

Mechanism of Ammonium Chloride Enhanced Microbial Degradation of Coal: A Metagenomic Perspective

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Abstract: Microbial anaerobic degradation of coal matrix to produce methane is an important way to realize green development and clean utilization of coal. However, the extremely high carbon-nitrogen ratio of coal leads to the limitation of microbial metabolic activity. In this study, a comparative experiment was set up between the basic inorganic salt medium (control) and the addition of 1.5 g/L ammonium chloride (treatment). Using metagenomic sequencing technology, the regulation of nitrogen source addition on coal degradation process was deeply analyzed from the two dimensions of species succession and metabolic functional gene abundance. The results showed that the community diversity was significantly improved, and the addition of nitrogen sources broke the nutritional bottleneck, and the Shannon index of the microbial community in the control group was significantly increased to 5.1. PCoA showed that the PC1 interpretation rate of the two groups of community structure was 75.72 %. Species composition analysis showed that the abundance of core degrading bacteria (*Clostridium*, *Bacillus*) and methanogens (*Methanosarcina*, *Methanotherix*) increased significantly in the treatment group. STAMP difference analysis showed that the Xylene degradation and Naphthalene degradation pathways directly related to coal skeleton degradation in the treatment group were significantly activated. At the same time, the enhancement of oxidative phosphorylation, cofactor biosynthesis and quorum sensing pathway provide enough energy support and enzyme system guarantee for the cleavage of coal macromolecules. This study elucidates the molecular mechanism of nitrogen source enhanced coal biotransformation, which has important reference value for optimizing the production technology of underground coalbed methane project.

Keywords: Coal; Anaerobic Degradation; Metagenomics; Exogenous Nitrogen.

1. Introduction

As China's main energy source, clean and efficient utilization of coal is an important way to achieve the goal of "double carbon" and ensure national energy security[1]. Traditional physical and chemical conversion of coal is often accompanied by high energy consumption and high pollution. In contrast, coal bioconversion (CB) technology[2], which uses microorganisms to convert coal matrix into clean energy such as methane under anaerobic conditions. With the advantages of mild conditions, environmental friendliness and low cost, it is regarded as the frontier technology of coal green development.

However, as an extremely complex biomacromolecular polymer, the core of coal is a hydrophobic structure composed of highly cross-linked aromatic rings[3]. In the process of degradation, microorganisms are difficult to completely degrade coal. In natural coal seams, the carbon-nitrogen ratio of coal itself is extremely high. The lack of available nitrogen sources has become a short board that limits microbial growth and metabolic activity. Nitrogen is the core component of microbial synthetase, protein and nucleic acid. The lack of nitrogen source will lead to the inhibition of dehydrogenase and oxygenase expression, thus inhibiting the cracking efficiency of coal from macromolecules[4].

Previous studies have shown that the addition of ammonium salt, urea, organic nitrogen and other exogenous nitrogen can significantly increase the production of coalbed methane. Among them, ammonium chloride, as a cheap and highly bioavailable available nitrogen source, has only been proved to have a good effect, but the deep molecular

mechanism is still in its infancy[4].

Based on this, in order to reveal its deep molecular mechanism, this study set up a comparative experiment between the basic inorganic salt medium (control) and the addition of 1.5 g/L ammonium chloride (treatment). The purpose of this study is to analyze the nitrogen source-enhanced coal degradation from multiple dimensions such as species composition and metabolic pathways.

2. Experimental Design and Method

China Huaibei bituminous coal was selected, the pulverized coal was crushed and sieved, and the pulverized coal with a particle size of < 0.2 mm was selected. Before the experiment, vacuum drying was used, and industrial analysis and elemental analysis were carried out. The long-term acclimated methanogenic enrichment culture was selected and pre-cultured under anaerobic conditions before inoculation to restore microbial activity. Refer to the classical anaerobic medium formula[5], including K_2HPO_4 0.5g/L, KH_2PO_4 0.5g/L, $MgCl_2 \cdot 6H_2O$ 1.2g/L, trace elements and vitamin solution 0.2mL/L.

The experiment was carried out in a 250 mL anaerobic fermentation bottle. The experiment was divided into two groups, with three biological replicates in each group. In the control group, the above-mentioned basic salt medium was used. Treatment was added with 1.5 g/L NH_4Cl on the basis of the basic medium and cultured in a constant temperature incubator for 45 days.

On the 10th, 25th and 45th day of the experiment, the fermentation broth was extracted, and the total DNA of microorganisms was extracted by kit. The concentration and

purity of DNA were detected by agarose gel electrophoresis and NanoDrop. The sequencing library of 350 bp insertion fragment was constructed and Paired-end sequencing was performed based on Illumina NovaSeq 6000. Fastp software was used to remove adaptors and low-quality sequences, MetaSPAdes was used for Contigs assembly, Prodigal was used for ORF prediction, and Diamond software was used to

align protein sequences to KEGG, CAZy (carbohydrate active enzyme), eggNOG and NCBI-NR databases. The abundance of community structure was calculated based on Kraken2 or gene markers. R language was used for significant difference analysis. The vegan package of R is used for Alpha and Beta diversity analysis and PCoA ranking map drawing[6].

Table 1. Industrial components and elemental components of experimental coal samples

Sample	Proximate analysis (%)				Ultimate analysis (%)				
	M_{ad}	A_{ad}	V_{daf}	FC_{ad}	C	H	O*	N	S
YM	1.74	9.67	32.37	56.22	73.13	6.32	18.82	1.34	0.39

O*: Differential method oxygen content.

3. Alpha and Beta Diversity Analysis

After splicing and filtering the original offline data, 413 512 high-quality raw reads were obtained. Alpha analysis based on Shannon index showed that the Shannon index of the control group was 2.0, and the treatment was significantly increased to about 5.1. There was a significant difference between the two groups ($p < 0.01$). On the surface of the results, the addition of exogenous nitrogen greatly enriched the diversity of microbial communities involved in coal degradation, broke the original nutritional restrictions, and promoted the growth of more functional flora.

Fig 1b shows the PCoA results based on species composition. The first principal component (PC1) explained 75.72 % of the total variance, and PC2 explained 8.84 %. Control and treatment showed obvious long-distance separation on the PC1 axis.

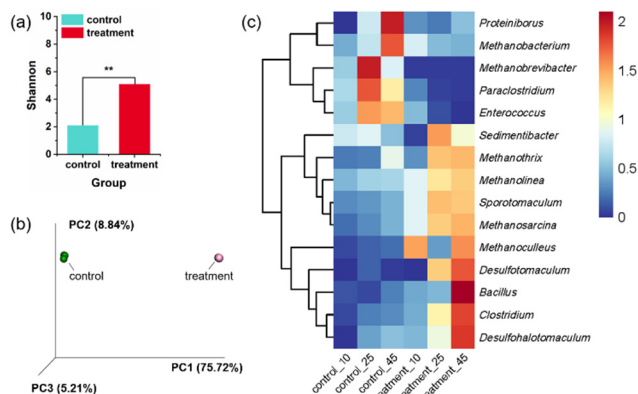


Fig 1. (a) The Shannon index of the fermentation broth at the peak period of the two groups; (b) PCoA analysis of the peak period of the two groups; (c) Two groups of microbial community composition based on genus level.

4. Species Composition Analysis

Fig.1c shows the species abundance changes and clustering of the two groups at the genus level at different stages. *Proteiniborus*, *Methanobrevibacter*, *Paraclostridium* and *Enterococcus* were mainly enriched in the control group during the whole experimental period. This shows that under the condition of nitrogen deficiency, the community function is relatively simple. With the passage of degradation time (especially 25d and 45d), the types of methanogens in the treatment group increased significantly, including acetic acid-utilizing methanogens (*Methanosarcina*, *Methanoxithrix*) and hydrogenotrophic methanogens (*Methanolinea*, *Methanoculleus*).

5. STAMP Differential Function Enrichment Analysis

Fig.2 shows the significant differences in the metabolic pathways of microbial communities between the two groups. The top 20 metabolic pathways with the most significant difference in abundance were screened by t-test. In general, the differences between the two groups are reflected in degradation pathways, energy metabolism and synthesis potential, fatty acid metabolism and intercellular collaboration. The addition of ammonium chloride significantly enriched Xylene degradation and Naphthalene degradation, and the surface directly enhanced the degradation ability of microorganisms to typical polycyclic aromatic hydrocarbons (PAHs) in coal structure, which proved the promotion effect of ammonium chloride on coal conversion at the molecular level[7]. Oxidative phosphorylation means that microorganisms have higher energy (ATP) production efficiency and can support more active life activities. Biosynthesis of cofactors and Pantothenate and CoA biosynthesis are necessary cofactors for the activity of various degrading enzymes, and their enrichment indicates that nitrogen source supplement provides material guarantee for the synthesis of key degrading enzymes. Fatty acid metabolism, Fatty acid metabolism and fatty acid biosynthesis pathways were also significantly enriched. The enhancement of these pathways is usually closely related to the repair and expansion of cell membranes and the metabolism of long-chain alkanes, the intermediate products of coal degradation[8].

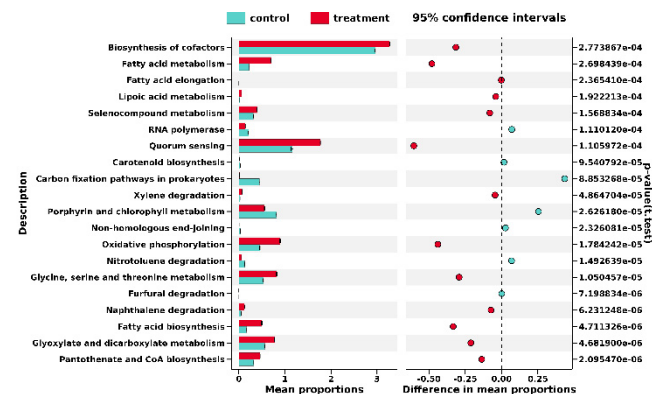


Fig 2. Two sets of STAMP-based differential functional enrichment analysis.

6. Conclusion

In this study, the effects of exogenous nitrogen sources on microbial anaerobic degradation of coal matrix and its

molecular mechanism were systematically explored by constructing an anaerobic fermentation system and combining metagenomic sequencing technology. The study found that the introduction of exogenous nitrogen sources effectively broke the metabolic bottleneck caused by the high carbon-nitrogen ratio of coal, significantly improved the α -diversity of the microbial community, increased the Shannon index from 2.0 to 5.1, and drove the directional succession of the community structure. The PC1 interpretation rate was 75.72 %. At the species level, the treatment group constructed a synergistic metabolic network composed of high-efficiency degrading bacteria such as *Clostridium* and *Bacillus*, and multiple methanogens such as *Methanosarcina* and *Methanothrix*, which realized a leap from a single methanogenic pathway to a dual pathway of acetic acid combined with hydrogen nutrition. At the functional level, STAMP difference analysis further confirmed that the addition of nitrogen sources significantly activated aromatic hydrocarbon degradation pathways such as xylene and naphthalene, and simultaneously enhanced oxidative phosphorylation energy metabolism and Quorum sensing activity.

In summary, ammonium chloride enhances the attack efficiency of microorganisms on the complex skeleton structure of coal and the level of electron transfer within the system, thereby significantly increasing the bioconversion rate of coal to methane, and providing an important molecular biological basis for the biological production of coalbed methane.

Acknowledgments

This study was supported by Jiaojiazhai Coal Mine Research Project [NO. MYXGJJZKJSFW20240063].

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