INTRODUCTION

The coal is still one of the indispensable fuels in the world. In recent years, 93% of electric power is produced from coal in South Africa, 79% in China, 78% in Australia, 68% in India, and 39% in America. However, a large amount of SO₂ and other sulfur-containing gases was released into the atmosphere in the process of high-sulfur coal combustion, resulting in environmental problems such as acid rain and haze. With the depletion of high-quality coal resources, the proportion of high-sulfur coal consumption is increasing. Therefore, developing an effective mechanism that is capable of reducing the sulfur content of high-sulfur coal becomes a hotspot in energy and environmental fields. In the past years, chemical and physical methods were applied to remove the sulfur from the coal; however, these mechanisms are high-cost, energy-intensive, and inefficient for removing organic sulfur. Thus, more and more attention has been focused on...
the biodesulfurization of high-sulfur coal because it offers a clean alternative method to remove sulfur from coal.6

The sulfur in coal is in the form of inorganic sulfur and organic sulfur.7 Inorganic sulfur is easily removed by flotation; however, organic sulfur in coal, in the form of heterocyclic ring, is too recalcitrant to take out by conventional physical and chemical methods. Biodesulfurization draws attention because of its special inorganic and organic sulfur removal ability.5,9 There are some desulfurizing bacteria, both organosulfur and inorganosulfur, discovered to be used in coal biodegradation. Examples of inorganosulfur bacteria such as Acidithiobacillus caldus, Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans,10–12 and examples of organosulfur desulfurizing bacteria such as Pseudomonas, Bacillus, and Rhodococcus were found.5,13,14 Dibenzothiophene (DBT) is one of the representatives of organic sulfur in coal.15 There are two pathways, Kodama and sulfur-specific (4S),16,17 that microbially degrade organic sulfur when the DBT is used as a model compound. The Kodama pathway uses microbial metabolism to cut carbon-carbon bonds in the DBT to convert it into a water-soluble organic compound, thereby achieving the purpose of desulfurization, which destroys the carbon structure and causes loss of coal calorific value. The 4S pathway desulfurization is the specific cutting of the carbon-sulfur bond in the DBT by the bacterial metabolism, without destroying the carbon skeleton, and the sulfur is removed in the form of sulfate, which would not reduce the calorific value of the coal.

In the previous experiments, it was effective to remove organic sulfur and inorganic sulfur in a stepwise manner, in which organic sulfur was mainly removed by bioleaching and inorganic sulfur by physical flotation.18 In this paper, it is envisioned that making use of desulfurizing bacteria, A. ferrooxidans GF, instead of flotation helps to lower costs and power consumption of the desulfurization process of coal. It has been demonstrated that A. ferrooxidans GF was capable of desulfurizing through oxidizing inorganic sulfur from coal such as FeS2 in the previous study.19 The concentration of coal slurry in shake flask experiments met the needs of practical industrial applications. Consequently, small or medium heap leaching methods are necessary for research. It was previously reported that the co-culture of various desulfurizing bacteria was used for the removal of sulfur to increase the desulfurization rate. However, the destabilization of co-culture was frequently observed in practical applications, in contrast, which may decrease the desulfurization efficiency. Furthermore, the biodesulfurization process of mixed bacteria is difficult to be controlled and repeated in industrial practice. Meanwhile in order to resolve the drawback of co-culture, “two-step” leaching desulfurization was proposed that organic sulfur desulfurizing bacterium and A. ferrooxidans GF were used to remove organic and inorganic sulfur from coal, respectively. In this paper, we attempted to utilize DBT as the sole sulfur source to separate an organic sulfur degradation bacterium from the petroleum-polluted soil and then for the “two-step” leaching test. The present study aimed to: (a) find a bacterium with the capability of degrading the DBT and (b) evaluate this approach of “two-step” leaching desulfurization process of coal.

2 | MATERIALS AND METHODS

2.1 | Petroleum-contaminated soil samples

Soil samples were collected 3~5 cm deep in the second oil production plant in Daqing Oilfield, Tuqiang West Street, Honggang District, Daqing City, Heilongjiang Province. The samples were collected and placed in a sterile plastic bottle and immediately returned to the laboratory for separation of desulfurizing bacteria, which were stored in a refrigerator at 4°C for further analysis.

2.2 | Chemical and coal sample

Dibenzothiophene and 2-hydroxybiphenyl (2-HBP) were purchased from Sigma-Aldrich Fluka, purum ≥98%.

The coal sample used in the experiment was collected from Liupanshui, Guizhou province, China. The sample was comminuted to <0.5 mm in size for shaking test and leaching test using a ball mill.20 X-ray diffraction (XRD) was used to analyze the component of the coal sample. The result is shown in Table 1. The characteristic of char residue of the coal is 4, and the caking index G 13, which explained why the coal sample was weakly cohesive. The gross calorific value as air-dried basis and as-received basis net calorific value of coal were 29.29 and 27.59 MJ/kg, respectively. Therefore, the

| TABLE 1 Analysis results of raw coal from Liupanshui, Guizhou |
|----------------------------------|-----------------|
| Project name | Result |
|----------------|--------|
| Total moisture Mt (%) | 2.7 |
| Air-dried moisture Mad (%) | 2.54 |
| Air-dried ash Aad (%) | 11.81 |
| Air-dried volatile Vad (%) | 26.68 |
| Characteristics of char residue (1-8) | 4 |
| Air-drying fixed carbon FCad (%) | 58.97 |
| High heat output of coal Qgr, v,d (MJ/kg) | 29.29 |
| Low heat output of coal Qnet,v,ar (MJ/kg) | 27.59 |
| Caking index G | 13 |
| Total sulfur (%) | 4.973 |
| Pyritic sulfur (%) | 2.896 |
| Organic sulfur (%) | 1.979 |
| Sulfatic sulfur (%) | 0.144 |
coal sample was classified as thermal coal. The total sulfur of coal is 4.973%, which was composed of 2.896% of pyritic sulfur, 1.979% organic sulfur, and 0.144% of \( \text{SO}_4^{2-} \). And it is about 60.205% being inorganic sulfur, whose main form is iron sulfide. In general, experimental coal samples belong to high-sulfur steam coal.

### 2.3 Medium

The basal salt medium (BSM) was utilized for the cultivation of strain SX-12, which contained 12.03 g of \( \text{Na}_2\text{HPO}_4\cdot12\text{H}_2\text{O} \), 2.44 g of \( \text{KH}_2\text{PO}_4 \), 2.0 g of \( \text{NH}_4\text{Cl} \), 0.36 g of \( \text{MgCl}_2\cdot6\text{H}_2\text{O} \), 0.004 g of \( \text{MnCl}_2\cdot4\text{H}_2\text{O} \), 0.001 g of \( \text{FeCl}_2\cdot6\text{H}_2\text{O} \), 0.001 g of \( \text{CaCl}_2 \), 1.62 g of glycerin, and 1 L of deionized water. The final pH value was controlled at 7.0.21 The basal salt DBT medium (BSDM) was prepared by mixing 0.3 mmol/L DBT into the BSM, with the DBT as the sole sulfur source.

The enrichment medium was prepared for the enrichment of oilfield soil microorganisms, which contained 5.0 g of \( (\text{NH}_4)_2\text{SO}_4 \), 2.0 g of glucose, 1.2 g of \( \text{KCl} \), 1.2 g of \( \text{NaCl} \), 0.029 g of \( \text{FeSO}_4\cdot7\text{H}_2\text{O} \), 1.5 g of \( \text{KH}_2\text{PO}_4 \), 1.5 g of \( \text{K}_2\text{HPO}_4 \), 0.5 g of yeast powder, 5.0 g of glycerin, and 1 L of deionized water. The final pH value was 7.5, adding 5 mL of trace element solution.22

*Acidithiobacillus ferrooxidans* GF was cultivated by 9K medium that contains 3.0 g of \( (\text{NH}_4)_2\text{SO}_4 \), 0.1 g of \( \text{KCl} \), 0.5 g of \( \text{K}_2\text{HPO}_4 \), 0.5 g of \( \text{MgSO}_4 \), 0.01 g of \( \text{Ca(NO}_3)\text{}_2 \), and 1 L of deionized water. The final pH value was 2.0.23

### 2.4 Separation and identification of organic sulfur desulfurizing bacteria

Fifteen grams of Daqing Oilfield soil was added to 90 mL of sterile water and the shaker shook for 2 hours. After standing for some time, 10 mL of the supernatant was taken to be filled with 90 mL of enrichment medium and incubated for 2 days at a constant temperature shaker (temperature 30°C, 170 rpm). The bacterial solution after enrichment was appropriately diluted, and the appropriate diluted suspension was subjected to plate coating separation on BSDM plates.21 Lastly, the final individual and distinguishable colonies were selected and conducted by applying the coal shaking desulfurization test individually to choose the most efficient bacterium. In addition, the target strain isolated was inoculated into the BSDM liquid medium, and the DBT degradation rate was determined by high performance liquid chromatography.

The DNA of isolated strain was extracted by using a TIANamp Bacteria DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer’s protocol, whose 16S rRNA sequence was amplified by universal primers 1492R and 27F. The PCR product mentioned above was subjected to agarose gel electrophoresis, and then gel recovery was performed by the E.Z.N.ATM Gel Extraction Kit (Omega Bio-tek, Inc.) and sequenced in the Shanghai Sangon Company (Shanghai, China). The 16S rRNA gene sequence of the isolated strain was uploaded to the GenBank. Subsequently, it was aligned with other sequences from the BLAST facility of the National Center for Biotechnology Information (NCBI) by Clustal (https://www.ncbi.nlm.nih.gov). And the phylogenetic tree was established based on the 16s rRNA gene with the MEGA 7.0.

### 2.5 Determination of the optimum growth conditions

pH- and temperature-controlled tests were applied to determine the optimum pH and temperature of the isolated strains. A pure culture of the strain was inoculated into the BSDM. The incubation was performed under different temperatures and different initial pH values. While determining the effect of initial pH on bacterial growth, the incubation temperature was set at 30°C, and the initial pH was set at 7.0 while exploring the effect of temperature on bacterial growth. In the temperature-controlled and pH-controlled experiments, the cell concentration was measured and compared in the whole growth cycle of 84 hours. The cells were incubated in BSDM at 170 rpm, and the cell growth was measured by hemocytometer cell counting.

### 2.6 “Two-step” biodesulfurization test

The strain SX-12, separated from petroleum-contaminated soil, was applied to the “two-step” high-sulfur coal column desulfurization experiment. Leaching columns, 20 cm height with a 20 cm internal diameter, were packed with 1 kg air-dried coals. The processing phase of “two-step” biodesulfurization adopted the intermittent leaching method with a 2.5 L cultured appropriately bacterial solution. The experimental system is shown in Figure 1. The optimal growth initial pH of *A. ferrooxidans* GF is pH 2.0. In view of an extremely low pH value of the system in the process of inorganic sulfur removal, it is not conducive to the growth and desulfurization of strain SX-12 in the later period. Therefore, the removal of organic sulfur was put in first step. At room temperature, the isolated strain was used to carry out for a period of 15 days of coal leaching off organic sulfur test, subsequently, the *A. ferrooxidans* GF for the 15-day follow-up of the inorganic sulfur removal test. The test group and the control group (no bacteria added) were, respectively, set up to measure the change of pH and Eh values. After the test was finished, the coal samples were washed and dried, and the total sulfur content was measured by the Eschka method to calculate the desulfurization rate. The two forms of sulfur content in experimental coal, inorganic sulfur and organic sulfur, were determined according to the GB/T214-2007.24
3 | RESULTS AND DISCUSSION

3.1 | Separation and identification of the target strain

One of the strains, designated SX-12 with large DBT degradation ability, was found to be cultured on the solid medium of BSDM. Colonies were round, raised, white and the surface was smooth and moist (shown in Figure S1). The bacteria, observed under a microscope, were motile and spherical in the liquid medium of BSDM, which could reduce the DBT concentration from 0.3 to 0.06 mmol/L within 48 hours. After 96 hours, the DBT concentration was reduced to 0.05 mmol/L, and the DBT degradation rate was 83.33%. And 2-HBP, an intermediate metabolite in the 4S pathway, was detected (from Figure 2A and Figure S2). In addition, the sulfur content of the raw coal reduces from 4.973% to 2.147%, total desulfurization rate of 56.83%, whose organic sulfur desulfurization is 85.87% after coal in shake flask experiments. And the desulfurization rate approaches the maximum value on the tenth day (from Figure 2B). It was demonstrated that strain SX-12 could effectively remove sulfur from coal in shake flask experiments, especially organic sulfur.

After the 16S rRNA gene sequence of strain SX-12 aligned with other sequences from the BLAST, a phylogenetic tree was established based on the 16s rRNA gene, as shown in Figure 3. As seen from the figure, the isolated strain SW-12 was closely affiliated with *Rhodococcus erythropolis* strain K85 (KF976880.1) and *R. erythropolis* strain N14 (Q435706.1) with 99% sequence similarity. Taking into account the morphological and physiological characteristics of strain SX-12, the strain was identified...
as most closely related to *Rhodococcus erythropolis*. *Rhodococcus erythropolis* was the earliest discovered one of fuel desulfurizing bacteria, which have been considered to be excellent coal desulfurization bacteria because of their sulfur-specific (4S) pathway and the capacity for producing a biosurfactant.\textsuperscript{25,26}

In order to further verify whether the bacteria isolated also possess a 4S organic sulfur pathway, we analyzed the PCR products of the gene sequences of the strains. According to the 4S pathway of the key gene sequence, it is necessary to design primers for amplification. The amplification products were sent by the gel recovery kit for recovery, the PCR product was sent to the Shanghai Sangon Company for sequencing, and then sequenced the results which were compared in the NCBI. The gel electrophoresis result of *Rhodococcus erythropolis* SX-12 desulfurization gene is shown in Figure S4. After sequencing, the sequence of the PCR product was 99% identical to the desulfurization gene of *Rhodococcus erythropolis* IGTS8 after BLASTX alignment. It was demonstrated that the dsz ABC, a desulfuration gene, is highly conserved and further suggests that the strain SX-12 is equipped with the 4S sulfur pathway. Of course, there may be other sulfur metabolic ways in the strain SX-12 to help remove inorganic sulfur of coal, which needed further study.

### 3.2 Effect of different initial pH and temperatures on strain growth

The results of the strain SX-12 culture in the BSDM of different initial pH are shown in Figure 4A. The results revealed that the initial pH value had a significant effect on the growth of bacteria. It was illustrated that the strain SX-12 could survive in a wide range of pH but it prefers a neutral environment. Bacterial growth was inhibited when the initial pH was above or below the optimum initial pH 7.0. Since the pH of the petroleum-contaminated soil from the Daqing Oilfield was always lower than 7.0, it could be explained as a possible reason for the little proportion of the strain in the microbial community of the petroleum-contaminated soil (shown in Figure S3).

The results of the strain SX-12 culture in the BSDM of different temperatures, shown in Figure 4B, indicate that the bacterial concentration of the strain SX-12 reached the highest value at 30°C of 42 hours. Overall, the bacteria had grown...
optimally at 30°C. The growth of the bacteria was affected in different extents when the culture temperature was lower or higher than 30°C. Interestingly, the bacteria could still grow well at 10°C. It demonstrated that the strain could tolerate the cold, which may be related to the isolation of the bacteria from the Heilongjiang province, northeast China, where the perennial air temperature is very low.

3.3 “Two-step” leaching biodesulfurization test

The results of the first step organic sulfur removal are shown in Figure 5A. The results exhibited that the pH value of the experimental group appeared to show a more obvious decline than the control group, but the Eh value had shown a more obvious rise. We deemed bacteria released H⁺ in the process of metabolizing sulfur is the reason for the pH value of the whole leaching system declining sharper than the control group. However, the pH value of the control group without bacteria declined sharply on the first day. This phenomenon could be attributed to the dissolution of acidic humic acid contained coal, thus causing the pH of the system to drop. It is also worth mentioning that the pH value of the control group still appeared to show a small decline. The oxidation of pyrite in coal during the longer leaching cycle was likely to cause pH decline. The acidic condition influenced bacterial growth at the later period of the experiment. Therefore, the pH control strategy should be added in future process design. The Eh value of the experimental group increased more significantly than that of the control group, indicating that the addition of bacteria increased oxidizability of the whole leaching system. From Figure 5B, the total desulfurization rate of 38.78% was obtained after 15 days of leaching experiment, removal of organic sulfur of 81.45%, and removal of inorganic sulfur of 10.38%. The desulfurization rate of organic sulfur was better than most of the reported results, which indicated that the strain SX-12 has a good ability of organic sulfur removal. However, the desulfurization rate is lower than the shake flask test result, which may be a more complete contact area between bacteria and coal in the shaking test.
The 9K medium containing *A. ferrooxidans* GF was added to the leaching system after the removal of organosulfur to carry out the second step of the inorganic sulfur extraction experiment, and the result is shown in Figure 6A. Compared with the control group, the pH value of the two-step leaching system showed a significant downward trend; the Eh value was significantly higher than the control group. *Acidithiobacillus ferrooxidans* oxidized pyrite to remove sulfur from coal, so that the pH and Eh in the leaching system vary. After a 30-day “two-step” biodesulfurization leaching test, the sulfur content of the raw coal reduces from 4.973% to 1.729%, whose pyritic sulfur of coal reduced to 1.828%, organic sulfur 0.367% and sulfatic sulfur 0.057%. And the total sulfur removal rate of 65.23% was obtained, in which the removal rate of organic sulfur was 81.45%, inorganic sulfur of 38.78%. Although pyritic desulfurization was worse than some of the reported results, total desulfurization of the two-step approach was better than the majority of other leaching test results, which demonstrated that two-step biodesulfurization could further expand in future. Compared with the total desulfurization rate of 67.83% of our previous flotation-biodesulfurization method, the “two-step” bioleaching method was slightly lower than it, but it was a more energy-saving and economical technology.

**CONCLUSION**

A strain of organic sulfur degradation, named SX-12, was isolated, which can remove 56.83% of the sulfur from the coal in shake flask experiments. The morphological, physiological characterizations and the analysis based on 16S rRNA sequence of strain SX-12 suggest that it is most closely related to *R. erythropolis*. Reportedly, *Rhodococcus* make use of the 4S organic sulfur metabolic pathway in desulfurization, and we confirmed *R. erythropolis* SX-12 too. The strain can grow at a broad range of pH and temperatures, but optimally at 30°C and pH of 7.0. In general, *R. erythropolis* SX-12 is considered to have good prospects for fossil fuel biodesulfurization applications.

Additionally, *R. erythropolis* SX-12 isolated and *A. ferrooxidans* GF were exploited together for the “two-step” biodesulfurization leaching of coal, total sulfur removal rate of 65.23%, the sulfur content of the raw coal reduces from 4.973% to 1.729% after a month of the leaching experiment. To the best of our knowledge, the coal leaching desulfurization with two various desulfurizing bacteria were employed for the first time. The “two-step” leaching test overcomes the shortcoming of co-culture and has a high desulfurization rate, which indicates that this desulfurization method with a good research value can be further expanded. Naturally, this method also has the disadvantage of a long processing period. To supply theoretical value and upgrade the direction for the pilot production, the scale expansion, process optimization, and strain improvement need to be researched in the future.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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