


ORIGINAL RESEARCH

Deciphering the initial products of coal during methanogenic bioconversion: Based on an untargeted metabolomics approach

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Abstract

Although the process of microbial degradation of coal to produce biomethane has got much attention from many research works, the products profiles of coal at the initial stage of methanogenic bioconversion are not clear yet. In this study, five coal-degrading bacterial strains (CD1, CD10, CD20, CD24, and CD25) from a methanogenic community were isolated and identified. Among them, CD1 and CD24 belong to *Paenibacillus* sp., CD10 and CD20 belong to *Bacillus* sp., and CD25 belongs to *Stenotrophomonas* sp. After biotreatment of lignite and bituminous coal, the kinds of newly produced compounds were 33 and 45, respectively. Metabolomics analysis showed that a large number of alkane compounds and heterocyclic aromatic compounds were produced after degradation of bituminous coal and lignite by isolated bacteria, and most of the compounds had been produced in a hydroxylated or acylated manner, indicating that the initial microbial treatment enhanced the bioavailability of coal. Some alkaloids and biosurfactants were also detected in the aforementioned products, such as glycerophosphocholine, proveratrol A, proveratrol B, surfactin, etc. These microbial metabolites may play an important role in solubilization during the degradation of coal. This study added to the understanding of the complicated metabolic process of methanogenic coal bioconversion and enabled effective production of biomethane with appropriate metabolic strategies.

KEYWORDS

biomethane, coal, metabolomics, methanogenic community, microbes, products

1 | INTRODUCTION

Burning of coal as a fossil fuel has caused detrimental effects on the environment while utilization of clean energy biomethane leads to significantly great greenhouse gas credits savings (Kim et al., 2019). The generation of biomethane from the microbial conversion of coal in situ has spurred interests to explore new ways of using coal cleanly (Strapoć et al., 2008, 2011). In the laboratory, many efforts have been devoted to

investigating how to achieve efficient coal bioconversion and optimize the production of clean energy biomethane (Ünal et al., 2015; Zhang et al., 2016) while the complicated interaction process between coal and the microbial communities is responsible for the methanogenic coal bioconversion.

Coal is a complex mixture composed of macromolecules consisting of condensed aromatic systems with benzene rings, aliphatic hydrocarbons, and heterocyclic rings as the core and side chains (Strapoć et al., 2011). Compared with the general

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macromolecular organic matter, it is more difficult for most microorganisms to consume coal (Welte, 2016). Biomethane production from coal involves many different types of microbial metabolism synergisms (He et al., 2020; Huang et al., 2018). The classical anaerobic fermentation theory proposed by Zinder has been used to interpret the process of methanogenic coal bioconversion (Zinder, 1993). In the first stage, complex organic compounds are converted into organic acids and alcohols by anaerobic fermentation bacteria. In the second stage, the hydrogen-producing and acetic acid-producing bacteria further decompose the organic acids and alcohols into H_2 , CO_2 , acetic acid, or formic acid and the corresponding salts. In the last stage, methanogens of different substrate-utilized types use formic acid, CO_2 , or acetic acid to produce methane (Zinder, 1993). To better clarify the specific process of methanogenic coal bioconversion, several studies tried to investigate the interaction mechanism between coal and microbial communities. Vick et al. identified the biodegradable components of coal by a native microbial community and the corresponding microorganisms involved in that scenario (Vick et al., 2019). Webster et al. evaluated the effect of different chemical oxidative treatments on coal to mimic microbial oxidation, and found that oxidative treatments of coal caused significant changes in chemical profiles compared to untreated coal (Webster et al., 2019). Also, numerous studies have verified the final stage of the methane-producing metabolic pathway of coal bioconversion, in which the polymers in coal are fermented to produce volatile fatty acids, organic acids (lactic acid, acetic acid, succinic acid, etc.), alcohols (methanol, ethanol, etc.), hydrogen, and carbon dioxide, which are then used by methanogens to produce methane (in't Zandt et al., 2017; Wei et al., 2013). The methanogenic coal bioconversion process has important implications for both energy production and our understanding of global carbon cycle (Vick et al., 2019) while a start-up procedure was necessary and important for the subsequent reactions. The initial stage of coal degradation accounts for great responsibility for the start of bioenergy production and carbon metabolic flux. However, little is known about which compounds are produced from coal at the initial stage of methanogenic bioconversion.

The advent of the metabolomics approach emerged as a powerful tool for studying microbial metabolisms (Shu et al., 2020). Currently, metabolomics techniques have been used in many studies involved in organic pollutant treatments (Mishra et al., 2019). Zheng et al. utilized metabolomics analyses to track the degradation of intermediates of refractory dye reactive black 5 by *Klebsiella* sp. KL-1 (Zheng et al., 2020). Crnkovic et al. investigated the metabolomic profiles of metabolites produced by three filamentous cyanobacterial strains (Crnkovic et al., 2018). Another study revealed the metabolomics profiles of organic pollutant dibutyl phthalate degraded by *Pseudomonas* sp. DNB-S1 (Yu et al., 2020). These studies fully supported that metabolomics could facilitate a greater understanding of the metabolic intermediates of coal in methanogenic biodegradation.

In this study, five coal-degrading bacterial strains were isolated and identified from a microbial community cultured in a previous study that refers to methanogenic coal bioconversion (Wang, Yu, et al., 2019). As we all know, a methanogenic microbial community consists of microbes with different functions which lead to the conversion of coal into methane in a synergistic manner. Since these isolated strains can degrade coal directly, they could play a crucial role in the initial stage of methanogenic coal bioconversion. Subsequently, the obtained strains were cultured with coal as the sole carbon source, and after the coal degradation process, the products were analyzed by metabolomics. The coal-derived products profiles between lignite and bituminous were also compared.

2 | MATERIALS AND METHODS

2.1 | Isolation of coal-degrading strains

The methanogenic microbial community and coal sample used to screen the coal-degrading strains were reported previously (Wang, Yu, et al., 2019). In short, this microbial community used for isolation was collected from the second week during successive cultivation, which was dominated by Actinomycetales (49.19%), Bacteroidales (14.3%), Bacillales (9.7%), and Clostridiales (25.25%) that can convert coal to methane synergistically and collaboratively. To ensure the consistency of the culture conditions, the basic medium used to screen the coal-degrading strains is almost the same as the methanogenic anaerobic medium shown in the previous study with slight changes (Strapoć et al., 2008). Vitamin solution was not added when preparing basic anaerobic medium in this study to reduce the influence of carbon substances on the strain screening and metabolites analyses. The coal was ground to coal powder using a mortar and pestle and sieved through a 200-mesh sieve to collect fractions containing particles <250 μm in diameter in an anaerobic chamber (XinmiaoYQX-11). The collected microbial community used to screen the coal-degrading strains was pretreated with a dilution gradient of 10^{-1} – 10^{-6} . The diluted concentration of 10^{-6} was spread to the basic anaerobic medium plates with coal powder (30 g/L) as the sole carbon source. Then the plates were cultured in an anaerobic incubator at 35°C. When the microbes grew on the plates, single colony was isolated for further study.

2.2 | Identification of coal-degrading strains by 16S rRNA analysis

Genomic DNA from the isolated strains was extracted using a DNA Extraction kit immediately (MP Biomedicals) according to the manufacturer's instructions, followed by

amplifying 16S rRNA genes by polymerase chain reaction (PCR) with DNA Taq Polymerase, dNTPs, and primers set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'; Kaur & Goyal, 2020). 16S rRNA genes of isolated strains were sequenced at Shenggong Ltd. To identify the isolated strains, the 16S rRNA gene sequences were compared with the NCBI database using a BLAST program (Altschul, 2012). Closely similar sequences were chosen to pairwise alignment (CLUSTALW). MEGA 7.0 software was used to construct a phylogenetic tree by a maximum likelihood method. Bootstrap analysis with 1000 replications was performed to evaluate the statistical support for the phylogenetic tree. The evolutionary distance was represented by P-distance. The 16S rRNA gene sequences of isolated coal-degrading strains were deposited in the NCBI GenBank database with accession numbers MT889989–MT889993.

2.3 | Biodegradation of coal by isolated strains and pretreatment of metabolites

Bacterial cells anaerobically grown in LB broth were collected in the late log phase via centrifugation at 7200 *g* and 4°C. The cell pellet was washed thrice with phosphate buffer saline solution; inoculum size was adjusted to 0.02 (optical density of 600 nm) with phosphate buffer saline solution and was then incubated in a basic anaerobic medium with lignite or bituminous (particles <250 μm ; 30 g/L) as a sole carbon source for 7 days at 35°C, 100 rpm in an incubator shaker. The lignite sample used for degradation in this study was obtained from previous research (Wang, Wang, et al., 2019). After cultivation, aliquots of culture were collected, respectively. 100 μl of each culture was taken out and 300 μl methanol (containing 5 $\mu\text{g}/\text{ml}$ 2-chloro-L-phenylalanine as internal standard) was added to the culture. The commixture was first vortexed for 2 min and then centrifuged at 16,200 *g*, 4°C for 10 min. The supernatant was transferred to sampler vials for metabolites detection. Six independent biological replicates were used for the metabolomics analysis.

2.4 | Liquid chromatograph-mass spectrometer (LC-MS) detection

Liquid chromatograph-mass spectrometer detection was referred to the previous literatures with appropriate adjustment based on the current study (Ma et al., 2020). Specifically, an Agilent 1290 Infinity II UHPLC system combined with an Agilent 6545 UHD and Accurate-Mass Q-TOF/MS was used for LC-MS detection. Vydamos® C18-P 100A (5 μm , 250 mm*2.1 mm) was used as the chromatographic column.

Mobile phase: A: aqueous solution with 0.1% formic acid. B: acetonitrile solution. Flow rate: 0.4 ml/min. Injection volume: 10 μl . Column temperature: 296 K. Gradient elution condition optimized: 0–0.5 min, 0% B; 0.5–4 min, 0–15% B; 4–5 min, 15% B; 5–20 min, 15–95% B; 20–21 min, 95–0% B; 21–23 min, 0% B. Post-time was set as 6 min for system balance. Mass spectrometry was operated in both positive and negative ion modes. The ion source used was the electrospray ionization source. The optimized parameters were set as follows. Drying gas flow: 10 L/min. Capillary voltage: 4 kV. Nebulizer pressure: 20 psig. Skimmer voltage: 45 V. Fragmentor voltage: 120 V. Ion source temperature: 400°C. Mass range: *m/z* 50–1000. Reference ions were used to ensure mass accuracy during the MS acquisition process. Reference ions in positive ion mode: 121.0509, 922.0098. Reference ions in negative ion mode: 112.9856, 1033.9981.

2.5 | Metabolites identification and metabolomics analyses

Agilent Masshunter Qualitative Analysis B.08.00 software (Agilent Technologies) was used to convert the raw data to the common (*mz*.data) format. In the R software platform, the XCMS program was used to perform retention time correction, automatic integration pretreatment, and peak identification. Visualization matrices containing sample name, peak area, and *m/z*–RT pair were acquired. The peak areas in chromatograms were used for relative content analysis in the current study. The qualitative method of metabolites is searched in the Metlin online database for accurate molecular weight comparison (Montenegro-Burke et al., 2020). Adduct manner: [M+H]⁺ was selected in positive mode, [M–H][–] in negative mode. Mass error value: 30 ppm. MetaboAnalyst 4.0 is used for comprehensive and integrative metabolomics data analysis (Chong et al., 2019).

3 | RESULTS

3.1 | Isolation and identification of coal-degrading strains

After 7 days of incubation on the screening culture, the colonies growing on the plate can be roughly divided into two forms. The colonies with a rough surface are larger and those with a smooth surface are smaller (Figure S1). The genomes of more than 20 strains were extracted, amplified by the 16S rRNA gene, and the PCR products were sequenced by Shenggong Biotechnology Ltd. Most of the sequences were divided into three different genera after comparison and five strains with different sequences were selected for subsequent experiments in this study (Table 1).

Isolate	Colony size (mm)	Surface	Identification (16S rRNA gene) ^a	Classification ^b
CD1	1–2	rough	<i>Paenibacillus macerans</i> (99%)	Firmicutes, Paenimataceae
CD10	1	smooth	<i>Bacillus cereus</i> (99%)	Firmicutes, Bacillaceae
CD20	1	smooth	<i>Bacillus idriensis</i> (99%)	Firmicutes, Bacillaceae
CD24	1–2	rough	<i>Paenibacillus barengoltzii</i> (99%)	Firmicutes, Paenimataceae
CD25	2–3	rough	<i>Stenotrophomonas maltophilia</i> (99%)	Proteobacteria, Xanthomonadaceae

^aIsolates were identified through partial sequencing of their 16S rRNA gene and subsequent BLAST searches of the NCBI database with this sequence.

^bThe classification of phylum and family levels of isolates.

Phylogenetic tree analysis indicated that the 16S rRNA gene of strain CD1 was 99% similar to the sequence of *Paenibacillus macerans* sp.; the sequences of 16S rRNA gene of strain CD10 were 99% similar to those of *Bacillus cereus* sp.; the 16S rRNA gene of strain CD20 was 99% similar to that of *Bacillus idriensis* sp.; the 16S rRNA gene of strain CD24 was 99% similar to that of *Paenibacillus barengoltzii* sp.; the 16S rRNA gene of CD25 was 98% similar to that of *Stenotrophomonas maltophilia* sp. (Figure S2). Therefore, in this study, strain CD1 was identified as *Paenibacillus* sp., strain CD10 as *Bacillus* sp., strain CD24 as *Paenibacillus* sp., and strain CD25 as *Stenotrophomonas* sp. Strains CD10 and CD20 belong to phylum Firmicutes and family Bacillaceae. CD1 and CD24 belong to the phylum Firmicutes and family Paenimataceae. CD25 belongs to the phylum Proteobacteria and family Xanthomonadaceae.

3.2 | Analysis of the overall differences in products profiles

In this study, a LC-MS-based metabolomics study was carried out in lignite and bituminous treated by the isolates and microbial community. Unweighted unifrac distance-based principal coordinates analysis (PCoA) was used to identify and reveal the differential products among non-biotreat control, microbial community, and isolates groups. The PCoA plots revealed a distinct clustering of products profiles between biotreat groups and non-biotreat control in both lignite- and bituminous-containing cultures (Figure 1), which indicated a remarkable difference in the products of coal after biodegradation. Score plots also showed that products profiles in microbial community groups were located far away from those in the isolates group. The results of PCoA score plots indicated that the products of coal by the microbial community were quite different from the isolates groups.

To identify the overall differences between the products of coal by the microbial community and by the isolates, the

TABLE 1 The basic properties of isolated strains that grown with coal as the sole carbon source

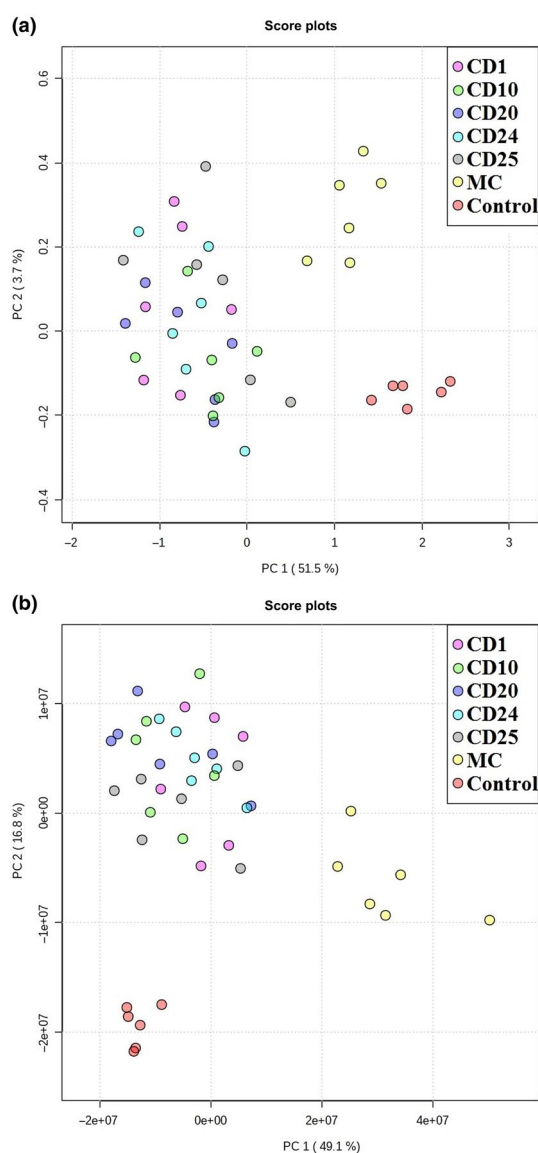


FIGURE 1 PCoA score plot based on the metabolic profiling of lignite (a) and bituminous (b) in non-biotreat control, microbial community, and isolates groups. MC, microbial community; PCoA, principal coordinates analysis

number of identified products in those groups was counted and partitioned according to the relative molecular mass. As shown in Figure 2a, the relative molecular mass of most products from lignite was converged in 600–900. However, the mass distributions of products in the microbial-community-treated groups were different from the isolates-treated groups. Specifically, the products with high relative molecular mass (600–1300) in the microbial-community-treated groups were less than those in isolates-treated groups, whereas the products with low relative molecular mass (0–600) in the microbial-community-treated groups were higher than those in isolates-treated groups. This is probably due to that the synergetic microbes in the microbial community further utilized the macromolecular products from initial coal-degrading strains once the initial products were produced. The relative molecular mass distribution of the products from bituminous was quite similar to those from lignite (Figure 2b). These results suggested that bacterial degradation behavior was different between single culture and co-culture with a synergetic methanogenic community. Therefore, to explore the initial products from coal, the comparative metabolomics analysis was focused on the products from direct coal-degrading isolates in this study.

3.3 | Comparative metabolomics analysis

The selected five strains of coal-degrading bacteria were, respectively, cultured in liquid medium with bituminous or lignite as the sole carbon sources and the medium without inoculating microbes as the control group. As shown in Figure 3, some commonly detected compounds from the biotreated and non-biotreated groups showed obvious decreased relative content, indicating that microbial degradation has occurred in the degradable component from coal. Interestingly, some commonly detected compounds from biotreated and non-biotreated groups showed obvious increased relative content, showing that the microbial activity may facilitate the dissolution of such matters.

Besides, after the microbial degradation of lignite, a total of 33 substances were identified in the positive and negative ion modes for newly produced compounds, whereas a total of 45 substances were detected in the positive and negative ion modes for newly produced compounds of bituminous. As shown in Figure 4, although there were differences between the content of products of five coal-degrading strains, most of the products were the same between those five bacteria. In

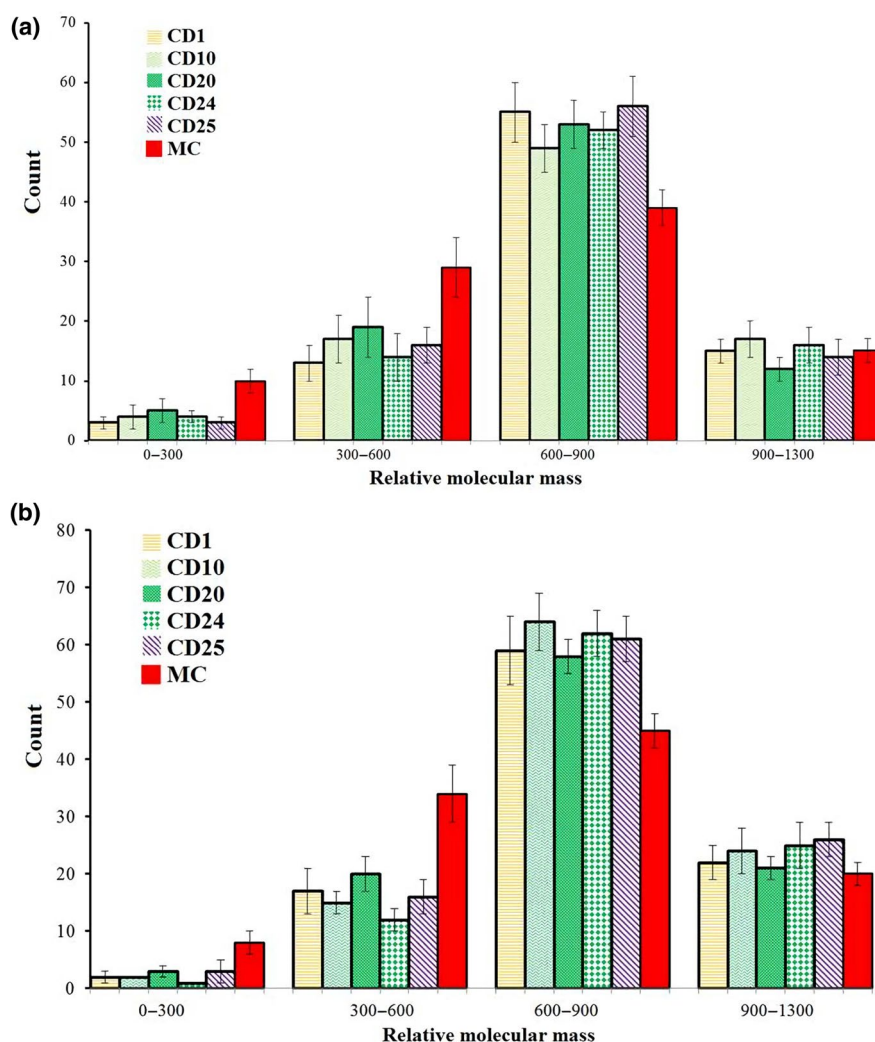


FIGURE 2 The counts and relative molecular mass distribution of the identified products from lignite (a) and bituminous (b) by the microbial community (MC) and isolates

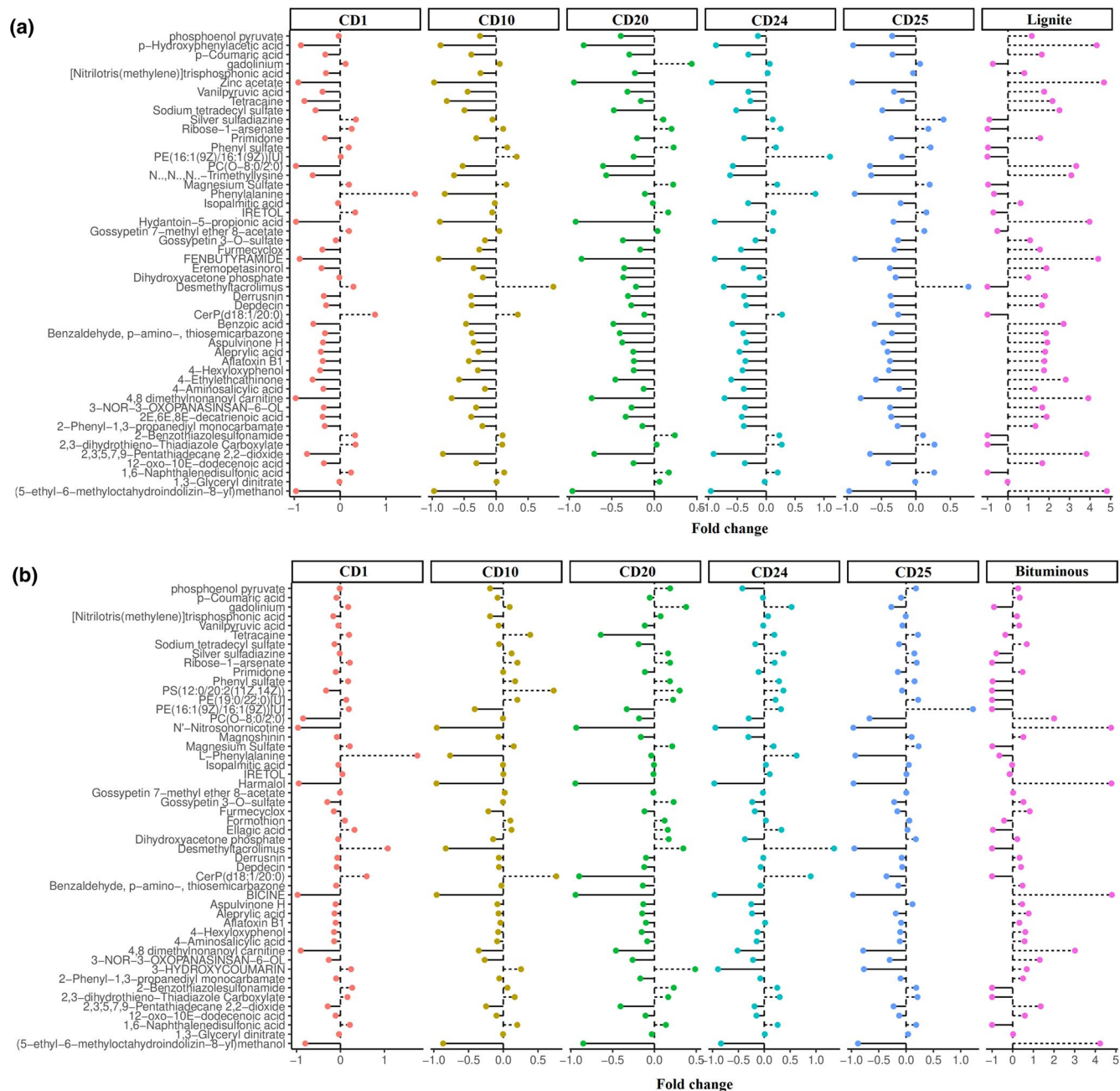


FIGURE 3 Changes in common products from the biotreated lignite (a) and bituminous (b) by five coal-degrading strains and non-biotreated lignite (a) and bituminous (b). The levels of compounds present in both groups were normalized to the level of internal standards. The dots with different colors represent the biotreated groups by corresponding coal-degrading strains while the rose ones represent the untreated control groups

general, these products could be divided into three categories according to their chemical construction. Those are alkane compounds, heterocyclic aromatic compounds, and microbial metabolism by-products.

3.4 | Initial products of lignite by coal-degrading strains

The degradation of lignite by five microorganisms led to the production of alkane compounds that include

17, 21-dimethylhenteontane, 3-oxotetradecanoyl-CoA, cabazitaxel, desmethyltacrolimus, didesmethyltacrolimus, glycinoprenol, N-(26-hydroxyhexacosanyl) Sphinganine, N-ornithinyl-35-aminobacteriohopane-32,33,34-trio 1, 5-phosphomevalonate, 3-hydroxytetradecanoy-CoA, 3-hydroxydodecanoy-CoA, 6,10,14-octadecatrienoic acid (6,10,14-octadecatrienoic acid), diaminopimelate, etc. Simultaneously, the heterocyclic aromatic compounds such as 26, 27-dinorcholecalciferol, acetyl podocarpic acid anhydride, 24, 25-dihydrosterol, 6 α -hydroxycortisol to cucurbitacin P, 3,7,cholest-5-en-3,7,12-triol, etc. were also

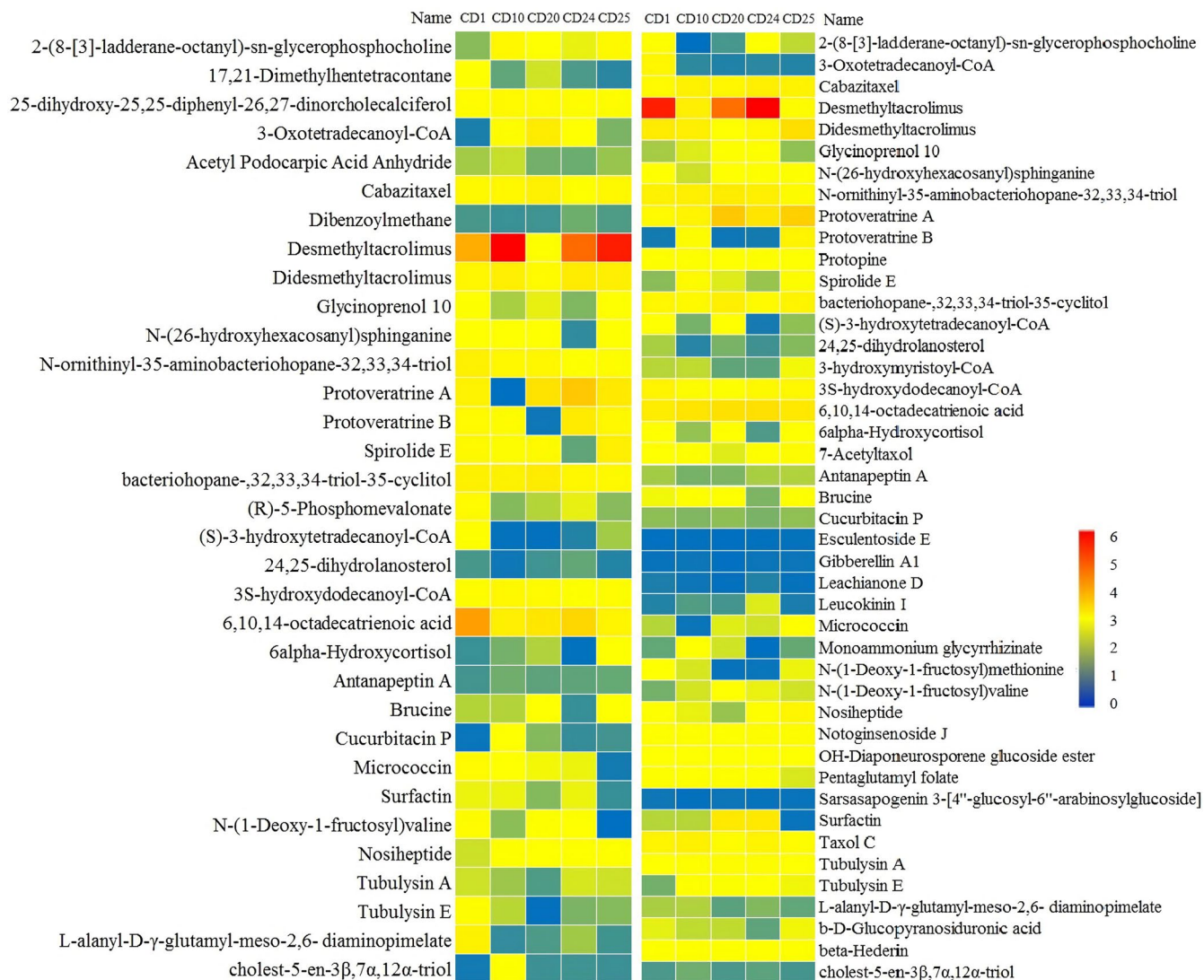


FIGURE 4 Heatmap of identified products of lignite (left) and bituminous (right) degraded by five isolated strains. The deeper the red color, the higher content in the tested sample; similarly, the deeper the blue color, the lower content in the tested sample. In this grid, each column represents the produced compounds from each coal-degrading strain and each row corresponds to the products

produced. In addition, the metabolic byproducts of lignite degradation by the five aforementioned strains mainly included some alkaloids and active proteins such as glycerophosphocholine, prooveratrine A, prooveratrine B, spirolide E, surfactin, antanapeptin A, nosiheptide, brucine, micrococcin, tubulysin A, tubulysin E, etc.

3.5 | Initial products of bituminous by coal-degraded strains

The alkanes produced during the degradation of bituminous coal by five microorganisms include 3-oxotetradecanoyl-CoA, cabazitaxel, desmethyltacrolimus, didesmethyltacrolimus, glycinoprenol, N-(26-hydroxyhexacosanyl) sphinganine, (S)-3-hydroxytetradecanoyl-CoA, N-ornithinyl-35-aminobacteriohopane-

32,33,34-triol, 7-acetylaxol, 3-hydroxymyristoyl-coA, 6,10,14-octadecatrienoic acid, diaponeurosporene glucoside ester, taxol C, and 2, 6-diaminopimelate. Simultaneously, the generated heterocyclic aromatic compounds after bituminous coal biodegradation include 6-hydroxycortisol, 24, 25-dihydrosterol, cucurbitacin P, esculentoside E, diterpene acid, gibberellin A1, leachianone D, monoglycyrrhizinate, notoginsenoside J, pentaglutamyl folate, sarsasapogenin, D-glucopyranosiduronic acid, and cholest-5-en-3 β ,7 α ,12 α -triol. Moreover, the produced metabolic by-products produced by the five microbial biodegradation of bituminous coal mainly include some alkaloids and active proteins such as glycerophosphocholine, prooveratrine A, prooveratrine B, protopine B, spirolide E, antanapeptin A, brucine, leucokinin I, micrococcin, nosiheptide, surfactin, tubulysin A, and tubulysin E.

3.6 | The produced common products from lignite and bituminous by five strains

It could be found that there were 24 common products from lignite and bituminous by five strains (Table 2). These compounds were distributed in all three classifications. ANOVA analysis was used to compare whether there was a significant difference between the relative abundances of commonly produced compounds from lignite and from bituminous. As shown in Table 2, the *p* value for both the produced alkane compounds and heterocyclic aromatic compounds from lignite and bituminous was higher than 0.05 except 6 α -hydroxycortisol, which implied that the relative abundance of common products from different coals had not too many differences. The common products from bituminous and

lignite were all probably originated from the same precursors in bituminous and lignite. One possible speculation is that the isolated strains showed similar and low degradation potential to these precursors, which resulted in displaying no differences in those common products between bituminous and lignite. Nevertheless, the metabolic by-products surfactin yields from bituminous (0.3901 ± 0.0027 SD) were higher than those from lignite (0.1479 ± 0.0012 SD).

4 | DISCUSSION

Coal is a complex organic polymer that is resistant to cracking by chemical and physical means (van Heek, 2000). Biogasification processes can convert the coal into methane

TABLE 2 ANOVA analysis for the relative abundances of common produced metabolites from lignite and bituminous

Common metabolites	Yield (%) ^a		SS ^b	df	MS ^b	F value	p value ^c
	Lignite	Bituminous					
2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine	0.1929 \pm 0.0289	0.3061 \pm 0.0033	0.0439	1	0.0439	2.7197	0.1377
3-Oxotetradecanoyl-CoA	0.2668 \pm 0.0138	0.0838 \pm 0.0002	0.0837	1	0.0837	1.0115	0.3439
Cabazitaxel	0.4074 \pm 0.0382	0.4809 \pm 0.0238	0.0135	1	0.0135	0.4347	0.5281
Desmethyltacrolimus	6.1993 \pm 1.9632	4.8111 \pm 1.8032	4.8273	1	4.8273	0.2558	0.6266
Didesmethyltacrolimus	0.6559 \pm 0.0537	0.6170 \pm 0.0201	0.0037	1	0.0037	0.0297	0.8673
Glycinoprenol 10	0.0903 \pm 0.0023	0.0807 \pm 0.0021	0.0002	1	0.0002	0.1025	0.7570
N-(26-hydroxyhexacosanyl)sphinganine	0.0871 \pm 0.0017	0.0949 \pm 0.0010	0.0001	1	0.0001	0.1093	0.7494
N-ornithinyl-35-aminobacteriohopane-32,33,34-triol	0.3803 \pm 0.0048	0.5979 \pm 0.0037	0.0118	1	0.0118	2.7592	0.1352
Protoveratrine A	0.4091 \pm 0.0129	0.1459 \pm 0.0046	0.1690	1	0.1690	1.9221	0.2030
Protoveratrine B	0.3396 \pm 0.0063	0.1281 \pm 0.0012	0.0112	1	0.0112	2.9404	0.1247
Spirolide E	0.6082 \pm 0.0046	0.4262 \pm 0.0025	0.0082	1	0.0082	2.2705	0.1702
bacteriohopane-,32,33,34-triol-35-cyclitol	0.0489 \pm 0.0005	0.0570 \pm 0.0001	0.0008	1	0.0008	0.0024	0.9615
3-hydroxytetradecanoyl-CoA	0.0188 \pm 0.0001	0.0285 \pm 0.0002	0.0002	1	0.0002	1.4603	0.2613
24,25-dihydrolanosterol	0.2777 \pm 0.0007	0.2966 \pm 0.0014	0.0008	1	0.0008	0.0813	0.7827
3S-hydroxydodecanoyl-CoA	1.6666 \pm 0.0219	1.0198 \pm 0.0408	1.0527	1	1.0527	0.9345	0.3619
6,10,14-octadecatrienoic acid	0.0403 \pm 0.0010	0.0603 \pm 0.0008	0.0010	1	0.0010	1.0694	0.3313
6 α -hydroxycortisol	0.0306 \pm 0.0003	0.0408 \pm 0.0005	0.0002	1	0.0002	6.3658	0.0356*
Antanapeptin A	0.0641 \pm 0.0009	0.0743 \pm 0.0006	0.0002	1	0.0002	0.3211	0.5864
Brucine	0.0327 \pm 0.0014	0.0367 \pm 0.0007	0.0005	1	0.0005	0.0007	0.9782
Cucurbitacin P	0.1355 \pm 0.0032	0.1703 \pm 0.0207	0.0030	1	0.0030	0.2526	0.6287
N-(1-Deoxy-1-fructosyl)valine	0.1549 \pm 0.0079	0.1703 \pm 0.0020	0.0006	1	0.0006	0.0118	0.9158
Surfactin	0.1479 \pm 0.0012	0.3901 \pm 0.0027	0.0014	1	0.0014	8.2819	0.0205*
Tubulysin A	0.0747 \pm 0.0076	0.0777 \pm 0.0020	0.0002	1	0.0002	0.0045	0.9476
Tubulysin E	0.1549 \pm 0.0079	0.1328 \pm 0.0026	0.0041	1	0.0041	0.0218	0.7541
cholest-5-en-3 β ,7 α ,12 α -triol	0.0411 \pm 0.0030	0.0227 \pm 0.0024	0.0008	1	0.0008	0.5535	0.4781

^aEach value shown represents the mean \pm SD of five individual experiments.

^bSS, between-groups sum of squares. MS, between-groups mean squares.

^cSignificant differences (*p* < 0.05) are indicated in asterisk (*).

step by step. A large number of studies have been done to understand the important intermediates in the late process of methanogenic coal bioconversion, such as acetate, carbon dioxide, hydrogen, methanol, and so on. In this study, the isolated microorganisms from a methanogenic community were used to degrade the coal alone, and the metabolomics method was used to reveal the produced chemical substances during coal degradation by those microbes. This study provides a new understanding of the metabolic intermediates of coal in the initial stage of coal bioconversion.

Coal-degrading strains isolated from the methanogenic microbial community play an important role in the start-up of coal bioconversion. The isolated strains growing on the basic anaerobic culture with coal as the sole carbon source belonged to three different genera, including *Bacillus*, *Paenibacillus*, and *Stenotrophomonas*. Interestingly, the genus *Stenotrophomonas* has never been reported to degrade coal before. *Stenotrophomonas* can degrade different xenobiotics and can convert refractory phenolic compounds into easily biodegradable compounds (Mukherjee & Roy, 2013; Piyali & Pranab, 2016). In a recent study, *Stenotrophomonas* was used to improve the start-up and the stability of sequential biofilm batch reactor for coal gasification wastewater treatment (Zhuang & Fang, 2020). Microorganisms belonging to genera *Bacillus* and *Paenibacillus* isolated from the coal mine environment have been widely reported to be able to degrade coal (Akimbekov et al., 2020; Deora et al., 2013; Jiang et al., 2013; Konishi et al., 1998; Pokorny et al., 2005; Steinbock et al., 2019). Most of the isolated strains also belong to the genera *Bacillus* and *Paenibacillus* in this study. However, it is hard to reveal uncultured taxa due to the limitations of traditional isolation and cultivation methods (Lewis et al., 2020). To fully unravel the metabolites in the initial stage of methanogenic coal bioconversion, further study could isolate more coal-degrading microbes with different modern innovative screening methods to achieve cultivation.

More compounds were significantly detected from bituminous treated by coal-degrading strains than from lignite. This extra part of metabolic products mainly includes alkane and polycyclic aromatic hydrocarbon (PAH) compounds of macromolecules, such as 7-acetyltaxol, C30 alkane glucoside ester, esculentoside E, diterpene acid, gibberellin A1, leachianone D, and monoglycyrrhizinate, notoginsenoside J, pentaglutamyl folate, sarsasapogenin, hederin, etc. This finding was probably due to the higher bioavailability of lignite than bituminous coal while the products of bituminous coal require more metabolic strategies for further degradation. However, there are still a large number of common products found in the treatment group of bituminous coal and lignite. These common compounds are mainly composed of long fatty carbon chains and their hydroxylated or acylated products, as well as PAHs, such as hydroxylated phenyl, naphthalene, etc. Some long-chain alkanes (C8–C30) have been

previously found in Australian bituminous coal (Youtcheff et al., 1983). PAHs, such as acylated or hydroxylated phenanthrene and terpenoids, have also been found in coal extracts of different coal ranks (Mastral et al., 1996). But there are more unreported compounds produced as shown in this study. More different metabolic strategies are needed to further degrade these newly produced metabolites by other synergistic microorganisms.

Interestingly, many microbial metabolic by-products including bioactive alkaloid and surfactin were also detected from the metabolites of coal treated by microorganisms. In previous studies, Maka et al. found that *Bacillus* sp. was able to secrete a polar water-soluble macromolecular alkaloid with a molecular weight between 3000 and 30,000, which could solubilize coal matrix (Maka et al., 1989). However, the chemical composition of this macromolecular alkaloid was not determined. Large amounts of alkaloids such as glycerophosphocholine, provera-trine A, provera-trine B, and brucine detected in the coal biometabolites in this study may play a key role in the solubilization of coal. In addition, surfactin detected from the products may play a role in promoting the bioavailability of coal. Surfactin is a type of cyclic lipopeptide with polar hydrophilic amino acid heads and hydrocarbon chain tails produced by bacteria, well known for its superior surfactant ability to reduce the surface tension of water from 72 mN m⁻¹ to 27 mN m⁻¹ (Peypoux et al., 1999). Solubilization of coal matrix with biosurfactants and remediation of PAHs pollutants has been widely reported (Breckenridge & Polman, 1994; Cameotra & Bollag, 2003). These microbial metabolic by-products might play an important role in the coal bioconversion process.

It is worth noting that a large number of alkanes and aromatic compounds were produced in an acylated or hydroxylated manner in bacterial coal degradation, indicating that the activity of microorganisms in the initial stage of methanogenic coal bioconversion could increase the bioavailability of coal. Hydroxylation of complex macromolecules is considered to play an important role in the anaerobic degradation process of coal; hydroxylated macromolecules can further react to produce metabolites such as fatty acids (Colosimo et al., 2016). The hydroxylated alkane (higher than 36 carbon atoms) in the metabolites can be further oxidized into carbon dioxide by *Arthrobacter* sp., *Acinetobacter* sp., *Candida* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., and *Bacillus* sp., while alkanes and aromatic compounds with less than 30 carbon atoms can be further degraded in the presence of terminal electron receptors such as nitrate, sulfate, and iron (Singh et al., 2012; Vogt et al., 2008), or syntrophic associations of various anaerobic hydrogen-using microorganisms such as methanogens or sulfate-reducing bacteria (Jackson et al., 1999). PAHs such as phenanthrene can be further degraded via 2-Naphthoyl-CoA intermediates through carboxylation or methylation under anaerobic conditions (Boll et al.,

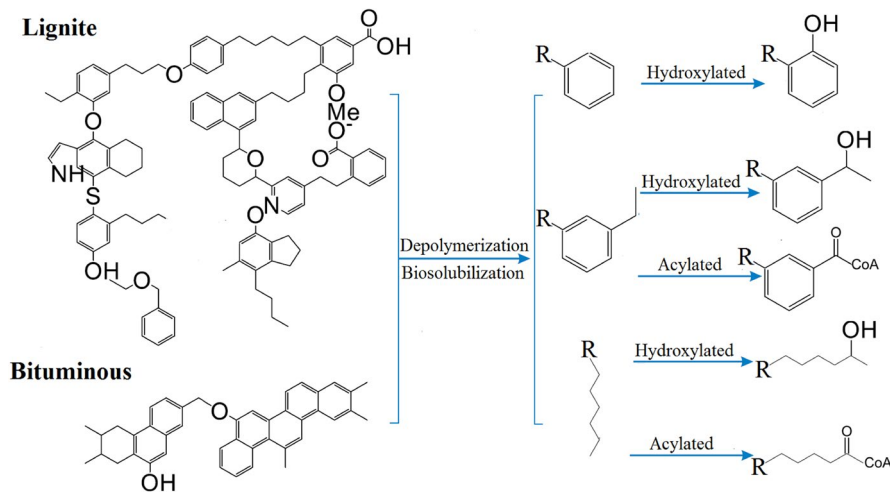


FIGURE 5 A proposed pathway of coal degradation by selected strains based on the chemical structure characteristics of the products from bituminous and lignite. Typical structural models of different ranks of coal were modified from Fakoussa and Hofrichter (1999). R represented the remainder of the chemical group in coal

2014). Strapoć and his collaborators have hypothesized a model of methanogenic coal bioconversion in which long-chain alkanes and PAHs are released from coal during the initial stages of coal degradation and are further converted by methanogenic microbial communities (Strapoć et al., 2011). In this study, the alkane and aromatic compounds produced by isolated strains from coal could be used as an appropriate substrate for further anaerobic degradation by the introduction of fumaric acid or carboxylation.

A proposal pathway of products from bituminous and lignite by selected strains is hypothesized based on the metabolomics analysis (Figure 5). Due to the recalcitrance, heterogeneity, and hydrophobicity of the complicated coal matrix, the degradation of coal requires a series of metabolic strategies by a community of microorganisms (Strapoć et al., 2011). Fakoussa and Hofrichter (1999) have suggested that the initial step of coal biodegradation is likely catalyzed by biosolubilization and extracellular enzymatic depolymerization under oxic conditions. In this study, under anoxic condition, biosurfactant and alkaloid which may involve in the biosolubilization of coal were also detected in the biotreated groups. Therefore, the biosolubilization of coal could also play an important role in the depolymerization of coal in the initial methanogenic bioconversion. The hydroxylation pathway involved in the anaerobic activation of coal components such as aromatic and aliphatic hydrocarbons has been suggested (Strapoć et al., 2011). In this study, the most prevalent products were those consistent with microbial hydroxylation of the aromatic core of coal, such as triol, sterol, and cyclitol, which did not appear in the non-biotreated groups. Furthermore, some of the products had been produced with an acyl-CoA structure. The product with an acyl-CoA structure was a temporary compound formed when coenzyme A (CoA) attaches to the end of the long-chain substrate. Then, the compound will form one or more molecules of acetyl-CoA. This, in turn, enters the citric acid cycle.

In summary, this study provided important insights into the initial products of coal during the biomethane

production process. Five coal-degrading strains (CD1, CD10, CD20, CD24, and CD25) from a methanogenic microbial community were isolated and identified. Among them, CD1 and CD24 belong to *Paenibacillus* sp.; CD10 and CD20 belong to *Bacillus* sp. and CD25 belongs to the *Stenotrophomonas* sp. Furthermore, the results of metabolomics showed that a large number of alkanes and heterocyclic aromatic compounds were produced from bituminous coal and lignite degraded by isolated bacteria, and most of the compounds had been hydroxylated or acylated. Also, microorganisms secreted some alkaloids and biosurfactants which inevitably play a positive role in coal degradation. These results indicate that the initial degradation of coal by microorganisms plays a significant role in the process of methanogenic coal bioconversion, and microorganisms can provide abundant substrates from coal for further degradation by utilizing various metabolic strategies. Further studies are required to explore which of these intermediate metabolites are biodegradable under the methanogenic condition and which members of the methanogenic community are responsible for its degradation to depict a more complete metabolic flux model of methanogenic coal bioconversion.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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