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Highlights

- A biochar to solution of 1:30 (w/v) and incubation for 24 h are the optimal immobilization condition.
- Biochar with high N content, large pore size and high WHC enhances bacterial immobilization.
- Immobilized bacteria accelerate PAH removal via adsorption and biodegradation.
- High moisture content and proper stimulants or surfactants act as regulatory factors.
- Biochar combined measures improve the bacterial abundance and soil enzyme activities.

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Characterization and PAH removal performance of microbe-immobilized biochars derived from different feedstocks

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Abstract

Microbial degradation is the primary mechanism for purifying polycyclic aromatic hydrocarbon (PAH) contamination in environments, and biochar immobilization is an effective technology to enhance biodegradation, but the process parameters of the immobilization technology still require further systematic evaluation. Here, biochars derived from pig manure (PM), bamboo (BB), rice straw (RS), and soybean straw (SS) were used as carriers of *Mycobacterium* sp. ZL7, and the optimal biochar dosage of 1:30 (w/v) and immobilization time of 24 h were determined. The immobilization effects followed the order of RS > SS > PM > BB. Scanning electron microscopy and physicochemical properties revealed that porous structures acted as shelters for bacteria, and high nitrogen content, large pore size and high-water holding capacity played important driving roles in immobilization. In the single-substrate system, pyrene removal rates of the PM-, RS- and SS-immobilized materials were greater than 96 %, which were significantly higher than those of the biochar alone or the free strains. An orthogonal design experiment in historically PAH-contaminated soil further revealed that, compared with free strain, immobilized materials combined with high moisture

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content and moderate salicylic acid or Brij 30 can effectively increase the abundance of bacteria and the *nidA* gene, and enhance the dehydrogenase and polyphenol oxidase activities. The removal rate of total PAHs increased by 8.53 %-30.45 % after 24 d. Moreover, biochar with strong immobilization capacity showed better PAH removal effects. This study provides a scientific basis and practical reference for biochar-immobilized microorganisms to enhance the self-purification of PAH-contaminated soil.

Keywords:

Polycyclic aromatic hydrocarbons (PAHs)

Self-purifying

Microorganism

Biochars

Immobilization

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Introduction

As social and economic centers, cities are subject to widespread contamination due to resource and energy consumption (Wang et al., 2022). Industrial activities, fossil or wood combustion produce environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) (Han et al., 2019). 16 PAHs with highly persistent and recalcitrant to biodegradation were listed as priority pollutants by the United States Environmental Protection Agency (US EPA) because of their mutagenic and carcinogenic properties (Mallah et al., 2022; Patel et al., 2020). PAHs are easily stored in urban soil through adsorption, deposition, and migration. Thus, as an important reservoir of PAHs, urban soil has attracted increasing attention (Zhu et al., 2023).

To address the growing problem of urban pollution, several technologies have been applied to remove PAHs from polluted environments. Compared to physical and chemical remediation technologies with high energy consumption, increasing attention has been given to the development of environmental self-purification ability (Dai et al., 2022; Mojiri et al., 2019). In the process of environmental self-purification, microorganisms act as decomposers to degrade pollutants for purifying contaminated water and soil (Patel et al., 2020). Although the presence of microbes possessing the proper degradative capabilities in polluted environments, undesirable conditions reducing microbial biomass and activity results in the slow natural attenuation

(Romantschuk et al., 2023). Thus, bioremediation technology aimed at enhancing microbial purifying capacity is becoming more and more important (Haritash and Kaushik, 2009; Peralta-Maraver et al., 2023). However, among the technologies that enhanced the purifying capacity of microorganisms, biostimulation (e.g., the addition of surfactants, activators, etc.) is easily constrained by the low number of indigenous functional bacteria, whereas bioaugmentation (e.g., the addition of exogenous functional bacteria) is highly susceptible to species competition and environmental conditions (Lopes et al., 2022). In contrast, microbial immobilization technology is considered as an effective augmentation measure because it can increase biomass, maintain high microbial activity and prevent cells from being damaged by the external environment (Wang et al., 2019; Zhang et al., 2019).

Ideal immobilization materials should be mechanically stable, nontoxic, easily available, compatible with microorganisms, and permit high bioload (Bouabidi et al., 2019). Biochar is one of the most appealing supports due to its relatively large specific surface area (SSA), high porosity, strong adsorption capacity, and it has been used in the practice of urban self-purification (Li et al., 2024; Liang et al., 2021; Zhao et al., 2024). Biochar not only enables microbial protection against environmental stress, but also improves microbial activity in degrading organic pollutants (Li et al., 2021; Li et al., 2022). Xiong et al. found *Mycobacterium gilvum* immobilized on rice straw biochar could remove 62.6 % ± 3.2 % of phenanthrene, 52.1 % ± 2.3 % of fluoranthene, and 62.1 % ± 0.9 % of pyrene (PYR), which were higher than those using *M. gilvum* alone (Xiong et al., 2017). Pine needle biochar could protect *Sphingomonas* sp. PJ2 from being harmed by the saline soil to reach the maximum PAH removal efficiency of 58.64 % (Song et al., 2021). A functional microbial consortium loaded on corn straw biochar presented a 9.25 % higher removal rate of total PAHs in soil than the control treatment (Zhou et al., 2024). However, different biochars often have certain differences in physicochemical properties, which affect the immobilization efficiency and microbial activity, and also affect the reasonable choice of biochars in practical applications. Although biochar characteristics such as SSA, pH, nutrients (C and N, etc.), and water holding capacity have been reported to affect microbial habitation (Bolan et al., 2023; Schommer et al., 2024), the selection of immobilization conditions and which physicochemical properties play a major role in promoting immobilization efficiency still need to be systematically evaluated for practical application reference.

In addition to the selection of ideal immobilization materials, choosing appropriate

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regulation methods to meet the requirements for highly effective removal of soil PAHs is crucial. However, few reports on the regulation methods of biochar and functional bacteria have limited the application of biochar-immobilized functional bacteria in enhancing soil self-purification. Soil moisture amendment, carbon supplementation, and surfactant addition are considered to facilitate bioremediation of PAH-contaminated soils by enhancing soil microbial activity. Water amendment is necessary during the bioremediation of PAH-contaminated soils because moisture content is an important factor in soil conditions (Teng et al., 2010). Co-metabolic carbon substrates such as salicylic acid can also enhance the biodegradation of PAHs in soil (Ahmad et al., 2021). Surfactants such as Brij 30 are beneficial for PAH bioremediation processes that are limited by low bioavailability (Adrion et al., 2016). However, reports on the interactions between these regulatory factors are still limited and the combination of advantages for enhanced soil self-purification by biochar-immobilized microorganisms is unclear.

A typical PAH-degrading strain, *Mycobacterium* sp. ZL7, isolated previously from PAH contaminated soil at a Beijing coking plant was used in this study. Four types of biochar made from different feedstocks (i.e., pig manure biochar (PM), bamboo biochar (BB), rice straw biochar (RS) and soybean straw biochar (SS), were selected as carriers of ZL7. The biochar dosage ratio and immobilization time were selected as the key parameters for optimizing the immobilization conditions. The aged PAH contaminated soil was selected as the remediation object to explore the effect of biochar combined with regulatory measures on actual PAH pollution. The main objectives of this study were: (1) to compare the immobilization time and dosage of different biochars for bacteria to determine the optimal conditions; (2) to analyze the physicochemical properties affecting microbial immobilization to determine the type of biochar with good immobilization effect; (3) to investigate the removal effect and regulation measures of immobilization materials on PAHs from actual contaminated soil, providing a theoretical basis and practical reference for the development of urban soil self-purification technology.

1. Materials and methods

1.1 Preparation and characterization of biochars

Four typical raw materials with different physicochemical properties were chosen for the preparation of biochars, namely, pig manure, soybean straw, rice straw and

bamboo (Kloss et al., 2012). All raw materials comprise organic wastes containing organic matter derived from agricultural or breeding activities. These materials are widely available, renewable, and their utilization can help reduce environmental pollution. Pyrolysis biochar at 500 °C can retain nutrients well and is conducive to microbial immobilization. Thus, all materials except for PM were prepared by slow pyrolysis at 500 °C for 5 h in a model FB600 muffle furnace (Instruments for Research and Medicals, Germany) under oxygen-limited conditions. PM was prepared at 500 °C for 0.5 h. All biochars were sieved through a 2 mm mesh.

The pH were analyzed by 1 g of biochar suspending in 10 mL of deionized water and shaking at 180 r/min for 3 h. and subsequently measured using a model 310p-01 pH meter (Thermo Fisher Scientific, USA) (Cayuela et al., 2013). The pore structure of the materials was determined via the Brunauer–Emmett–Teller (BET) method using a model ASAP 2020M+C specific surface and porosity analyzer (Micromeritics, USA) (Xiong et al., 2017). The elemental composition, including total carbon (C) and total nitrogen (N), was measured by a model Vario MAX elemental analyzer (Elementar, Germany) (Xiong et al., 2017). The water holding capacity (WHC) of the biochars was determined by saturating the materials with water for 8 h and then air drying for 1 h. The %WHC was calculated using the mass of water retained in the material per g dry material×100 (Hale et al., 2015).

1.2 Contaminated soil

Soil samples were collected from the 0-20 cm surface soil at the edge of a abandoned Beijing coking plant, and mixed evenly after discarding gravel and plant root residues. A part of soil samples was sealed and stored through a 2 mm sieve after natural air drying, which was used for the orthogonal culture experiments. Others were screened by 2 mm, 0.15 mm and 0.25 mm sieve for testing pH, elemental analysis and organic matter, respectively. The physicochemical properties of the tested soil were as follows: soil pH value was 8.61 ± 0.60, total nitrogen content was 0.22 % ± 0.02 %, total carbon content was 16.75 % ± 0.53 %, total sulfur content was 0.63 ± 0.01 %, and the organic matter content was 1.66 % ± 0.17 %. The total PAH content was (329.93 ± 12.62) μg/g.

1.3 Culture and immobilization conditions for the ZL7 strain

The strain *Mycobacterium* sp. ZL7 was successfully enriched and screened from

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PAH contaminated soil and classified as a Gram-positive bacterium. It exhibits a broad spectrum of substrates and can utilize various PAHs, including phenanthrene, pyrene, benzo[a]pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, and benzo[k]fluoranthene as a single substrate for metabolic degradation. Microbial cultures were grown at 37 °C in a rotary shaker with shaking at 180 r/min using Luria Bertani medium (LB: 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl in 1 L of distilled water). To determine the degradation ability of the strain ZL7, PAHs were used as a sole carbon source and incubated at 37 °C at 180 r/min in the minimal salt media (MSM: 1.1 g of NH_4Cl, 1.0 g of K_2HPO_4, 0.5 g of NaCl, 0.2 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of $FeSO_4$, 0.01 g of $CaCl_2$ in 1 L of distilled water). PYR (≥ 99% purity) were purchased from Sigma Aldrich, USA.

To obtain the cell suspension, the ZL7 culture was centrifuged at 3800 r/min for 10 min and then resuspended in sterilized fresh LB medium to obtain a cell suspension with OD_{600} of 1.4. The biochar and cell suspension were mixed to prepare immobilized materials with solid-liquid ratios of 1:5, 1:10, 1:20, 1:30 and 1:40 (w/v). The immobilized materials were placed on a shaker at 180 r/min at 37 °C for 6, 12, 18, 24, 36 or 48 h. The mixtures were separated with a 75 μm sieve and washed twice with sterile fresh LB to remove the free bacteria. The immobilized materials were collected and stored at 4 °C. The *nidA* gene copy number and dehydrogenase activity were measured to quantify the biomass and microbial activity of the immobilized bacteria. The optimal immobilization conditions were determined by evaluating the effect of immobilization time (0-48 h) and the ratio of biochar dosage to cell suspension volume (w/v, from 1:5 to 1:40).

1.4 Quantitative polymerase chain reaction (qPCR)

DNA was extracted from 0.5 g of the immobilized materials using the FastDNA SPIN Kit for Soil (MoBio Laboratories, USA) according to the manufacturer's protocol. The bacterial abundance was estimated using real-time PCR assays targeting the *nidA* gene (DeBruyn et al., 2007). The forward and reverse sequences of primers used were 5'-TTCCCGAGTACGAGGGATAC-3' and 5'-TCACGTTGATGAACGACAAA-3', respectively. The real-time PCR used Light Cycler FastStart DNA Master Hybridization Probes (Roche) and were performed using the following protocol: 50 °C for 5 min, 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and lengthening at 72 °C for 30 s. Soil bacterial abundance was estimated using real-

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time PCR of the 16S rRNA gene. The forward and reverse sequences of the primers used are 5'-GGGTTGCGCTCGTTGC-3' and 5'-ATGGYTGTCGTCAGCTCGTG-3', respectively. The PCR protocol consisted of an initial denaturation step at 95 °C for 5 min, 95 °C for 15 s, 60 °C for 1 min, 72 °C for 20 s). The standard curve was developed by amplifying a tenfold diluted standard plasmid containing the target gene. All qPCR involving sample DNA, control DNA templates or negative controls were prepared in duplicate.

1.5 Enzyme assay

Dehydrogenase (DH) activity was measured by the methods described in Tabatabai (Tabatabai and Dick, 2002). Based on this method, 0.5 g of sample was completely mixed with 0.2 mL of Tris-HCl buffer solution (0.2 mol/L, pH=7.6). After incubating at 37 °C in the dark for 24 h, 2 drops of concentrated sulfuric acid were added to terminate the reaction. Then, 0.5 mL of methylbenzene was added, and the mixture was shaken at 30 °C for 30 min. After holding, the cell suspension was centrifuged at 400 r/min for 10 min. The obtained supernatant was separated, and the proportion of triphenyl formazan (TPF) in the filtrate was measured using a model UV-6300 spectrophotometer (Mapada, China) at 485 nm. A control without the addition of 2,3,5-triphenyltetrazolium chloride (TTC) was included for each sample.

Polyphenol oxidase (PPO) activity were measured using the methods of Perucci et al. with some modifications (Perucci et al., 2000). 1 g of fresh soil samples stored at 4 °C was weighed into centrifuge tubes, 3 mL of reaction solution (0.2 mol/L catechol and 0.2 mol/L proline 1:1 mixture) and 2 mL of 0.1 mol/L phosphate buffer (pH=6.5) were added to it. Samples were in a 30 °C water bath for 10 min. after the reaction, all samples were then transferred to an ice box and added 5 mL of absolute ethanol to stop the reaction. Samples were centrifuged at 4 °C, 5000 r/min for 5 min. Supernatant was taken to measure the absorbance at a wavelength of 525 nm. The activity of PPO was expressed as the amount of *o*-diphenol oxidized in 1 g of dry weight soil within 10 min.

1.6 Scanning electron microscope (SEM) analysis

The pore structures of the biochars and biochars inoculated with ZL7 cells were observed via a model S-4800 SEM (Hitachi, Japan). The samples were fixed with 2.5 % glutaraldehyde in 0.1 mol/L phosphate-buffered saline (PBS, pH=7.2) for 1 h and rinsed twice with 0.1 mol/L PBS. The samples were then dehydrated in 30 %, 50 %, 70 %,

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90 %, or 100 % ethanol for 20 min each. After dehydration, the samples were dried to the critical point with carbon dioxide in a Samdri-PVT apparatus. Images were recorded via SEM operated at 5 kV.

1.7 PAH removal detection

Determination of PYR removal from water. The biochar as an immobilization material was weighed at 1:30 (w/v) into a 150-mL flask containing 60 mL of MSM at a concentration of 50 mg/L PYR. Moreover, the experimental groups included an MSM control group, a free-strain treatment group and a biochar-immobilized material treatment group. The cells in each group were incubated at 180 r/min and 37 °C in a shaker in triplicate. After 8 days of incubation, destruction sampling was used to determine the residual PYR concentration. The removal of PYR by the biochars and immobilized materials were evaluated based on the mass balance of PYR. The concentrations of PYR in the water and in the carrier were analyzed according to previous methods (Xiong et al., 2017). Briefly, HPLC grade hexane (TEDIA, USA) was used as the extraction agent for extraction of PAHs in water, and the proportion of extraction agent to solution was 3:10. Then, all the plates were shaken at 180 r/min for 30 min within separatory funnels. The organic phase was collected for PYR analysis. The extraction process was performed twice. The organic phase was collected and purified for GC-MS analysis (7890A, Agilent, USA).

Determination of removal of PAHs in soil slurry systems. All samples were incubated at 37 °C, 180 r/min in a shaker protected from light for 24 days. All samples were then transferred to a centrifuge tube and centrifuged at 5000 r/min for 20 min to separate the soil and supernatant. The determination of PAHs in liquid phase is the same as above. The original Erlenmeyer flasks were washed with the supernatant in three times, and the washed supernatant was transferred to the corresponding centrifuge tube and centrifuged again at 5000 r/min for 20 min. Liquid samples were stored at -20 °C for the determination of PAHs in the liquid phase. The solid phase samples were mixed evenly and stored at 4 °C for the determination of DH and PPO activities and gene abundances, and a part was freeze-dried for the determination of PAHs. PAHs were extracted from soil samples using a model ASE350 solvent extractor (Thermo Fisher Scientific, USA), the extracts were purified using the US EPA 3630C method, and the content of PAHs in the samples was determined by GC-MS.

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1.8 Orthogonal design experiment

Ultrapure water was added to the tested soil to make its moisture content at about 60 % for 10 days. 10 g of activated PAH-contaminated soil was weighed in 250 mL Erlenmeyer flasks. Soil-water ratios in different gradient levels were configured, bioremediation materials were added according to different inoculation methods, and different concentrations of salicylic acid and Brij 30 were added in samples. The codes and levels of the four factors are shown in **Table 1**. The full experimental settings are shown in **Appendix A Table S1**, and three replicates were set for the experiments. The orthogonal experiment number for RS was R, and the orthogonal experiment number for PM was P. Meanwhile, the changes of PAH removal ability, bacterial abundance, and soil enzyme activity were analyzed.

1.9 Statistical analysis

Microsoft Excel software (version 2010) was used to perform the statistical analysis. The Statistical Package for Social Sciences (SPSS) v17.0 software (SPSS, Inc., IL, USA) was used to determine whether there were significant differences between the treatments at different factor levels. ANOVA results with $P < 0.05$ were considered to indicate statistical significance. Principle component and linear regression analyses were also performed using SPSS.

2. Results

2.1 Optimization of immobilization conditions

To determine the optimal immobilization conditions for ZL7 on the four types of biochar, the optimal immobilization dosage and time were determined by the *nidA* gene copy number and DH activity as indicators. A higher number of gene copies or DH activity indicates a greater immobilization capacity, and a significant positive correlation between the abundance of the *nidA* gene and DH activity is shown in **Appendix A Fig. S1** (R^2=0.83, $P < 0.001$).

The results showed that the copies of the *nidA* gene was significantly affected by adjusting the biochar dosage-to-cell suspension ratio ($P < 0.05$) (**Fig. 1**). The abundance of the *nidA* gene at ratios of 1:20, 1:30 and 1:40 were significantly greater than that at ratios of 1:5 and 1:10 in all the treatments. Furthermore, in the BB and RS treatments, the number of *nidA* copies at ratios of 1:30 and 1:40 were significantly greater than that at a ratio of 1:20 (**Fig. 1b and c**). There were no significant differences in *nidA* gene

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abundance between the 1:30 and 1:40 ratios in all treatments. The changes in DH activity at different immobilization ratios were similar to the changes in *nidA* gene abundance. Among all the immobilization ratios, the 1:30 and 1:40 ratios had the highest DH activity. In addition, the immobilization efficiency of RS and SS were greater than that of the other biochars ($P < 0.05$) (**Fig. 1c and d**). Since there was no significant difference in the immobilization capacity of biochars tested between 1:30 and 1:40, considering resource utilization and cost factors, a ratio of 1:30 was selected as the optimal immobilization ratio for further research.

The immobilization efficiency was also affected to some extent by the immobilization time. The relationship between the immobilization efficiency and time (within the range of 0-48 h) at an immobilization ratio of 1:30 was investigated (**Fig. 2**). According to the *nidA* gene abundance data, the biomass of the immobilized bacteria significantly increased with increasing immobilization time for most biochars in the first 24 h, except for RS, which reached the highest biomass in the first 18 h ($P < 0.05$). In the latter 24 h of the immobilization process, the biomass of BB and RS was stable, whereas that of PM and SS was significantly lower ($P < 0.05$). The DH activity results suggested that the activity of the PM-, RS- and SS-immobilized bacteria reached a maximum at 18 h, and the peak activity of BB was observed at 24 h. After the activity reached its highest level, the activities of all immobilized cells were stable at later times. Based on the above results, the optimal immobilization time was 24 h.

Moreover, both the immobilization ratio and time analysis suggested that the order of the immobilization capacity for the different biochars was RS > SS > PM > BB under the optimal conditions.

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2.2 Physicochemical characteristics of the biochars

Images of the biochars and immobilized samples were observed via SEM (**Fig. 3**). **Fig. 3a1-d1** showed that all the biochars were full of pores, indicating good potential for immobilizing bacteria. The pore structures of BB, RS and SS were clear, varied and aligned, while the surface of PM was rough with granular and unsharp pore structures. After 24 h of immobilization, the adsorption of ZL7 cells on the different biochars was observed (**Fig. 3a2-d2**). The cells were immobilized on the surface or inside the pores of all the immobilized materials. In addition, the surface of the PM was obviously covered by biofilms, indicating that the PM might promote the secretion of biofilms by bacteria.

To analyze the factors affecting the immobilization of the four biochars, their basic physicochemical properties were evaluated (**Table 2**). The pH, C and N contents, %WHC, SSA and pore structure of the four biochars produced from different feedstocks exhibited high variability. All the biochars used in this study were alkaline, with pH values between 9.76 and 10.67. The C and N contents were significantly affected by the biochar feedstocks ($P < 0.05$). The C content of BB (78.1 ± 0.5 %) was greater than that of the other three biochars (17.3 ± 0.8 %-67.6 ± 0.6 %), and its C:N ratio (110.64) was also significantly greater than that of the other biochars (21.72-65.26). The biochar samples contained a small amount of N, which varied from 0.71 % to 1.47 %, and the RS sample had the highest N content among all the biochar samples in this study. The %WHC of the four biochars clearly varied from 288.18 to 915.10 ($P < 0.05$), and the RS had the highest %WHC among the four biochars. The SSA of the studied biochars increased from 1.17 to 22.33 m^2/g, and that of PM was significantly greater than that of the other biochars. The pore structure of the different biochars also varied greatly, with pore volumes ranging from 8.1×10^{-4} to 3.50×10^{-2} cm^3/g and pore sizes ranging from 2.80 to 8.59 nm. Among all the biochars, PM had the largest pore volume, whereas RS had the largest pore diameter.

The relationships between the physicochemical properties of the biochars and their immobilization capacity were further revealed via principal component analysis (PCA) (**Fig. 4**). The results showed that the N content, pore size, and %WHC were positively related to the immobilization capacity of the biochars and that the C:N ratio and pH were negatively correlated with the immobilization capacity, which explained why the immobilization capacities of the RS and SS were greater than those of the other types of biochars. The C content, pore volume and SSA did not play decisive roles in the differences of immobilization capacity of the four biochars.

2.3 Removal of PYR from solution by biochars and immobilized materials

To evaluate the ability of the four immobilized materials to remove PAHs, the removal rates of PYR in solution were examined (**Fig. 5**). The results showed that the free strain could efficiently remove 60.43 ± 3.0 % PYR in solution. Biochar alone also effectively removed PYR, and the removal rates of PB, BB, RS and SS were 71.19 ± 1.5 %, 47.63 ± 1.9 %, 58.99 ± 4.7 % and 70.78 ± 3.7 %, respectively, indicating that the adsorption capacity of the biochars contributed to PYR removal. Among them, PB and SS exhibited high adsorption capacities for PAHs. Significantly greater removal

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rates of PYR were detected in solution after 8 days of incubation when the biochars were used as carriers for ZL7 than the corresponding biochars were added directly ($P < 0.05$). The removal rates of PYR by immobilized materials, IMPB, IMBB, IMRS and IMSS, were 100 %, 72.12 ± 2.9 %, 96.78 ± 5.6 % and 97.58 ± 4.2 %, respectively, and the removal rates of all the immobilized materials were significantly higher than those of the free strains and biochars ($P < 0.05$). The above results revealed the positive impact of the immobilized materials on the removal of PYR in solution compared with that of the biochar alone and free strains.

2.4 Immobilized material combined technology to remove PAHs from contaminated soil

To confirm the effect of immobilized materials on purifying the actual PAH contaminated soil, an orthogonal experiment was carried out in historically contaminated soil and optimized the removal effect. RS and PM, which significantly differ in their immobilization effects but have PYR removal effects exceeding 90 %, were selected. And IMRS and IMPM have stable immobilization effects, indicating that they can be used for long-term soil purification (**Appendix A Fig. S2**).

The results of the orthogonal design experiment indicate that different combinations significantly enhanced the removal efficiency of the soil PAHs, demonstrating effective removal for both low molecular weight (LMW, two to three aromatic rings) and high molecular weight (HMW, four and above aromatic rings) PAHs. After 24 days of incubation, R9 exhibited the highest removal efficiency of PAHs (56.70 % ± 11.33 %) among the RS treatments, which was more than double that of the lowest R4 (25.05 % ± 3.47 %) (**Fig. 6a**). The removal rates of LMW and HMW PAHs by R9 reached 62.70 % ± 8.51 % and 55.54 % ± 11.89 %, respectively (**Fig. 6b**). Among all the PM treatments, P3, P6, P8 and P9 showed relatively high removal rates ((34.78 ± 6.19) %-(43.89 ± 1.53) %), which were significantly higher than those of the other treatments ((25.05 ± 3.47) %-(28.69 ± 4.56) %) ($P < 0.05$) (**Fig. 6c**). P9 had the highest removal rates of LMW and HMW PAHs, which were 53.88 % ± 2.24 % and 41.95 % ± 1.97 %, respectively (**Fig. 6d**). Compared with the PM treatments, the RS treatments resulted in better removal efficiency, suggesting that biochar with excellent immobilization effect was more conducive to purifying PAHs in soil.

Four factors affecting the removal of total PAHs in the soil were analyzed (**Table S4-S6**). For both RS and PM treatment groups, the inoculation method was the most

important factor in soil PAH removal ($P < 0.0001$), and the immobilized strains were more efficient than free strains in purifying soil PAHs, which was also consistent with the removal effect of soil total PAHs (**Fig. 6a and c**). The other three factors strongly differed between the two biochar treatment groups. In the RS treatments, the soil-water ratio (P=0.1949), salicylic acid (P=0.0546) and surfactant (P=0.0598) had little effect on PAH removal. The increasing moisture content aided in the removal of PAHs, as did the appropriate addition of salicylic acid, while the increase in the concentration of the surfactant Brij 30 played an inhibiting role. In the PM treatments, the soil-water ratio (P=0.0018) and salicylic acid (P=0.0104) had significant effects on the removal of PAHs, and Brij 30 (P=0.4748) had little effect on PAH removal. With the increase of moisture content, the removal of PAHs was significantly enhanced, but salicylic acid markedly reduced the PAH removal. However, increasing the concentration of Brij30 had a positive effect on the removal of PAHs. These results showed that the effect of biochar-immobilized bacteria in purifying PAH-contaminated soil was significantly better than that of free bacteria, and the biochar from different feedstocks would affect the adaptability of bacteria to other enhanced remediation measures.

Overall, the optimal conditions for the purification of soil PAHs by RS carrier was a 1:10 (w/V) soil-water ratio, immobilized bacteria, 2 g/kg salicylic acid, and no surfactant Brij 30. The optimal combination of PM carriers was a 1:10 (w/V) soil-water ratio, immobilized bacteria, the addition of salicylic acid, and 10 g/L surfactant Brij 30.

2.5 Changes in functional gene abundance and enzyme activity in soil

The effects of biochar combined with regulatory measures on functional microorganisms were further investigated. The abundance of the 16S rRNA gene could indirectly reflect the number of microorganisms in the soil. After 24 days of incubation, the abundance of the 16S rRNA gene in the RS treatment group ranged from (9.32 ± 1.03) to (143.14 ± 17.43) copies/g_{soil}, and that in the PM treatment group ranged from (9.32 ± 1.03) to (129.40 ± 15.87) copies/g_{soil} (**Fig. 7a and b**). The abundance of the 16S rRNA gene in the combined biochar treatments was markedly higher than that in the treatments without biochar, indicating that the addition of biochar could increase total bacteria number in the contaminated soil. Moreover, a significantly positive correlation between the abundance of the 16S rRNA gene and the removal rate of total PAHs was detected in the two treatment groups (RS: R^2=0.4671, $P < 0.0001$; PM: R^2=0.5025, $P < 0.0001$) (**Appendix A Fig. S3**), suggesting that the number of microorganisms played

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an important role in purifying soil PAHs.

The abundance of functional bacteria was characterized by the representative PAH-catabolic gene *nidA* of gram-positive bacteria. RS treatment group showed that the highest abundance of the *nidA* gene in R9 was $(8.96 \pm 2.10) \times 10^8$ copies/g_{soil}, which was remarkably higher than that in the other RS treatments ($P < 0.05$) (**Fig. 7a**). Similarly, P9 soil showed the highest abundance of the *nidA* gene ($(8.05 \pm 1.75) \times 10^8$ copies/g_{soil}) that was markedly higher than the others ($p < 0.05$) (**Fig. 7b**). These results suggested that in addition to biochar, other regulatory factors also had important impacts on the survival of immobilized functional bacteria in contaminated soil. The abundance of the *nidA* gene was significantly positively correlated with the removal rate of total PAHs in soil (RS: R^2=0.4119, P=0.0003; PM: R^2=0.4490, P=0.0001) (**Appendix A Fig. S3**), indicating that the number of immobilized functional microorganisms is an important factor in purifying soil PAHs.

The activity of soil DH is an important indicator for evaluating soil biological activity. The DH activity of R9 was the highest in the RS treatment, at (13.29 ± 6.18) μg/(g_{soil}·h), which was significantly higher than that in the other treatments. Similarly, the highest DH activity of P9 was (7.88 ± 1.85) μg/(g_{soil}·h) (**Fig. 7c and d**). The trend of DH activity was consistent with the *nidA* gene abundance, indicating that immobilized bacteria may be a major contributor to soil enzyme activity. PPO has been reported to be involved in the decomposition of aromatic compounds. Compared with that in the treatments with free bacteria alone, the PPO activity in the RS treatment group increased by 24.78 %-115.55 %, and that in the PM treatment group increased by 34.88.5 %-118.35 % (**Fig. 7c and d**). However, compared with that of DH, the variability of PPO was relatively stable. In addition, R2 and R3 also showed high PPO activities, which were (0.670 ± 0.13) μmol/(g_{soil}·10 min) and (0.798 ± 0.09) μmol/(g_{soil}·10 min), respectively, indicating that they may be among the reasons for higher removal effects of R2 and R3. There was also a certain correlation between soil enzyme activity and PAH removal efficiency (**Appendix A Fig. S4**). Therefore, increased soil biological activity played a role in PAH biodegradation.

In general, the combined remediation technology of immobilized materials is capable of enriching functional microorganisms, stimulating the activities of soil dehydrogenase and polyphenol oxidase, thereby promoting the purification of PAH-contaminated soil.

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3. Discussion

3.1 Factors influencing biochar immobilization capacity

Previous studies have utilized immobilization methods for PAH removal, but further details about the immobilization conditions have rarely been mentioned (Chen et al., 2021; Xu et al., 2019). Here, the optimal immobilization ratio of biochar dosage to cell suspension was 1:30 for all the tested biochar types. This suggests that with increasing cell density, the immobilization efficiency increased because of increasing contact opportunities for binding sites and cells. When the binding sites of cells reached saturation, the excess cells competed for oxygen and nutrients, which limited the growth space and inhibited bacterial activity. Within a few hours of immobilization, the initial adsorption of ZL7 by the carriers was complete. With increasing numbers of immobilized cells, the biochar reaches saturation within a certain period. According to our results, the immobilization of PM and SS tended to decrease after 24 h, possibly because the biomass of the immobilized strains exceeded the adsorption capacity of the biochars, resulting in the shedding of some bacteria (Zhang and Wang, 2021).

All the biochars tested here were shown to be useful carriers for immobilizing strain ZL7, but their efficiencies varied (RS > SS > PM > BB). This difference was due to the different physicochemical properties of the various biochars. PM had a much larger SSA and pore volume than the other biochars did, which may be the reason why PM still has a high PYR removal rate despite its low immobilization efficiency. A proper pore size distribution provides mass transfer pathways for PAHs, and a large SSA provides more active sites for PAH adsorption, thereby increasing the adsorption capacity (Li et al., 2020). The SSA, pore size, and pore volume of BB were much lower than those of the other biochars, resulting in the lowest immobilization and PAH adsorption capacity, further leading to the lowest PYR removal rate. RS and SS exhibited high levels of immobilization, and both had a high N content, large pore size, and high %WHC, suggesting their promoting role in bacterial immobilization.

N content was the key property affecting the immobilization efficiency of the four biochars in this study. Studies have revealed that some elements, most notably N, possess the remarkable ability to foster microbial growth and could promote a greater bacterial density (Wei et al., 2022; Wu et al., 2021). A larger pore size could provide bacteria access to the internal structure of the support, thus improving the mass transfer process and improving the immobilization yield (Pandey et al., 2020). In addition to nutrients and porosity, the features that favor microbial habitation include high %WHC

(Wu et al., 2021) (Adhikari et al., 2023), which is similar to our findings. These results demonstrated that the physicochemical properties promoting bacterial colonization and survival play more important roles in bacterial immobilization than SSA does, suggesting that more factors contributing to bacterial growth in the selection of biochar should be considered.

Other variables associated with immobilization efficiency were the C:N ratio and pH (**Fig. 4**). Jindo et al. reported a negative correlation between the C:N ratio and bacterial biomass in biochar-compost mixtures, which is in agreement with our findings (Jindo et al., 2012). However, another study demonstrated a positive relationship between the C:N ratio of biochar and its immobilization efficiency (Muhammad et al., 2014). These findings indicated that variations in the C:N ratio might have an important effect on bacteria but this effect may be inconsistent across different kinds of environments. A relatively high pH had a negative effect on the immobilization capacity of the biochars. Studies have shown that biochar pH is often neutral to slightly alkaline and plays an important role in supporting the survival of microorganisms by buffering pH levels (Shi et al., 2018; Shi et al., 2019; Zhang et al., 2022a). This finding also indicates that lowering the high pH of the BC biochars can further improve the microbial immobilization capacity (**Appendix A Fig. S5**).

3.2 Effect of biochar combined with bioremediation technology on PAH removal

Our results indicated that four types of biochar enhanced the removal efficiency of PYR in aqueous solution. The adsorption and accumulation of PAHs on the surface of biochars facilitate their transport and increase the contact between PAHs and microorganisms (Zhang et al., 2022b). Meanwhile, microorganisms play a key role in the purification process. Compared with biochar alone, biochar with NaN_3 inhibited microbial activity in soil leading to a 30 % reduction in PAH degradation (Kong et al., 2018). The combination of these two methods could simultaneously achieve the adsorption and degradation of PAHs and increase processing efficiency through their interaction.

Orthogonal experiments revealed the effects of different regulatory methods on the purification of PAH-contaminated soil. It has been reported that improving the water-to-soil ratio to some extent could increase the mineralization level of PAHs (Doick and Semple, 2003). For example, the maximum degradation rate achievable by PAHs occurs at 60 %-80 % moisture content in aged coal tar-contaminated soil

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(Antizar-Ladislao et al., 2006). Other reports have suggested that soil microbial counts and PAH degradation were lower in submerged soil (Teng et al., 2010). Here, the results indicated that increasing the moisture content enhanced PAH removal, and biochars may improve the biodegradation of PAHs under submerged conditions.

The biodegradation rate of PAHs could be enhanced by adding intermediate catabolites, as they can stimulate growth and improve enzyme expression (Gottfried et al., 2010). Salicylic acid is often used as a pathway inducer to increase the initial rate of PAH removal (Ahmad et al., 2021; Xie et al., 2023). In this study, salicylic acid had some extent of facilitating effect only in the RS treatments, which may be due to the competitive effect. Salicylic acid might compete with PAHs for binding sites on biochars, resulting in reduced microbial utilization of PAHs, thereby affecting PAH degradation.

Surfactant is another measure to promote degradation by increasing the bioavailability of PAHs (Subashchandrabose et al., 2019). Some research have shown that Brij 30 is particularly effective in enhancing the biodegradation of four- and five-ring PAHs (Adrion et al., 2016). However, Brij 30 has also been reported to inhibit the adsorption of organic pollutants to biochar because of its high solubility (Xiong et al., 2017). Brij 30 had completely different effects on the removal of PAHs in RS and PM treatments, which may be related to the mass transfer capacity of the biochar and the solubility of Brij 30. PM and Brij 30 mutually promoted on removal, suggesting that the interaction between them in a certain concentration range could improve the transfer between PAHs and bacteria. These findings suggested that further investigation of the interaction of biochar with stimulants and surfactants is essential to optimize biochar enhanced microbial purification.

The 16S rRNA gene, *nidA* gene, DH activity, and PPO activity were positively correlated with the removal of soil PAHs, which is similar to previous reports (Cao et al., 2016; Guo et al., 2021; Lu et al., 2019; Zhou et al., 2024). However, the correlation between each indicator and PAH removal was not extremely strong, indicating that the removal of PAHs was the result of a combination of factors. Here, the correlation between soil enzyme activity and PAH removal was not as pronounced as the abundance of the 16S rRNA and *nidA* genes, which seems to suggest that they are not as specific as functional genes as indicators of PAH removal capacity. However, owing to the wide variety of PAH degradation genes, a typical PAH degrading gene may not represent the full degradation potential of a sample. Therefore, the combined analysis

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of multiple genes can be considered when evaluating the potential of soil microbial decontamination of PAHs.

4. Conclusions

The use of bioengineered biochars via cell immobilization can improve the likelihood of success of bioaugmentation strategies, especially for recalcitrant PAHs. The immobilization conditions and properties of the carriers had significant effects on the immobilization of bacteria. Considering efficiency and cost, the optimized condition was immobilization at a ratio of 1:30 (w/v) for 24 h in this study. Among the immobilized biochars used, the immobilization capacity of the biochars under the optimal conditions was as follows: RS > SS > PM > BB. Biochars with high N contents, large pore sizes and high %WHCs were more suitable supports for immobilization. Compared with biochar alone or free strains, bacteria-immobilized materials were more efficient at removing PAHs due to simultaneous sorption and biodegradation processes. IMRS and IMPM, combined with the high moisture content and the moderate biostimulant salicylic acid or surfactant Brij 30, can effectively increase the functional microbial abundance and soil enzyme activities in aged PAH-contaminated soil, thereby enhancing the removal effect. IMRS, with higher immobilization efficiency showed, resulted in better removal of soil PAHs than IMPM did, with a maximum removal rate of 56.7 ± 11.33 % within 14 days.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A Supplementary data

Supplementary data associated with this article can be found in the online version at xxxxxx.

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Table 1 Code and level of variables of four factors in Orthogonal Experiment Design

Factor	(A) Soil-water ratios (*w*/*V*)	(B) Inoculation	(C) Salicylic acid (g/kg)	(D) Surfactant Brij 30 (g/L)
Level 1	2:5	FS	0	0
Level 2	1:5	FS+RS/PM	2	5
Level 3	1:10	IM	5	10

FS: Free strain; IM: Immobilized material.

Table 2 Physicochemical properties of the biochars

Biochar	PM	BB	RS	SS
pH	9.76 ± 0.10	10.67 ± 0.04	10.03 ± 0.03	10.42 ± 0.03
C (%)	17.30 ± 0.80	78.13 ± 0.51	47.62 ± 0.23	67.57 ± 0.62
N (%)	0.80 ± 0.03	0.71 ± 0.05	1.47 ± 0.00	1.04 ± 0.01
C/N	21.72	110.64	32.29	65.26
%WHC (w/w)	288.18 ± 11.37	651.44 ± 6.81	915.1 ± 83.17	727.41 ± 7.01
BET surface area (m^2/g)	22.33	1.17	2.98	2.04
pore volume (cm^3/g)	3.50×10^{-2}	8.18×10^{-4}	6.41×10^{-3}	3.30×10^{-3}
pore diameter (nm)	6.27	2.80	8.59	6.47

PM, BB, RS and SS represent pig manure biochar, bamboo biochar, rice straw biochar and soybean straw biochar, respectively

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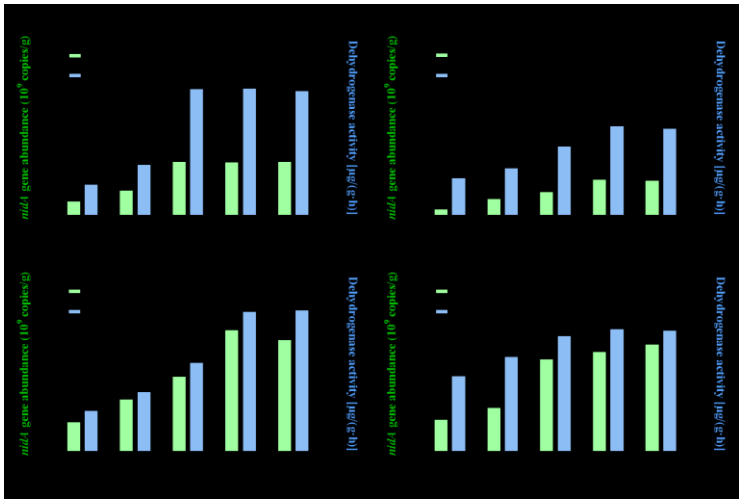

Fig. **1** Effects of the biochar dosage to cell suspension ratio (w/v) on the immobilization capacity of different biochars. (a) pig manure biochar (PM); (b) bamboo biochar (BB); (c) rice straw biochar (RS); (d) soybean straw biochar (SS). The lowercase and uppercase letters in the figure show the differences in the abundance of *nidA* gene and dehydrogenase activity, respectively ($P < 0.05$).

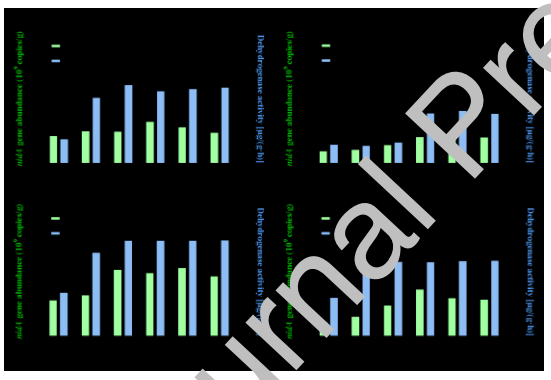

Fig. 2 Effects of immobilization time on the immobilization capacity of different biochars. (a) pig manure biochar (PM); (b) bamboo biochar (BB); (c) rice straw biochar (RS); (d) soybean straw biochar (SS). The lowercase and uppercase letters in the figure show the differences in the abundance of *nidA* gene and dehydrogenase activity, respectively ($P < 0.05$).

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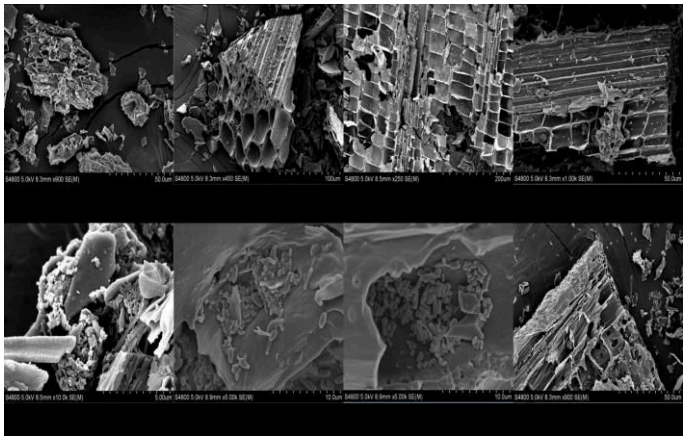

Fig. 3 SEM images of different biochars (a1-d1) and immobilized samples collected after incubation for 24 h (a2-d2). IMPM, PM for immobilized bacteria; IMBB, BB for immobilized bacteria; IMRS, RS for immobilized bacteria; IMSS, SS for immobilized bacteria.

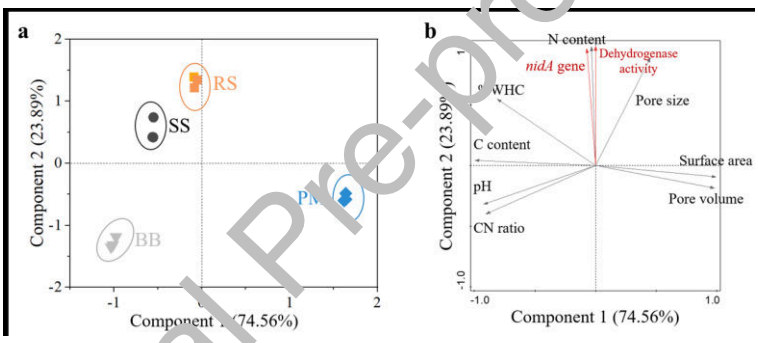

Fig. 4 Principal component analysis of the physicochemical properties of the biochars and their immobilization capacity.

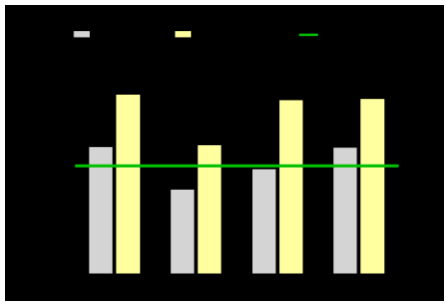

Fig. 5 Removal rates of PYR by the free strain, different biochars and immobilized materials in solution. The different letters indicate significant differences between treatments ($P < 0.05$).

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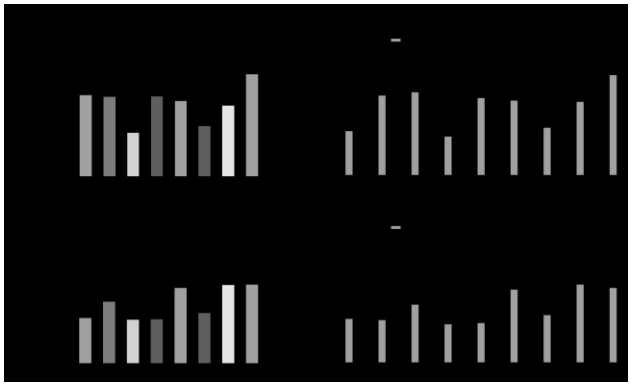

Fig. 6 Removal rates of (a) total PAHs and (b) LMW and HMW PAHs in soil after 24 d of various RS treatments. Removal rates of (c) total PAHs and (d) LMW and HMW PAHs in soil after 24 d of various PM treatments. The lowercase and uppercase letters show significant differences between treatments ($P < 0.05$).

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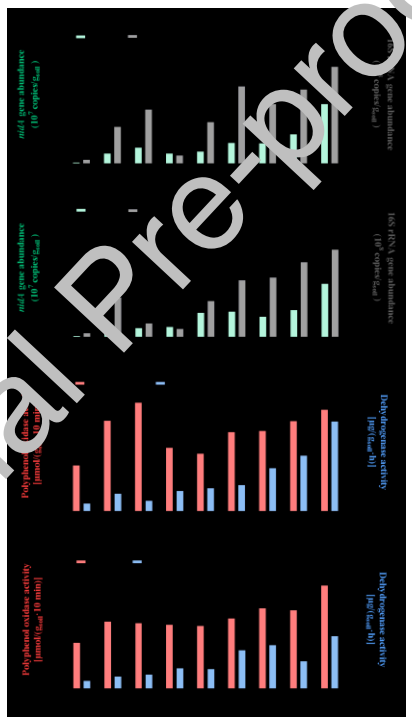
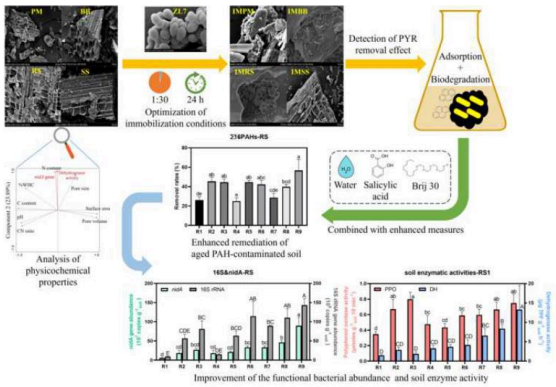


Fig. 7 Effects of all the treatments on the function genes and enzyme activities in soil. (a) the abundance of the *nidA* and 16S rRNA genes in the RS treatments; (b) the abundance of *nidA* and 16S rRNA gene in the PM treatments; (c) the polyphenol oxidase (PPO) and dehydrogenase (DH) activities in the RS treatments; (d) the PPO and DH activities in the PM treatments. The lowercase and uppercase letters indicate significant differences between treatments for different indicators ($P < 0.05$).

Graphical Abstract



Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Characterization and PAH removal performance of microbe-immobilized biochars derived from different feedstocks

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