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Article

The impact of biochar-entrapped microorganisms on enzymatic activity and the cleanup of petroleum hydrocarbons in polluted soil

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Abstract: The high-temperature pyrolysis oxidation process was utilized to produce reed biochar, which was then employed as a carrier for immobilizing the predominant petroleum hydrocarbon-degrading bacteria. The study investigated the effects of this biochar-bound microbial immobilization on the remediation efficacy and enzymatic activity of soil contaminated with petroleum hydrocarbons. The findings indicated that after 40 days of treatment with reed biochar-immobilized microorganisms, the soil's petroleum hydrocarbon removal rate reached 55.01%, markedly surpassing the removal rates of biochar (45.82%) and the untreated control group (24.83%). Additionally, it was observed that the biochar-immobilized microorganisms significantly enhanced the activities of soil enzymes such as dehydrogenase, catalase, urease, and polyphenol oxidase. The application of biochar-bound microbial immobilization technology not only boosts the cleanup efficiency of petroleum hydrocarbon-contaminated soil but also substantially elevates the soil's biological enzyme activity.

Keywords: biochar; immobilized microorganism; petroleum hydrocarbon; enzymatic activity

Ground crude oil is one of the main sources of petroleum hydrocarbon pollution in the soil of oil field production sites [1]. Taking the oil field as an example, the polluted land area of each oil well is generally about 200–500 m^2. If the current 200000 oil wells in China are estimated, the polluted area of petroleum hydrocarbon soil in only the oil field production areas in China can reach 8×10^7 m^2 [2]. The investigation conducted by our research group in Daqing Oilfield and Liaohe Oilfield in Northeast China shows that in the heavily polluted area of the oilfield, the content of soil crude oil is more than 10,000 $mg \cdot kg^{-1}$, which is 20 times the critical value [3]. Crude oil has strong adhesion. After entering the soil environment, it is adsorbed, retained, migrated or decomposed by the soil in physical, chemical and biological ways. It often blocks the soil pores, affects the soil water flow and degrades the soil biological and chemical properties [4]. The components of petroleum hydrocarbons that have not been degraded and absorbed by soil enter the water environment through rainfall scouring, which directly leads to water environment pollution. In addition, petroleum hydrocarbon components or their degradation products enter animals and plants through the food chain, directly affecting food safety and ultimately threatening human health [5,6].

Petroleum hydrocarbon pollutants have stable chemical properties, strong persistence and difficult degradation in soil environment. These characteristics determine that it is difficult to repair their pollution. At present, the remediation technologies of petroleum hydrocarbon contaminated soil mainly include physical remediation, chemical remediation and biological remediation [7–9]. Among them, bioremediation technology has attracted the attention of environmental protection workers because of its advantages of green environmental protection, no secondary pollution and low input cost [10–12]. However, when free microorganisms are added to polluted soil, they need to adapt to new environmental conditions, and they compete with indigenous microorganisms in the soil, which makes it difficult for them to play a normal role in the biodegradation of petroleum hydrocarbons, often resulting in poor remediation effect.

Biochar has the advantages of developed pore structure, large specific surface area and rich active functional groups. It is widely used in the field of environmental pollution control because of its wide source of raw materials and relatively low cost. Using biochar as a carrier to immobilize microorganisms can not only effectively increase the number of microorganisms per unit volume or weight, but also improve microbial activity and long-term effectiveness. Biochar can protect the environment from dominant degrading microorganisms in polluted soil, reduce the damage of toxic substances, and better play the role of biodegradation of pollutants [13]. In the process of fixing petroleum hydrocarbon contaminated soil by immobilized microorganisms, microorganisms biodegrade petroleum hydrocarbon pollutants, and the immobilized carrier can also adsorb some petroleum hydrocarbon pollutants [14]. Therefore, the selection of microbial immobilized carriers with strong adsorption capacity can effectively improve the removal effect of petroleum hydrocarbon pollutants. As a green immobilized carrier, biochar can adsorb petroleum hydrocarbons in soil and prevent the diffusion of pollutants to water and atmospheric environment. On the other hand, biochar can increase the contact area between microorganisms and pollutants by adsorbing petroleum hydrocarbon pollutants into the body, so as to promote the biodegradation of petroleum hydrocarbon pollutants [15]. Wang et al. [16] conducted a remediation test on petroleum hydrocarbon contaminated soil in Karamay oil field, which showed that after 63 days of remediation test using immobilized microbial technology, it was found that the oil removal rate of immobilized microbiome could reach 78.7%, which was 49.5% higher than that of the control group, and the toxicity of contaminated soil was significantly weakened after microbial remediation.

Therefore, the petroleum hydrocarbon contaminated soil in Liaohe oil field is selected as the research object in this study, and reed biochar is used as the immobilization material of petroleum hydrocarbon dominant degrading bacteria to study the influence of biochar immobilized microorganisms on the enzyme activity and remediation effect of petroleum hydrocarbon contaminated soil, so as to provide technical support for improving the bioremediation efficiency of petroleum hydrocarbon contaminated soil.

1. Materials and methods

1.1. Test soil samples

The test soil was collected from the oil production area of Liaohe Oilfield, Panjin City, Liaoning Province. Place the collected soil sample in a dark place for natural air drying, grind it, sift it through 10 meshes, and collect it in a fresh-keeping box for sealed storage. Soil porosity is 39.51% and water holding capacity is 12.14% [17]. The content of total petroleum hydrocarbons (TPHS) in soil samples was determined by ultrasonic extraction gravimetric method [18], and its content was 9.62 $g \cdot kg^{-1}$. The soil ph is 8.92, and the organic carbon content is 22.43 $g \cdot kg^{-1}$ [19].

1.2. Preparation of reed biochar

Reed biochar was prepared by pyrolysis and oxidation at high temperature. Remove impurities from reed biomass materials, wash them with deionized water to remove adhesive substances, cut them into 3–5 cm segments after natural air drying, and dry them in a 60 ℃ oven for 3 hours. After crushing, they are sieved and sealed for storage. Put the treated reed material into the crucible, compact it, place the cover in the atmosphere furnace, vacuum it, connect the water pump, heat it up to 500 ℃ at the rate of 5 ℃ min^{-1}, and conduct pyrolysis at constant temperature for 3 hours to obtain reed biochar. The yield of biochar was 34.62% and the content of C was 80.14%.

1.3. Preparation method of biochar immobilized microorganisms

In this experiment, biochar was prepared by adsorption method to immobilize microorganisms, and the prepared reed biochar was used as the carrier for microbial immobilization. Select 3 g of mixed microbial agent for petroleum hydrocarbon degradation (composition: actinomycetes 47.30%, campylobacter viridis 19.83%, proteus bacteria 14.67%, acidobacteria 7.83%) to be awake for 48 hours, take 15 g of biochar and inoculate and mix it with the bacterial solution after awakening, and place it in a constant temperature oscillator at 32 ℃ and 130 r/min to mix it for 24 hours, then you can get a biochar immobilized microorganism with an inoculation amount of 2% (expressed by the ratio of petroleum hydrocarbon degrading bacteria/biochar), the quantity of microorganism inoculation shall be determined according to the design requirements of experimental treatment.

1.4. Experimental design of bioremediation of petroleum hydrocarbon contaminated soil by biochar immobilized microorganisms

There are three types of experiments, as shown in **Table 1**. Among them, ta was the control treatment, reed biochar was treated with TB, and reed biochar immobilized microorganism was treated with TC. Each treatment was repeated for 3 times. The petroleum hydrocarbon contaminated soil was repaired for 40 days.

Table 1. Experimental treatments.

Processing group	Treatment method	Biochar content /%	Immobilized microorganism content /%
Ta	Nothing	0	0
Tb	Reed biochar	10	0
Tc	Immobilized microorganisms on reed biochar	10	2

1.5. Experimental design of effect of biochar immobilized microorganisms on enzyme activity in petroleum hydrocarbon contaminated soil

In order to analyze the effect of inoculum biomass and biochar particle size on petroleum.

The enhanced remediation effect of hydrocarbon contaminated soil, and the remediation parameters of petroleum hydrocarbon contaminated soil in Liaohe Oilfield by immobilized microorganisms of reed biochar are further optimized. The experimental treatment scheme is set as shown in **Table 2**. The activities of soil dehydrogenase, catalase, urease and polyphenol oxidase were measured on the 30th day of remediation experiment, and the removal rate of petroleum hydrocarbons was measured on the 60th day.

1.6. Determination of TPHS content in soil

The content of TPHS in soil was determined by ultrasonic extraction gravimetric method [20]. Put 10 g of soil sample into a 50 ml centrifuge tube, add 20 ml of methylene chloride solvent, conduct ultrasonic extraction at 60 W and 30 °C for 15 min, centrifuge at 4000 R · min^{-1} for 10 min, and pour the supernatant into a 100 ml conical flask. Repeat this step three times. Evaporate the total petroleum hydrocarbon solution in the conical flask in a water bath at 54 °C, bake it at 54 °C until all the solvent volatilizes, weigh it, and calculate the TPHS content.

1.7. Determination of soil enzyme activity

In this study, 2,3,5-triphenyltetrazolium chloride (TCC) spectrophotometry was used to determine the content of soil desorbed water

Table 2. Experimental treatments.

Processing group	Soil volume/g	Carbon content/g	Particle size/mesh	Bacterial dosage/%
T1	150	15	1.5	1
T2	150	15	1.5	2
T3	150	15	1.5	3
T4	150	15	10	1
T5	150	15	10	2
T6	150	15	10	3
T7	150	15	60	1
T8	150	15	60	2
T9	150	15	60	3
T10	150	-	-	0

Hydrogenase activity; the activity of catalase was determined by potassium permanganate titration; urease activity was determined by indophenol blue colorimetry; polyphenol oxidase activity was determined by pyrogallol colorimetry [21,22].

1.8. Scanning electron microscope determination of immobilized microorganisms in biochar

The scanning electron microscope (SEM) of biochar immobilized microorganisms was determined by Hitachi s-4200 scanning electron microscope of Japan Electronics Co., ltd.

2. Results and discussion

2.1. SEM morphology analysis of biochar and immobilized microorganisms

The SEM results of the prepared samples are shown in **Figure 1**.

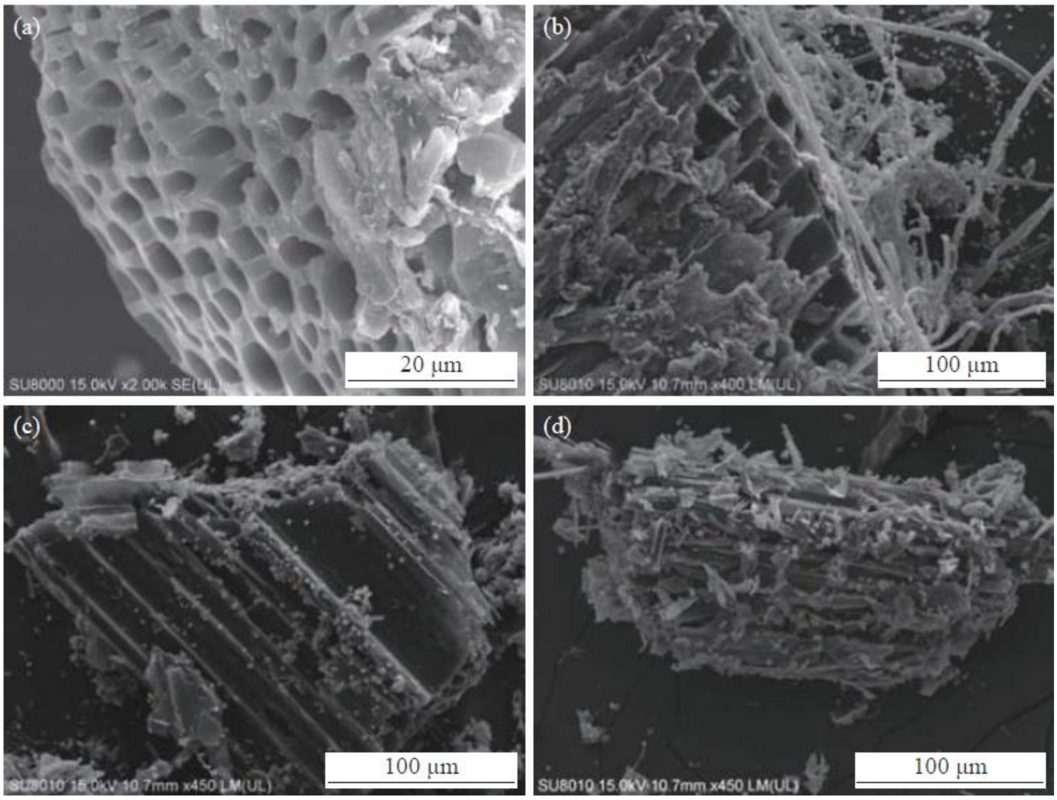

Figure 1. SEM images. **(a)** Reed biochar; **(b)** segmental reed biochar immobilized microorganisms; **(c)** immobilized microorganisms on granular reed biochar; **(d)** immobilized microorganisms on 60 mesh reed biochar.

Figure 1a shows that the prepared reed straw biochar has an obvious tubular structure, and the pore structure of the cross section is honeycomb, closely arranged and irregular, with different sizes and a certain depth. **Figure 1b** shows the immobilized microorganism of 1 cm segmental reed biochar, with clear and complete microbial morphology, and the main immobilized area is porous structure. **Figure 1c** shows the immobilized microorganisms of granular reed biochar. The reed biochar has obvious pore structure, but the edge may be broken during the screening process. No complete microbial appearance is found on the surface of reed biochar, and some broken microorganisms are scattered on the surface. **Figure 1d** shows the immobilized microorganisms of reed biochar passing 60 mesh sieves. There are fine biochar residues on the surface of reed biochar and attached with cataclastic microorganisms. The SEM results show that the segmented biochar has a better protective effect on microorganisms when immobilizing microorganisms, so that it can completely retain the structural characteristics of microorganisms, and provide good remediation conditions for petroleum hydrocarbon contaminated soil.

2.2. Bioremediation of petroleum hydrocarbon contaminated soil by biochar immobilized microorganisms

The removal effect of TPHS in the soil of the oil production area is shown in **Figure 2**.

At the initial stage of remediation, the remediation efficiency of each treatment group gradually showed differences. The removal rate of TPHS in TA treatment group increased slowly. The removal rate of soil TPHS in TB and TC treatment groups increased significantly in the first 20 days, and still showed an upward trend in the later stage of remediation. Through the 40 day remediation test, the mass fraction of soil TPHS in TA treatment group decreased from the initial value of 9.62 $g \cdot kg^{-1}$ to 7.23 $g \cdot kg^{-1}$, and the total petroleum hydrocarbon removal rate was 24.83%; in TB treatment, the concentration of TPHS decreased to 5.21 $g \cdot kg^{-1}$, and the removal rate was 45.82%, which was significantly higher than that in TA treatment. The concentration of TPHS in TC treatment group decreased to 4.33 $g \cdot kg^{-1}$, and the removal rate was 55.01%, which was 2.2 times that of TA treatment. The results show that biochar has obvious removal effect on petroleum hydrocarbon pollutants in contaminated soil, and biochar immobilized microorganisms can effectively enhance the remediation efficiency of petroleum hydrocarbon contaminated soil.

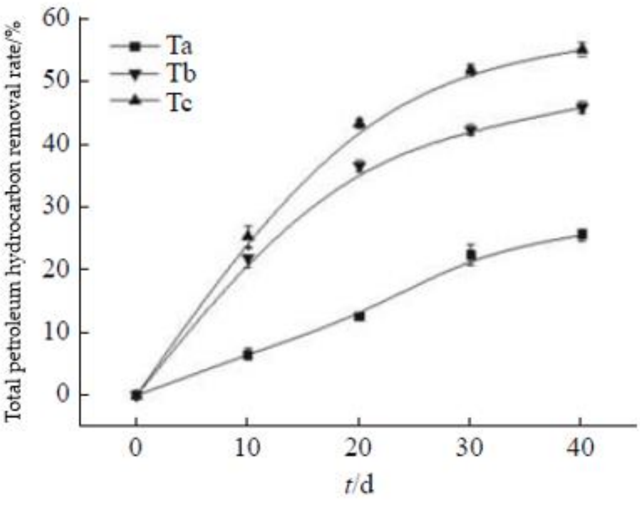

Figure 2. Removal rate of tphs in soil during different treatment.

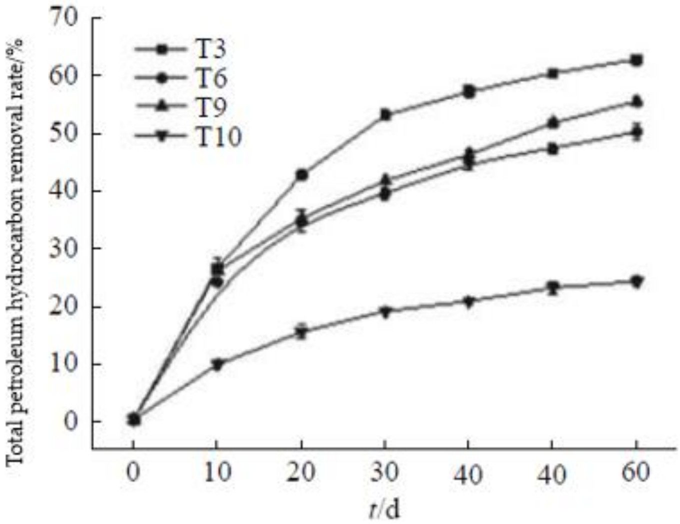

Figure 3. Removal rate of tphs in soil during different period.

2.3. Effect of biochar particle size and microbial inoculum on remediation of petroleum hydrocarbon contaminated soil

2.3.1. Different effects of biochar particle size on the remediation effect of petroleum hydrocarbon contaminated soil

The total petroleum hydrocarbon removal rate during the repair period is shown in **Figure 3**. In the 60 day remediation process, with the extension of remediation time, compared with the control (T10) treatment and the reed biochar immobilized microbial remediation treatment (t1–t9), the addition of reed biochar immobilized microbial treatment significantly improved the remediation efficiency of petroleum hydrocarbon pollutants in soil, which is related to the adsorption of petroleum hydrocarbons by biochar And the improvement of soil properties and the promotion of remediation by dominant petroleum hydrocarbon degrading bacteria. Under the condition that the microbial inoculation amount was all 3%, the remediation effect of 1 cm segment reed biochar immobilized microbial treatment group (T3) was the best after 60 days, and the total petroleum hydrocarbon removal rate was 62.59%, the total petroleum hydrocarbon removal rate of granular reed biochar treatment group (T6) was 50.03%, and the total petroleum hydrocarbon removal rate of powdered reed biochar treatment group (T9) was 54.35%.

Based on the SEM analysis of immobilized microorganisms of reed biochar in **Figure 1**, the reason for the difference in the above remediation effects may be related to the better protection of 1 cm segment biochar against microorganisms when immobilized microorganisms. After the segmental reed biochar is applied to the petroleum hydrocarbon contaminated soil in the exploitation area of Liaohe Oilfield, it can enhance the permeability of the soil, better provide a suitable breeding space for microorganisms in the soil, promote the biological transformation of nutrients in the soil environment, and effectively improve the physical and chemical properties of the petroleum hydrocarbon contaminated soil in Liaohe Oilfield. SEM observation shows that there is no significant difference between T6 and T9 in terms of microbial immobilization effect, but the removal effect of petroleum hydrocarbon pollutants in T9 soil is higher than that in T6 soil sample.

This may be because the powdered biochar is more evenly mixed with the soil, which improves the soil properties as a whole, thus enhancing the removal effect of petroleum hydrocarbons in the soil. The above remediation mechanism needs to be further explored and clarified in the follow-up tests.

2.3.2. Effect of microbial inoculation amount on remediation effect of petroleum hydrocarbon contaminated soil

Taking the 1 cm segmental reed biochar immobilized microbial treatment (T1, t2, t3) as an example, the removal effect of different inoculants (1%, 2%, 3%) of reed biochar immobilized on petroleum hydrocarbon pollutants in soil was investigated. The experimental results are shown in **Figure 4**.

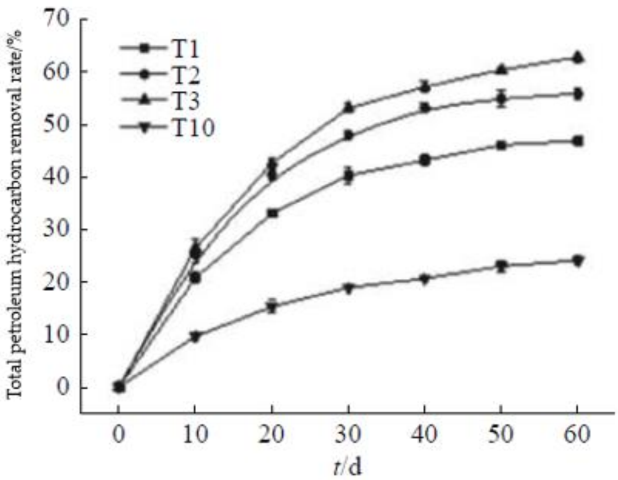

Figure 4. Removal rate of tphs in soil during different period.

The removal efficiency of TPHS in soil by immobilized microorganisms of reed biochar increased with the increase of microbial inoculation. When the microbial inoculation amount increased from 1% to 2%, the removal rate of soil TPHS increased significantly from 46.70% to 55.68%. When the microbial inoculation amount was 3%, the removal rate of soil TPHS increased to 62.59%, but the growth rate of TPHS removal rate decreased. This may be because in a certain soil environment, with the proliferation of immobilized microorganisms, the density of microbial flora in the unit soil increases, and the competition between oxygen and nutrients required for microbial growth and metabolism is fierce, which limits the growth of microorganisms. It is necessary to optimize the regulation of soil nutrition in the process of remediation, so as to create a good nutritional state for microorganisms to give full play to the role of remediation.

2.4. Effect of biochar immobilized microorganisms on enzyme activity in petroleum hydrocarbon contaminated soil

2.4.1. Dehydrogenase activity

Oxidative decomposition plays an important role in the remediation of petroleum hydrocarbon contaminated soil

The prerequisite and important role of microorganisms in the successful decomposition and utilization of petroleum hydrocarbons. Soil dehydrogenase can improve the activity of hydrogen atom, make it combine with hydrogen acceptor successfully, and then promote the degradation of petroleum hydrocarbons. The change results of soil dehydrogenase activity with time during the remediation process are shown in **Figure 5**.

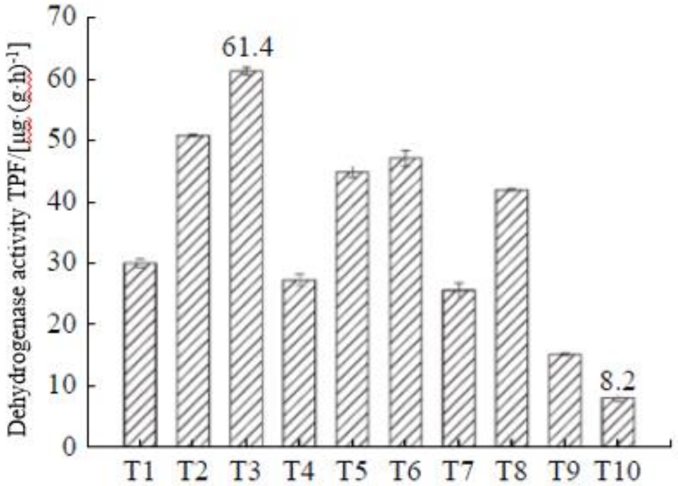

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Figure 5. Variation of catalase activity in soil.

It can be seen from **Figure 5** that the dehydrogenase activity in the soil of each treatment (t1-t9) added with reed biochar immobilized microorganisms was higher than that of the control group (T10), and the dehydrogenase activity of T3 treatment was the highest, reaching 61.4 μ g/(g h), while the soil dehydrogenase activity of the control group was only 8.2 μ g/(g·h). The dehydrogenase activity in the soil treated with T3 is more than 7 times that of T10, which may be related to the reduction of the invasion of toxic and harmful substances such as petroleum hydrocarbons in the soil by microorganisms fixed with reed biochar, and the increase of microbial activity [23], thereby increasing the dehydrogenase activity. The results showed that biochar immobilized microorganisms could significantly enhance the activity of dehydrogenase in soil, and then enhance the oxidative decomposition process of petroleum hydrocarbon pollutants in soil.

2.4.2. Catalase activity

Catalase can promote the catalytic decomposition of hydrogen peroxide and can be used to characterize the strength of soil biochemical oxidation process. Good respiration is the premise and guarantee for microorganisms to successfully decompose and utilize petroleum hydrocarbons. The adhesion of petroleum hydrocarbons often leads to the decrease of soil permeability and the inhibition of microbial respiration. Excessive hydrogen peroxide content will hinder microbial respiration. The change of catalase activity during the repair process is shown in **Figure 6**.

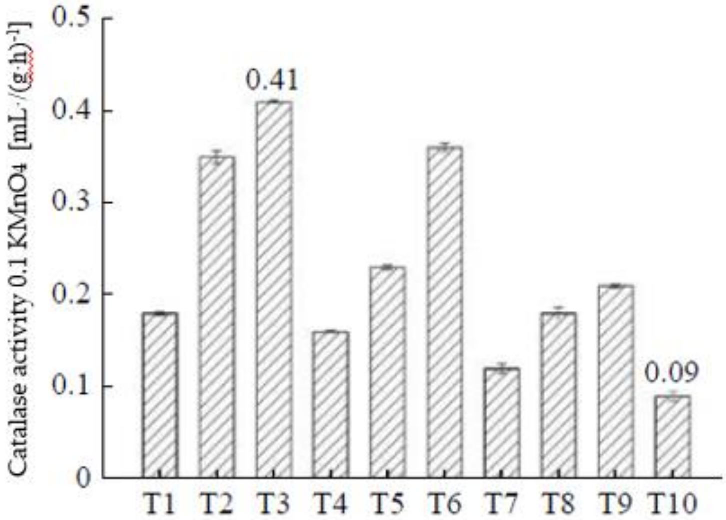

Figure 6. Variation of catalase activity in soil.

It can be seen from **Figure 6** that the addition of reed biochar immobilized microorganisms can effectively improve the catalase activity in petroleum hydrocarbon contaminated soil. The catalase activity of T3 treatment soil is the highest, 4.5 times that of the control group (T10), indicating that reed biochar immobilized microorganisms can effectively reduce the toxicity of hydrogen peroxide to microorganisms in petroleum hydrocarbon contaminated soil, which is more conducive to the survival of microorganisms in soil [24]. Catalase activity in soil is closely related to organic matter and nitrogen in soil. Studies have shown that there is a very significant positive correlation between catalase activity and soil organic matter content and soil total nitrogen [25]. Graham et al. [26] found in the test that in the soil polluted by petroleum hydrocarbons, when the optimal ratio of carbon, nitrogen and phosphorus in the soil is 100:15:1, microorganisms have a better effect on the degradation of petroleum hydrocarbons. In the control treatment (T10), the catalase activity was low, which may be due to the imbalance of the proportion of nutrients ($C:n:P$) in the petroleum hydrocarbon contaminated soil, which is not conducive to the growth of microorganisms. Regulating the proportion of nutrients in the contaminated soil is an important measure to improve the remediation efficiency.

2.4.3. Urease activity

Urease is directly involved in the transformation of organic nitrogen into alkali hydrolyzed nitrogen in soil. It is an amidase related to the hydrolysis of carbon nitrogen bond (CO-NH) in pollutants. It plays an important role in soil nitrogen cycle. Soil urease activity can be used to characterize the utilization efficiency of microorganisms for alkali hydrolyzed nitrogen in soil, so it can reflect the metabolic degree of microorganisms for petroleum hydrocarbon pollutants to a certain extent. Urease in soil is closely related to the transformation ability of nitrogen. The greater the urease activity, the higher the transformation rate of soil nitrogen. Zhang et al. Also showed that urease activity in petroleum hydrocarbon contaminated soil has a great correlation with the removal rate of total petroleum hydrocarbons [27]. **Figure 7** shows the soil urease activity in each treatment group.

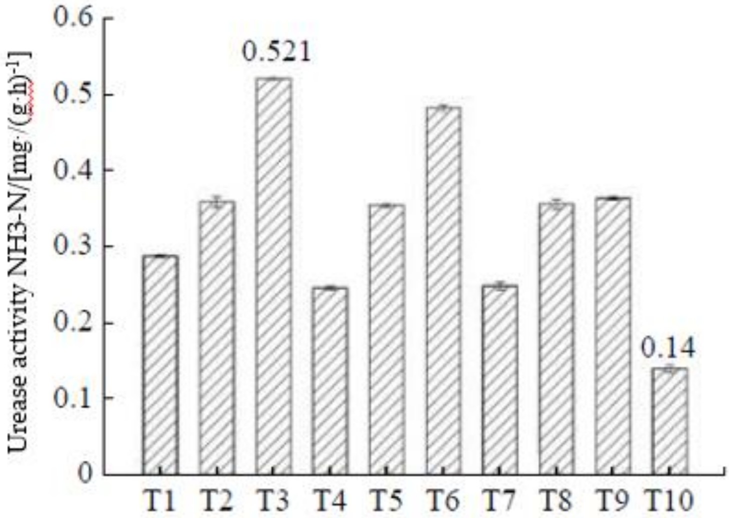

Figure 7. Variation of catalase activity in soil.

It can be seen from **Figure 7** that the soil urease activity in the reed biochar immobilized microorganism treatment (t1t9) is significantly higher than that in the control group (T10), which indicates that after adding the reed biochar immobilized microorganism to the petroleum hydrocarbon contaminated soil, the biological transformation of nitrogen in the soil environment is more active. The results also showed that soil urease activity increased with the increase of inoculum amount. In the treatment of 1 cm segment reed biochar immobilized microorganisms, the sequence of urease activity was t3 > t2 > t1, which may be that reed biochar provided a suitable environment for immobilized microorganisms, making it more active and metabolic than non-immobilized microorganisms in petroleum hydrocarbon contaminated soil.

2.4.4. Polyphenol oxidase activity

During the degradation of petroleum hydrocarbon pollutants, phenols will be produced, which will promote soil microorganisms to secrete polyphenol oxidase, resulting in a significant increase in polyphenol oxidase activity. Therefore, in the process of petroleum hydrocarbon contaminated soil remediation, the more polyphenol oxidase secreted by microorganisms, the better the degradation effect of petroleum hydrocarbons. **Figure 8** shows the activity of soil polyphenol oxidase in each treatment group.

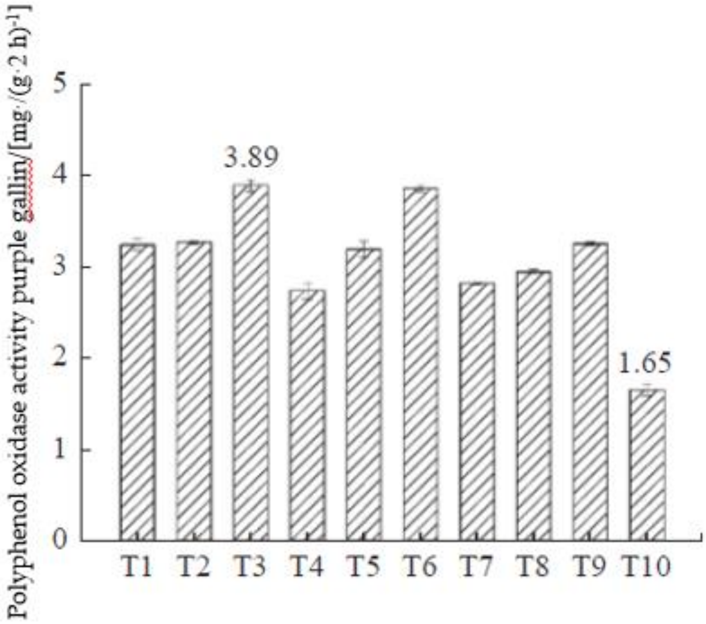

Figure 8. Variation of catalase activity in soil.

It can be seen from **Figure 8** that after 40 days of remediation of petroleum hydrocarbon contaminated soil, the polyphenol oxidase activity in T3 treatment soil is the highest, about 5 times that of the control treatment soil, indicating that T3 treatment soil has higher microbial activity, which makes it secrete more polyphenol oxidase. This result is consistent with the better petroleum hydrocarbon remediation effect of T3 treatment.

3. Conclusion

(1) Biochar has a good remediation effect on petroleum hydrocarbon pollutants in contaminated soil, and biochar immobilized microorganisms can effectively enhance the remediation efficiency of petroleum hydrocarbon contaminated soil.

(2) The shape of biochar has a significant effect on petroleum hydrocarbon contaminated soil. The removal rate of petroleum hydrocarbon pollutants by segmental reed biochar immobilized microorganisms was higher than that by granular reed biochar immobilized microorganisms.

(3) Biochar immobilized microorganisms can effectively improve the biological activity of enzymes in petroleum hydrocarbon contaminated soil. The activities of dehydrogenase, catalase, urease and polyphenol oxidase in soil were significantly increased by the treatment of segmental reed biochar immobilized microorganisms, and the growth and metabolism of microorganisms were promoted.

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