Degradation Performance of Petroleum-Hydrocarbon-Degrading Bacteria and its Application in Remediation of Oil Contaminated Soil

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Abstract. Three petroleum degrading strains were obtained from oily sludge and denoted as A2 (Pseudomonas putida), A4 (Acinetobacter calcoaceticus) and L5 (Sphingomonas sp). Crude oil degradation experiments were conducted to investigate the degradation performances of the strains and their bacterial consortium(C) on crude oil. The petroleum degradation rates of A2, A4, L5, and C were 42.8%, 48.01%, 26.56%, and 81.07%, respectively, after 28 days of cultivation, illustrating the removal of petroleum hydrocarbons by the bacterial consortium had a synergistic effect. The bacterial consortium was used to remediate oil contaminated soil in laboratory and the remediation results of the different microbial addition methods–free bacterial consortium (TB), biochar-free bacterial consortium (TB-BC), and biochar-immobilized bacterial consortium (CTB) were compared. The results showed that biochar was beneficial to the colonization of degrading bacteria in the soil, and the number of microorganisms, dehydrogenase activity, and intensity of soil respiration in the CTB treatment were considerably improved compared with those in the other treatments. The removal rates of petroleum hydrocarbons were 18.0% and 32.51% higher than those of the TB-BC treatment and the TB treatment, respectively, indicating that the biochar-immobilized bacterial consortium had a synergistic mechanism of bioaugmentation and biostimulation for the removal of petroleum hydrocarbons.

1. Introduction
Petroleum can enter the soil in various manners during its use, and the soil pollution problems caused by petroleum have become increasingly prominent[1]. Therefore, the remediation of petroleum contaminated soil has attracted increased attention [2]. Bioremediation technology will not cause changes in soil structure and secondary pollution and is widely used in the remediation of oil contaminated soils [3]. Due to the slow degradation rate of indigenous microorganisms, it is often necessary to add exogenous microorganisms to accelerate the pollutant degradation [4,5]. However, it is difficult to achieve complete degradation by an individual microbial strain, and the degradation rate tends to decrease over time. The construction of bacteria consortiums can take advantage of the synergistic effects of different strains to obtain a better effect on degradation of petroleum hydrocarbons, and its degradation performance can be significantly higher than that of individual bacteria [6-8].

However, most of the exogenous degrading bacteria entering the soil in the form of free bacteria are susceptible to changes in the soil microenvironment and pollutants themselves, and it is not easy for them to colonize; thus, it is difficult to achieve the expected remediation results. In addition, petroleum
hydrocarbons have strong hydrophobicity, which cannot be directly used by microorganisms. Hence, the bioavailability of petroleum hydrocarbons is reduced, and biological toxicity is easily produced. A microbial immobilization technology enables microorganisms to proliferate rapidly and maintains biological activity under suitable conditions by immobilizing microorganisms on corresponding carriers [9,10]. The selection of a suitable immobilization carrier can also have a certain water retention effect on the soil, increase the content of soil nutrients, and enhance the utilization of organic matter, total nitrogen, and available phosphorus by microorganisms. For example, adding biochar to contaminated soil can achieve the rapid adsorption and desorption of pollutants, change the physicochemical properties of the soil, help improve the bioavailability of pollutants, and promote the biodegradation of pollutants [11].

The purpose of this study is 1) to investigate the removal characteristics of petroleum hydrocarbons using three strains of petroleum-hydrocarbon-degrading bacteria; and 2) to evaluate the effect on the remediation of oil contaminated soil by different microbial addition methods: a free bacterial consortium (TB), a biochar-free bacterial consortium (TB-BC), and a biochar immobilized bacterial consortium (CTB). This research was expected to provide a scientific basis for the application of related strains to oil contaminated soil.

2. Materials and methods

2.1. Soil, pollutants and bacteria

The pristine soil was collected in Zhangwu, Liaoning, China, from unpolluted 20-40 cm soil layer, then air-dried and passed through a 2-mm sieve prior to use. The properties of the soil were as follows: pH 8.22, 0.75% organic mater, 0.019% total nitrogen, 0.029% total phosphorus, a particle size distribution of 13.7% sand, 56.5% fine, 29.8% clay.

The soil was artificially polluted with crude oil at a final concentration of 44.32 g/kg. The crude oil used was from Daqing oilfield with a density of 0.858 g/cm3 (20°C), a freezing point of 25.6°C, and a viscosity of 20.9 mPa·s (50°C). The proportion of alkanes, aromatics, and resin asphaltenes in the petroleum were 60.5%, 33.5%, and 6.0%, respectively.

Three bacterial strains (labelled as A2, A4, and L5) and their bacterial consortiums (labelled as C) were used as the experimental bacteria. The bacteria were previously screened from the oily sludge and were identified to be Pseudomonas putida, Acinetobacter calcoaceticus, and Sphingomonas sp., respectively by 16S rDNA sequence analysis.

2.2. Experimental method

2.2.1. Crude oil degradation experiment. The bacterial suspensions of the three strains of individual bacteria were mixed according to the optimal inoculation ratio under the orthogonal optimization conditions to obtain a bacterial consortium agent (C) which consisted of 33.77% of A2, 46.12% of A4 and 20.11% of L5. Then, 2 ml suspension was activated to the logarithmic growth phase (the same biomass, approximately $3.23 \times 10^7$ cfu/ml), and was inoculated into 100 mL of inorganic salt medium containing 0.5% petroleum hydrocarbons. The inorganic salt medium consisted of (g/L): MgSO$_4$·7H$_2$O 0.5, CaCl$_2$ 0.02, KH$_2$PO$_4$ 1.0, NH$_4$Cl 2.0, FeCl$_3$ 0.05. The pH was adjusted to 7.0. The bacteria were cultivated on a constant-temperature shaker at 30°C and 180 r/min for 28 days, and the 100 mL of uninoculated inorganic salt medium containing 0.5% petroleum hydrocarbon was used as a control. Three replications of each treatment were performed.

2.2.2. Preparation of the immobilized bacteria. The adsorption immobilization method was adopted. 2.0 g of biochar with a particle size of 0.25 mm was used as a carrier and added to 100 ml of Lysogeny broth (LB) medium (peptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar powder 15 g/L, pH = 7.2) after autoclaving. In addition, 6 ml of the bacterial consortium suspension was inoculated. The bacterium was cultivated at 30°C and 150 r/min for 36 h. The bacterial cells obtained by
centrifugation were washed twice with physiological saline solution to obtain the immobilized bacterial agents.

2.2.3. The oil contaminated soil remediation experiment. Pot simulation experiments were used. 1 kg of sterilized oil contaminated soil was added into a pot (diameter 18 cm; height 25 cm), and the soil C:N:P mass ratio was adjusted to 100:10:1 with KH₂PO₄ and NaNO₃. Five treatments were conducted: sterilized oil contaminated soil (CK), soil added with biochar (BC), soil added with free bacterial consortium (TB), soil added with immobilized bacteria (CTB), and soil added with free bacterial consortium-biochar (TB-BC). Among them, the TB, CTB, and TB-BC treatments contained the same biomass (approximately 5.73 × 10⁷ cfu/g), the amount of biochar added for each treatment with additions of biochar was the same, and three parallels were set for each treatment. During the remediation process, the weight method was used to adjust the moisture content of each treatment to maintain it at approximately 30%. Sampling was conducted every 7 days to determine the intensity of soil respiration, the number of microorganisms, the activity of soil dehydrogenase, and the contents of petroleum hydrocarbons and their components. The experimental period was 28 days.

2.3. Index determination

2.3.1. Biomass. The biomass was determined using the gradient dilutions of soil suspensions in sterilized water. 10 g of wet soil was suspended in 10 ml of sterile water and agitated for 1 min, then the suspension was plated on LB plates and incubated at 30℃ for 3 days.

2.3.2. Intensity of soil respiration. The intensity of soil respiration was measured using the absorption method. After 10 g of fresh soil was added in a 200 mL beaker, a small beaker containing 5 mL of NaOH (2 mol/L) solution was inserted, and the system was sealed and cultivated at 30℃ for 24 h. Subsequently, the solution was transferred to a volumetric flask and then diluted to the mark. With phenolphthalein and methyl orange used as indicators, the solution was titrated with a 0.1 mol/L HCl solution, and the amount of CO₂ [mg/(kg ⋅ h)] produced by soil respiration was calculated based on the consumption.

2.3.3. Soil dehydrogenase activity. After 5 g of soil was mixed with 2 mL of Tris-HCl buffer (pH 7.6), 2 mL of 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution, and 2 mL of distilled water, the mixture was shaken sufficiently and cultivated at 37℃ in the dark for 24 hours. After the cultivation, 5 mL of methanol was added to terminate the reaction, and the mixture was vigorously shaken for 1 min. The supernatant solution was collected, and the absorbance was measured at 485 nm. TPF (1,3,5-triphenylformazan) produced per gram of dry soil was used as an activity unit of dehydrogenase, and the result was calculated according to the TPF standard curve.

2.3.4. The total petroleum hydrocarbon (TPH) content. The soil sample was air-dried and passed through a 40-mesh sieve, 10 g of the soil sample was mixed with 30 mL of dichloromethane, gently shaken for 1 min, and ultrasonically extracted for 30 min, then centrifuged at 8000 rpm for 2 min. The supernatant was filtered and dehydrated with anhydrous sodium sulfate. The precipitate was extracted three more times, and the extracts were combined. The TPH content was determined gravimetrically after evaporation of the solvent.

2.3.5. Separation and determination of the petroleum components. The separation and determination of the petroleum components was based on the “Analysis Method for Family Composition of Rock Extract and Crude Oil” SY/T5119-2016 [12]. The content of each component was determined using infrared spectrophotometry.
3. Results and discussion

3.1. Degradation performance of petroleum-degrading bacteria on crude oil

As shown in Fig. 1, all three strains could grow with petroleum hydrocarbons as the sole carbon source and energy source. The petroleum hydrocarbon degradation rates of strains A2, A4, L5, and C after 28 days of cultivation were 42.8%, 48.01%, 26.56%, and 81.07%, respectively. According to the analysis of the removal rates of the different petroleum components (Fig. 2), it can be seen that strains A2 and A4 primarily degraded the saturated hydrocarbons, and strain L5 showed a higher degradation ability on aromatic hydrocarbons than A2 and A4. The bacterial consortium degraded each petroleum component more evenly, and the removal rates of the saturated hydrocarbons and aromatic hydrocarbons reached 89.93% and 82.08%, respectively. This phenomenon may be related to the selective degradation of different petroleum components by different strains. For example, *Pseudomonas* species have been proved to be able to utilize various petroleum components, while the degradation abilities of *Acinetobacter* species on petroleum components are in the order of alkanes > cycloalkanes > aromatic hydrocarbons [13]. In addition, *Sphingomonas* is considered to have a high-efficiency degradation ability on a variety of aromatic compounds [14]. The combination of the three strains of bacteria can achieve the effect of the synergistic removal of petroleum hydrocarbons, which is beneficial to improving the removal rate of petroleum hydrocarbons.

![Fig. 1 Degradation rate of crude oil](image1.png)

![Fig. 2 Removal rates of different components of petroleum hydrocarbon](image2.png)

3.2. Changes in bacterial counts

After the remediation, the counts of bacteria in each treatment was shown in Fig. 3. The bacteria of each treatment were effectively colonized in the contaminated soil. In CTB treatment the number of microorganisms was 7.2×10^8 CFU/g dry soil, which was increased by an order of magnitude compared to the initial counts. The number of microorganisms in the TB treatment was reduced by an order of magnitude compared with the initial counts, and the number in the TB-BC treatment was slightly lower than the initial counts. The above results suggested that the addition of biochar was favorable for the colonization of degrading bacteria in the soil, and addition of the bacteria using an immobilization carrier was the most favorable to increasing the number of degrading bacteria. This may have been due to the fact that as a carrier biochar provides a suitable microenvironment for degrading bacteria, which reduces the toxic effect of intermediate products of petroleum hydrocarbon degradation on microbial cells [15], thereby being conductive to the colonization of the degrading strains.
3.3. Intensity of soil respiration and dehydrogenase activity

Soil microbial activity is the primary source of soil respiration, and determining the intensity of soil respiration can measure the total activity of soil microorganisms. Dehydrogenase is considered a type of biocatalyst and can effectively promote the biological decomposition of organic matter in soil. During the degradation process of petroleum pollutants, hydrogen atoms can be activated by dehydrogenase and transferred to specific hydrogen acceptors to realize the oxidative decomposition of petroleum pollutants; thus, the effect of biodegradation on petroleum pollutants can be reflected by determining the dehydrogenase activity of the soil. As shown in Fig. 4 and Fig. 5, the intensity of soil respiration and dehydrogenase activity both increased constantly. In the early stage of remediation, the number of exogenous degrading bacteria increased rapidly after a short adaptation period, the growth and metabolism were vigorous. Accordingly, the respiratory intensity and soil dehydrogenase activity were also greatly improved. The number of microorganisms, intensity of soil respiration, and dehydrogenase activity in the CTB and TB-BC treatments were significantly higher than those in the TB treatment, indicating that the addition of biochar could promote soil respiration and microbial activity. This was because the large quantities of nutrients and porous characteristics of the biochar can not only meet the needs for the growth, metabolism, and reproduction of microorganisms, but also shield from the harmful soil environment. In addition, the immobilization method can prevent microorganisms from being exposed to toxic effects from the outside and ensure that the microorganisms have high cell activity, so the ability to secret enzymes is correspondingly high, and the ability of microorganisms to uptake and degrade petroleum hydrocarbons is improved, which was shown in Fig. 6.
3.4. TPH degradation

Fig. 6 shows the TPH degradation extent during the remediation of oil contaminated soil. After the remediation, the average degradation rates of petroleum hydrocarbons in each treatment were in an order of CTB > TB-BC > TB > BC > CK. The average TPH degradation rate in BC test was only 45.81%, while in the crude oil degradation experiment with the same biomass, the degradation rate by the free bacterial consortium was 79.73%. That’s because, the degradation ability of microorganisms on petroleum hydrocarbons was greatly affected by the microenvironment. Comparing the three treatments of TB-BC, TB, and BC, the simple mixing of the bacterial consortium and biochar could improve the removal rate of petroleum hydrocarbons to a certain extent, and the removal rate was higher than the sum of the removal rates of the TB and BC treatments, suggesting that the combined use of TB and BC had a synergistic effect on the removal of petroleum hydrocarbons. The removal rate of petroleum hydrocarbons in the CTB treatment was 78.32%, which was 18.0% or 32.51% higher than that in the TB-BC or the TB treatment, respectively. It was thus inferred that the reason might be that biochar, as an immobilization carrier, can adsorb not only a large number of bacteria, but also petroleum hydrocarbons in the pores of biochar, increasing the contact between degrading bacteria and petroleum hydrocarbons. Additionally, the functional groups, easily decomposable carbon sources, and nitrogen sources on the surface of biochar help to enhance the activity of degrading bacteria, thereby increasing the degradation rate of petroleum hydrocarbons [4].

4. Conclusions

(1) All three petroleum-hydrocarbon-degrading strains could grow with petroleum hydrocarbon as the sole carbon source and energy source. After 28 days of cultivation, the petroleum hydrocarbon degradation rates of A2, A4, L5, and the bacterial consortium were 42.8%, 48.01%, 26.56%, and 81.07%, respectively. Strains A2 and A4 possessed a strong ability to remove saturated hydrocarbons, and L5 possessed a strong ability to remove aromatic hydrocarbons. The bacterial consortium had a synergistic effect on the removal of petroleum hydrocarbons.

(2) The number of microorganisms, soil respiration, and dehydrogenase activity all showed differences under different microbial addition methods, and the values were in an order of CTB > TB-BC > TB. Biochar was beneficial to the colonization of degrading bacteria in the soil. The addition of biochar as an immobilization carrier to the soil was the most favorable for increasing the activity and number of degrading bacteria.

(3) The combined use of biochar and the bacterial consortium had a synergistic effect on the removal of petroleum hydrocarbons. The biochar-immobilized bacterial consortium had the optimal remediation effect on oil contaminated soil, and the removal rate of petroleum hydrocarbons was 78.32% after 28 days of remediation.

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