Determination of the best conditions for modified biochar immobilized petroleum hydrocarbon degradation microorganism by orthogonal test

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Abstract. Modified wheat biochar and modified peanut shell biochar is immobilized carrier, studying its physical and chemical properties, and preparing immobilized microorganisms by adsorption method. The optimum conditions for the preparation of immobilized microorganisms were determined by orthogonal experiment. The effects of biochar types, adsorption time, the amount of biochar and the rotating speed on the amount of bacteria were studied. The results showed the physical and chemical properties of the two modified biochar were beneficial to the adsorption of microorganisms, and when the volume ratio of bacteria liquid and modified peanut shell biochar was 20:1, stereo adsorption for 2 hours that the optimum immobilization rate was 72.38%.

1. Introduction

A large amount of petroleum leads to the destruction of soil structure, the reduction of soil permeability, the damage to soil microbes, the reduction of available nitrogen and phosphorus in soil which causes a decrease in soil fertility. In addition, petroleum hydrocarbons and polycyclic aromatic hydrocarbons enter the body through the food chain (Yin et al. 2011), which threatens human health seriously.

The use of biochar to repair contaminated soil is a new research direction in recent years. Biomass materials (such as wood waste, animal manure, crop waste, sludge) are pyrolyzed under anoxic or anaerobic conditions to produce biochar (Grossman et al. 2010). Pietikainen (Pietikainen et al. 2000) have found that biochar was alkaline, which could increase the rate of respiration and metabolism of microorganisms, influence and improve the utilization of substrate, and improve soil fertility. Graber (Graber et al. 2010) have found that biochar can provide favorable conditions for microbial growth and increase microbial biomass. Zhu (Zhu et al. 2014) found that biochar made from wheat straw can effectively adsorb polycyclic aromatic hydrocarbons in petroleum contaminated soil and obtain better remediation effects. Biochar can adsorb microorganisms, with larger specific surface area and porous properties that can provide adhesion sites for microorganisms. Some researchers found that biochar can play a role in the adsorption and inhibition of soil PAHs. White rot fungi can adsorb and degrade PAHs, and the adsorbed PAHs on biochar can be biodegraded by immobilized white rot fungi. However, the specific surface area of biomass carbon is much smaller than that of activated carbon, and the adsorption capacity is limited. Cui et al. (Cui et al. 2016) found that the introduction of MgCl₂ significantly increased the specific surface area of biochar (116.2m²·g⁻¹). And the incorporation of alginate/chitosan and the modification of magnesium improve the adsorption of phosphate on biochar.

At present, there are more reports on the use of biochar adsorption as a stimulus to repair contaminated soil at home and abroad (Khaled et al.2011, Meynet et al.2014, Chen et al.2011, Zhang et
al. 2014), but the research of biochar immobilized microbial is less. Therefore, it is valuable to improve the specific surface area of biochar, and magnesium chloride was used to modify biochar to increase its specific surface area. Orthogonal test was used to determine the optimal parameters of immobilization.

2. Experimental part

2.1 Experimental strains
The experimental strains are prescreened hydrocarbon degrading bacteria H2 and H4. H2 was identified as Bacillus, and H4 was Bacillus.

2.2 Preparation of biochar
2.2.1 Raw materials. Crop waste materials, wheat straw and peanut shells are cleaned, dried and trimmed to 0.5 cm fragments.

2.2.2 Preparation of magnesium modified biological carbon. The wheat straw and peanut shell are washed and dried, then cut to below 0.5 cm. Wheat stalk biochar (WB) and peanut shell biochar (PB) were obtained by pyrolysis of 3 h at a temperature of 400°C in a tube furnace with a certain amount of wheat straw and peanut charcoal and were milled in 80 mesh sieves, then placed in a dry dish. Some of the WB and PB were soaked in 1 mol L⁻¹ MgCl₂ solution (the ratio of magnesium to carbon was 0.24: 1) and dried at 105°C, then magnesium modified wheat straw carbon (M-WB) and magnesium modified peanut shell biochar (M-PB) were obtained by pyrolysis of 3 h at a temperature of 400°C in a tube furnace.

2.2.3 Determination of basic physical and chemical properties of biochar and modified biochar. Test methods of wooden activated carbon: Determination of pH (GB/T 12496.7-1999) was used to determine the pH of biochar; Test methods of wooden activated carbon: Determination of ash content (GB/T 12496.3-1999) was used to determine the ash content; The content of functional groups of biochar was determined by Boehm titration; The specific surface area was measured by ASAP 2020M physical adsorption apparatus; Scanning electron microscopy (SEM) was used to observe the surface morphology.

2.3 Immobilization of microorganisms on biochar
Orthogonal experiment designs with SPSS20.0 software, the effects of biological carbon types, adsorption time, biochar amount and rotating speed on the amount of bacteria absorbed by the carrier were studied by orthogonal experiment and the adsorption capacity of the adsorbent was measured. The optimum conditions for the preparation of immobilized microorganisms were obtained by analysis of variance.

2.3.1 Orthogonal experimental design. SPSS 20.0 software was used to design orthogonal experiments of four factors at different levels, then author did the immobilized experiment. The optimum conditions for the preparation of immobilized microorganisms were obtained by multivariate analysis of variance. The modified biochar immobilized rate under the optimum conditions was also verified. Four factors at different levels experiment are shown in Table 1.

| Multiple factors | Modified biochar types | Volume ratio | adsorption time (h) | Shaking speed | table |
|------------------|------------------------|--------------|---------------------|---------------|-------|
| Level 1          | M-WB                   | 1: 20        | 2                   | 0             |       |
| Level 2          | M-PB                   | 1: 30        | 6                   | 120rpm        |       |
| Level 3          |                        | 1: 40        | 12                  | 160rpm        |       |
| Level 4          |                        |              | 24                  | 180rpm        |       |

2.3.2 Immobilization of microorganisms by adsorption method. The bacterial fluid after activation of 20 h was centrifuged at 3000rpm and 10 min, then the supernatant was washed down and the bacterial suspension was prepared by physiological saline. The OD600 value of bacterial suspension was 0.40.
The immobilized microorganisms were prepared by the adsorption of biochar and bacterial suspension in a conical flask in accordance with the designed orthogonal test. After adsorption, the mixture of immobilized microorganisms and free bacteria was transferred to the centrifuge tube with physiological saline. The mixture is centrifuged at 1000 rpm 10 min and the supernatant was collected in volumetric flask. In the end, the OD600 value of supernatant was measured. The test was repeated 3 times. The precipitation was immobilized microorganisms, and the immobilization rate of immobilized microorganisms on biochar was obtained by subtraction. The formula (1) is following.

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\text{Immobilization rate of microorganisms on biochar (\%)} = \frac{(P_0-P_1)}{P_0} \times 100\% \quad (1)
\]

P0, OD600 value of initial bacterial suspension; P1, OD600 value of supernatant.

3. Results and Discussion

3.1 Physical and Chemical Properties of Biochar and Modified Biochar

The pH, ash, Acid and alkali functional groups, specific surface area of WB, PB, M-WB, M-PB were determined as shown in Table 2.

The surface morphological changes were observed by scanning electron microscopy (SEM), as shown in Figure 1.

### Table 2. Basic physical and chemical properties of biochar and modified biochar

| Sample | pH   | ash | Carboxyl groups mmol·g⁻¹ | Ester groups mmol·g⁻¹ | Phenolic hydroxyl groups mmol·g⁻¹ | alkali functional groups mmol·L⁻¹ | BET (m²·g⁻¹) | SA |
|--------|------|-----|--------------------------|----------------------|---------------------------------|---------------------------------|--------------|----|
| WB     | 9.71 | 19.51 | 0.09                       | 3.18                 | 2.89                             | 1.86                             | 6.5392       |    |
| PB     | 10.17 | 6.13  | 0.10                       | 2.81                 | 2.86                             | 1.83                             | 4.5172       |    |
| M-WB   | 8.81 | 26.24 | 0.41                       | 11.37                | 9.98                             | 2.48                             | 275.1356     |    |
| M-PB   | 9.06 | 15.87 | 0.32                       | 6.48                 | 6.40                             | 2.48                             | 152.5155     |    |

As can be seen from table 2, the pH of all four biochars are alkaline, which is beneficial to improve the pH of the soil. After modification, the pH of M-PB and M-WB has been reduced and the alkalinity is weak. Pietikainen (Pietikainen et al. 2011) found that because of weak alkaline of the biochar, use it as the carrier of immobilized microorganism could increase the respiration rate of microorganisms and facilitate the growth of microorganisms. So M-PB and M-WB can provide a living environment conducive to the growth and metabolism of microorganisms. Before modification, the ash content of WB is higher than that of PB, which is related to the content of lignin, cellulose and hemicellulose of wheat straw and peanut shells, which is also related to hardness of the raw material. The higher hardness is, the less biochar ash content is. After modification, ash content of the M-PB and M-WB is increased, this is due to the modified carbon load magnesium; and specific surface area of the M-PB and M-WB is much higher than biochar before modification, most microbial adsorbed on the surface of the adsorbent, therefore, in a certain range, the bigger the specific surface area, the more microbial adsorption immobilized. Modified carbon is more advantageous to immobilize microorganism; It can be
seen from Figure 1 that after modification, the surface of the biochar can be roughened, the gap became smaller and the number of micropores increased, which is closely related to the increase of surface area.

Alkaline functional group content can be seen in table 2, there were no significant difference in PB and WB alkaline functional groups content. After modification, the two functional groups on the surface of the carbon content is also quite (both are 2.48 mmol/L), and higher than the unmodified biochar; however, the contents of the modified M-WB and M-PB surface acidic functional groups increased significantly, especially the M-WB, the carboxyl group reached 0.41 mmol/g, and the phenolic hydroxyl group reached 9.98 mmol/g. Research (Zhang et al. 2010, Sun et al. 2009) show that the carboxyl group and the hydroxyl group and other functional groups on the surface of the carrier can be better combined with the microbial surface, which is beneficial to the rapid immobilization of microorganisms and improve the binding strength between the carrier and the microorganism. At the same time, more surface acidic functional groups are conducive to the adsorption to bacteria. Therefore, biochar is more conducive to the adsorption to microorganisms after modification, and the modified wheat straw carbon and modified peanut shell charcoal with more surface acidic functional groups are more suitable for the preparation of immobilized microorganisms.

### 3.2 Orthogonal test determines the optimum conditions for immobilization of microorganisms by modified biochar

First, H2 and H4 were adsorbed and immobilized with WB, PB, M-WB and M-PB at a volume ratio of carbon to bacterial liquid 1:40, rotational speed 160 rpm and constant temperature 30℃. The immobilized bacteria were expressed indirectly by OD600 and the rate of immobilization are shown in Table 3.

| Sample | Immobilized rate of H2 | Immobilized rate of H4 |
|--------|------------------------|------------------------|
| PB     | 20.88%                 | 14.93%                 |
| WB     | 17.67%                 | 17.54%                 |
| M-PB   | 48.50%                 | 32.75%                 |
| M-WB   | 39.00%                 | 49.50%                 |

It can be seen from Table 3 that the immobilized rate of M-PB and M-WB is improved obviously, and the degradation of petroleum hydrocarbon is directly related to its density. The more the number of microorganisms are in certain space, the stronger the degradation of petroleum hydrocarbon is (Li et al. 2009). M-PB and M-WB are more suitable for the preparation of immobilized microorganisms because of the better degradation of petroleum hydrocarbons and polycyclic aromatic hydrocarbons. The results of orthogonal experiments are shown in Table 4.

The variance analysis of orthogonal experiment results was carried out by using spss20.0 software to obtain the following results.

The results of the inter-subjective tests are shown in Table 5.

### Table 3. Immobilized rate of biochar and modified biochar to H2 bacteria and H4 bacteria

| Sample | Immobilized rate of H2 | Immobilized rate of H4 |
|--------|------------------------|------------------------|
| M-WB   | 36.50%                 | 28.25%                 |
| M-PB   | 70.50%                 | 72.25%                 |
| M-PB   | 61.50%                 | 48.25%                 |
| M-PB   | 77.50%                 | 51.75%                 |
| M-WB   | 52.75%                 | 39.50%                 |
| M-PB   | 61.50%                 | 46.00%                 |
| M-PB   | 74.75%                 | 72.38%                 |
| M-WB   | 39.00%                 | 39.50%                 |
| M-WB   | 49.50%                 | 43.25%                 |

### Table 4. Orthogonal experimental results

| Biological carbon types | Bacteria liquid / charcoal (v/v) | Rotating speed /rpm | Time/h | Immobilized rate of H2 | Immobilized rate of H4 |
|------------------------|---------------------------------|---------------------|--------|------------------------|------------------------|
| M-WB                   | 30                              | 120                 | 12     | 36.50%                 | 28.25%                 |
| M-PB                   | 20                              | 120                 | 6      | 70.50%                 | 72.25%                 |
| M-PB                   | 40                              | 120                 | 2      | 61.50%                 | 48.25%                 |
| M-PB                   | 20                              | 160                 | 12     | 77.50%                 | 51.75%                 |
| M-WB                   | 40                              | 0                   | 12     | 52.75%                 | 39.50%                 |
| M-PB                   | 40                              | 180                 | 24     | 61.50%                 | 46.00%                 |
| M-PB                   | 20                              | 0                   | 6      | 74.75%                 | 72.38%                 |
| M-WB                   | 40                              | 160                 | 6      | 39.00%                 | 39.50%                 |
| M-WB                   | 20                              | 0                   | 24     | 49.50%                 | 43.25%                 |
Table 5. Tests of inter-subjective effects

| Source                     | Dependent variable      | Mean square | F       | Sig. |
|----------------------------|-------------------------|-------------|---------|------|
| Modified biochar types     | Immobilized rate of H4  | 0.112       | 11.123  | 0.013|
|                           | Immobilized rate of H2  | 0.225       | 39.427  | 0    |
| Amount of modified biochar | Immobilized rate of H4  | 0.055       | 5.426   | 0.038|
|                           | Immobilized rate of H2  | 0.028       | 4.922   | 0.046|
| Shaker speed               | Immobilized rate of H4  | 0.006       | 0.559   | 0.659|
|                           | Immobilized rate of H2  | 0.007       | 1.268   | 0.357|
| Adsorption time            | Immobilized rate of H4  | 0.01        | 0.944   | 0.469|
|                           | Immobilized rate of H2  | 0.009       | 1.647   | 0.264|

When the "Sig." Value is less than 0.05, it means that the index has a significant impact, less than 0.01, said the impact of the index is very significant. It can be seen from Table 5. The sig values of modified biochar types and modified biochars are less than 0.05, indicating that these two factors have a significant effect on the immobilized rate of H2 bacteria and H4 bacteria. The effect of modified biochar types on H2 immobilized rate is very significant, so we consider the factor priority level should first consider the types of modified biochar and modified biochar dosage, after all, sig values of rotating speed and adsorption time are greater than 0.05, indicating that these two factors have little effect on the immobilized rate of degrading bacteria. In the Table 4, the highest immobilized rate of H2 bacteria was 77.50%, and the immobilized rate of H4 bacteria was 72.38%. All of them were treated with M-PB as adsorbent and volume ratio of 20:1, but the rotating speed and time have a different effect. In order to obtain the best immobilized conditions for biochar immobilizing microorganisms, further analysis to determine the best level of various factors, the experimental results were shown in Table 6 and Table 7.

Table 6. Immobilization results analysis table of H4

| Immobilization results analysis of H4 | 1   | 2   | 3   | 4   |
|--------------------------------------|-----|-----|-----|-----|
|                                       | 41.09% | 57.30% | 53.53% | 44.63% |
|                                       | 59.20% | 42.69% | 46.81% | 47.00% |
|                                       | 41.20% | 46.80% | 46.80% | 53.03% |
|                                       |       | 48.75% |       | 55.94% |
| R                                     | 18.11% | 14.61% | 6.73%  | 11.31% |
| Better level                          | A2   | B1   | C1   | D4   |
| Factor primary and secondary          | 1    | 2    | 4    | 3    |
Table 7. Immobilization results analysis table of H2

| Immobilization results analysis of H2 | 1 | 2 | 3 | 4 |
|--------------------------------------|---|---|---|---|
|                                      | 45.56% | 62.97% | 60.88% | 56.25% |
|                                      | 67.81% | 51.94% | 52.19% | 60.63% |
|                                      | 52.65% | 60.25% | 52.75% | 52.75% |
|                                      | 55.31% | 60.88% | 60.88% | 60.88% |
| R                                    | 22.24% | 11.03% | 8.69% | 8.12% |
| Better level                         | A2 | B1 | C1 | D4 |
| Factor primary and secondary         | 1 | 2 | 3 | 4 |

According to the experimental results of Table 6 and Table 7, it can be seen that the optimal level of H4 and H2 bacteria is M-PB as the adsorption carrier, the second important factor is the volume ratio of bacteria to biochar of 20: 1, which are consistent with the analysis results in Table 6. So we can obtain the best immobilization conditions are in the bacteria liquid and M-PB volume ratio of 20: 1, adsorption for two hours, rotating speed of 0 rpm. Under these conditions, the immobilization rate of H2 bacteria was 78.9%, the fixation rate of H4 bacteria was 72.41%, and the value was higher than or equal to the maximum immobilization rate of orthogonal test. Indicating that the optimum immobilization conditions for microorganisms determined by variance analysis and optimum levels are in line with the actual situation.

3.3 M-PB immobilized microorganisms

Figure 2. Scanning electron microscopy of M-PB immobilized microorganisms scanning electron microscopy

a, M-PB immobilized H2 bacteria; b, M-PB immobilized H4 bacteria

From Figure 2, the microorganism adsorption on the M-PB surface and the aggregation effect is more obvious, but careful observation can be found in the pores are also distributed within the H2 and H4, indicating that increased pore size may increase the immobilization rate of microorganisms. Preparation of immobilized microorganisms to remediate petroleum contaminated soil.

4. Conclusion

The optimal immobilization conditions of M-PB immobilized H2 bacteria and H4 bacteria were as follows: the volume ratio of bacteria liquid to M-PB was 20: 1, the adsorption was 2 hours, the rotating speed was 0 rpm. Under the optimum conditions, the M-PB immobilization rate of bacteria was 77.50%, and the M-PB immobilization rate of H4 bacteria was 72.38%.

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