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ORIGINAL ARTICLE

Toxicity evaluation and biodegradation of phenanthrene by laccase from *Trametes polyzona* PBURU 12

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Abstract

The newly isolated *Trametes polyzona* PBURU 12 demonstrated a high tolerance and potential for the degradation of phenanthrene. The fungal isolate was able to tolerate 100 ppm of phenanthrene with 45% relative growth. The crude laccase produced by *Trametes polyzona* PBURU 12 was able to degrade phenanthrene by up to 98% within 24 h. The degradation metabolites showed the absence of toxic compounds. Microbial viability tests using *E. coli* and *B. subtilis* revealed that the treated phenanthrene was less toxic than untreated phenanthrene. Phytotoxicity and genotoxicity tests, using *Vigna radiata* and *Allium cepa*, indicated that the treated phenanthrene was less toxic to the plants. No mutagenic activity was found in the Ames test. The crude laccase from *Trametes polyzona* PBURU 12 was demonstrated as a potential tool for the biodegradation of PAHs (phenanthrene), with low toxic effects after the degradation.

Keywords Biodegradation · Phenanthrene · Lignolytic enzyme · White-rot

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of several hundred organic compounds formed during the pyrolysis and incomplete combustion of coal, wood, oil, gas, and organic materials (Anastasi et al. 2009). They are recognized by the Stockholm Convention as the most dangerous pollutants to the environment. The possible fate of PAHs in the environment depends not only on physicochemical processes but also on their biotic transformation and degradation. A large number of microorganisms, such as bacteria, fungi, and algae, that are capable of degrading different PAHs have been isolated from various environments (Haritash and Kaushik 2009). As a result of their different transformation/degradation mechanisms, several PAH derivatives are produced. Such compounds may interact with the biotic and abiotic surroundings, entering and moving along food chains and so could exert a negative influence on a wide range of living organisms.

Several fungi are known for their ability to transform persistent pollutants by producing various enzymes, such as lignin-modifying enzymes (LMEs), which have been intensively studied (Lee et al. 2014, 2020). Among the LME producers, white-rot fungi (WRF), a group of basidiomycetous fungi, have received considerable attention due to their ability to degrade a wide range of recalcitrant organic compounds possessing complex structures. Many WRF produce all three groups of LMEs (laccase, manganese peroxidase, and lignin peroxidase), which enables them to attack several complicated molecules that most other microorganisms cannot degrade. Among the hundreds of known WRF that display LME activity, *Phanerochaete chrysosporium, Bjerkandera adusta, Pleurotus ostreatus, Trametes versicolor*, and *Irpex lacteus* have been the most intensively studied for their ability to transform various PAHs (Pozdnyakova 2012). Compounds such as quinones, hydroxyl- and

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dihydroxy-PAHs have been identified as the biotransformed products, but it is not clear whether they accumulate as dead-end products.

A number of microbial PAH degradative pathways have been thoroughly investigated and found to differ depending on the taxonomic relations of the microorganisms. Thus, different groups of intermediate compounds are produced when PAHs are exposed to different microbial communities. Moreover, even a single PAH may yield several different intermediates when degraded by a single microbial species. For example, phenanthrene degradation by *P. ostreatus* led to the formation of 9, 10-phenanthrenequinone, 2, 2-diphenic, and salicylic acid (Pozdnyakova et al. 2018). While, phenanthrene degraded by *Pycnoporus sanguineus* 14 yielded 9,10-phenanthrenedione and 2-methylphenol (Li et al. 2018).

From an ecological viewpoint, PAHs are of concern due to their toxicity. However, many reports have revealed that their derivatives may be even more toxic (Bekki et al. 2013). Therefore, it is very important to study the fates of PAHs when released into the environment, because the accumulation of their dead-end and toxic metabolites may cause deleterious impacts on the growth and activities of all exposed living organisms, especially microorganisms and plants.

Phenanthrene is a three-ring PAH compound that has frequently been used as a surrogate model for studying the removal of PAHs in soil (Bezalel et al. 1996). Phenanthrene is one of the 16 PAHs listed as priority pollutants by the Environmental Protection Agency (USA) and is commonly found in soil and river sediments (Ting et al. 2011). Like most PAHs, phenanthrene is used to make dyes, plastics, pesticides, explosives, and drugs. It has also been used to make bile acids, cholesterol, and steroids. It is known to be a human skin photosensitizer and mild allergen (Janbandhu and Fulekar 2011).

In the present study, the fate of phenanthrene and its degradation derivatives was studied using a crude extracellular laccase preparation from *Trametes polyzona* PBURU 12, a WRF isolated from East Kalimantan, Indonesia. Their bacterial mutagenicity, cytotoxicity, and phytotoxicity were investigated. The results could lead to a better understanding of how phenanthrene is changed by the microbial community and how its derivatives might affect living organisms in contaminated areas.

Materials and methods

Microorganism

The WRF used in this study, *Trametes polyzona* PBURU 12, *Ceriporia inflata* PBURU R1, *Ceriporia lacerata* PBURU 141, *Coriolopsis* sp. PBURU 11, *Coriolopsis* sp. PBURU 1, *Ganoderma* sp. PBURU 21, *Ganoderma* sp. PBURU 5, *Phellinus noxius* PBURU 2, *Pycnoporus sanguineus* PBURU 60, *Schizophyllum commune* PBURU 13 were isolated from Samarinda Botanical Garden, East Kalimantan, Indonesia and deposited in the cultures collection of the Plant Biomass Utilization Research Unit (PBURU), Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Cultures were maintained on malt extract agar (1.5% (w/v) agar), stored at 4 °C, and sub-cultured monthly. Bacterial strains of *Escherichia coli* and *Bacillus subtilis* were sub-cultured from PBURU culture collection. The bacteria strains were maintained on 2% nutrient agar. *Vigna radiata* and *Allium cepa* were purchased from Tesco Lotus market, Bangkok, Thailand. *Salmonella typhimurium* (AMES test) strain TA98 and TA100 were obtained from the Thailand Institute of Scientific and Technological Research (TISTR) Bangkok, Thailand and maintained on 2% nutrient agar.

Phenanthrene tolerance test

Phenanthrene tolerance test was evaluated by a plate assay, comprised of 0.5% (v/v) of 20 mg/ml phenanthrene in acetonitrile solvent (100 ppm final) on a potato dextrose agar (PDA) plate. Mycelial growth was measured each day for 7 days in a dark room. Growth inhibition (%) was calculated relative to that of the control cultures that had only the solvent (acetonitrile) without phenanthrene addition (Lee et al. 2014).

Laccase production and assay

Ten agar plugs from a fresh fungal culture on PDA were used as an inoculum in 100 ml laccase production medium (Revankar and Lele 2006). The culture was grown in a 250-ml Erlenmeyer flask and incubated at room temperature (28 ± 2 °C) with continuous shaking at 150 rpm. The 7-day-old culture was filtered through Whatman No. 1 filter paper and the supernatant was harvested following centrifugation at 9072 × g at 4 °C for 15 min and then concentrated by ultrafiltration (10 kDa molecular cut-off, VivaFlow, Sartorius, Germany). The concentrated supernatant was determined for laccase activity (Thongkred et al. 2011), freeze-dried and kept at 4 °C. For each following experiment, the crude laccase preparation was freshly prepared from the supernatant powder by dissolving in 50 µM sodium citrate buffer and its laccase activity was determined. One unit (U) of laccase activity was defined as the amount of enzyme that oxidized 1 µM 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) per min.

Phenanthrene degradation by laccase

For phenanthrene degradation, a reaction mixture containing 10 U/ml crude laccase in 20 ml of 50 µM sodium citrate buffer (pH 4.5) and 0.5% (v/v) of 20 mg/ml phenanthrene dissolved in acetonitrile (100 ppm and 0.5% (v/v) final phenanthrene and acetonitrile concentration, respectively) was shaken at 150 rpm at room temperature in the dark for 24 h prior to analysis. Only citrate buffer and phenanthrene (in acetonitrile) were added in the control treatment. At the end of the incubation period, the residual phenanthrene and all derivatives were acidified to pH 3 using 1 N hydrochloric acid (HCl) and extracted using ethyl acetate (Bezalel et al. 1996). The extracted compounds were analyzed using high-performance liquid chromatography (HPLC) (C18 reverse-phase HPLC column (4.6 × 150 mm) with a 7:3 (v/v) acetonitrile: water as the mobile phase at a flow rate of 1 ml/min) (Hadibarata and Tachibana 2010). Phenanthrene was determined by UV detection at 250 nm. Structures of the derivatives were analyzed and identified by gas chromatography–mass spectrometry (GC–MS).

GC–MS analysis

The degradation of phenanthrene and identification of its products was performed by gas chromatography–mass spectrometry (GC–MS) analysis (Agilent 6890 and Agilent 5973, respectively) to determine degradation products. The GC–MS was equipped with a DB-1MS capillary column (0.25 mm diameter, 0.25 mm film thickness and 30 m length). The column temperature was held at 60 °C for 2 min, raised to 150 °C at 10 °C per min, raised to 300 °C at 20 °C per min and held at 300 °C for 10 min (total runtime of 28.50 min). The GC–MS was operated using a mass selective detector over a mass range of 30–500 atomic mass unit (amu). The mass spectra from the samples were identified by comparison with those of standard compounds and the Wiley 7n.l mass spectra databases (John Wiley & Sons, Inc., US).

Microbial viability test

Cultures of *E. coli* and *B. subtilis* were incubated in 2% (w/v) nutrient broth on a rotary shaker (150 rpm) at room temperature (28 ± 2 °C) for 24 h. Five-ml aliquots of each culture were separately transferred to fresh NB medium (total 20 ml), along with one of 100 µl of acetonitrile solvent (control), 100 µl of 20 mg/ml phenanthrene solution (in acetonitrile) or 100 µl of laccase-treated phenanthrene solution, the latter two to a final concentration of 100 ppm. Growth of the bacteria was determined by measuring the absorbance at 600 nm at 1 cm light path every 2 h (Sowada et al. 2017).

Phytotoxicity test

Dry mung beans (*Vigna radiata*) were hydrated for 24 h by submersion in distilled water. The external skin was removed to expose the embryo and the zone of root growth. Ten seeds were then placed per petri dish (90 mm diameter) containing a Whatman filter paper (No. 1). The dishes were filled up to ¾ of their capacity (5 ml) with either distilled water (control) or the test solution and incubated for 7 days at 25 °C in the dark. Distilled water was added every 2 days to keep the liquid level. Three different treatments of (1) control (distilled water), (2) 0.5% (v/v) of 20 mg/ml phenanthrene in acetonitrile in distilled water (100 ppm final phenanthrene concentration) and (3) the laccase-treated phenanthrene (100 ppm initial phenanthrene), were conducted. The germination (%) was calculated as seed germinated/total seed × 100% (Agrawal and Shahi 2017).

Genotoxicity test

Healthy commercial shallot (*Allium cepa* var. *aggregatum*) bulbs of equal size were used to evaluate toxicity of phenanthrene and its laccase-derived derivatives (Taranath et al. 2015). The outer scales of the shallot bulb were cleaned, and the bottom plates were cut to expose the root primordia. The bulbs were placed in 50-ml test tubes containing phenanthrene solution (100 ppm final concentration). 2-Aminofluorene (0.01 ppm final concentration) was used as the positive control, whereas distilled water was used as the negative control. The solvent for both phenanthrene and 2-aminofluorene was acetonitrile (0.5% (v/v) final concentration). The experiment was conducted in the dark to prevent phenanthrene auto-oxidation. The solutions were freshly prepared and changed every 24 h. After 3 days in the test solution, the number and length of the roots were recorded.

To observe chromosome aberration, five root tips were randomly cut from each onion bulb. The cut root tips were washed thoroughly in distilled water and fixed in Carnoy's fixative (1:3 (v/v) glacial acetic acid: 95% (v/v) ethanol) for 24 h. The root tips were then subjected to a conventional squash preparation using 1 N HCl to hydrolyse the cellulosic cell wall for 5 min. Staining was done in 2% (w/v) aceto-orcine in 45% (v/v) glacial 1 N HCl. Mitotic cells were detected under light microscopy (Olympus BX51, Canada) with magnifications of 400 × and 1000 ×. The mitotic index (MI) was calculated from 1000 cells in each slide (Bilal et al. 2016).

Ames mutagenicity test

Mutagenicity was determined by the Ames test using *Salmonella typhimirium* TA98 and TA100 strains (Rainho et al. 2013). Dimethyl sulphoxide (DMSO; 100 µ1) was used as a negative control, with the standard mutagens of 4-nitroquinoline-1-oxide (10 µg/plate) and sodium azide (10 µg/plate), or 2-aminoflourene (10 µg/plate) and methyl methane sulfonates (10 µl/plate) as positive controls for the TA100 and TA98 strains, respectively. Revertant colonies were scored after 48 h incubation at 37 °C and the results are expressed as the number of revertant colonies per plate.

Statistical analysis

Results are presented as mean ± one standard deviation (SD) derived from three replications. When appropriate, ANOVA and *t* test or LSD tests were employed to assess significant differences among means at the 5% level ($p \leq 0.05$). All statistical analyses were performed with the Statistical Product and Service Solutions (SPSS) 20 program from StatSoft, IBM, USA.

Results and discussion

Fungal isolate

T. polyzona PBURU 12 was submitted into the fungal section of the Professor Kasin Suvatabhandhu Herbarium, Chulalongkorn University, Bangkok, Thailand, with the herbarium number 0073 BCU. The internal transcribed spacer (ITS) and large subunit ribosomal RNA (nLSU) accession numbers were listed in GenBank as KY234233 and KY234231, respectively.

Phenanthrene degradation by *T. polyzona* PBURU 12

T. polyzona PBURU 12 was found to be relatively tolerant to phenanthrene at 100 ppm compared to other fungi, with a relative growth of 45% of the control after 7 days of cultivation (Table 1). The result was in line with previously report, that *T. polyzona* was able to grow and tolerate phenanthrene (Teerapatsakul et al. 2017). *T. polyzona* PBURU 12 produced laccase and manganese peroxidase (MnP) as ligninolytic enzymes, whereas no lignin peroxidase was detected. However, MnP activity in the culture filtrate of *T. polyzona* PBURU 12 was found to be very low compared to laccase. In the submerged laccase production medium, the isolate produced extracellular laccase at 1.13 ± 0.03 U/ml, while only 0.019 ± 0.006 U/ml MnP was found after 7 days of cultivation. *T. polyzona* has been previously reported

Table 1 Effect of phenanthrene on the growth of selected WRF strains

Fungal strain	Relative growth (%)[a]
Ceriporia inflata PBURU R1	nd[b]
Ceriporia lacerata PBURU 141	16.18 ± 1.04
Tramates polyzona PBURU 12	45.26 ± 2.87
Coriolopsis sp. PBURU 11	33.79 ± 4.16
Coriolopsis sp. PBURU 1	22.22 ± 1.15
Ganoderma sp. PBURU 21	20.54 ± 3.21
Ganoderma sp. PBURU 5	21.21 ± 1.75
Phellinus noxius PBURU 2	9.60 ± 1.15
Pycnoporus sanguineus PBURU 60	32.15 ± 2.30
Schizophyllum commune PBURU 13	14.88 ± 1.75

The fungi were grown on PDA supplemented with 100 ppm phenanthrene at room temperature for 7 days

[a]Relative growth was calculated as the percentage of those on PDA without phenanthrene addition. Data are shown as the mean ± 1SD, derived from three repeats

[b]nd = not detectable

able to produce LMEs. The fungal produced two kinds of ligninolytic enzymes as laccase and MnP, but not LiP (Chairin et al. 2014; Teerapatsakul et al. 2017; Lueangjaroenkit et al. 2018, 2019). When added to the culture at 100 ppm, the fungus could transform up to 88% of the 100 ppm phenanthrene within 15 days of incubation (data not shown). Faster phenanthrene degradation was found when the crude laccase was used, with 98% of the phenanthrene (initial 100 ppm) oxidized after 24 h of incubation using 10 U/ml of crude laccase (Fig. 1). Previously, many studies have been reported the use of white-rot fungi to degrade phenanthrene (Table 2). Teerapatsakul et al. (2017) reported the ability of *T. polyzona* to degrade phenanthrene. That study reported the complete degradation (100%) of 100 mg/l phenanthrene within 18 days of incubation at 30 °C. Hence, *T. polyzona* PBURU 12 showed faster degradation when crude laccase was used. To the best of our knowledge, this is the first study to report the use of a crude laccase of *T. polyzona* PBURU 12 to degrade phenanthrene.

Determination of the phenanthrene degradation products

Phenanthrene degradation by microorganisms has been extensively studied over the past few decades. Diverse fungi capable of utilizing PAHs have been investigated as well. Some filamentous fungi, basidiomycetes, WRF, and deuteromycetes have been shown to remove PAHs more competently than bacteria. This occurs via dioxigenase enzymes, such as cytochrome P-450 and peroxidases (Peng et al. 2008). Hence, fungi do not utilize phenanthrene as the sole source of carbon and energy but, instead, co-metabolize the

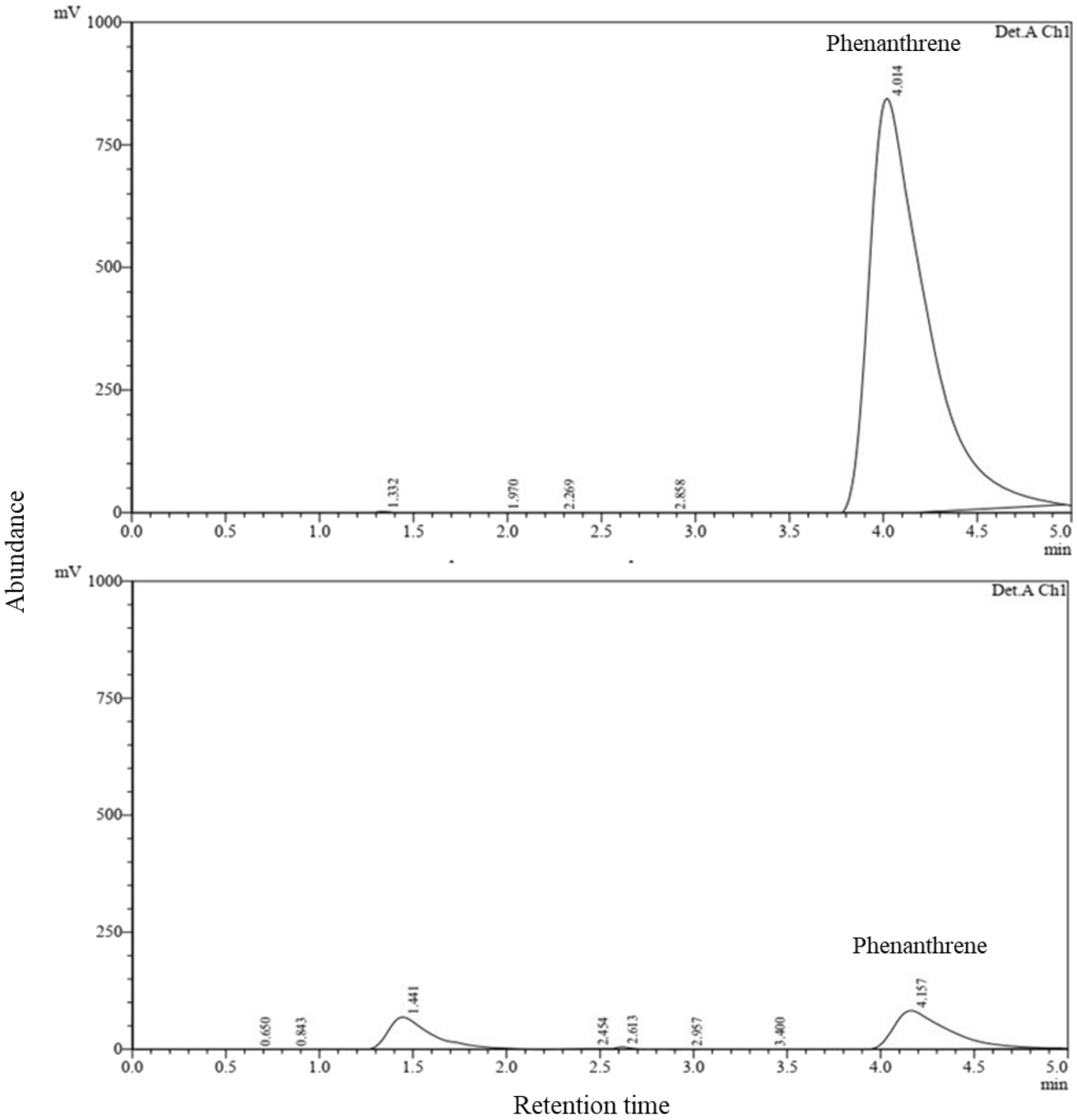

Fig. 1 HPLC chromatogram showing before and after phenanthrene (100 ppm) degradation by *T. polyzona* PBURU 12 crude laccase (10 U/ml) after 24 h incubation at room temperature in the dark with shaking at 150 rpm. The chromatograph shown is representative of that seen in three independent trials

phenanthrene to a hydroxylated product (Cerniglia 1997). *Phanerochaete chrysosporium* was previously shown to be able to mineralize phenanthrene, oxidizing it initially to phenanthrene-9,10-quinone and then to the ring cleavage product 2,2–9-diphenic acid under ligninolytic conditions. Interestingly, under non-ligninolytic conditions, *P. chrysosporium* metabolized phenanthrene to phenanthrene *trans*-3,4- and *trans*-9,10-dihydrodiols, 3-, 4-, and 9-phenanthrols, and a glucoside conjugate of 9-phenanthrol. *Pleurotus ostreatus* was found to be able to oxidize phenanthrene by cytochrome P-450 monooxidase, manganese peroxidase, and laccase (Bezalel et al. 1996). Furthermore, phenanthrene degradation by ligninolytic enzymes of *Polyporus* sp. S133 identified the metabolites 9,10-phenanthrenequinone,

Table 2 Phenanthrene degradation by WRF

Fungal species	Condition	Concentration	Degradation (%)	Time (d)	References
Trametes polyzona PBURU 12	Live culture (submerged) Crude enzyme (10 U/ml)	100 ppm 100 ppm	88 98	15 1	This study
Trametes polyzona RYNF13	Live culture (submerged)	100 ppm	84–100	11–18	Teerapatsakul et al. (2017)
T. versicolor	Live culture (submerged)	20 ppm 100 ppm	75 46–75	11	Collins and Dobson (1996)
Pycnoporus coccineus Thongkerd 013 BCU	Live culture (submerged) Crude enzyme (1 U/ml)	100 ppm 100 ppm	67.7 50.5	1 1	Thongkred et al. (2011)
Phanerochaete chrysosporium	Live culture (submerged)	100 µM	80	7	Bogan and Lamar (1996)
P. sordida KUC8370	Live culture (submerged)	50 ppm	66.9	14	Lee et al. (2010)
Pheniophora incarnata KUC8836	Live culture (submerged)	25 ppm	95.3	10	Lee et al. (2014)
Pleurotus ostreatus	Live culture (submerged)	2.5 mg	94	11	Bezalel et al. (1996)
Ganoderma lucidum	Live culture (submerged)	100 ppm	50	10	Ting et al. (2011)
Phlebia brevispora	Live culture (submerged)	0.025 mM	95	15	Harry-asobara and Kamei (2019)

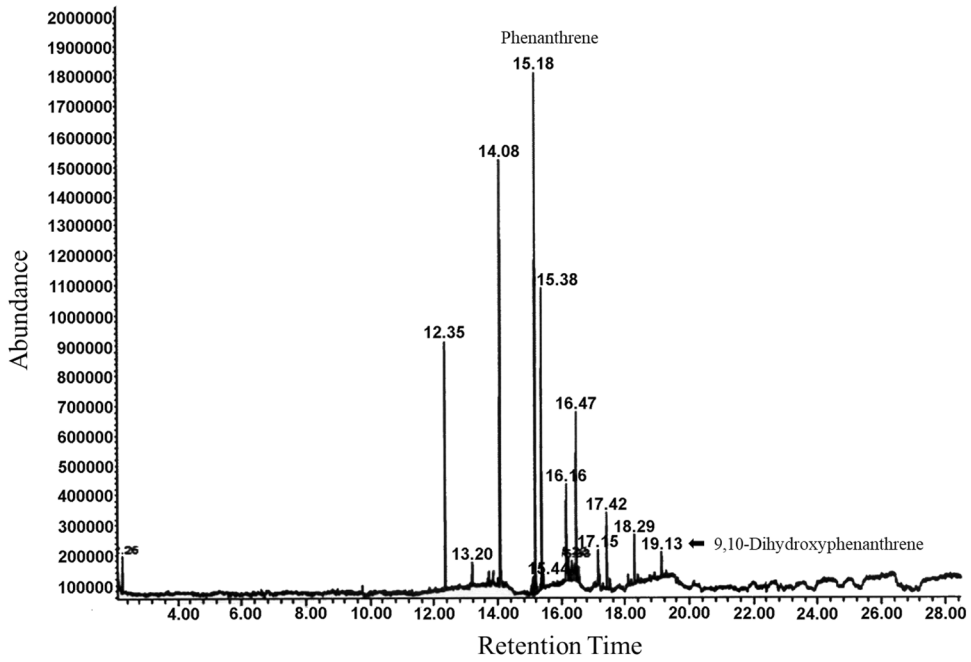

Fig. 2 GC chromatogram of the oxidation products obtained from phenanthrene degradation by *T. polyzona* PBURU 12. The chromatograph shown is representative of that seen in three independent trials

2,2′-diphenic acid, phthalic acid, and protocatechuic acid (Hadibarata and Tachibana 2010).

An analysis of the ethyl acetate-extracted compounds formed from phenanthrene by the crude laccase of *T. polyzona* PBURU 12 is shown in Fig. 2. Phenantrene was detected at retention time (RT) of 15.18 min. Metabolites such as 9,10-Dihydroxyphenanthrene (RT 19.14) pentadecane, hexadecanoic acid, octadecanoic acid, hexadecane, and nonacosane, were detected. However, phenantrols, phthalic acid, and diphenic acid were not detected. This result was in accordance with the previous study reported phenanthrene degradation by *Ganoderma* sp. which resulted the formation of *trans*-9,10-dihydroxyphenanthrene, protocatechuic acid and phthalic acid (Torres-Farrada et al. 2019). A phthalic derivative is produced as one of the ring fission products of PAHs by WRF (Haritash and Kaushik 2009). The derivatization of phthalate results in the production of CO_2 or highly polar metabolites, where the ligninolytic enzymes and ozonation/photocatalytic oxidation act by free radical attack on the organopollutants (Machado et al. 2000). Thus, the intermediates of these three methods are ring-opening phthalic derivatives and aliphatics, such as pentadecane, hexadecane, and nonadecane (Machado et al. 2000; Juhasz et al. 2010).

Microbial viability

Microbial viability was determined using *E. coli* and *B. subtilis* bacteria (Fig. 3), since they are representative

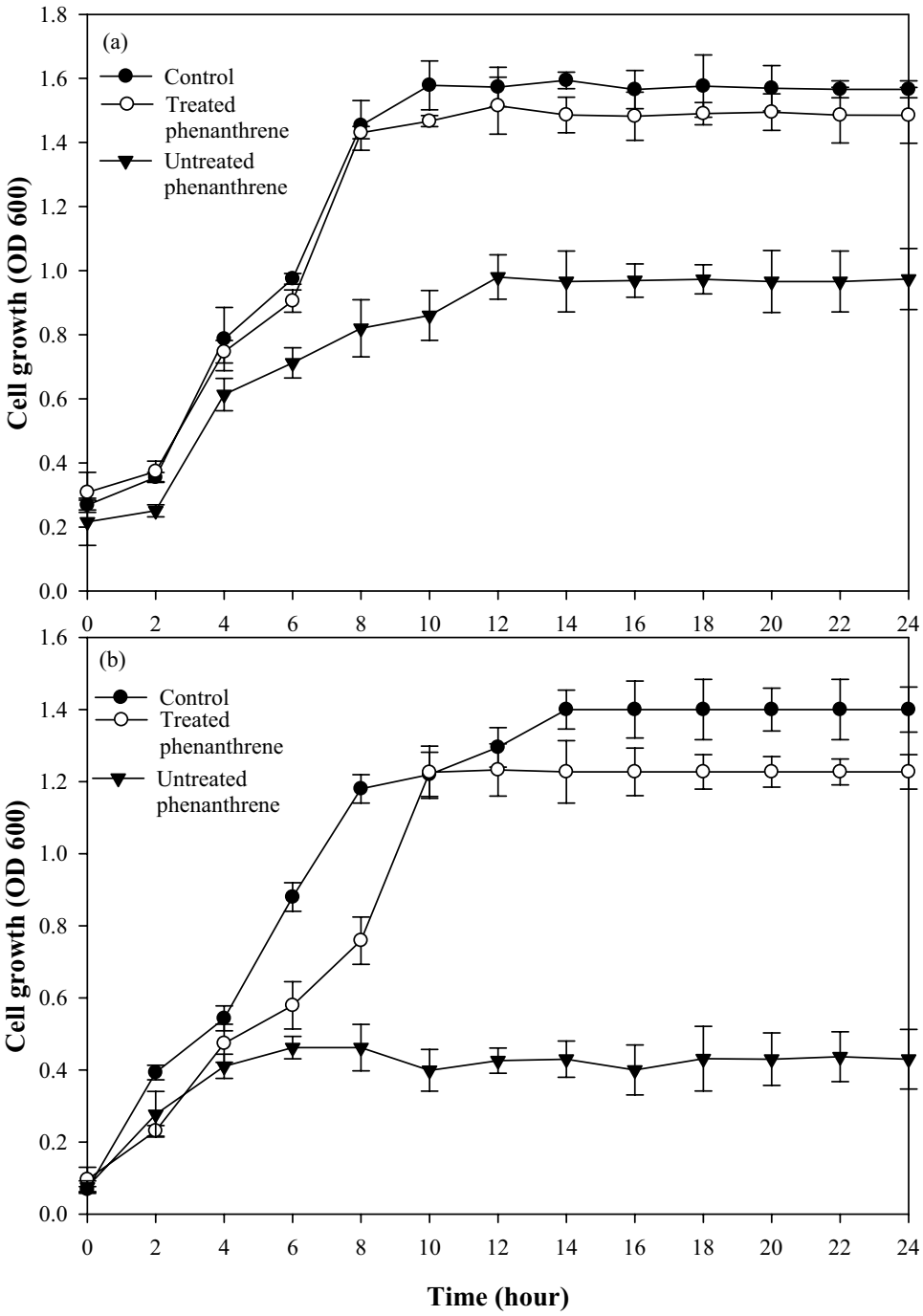

Fig. 3 Viability of **a** *E. coli* and **b** *B. subtilis*. Data are shown as the mean ± SD of three replicates

Gram-negative and Gram-positive bacteria, respectively, that are commonly found in soil. The growth of *E. coli* was reduced by up to 18% of the control at 8 h in the presence of 100 ppm phenanthrene and remained at this level until at least 24 h, indicating that *E. coli* hardly grew in the presence of phenanthrene in the medium. In contrast, the growth of *E. coli* in the laccase-treated phenanthrene medium was not significantly different from that of the control. The same trend was observed for the growth of *B. subtilis*, where a significantly reduced growth (up to 30% of the control at 10 h) in the presence of 100 ppm phenanthrene was observed and then remained at this level until at least 24 h. Likewise, the growth of *B. subtilis* in laccase-treated phenanthrene medium was not significantly different from that in the control. Thus, the phenanthrene metabolites were not toxic to either of these bacterial species. Wu et al. (2018) and Jang et al. (2007) studied the toxicity of phenanthrene to *E. coli* and *Pseudomonas putida*. Both studies' findings suggested that the cell viability decreased with the increase in phenanthrene concentration. The low viability for the intermediate

phenanthrene may have been due to instability of the cell growth and microbial stress, which were very sensitive to the experimental conditions. However, it can be deduced that toxicity would be greater if phenanthrene directly contacts the surfaces of cells at concentrations greater than solubility (Jang et al. 2007). It may be explained for both strains of *E. coli* and *B. subtilis* that the growth was inhibited by the presence of phenanthrene due to the high concentration of phenanthrene (100 ppm) in the media. In accordance with this, it was previously reported that the toxicity of phenanthrene toward the bacterium *Vibrio fischeri* and its metabolites after a 10-d degradation by the halophilic *Martelella* sp. AD-3 was markedly reduced, indicating the transformation of phenanthrene to less toxic or harmless by-products (Feng et al. 2012).

Phytotoxicity tests

The phytotoxicity of the phenanthrene degradation products was assessed by observing its effects on the germination and seedling development of *V. radiata* (Table 3). According to the Terrestrial Plant Test, the seedling emergence must be at least 70% in the negative control for the test to be considered valid. The seed germination of *V. radiata* after soaking with 100 ppm phenanthrene ranged from 50–90%, although the average was above 70%, while the degradation products of phenanthrene (i.e., the laccase-treated phenanthrene) were apparently not toxic (80–90% germination). Previous studies have reported that seedling germination was not significantly affected by similarly structured aromatic compounds, such as PAHs (Juhasz et al. 2010; Khan et al. 2012). Furthermore, it has been suggested that seedling germination alone may not be predictive of PAH toxicity (Sverdrup et al. 2003), which is also in accord with the result from this study.

Compared to the control, *V. radiata* appeared to be sensitive to phenanthrene (100 ppm) in terms of the root and shoot elongation. The addition of phenanthrene (100 ppm) significantly decreased the shoot length of *V. radiata* by twofold compared to the control. In contrast, the laccase-treated phenanthrene showed only a 1.1-fold decreased shoot length compared to the control, which was not significant. Likewise, phenanthrene significantly decreased the root length (2.3-fold) of *V. radiata* compared with control, whereas the laccase-treated phenanthrene appeared to be non-toxic to root elongation, with no significant difference in comparison to the control.

Table 3 Phytotoxicity against *V. radiata* of phenanthrene (100 ppm) and its laccase-treated degradation products

Parameter	*Vigna radiata*		
	Water	Phenanthrene (100 ppm)[A]	Laccase-treated phenanthrene[B]
Germination (%)	100	80 ± 1.09	90 ± 1.22
Shoot length (mm)	40.13[a] ± 1.85	19.93[b] ± 1.02	36.63[a] ± 9.71
Root length (mm)	18.20[a] ± 4.31	7.83[b] ± 0.69	12.17[a] ± 7.26

Values are the mean ± SD of three replicates. Values followed by different superscript letters ([a], [b] and [c]) in the same row are significantly different from the control (seeds soaked with water) at $p < 0.05$ by one-way analysis of variance (ANOVA) with Tukey's test

[A]Seeds soaked in phenanthrene (100 mg/l)

[B]Seeds soaked in biotransformed products of phenanthrene (100 mg/l) from *T. polyzona* PBURU 12 crude laccase

The metabolism of PAH in different plant species involves different metabolites and so different toxic effects. The myriad effects of PAHs on plant cells are reflected in the wide assortment of plant- and contaminant-specific phytotoxic effects of PAH reported in the literature. For example, phenanthrene at concentrations greater than 50 µM was reported to adversely affect the growth of *Arabidopsis thaliana* in vitro, as reflected by a reduced shoot and root growth, deformed tricomes, reduced root hairs, chlorosis, late flowering, and the appearance of white spots on leaves (Alkio et al. 2005). Overall, the toxicity of phenanthrene was eliminated after treatment with *T. polyzona* PBURU 12. The fungal bioremediation of PAHs has been noted, including pyrene degradation in soil after treatment with a consortium of three WRF (*Trametes versicolor*, *Bjerkandera fumosa*, and *Lopharia spadicea*), which relieved the inhibition of the seed germination of *Lepidium sativum* L. (Anastasi et al. 2009). The degradation of PAHs by *T. polyzona* PBURU 12 can potentially be developed as a useful bioremediation process, since the result from this study showed its potential application to reduce the toxicity of phenanthrene after treatment with the *T. polyzona* PBURU 12 crude laccase.

Genotoxicity test

Bioassays with *Allium cepa* also provided useful results for evaluating the effects of organic xenobiotics, including petroleum hydrocarbons, PAHs, pesticides, and other chemical agents in complex mixtures (Geremias et al. 2011). In terms of the damage index of *A. cepa* root tips, exposure to 100 ppm phenanthrene increased the damage index compared to the control (0.00) and was the same as the positive control. However, the laccase-treated phenanthrene had a twofold and significantly lower damage index than phenanthrene. The damage index due to chromosome aberrations is characterized by changes in either the chromosomal structure or the total number of chromosomes, which can occur both spontaneously and as a result of exposure to physical or chemical agents (Rainho et al. 2013; Leme and Marin-Morales 2009). Formation nuclei abnormalities, such as of micronuclei and binuclei, were found to be scattered when the root was exposed to phenanthrene, while chromosome bridge was found in treated phenanthrene. These results

were in agreement with another study (Rainho et al. 2013) that suggested a complex mixture of hydrocarbons may present clastogenic and aneugenic activities or even induce cell death in *A. cepa* genetic material.

The mitotic behavior of chromosomes in the root tips of *A. cepa* was used as a cytotoxicity test. Table 4 shows the percentage of the mitotic index (MI) and damage index observed when *A. cepa* root tips were treated with 100 ppm phenanthrene solution. The root tips exposed to 100 ppm of phenanthrene showed a significant 1.37-fold decreased MI compared to the control and this was not significantly different to that of the positive control. However, although the laccase-treated phenanthrene caused a less marked MI reduction than phenanthrene (1.15- vs. 1.37-fold), this was still significantly lower than in the control.

Phenanthrene at 100 ppm showed a high cytotoxic effect toward *A. cepa* root tips compared to the control. The reduction in MI, that is the percentage of mitotic cells in certain stages of the cell cycle, may indicate the need to disrupt the mitotic cycle to repair the damage caused by the components of phenanthrene toxicity. Benzo(a)pyrene at 1.0–5.0 µg/ml was previously reported to cause a decreased MI and an increased frequency of abnormal mitosis compared to the control, which was a result of the change in the metaphase/prophase (M/P) ratio, with a tendency for the M/P ratio to decrease with increasing benzo(a)pyrene concentrations and exposure times, leading to inhibition of the cell cycle in prophase (Cabaravdic 2010).

Mutagenicity (Ames) test

Four treatments (negative control (DMSO), positive control, phenanthrene addition (100 ppm), and laccase-treated phenanthrene) were tested for mutagenicity in the Ames test using both the TA98 and TA100 strains of *S. typhimurium*, with the results summarized in Table 5. A compound is considered a mutagen if it induces a reproducible, dose-related increase in the number of revertant colonies in one or more strains, and is considered a weak mutagen if the increased number of revertants is not double the background number of colonies (Mortelmans and Zeiger 2000). Most mutagens are toxic to the bacteria at some concentration, and this is indicated by the decreased number of revertant colonies with increasing concentrations of the mutagen as a result of cell death. In this study, the highest activity appeared with the positive controls: 4-nitroquinoline-oxide and sodium azide at 1.5- to 1.6-fold higher activity than the control for strain TA100, and with 2-aminoflourene and methyl methane sulfonate at 2.3- to 2.8-fold higher activity than the control for strain TA98. The addition of phenanthrene to the media did not increase the number of revertants, and so phenanthrene was not mutagenic in this assay. Likewise, the laccase-treated phenanthrene also showed no mutagenic activity in this assay. These results are consistent with a previous report that phenanthrene showed no mutagenic response in the Ames test with or without metabolic activation from rat or human liver S9 (cytosol containing microsome) fractions (Hakura et al. 2005). Indeed, phenanthrene is generally considered as non-carcinogenic. In that study (Hakura et al. 2005), when the phenanthrene dose was increased up to 500 µg/plate, the TA100 strain showed a decreasing number

Table 5 Mutagenic study of phenanthrene (100 ppm) and its laccase-treated degradation products using *S. typhimurium* strains TA98 and TA100

Chemical	Mutagenic activity (revertants/µg/plate)	
	TA98	TA100
Negative control		
DMSO (100 µl)	$36^{b} \pm 5.29$	$112^{ab} \pm 7.55$
Positive controls		
4-Nitroquinoline-oxide (10 µg)	–	$183^{a} \pm 13.31$
Sodium azide (10 µg)	–	$173^{a} \pm 16.04$
2-Aminoflourene (10 µg)	$102^{a} \pm 4.58$	–
Methyl methane sulfonates (10 µl)	$83^{a} \pm 13.45$	–
Test compounds		
Phenanthrene (100 ppm)	$15^{b} \pm 1.53$	$95.33^{b} \pm 15.18$
Laccase-treated phenenthrene (100 ppm)	$23^{b} \pm 18.48$	$63^{b} \pm 30.92$

Data are shown as the mean ± SD of three replicates. Values followed by superscript letters (^{a}, ^{b} and ^{c}) in the same column are significantly different from the control at $p < 0.05$ by one-way analysis of variance (ANOVA) with Tukey's test

Table 4 Genotoxicity of phenanthrene (100 ppm) and its laccase-treated degradation products against *A. cepa* root tip cells

Treatment	No. cells	No. dividing cells	Mitotic index (%)	Damage index (%)
Negative (distilled water)	$325.80^{a} \pm 77.85$	$55.00^{b} \pm 7.00$	$18.52^{a} \pm 8.89$	0.00^{a}
Positive (2-aminoflourene)	$100.60^{b} \pm 12.18$	$12.40^{c} \pm 5.17$	$12.95^{a} \pm 4.94$	$0.06^{c} \pm 0.02$
Phenanthrene (100 ppm)	$144.40^{b} \pm 26.96$	$16.20^{c} \pm 3.11$	$13.56^{a} \pm 4.02$	$0.06^{c} \pm 0.02$
Laccase-treated phenanthrene (100 ppm)	$289.40^{a} \pm 34.82$	$71.6^{a} \pm 53.19$	$16.14^{a} \pm 2.29$	$0.03^{b} \pm 0.01$

Values are shown as the mean ± SD of three replicates. Values followed by superscript letters (^{a}, ^{b} and ^{c}) in the same column are significantly different from the control at $p < 0.05$ by one-way analysis of variance (ANOVA) with Tukey's test

of revertants, which likely reflected the loss of viable cells due to the toxicity of phenanthrene, rather than a reduced proportion of revertants in the viable cell population. Likewise, in this study, the number of revertants was lower in the presence of phenanthrene or the laccase-treated phenanthrene compared to the control for both the TA100 and TA98 strains, likely reflecting the toxic effect to both strains. However, mutagenic activity in the Ames test has previously been observed for phenanthrene metabolites produced during the biological degradation of phenanthrene, and these were also found to be harmful for selected representatives of aquatic ecosystems (Bücker et al. 1979; Traczewska 2000). This may represent the mutagenic potential of other metabolites from different pathways in contrast to that produced here by the laccase from *T. polyzona* PBURU 12.

Conclusions

The fungal biodegradation of phenanthrene using the crude extracellular laccase enzyme from the WRF *T. polyzona* PBURU 12 can potentially be a useful tool. This WRF was found to be tolerant to phenanthrene compared to other isolates. The degradation of phenanthrene occurred within 24 h of incubation, achieving up to 90% phenanthrene degradation (from 100 ppm initial concentration) when 10 U/ml crude laccase was used. The phenanthrene degradation metabolites were significantly less toxic to both *E. coli* and *B. subtilis* than phenanthrene and also showed a reduced (to undetectable) phytotoxicity. This is the most positive finding in regard to their bioremediation potential in PAH degradation. The laccase-treated phenanthrene metabolites showed no significant cytotoxic or Ames test mutagenic activities.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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