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Original Research

Biodegradation of PAHs by *Trametes hirsuta* zlh237 and Effect of Bioaugmentation on PAHs-Contaminated Soil

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Abstract

This study aimed to investigate the biodegradation of three distinct PAHs: Phenanthrene (Phe), Pyrene (Pyr), and Benzo[a]pyrene (BaP) by using fermentation broth from *T. hirsuta* culture medium. The results revealed that 2,5-xylidine ameliorates laccase production in *T. hirsuta* fermentation medium. After 6 days of incubation, *T. hirsuta* zlh237 fermentation broth (6-day-FFB) showed a higher degradation rate for three PAHs than 11-day fermentation broth (11-day-FFB). Final Phe, Pyr, and BaP degradation rates of 6-day-FFB were 89.35%, 85.92%, and 89.45%, respectively, on the 5th day of incubation. The PAHs biodegradation rate of 6-day-FFB in natural soil samples were lower than in sterilized soil. The Phe, Pyr, and BaP degradation rates of 6-day-FFB were 54.49%, 46.76%, and 51.93% in sterilized soil samples, and 39.37%, 34.00%, 33.99% in natural soil samples, after 15 days incubation, respectively. The high-throughput sequencing analysis revealed that the 6-day-FFB altered the bacterial community structure and enhanced microbial biodiversity of contaminated soil. The PAHs-contaminated soils incubated with 6-day-FFB showed significant increase in well color development than the controls. Principal Component Analysis of Biolog data differentiated the effect of 6-day-FFB and sterilized 6-day-FFB on contaminated soils. It implied that *T. hirsuta* zlh237 restored the microbiological functioning of the PAHs contaminated soils.

Keywords: biodegradation, microbial functional diversity, *Trametes hirsuta,* bioaugmentation

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are aromatic hydrocarbons derived from natural or anthropogenic sources and widely distributed in terrestrial and aquatic environments [1]. Natural PAH sources include forest and rangeland fires, oil seeps, volcanos, and tree exudates. A remarkable amount of PAHs are released from anthropogenic sources such as petroleum [2]. Apart from being ubiquitous toxic pollutants, PAHs are mutagenic as well as carcinogenic and pose a significant threat to the environment [3, 4].

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The PAHs are primarily degraded by the microbes; however, other degradation processes, such as adsorption, volatilization, photolysis, and chemical degradation, also play a substantial role [5, 6]. Interestingly, ligninolytic fungi, specifically white-rot fungi (WRF), degrade PAHs effectively due to their extracellular enzymes with low substrate specificity [7]. The degradation activity is dependent on the lignin-degrading system of these fungi. The ligninolytic system primarily comprises of laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and hydrogen peroxide generating oxidases. but the oxidation of PAHs by laccases is considered a critical step in this process [8].

Laccases (p-diphenol oxidase, EC 1.10.3.2) belong to a blue multi-copper oxidase group of enzymes widely distributed in higher plants, bacteria, fungi, and insects. Laccase catalyzes monoelectronic oxidation of a broad spectrum of substrates with the concomitant reduction of oxygen to water without the formation of harmful intermediates. Thus, laccases effectively treat or remove industrial and environmental pollutants [9] released due to textile dye transformation, food technological uses, biosensors, waste detoxification, PAHs degradation, and so on [10, 11]. All these characteristics make laccase an exciting option for the bioremediation of pollutants [12].

The genus *Trametes* of white-rot fungi is one of the most efficient lignin-degrading genera. *Trametes versicolor* degrades PAHs effectively. Previous reports have accounted for 12 distinct PAHs' oxidation by this fungus [13]. *Trametes hirsuta*, notably, is a promising candidate for laccase production [14]. The *T. hirsuta* culture under the optimal fermentation parameters, after an 3-day incubation period, demonstrated maximum laccase activity of 65.4 U/mL. *Trametes hirsuta* laccases have been used to decolorize and detoxify textile dyes [15]. *Trametes hirsuta* D7, a new fungus isolated from Indonesian peat swamp forest, capable of degrading phenanthrene, chrysene, and BaP. The strain showed good performance in the metabolism of phenanthrene [16].

In the current study, we investigated the fermentation broth from *Trametes hirsuta* zlh237 and PAH's degradation by *T. hirsuta* zlh237. Microbes in contaminated soil enhance the degradation process; however, the efficiency of this process depends on the composition and catabolic activity of bacterial communities [17]. The soil microbial diversity serves as a useful index for assessing the impact of pollution on soil health [18]. However, the changes in the microbial community structure and diversity that occur when laccase is added to contaminated soil for PAHs remediation remains unexplored. Therefore, in this study, we employed the Biolog plate technique to examine the bioaugmentation effect of *T. hirsuta* zlh237 inoculum on microbial activities in the PAH-contaminated soil. The altered microbial community structure, as a function of treatment type, was assessed using high-throughput sequencing. This study demonstrates the preliminary assessment of white-rot fungus (*T. hirsuta* zlh237) on PAH degradation.

Materials and Methods

Fungal Strains for Laccase Production

T. hirsuta zlh237 (CGMCC No. 11811) was screened from white-rot fungi cultures. *T. hirsuta* zlh237 culture was confirmed through the ITS internal sequencing analysis coupled with the morphological characterization of fungal cultures. This *T. hirsuta* zlh237 culture was used further in this study for laccase production and PAH biodegradation.

Liquid Culture Conditions for the Fermentation Broth Preparation

The *T. hirsuta* zlh237 fermentation medium was prepared using laccase liquid-state fermentation medium. The fermentation medium contained $NaH_2PO_4 \cdot 12H_2O$ (0.39 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), sodium succinate (1.18 g), $FeSO_4 \cdot 7H_2O$ (0.0315 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $MnSO_4 \cdot H_2O$ (0.035 g), $CH_3COONa \cdot 3H_2O$ (0.408 g), $CoCl_2 \cdot 6H_2O$ (0.06 g), $ZnSO_4 \cdot 7H_2O$ (0.028 g), $CuSO_4 \cdot 5H_2O$ (0.168 g), cornflour (40.0 g), ammonium tartrate (0.03 g), 2,5-dimethyl aniline (0.25 mL), vitamin B1 (10 µg), vitamin B2 (5 µg), vitamin B6 (5 µg) in 1 L of distilled water. Potato dextrose agar (PDA) was used to culture *T. hirsuta* zlh237 at 30ºC for 96 h in an incubator. 50 mL fermentation medium in the Erlenmeyer flask (250 mL) was inoculated with the five (1 mm × 1 mm*)* agar plugs from these PDA plates and incubated overnight at 30ºC with shaking at 180 rpm in the dark. Later, this medium was centrifuged at 15000 rpm at 4ºC for 5 min, and the supernatant obtained was treated as a crude laccase. 6 days (6-day-FFB) and 11 days (11-day-FFB) fungus fermentation broth was obtained by shake-flask fermentation without centrifugation.

Analysis of Laccase Activity

Spectrophotometric determination of laccase activity was performed by using 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate and absorbance of the oxidized product was measured at 420 nm. The assay mixture (2 mL) contained 500 µM ABTS (0.2 mL), 100 mM tartaric acid buffer (1 mL) (pH 4), water (0.78 mL), and 20 µL laccase enzyme. One unit of enzyme activity equates to enzyme quantity that oxidizes 1 µmol ABTS per min at 30ºC [19].

Biodegradation of PAHs Using the Fungal Fermentation Broth

Biodegradation of the three PAHs (Pyr, Phe, BaP) was performed using 6-day-FFB and 11-day-FFB. The laccase activity in the fermentation broth was 50 U/mL, and the initial concentration of all three PAHs was 150 mg/L. The required quantities of Pyr, Phe, or BaP was suspended in 1 mL of n-hexane and added to autoclaved brown glass vials. The n-hexane in these vials was evaporated under a fume hood, and later 3 mL FFB was added to these brown glass vials and incubated at 30ºC on a rotary shaker set at 180 rpm. During this degradation process, FFB was sampled at regular intervals to quantitate PAH concentration. Biodegradation of the five different concentrations (12.5, 25, 50, 100, and 150 mg/L) of each of the three PAHs was evaluated in the 6-day-FFB. All experiments were carried out in triplicates.

Preparation of PAHs-Contaminated Soil

The soil samples without PAHs (CK) for the experiment were collected from Zhengzhou, Henan province, northern China. The collected soil was air-dried and sieved (2 mm) to remove stones and plant debris. The physical and chemical properties of the soil were measured. Soil pH was 8.25. Soil organic matter (OM), alkali-hydrolyzable nitrogen, available potassium (AN) and available phosphorus (AP) contents were 24.1g/kg, 91.0 mg/kg, 57.0 mg/kg and 30.1 mg/kg, respectively. Soil was added to the brown glass vial and autoclaved at 121ºC for 20 min. These sterilized soil samples were spiked respectively with stock solutions of Pyr, Phe, and BaP in hexane to give nominal concentrations of 50 mg/kg soil. Then, the spiked soils were left under a fume hood for 48 h to allow hexane to evaporate. Finally, the contaminated soils were equilibrated below 25ºC for 2 weeks. The natural soil samples were not autoclaved.

Biodegradation of PAH in the Sterilized and Natural Soil Samples by *T. hirsuta* zlh237

Each of these sterilized soil samples (50 g each) was inoculated with 25 mL of 6-day-FFB, aseptically. Control microcosms were inoculated with 25 mL sterilized fermentation broth. The water holding capacity of the soil was adjusted to 60% by using sterile water. These samples were incubated at 30ºC for 15 days in the dark.

The natural soil samples (50 g each) were placed in a 15 cm diameter flowerpot. 25 mL of 6-day-FFB was inoculated to each of these artificially PAH-contaminated soil samples (PheBA, PyrBA and BaPBA). 25 mL of sterilized 6-day-FFB was aseptically inoculated to soil samples (PheCK, PyrCK and BaPCK), and it was treated as control. The water holding capacity of the soil was adjusted to 60% by using sterile water. These samples were incubated at 30ºC for 15 days in the dark.

PAH Extraction and Analysis

The residual Pyr, Phe, BaP in the vials were extracted with 7 mL n-hexane. The extracts were mixed first by vortexing for 10 min and then on a rotary shaker at 180 rpm for 30 min. This solution was allowed to stand for 30 min at room temperature, and later 1 mL upper organic phase was aliquoted for further analysis. PAHs in 1 g of bulk soil samples were extracted using Soxhlet extractor and n-hexane [20].

The concentrations of each PAH in the extracts were determined using HPLC analysis. Around 20 µL of PAHs extract was injected into an injection port (HPLC, Agilent technologies, 1200 series), equipped with a U.V. detector set to 254 nm (column temperature was 30ºC). The extract was separated using a ZORBAX SB-C18 column (0.46 mm in diameter and 150 mm in length). The mobile phase was an 80:20 (v/v) ratio acetonitrile: water mixture and the run was set to a flow rate of 1 mL/min.

The percentage of PAH degradation (D%) was calculated using the following formula:

$$D\% = 100 \times \left[\left(M_{\mathrm{i}} - M_s \right) \div M_i \right]$$

where M_s represents PAH concentration in each treatment, and M_i represents initial PAH concentration.

High-Throughput Sequencing and Biolog Biodiversity Analysis

DNA was extracted from the soil samples(0.5 g) using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions. The quality of DNA was checked by 0.8% agarose gel electrophoresis. The V3-V4 region of the bacteria 16S rRNA gene were amplified using 338f/806r primer. These primers contained a set of 8-nucleotide barcodes sequence unique to each sample. The amplicon mixture was applied to the MiSeq Genome Sequencer (Illumina, San Diego, CA, USA).

The extraction of high-quality sequences was firstly performed with the QIIME package (Quantitative Insights Into Microbial Ecology) (v1.2.1). Raw sequences were selected based on sequence length, quality, primer and tag. The raw sequences were selected and the low-quality sequences were removed. The unique sequence set was classified into operational taxonomic units (OTUs) under the threshold of 97% identity using UCLUST. Relative abundance (%) of individual taxa within each community was estimated by comparing the number of sequences assigned to a specific taxon versus the number of total sequences obtained for a sample. Alpha diversity indices (including No. of OTUs, Chao1 and Shannon) were calculated on a subset of 24,684 randomly selected sequences from each sample.

Soil microbial functional diversity and bacterial activity were examined using Biolog EcoPlates (Biolog, Hayward, CA, USA), as described by Liao et al. [21]. These plates test 31 kinds of C substrates [22]. Briefly, 10 g of fresh soil was added to 90 mL of sterilized saline water (0.9% NaCl w/v) in the 250 mL Erlenmeyer flask and shaken for 30 min at 28ºC and diluted to 10^{-3}. 125 µL of this diluted solution was inoculated into the Biolog EcoPlates and incubated at 25ºC for 7 days. Color development was measured as optical density (O.D.) at 590 nm with a microplate reader at regular 24 h intervals. Average well color development (AWCD) was used to evaluate microbial activity in each microplate. AWCD was calculated using the following equation: $AWCD = \Sigma OD_i/31$, where OD_i represents the optical density value of each well. Principal Component Analysis (PCA) was performed on Biolog data at 72 h using the Canoco software.

Data Analysis

Analysis of variance was performed using the SPSS software version 19.0 (IBM), and PCA was analyzed using the Canoco software version 4.5.

Results

Laccase Production from *T. hirsuta* zlh237

T. hirsuta zlh237 mycelium formed white cottony mass on the PDA plates at 30ºC after 4-5 days incubation. Mycelia appeared 2-3 days after inoculation and covered the entire plate after 4-5 days. Mycelia appeared dense and white floss shaped. The laccase production from *T. hirsuta* zlh237 reached its peak on the 11th day (Fig. 1) *T. hirsuta* zlh237 produced around 20 U/mL of laccase when cultured in fermentation medium without inducer, whereas fermentation medium supplemented with 2,5-xylidine as an inducer showed tripled laccase production (Fig. 1), which peaked to 59.8 U/mL on day 11. An increase in laccase activity intensified the red color of 2,5-xylidine supplemented fermentation medium. It suggests that the 2,5-xylidine supplemented fermentation medium might be correlated to laccase activity.

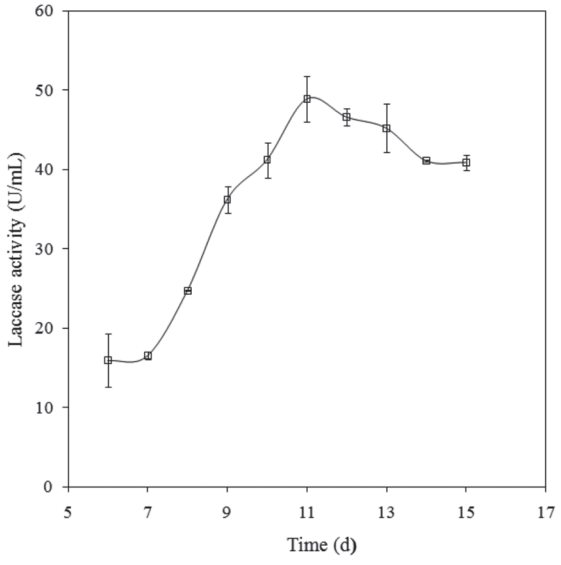

Fig. 1. *T.hirsuta* zlh237 laccase production profile when cultured in fermentation medium supplemented with 2,5-xylidine as an inducer. Data points represent the average of three replicates. Vertical bars represent standard errors.

Biodegradation of PAHs Using *T. hirsuta* zlh237 Fermentation Broth

The two different treatments, i.e., 6-day-FFB or 11-day-FFB, degraded Phe, Pyr, and BaP substantially during five days incubation period (Fig. 2). As shown in Fig. 2a), 6-day-FFB, and 11-day-FFB treatment decreased the Phe concentration to 6.92 and 13.94 mg/L, respectively, on the 5th day. The differences in the degradation rate between these two treatments were significant ($P<0.05$). It was significantly higher with 6-day-FFB as compared to 11-day-FFB on the 5th day. On the first day of incubation, we observed that 6-day-FFB had rapidly degraded Phe and Pyr; however, BaP was not degraded as effectively as Pyr and Phe (Fig. 2b-c). Around 50% of Phe and Pyr were degraded after one-day incubation. The maximum 6-day-FFB and 11-day-FFB treatment degradation rate for Phe, Pyr, and BaP was around 85% and 80%, respectively, on day 5. The degradation rate of 6-day-FFB treatment for Phe, Pyr, and BaP was significantly higher than 11-day-FFB. Overall, the results suggest a high degradation efficiency of 6-day-FFB as compared to 11-day-FFB.

Fig. 3 depicted the effects of 6-day-FFB on Phe, Pyr, and BaP biodegradation rate. The initial PAHs concentrations ranged from 12.5-150 mg/L with different biodegradation rates. The higher Phe, Pyr, and BaP concentration resulted in a lower degradation rate. 96.45%, 89.42%, 84.29%, 74.49%, 52.33% of Phe, and 80.35%, 80.13%, 64.38%, 63.20%, 62.27% of BaP were degraded in the Phe and BaP contaminated samples with initial concentrations of 12.5, 25, 50, 100, and 150 mg/L, respectively (Fig. 3). The different concentrations of Phe and BaP demonstrated more than 50% degradation rates. The degradation rate of different Pyr concentrations, i.e., 12.5, 25, 50, 100, and 150 mg/L were 86.33%, 82.33%, 83.18%, 48.42%, and 35.98%, respectively. The outcomes of our analysis indicate that initial PAH concentration influences the biodegradation rates.

PAHs Biodegradation by *T. hirsuta* zlh237 in Sterilized and Natural Soil Samples

T. hirsuta zlh237 increased PAH biodegradation when 6-day-FFB was added to PAH contaminated soil samples from Henan province. This study evaluated

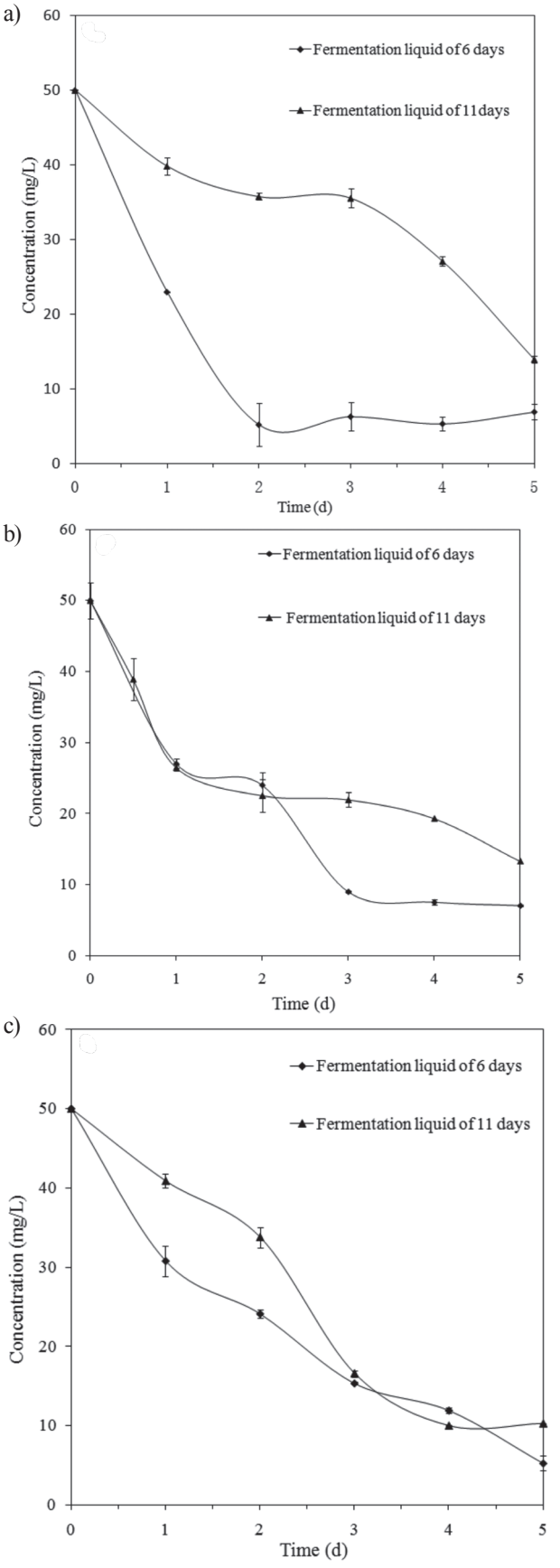

Fig. 2. Biodegradation of the three distinct PAHs (a: Phe, b: Pyr, c: BaP) by *T.hirsuta* zlh237' 6 days (6-day-FFB) and 11 days (11-day-FFB) fermentation broth. Data points represent the average of three replicates. Vertical bars represent standard errors.

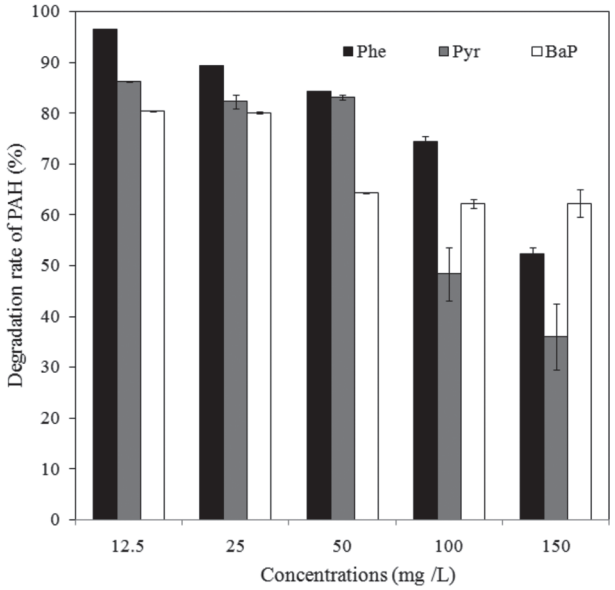

Fig. 3. Phe, Pyr, and BaP degradation rate after 3 days incubation (a, b, c, d, and e represent 12.5, 25, 50, 100, and 150 mg/L, respectively). Data points represent the average of three replicates. Vertical bars represent standard errors.

the degradation effect of 6-day-FFB on these contaminated soil samples. Fig. 4 demonstrated the biodegradation effect of PAHs on sterilized soil samples. Soil media influenced the degradation effect of PAHs as compared to the direct application of PAHs. We observed that approximately 54.49%, 46.76%, and 51.93% of Phe, Pyr, and BaP were degraded after 15 days of incubation, respectively (Fig. 4).

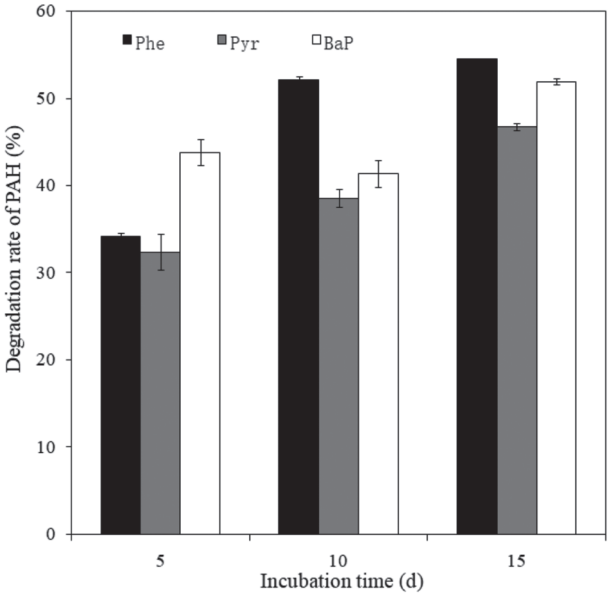

Fig. 4. Phe, Pyr, and BaP degradation rate of 6-day fermentation broth (6-day-FFB) in the sterilized soil after 5-, 10- and 15-days incubation. Data points represent the average of three replicates. Vertical bars represent standard errors.

Fig. 5 demonstrated the *T. hirsuta* zlh237 induced PAHs biodegradation effect on the natural soil in the laboratory-scale test. The degradation rate of Phe, Pyr, and BaP reached 39.37%, 34.00%, and 33.99% after 15-day treatment, respectively, as compared to control (Fig. 5).

Analysis of Microbial Community Composition and Functional Diversity

The taxonomic distribution at the genus level within different samples was determined, as depicted in Fig. 6. The bacterial diversity at genus level was found to be dramatically different in the PAHs contaminated soil samples incubated with sterilized 6-day-FFB and 6-day-FFB. The bacterial community of CK was mainly composed of *Sphingomonas*, *Skermanella*, and *Blastococcus*, *Roseiflexus*, *Gaiella*, *Solirubrobacter*, *Rubrobacter*, *Microvirga*, *Streptomyces*, *Pseudarthrobacter*, *Asanoa*, and *Blastococcus*. *Rubrobacter* was the most abundant genus in CK, with an average relative abundance of 7.88%. The relative abundance of *Phenylobacterium*, 7.44% and 6.45%, was found to be most in the Phe and Pyr contaminated soil samples, respectively, supplemented with sterilized 6-day-FFB (PheCK, PyrCK). The bacterial community structures changed significantly in the Phe and Pyr contaminated soil supplemented with 6-day-FFB (PheBA, PyrBA). *Sphingomonas* sp. accounted for 5.25% and 5.27% of relative abundances in PheBA and PyrBA, respectively. As per the average relative abundance, the dominant genera of BaP contaminated soil supplemented with 6-day-FFB (BaPBA) were *Sphingomonas*, *RB41*, *Skermanella*, *Blastococcus*, *Gaiella*, *Solirubrobacter*, *Rubrobacter*, *Microvirga*, *Streptomyces*, *Pseudarthrobacter*, and *Nocardioides*. The *Sphingomonas* was the most abundant genus in BaPBA, with an average relative abundance of 8.51%. Besides, the *Pseudomonas* showed the most relative abundance of 19.17% in the BaP soil supplemented with sterilized 6-day-FFB (BaPCK). In comparison, they accounted for 2.85% of the original soil CK, and 1.19% in PyrCK. Conversely, it was not detected in PheCK and BaPCK. *Phenobacillus* was found to be the dominant genus with relative abundances of 7.44% and 6.45% in the PheCK and PyrCK, respectively. *Pseudomonas* was the dominant genus in the BaPCK soil samples with a relative abundance of 19.17%.

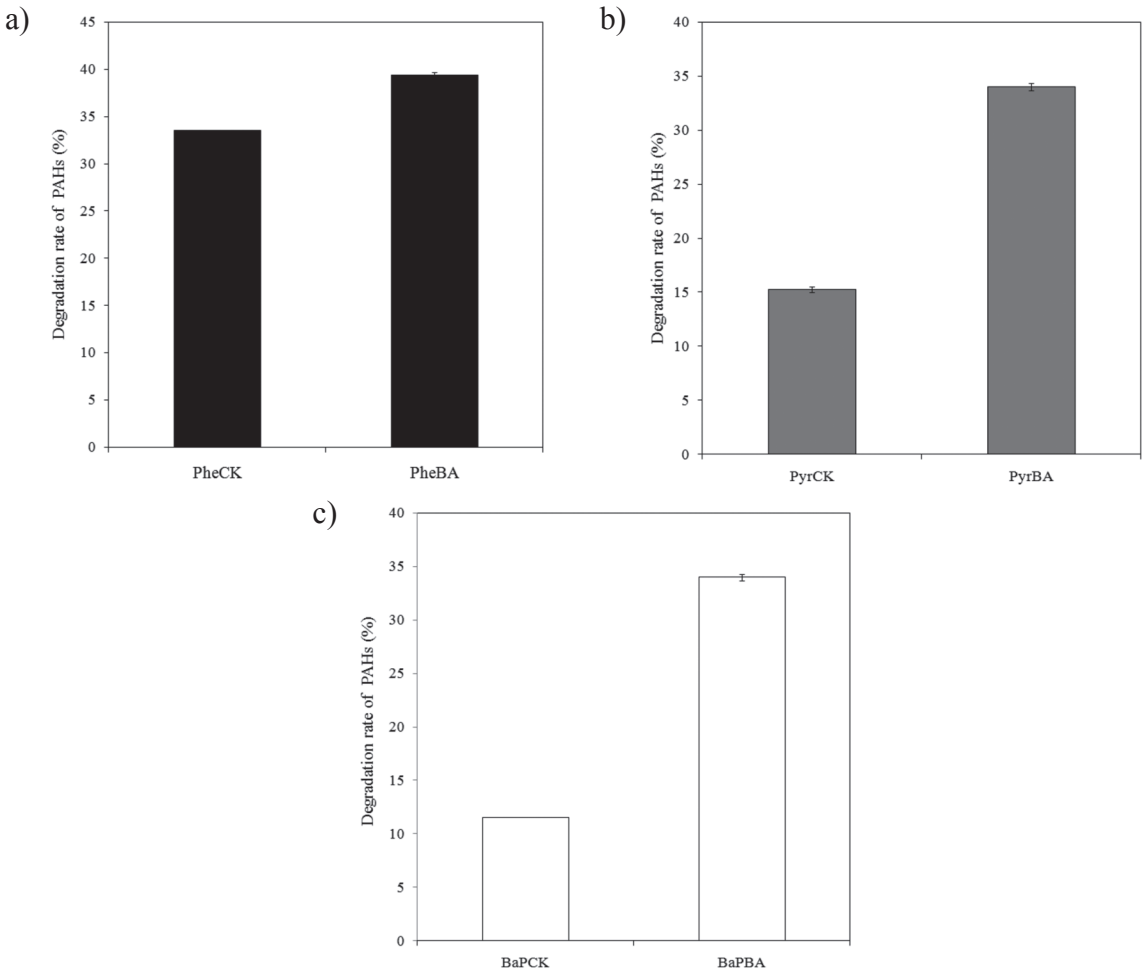

Fig. 5. a) Phe, b) Pyr, and c) BaP degradation rate of 6-day fermentation broth in the natural soil after 15 days incubation. Data points represent the average of three replicates. Vertical bars represent standard errors.

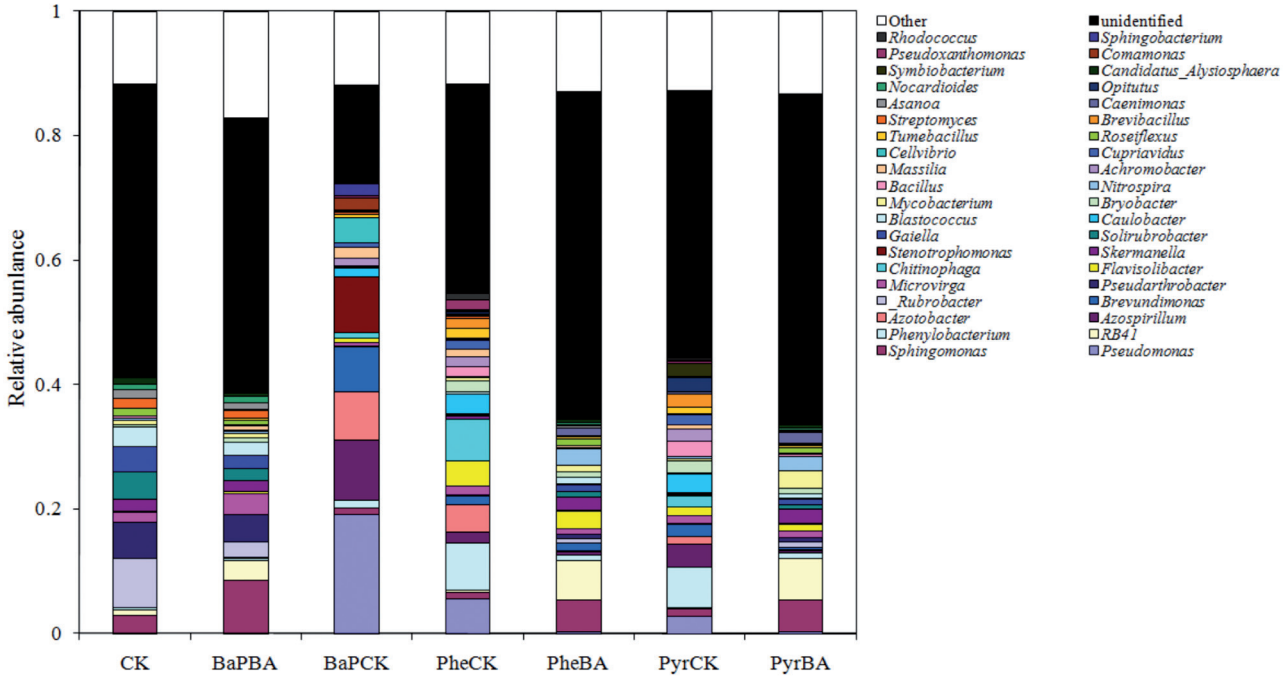

Fig. 6. Bacterial communities and distribution of the microbial genus in the soil samples.

The α-diversity indices of bacteria within different samples are shown in Table 1. After removing low-quality reads, 37359, 28609, 29249, 20686, 37996, 12685, and 17974 bacterial sequences were obtained in the CK, PheBA, PheCK, PyrBA, PyrCK, BaPBA, and BaPCK samples, respectively. The coverage was higher than 96% for all the samples, indicating that the bacterial sequencing depths were rational and that more sequencing is likely to yield a few additional species.

The sequences were then classified into OTUs at a clustering threshold of 97% using the CD-HIT method. The results indicated that among all the samples, the bacterial OTU numbers were highest in the Phe and Pyr treatments.

The α-diversity indices were calculated using the mothur program. The highest values of the biodiversity indices, including Chao1 and Shannon, were obtained for the Phe, Pyr, and BaP contaminated soils supplemented with 6-day-FFB. It suggests that the microbial biodiversity of the contaminated soil treated with PheBA, PyrBA, and BaPBA microcosms was better than the other treatments. Thus, *T. hirsuta* zlh237 fermentation broth addition increased the α-diversity of the bacterial communities in PAH-contaminated soil.

Table 1. Effective number and α-diversity indices of soil microbial communities in different soil samples.

Soil samples	Final_tags	OTUs	Chao1	Shannon	Coverage rate
CK	37359	1498	1729.77±46.54b	7.97±0.16b	0.97
PheBA	28609	1178	2044.73±12.55a	8.66±0.23a	0.96
PheCK	29249	1765	1387.11±86.90c	6.85±0.23c	0.97
PyrBA	20686	970	2044.08±23.69a	8.62±0.09a	0.96
PyrCK	37996	1884	1307.63±112.80c	6.70±0.53c	0.97
BaPBA	12685	532	1941.91±26.12a	8.48±0.08a	0.98
BaPCK	17974	1429	824.49±83.59d	5.42±0.26d	0.99

Note: CK: initial soil samples; PheCK: Phe-contaminated soil incubated with sterilized 6-day-FFB; PyrCK: Pyr-contaminated soil incubated with sterilized 6-day-FFB; BaPCK: BaP-contaminated soil incubated with sterilized 6-day-FFB; PheBA: Phe-contaminated soil incubated with 6-day-FFB; PyrBA: Pyr-contaminated soil incubated with 6-day-FFB; BaPBA: BaP-contaminated soil incubated with 6-day-FFB. Different lowercase letters in a column represented significant difference ($P<0.05$).

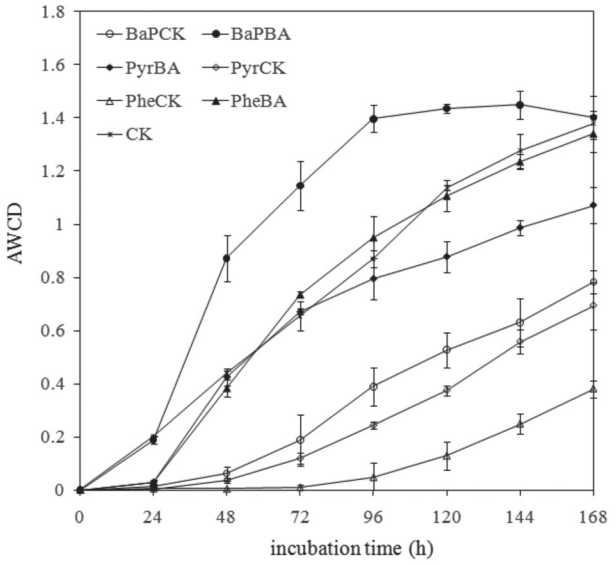

Fig. 7. AWCD for soil samples from the different treatments after 15 days of bioaugmentation. CK represents initial soil samples. Data points represent the average of three replicates. Vertical bars represent standard errors.

AWCD of soil samples with different treatments is represented in Fig. 7. The PAH-contaminated soil samples incubated with 6-day-FFB showed a higher AWCD than PAH-contaminated soil samples incubated with sterilized 6-day-FFB. BaPBA exhibited the highest AWCD. The results implied that the microbial communities in BaPBA, which was remediated by the *T. hirsuta* zlh237 strain, were metabolically more active as far as the utilization of carbon substrates is concerned. The AWCD values were BaPBA>PheBA>PyrBA in the treatments of PAH-contaminated soil supplemented with 6-day-FFB. The AWCD of PAH-contaminated soil supplemented with sterilized 6-day-FFB was relatively low.

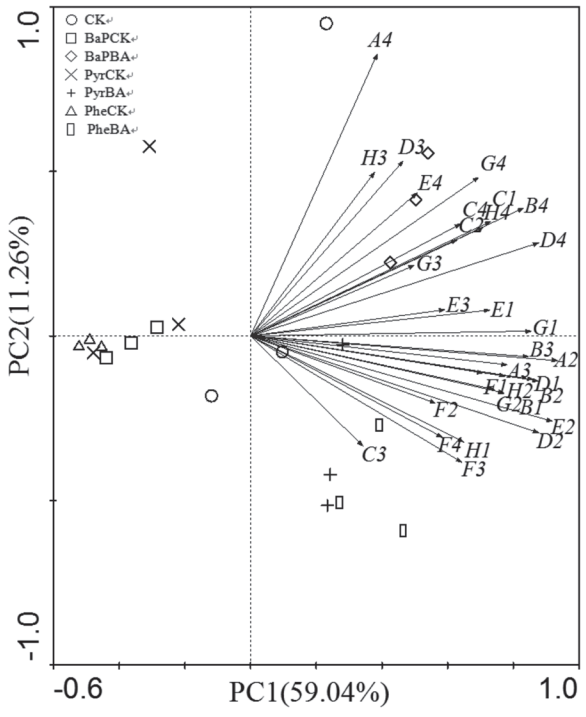

Fig. 8. Principal Components Analysis (PCA) of Biolog data at 72 h from soil samples after 15 days incubation. CK represents initial soil samples.

Principal Component Analysis (PCA) was performed to compare different treatments (Fig. 8). The first and second principal components (PC1 and PC2) were used to describe the information from 31 carbon sources. Fig. 8 demonstrates the separation of several locations: CK, BaPCK, PheCK, PyrCK, BaPBA, PheBA, PyrBA. PC1 and PC2 explained 59.4% and 11.2% of the variance in the functional diversity of carbon utilization. The BaPCK, PheCK, and PyrCK soil samples were clustered together, whereas PheBA and PyrBA were clustered together in another sector. The BaPBA samples were independently clustered in the chart, which suggests that the microbial community incubated with 6-day-FFB and sterilized 6-day-FFB were completely different. Soil microbial communities in BaPBA metabolized the substrates, such as 4-Hydroxybenzoic acid (D3), D-Malic acid (H3), L-Threonine (E4), phenylethylamine (G4), L-Phenylalanine (C4), a-Ketobutyric Acid (G3), i-Erythritol (C2), putrescine (H4), tween 40 (C1), L-Asparagine (B4), L-Serine (D4). Soil microbial communities from PheBA and PyrBA mainly utilized 2-Hydroxy Benzoic Acid (C3), itaconic acid (F3), glycyl-L-glutamic acid (F4), α-D-Lactose (H1), D-Galacturonic acid (B3), β-Methyl-D-glucoside as substrates. The samples incubated with 6-day-FFB showed increased utilization level of the substrates as compared to PAH-contaminated soil samples incubated with sterilized 6-day-FFB. PAH-contaminated soil samples supplemented with 6-day-FFB showed more extensive and higher metabolic activity than the soil samples supplemented with sterilized 6-day-FFB.

Discussion

The adeptness of fungal laccases in oxidizing a wide range of aromatic compounds makes them conspicuous determinants in the environmental bioremediation process [23]. Laccases from different microorganisms have been employed for biodegradation of PAHs, such as a yellow laccase from *Leucoagaricus gongylophorus* [24], CueO from *Escherichia coli* [25], and laccase from *Trametes villosa* [26]. Till now, prior studies had primarily focused on the degradation capacity of laccase determined by the shake flask method. In the current study, we investigated the effect of *T. hirsuta* zlh237' 6-day-FFB and 11-day-FFB in the PAHs degradation (Phe, Pyr, and BaP) in sterilized and natural soil samples. Changes in the microbial community structure in natural soil samples were also assessed.

The components in fungal culture medium, such as carbon, nitrogen, temperature, pH, and so on, influences laccase production. The addition of various supplements could increase the low yield of laccase. As reported in previous studies, 2, 5-xylidine [27], lignin [28], and veratryl alcohol induces the laccase activity. The outcomes of the current study demonstrated that 2, 5-xylidine supplementation ameliorated *T. hirsuta* zlh237 growth. Furthermore, an increase in laccase activity intensified the red color of 2,5-xylidine supplemented fermentation medium. It suggests that the 2,5-xylidine supplemented fermentation medium might be correlated to laccase activity. The previous report has accounted for a similar phenomenon in the *Pynoporus sanguineus* culture medium [27].

In the current study, we found that *T. hirsuta* zlh237 FFB retained its biodegradation capacity until day 5. It might be due to the active *T. hirsuta* zlh237 in brown bottles containing PAH. The *T. hirsuta* zlh237 started forming mycelium after 24 hours of incubation. Besides, we found that the degradation rate of PAHs by 6-day-FFB was much higher than that of 11-day-FFB. We speculated that it might be due to the presence of other extracellular enzymes, such as lignin peroxidase and manganese, dependent-peroxidases in 6-day-FFB. Previous studies have stated that these enzymes degrade PAHs efficiently [29]. Natural laccase mediators in the 6-day-FFB might be another reason behind the higher degradation rate of PAHs under lower laccase activity [30].

The outcomes of our analysis indicate that initial PAH concentration influences the biodegradation rates. The degradation rate of PAHs by 6-day-FFB of *T. hirsuta* zlh237 decreased as the initial concentration (of PAH) increased, which might be due to the toxicity of aromatic hydrocarbons [31]. In summary, *T. hirsuta* zlh237 emerged as a promising agent for PAH degradation. Further studies on the *T. hirsuta* zlh237 mediated degradation of PAH-contaminated soil would be of interest to ensure the efficacy of *T. hirsuta* zlh237 for soil bioremediation on a large scale.

In this study, we assessed *T. hirsuta* zlh237 mediated PAHs biodegradation in sterilized and natural soil samples supplemented with 6-day-FFB. The soil media influenced the degradation effect of PAHs. It might be due to the decreased microbial exposure [32] and oxygen concentration caused by the adsorption of PAH on the soil particles, which might have inhibited the laccase activity [33]. Moreover, soil temperature, moisture, redox potential, nutrient availability also hindered the PAH biodegradation [34].

In this study, we observed that the mycelial growth covered the entire soil surface after three days of incubation. It suggests that *T. hirsuta* zlh237 could remain active in the natural environment and compete with the autochthonous microbial population. The *T. hirsuta* zlh237 mediated PAH degradation rate in natural soil was lower as compared to the sterilized soil. It might be due to the complex biological structure of the soil that might have mitigated the inoculated microbial population by predation and antagonism from the autochthonous populations [35]. However, biodegradation by introduced fungus demonstrated a higher degradation rate than the natural rate of decontamination and natural attenuation. The degradation rate of Phe, Pyr, and BaP increased to 5.87, 18.73, 22.44%, respectively, as compared to control soil, after 15-day treatment with 6-day-FFB (Fig. 5). 6-day-FFB showed a better degradation effect on BaP.

The composition of bacterial genera in the community varied with different treatments. In the present study, *Sphingomonas* and *Bacillus* been characterized as potentially dominant genera and able to degrade PAHs in contaminated soils [36]. The role of *Sphingomonas* sp in the biodegradation of aromatic compounds, specifically its efficiency in the low-ring and high-ring PAHs biodegradation, was identified [37]. *Sphingomonas* sp. accounted for 5.25%, 5.27%, and 8.51% of relative abundances in Phe, Pyr, and BaP contaminated soil samples inoculated with 6-day-FFB, respectively. In comparison, they accounted for 2.85% of the original soil CK, and 1.19% in Pyr contaminated soil samples inoculated with sterilized 6-day-FFB. Conversely, it was not detected in Phe and BaP contaminated soil samples inoculated with sterilized 6-day-FFB. *Phenobacillus* was found to be the dominant genus with relative abundances of 7.44% and 6.45% in the Phe and Pyr contaminated soil samples inoculated with sterilized 6-day-FFB, respectively. *Pseudomonas* was the dominant genus in the BaP soil samples with a relative abundance of 19.17%. This study also found that the species and proportion of PAHs degrading bacteria were higher in the contaminated soil inoculated with sterilized 6-day-FFB as compared to 6-day-FFB, but with reduced microbial diversity. It might be due to stress created by high PAHs concentration, which might have hampered low-tolerant microbes due to their inability to adapt to the changing environment, resulting in reduced microbial diversity. Only a few microbial strains that were resistant to PAHs survived and became the dominant bacteria in the soil.

PAHs are toxic environmental pollutants, which negatively influences the microbial activity. Previous studies have investigated the metabolic response of microbial communities using Biolog EcoPlates [38]. The microbial community-level physiological profiles (CLPPs) serves as an apt indicator of the functional dynamics of microbiota in the soil. CLPPs revealed the response of the cultivable portion of the heterotrophic microbial community to carbon substrates. In this study, the physiological profiles of the microbial community indicated differences in functional diversity of soil microbial community when supplemented with sterilized 6-day-FFB and unsterilized 6-day-FFB after 15 days of the remediation process(Fig. 7). These outcomes indicate that the 6-day-FFB supplementation induced higher AWCD in PAH-contaminated soil samples and highest in BaP-contaminated soil samples

with the highest functional diversity in carbon substrate utilization. In conclusion, bioaugmentation with *T. hirsuta* zlh237 significantly restored the microbiological diversity of PAHs-contaminated soil.

Conclusions

6-day-FFB of *T. hirsuta* zlh237, a white-rot fungus, efficiently degraded the Phe, Pyr, and BaP in contaminated soil. The 6-day-FFB Phe, Pyr, and BaP degradation rate was 39.37%, 34.00%, and 33.99% in the natural PAHs-contaminated soil after 15-dayincubation. *T. hirsuta* zlh237 can increase soil microbiological activity and restore the microbial functioning of the PAHs-contaminated soil. Thus, the outcomes of this investigation suggested that *T. hirsuta* zlh237 is an excellent candidate with a promising bioremediation strategy for PAHs-contaminated soil.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Material

Table S1. The specific carbon sources in 6 groups from Biolog-Eco plate experiment.

No.	Carbon group	No.	Carbon source
1	Amines/amides	G4	Phenylethylamine
		H4	Putrescine
2	Amino acids	A4	L-Arginine
		B4	L-Asparagine
		C4	L-Phenylalanine
		D4	L-Serine
		E4	L-Threonine
		F4	Glycyl-L-glutamic acid
3	Carbohydrates		Pyruvic acid methyl ester
		H1	α-D-Lactose
		A2	β-Methyl-D-glucoside
		B2	D-Xylose
		C2	i-Erythritol
		D2	D-Mannitol
		E2	N-Acetyl-D-glucosamine
		G2	Glucose-1-phosphate
		H2	D,L-α-Glycerol phosphate
		G1	D-Cellobiose
4	Carboxylic acids	E3	γ-Hydroxybutyric acid
		F3	Itaconic acid
		G3	α-Ketobutyric acid
		H3	D-Malic acid
		F2	D-Glucosaminic acid
		B3	D-Galacturonic acid
		A3	D-Galactonic acid-γ-lactone
5	Miscellaneous	C3	2-Hydroxybenzoic acid
		D3	4-Hydroxybenzoic acid
6	Polymers	C1	Tween 40
		D1	Tween 80
		E1	α-Cyclodextrin
		F1	Glycogen