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Bioremediation of Diesel-Contaminated Soil by Fungal Solid-State Fermentation

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Abstract

To address the poor removal of diesel in soil by indigenous microorganisms, we proposed a fungal solid-state fermentation (SSF) method for bioremediation. We screened *Pycnoporus sanguineus* 5.815, *Trametes versicolor* 5.996, and *Trametes gibbosa* 5.952 for their diesel-degrading abilities, with *Trametes versicolor* 5.996 showing the most promise. The fungal inoculum was obtained through SSF using wood chips and bran. *Trametes versicolor* 5.996 was applied to two treatments: natural attenuation (NA, diesel-contaminated soil) and bioremediation (BR, 10% SSF added to diesel-contaminated soil). Over 20 days, NA removed 12.9% of the diesel, while BR achieved a significantly higher 38.3% degradation rate. BR also increased CO_2 and CH_4 emissions but reduced N_2O emissions. High-throughput sequencing indicated SSF significantly enriched known diesel-degrading microorganisms like Ascomycota (83.82%), Proteobacteria (46.10%), Actinobacteria (27.88%), Firmicutes (10.35%), and Bacteroidota (4.66%). This study provides theoretical support for the application of fungal remediation technology for diesel and improves understanding of microbiologically mediated diesel degradation and soil greenhouse gas emissions.

Keywords Bioremediation · Diesel-contaminated soil · Greenhouse gas · Solid state fermentation · White rot fungal

Diesel, a volatile fuel derived from crude oil, enters the environment during various stages, disrupting soil and posing ecological risks (Logeshwaran et al. 2018). The railroad refueling station in Wegliniec, Poland, witnessed diesel contamination from 1970 to 2000, and natural attenuation processes were documented at the site (Sutton et al. 2013). 78% of soil contamination in São Paulo state is attributed to fuel spills originating from roadside gas stations. (Villa et al. 2010). In Madrid, Spain, a train maintenance facility faced diesel soil contamination due to leaking underground storage tanks, with soil TPH concentrations reaching approximately 5000 mg/kg (Lominchar et al. 2018). Diesel is classified as a major health risk due to its carcinogenic components (Cogliano et al. 2011). Urgent remediation of diesel-contaminated soil is crucial.

Diesel-contaminated soils are often remediated using chemical oxidation processes such as Fenton's method (Villa et al. 2010), activated persulfate (Lominchar et al. 2018), and electrokinetic remediation (Marta Pazos et al. 2011). These techniques have the disadvantages of high energy consumption and high costs. Bioremediation, particularly in-situ methods, is a cost-effective, practical, and eco-friendly solution. Microbial inoculation and nutrient addition enhance diesel degradation. Fungi, like white rot fungi (WRF), are advantageous due to their extensive hyphal structures, enabling them to reach inaccessible areas (Gao et al. 2022). WRF's unique ligninolytic system and CYP450 enzymes are effective for petroleum hydrocarbon degradation (Daccò et al. 2020).

Current studies often use unrealistic conditions like synthetic media or sterilized soil (Yanto et al. 2017; Al-Hawash et al. 2019), which don't represent actual contaminated sites where exogenous fungi face competition from indigenous microorganisms. Solid-state fermentation (SSF) provides a natural habitat for fungi, allowing them to secrete enzymes

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that break down solid substrates and environmental pollutants effectively (Kaewlaoyoong et al. 2020).

Increasing greenhouse gas emissions due to global warming is a concern. Diesel contamination also affects soil greenhouse gas emissions. Monitoring these emissions serves as an indicator of microbial communities and remediation progress. Microorganisms produce CO_2 during organic matter consumption, reflecting their metabolic activity. Anaerobic bioremediation may produce CH_4, indicating the presence of methanogenic microorganisms. N_2O, a potent greenhouse gas, can result from denitrification. Traditional chemical remediation methods have high energy consumption and emissions detrimental to climate change mitigation. Despite extensive studies on microbial communities and diesel biodegradation (Kundu et al. 2023; Yin et al. 2023), the impact of bioremediation on greenhouse gas (GHG) emissions and their link to microbial community changes remain unexplored.

This study aimed to remediate diesel-contaminated soil using white rot fungi and SSF, assess its impact on greenhouse gas emissions, and examine changes in microbial community structure. White-rot fungi (*Trametes versicolor* 5.996, *Trametes gibbosa* 5.952, and *Pycnoporus sanguineus* 5.815) were evaluated for diesel degradation. The most effective fungus was selected for remediation using SSF. Parameters related to diesel residues, greenhouse gas emissions, and relevant microbial flora were monitored, providing valuable insights into the relationship between greenhouse gas emissions, diesel degradation and microbial communities in the remediation process.

Materials and Methods

Soil samples from Beijing University of Civil Engineering and Architecture (39°45′N, 116°16′E) were sieved (0.25 mm aperture) to remove large particles. Diesel from a Beijing Sinopec station (density: 0.791 g/mL) was mixed into soil. After 15 days of aging, diesel concentration was 5592.98 mg/kg, soil pH was 8.16, and total organic carbon (TOC) was 0.985%. TOC was determined by an element analyzer (Vario EL cube, Elementar, Germany) (Liu et al. 2019). Soil retained indigenous microorganisms.

Fungal strains (*Pycnoporus sanguineus* 5.815, *Trametes versicolor* 5.996, *Trametes gibbosa* 5.952 were sourced from the China General Microbiological Culture Collection Center. They were maintained at 4°C on potato dextrose agar. Liquid fermentation methods were used to assess their degradation capacity (Wen et al. 2011). Wood chips and bran were obtained from a Chinese agricultural waste processing plant for white rot fungus solid-state fermentation (SSF). The SSF method for white rot fungus is as follows: 55% wood chips, 40% bran, 1% glucose, 2.5% $(NH_4)_2SO_4$, 0.48% $CaCl_2$, 0.5% KH_2PO_4, 0.5% $MgSO_4{\cdot}7H_2O$, 0.02% $CuSO_4$, and sufficient water to achieve a moisture content of 60%. Wood chips are untreated fragments of pine wood, produced through mechanical cutting or grinding, while bran is a byproduct of grain husks, rich in fiber content. After autoclaving and inoculation with white rot fungi, it was cultured at 26°C until white mycelium colonized the substrate for soil remediation experiments.

After 4 days of liquid fermentation, 0.9% (v/v) membrane-treated diesel was introduced to the culture system of three white rot fungi. They were degraded over 7 days at 30°C and 140 rpm. Soil remediation tests used 50 g dry-weight contaminated soil in 250 mL brown bottles. The control (natural attenuation: NA) had only contaminated soil, while bioremediation (BR) had 10% (w/w) SSF. All had 20% moisture content and were incubated at 26°C for 20 days. Residual diesel in the soil was sampled at intervals (0, 3, 6, 10, 15, and 20 days) using a destructive method for accurate measurement.

The concentration of diesel was quantified in terms of the total petroleum hydrocarbons (TPH). The diesel extraction process involves taking 10 mL of petroleum ether (60–90°C) and adding it to the respective sample, which is 50 mL of liquid fermentation broth or 2 g of soil. For liquid fermentation broths, after shaking and allowing it to stand for 2 min, transfer the upper organic layer to a 25 mL cuvette. Repeat this process twice to bring the combined extraction solution to 25 mL. In the case of soil samples, after sonication at 170 W for 40 min, the resulting extract is separated into a 25mL cuvette with a stopper. This operation is also repeated twice, and the extracts are combined and adjusted to 25mL. The recovery rate of diesel from soil was 87.1%±2%. TPH concentration in the extract was analyzed using UV–Vis spectroscopy at 225.00 nm (Huang et al. 2021). The removal of TPH from diesel fuel was calculated according to Eq. 1:

$$TPH\ removal = \frac{C_0 - C_1}{C_0} \times 100\% \quad (1)$$

where C_0 is the initial TPH content in diesel and C_1 is the final TPH content after diesel degradation in the liquid medium/soil.

Weigh 10 g of soil and add 25 mL of distilled water that has been boiled and cooled. Seal the flask with a film, shake vigorously for 2 min, let it stand for 30 min, and then measure the soil pH. Soil pH was measured using a potentiometer (PHS-25, Rex Electric Chemical, China).

The experiment included US (uncontaminated soil), US + WRF (uncontaminated soil with 10% SSF), NA, and BR. A 50 g soil sample was packed into a 60 mm diameter by 135 mm high 200 mL glass bottle with a permeable membrane. The soil in each glass bottle was moistened to approximately 20% water holding capacity by adding

distilled water. Soil samples were incubated in a dark climate chamber at 26°C for 14 days. Gas emissions were measured for each sample on alternate days. The lids were sealed before sampling, and gas samples were obtained using a 25 mL gas-tight syringe at 0 and 5 h of incubation (Sial et al. 2019). GHG (CO_2/CH_4/N_2O) concentration was measured by gas chromatograph (Agilent Technology 8890, USA). The data was transferred into an Excel sheet with calculated gas emission rates and cumulative emissions.

DNA was extracted from NA and BR soil after 20 days, using a soil DNA extraction kit (SPIN easy DNA Kit for Soil). DNA concentration was measured with a NanoDrop. Bacterial 338F_806R and fungal ITS1F_ITS2R were amplified, 338 F: ACTCCTACGGGAGGCAGCAG, 806R: GGACTACHVGGGTWTCTAAT, ITS1F: CTTGGTCATTTAGAGGAAGTAA and ITS2R: GCTGCGTTCTTCATCGATGC were used for pyrosequencing. Polymerase Chain Reaction (PCR) products were sequenced using an Illumina MiSeq PE300 platform and analyzed on Majorbio Cloud Platform (www.majorbio.com). The sequences in this study were deposited in the NCBI Sequence Read Archive database under the PRJNA975535 (https://www.ncbi.nlm.nih.gov/sra/PRJNA975535).

All treatments were replicated three times, and the results are presented as mean ± standard deviation (SD). The data were subjected to normality testing using SPSS, which indicated that $P > 0.05$. Analysis of variance (2way-ANOVA) was conducted using GraphPad Prism 7.04, and statistical significance was considered at $P < 0.05$. This analysis aimed to evaluate the differences between groups at various time points.

Results

All three white rot fungi effectively reduced diesel concentrations (Fig. 1). *Trametes versicolor* 5.996 was the most efficient, degrading 65.1% of diesel within 7 days, while *Trametes gibbosa* 5.952 initially eliminated 35.43% in 5 days but slowed later.

The contaminated soil underwent treatment with *Trametes versicolor* 5.996-containing SSF, and TPH levels were monitored over time (0, 3, 6, 10, 15, and 20 days) using UV spectrophotometry. Bioremediation (BR) with SSF and indigenous microorganisms significantly outperformed natural attenuation (NA) (Fig. 2). After 20 days, BR reduced TPH from the initial 5592.98 mg/kg to 3451.89 mg/kg (38.3% diesel degradation), a 25.4% improvement over NA. Initially, diesel degradation was delayed, followed by a gradual increase, and more effective degradation from day 10. Soil pH consistently increased in both strategies, with SSF addition lowering pH compared to the original contaminated soil.

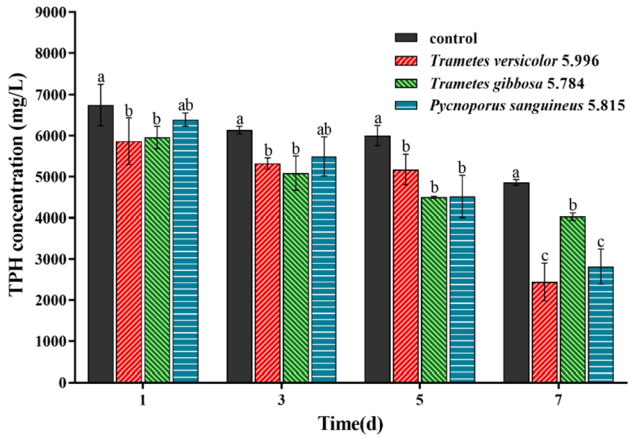

Fig. 1 The TPH in diesel degradation status by different white rot fungi at 30°C and 140 rpm for 7 days. The data are the mean of three replicates. The error bars represent standard deviations of the mean for triplicates. Different letters in the same day represent a significant difference at $P < 0.05$

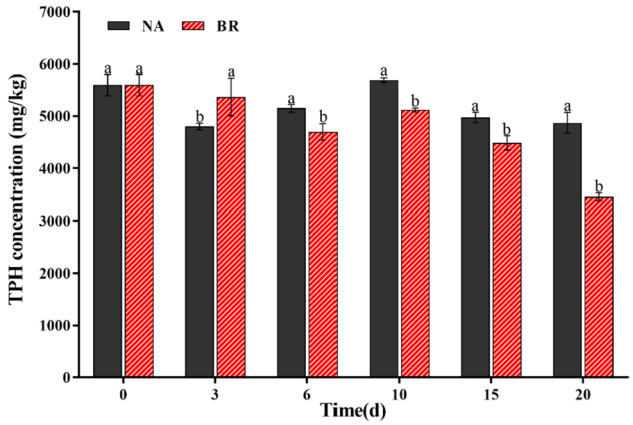

Fig. 2 The degradation efficiencies of TPH in diesel in natural attenuation (NA) and bioremediation (BR) treatments at 26℃ for 20 days. The data are the mean of three replicates. The error bars represent standard deviations of the mean for triplicates. Different letters in the same day represent a significant difference at $P < 0.05$

Diesel and SSF noticeably increased soil CO_2 emission rates (Fig. 3a). In SSF-treated groups (BR, US + WRF), rates peaked on day 2 and stabilized after day 6. CO_2 emission in untreated soil (US + WRF) was slightly lower than in diesel-contaminated soil (BR) from day 4. BR had nearly five times higher CO_2 emissions than NA, with BR's cumulative CO_2 production 9.5 times greater (Fig. 3b). SSF in uncontaminated soil increased N_2O emissions, with the highest cumulative production occurring in US + WRF (Fig. 3c, d). CH_4 emissions in BR and US + WRF increased after 8 days with SSF inoculation. By day 14, BR had 5.1 times higher CH_4 emissions than US + WRF, with cumulative CH_4 production 5.4 times

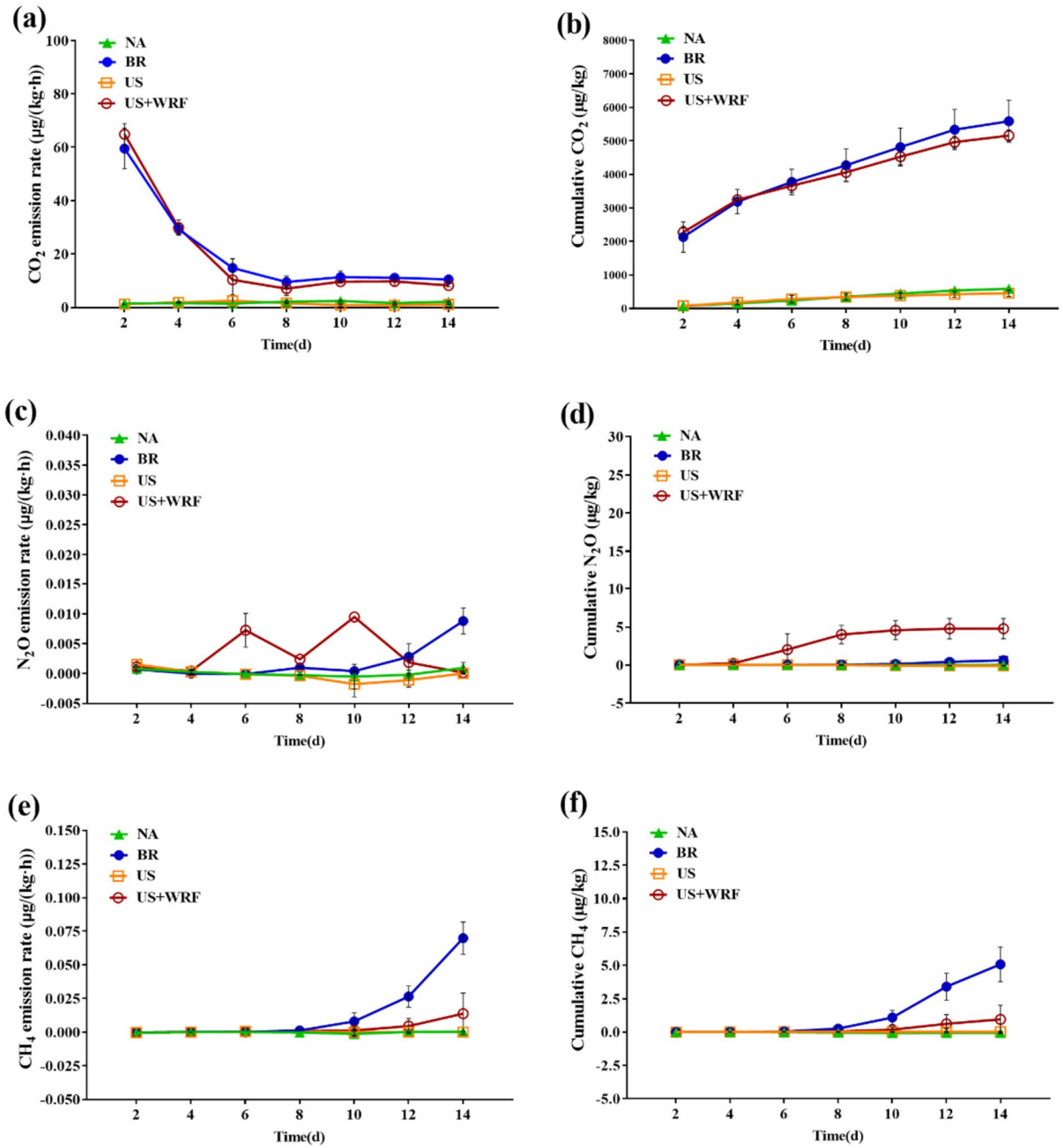

Fig. 3 Greenhouse gas emissions rate and cumulative greenhouse gas production in contaminated soil during 14 days of incubation at 26℃ using different bioremediation strategies.NA, natural attenuation; BR, bioremediation; US, uncontaminated soil; US + WRF, uncontaminated soil + SSF. **a** CO_2 emissions rate **b** Cumulative CO_2 production **c** N_2O emissions rate **d** Cumulative N_2O production **e** CH_4 emissions rate **f** Cumulative CH_4 production. The data are the mean of three replicates. The error bars represent standard deviations of the mean for triplicates

greater in BR. Emissions remained stable in treatments without SSF (Fig. 3e, f).

Alpha diversity was evaluated using Chao for richness and Shannon for diversity. Bacterial richness and diversity decreased significantly in BR compared to NA. Fungal community abundance was similar, but BR had lower fungal diversity, especially after SSF inoculation (Fig.S2).

In the NA soil, we detected 2373 bacterial ASVs, with the top five phyla being Proteobacteria (31.61%), Actinobacteria (29.36%), Acidobacteriota (13.96%), Patescibacteria (8.51%), and Chloroflexi (6.09%). Dominant genera included *Ramlibacter* (4.12%), *RB41* (3.66%), *Nocardioides* (3.39%), and *Norank_f_LWQ8* (3.30%). BR exhibited significant microbial community changes, with

Proteobacteria, Firmicutes, and Bacteroidota increasing to 46.10%, 10.35%, and 4.66%, respectively. Actinobacteria remained dominant, while *Bacillus* and *Luteimonas* notably increased. Conversely, *Ramlibacter* and *Norank_f_LWQ8* decreased (Fig. 4). Regarding fungi, we detected 405 fungal ASVs in NA, with Ascomycota (44.73%), Mortierellomycota (28.45%), Basidiomycota (14.32%), and unclassified_k_fungi (11.41%) as the main phyla. During degradation, Ascomycota (83.82%) and unclassified_k_fungi (12.88%) increased, while Mortierellomycota (1.42%) and Basidiomycota (1.76%) decreased significantly. At the genus level in NA, *Mortierella* (26.03%), *Neocosmospora* (12.10%), *unclassified_k_fungi* (11.41%), *Lepiota* (8.07%), and *Scytalidium* (4.64%) were dominant. In BR, *Chaetomium* (58.59%) became dominant, while *Mortierella*, *Scytalidium*, and *Lepiota*, dominant in NA, were significantly reduced by bioremediation. Basidiomycota genera, particularly *Lepiota*, *Cystofilobasidium*, and *Tausonia*, showed significant changes, with *Trametes* becoming dominant in BR, differing from NA (Fig. 5).

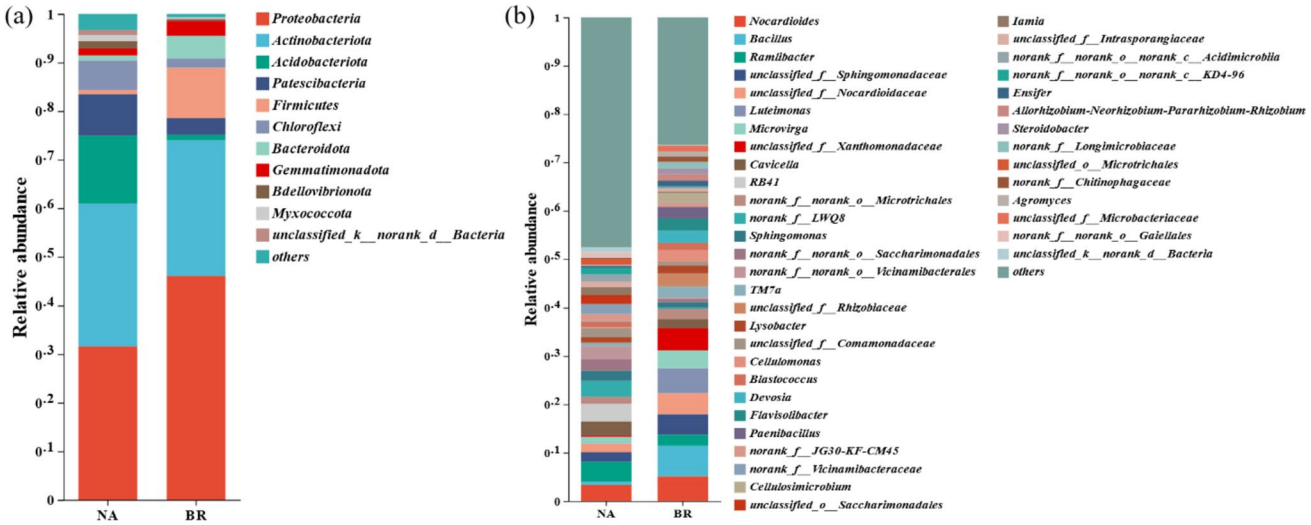

Fig. 4 Relative abundances of bacteria in contaminated soils under different bioremediation strategies after 20 days of incubation. NA, natural attenuation; BR, bioremediation. **a** Phylum **b** Genus

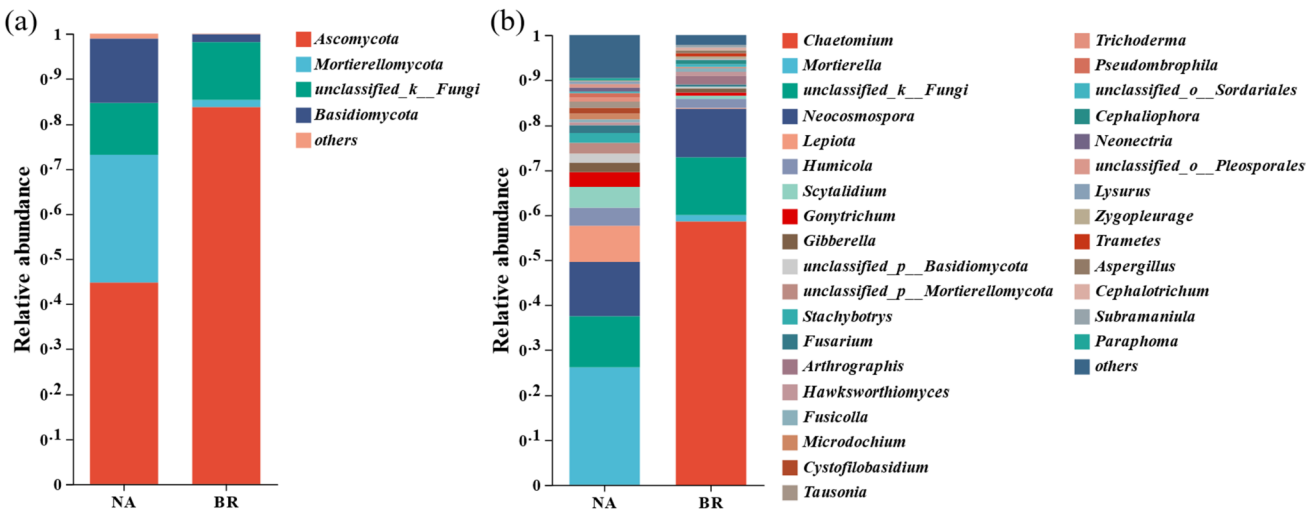

Fig. 5 Relative abundances of fungi in contaminated soils under different bioremediation strategies after 20 days of incubation. NA, natural attenuation; BR, bioremediation. **a** Phylum **b** Genus

Discussion

White rot fungi are of interest for their wide range of adaptability, efficiency, eco-friendliness, and self-adaptability in the degradation of diesel. This study demonstrates that *Trametes versicolor* 5.996 has a significant advantage in diesel degradation. *Trametes versicolor* 5.996 can degrade different components of petroleum hydrocarbons through the cytochrome P450 oxidase and lignin hydrolase systems (Zhuo and Fan 2021).

Agricultural waste effectively lowers soil pH contaminated by petroleum hydrocarbons because of acidic functional groups such as carboxyl and nitro groups present in agricultural waste (Bilal et al. 2020). *Trametes versicolor* 5.996 supplements soil with secondary metabolites, serving as an extra carbon source for microorganisms (Daccò et al. 2020). Solid-state fermentation (SSF) introduces foreign microorganisms and agricultural waste as nutrients, enhancing microbial activity for total petroleum hydrocarbon (TPH) degradation (Bao et al. 2022). Nonetheless, SSF-based bioremediation faces a delay during the adaptation period.

Bioremediation alters soil greenhouse gas emissions. Diesel degradation via aerobic and anaerobic pathways emits CO_2 and CH_4. Adding SSF boosts CO_2 emissions due to higher soil organic carbon, promoting methanogenic bacteria and anaerobic diesel degradation. CH_4 emissions are notably higher in petroleum-contaminated soils (Yang et al. 2018) as diesel adsorption limits soil respiration, fostering anaerobic conditions and methanogenic bacteria. This stimulates diesel-degrading and methanogenic bacteria, elevating CH_4 emissions. SSF plays a crucial role, as the NA group lacked this effect. Higher CO_2 and CH_4 emissions in contaminated soils suggest diesel metabolism. Diesel contaminants inhibit N_2O emissions, linked to nitrification-denitrification processes. Functional genes associated with N_2O emissions may decrease in hydrocarbon-contaminated soils (Yang et al. 2018).

In this study, fungi played a crucial role in reshaping microbial communities and population densities. Indigenous microorganisms were pivotal for soil restoration, working in synergy with introduced strains. Notably, bacterial and fungal compositions differed significantly between NA and BR, impacting diesel degradation efficiency. The addition of SSF brought about substantial changes in the microbial community of contaminated soil, creating a more conducive environment for diesel degradation. BR exhibited a notable enrichment and dominance of renowned diesel-degrading bacteria, including Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidota, well-known for biodegrading hydrocarbons (Wang et al. 2021). Proteobacteria, the dominant phylum in contaminated sites, played a significant role in diesel removal (Ehiosun et al. 2022). Actinobacteria were the primary contributors to CH_4 production, followed by Firmicutes (de Sousa Pires et al. 2021). Bacteroidota, essential for petroleum hydrocarbon degradation, thrived in the locally formed anaerobic soil microenvironment due to oxygen depletion and nutrient availability, promoting diesel degradation. Elevated cumulative CH_4 emissions in the BR group were likely linked to the enrichment of Actinobacteria, Firmicutes, and Bacteroidota. At the genus level, *Nocardioides* efficiently adapted to diesel-induced stress and acted as a proficient diesel degrader. *Bacillus* secreted biosurfactants (Cerqueira et al. 2012), while *Luteimonas* effectively degraded aliphatic compounds (Ling et al. 2023), contributing to diesel degradation. These microorganisms are commonly found in petroleum hydrocarbon environments due to their specific degradation pathways. Post-bioremediation, diesel degrades remained enriched and dominant in contaminated soils, indicating the effectiveness of the bioremediation process.

After SSF addition, fungal diversity decreased, and Ascomycetes abundance increased. Ascomycetes and Basidiomycota, effective in degrading aliphatic compounds, are common in petroleum-contaminated sites (Andreolli et al. 2015; Daccò et al. 2020). Ascomycetes and Basidiomycota were initially present, but Ascomycetes, particularly *Chaetomium*, dominated (58.59%) after *Trametes versicolor* 5.996 inoculation. *Chaetomium*, likely diesel-tolerant, maybe the primary fungal diesel degrader, a rare finding in previous studies. During bioremediation, most Basidiomycota genera declined or became inactive. *Trametes* in BR differed from NA, likely the introduced *Trametes versicolor* 5.996. Even after 20 days, it remained predominant among Basidiomycota, showing remarkable environmental adaptability. SSF effectively sustained its activity. *Trametes versicolor* 5.996, in conjunction with SSF, fostered diesel-degrading bacteria proliferation. Indigenous microorganisms likely synergized with it for diesel degradation (Li et al. 2021).

In this study, *Trametes versicolor* 5.996 demonstrated promise in the remediation of diesel-contaminated soil. However, it still presents challenges in practical engineering applications. As an exogenous microorganism introduced through inoculation, *Trametes versicolor* 5.996 exhibits slower growth compared to bacteria and encounters difficulties in colonization. In this 20-day study, we aimed to simulate short-term diesel-contaminated soil remediation using *Trametes versicolor* 5.996. It's essential to acknowledge that this short-term approach may not fully represent the temporal effects of fungal remediation over longer time scales. While we made every effort to replicate real contamination scenarios, there are still disparities with actual field conditions, leaving room for improvement in this study.

Conclusion

During the bioremediation of diesel-contaminated soil, the introduction of *Trametes versicolor* 5.996 significantly enhanced the degradation of diesel in the soil. This addition also acted as a stimulant for the proliferation of indigenous microorganisms, which predominantly included Ascomycetes (83.82%), Proteobacteria (46.10%), and Actinobacteria. This shift in the microbial community composition favored a more conducive environment for diesel removal. Moreover, the incorporation of *Trametes versicolor* 5.996 had an impact on the release of greenhouse gases from the soil. This study establishes a theoretical foundation for applying fungal remediation techniques in diesel-contaminated soils. It also yields novel insights into the interactions among bioremediation of diesel-contaminated soils, alterations in microbial communities, and greenhouse gas emissions. Future research should investigate alternative substrates to enhance the colonization rate of *Trametes versicolor* 5.996 in contaminated soils. Furthermore, our forthcoming studies will shift their focus to assess the long-term remediation potential of *Trametes versicolor* 5.996 in authentic diesel-contaminated sites. This approach will enhance our capacity to accurately anticipate the practical applications of fungal remediation.

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Declarations

Competing Interests The authors declare that they have no competing interests.

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