 new metabolites, whereas the addition of enriched microorganisms increased the bioavailability of 778 compounds in bituminous coal, of which the enriched flora in the BC group promoted the degradation of 339 compounds and produced 298 metabolites in bituminous coal; the enriched flora in the AC group promoted the degradation of 452 compounds in bituminous coal and produced 327 new metabolites, and the enriched bacterial colony in the CC group promoted the degradation of 413 compounds in bituminous coal and produced 167 metabolites. Detailed information on compounds are provided in Table S3.

Based on the metabolomics analysis process, 55 compounds were labeled as significantly different metabolites associated with bituminous coal degradation (Table S2), including 26 compounds with decreased levels and 29 compounds with increased levels. These compounds were further categorized into four major groups based on the carbon framework structure (Figure 7): heterocyclics, benzenoids, aliphatic acids,

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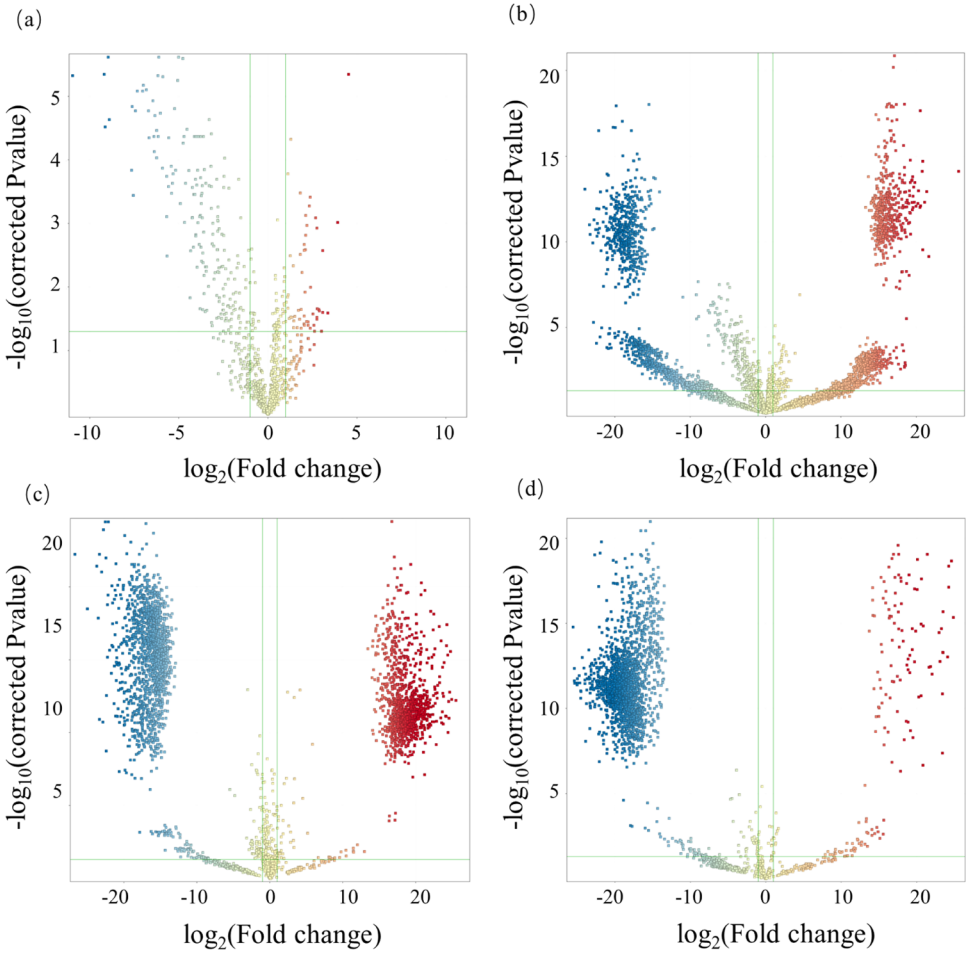

Figure 6. Changes in metabolites before and after culture. (a) Different metabolites in CK. (b) Different metabolites in BC. (c) Different metabolites in AC. (d) Different metabolites in CC.

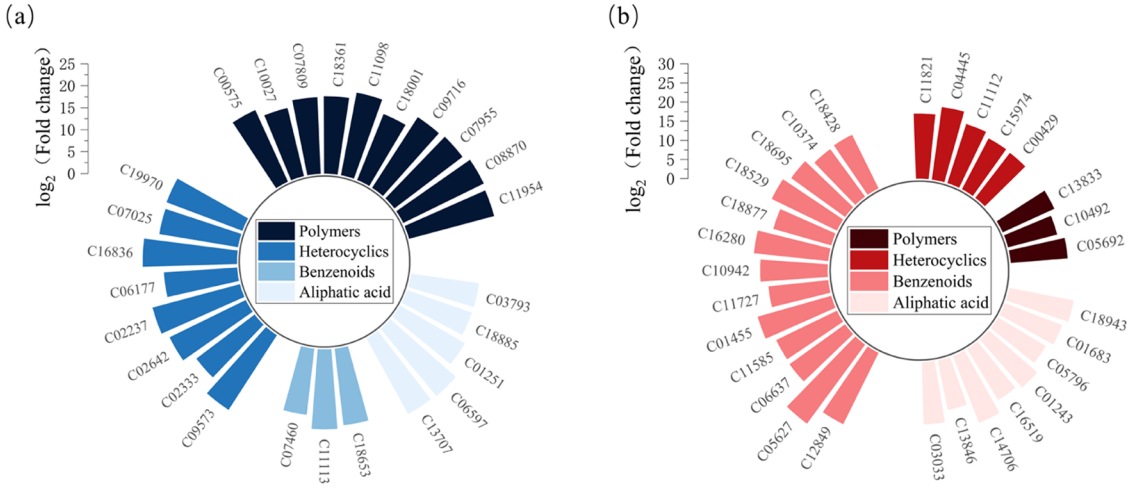

Figure 7. Significant differential metabolites in three experimental groups. (a) Significant reduction ($p < 0.05$). (b) Significant increase ($p < 0.05$).

and polymers (mass charge ratio >400). There were 13 significantly different heterocyclic compounds in the three experimental groups, including a significant increase in five heterocyclic compounds, such as homostachydrine, neomycin B, and 5-hydroxyisourate, and a significant decrease in eight heterocyclic compounds, such as lophophorine, retronecine, and xanthopterin-B2 compounds; 16 significantly different benzene ring derivatives, including a significant decrease in 3 benzene ring derivatives, such as chloraminophenamide, methaphenilene, and DNOC, and a significant increase in 13 benzene ring derivatives such as oryzalin, okenone, and inabenfide; and 13 significantly different differences in polymers, including a significant decrease in 10 polymers, such as fenpyroximate, mafoprazine, and flavoxate, and a significant increase in 3 polymers, such as Se-adenosylseleno-homocysteine, salvianolic acid A, and NS 1619; 13 aliphatic

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compounds, including a significant decrease in five aliphatic compounds, such as propamocarb, Nε,Nε,Nε-Trimethyllysine, and RU-0211, and a significant increase in eight aliphatic compounds, such as prothiocarb, diethyl sulfate, and ornaline.

4. DISCUSSION

In this study, it was found that the enrichment of target flora from the native flora of different coal seams could all significantly enhance the methane production potential of high-volatile bituminous coals. In addition, the different strain compositions of different microorganisms resulted in differences in their methane production potential. Biomethane production is the result of cometabolism of multiple functional flora in an anaerobic environment, which is subject to a variety of endogenous influences, including strain composition and diversity.[27] Currently, most biocoalbed methane research focuses on increasing microbial diversity with the aim of enhancing interactions between microorganisms to promote coal degradation and provide more metabolic substrates for methanogen flora.[28,29] This study also found that enrichment of in situ microorganisms in coal seams significantly increased their diversity, which in turn enhanced bacterial biodegradation of bituminous coal and produced abundant aromatic, aliphatic, and alkane compounds, which in further degradation provided methanogens with the necessary metabolic substrates and ultimately increased methane production. However, comparing the three experimental groups, the methane production of the more diverse CC group was lower than that of the less diverse BC group, which could be attributed to the fact that the enrichment targeting the methanogenic-associated microbial communities in the coal beds made the methanogenic metabolism functional flora become the main dominant flora in the culture system. In addition, sulfate-reducing bacteria (SRB) were enriched in the CC group. Previous studies have shown that SRB and methanogen coexist in a symbiotic manner in the metabolic pathway of microbial methane production in coal seams.[30] These SRB can utilize casein as a terminal autoreceptor or shuttle to metabolize acetic acid or other simple fatty acids, an important step in coal degradation.[31] However, some studies have found that toxicants in the substrates of SRB metabolism, such as ammonia nitrogen compounds, H_2S, and sulfate, can stress the succession of methanogenic bacterial communities.[32] The results of the present study also revealed changes in the structure of the methanogen community. In the sulfur metabolic pathway, SRB forms APS by utilizing sulfate activated by sulfate adenylyltransferase, APS reductase reduces APS to HSO_3^-, and finally, sulfite reductase reduces HSO_3^- to H_2S.[33] These S^{2-} ions, produced by SRB metabolism, further inhibit the metabolic activity of methanogens in CC by inhibiting electron donors to the mitochondrial respiratory chain.[34,35]

Methanogens are the true producers of biomethane and dominate the final segment of the pathway for biomethane formation, converting low-molecular-weight intermediates (CO_2, H_2, Acetate, etc.) to methane.[36] Previous studies have classified methanogens into three major groups based on their metabolic substrates, including hydrogenotrophic archaea that convert H_2 and CO_2 to methane, acetoclastic archaea that convert acetate compounds to methane, and methylotrophic archaea that convert methyl compounds to methane.[37] Dominant methanogenic archaea such as methanosarcina identified in this study are closely related to methylotrophic archaea. These archaea usually do not require organic growth factors and can produce methane in a variety of environments through different pathways, including disproportionation to CH_4 and CO_2 and NH_3 when methylamine and methanol are present in the substrate; when H_2 is present in the environment, methanol and methanol are reduced to CH_4; and also, the substrates that are available include H_2 + CO_2 or acetic acid analogues.[38,39] This diversity of metabolic pathways enables the strain to adapt to the time-changing culture environment in the laboratory system and has excellent methanogenic potential.

The effect of microbial metabolism and degradation on organic matter fractions has been an unelucidated research hotspot due to the complex structure of the macromolecular skeleton in coal. In this study, genomic and metabolomic analyses revealed that the enrichment of enriched microorganisms directly promoted the degradation of macromolecules in bituminous coal to produce a large number of intermediate metabolites, such as heterocyclic compounds, benzene compounds, and aliphatic compounds, and further facilitated the fracture and hydrolysis of these intermediates on branched chains to produce organic acids, lipids, and amides, which are the prerequisites for the production of central metabolic intermediates (acetate-CoA) of biomethane, and also important signaling molecules for microbial anaerobic fermentation.[40] Furthermore, unlike in other studies, the degradation of nitrogen-containing heterocyclic compounds in bituminous coal analyzed in this study is likely to be a key pathway for methane formation. During the degradation of nitrogen-containing heterocyclic compounds, 2-oxoisocaproate dehydrogenase [EC:1.2.4.4], uric acid oxidase [EC:1.7.3.3], and dihydropyrimidine dehydrogenase [EC:1.3.1.2] act on the reductive cleavage, and dihydropyrimidinase [EC:3.5.2.2] acted on hydroxylation reactions to open nitrogen-containing heterocyclic compounds in the closed state, yielding organonitrogen compounds such as formiminoglycine, 3-ureidopropionate, and others.[41] Meanwhile, this also indicates that the acquisition of nitrogen is an important process in microbial metabolism, and the low content of nitrogen in bituminous coal may limit microbial metabolism and growth.[42] Shi et al.[43] found that microbial degradation of organic pollutants was related to nitrogen response and further determined that controlling organic nitrogen content could effectively influence the degradation of phenol, acetaminophen, and sulfamethoxazole in industrial wastewater. Wild et al.[44] found that increasing nitrogen levels in the soil can promote soil microbial metabolic activity at low temperatures and further lead to the entry of soil carbon into the carbon cycle. These studies indicate that nitrogen regulates the bioavailability of organic matter by microorganisms and further affects their potential for anaerobic fermentation to form methane.

Another important finding is that low bioavailability of compounds in bituminous coal is an important factor affecting the rate of biomethane production. Hu et al.[45] found that in anaerobic environments, it is difficult to degrade most of the benzene ring compounds in wastewater by microbial self-metabolism alone. Similarly, the present study also revealed the presence of several benzene ring derivatives in the culture broth at the later stage of the experiment. This suggests that the carbon ring structure of the benzene ring itself is relatively stable in anaerobic environments and that enrichment of microorganisms merely promotes the removal of functional groups such as alkyl side chains, carboxyl groups, or amino

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groups from the benzene ring derivatives, and the breaking of aromatic compounds at the branching, and leads to the accumulation of NH_3, and the benzene ring in the culture system. This may be the main reason for the change in pH in the culture solution under anaerobic conditions. The production of organic acids and carbonation of CO_2 during degradation first reduced the pH value of the fermentation broth, and the metabolic utilization of organic acids and hydrolysis of NH_3 contributed to the alkaline tendency of the fermentation broth.[46] To counteract the acidic conditions in the culture broth, functional strains had to devote more of their energy to maintaining the expression drive of genes encoding ribosomal proteins, membrane transport, energy metabolism, and replication and repair.

In conclusion, this study confirmed that the enrichment of microorganisms from all three coal seams, bituminous, coking, and anthracite, could promote the anaerobic degradation of highly volatile bituminous coal and the production of biomethane. Degradation of nitrogen-containing heterocyclic compounds in bituminous coal may be a key reaction in the methanogenesis process, and the degradation of such compounds produces acetyl cofactor and lysine, which are important metabolic substrates for the two methanogenic pathways acetate ≥ methane (M00357) and CO_2 ≥ methane (M00567). This study is expected to provide functional microbial strains for coalbed methane extraction and enhance the development and utilization of coalbed methane resources.

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request. All concerned data are given in this manuscript.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09742.

Recognizable metabolism in coal (XLSX)

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B.L., T.Z., S.X., J.C., X.Z., and J.W. conducted the bulk of the data analysis for the study and cowrote the manuscript. B.L. and S.X. provided the funding for the study, were involved in the conceptualization of the study, and assisted in the writing of the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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