Impact of lignocellulosic waste-immobilised white-rot fungi on enhancing the development of ^{14}C-phenanthrene catabolism in soil

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Abstract

In this study, the impact of white-rot fungi on enhancing the development of ^{14}C-phenanthrene catabolism was investigated in soil amended with phenanthrene over time (1, 25, 50, 75 and 100 d). The WRFs were immobilised on spent brewery grains (SBG) prior to inoculation to the soil. The results showed that SBG-immobilised WRF-amended soils reduced the lag phases and increased the extents of ^{14}C-phenanthrene mineralisation. Greater reductions in the lag phases and increases in the rates of mineralisation were observed in immobilised *Trametes versicolor*-amended soil compared to the other soil conditions. However, the soil amendment with *Pleurotus ostreatus* and *Phanerochaete chrysosporium* influenced biodegradation more strongly than the other fungal species. In addition, fungal enzyme activities increased in the amended soils and positively correlated with the extents of ^{14}C-phenanthrene mineralisation in all soil amendments. Maximum ligninolytic enzyme activities were observed in *P. ostreatus*-amended soil. Microbial populations increased in all amended soils while PAH-degrading fungal numbers increased with increased soil-PAH contact time and strongly positively correlated with fastest rates of mineralisation. The findings presented in this study demonstrate that inoculating the soil with these immobilised WRFs generally enhanced the mineralisation of the ^{14}C-phenanthrene in soil. This has the potential to be used to stimulate or enhance PAH catabolism in field-contaminated soils..

Key words: White-rot fungi, Immobilisation, lignocellulose, enzymes, phenanthrene, soil

1. Introduction

The fate and degradation of polycyclic aromatic hydrocarbons (PAHs) in soil are influenced by the physicochemical properties of the PAH molecules (Wilson and Jones, 1993). As the ring number increases, PAHs become less mobile and biodegradable, thereby increasing their persistence in soil (Abdel-Shafy and Mansour, 2016; Ghosal et al., 2016). The PAH properties include solid-liquid partition (Kd) and organic carbon-water partition coefficients (Koc), hydrophobibity, lipophilicity, and low aqueous solubility (Semple et al., 2003; 2007; Haritash and Kaushik, 2009; Couling et al., 2010; Yu et al., 2018). PAH molecules also display toxic, mutagenic and carcinogenic properties (Balmer et al., 2019), thus their presence poses a risk to human and environmental health (Lawal, 2017; Tongo et al., 2017; Sakshi et al., 2019).

Microbial degradation has been demonstrated as an important mechanism for the removal of PAHs and other organic contaminants in soil (Kim and Lee, 2009; Haritash and Kaushik, 2009; Ghosal et al., 2016). Bacterial and fungal populations have been reported to play major roles in PAH degradation (Ghosal et al., 2016; Fernández-Luqueño et al., 2011). However, fungal degradation of PAHs is more effective technique for soil biodegradation owing to their vast hyphal network, extracellular enzymes, high surface area to volume ratio, adaptability to changes in pH and temperature changes (Akhtar and Mannan, 2020). In addition, bacterial degradation of contaminants in soils often requires uptake of PAHs into the cell, which is controlled by the mobility of the molecules and can be rather slow (Schamfuß et al., 2013; Czaplicki et al., 2018). However, this can be overcome through the support of some hydrophilic filamentous fungi by active transportation of the contaminant through their hyphal network (also called 'fungal highways') over a greater range of distance compared to bacteria (Kohlmeier et al. 2005; Schamfuß et al., 2013; Czaplicki et al., 2018). For example, the fungal mycelia can cross the air-water interface and allow motile bacteria to move along

the fungal hyphae in unsaturated soil and to cross air-gaps in the soil or the fungal hyphae can transport PAHs inside of cytoplasmic vesicles (Kohlmeier et al. 2005; Schamfuß et al., 2013). Fungal highway-like associations can also increase frequency of bacterial-PAH contact and facilitate the movement of indigenous bacteria; for example, support the movement of PAH-degrading bacteria into micro-zones within contaminated soil for efficient PAH biodegradation (Wick et al., 2007; Furuno et al., 2012; Simon et al., 2015).

The basidiomycetes, especially the white rot fungi (WRF), have dual characteristics, being not only proficient degraders of lignocellulosic biomass, but can also be active degraders of PAHs; this is in part due to the aromatic structural similarities between PAHs and lignin (Memić et al., 2020). Several species of WRF have been reported to efficiently degrade and mineralise PAHs, especially those of the genera *Trametes*, *Bjerkandera*, *Phanerochaete*, *Irpex* and *Pleurotus,* as well as the hyphomycetes *Penicillium* and *Aspergillus* (Quintella et al., 2019; Gao et al., 2010). Lignin is degraded through ligninolytic mechanisms involving extracellular peroxidase enzymes (lignin peroxidase, manganese peroxidase, versatile peroxidase), and phenol oxidases (laccases), owing to their non-specific and oxidative properties, including the production of hydroxyl free radicals (Winquist et al., 2014; Akhtar and Mannan, 2020). Ligninolytic fungi have been shown to degrade low and high molecular weight PAHs, as well as other organic pollutants including synthetic dyes, organochlorine pesticides, and polychlorinated biphenyls (Young et al., 2015; Kadri et al., 2017; Al-Hawash et al., 2018; Pozdnyakova et al., 2018).

Lignocellulosic wastes can be applied to soil to deliver organic carbon, nitrogen and other nutrients to improve soil quality and stimulate microbial degradation of organic contaminants, such as PAHs (Lladó et al., 2015; Cao et al., 2016; Sigmund et al., 2018; Omoni et al., 2020b). Among the lignocellulosic waste materials, wheat straw, saw dust, sugarcane bagasse and corn cobs have been reported to stimulate both microbial and lignolytic activity (Dzionek

et al., 2016). These waste residues can also enhance the survival of the fungal mycelia, optimizes enzyme secretion, protect both the fungi and enzyme from harsh environmental conditions and increase their viability and activity in soil (Dzionek et al., 2016). Previous studies, however, have focused more on WRF immobilised on lignocellulose residues for the degradation of chemical contaminants in wastewater treatment and agricultural pesticides (Gao et al., 2010; Dzionek et al., 2016). Only a very few studies have investigated WRF immobilisation on lignocellulosic wastes (wheat straw, composted green wastes, saw dust and sugarcane bagasse) particularly for PAH degradation in soil (Dzul-Puc et al., 2005; Mohammadi and Nasernejad, 2009; Covino et al., 2010; Winquist et al., 2014). Further, to the authors' knowledge, there have been no published studies focussing on the the biodegradation of PAHs in soil by WRF immobilised on spent brewery grains (SBGs). SBGs are non-hazardous and biodegradable lignocellulosic wastes with a high nutritive value which are composed of rich fibres (cellulose, hemicellulose and lignin) and protein. As a result of their rich chemical composition, they have been useful in agriculture and sustainable energy production (bioenergy) and as animal feed (Mussatto et al., 2006; Maqhuzu et al., 2021). It is estimated that approximately 40 million tons of SBG is produced globally as a waste from the beer brewing inductry (Sahin et al., 2021). These organic wastes are rich in microbes, used for the cultivation of microorganisms, enzyme production, and as putative amendments for the bioremediation of organic contaminants in soil (Omoni et al., 2021; Girelli and Scuto, 2021). SBG has also been reported as absorbent material for heavy metals and the treatment of wastewater (Mussatto, 2014).

The study hypothesised that the addition of SBG-immobilised WRF would (i) enhance the development of catabolism of ^{14}C-phenanthrene in soil over time; (ii) stimulate microbial and enzymatic activities in phenanthrene-amended soils over time, and (iii) support efficient

mineralisation of ^{14}C-phenanthrene by promoting a favourable carbon to nitrogen ratio and pH in PAH-amended soils over time. Therefore, to address these hypotheses, the aim of this study was to investigate the impact of SBG-immobilised WRF on the biodegradation of ^{14}C-phenanthrene in soils amended with five different lignin-degrading WRFs inocula: *Irpex lateus*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor* and *Bjerkandera adusta*, respectively (Omoni et al., 2021). In addition, the impact on the soil biological (enzyme and microbial activities) and physicochemical properties (soil pH and C:N ratio) were investigated after 1, 25, 50, 75 and 100 d soil-PAH contact time.

2. Materials and methods

2.1 Soil and lignocellulosic waste collection

A pristine soil (Dystric Cambisol, 5 –20 cm) from Myerscough Agricultural College, Preston, UK was used for this study. Soil was partially air-dried and then homogenised after sieving through a 2 mm mesh. Sieved soil was stored at a temperature of 4 ºC in the dark before use. The microbial and physicochemical properties have been determined previously (Couling et al., 2010a) and are presented in Table S1. Fresh SBGs were obtained from Lancaster Brewery, Lancaster, UK; the general properties are presented in Table S2 (Omoni et al., 2020b). The SBGs were stored at a temperature of 4 °C in a sterile sealed high-density polyethylene bag.

2.2 White rot fungi, culture conditions and preparation for immobilisation

All of the white rot fungi, which demonstrated a high capacity for both ligninolysis and PAH mineralisation, were supplied by the Czech Republic's Culture Collection of Basidiomycetes

(CCBAS), Institute of Microbiology, Prague, Czech Republic. Strains included *Pleurotus ostreatus* (CCBAS 473), *Trametes versicolor* (CCBAS 614), *Phanerochaete chrysosporium* (CCBAS 570), *Irpex lateus* (CCBAS 196), and *Bjerkandera adusta* (CCBAS 232). The fungi were maintained on potato dextrose agar (PDA, 39 g l^{-1} pH 5.0) and incubated for 7 days (25 ± 1°C). The fungal isolates were subcultured routinely to maintain viability at every 15-20 days. Homogenised mycelial (Ultra-Turrax homogenizer at 10,000 rpm for 60s) were prepared from four mycelial plugs of 5 mm in diameter excised from actively growing edge of the fungus and then transferred into 100 ml of potato dextrose broth (PDB, 24 g l^{-1} pH 5.0). The medium was devoid of nitrogen sources so that the fungal strains can effectively utilise the available N sources in SBG after immobilisation for efficient metabolism of the target contaminant. Mycelial suspensions were incubated at (25 ± 1°C) under a rotary shaker at 150 rpm in the dark and mycelial pellets were harvested by centrifuging at 3500 x *g*, for 10 mins at 4°C (Thermo Scientific™, Sorvall TX-40R Cell Culture Centrifuge, UK). The supernatant was decarded and then the pellets were washed three times with sterile distilled water after several resuspension of mycelial cells and centrifugation. The fungal biomass was measured gravimetrically (dry weights, dw) after incubation in an oven at 60°C.

2.3 White-rot fungi immobilisation

The immobilisation of fungal hyphae onto the SBG was performed using a homogenised fungal mycelial suspension. The suspension was prepared by blending each fungal mycelium (0.01 g/g SBG dw) with sterile distilled water. Fresh brewery grains (250 g dw, autoclaved for 15 mins) were then mixed with mycelial suspensions (1:1, w/v) in a 500 ml Erlenmeyer flask and incubated for 24 hrs at 28 °C for sorption of fungal mycelia onto the SBGs. A moisture content of 60% was maintained in mycelial-SBG mixture before mixing into soil. The final

moisture content was selected based on a pre-study carried out to observe the marked cumulative CO_2 production, resulting from fungal growth (biomass), degradation and metabolism at various moisture contents (50, 60, 70, and 80%) of mycelial-SBG mixture over a 20 d incubation period (Fig. S1). Fungal respiration was measured by CO_2 production using a Micro-Oxymax respirometer (automated Columbus Instrument), all of which was replicated in with triplicate incubations.

2.4 Soil spiking and immobilised spent brewery grains amendment

Sieved soil was rehydrated to 60% water holding capacity with deionized water and spiked with ^{12}C-phenanthrene (> 96%, HPLC-grade, Sigma-Aldrich, Germany) according to the method, as previously described (Doick et al., 2003). Soil was amended with ^{12}C-phenanthrene (100 mg/kg, dw) and homogenously mixed with 20% of an SBG-immobilised fungus (1:5 SBG:soil), as reported previously (Omoni et al., 2020b). The SBG-immobilised fungus:soil mixtures, including the controls and blanks, were transferred to amber glass bottles and incubated in the dark (21 ± 1°C) and aerated with humidified and nonsterile forced air at a flow rate of 0.35–0.40 ml min^{-1} g^{-1} soil. The soil conditions were incubated for 100 d and sampled at 1, 25, 50, 75 and 100 d. Non-destructive sampling was used in this experiment to maintain the same biological function and soil properties in all the soils at the various time points throughout the study. Respirometric soil-slurry incubations were conducted to evaluate the influence of the SBG-immobilised fungi on the mineralisation of ^{12}C-/[9-^{14}C]-phenanthrene at each of the sample times (1, 25, 50, 75 and 100 days) in modified 250 ml Schott bottles (Teflon-lined screw cap) following the well-established method described by Semple et al. (2006). Briefly, at each contact time, 10 g ± 0.2 g (dw) of incubated soil (^{12}C-phenanthrene-spiked soil) was transferred into the respirometer, which contained 30 ml of

deionized water (1:3 soil:liquid ratio) and added [9-^{14}C] phenanthrene standard (98.2 Bq g^{-1} soil) and incubated in the dark at 21 ± 1°C (n = 3). The respirometers (n = 3) contained $^{14}CO_2$ traps of 1 ml of 1 M NaOH solution and incubated by shaking at 100 rpm on a flat-bed orbital shaker in the dark at 21 ± 1°C for 18 days, though the respirometers were opened and sampled daily to replace the $^{14}CO_2$ traps, allowing an exchange of air into the respirometer.

The ^{14}C-phenanthrene activity in each of the $^{14}CO_2$ traps was quantified bi-hourly for 1 d and then daily by adding 5 ml of liquid scintillation counter (Goldstar-Meridian, UK) using the liquid scintillation analyzer (LSC, Canberra Packard Tri-Carb2250CA). Analytical blanks contained ^{12}C-phenanthrene (without amendment and ^{14}C-PAH), abiotic controls (autoclaved-sterilized soils) with ^{12}C-PAH but without ^{14}C-PAH and fungus) and controls with both ^{12}C and ^{14}C-phenanthrene (without organic amendments and fungus) were also prepared alongside the treatments. Here, the unamended control with ^{12}C and ^{14}C-phenanthrene was used as the control throughout the experiment.

2.5 Soil pH and C and N determinations

The changes in the soil pH for all soil conditions in soils were determined from 1:5 w/v (soil:water) slurry by a calibrated Jenway Model 3504 pH/conductivity meter. Carbon to nitrogen (C:N) ratios were analysed in soil samples by dry combustion method using an elemental analyser (Vario EL Cube, UK) according to the methodology described by Wilke, (2010).

2.6 Soil extraction and enzymes analyses

At each time point, 10 g (dw) of soil was homogenised in 25 ml potassium phosphate buffer (100 mM, pH 7.0), shaken on ice at 100 rpm for 60 mins and then the homogenised

suspensions were centrifuged at 15,000 x g for 15 mins at 4°C and repeated to completely remove cell debris (5000 x g, 15 mins, 4°C), as previously described (Baldrian et al., 2000; Lang et al., 1998) with some minor modifications. The enzyme extract was then filtered with sterile hydrophobic polytetrafluoroethylene syringe filter (0.45 µm pore size) and the transparent filtrate wass assayed immediately for enzymes activity.

Ligninolytic enzyme activity in soil, including laccase (LAC), lignin peroxidase (LiP) and manganese peroxidase (MnP), was determined spectrophotometrically. LAC enzyme activity was assayed by ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) oxidation, as described by Bourbonnais and Paice (1990- modified). Lignin peroxidase (LiP) activity was measured by veratryl alcohol oxidation method, as described by Tien and Kirk (1988). Manganese peroxidase (MnP) activity was determined by the oxidation of the phenol-red as substrate as described previously (Chan-Cupul, Heredia-Abarca, & Rodríguez-Vázquez, 2016). β-Glucosidase (βGA) activity was assayed using *p*–nitrophenyl–β–D-glucopyranoside (PNG) as substrate (Eivazi and Tabatabai, 1988), and phosphatase enzyme was measured using *p*–nitrophenyl phosphate (PNP) as substrate (Tabatabai and Bremner, 1969). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of the substrate per minute.

2.7 Quantification of microorganisms

Microbial numbers were measured in soils by dilution and spread plate method as described previously (Kästner et al., 1994; Okere et al., 2012) and presented as colony forming unit counts (CFUs g^{-1} soil dw). At each sample time point, soil (1.0 ± 0.01 g, dw) was collected and weighed; this was used for the enumeration of both total heterotrophic and PAH-degrading microbial numbers, respectively. The fungal numbers (heterotrophs and PAH-

degraders) were quantified by the method described by Kästner et al. (1994), with some modifications. For PAH-degrading fungal CFUs, the minimal basal salt (MBS) medium was enriched with 50 mg l^{-1} ^{12}C-phenanthrene as the sole carbon source before inoculation. All inoculated plates were incubated in triplicate and supplemented with antibacterial (penicillin-streptomycin-glutamate) and antifungal (fungizone) agents, based on the target organism (Omoni et al., 2020a).

2.8 Statistical analyses

Data were statistically analysed using IBM SPSS version 25.0. Homogeneity of variance in the data were performed using Levene's and Shapiro-Wilk tests to check the normality of the data (Razali and Wah, 2011; Field, 2013). The data were transformed when necessary to ascertain ANOVA assumptions. Univariate analysis of variance (ANOVA) and the student's t-test were used to identify significant differences in the kinetics of ^{14}C-phenanthrene mineralisation (lag phases, fastest rates and extents), as well in the soil biological and physicochemical physical properties in immobilised WRF-amended soils. Changes in these parameters over time were further analysed by Tukey's HSD post-hoc and Games-Howell tests. Pearson's correlations and linear regressions were applied to determine the relationships between the tested parameters in soil over time. Graphs were plotted with SigmaPlot 10.0 software.

3. Results

3.1 Impact of BSG-immobilised WRF on the mineralization of ^{14}C-phenanthrene in soil

The impact of SBG-immobilised WRF on the mineralisation of ^{14}C-phenanthrene was measured in soils at 1, 25, 50, 75 and 100 d of soil-PAH contact time (Table 1 and Fig. 1). The lag phases were significantly reduced in most of the amended soils at all time points ($p < 0.05$), as compared to control soil. Following 1 d of soil-PAH contact time, the measured lag phases in all amendments were statistically similar ($p > 0.05$), except in soils amended with *B. adusta* and *P. ostreatus,* which displayed significantly shorter lag phases ($p < 0.05$). The amended soils showed longer lag phases of 2.93 d – 5.97 d ($p < 0.05$) after 1 day, compared to the other time points (25 d –100 d), where marked decreases were observed. In particular, the soil amended with *B. adusta* after 25 d showed statistically shorter lag phases compared to the other WRF amendments and control. Similarly, SBG-immobilised *T. versicolor* reduced the length of the lag phases after 75 d and 100 d soil-PAH contact time. Furthermore, it was also observed that the soil amended with SBG-immobilised *T. versicolor* displayed both the longest and shortest lag phases, 5.97 ± 0.60 d and 0.19 ± 0.00 d after 1 d and 75 d, respectively. Specifically, the shortest lag phases lag phases for all SBG-immobilised fungal amendments were recorded at 75 d of soil incubation.

The fastest rates of mineralisation were also measured in soils amended with SBG-immobilised WRF at each time point (Table 1 and Fig. 1). In general, the rates of mineralisation significantly increased ($p < 0.05$) as soil-PAH contact time increased in all of the amendment conditions. After 1 d contact times the rates for the control soils were faster than those of the WRF amended soil; after 25 d contact time, the differences between the control and the WRF amended soils were similar.But as soil contact time increase, the fastest rates in the control soil tended to be greater than those of the WRF amended soils ($p < 0.05$). The soil amended with *T. versicolor* recorded the highest rate of 4.38% d^{-1} closely followed

283 by *B. adusta* (2.64% d^{-1}) at 75 d, while the lowest rate of 0.44% d^{-1} was observed in the SBG-
284 immobilised *P. chrysosporium* amended soil at 1 d incubation time. Fastest rates of
285 mineralisation were observed at 50 d (1.02 – 2.27 % d^{-1}) and 75 (1.32 – 4.38 % d^{-1}) with an
286 average increase of 245 and 344% increase compared to 1-d soil incubation. In contrast,
287 control soils showed significantly faster rates of mineralisation ($p < 0.05$) for most time points
288 (1, 75 and 100 d), when compared to amended soils, except in soil amended with SBG-
289 immobilised *B. adusta*, which displayed significantly faster rates at 75 d soil-PAH contact
290 time ($p < 0.05$). After 50 d soil incubation, *I. lateus* and *P. chrysosporium* displayed higher
291 rates of mineralisation compared to control soil.

292 The effects of the different WRF amendments on the overall extents of ^{14}C-phenanthrene
293 mineralisation in soils were also measured over a 100 d incubation period (Table 1 and Fig.
294 1). The greatest extents of ^{14}C-phenanthrene mineralisation were observed for all amended
295 soils at 25 d (53.8–61.2%); immobilised *P. ostreatus* displayed the greatest extent of
296 mineralisation (61.2%). However, out of the five WRFs, *B. adusta* and *P. chrysosporium*
297 exhibited significantly greater extents of mineralisation ($p < 0.05$) in most time points
298 compared to the other WRFs and the control incubations. Greater extents of mineralisation
299 were observed at almost all soil-PAH contact time points compared to the control soil ($p <$
300 0.01). For the WRF-amended soils, the extents of mineralisation increased between 1 d to 25
301 d contact time, then decreased through 50 d and 75 d soil incubation. However, greater
302 extents of mineralisation ($p < 0.05$) were observed in the WRF-amended soils after 100 d
303 incubation, particularly in *T. versicolor* and *I. lateus* amended soils with significant increases
304 by 30% and 25%, respectively, as compared to those observed at 75 d of soil-PAH contact
305 time.

306

307 *3.2 Influence of SBG-immobilised WRF on soil physicochemical properties*

308 The impoact of the SBG-immobilised WRF on the soil physicochemical properties (C:N ratio,
309 and pH) were also measured (Figures. 2 and 3). The soil C:N ratio was found to be 11.3
310 before the application of SBG-immobilised fungi and subsequently, the C:N significantly
311 increased ($p < 0.05$) in most amendment conditions (especially in soil amended with
312 immobilised *P. chrysosporium*) and at most time points (1 d and 25 d) compared to the
313 unamended (control) soil (Fig. 2). For example, C:N ratios were significantly higher in the
314 soils containing SBG-immobilised *P. chrysosporium* followed by *B. adusta* after 1 d and 25 d
315 soil-PAH contact time, respectively, when compared to the other amendment conditions and
316 control soils. However, the soil C:N ratios were not significantly different among the
317 amendment conditions and between the amendment conditions and control within and across
318 most time points, except for the SBG-immobilised *P. chrysosporium* amended soil, which had
319 a significantly higher C:N ratio in all of the soil-PAH contact time points ($p < 0.05$). Further,
320 the SBG-immobilised WRFs showed significantly higher C:N ratios ($p < 0.05$) at most
321 sampling times in the amended soils, especially *P. chrysosporium*-amended soils. Similarly,
322 the highest C:N ratio (22.4) was found in soil amended with *P. chrysosporium* after 25 d
323 aging. Significant decreases in the C:N ratios ($P<0.05$) in all amended and control soils were
324 measured between 50 d and 100 d.

325 The SBG-immobilised WRFs were found to influence the pH in the amended soils (Fig. 3). It
326 was observed that soil pH generally decreased in all of the amendment conditions with
327 increased soil-PAH contact time, ranging from a maximum to a minimum pH value of 7.52 to
328 4.68. The pH in the control inclubations did not show this behaviour, reamining above pH 6
329 for the 100 d incubation. After 1 d incubation, the pH values for all WRF soil conditions were
330 significantly higher than the control ($p < 0.05$); however this changed after 75 d and 100 d,
331 where the pH decreased below that of the non-amended control soil ($p < 0.05$). *T. versicolor*

amended soil exhibited a higher pH value at most time points in the experiment (50 d, 75 d and 100 d), followed by *P. chrysosporium* (50 d and 75 d). However, most time points did not show statistically different pH values ($p > 0.05$) in soils amended with WRFs. Furthermore, apart from *T. versicolor*-amended soil, all other soil conditions showed significant decreases ($p < 0.01$) in pH values over time. Moreover, the greatest ^{14}C-phenanthrene mineralisation was found at mean pH value of 6.3. It should be noted however that soil pH was strongly positively correlated with the lag phases except for *T. versicolor* and negatively correlated with fastest rates of mineralisation in virtually all amended soils (Table S3-S7). The pH of soil amended with immobilised fungi (*B. adusta* and *P. ostreatus*) had significant positive correlations with overall extents of mineralisation (Table S4 and S6).

3.3 WRF enzyme activities in soil

The production of ligninolytic enzyme activities was examined in phenanthrene-spiked soils over a 100 d study period (Fig. 4a-c). The ligninolytic systems (LAC, LiP and MnP) showed variations for all SBG-immobilised WRF-amended soils. For LAC enzyme, higher levels of activity were observed for all amended soils compared to the control, at most time points during the study (Fig. 4a). The highest laccase activity was detected for *P. ostreatus* (2.52 U g^{-1}), closely followed by *T. versicolor* (1.61 U g^{-1}). After 1 day of soil incubation, LAC activity was significantly higher in *T. versicolor*-amended soil when compared to other treatments and the control, with no enzyme activity being detected for the treatments *I. lateus* and *B. adusta*, and negligible enzyme activity in *P. chrysosporium*-amended soil. However, LAC activity subsequently increased at 25d, when all of the WRF demonstrated maximum activity, followed by significant decreases from this point onward (50 d –100 d). No LAC activity was detected in the soil after 100 d incubation.

356 LiP activities of all SBG-immobilised WRF-amended soils were 5.76-fold higher after 25 d
357 when compared to 1 d aging, especially in SBG-immobilised *P. ostreatus* amended soil with
358 14.3-fold higher than the other soil conditions. Higher LiP activities were measured in
359 amended soils than the control ($p < 0.05$) for almost all time points (Fig. 4b). *P.*
360 *chrysosporium* (2.00 U g^{-1}) closely followed by *P. ostreatus* (1.10 U g^{-1}) soil conditions
361 showed the highest LiP activities after 1 d incubation. Although LiP activities markedly
362 varied in the different amendment conditions the enzyme levels obtained were much lower
363 than those observed for LAC. It should be noted that only *B. adusta* produced LiP activity at
364 all time points. Moreover, after 100 d, LiP activities further increased in all amended soils
365 compared to 75 d soil-PAH contact time.

366 Compared to LAC and LiP enzymes measured, MnP activities were generally higher in SBG-
367 immobilised WRF-amended soils; although, MnP activities showed similar patterns with
368 those observed for LiP. The highest MnP activity of 3.84 U g^{-1} was observed in the presence
369 of SBG-immobilised *P. ostreatus*. No MnP activity was measurable in the soils amended soils
370 with *T. versicolor*, *P. chrysosporium* and *B. adusta* at the onset of incubation (1 d). However,
371 MnPs showed sharp increases from 1 d to 25 d incubation then decreased after 50 d –75 d
372 followed by an increase at 100 d soil incubation. Further, MnP activity was detected in
373 amended soils after 100 d incubation, while significantly higher MnP activities of more than
374 2.44-fold increase were detected in amended soils in comparison with 75 d soil-PAH contact
375 time. Linear correlations and regressions showing the relationships of the ligninolytic enzyme
376 activities in soils with the kinetics of mineralisation are presented (Table S3-S7, Fig. S3-S7).
377 There were no correlations between the ligninolytic enzyme activities and the lag phases in
378 amended soils, except for soil inoculated with *T. versicolor* for only laccase ($r = 0.54$, $p <$
379 0.05). Also, ligninolytic enzymes showed non-significant and weak negative correlations
380 with fastest rates ($r = –0.16–0.58$) except *T. versicolor* and *I. lateus*-amended soils, while the

overall extents of ^{14}C-phenanthrene mineralisation were strongly positively correlated with ligninolytic enzymes (r = 0.55–0.90; p < 0.05–0.001).

Non-ligninolytic enzymes (βGA and ACP) levels were also measured over a 100 d period in amended soils and the results are presented in Fig. S2 (a-b). The highest βGA activity were found in soils amended with *T. versicolor* (13.5 U g^{-1}). No significant differences were observed in βGA activity among the amendment conditions at most time points during the study (Fig. S2a). For all amended soils, elevated βGA activities were observed over time compared to control soil (p < 0.001). In contrast to the ligninolytic enzymes, increases in soil-PAH contact time did not affect the βGA activity, which peaked at 50 d soil incubation. The ACP activity was significantly higher (p < 0.05) in all amended soils compared to the control (p < 0.05) (Fig. S2b). Immobilised *B. adusta* and *P. chrysosporium* resulted in the highest ACP activity of 32.2 and 22.7 U g^{-1}, respectively. However, ACP activity was significantly decreased (p < 0.05) over time in amended soils. Furthermore, ACP activity in amended soils generally showed stronger correlations with the extent of mineralisation compared to βGA activity. Both enzymes displayed significantly positive correlations with the extents of mineralisation in almost all amended soils. Finally, ACP activity positively correlated with the lag phases (r = 0.52–0.86); *P. chrysosporium* amended soil showed the highest correlation coefficient (r = 0.86, p < 0.001) closely followed by *B. adusta* (r = 0.76, p < 0.001); but negatively correlated with fastest rates (r = –0.52–0.67) in all amended soils (Table S3-S7).

3.4 Enumeration of culturable microbial numbers in soil

3.4.1 Bacterial populations

Bacterial numbers (CFU g^{-1} soil) were markedly influenced by the presence of SBG-immobilised WRF and soil-PAH contact time (Table 1). Furthermore, both heterotrophic and

PAH-degrading bacterial numbers were consistently higher in all amended soils than the control soil over time (1 d –100 d); further, soils amended with *B. adusta* and *T. versicolor* presented the highest numbers of heterotrophs and PAH-degraders after 25 d and 50 d, respectively. Generally, the CFUs of total heterotrophs and PAH-degraders varied within and across BSG immobilised WRF-amended soils. At 25 d and 50 d soil incubations, the heterotrophic and PAH-degrading bacterial CFUs were significantly higher ($p < 0.05$) compared to other incubation periods, respectively, but not after 75 d –100 d where both heterotrophic and PAH-degrading bacterial numbers were significantly reduced ($p < 0.05$) in all amended soils. However, PAH-degrading bacterial numbers were significantly higher ($p < 0.05$) in all amendment conditions over time (Table 1). Moreover, PAH-degrading bacterial numbers moderately positively correlated with the extents of ^{14}C-phenanthrene mineralised in *B. adusta* ($r = 0.45$, $p < 0.05$) and *P. ostreatus* ($r = 0.57$, $p < 0.05$) amended soils, while no correlations were observed between PAH-degraders with lag phases and fastest rates in all WRF-amended soils, respectively (Table S3-S7). Additionally, no positive correlations between phenanthrene degrader CFUs and mineralisation kinetics were observed in *T. versicolor*-amended soil, despite the stimulated bacterial numbers (Table S3).

3.4.2 Fungal populations

The addition of SBG immobilised-WRF amended soils significantly stimulated the heterotrophic and PAH-degrading fungal numbers ($P<0.05$) compared to the control soil throughout the investigation period (Table 1). *B. adusta* and *P. chrysosporium* amended soils exhibited the highest fungal CFUs (heterotrophs and PAH-degraders) in soil after 24 h incubation, respectively. The heterotrophic fungal numbers were significantly reduced after 25 d for the rest of the study period. In contrast to the heterotrophic bacterial CFUs, PAH-degrading fungal CFUs significantly increased ($P<0.05$) in amended soils over time compared

to 1 d soil-PAH contact time. CFUs of heterotrophs and PAH degraders did not differ among the SBG-immobilised WRF amended soils at most of the time points. As observed in the bacterial numbers, *T. versicolor*-amended soil showed significantly higher heterotrophic fungal CFUs over time compared to the other amendment conditions. PAH-degrading fungal numbers had strong negative correlations with lag phases ($r = –0.63–0.79$), moderately correlated with the extents of ^{14}C-phenanthrene mineralisation ($r = –0.30–0.66$) in amended soils (Table S3-S7). However, significant, positive correlations of PAH-degrading fungal numbers with fastest rates of mineralisation ($p < 0.01$) were observed; these linear relationships were stronger in soils amended with *T. versicolor* ($r = 0.90$, $p < 0.001$) closely followed by *P. ostreatus* ($r = 0.82$, $p < 0.001$).

Discussion

This study investigated the impact on the addition of SBG-immobilised WRFs on enhancing the development of ^{14}C- phenanthrene catabolism in soil over time. The main reasons for immobilisation of the fungal inocula were (i) ease of inoculation of the fungi to the soil; (ii) to offer physical protection to the fungi in potentially hostile soil environment (particularly the case for genuinely contaminated soils) and offer an ecological niche for the indigenous degradative soil bacteria; (iii) as the SBG is decomposed in the soil, offer soil microflora carbon, nitrogen and other nutrients allowing the stimulation and/or enhancement of PAH degradation in soil, and (iv) the SBG and associated nutrients generally enhance fertility and improve the the structure and physico-chemical properties of the soil.

453 *4.1 Mineralisation of ^{14}C-phenanthrene in amended soils using SBG-immobilised WRF*

454 Mineralisation of the ^{14}C-PAH was quantified by measuring changes in lag phases prior to the
455 onset of mineralisation, increases in the fastest rates and extents of mineralisation of the ^{14}C-
456 PAH in soil. This study has shown that the addition of immobilised WRFs resulted in
457 significantly shorter lag phases in all amended soils over time, especially in soils amended
458 with *T. versicolor* and *B. adusta*. These strains of WRFs have been reported as the more
459 efficient lignocellulose decomposers (Sánchez, 2009; Chen et al., 2010). Whether the fungal
460 inocula were actively contributing to the mineralising the ^{14}C-PAH or stimulating the
461 indigenous catabolically active soil microflora by releasing nutrients into the soil, remains
462 unclear and was beyond the scope of this study.

463 This present study showed that the fastest rates of ^{14}C-phenanthrene mineralisation in
464 amended soils tended to be lower than those in the unamended soil, but did increase with soil-
465 PAH contact time. However, higher rates of mineralisation were generally seen after 50 d of
466 soil incubation in amended soils, suggesting higher SBG decomposition and release of
467 available nutrients for phenanthrene catabolism in soils. Oyelami et al. (2013) proposed that
468 nutrient enrichment and microbial richness can enhance the rates of PAH biodegradation in
469 soil. It must be noted, however, that higher rates of mineralisation were found in soils
470 amended with immobilised *T. versicolor* followed by *B. adusta*, indicating the potential of
471 these fungal species in stimulating the mineralisation of ^{14}C-phenanthrene in soil. Although
472 fungal hyphae have been shown to enhance biodegradation rate by increasing bioavailability
473 of entrapped PAHs in soil aggregates by hyphal penetration and reducing the distance for soil
474 indigenous bacteria to the contaminant (Wick et al., 2007), this is unlikely in this case given
475 the aggressive shaking of the respirometers in measuring the mineralisation of the ^{14}C-PAH.
476 This physical mixing would inhibit the development of stable fungal hyphae in the
477 microcosms.

Enhancement of the extents of ^{14}C-phenanthrene mineralisation were hypothesised to depend on the ligninolytic potential of each fungus and their positive synergistic fungal-bacterial interactions (Borràs et al., 2010; Wick et al., 2007). In this study, the addition of immobilised WRFs to PAH spiked soil increased the extents of ^{14}C-phenanthrene mineralisation in most contact points compared to a previous study without fungal immobilisation (Omoni et al., 2020b). The extent of mineralisation was greatest for *P. ostreatus*-amended soil; it was also found that *B. adusta* and *P. chrysosporium* in soils consistently displayed higher extents of mineralisation than their counterpart treatments in almost all contact points. This might be attributed to the WRFs releasing available and metabolisable carbon and nutrients to the indigenous soil microflora, thereby enhancing the mineralisation of the ^{14}C-PAH (Han et al., 2017; Harms et al., 2011). Some studies have reported enhanced PAHs biodegradation after the addition of WRFs with organic materials (Dzul-Pul et al. 2010; Lladó *et al.*, 2013; Winquist *et al.*, 2014; Andriani and Tachibana, 2016) and without organic materials amendments to contaminated soils (Pozdnyakova, 2012; Ghosal *et al.*, 2016; Quintella *et al.*, 2019; Cao *et al.*, 2020).

4.2 Effects of soil physico-chemical properties on ^{14}C-phenanthrene mineralised using SBG-immobilised WRF

The C:N ratio is a primary factor in determining the nutrient effect of PAH assimilation into microbial biomass during soil remediation (Leys et al., 2005). Lignocellulosic materials amended to soils would increased the C:N ratio (Hubbe et al., 2010) and C:N ratio around 9–10 promoted optimal PAH degradation (Riser-Roberts, 1998). In this study, soil C:N ratios increased in most of the amended soils; however, varied in amended soils over time, while

soils amended with immobilised *P. ostreatus*, *B. adusta* and *P. chrysosporium* with C:N ratio of 12.9, 18.3 and 22.4, respectively, had the greatest extents of mineralisation after 25 d soil incubation. Furthermore, Teng et al. (2010) reported that a C:N ratio of 10:1 resulted is greater PAH degradation in aged contaminated soils than 25:1 or 40:1. This supports the results reported here where a C:N ratio of 12.9:1 resulted in higher extents of mineralisation than in the higher C:N ratios of 18.3 or 22.4. In addition, after 25 d incubation, the high C:N ratio found in the soil amended with immobilised *P. chrysosporium* showed more efficient delignification of the organic waste materials and release organic nutrients to the soil.

In contrast, the soil pH changed from neutral to acidic conditions following the amendments with the immobilised WRFs; the highest rates and extents of mineralisation were seen in the soils with an acidic pH range of between 5.8–6.3; this is in agreement with a study by Bishnoi et al. (2008), who reported similar results for enhanced PAHs biodegradation in soils displaying a similar acidic pH range (Bishnoi et al., 2008). In addition, it was also found that the soil pH decreased as soil-PAH contact time increased, which may be due to production of hydroxylated and carboxylated intermediates of PAH biodegradation (Ghosal et al., 2016); the production of tannic and humic acids during lignin biodegradation (Clemente, 2001), as well as greater amounts of CO_2 dissolving in soil water to form weak acidic conditions (Angert et al., 2015).

4.3 Influence of enzyme activities on the mineralisation of ^{14}C-phenanthrene in SBG-immobilised WRF-amended soil

In this study, ligninolytic enzymes (LAC, LiP and MnP) were all stimulated in amended soils. In particular, LiP enzyme was produced by the WRF evaluated (Rogalski et al., 1991; Nakamura et al., 1999; Rothschild et al., 2002; Asgher et al., 2011). However, MnP was the

predominant ligninolytic enzyme in amended soils, which is in agreement with previously reported results (Diez, 2010). Therefore, the enzyme activities were likely influenced by the presence of SBG amendment. Depending on either high or low nutrient availability, the WRFs may change their enzyme production to increase or decrease the enzyme pool in a system to maintain their supply of inorganic nitrogen, phosphorus and carbon (Olander and Vitousek, 2000). Ligninolytic enzymes also promoted the extents of PAH biodegradation in soils (Novotný et al., 2004; Kadri et al., 2017). In amended soils, for example, the extents of ^{14}C-phenanthrene mineralisation decreased after 75 d soil incubation when compared to the other time points which were associated with the decline in ligninolytic enzymes activities. This was further evidenced by the positive relationships found between the ligninolytic enzymes and the extents of $^{14}CO_2$ mineralised in soils. Similar results have been reported in other studies, but this remains an under-explored aspect of PAH biodegradation (Novotný et al., 1999; Pozdnyakova et al., 2010). Additionally, LAC activities correlated positively with the C:N ratios for all amended soils, indicating the importance of C:N ratios in fungal laccase synthesis by WRF (Knežević et al., 2013).

4.4 Influence of microbial populations on the mineralisation of ^{14}C-phenanthrene in SBG-immobilised WRF-amended soil

The microbial numbers (heterotrophs and PAH-degraders) significantly increased in all amended soils following the application of SBG-immobilised fungi. This may be due to the release fo C, N and other nutrients from the decomposition of the SBG, allowing the proliferation of heterotrophic and PAH-degrading populations (Awad et al., 2018). When soil is amended with organic substrates, respiration increases while microbial growth and activities are stimulated (Tejada et al., 2008; Omoni et al., 2020b). Some studies have observed increases

in microbial numbers, changes and improves microbial diversity and structure following the addition of organic amendments after pre-treatment with WRF in PAHs contaminated soils (Bao et al., 2019; Wu et al., 2020; Omoni et al., 2021). High microbial numbers also suggest the presence and availability of C, N and other nutrients in the amended soils. Microbial numbers were generally higher in soils amended with immobilised *T. versicolor* compared to the other WRF-amended soils; however there was no positive relationship between the PAH-degrading bacterial numbers and the extents of mineralisation, but PAH-degrading fungal numbers showed a strong linear relationship. Moreover, PAH-degrading fungal numbers increased following increases in soil-PAH contact time in amended soils with the exception of 100 d soil incubation.

Conclusions

We investigated the impact of WRF immobilisation on SBG for enhanced physicochemical and biological properties in PAH contaminated soils. Although the different immobilised WRF amended to soils were found to promote mineralisation, variations were noticeable in the mineralisation kinetics at different sampling points, indicating that each fungus possess intrinsic capacity for SBG delignification and PAH biodegradation. The two fungi *T. versicolor* and *B. adusta* displayed shorter lag phases and higher rates of mineralisation. The greatest extents of mineralisation were found in SBG-immobilised *P. ostreatus* and *P. chrysosporium*-amended soils, indicating the release of metabolisable carbon and nutrients to the indigenous soil microflora, which may have promoted the mineralisation of the ^{14}C-PAH. Soil amendment with immobilised WRF also influenced the soil C:N ratios and pH level, which may also have contributed to the soil biology. However, soil amended with SBG-immobilised *P. chrysosporium* showed higher soil C:N ratio compared to the other soil

574 treatments. Optimum mineralisation was found at soil C:N ratio of 12.9 and pH 6.3, which are
575 within the optimum values at which biodegradation is effective in the removal of organic
576 contaminants in soil. Enzyme activities (ligninolytic and non-ligninolytic enzymes) increased
577 in amended soils but decreased over time. However, ligninolytics were positively correlated
578 with the extents of $^{14}CO_2$ mineralised in all WRF-amended soils (Higher in *P. ostreatus*-
579 amended soils), suggesting the importance of these enzymes in PAH removal from
580 contaminated soil. Increasing the length of soil-PAH interaction resulted in reduced extents of
581 phenanthrene mineralisation and ligninolytic enzymes in amended soils. Overall, microbial
582 populations increased in all amended soils while PAH-degrading fungal numbers increased
583 with increased soil-PAH contact time. Therefore, the immobilisation of white-rot fungi on
584 spent brewery grains can provide a cost-effective waste management system for the large
585 brewery industry in Nigeria and other parts of the world as well further provide a promising
586 cheap and sustainable remedial strategy for contaminated soils. In addition, this technique
587 may be useful for *in situ* remediation treatments for large and heavily contaminated soils such
588 as the Niger-Delta region of Nigeria. Further research is required in order to test the addition
589 of surfactants to amended soils for increase bioavailability of the target contaminant in soil.

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Figure Legend

Figure 1. Catabolism of ^{14}C-phenanthrene in soils amended with immobilised fungi on spent brewery grains after 1, 25, 50, 75, and 100d soil-PAH contact time. Immobilised fungi: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P. chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and (□) control (unamended). Values are mean ± SE (n = 3).

Figure 2. Changes in C:N ratio in amended soils with immobilised fungi on spent brewery grains after 1, 25, 50, 75, and 100d soil-PAH contact time. Values are mean ± SE (n = 3).

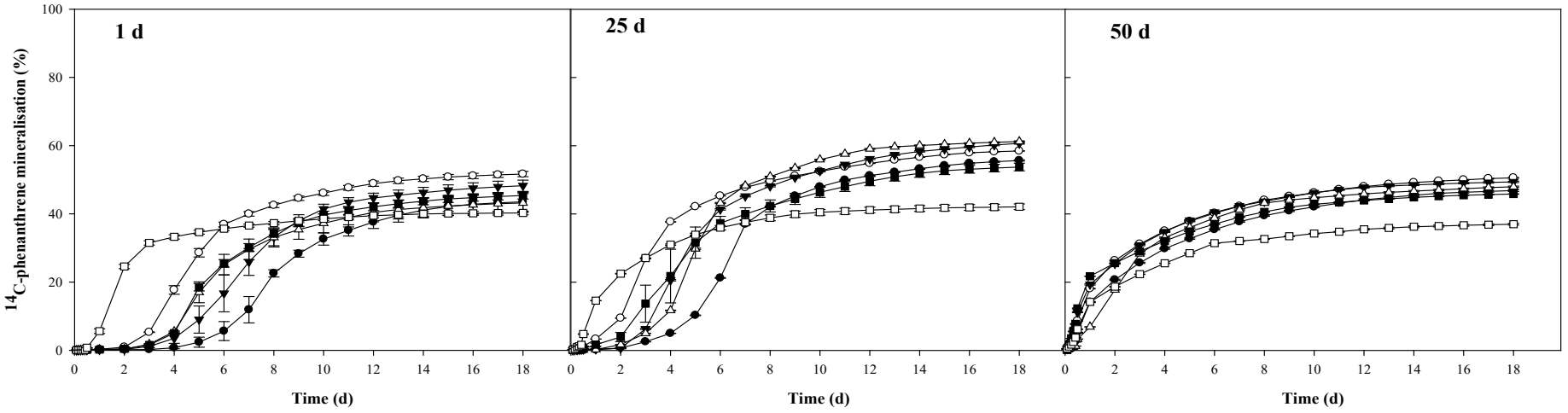
Figure 3. Changes in pH value in amended soils with immobilised fungi on spent brewery grains after 1, 25, 50, 75, and 100d soil-PAH contact time. Values are mean ± SE (n = 3).

915

916 **Figure 4**. Immobilised fungi on spent brewery grains on the enzyme activities (a) Laccase;
917 (b) Lignin peroxidase; (c) Manganese peroxidase in amended soils after 1, 25, 50, 75, and
918 100d soil-PAH contact time. Immobilised fungi: (●) *T. versicolor*, (○) *B. adusta*, (▼) *P.*
919 *chrysosporium*, (△) *P. ostreatus*, (■) *I. lateus,* and (□) control (unamended). Values are mean
920 ± SE (n = 3).

921

922 **Figure 1**



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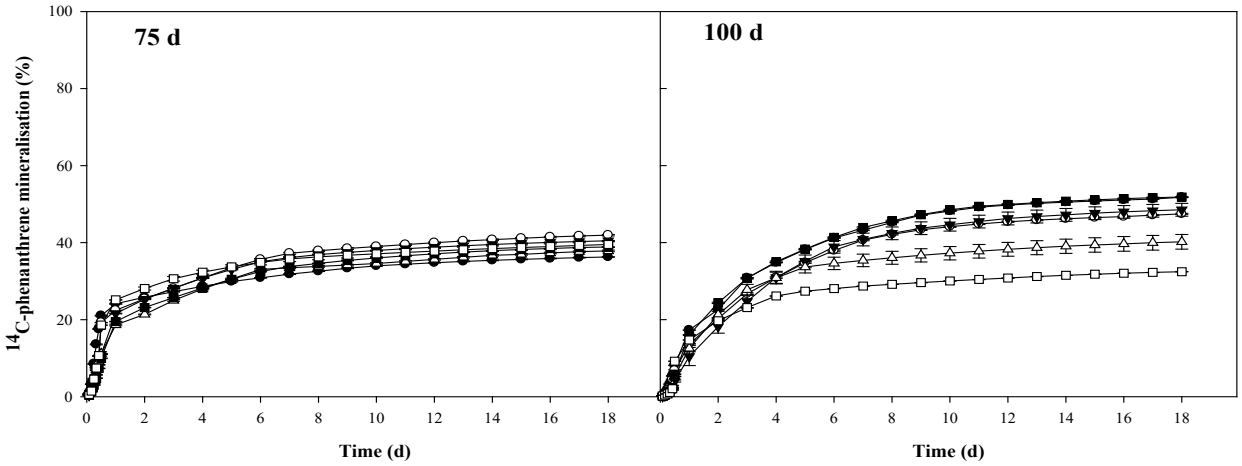

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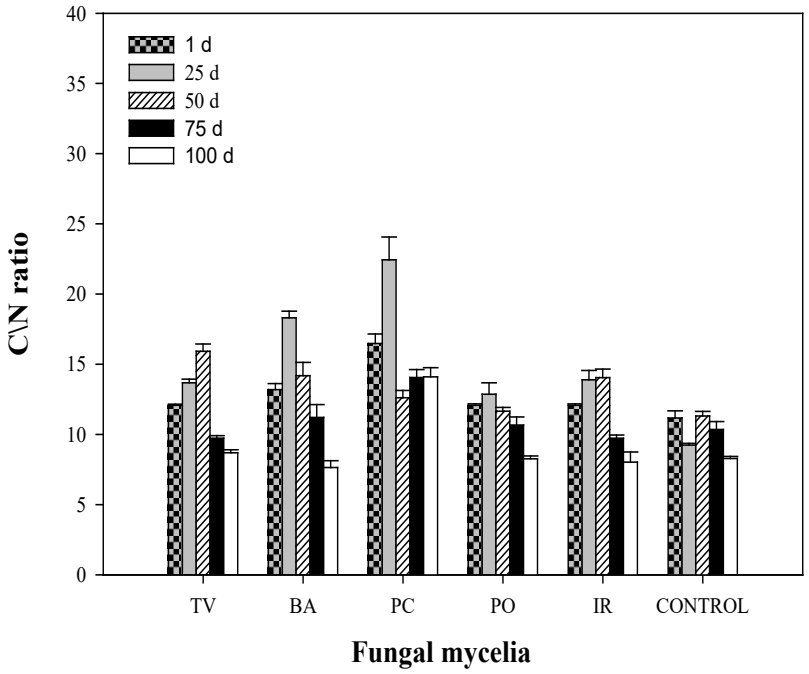
925 **Figure 2**



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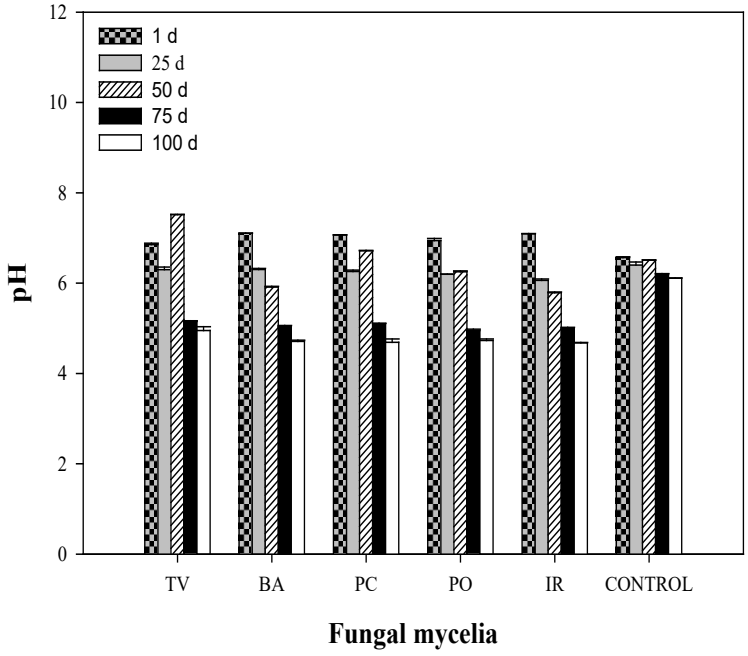
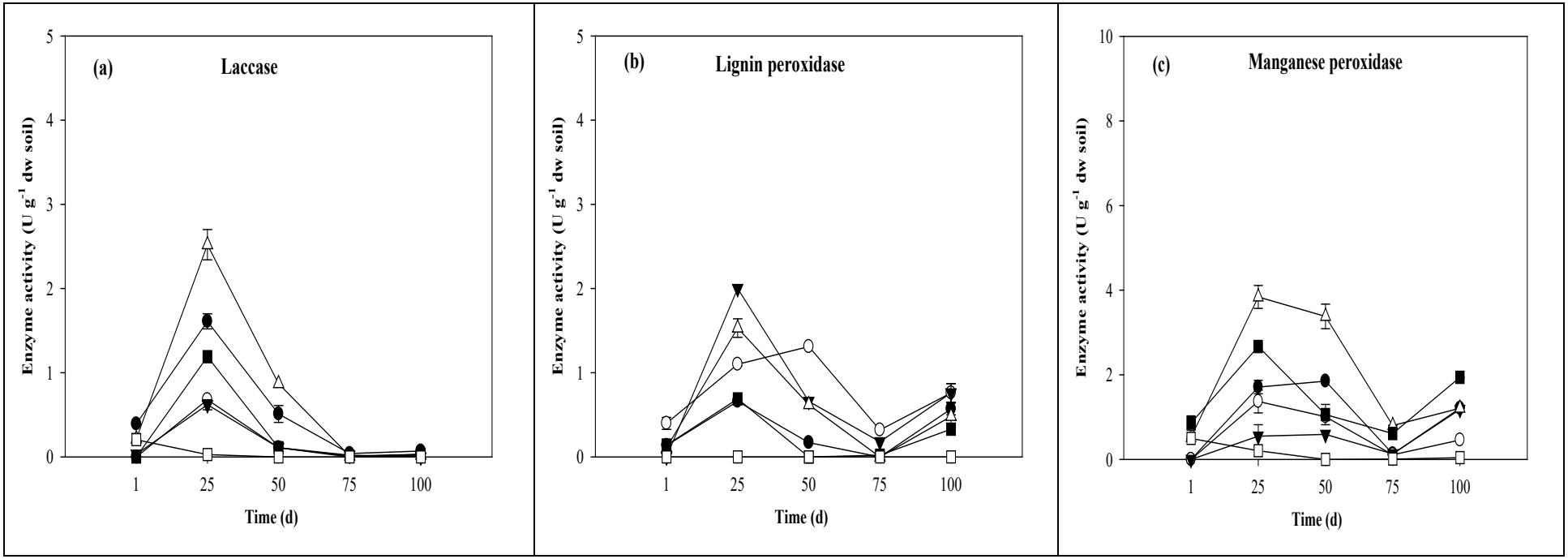
928 **Figure 3**



929

930 **Figure 4**

931



932

933 **Table 1.** Summary of the catabolic activity and microbial numbers (CFU/g) in soil amended with SBG-immobilised fungi after 18 days
934 respirometric assay for different ageing periods after spiking with $^{12}/^{14}$C-phenanthrene. Values are mean ± standard error (n = 3).
935

Contact time (d)	Ligninolytic fungi	Lag phase (d)	Fastest rate (%$^{14}CO_2$ d^{-1})	Cumulative Extent (%)	**Bacteria**		**Fungi**	
					Total Heterotrophs	PAH-degraders	Total Heterotrophs	PAH-degraders
					CFU x 10^7 g^{-1} soil dw		CFU x 10^7 g^{-1} soil dw	
1	*T. versicolor*	5.97 ± 0.60d3	0.47 ± 0.14a3	43.52 ± 2.30ab3	1.52 ± 0.02d3	0.31 ± 0.00d3	52.8 ± 1.19e1	0.08 ± 0.00d5
	B. adusta	2.93 ± 0.02b4	0.52 ± 0.04a5	51.67 ± 0.77a2	1.67 ± 0.01c3	0.34 ± 0.00b2	97.9 ± 0.87a1	0.09 ± 0.00c5
	P. chrysosporium	4.53 ± 0.52cd3	0.44 ± 0.05a3	48.24 ± 1.54a2	1.48 ± 0.02d3	0.35 ± 0.01a3	85.5 ± 0.33c1	0.08 ± 0.00d5
	P. ostreatus	3.86 ± 0.04bc5	0.51 ± 0.10a4	43.20 ± 2.75ab2	1.86 ± 0.01a3	0.32 ± 0.00c3	76.6 ± 1.20d1	0.10 ± 0.00b4
	I. lateus	3.99 ± 0.01bc4	0.56 ± 0.04a4	45.37 ± 1.06ab2	1.75 ± 0.02b2	0.31 ± 0.00d2	97.2 ± 0.31b1	0.11 ± 0.00a4
	Control	6.12 ± 0.39a3	0.79 ± 0.01a5	40.27 ± 0.75b1,2	1.08 ± 0.00e2,3	0.01 ± 0.01e4	0.01 ± 0.00f3	0.05 ± 0.00e1
25	*T. versicolor*	4.64 ± 0.32d2	0.66 ± 0.00abc3	55.68 ± 0.17bc1	11.7 ± 0.18e1	0.34 ± 0.00a2	9.62 ± 0.15a2	0.33 ± 0.00a3
	B. adusta	1.27 ± 0.00a2	0.73 ± 0.01a4	58.48 ± 0.03ab1	28.9 ± 0.18a1	0.28 ± 0.00d3	7.70 ± 0.12b2	0.22 ± 0.00d4
	P. chrysosporium	2.77 ± 0.04c2	0.59 ± 0.09c3	60.70 ± 0.20a1	21.4 ± 0.29b1	0.30 ± 0.00c2	6.68 ± 0.14c2	0.18 ± 0.00e4
	P. ostreatus	3.00 ± 0.05c4	0.75 ± 0.00a3	61.24 ± 0.13a1	17.1 ± 0.17d2	0.34 ± 0.00a2	9.72 ± 0.10a2	0.24 ± 0.01c3
	I. lateus	1.83 ± 0.09ab4	0.62 ± 0.05bc4	53.76 ± 1.11c1	18.5 ± 0.25c1	0.32 ± 0.00b2	6.81 ± 0.12c2	0.26 ± 0.00b3
	Control	2.56 ± 0.34bc2	0.70 ± 0.01ab3	42.10 ± 0.61d1	1.27 ± 0.02f1	0.01 ± 0.00e2	0.03 ± 0.00d2	0.01 ± 0.00f3
50	*T. versicolor*	0.49 ± 0.03b1	1.32 ± 0.01c2	46.86 ± 0.27bc2,3	8.00 ± 0.12a2	1.47 ± 0.01a1	4.23 ± 0.09a3	0.53 ± 0.02b2
	B. adusta	0.41 ± 0.03ab2	1.78 ± 0.01b2	50.60 ± 0.11a2	4.30 ± 0.11b2	0.53 ± 0.03c1	2.61 ± 0.09c3	1.32 ± 0.02a1
	P. chrysosporium	0.38 ± 0.00a1	2.19 ± 0.05a2	49.40 ± 0.20ab2	2.18 ± 0.12c2	0.65 ± 0.02b1	1.98 ± 0.11d4	0.51 ± 0.01b2
	P. ostreatus	0.73 ± 0.06c3	1.02 ± 0.00d2	47.97 ± 0.20abc2	2.31 ± 0.09c3	0.52 ± 0.00c1	3.73 ± 0.07b3	0.40 ± 0.01c2
	I. lateus	0.31 ± 0.00a1	2.27 ± 0.02a1	45.86 ± 0.06c2	1.65 ± 0.09d2	0.41 ± 0.02d1	3.67 ± 0.15b3	0.37 ± 0.01c2
	Control	0.45 ± 0.04b2	1.22 ± 0.03c4	36.98 ± 2.89d2	0.04 ± 0.00e2	0.03 ± 0.00e1	0.02 ± 0.00e4	0.01 ± 0.00d5

75	*T. versicolor*	0.19 ± 0.00a1	2.64 ± 0.00c1	36.28 ± 0.09e4	0.94 ± 0.01a4	0.10 ± 0.00b4	4.56 ± 0.10a3	0.84 ± 0.01b1
	B. adusta	0.27 ± 0.06b1	4.38 ± 0.01a1	41.95 ± 0.18a4	0.27 ± 0.00c4	0.12 ± 0.00a4	1.29 ± 0.06cd3,4	0.96 ± 0.01a2
	P. chrysosporium	0.31 ± 0.03c1	1.37 ± 0.01c2	40.47 ± 0.11ab3	0.41 ± 0.01b4	0.08 ± 0.00d4	3.50 ± 0.07b3	0.85 ± 0.01b1
	P. ostreatus	0.26 ± 0.06b1	1.39 ± 0.07b1	37.87 ± 0.21d3	0.41 ± 0.01b4	0.08 ± 0.00d4	1.42 ± 0.03c4	0.56 ± 0.02c1
	I. lateus	0.34 ± 0.03d1,2	1.32 ± 0.00d3	38.87 ± 0.07cd3	0.25 ± 0.00c3	0.09 ± 0.00c3	1.02 ± 0.03de4	0.58 ± 0.02c1
	Control	0.26 ± 0.05b1	3.96 ± 0.02b1	39.53 ± 1.01bc1,2	0.02 ± 0.00d2,3	0.01 ± 0.00e3	0.03 ± 0.01e1	0.02 ± 0.00d2
100	*T. versicolor*	0.40 ± 0.01a1	1.39 ± 0.01c2	51.78 ± 0.06a1,2	0.53 ± 0.00a5	0.03 ± 0.00b5	0.32 ± 0.00a4	0.25 ± 0.00b4
	B. adusta	0.44 ± 0.04a2	1.17 ± 0.04c3	47.47 ± 0.13a3	0.49 ± 0.00b4	0.02 ± 0.00d5	0.24 ± 0.01b4	0.32 ± 0.00a3
	P. chrysosporium	0.62 ± 0.07b1	1.15 ± 0.13c2	48.52 ± 1.67a2	0.31 ± 0.01c4	0.03 ± 0.00a5	0.14 ± 0.00d5	0.26 ± 0.01b3
	P. ostreatus	0.49 ± 0.00a2	1.18 ± 0.04c1,2	40.22 ± 1.90b3	0.49 ± 0.00b4	0.02 ± 0.00e5	0.17 ± 0.00c4	0.24 ± 0.00c3
	I. lateus	0.48 ± 0.00a2	1.71 ± 0.02b2	51.83 ± 0.15a1	0.60 ± 0.01a3	0.03 ± 0.00c4	0.11 ± 0.00e5	0.15 ± 0.00d4
	Control	0.45 ± 0.03a2	3.58 ± 0.01a2	32.45 ± 0.01c3	0.01 ± 0.00d3	0.01 ± 0.00f4	0.01 ± 0.00f5	0.01 ± 0.00e4

*Mean values with the same lower-case letters within each aging time in amended soils are significantly different ($p < 0.05$) while diifeerent lower-case letters indicate significant differences ($p < 0.05$) within each aging time in amended soils (1d–100d).

*Mean values with the same numbers across the samping points are significantly different ($p < 0.05$) while different numbers indicate significant differences ($p < 0.05$) across the samping points in amended soils (1d–100d).