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Biodegradation of chrysene and benzo[a]pyrene and removal of metals from naturally contaminated soil by isolated *Trametes versicolor* strain and laccase produced thereof

Ziva Vipotnik, Michele Michelin *, Teresa Tavares

Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

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ABSTRACT

The objective of this study was to assess the degradation rates of chrysene and benzo[a]pyrene, as well as the removal of aluminium and iron from contaminated soil collected in the upper layer (0–30 cm) in Lagos, Southwest Nigeria. *Trametes versicolor* was isolated from this soil and used in degradation experiments, with plantain peels as support. After 8 weeks, 81.0% of chrysene degradation was achieved by *T. versicolor*, and by adding support this increased to 91.0%. Benzo[a]pyrene was less degradable, with 38.0% and 49.1% of degradation, respectively. *Trametes versicolor* was also capable of accumulate 46.1% of aluminium and 57.2% of iron. By adding plantain peels, these amounts increased to 48.2% and 61.8%, respectively. At the same time, laccase was produced by *Trametes versicolor* on plantain peels, achieving 37.8 U/g of crude laccase during SSF at 30 °C for 3 weeks. Laccase degradation experiments were set up in packed-bed reactor (PBR), with a constant feed of 21.6 mL/day of laccase, with and without mediators. In 35 days, 75.8% degradation of chrysene was achieved by laccase. The highest degradation was observed with ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) as mediator, 87.9%. Benzo[a]pyrene degradation with laccase reached 35.6%, raising to 38.8 % with ferulic acid as mediator. In addition, 99.2% of iron and 99.6% of aluminium was removed by laccase, being the treatment for this last mediated with ABTS.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are some of the main pollutants typically present in contaminated soils, usually in combination with heterocyclic aromatic rings in which carbon atoms are substituted by nitrogen, sulphur or oxygen atoms. PAH substituted by alkyl groups are also common co-pollutants (Idowu et al., 2019), together with heavy metals, defining complex mixtures.

They are a group of organic pollutants related to anthropogenic activities and industrial development. With the rapid industrialization and demand for crude oil in developing countries, toxic chemicals and metals are spreading and becoming a threat to the environment and the food chain. Most of times, organic pollutants are present mixed with heavy metals (Okonofua et al., 2019). Their concentration is approximately 2–10 times higher in urban areas, where they are adsorbed and accumulated in the upper surface layer of the soil, finding their way into the ecosphere. Moreover, the soil appears

* Corresponding author.
E-mail address: mimichelin@ceb.uminho.pt (M. Michelin).

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to act as a long-term storage area for PAH as they are deposited there. In Nigeria, the negative effects of oil includes pipeline leakages, indiscriminate dumping of hydrocarbon wastes, leakages from transporting vessels/vehicles moving all over the country, with different effects on environment and impacts on health (Okonofua et al., 2019). Another contaminating factor is the open low temperature wastes burning on dumping sites, creating anthropogenic contamination from petrogenic and pyrogenic sources.

High molecular weight (HMW) PAH sorb strongly in soils and sediments and are more resistant to microbial degradation due to their high molecular weight, hydrophobicity and toxicity towards microbial cells (Bisht et al., 2015; Sikkema et al., 1995). Heavy metals are also considered as hazardous element, their non-degradable nature causes them to accumulate in the environment and pose a threat to eco-system. Several studies report microbial approaches to diminish the toxicity of some heavy metal ions or transform them to less harmful (Enayatizamir et al., 2020; Essa et al., 2012; Park et al., 2011; Zhou et al., 2013). Iron can act as co-factor and benefit cellular growth, however in excess amount it becomes toxic. On the other side aluminium has no biological function (Baldrian, 2003).

Bioremediation is an environmentally friendly, economic and efficient alternative to degrade and transform PAH into non-toxic compounds and has been classified as a soil clean-up technique. However, studies have shown that the success of PAH bioremediation has been limited to low molecular weight (LMW) PAH (Ogbonna et al., 2012). The major drawback for the bioremediation of PAH is their low water solubility and subsequent low degradation rates. PAH degradation rate is reduced with increasing benzene rings. Therefore, with increasing molecular weight also toxicity increases (Li et al., 2010a). Microbes require special conditions, as the toxicity of heavy contamination may also damage them (Bamforth and Singleton, 2005). Enzyme bioremediation may be another option that should be considered. Laccase are versatile enzymes with the ability to oxidize a wide range of aromatic and non-aromatic compounds, along with inorganic ions (Jacob et al., 2018) and has high stability and very low substrate specificity that makes it suitable for PAH degradation (Fernández-Fernández et al., 2013). Despite the high decomposition efficiency of enzymatic catalysis associated with the low toxicity of enzymes, their low redox potential may be a limiting factor, and to overcome it, redox mediator like ABTS or HBT (1-Hydroxybenzotriazole) might be used (Upadhyay et al., 2016).

The present study reports the potential of a fungus isolated from heavily polluted soil to degrade chrysene and benzo[a]pyrene, as well as to remove metals, present in this soil. The production of relevant enzyme by this microorganism, namely laccase, was also evaluated, as well as its ability to degrade soil contaminants in batch and packed bed reactor. Unlike PAHs remediation, there are no reports addressing the fate of heavy metals in contaminated soil during the enzymatic remediation process by laccase. Moreover, to evaluate bioaugmentation (by *T. versicolor*) – biostimulation (plantain peels) effect of selected soil, the contaminated soil in any assay was not sterilized.

2. Material and methods

2.1. Chemicals

Acetonitrile (HPLC grade) and nitric acid (69.5%) were purchased from Sigma-Aldrich as well as UHPLC and ICP standards; PAH Calibration Mix and ICP multi-element standard solution IV. ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), ferulic and coumaric acid were acquired from Alfa Aesar.

2.2. Soil collection and characterization

Soil used in this study was collected in Lagos (6°32'46.1″N 3°16'09.0″E - Lagos, Nigeria), in a condensed neighborhood. It has been collected at the end of November (dry season), with an average temperature 32 °C. Samples were collected from soil surface (0–30 cm depth) and kept into polyethylene bags at 4 °C until use.

The soil samples were dried for 2 days at 60 °C and sieved in a 2.0 mm mesh. The pH of the samples was measured in a soil/ H_2O ratio of 1:1.5. The moisture content of the sample was assessed in a hot air incubator at 105 °C and organic matter was determined as the percentage loss on ignition of 2.0 g of soil in an oven at 450 °C for 4 h.

Metals were extracted by putting 1 g of each sample into digestion tubes with 10 mL of aqua regia (concentrated hydrogen chloride and nitric acid, ratio 3:1) (US EPA method 3050b) (US EPA, 2012). Concentration of iron and aluminium were measured by an ICP-OES (Optima 8000, PerkinElmer), with detection of iron at 238.204 nm and aluminium at 396.153 nm, and operating conditions of 1300 W RF power, 8 L/min argon plasma flow, 0.2 L/min auxiliary gas flow and 0.5 L/min nebulizer gas flow.

For PAH extraction in all assays, 1.5 mL of acetonitrile was added to 0.5 g of soil and extraction was carried out using rotating shaker at 160 rpm for 30 min and for another 10 min in 40 kHz Sonicator. The samples were centrifuged at 8000 g and the supernatant was transferred to 2 mL vials. The quantification of the selected PAHs was performed by ultra-high-performance liquid chromatography (UHPLC), using a Shimadzu Nexera X2 (Shimadzu, USA) with one multi-channel pump (LC-30AD), an autosampler (SIL30AC), an oven (CTO-20AC), a diode array detector (M-20 A) and a system controller (CBM-20 A) with built-in software (LabSolutions). For the PAHs quantification, a Kinetex PAH C18 column (Phenomenex, Inc. CA, USA) was used. The mobile phase was ultrapure water (pump A) and acetonitrile (pump B). Starting mobile phase composition was 51% A, decreased to 4.5% A in 12.03 min, remaining in this percentage until 16.3 min and increased again to 51% (17.25 min) and remaining in this percentage for 2.35 min. The flow rate was 0.6 mL/min, and samples were monitored by a diode array detector from 190 to 400 nm, and chromatograms were extracted at 252 nm. Column oven was set at 25 °C, and the injection volume was 15 μL.

2.3. Fungal collection and isolation

Fungal strains were isolated from soil by the serial dilution technique, prepared by mixing in vortex 1 g of soil with 10 mL of distilled water, and further diluted to 10^{-6}. A volume of 0.1 mL was pipetted onto plates with Rose Bengal agar and Sabouraud agar, incubated at 28 °C. The pure culture obtained was transferred to MEA (malt extract agar) and kept at 4 °C for further use.

2.4. Laccase screening of isolated fungi and identification

For a preliminary screening of laccase production, 20 dried ABTS-impregnated discs were placed into an empty standard flat-bottom 96-well microplate (Dias et al., 2017). The screening of laccase activity was started by the adding 10 μL aliquots from each sample (in this work, 48 h old fungi biomass from malt broth) to discs and left for 10 min at 30 °C. Samples with laccase production developed green-bluish color and were further tested on laccase plate assay, where a diameter of 1 cm of mycelium from each isolated strain was inoculated into MEA, containing 10 mL of 20 mM ABTS and 1 mL of 100 mM $CuSO_4$ per 1 L medium. The formation of halo in the plates supplemented with ABTS indicated a positive laccase secretion. The diameters of the halo zones and of the mycelium were measured at regular intervals of time for 5 days, after the organisms were selected for benzo[a]pyrene and chrysene degradation.

The identification of the selected fungus was performed molecularly by DNA amplification and sequencing. Fungus was grown on Potato Dextrose Agar for 5 days at 28 °C and genomic DNA extraction was performed as described (Rodrigues et al., 2009). PCR amplification was achieved with universal primers ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and PCR reactions were carried out in a thermal cycler BioRad Mycycler, in a final volume of 50 μL, containing 10 μL of 5x Go-Flexi Taq $MgCl_2$-free reaction buffer (Promega), 1.5 mM $MgCl_2$, 1.25 U of Go-Flexi Taq polymerase (Promega), 200 μM of each Primer, 1 μL dNTP (Bioron) and 2 μL of genomic DNA. Amplifications were carried out in a Bio-Rad MYCYCLER thermal cycler using a temperature gradient protocol as described (Rodrigues et al., 2009). The sequencing was conducted by Microsynth (Switzerland) and manually adjusted by chromatogram comparison and then aligned with the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm.

2.5. Laccase production by solid state fermentation (SSF)

The fungal inoculum was prepared by cutting four agar plugs (5 mm × 5 mm) from the malt extract agar plates. These were extruded through a syringe into 500 mL Erlenmeyer flasks containing 200 mL of sterile malt extract (2% w/v). Fungus was cultivated at room temperature, with continuous agitation at 120 rpm, for 5 days. Plantain peels were used as substrate for SSF. For this, they were cut to 1 cm^2 and pretreated with 83 mM of KOH at room temperature (≈28 °C) for 20 min, in a ratio of 1:3, to neutralize organic acids (Stredansky and Conti, 1999). After that, they were washed twice with deionized water, dried at 60 °C for 45 min, and stored for use. The final moisture content of the peels was 60%.

The 500 mL Erlenmeyer flasks were filled with 200 g of the pretreated plantain peels, autoclaved at 121 °C for 15 min, inoculated with 5 mL of fungal biomass per flask and left at room temperature for 3 weeks. After that, the mixture was suspended in 400 mL of 50 mM sodium acetate buffer (pH 4.5) and mixed continuously (100 rpm) for 1 h at room temperature. This suspension was filtered through a nylon cloth and the filtrate was centrifuged at 7500 g for 15 min and used for enzymatic assays. SSF was performed in duplicate.

2.6. Laccase activity assay

Laccase activity was measured in the spectrophotometer (BioTek Synergy HT) at 420 nm by the oxidation of ABTS in 0.1 M sodium acetate buffer, pH 5.0, at 30 °C (Bourbonnais and Paice, 1990). One unit of enzyme activity was defined as 1 μmol of substrate oxidized per minute and expressed in U/g.

2.7. Soil rehabilitation

PAH degradation and metals removal were evaluated in soil using two strategies: degradation by the isolated fungus, namely *Trametes versicolor,* in amber laboratory bottle, and enzymatic degradation by laccase produced from *T. versicolor* in packed bed reactor (PBR). Laccase mediator system (LMS) was evaluated in the second strategy.

2.7.1. Trametes versicolor degradation

Batch fungal degradation assay was set up in 100 mL amber laboratory bottles with 50 g of naturally contaminated soil, 10 g of plantain peels and 1 mL of fungal inoculum. Experiment was set up for 8 weeks at room temperature, by taking samples once a week and, at the same time, each bottle was sprayed with air, at a flow rate of 0.02 ml/min, for 1 min. Experiments were performed in the dark and in triplicate.

2.7.2. Laccase degradation

Batch laccase degradation assay was set up into 100 mL amber laboratory bottles with 20 g of contaminated soil, 10 mL of laccase load at 2 U/g and 6 U/g, and mediators (ABTS, coumaric acid, ferulic acid) at concentrations of 0.5, 1 and 2 mM. Experiment was set up for 7 days in triplicate, at 30 °C and in the dark. Boiled enzyme was used as control.

Laccase-fed degradation was set up in packed-bed bioreactor (PBR) consisting of a vertical glass column (25 cm length, 3 cm Ø, and 3.5 cm width) filled with 300 g of contaminated soil. A load of 2 U/g of laccase and 1 mM of mediator was used, in a continuous flow of 0.015 mL/min, using a laboratory peristaltic pump (Masterflex, Cole-Parmer). The laccase flow was refreshed every five days. This procedure was done in triplicate and performed during 35 days at room temperature (28 °C) in the dark. Soil samples were taken every 5 days for measuring the removal efficiency of the pollutants.

2.8. Statistical analysis

GraphPad Prism® software (version 8.0; graphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses. The level of significance was determined by two-ways ANOVA followed by Tukey's test for multiple comparisons. Significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. Soil characterization and fungal screening

The physicochemical characteristics of the Nigerian soil are reported in the supplementary material (**Table S1**). The major pollutants considered in the present study were HMW-PAH, mainly chrysene and benzo[a]pyrene, with 367.94 mg/kg and 11.74 mg/kg in samples, respectively. This soil is classified as sandy loam, with 64% of sand, 16.2% silt and 19.8% of clay. The pH value of 8.3 ± 0.2 indicates that the soil was alkaline in the studied area, with 18% moisture at the beginning of the experiments. As expected, there were also 625.37 mg/kg of iron and 238.33 mg/kg of aluminium. The main reason for such high amounts is the location where the samples were collected, with high PAH and metal concentrations as a result of their accumulation from surface run-off, municipal and industrial waste discharges and aerial deposition from industrial pipes, probably with evident fluctuations depending on of the dry and wet season cycles.

Oketola & Akpotu and Adeyi & Oyeleke studies reported the formation of leachates from municipal solid waste dumpsites in Nigeria, containing high concentrations of metals, PAH and PCB, which are further distributed into soil, water and sea, and from there entering into food chain (Adeyi and Oyeleke, 2017; Oketola and Akpotu, 2015). The improper handling of different residues is contributing to the addition of metals and PAHs in the air and mainly in the top layer of soil. The efficiency of the polluting compounds degradation in soil differ randomly and it is more complex than in liquid media, mainly due to the low bioavailability of substrates, however, there are several factors that need to be considered in the bioremediation. One of them is organic matter content of topsoils, whose are also responsible for retaining concentrations of the contaminants in soils. Biomass waste as biochar are desirable, but can also affect the ability of biochar to sorb organic contaminants. Moreover, the presence of co-contaminants may affect the sorption, desorption, bioaccessibility and biodegradation of the target compound (Ogbonnaya and Semple, 2013). Adeyi and Oyeleke (2017) observed the migration of metals to the topsoil. In most cases, metal concentrations were higher in the topsoil, which is evidence of recent anthropogenic contamination; with limited evidence of migration to the subsoil, which also indicates that there is little risk of groundwater contamination. At the same time, it was also observed that concentrations of individual PAH were higher in soil at the 0–15 cm level compared to soil at the 15–30 cm level.

One of the aspects of the bioremediation of soils contaminated by oil derivatives with autochthonous microorganisms is the isolation and identification of fungal strains from polluted soil in order to choose the most active to degrade or remove them. In this sense, seventeen pure fungal cultures were isolated from the soil samples collected, and further screened for the laccase production in 96-well microplate (Fig. 1A). Among them, two rapidly showed greenish blue color (fungal strain 3 and 14), indicating laccase activity, and therefore they were selected for further laccase screening in plate (Fig. 1B,C). The one with the highest laccase production (4.5 ± 0.27 cm — determined through halo formation) was selected for the present study.

3.2. Molecular identification of fungal strain

Molecular identification revealed 99.31% sequence similarity of the fungal strain selected for this work with *Trametes versicolor* (**Table S2**). *Trametes versicolor* is well known by its ability to degrade different organic pollutants. They have the ability to efficiently degrade most PAH using them as an exclusive carbon source (Bhattacharya et al., 2014; Hadibarata et al., 2009). It has been recognized that white-rot fungi degrade PAHs by the synthesis of lignin modifying enzymes, as laccases. *Trametes versicolor* can secrete high levels of laccase; because of its oxidoreductive nature, this enzyme can oxidize various types of toxic chemical compounds into nontoxic ones (Brijwani et al., 2010), making *Trametes versicolor* an important contributor in bioremediation research. These enzymes usually catalyze the first attack on PAH molecules degradation (Steffen et al., 2003). *T. versicolor* has also been reported on biosorption studies of heavy metals (Bayramoğlu et al., 2003; Manna et al., 2018).

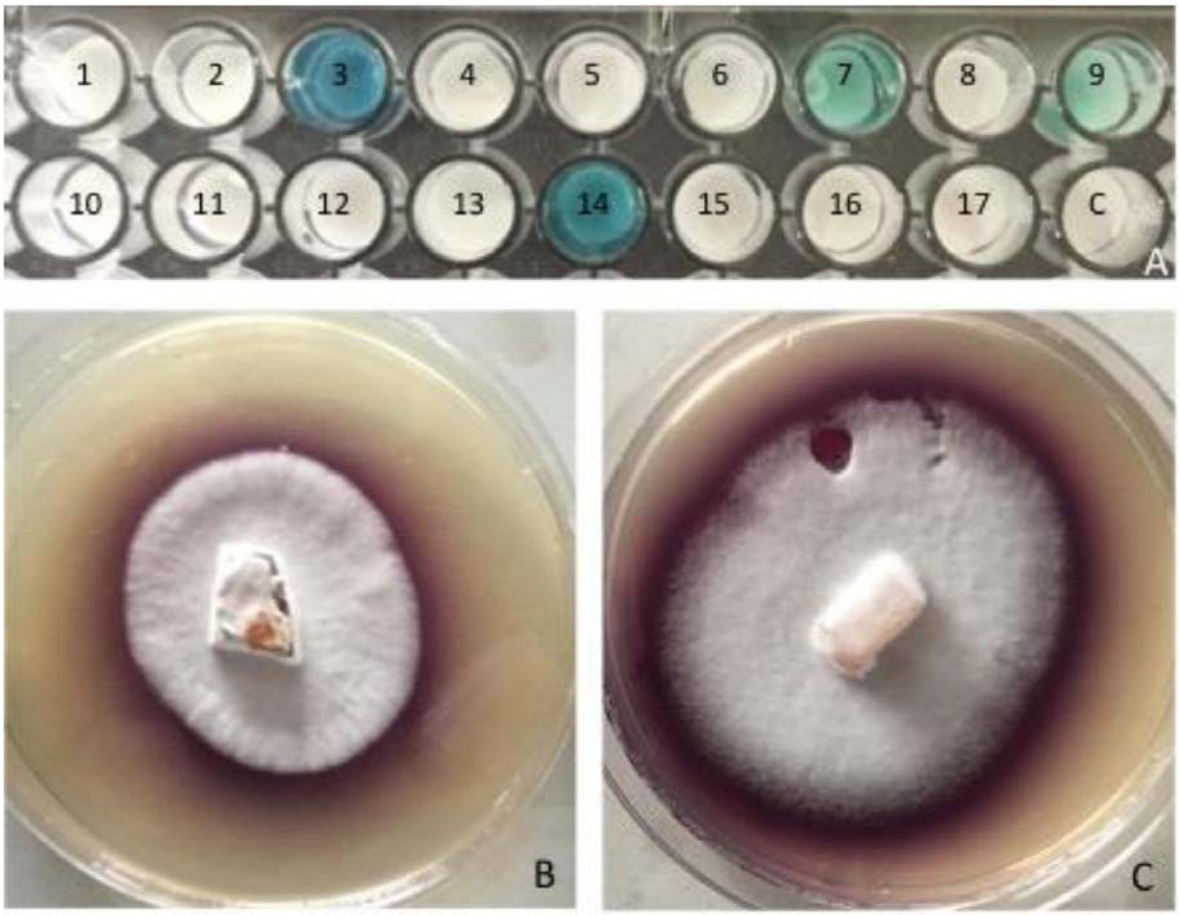

Fig. 1. Laccase screening of fungi isolated from Nigerian soil. ABTS-impregnated discs for 17 isolated fungi (**A**), laccase plate assay on MEA with 20 mM ABTS and 100 mM $CuSO_4$ for fungal strains nº3 (B) and nº14 (C) that showed the highest laccase oxidation. Fungus nº14 was identified as *Trametes versicolor*.

3.3. Evaluation of laccase production

Laccase production was performed by SSF for 6 weeks. The highest amount of laccase on plantain peels was 38.8 U/g after 3 weeks of fermentation (**Fig. S1**). Plantain peel represents a local agricultural waste in Nigeria, and such residue contains polysaccharides and phenolic compounds that can stimulate both the fungal growth and subsequent laccase production. Osma et al. (2007) achieved 63 U/L of laccase with 3 days of fermentation and 1570 U/L with 20 days, by cultivation of *Trametes pubescens* on banana peels (7 g of substrate with 20 mL of culture medium).

The dependence of laccase activity on temperature and pH is shown in the supplementary material (**Fig. S2**). Maximal laccase activity was reached at pH 6 and 30 °C. A number of reports have indicated that the optimal pH for fungal laccase activities varies from 3 to 7, depending on the fungal species, implying that laccase remediation is unsuitable for alkali soil (Li et al., 2010b; Vandelun Ado et al., 2019). However, it is important to highlight that the microorganisms isolated from contaminated environments are capable of degrading PAH, due to their increased cell affinity to hydrophobic substances that enable them to absorb and utilize the PAH and accumulate heavy metals. They are also capable of producing a variety of enzymes, including laccase, lignin peroxidase and manganese peroxidase, which transform PAH and heavy metals into less harmful and simpler forms (Ani et al., 2018; Camarero et al., 2008; Enayatizamir et al., 2020; Haritash and Kaushik, 2009; Xu et al., 2018).

An interesting behavior observed was that the produced laccase reached its maximal activity at pH 6 for temperature between 25–35 °C, but at higher temperatures the optimum pH was 4. Enzyme showed higher activity at lower temperatures and less acid pHs, but at higher temperatures, a more acid pH was more favorable. One of the limiting factors in laccase production is temperature. In the presence of light, the temperature of 25 °C is generally accepted as optimum, but in dark conditions the optimum temperature is generally 30 °C (Bamforth and Singleton, 2005; Pointing, 2001; Thurston, 1994), reducing the production when fungi are cultivated at temperatures higher than 30 °C (Lang et al., 2000). However, the optimal temperature and pH of laccase production diverse from one to another fungal strain.

The effect of temperature and pH on laccase stability was observed for 7 days, since the pollutant degradation assays by laccase was performed in this period of time. Regarding temperature stability, it was higher at 30 °C, with 54% of activity

remaining after 7 days. With increasing temperature, stability decreases. Laccase showed the highest stability at pH 6, followed by pH 5. The pH stability decreased during the incubation time. After 7 days there were still 41.7% and 37.4% of residual activity at pH 6 and pH 5, respectively. Considering the removal of PAHs and heavy metals in contaminated soil, the higher stability of the enzyme at room temperature, as well as in pH from 6.0 to alkaline, is particularly important for its application herein foreseen, as soil pH was 8.3 ± 0.2.

3.4. PAHs degradation and metals removal

3.4.1. Fungal treatment

The longer PAH remain in contact with the soil, the more irreversible their sorption is and the lower the chemical and biological extractability of the contaminants (Ghosal et al., 2016; Luo et al., 2012; Martin, 2000). Biodegradation of PAH using microorganisms has been proven to be an efficient way to degrade PAH into less toxic forms. This method is relatively cheap, easily managed, and eco-friendly. However, the presence of organic and inorganic contaminants on the same site can affect the efficiency of bioremediation.

Fig. 2 presents the degradation of chrysene (A) and benzo[a]pyrene (B) in nonsterile soil by *Trametes versicolor*, with and without plantain peels as support. Soil was not initially sterilized by choice, in order to make the process more competitive, and previous work excluded the influence of any other microbe with high degrading capability (data not shown). Around 81% of chrysene was degraded only by *T. versicolor* and, using plantain peels as support, the degradation increased to 91%, both in 8 weeks (Fig. 2A). Benzo[a]pyrene was less degradable. In 8 weeks, 38% degradation was reached only with *T. versicolor* and 49.13% with the support (Fig. 2B). The degradation rates by *T. versicolor* over time showed that the fungus was capable of commencing PAH degradation from the first week when plantain peels were used as support, mainly because of support-induced laccase production. When no support was used, the fungus took 2 weeks to start degrading the compounds. Biache et al. (2017) also reported a higher degradation rate for chrysene than benzo[a]pyrene by the microbial community, that is probably related with its lower molecular weight. Borràs et al. (2010) reported similar degradation rates with *T. versicolor,* which were capable of a faster and more extensive removal of PAH in artificially spiked soil, despite its weaker growth. The removal of a total of 16 priority PAHs from USEPA by *T. versicolor* was 49% in 10 weeks. Rama et al. (2001) reported similar results with degradation of 16 priority PAH from USEPA, which was 38% in 20 weeks. However, their study was carried out on industrially contaminated soil, using agricultural waste peels for cultivation. On the other hand, Baltrons et al. (2018) reported the biodegradation of 3–4 rings PAH (phenanthrene, fluoranthene and pyrene) was lower, as the concentration of metals increased, but no important effect on the biodegradation of HMW-PAH (benzo[b]fluoranthene and benzo[a]pyrene) was observed at the different concentrations of metals studied.

Aluminium and iron load biosorption by *T. versicolor* are presented in Fig. 3. In 8 weeks, it was capable of accumulate 46.1% of aluminium and 57.22% of iron. By adding plantain peels, these amounts increased 2.04% and 4.61%, respectively. Bamforth and Singleton (2005) reported that some metals may be too toxic for white-rot fungi and may have a negative effect on the activity of their ligninolytic enzymes. However, many of these metals naturally exist in soil in trace concentrations. *T. pubescence* was able to withstand 1000 mg/L of Pb and Ni, removing 99% of Pb and 8.6% of Ni (Enayatizamir et al., 2020); while *T. versicolor* was able to absorb almost 0.300 mg/g of Cd from contaminated effluent (Manna et al., 2018). Biosorption of Cu, Pb and Zn by immobilized *T. versicolor* was also reported by Bayramoğlu et al. (2003).

3.4.2. Laccase treatment in batch

Enzymatic treatment of contaminated soil may be considered as an alternative and/or as a supplement to microbial bioremediation. The main advantages include high reaction activity, low sensitivity to high pollutant concentration, coverage of a wide range of physicochemical gradients in the environmental matrix, therefore being easy to control. PAH degradation by ligninolytic enzymes produced by white-rot-fungi, such as laccases, have been reported by other authors (Agrawal et al., 2018; Agrawal and Shahi, 2017; Ike et al., 2019; Li et al., 2014). However, there is still a limited number of published reports dealing with enzymatic remediation of soil, mainly due to the high cost of large-scale production of commercial laccase and the high amount needed. Utilizing bio-wastes to produce an enzyme can reduce the production costs while generating high concentrations of products (Panda et al., 2016). Agricultural wastes contain polysaccharides and phenolic compounds that might stimulate fungal growth and enzyme production.

The efficiency of laccase in degrade chrysene and benzo[a]pyrene was evaluated in batch using two laccase loads **(Fig. S3)**. After 7 days, the highest chrysene degradation rates were obtained with 6 U/g of laccase with 57.9% of degradation, being that of 55.6% by 2 U/g **(Fig. S3-A)**. The most efficient benzo[a]pyrene degradation was achieved with 2 U/g with 8.7% of degradation, followed by 6.5% with 6 U/g **(Fig. S3-B)**.

Wu et al. (2008) studied the effect of 3 different initial concentrations of laccase DAIWA Y120 (from *Trametes*, obtained by Amano Enzyme Inc) (1, 3 and 10 U/g) on degradation of 15 PAHs in soil with concentration of 10,834.65 μg/kg, during 14 days. After 14 days, laccase activity was not detected in the soil samples and during this time the degradation of total PAHs was 17.6% with 3 U/g, 32.4% with 1 U/g and 31% with 10 U/g.

The obtained data indicate that laccase transforms PAH efficiently with low initial laccase load, thus it was evaluated the PAH degradation using 2 U/g laccase in combination with 0.5, 1 and 2 mM of mediators (Table 1). The highest degradation was observed with 0.5 mM of ABTS and ferulic acid, and with 1 mM of coumaric acid. After 7 days, the highest

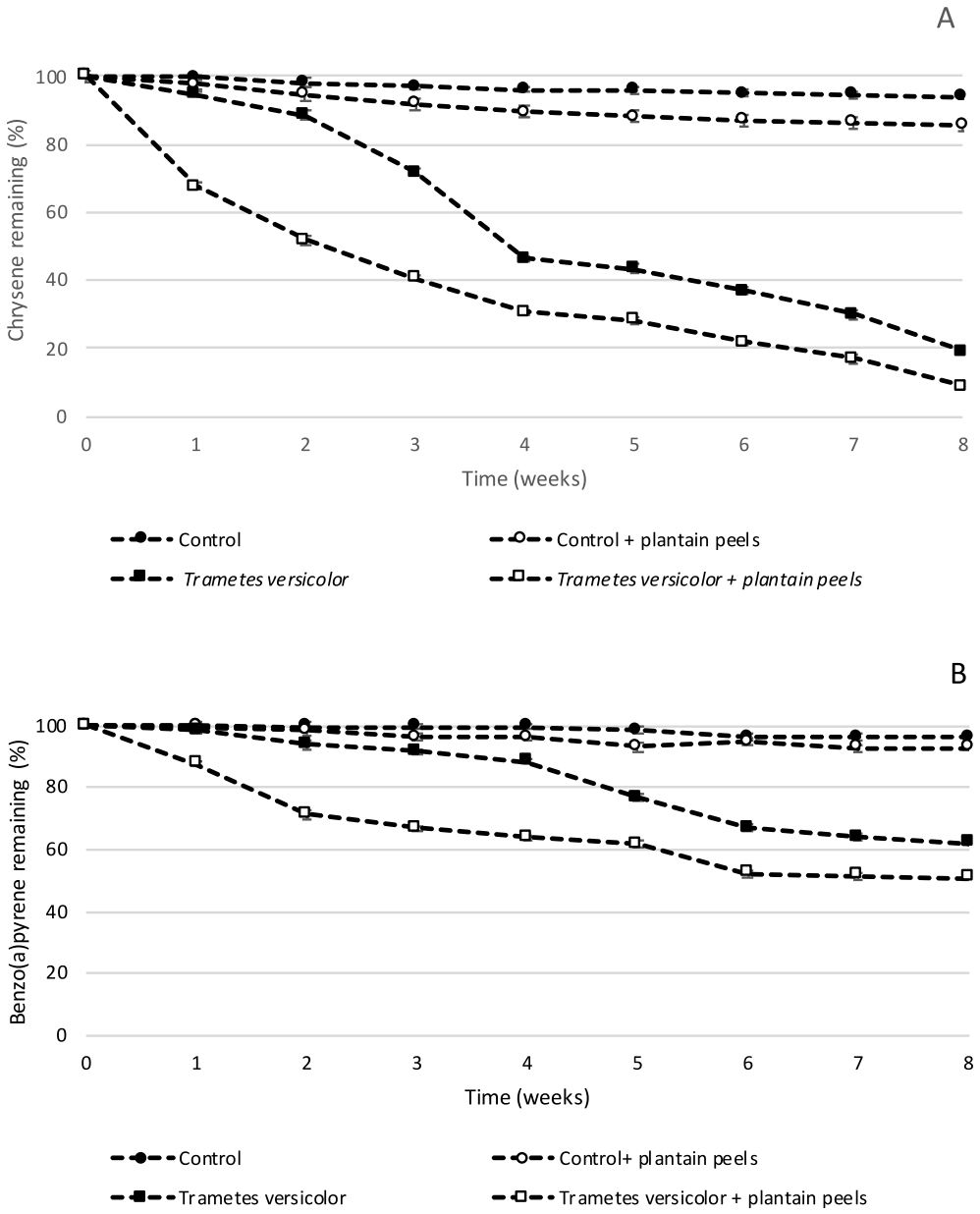

Fig. 2. Degradation of chrysene (A) and benzo[a]pyrene (B) in batch by *Trametes versicolor* with and without plantain peels as support at room temperature for 8 weeks in triplicate. Plotted values are the mean ± SD.

Table 1
Removal (%) of chrysene and benzo[a]pyrene with 2 U/g laccase and different concentrations of mediator.

Treatment	Chrysene removal			Benzo[a]pyrene removal		
	3 days	5 days	7 days	3 days	5 days	7 days
Laccase	33.73 ± 0.35	43.90 ± 0.40	55.58 ± 0.35	5.41 ± 0.42	6.40 ± 0.67	8.65 ± 0.64
L + 0.5 mM ABTS	37.42 ± 0.43	46.89 ± 0.33	67.41 ± 0.14	4.68 ± 0.84	10.37 ± 0.46	13.60 ± 0.15
L + 1 mM ABTS	26.32 ± 0.13	34.68 ± 0.04	49.94 ± 0.03	2.15 ± 0.19	4.73 ± 0.52	8.38 ± 0.42
L + 2 mM ABTS	19.66 ± 0.4	50.84 ± 0.16	53.35 ± 0.02	3.91 ± 0.45	5.20 ± 0.51	6.23 ± 0.14
L + 0.5 mM FA	32.69 ± 0.02	53.90 ± 0.03	65.15 ± 0.03	8.16 ± 0.62	8.59 ± 0.19	10.20 ± 0.52
L + 1 mM FA	16.33 ± 0.23	31.94 ± 0.21	37.00 ± 0.23	4.32 ± 0.25	5.12 ± 0.09	6.21 ± 0.31
L + 2 mM FA	14.55 ± 0.04	19.58 ± 0.42	26.79 ± 0.03	2.91 ± 0.16	3.50 ± 0.19	6.87 ± 0.41
L + 0.5 mM CA	9.23 ± 0.53	17.95 ± 0.04	24.23 ± 0.12	0.57 ± 0.32	2.23 ± 0.32	3.94 ± 0.52
L + 1 mM CA	26.81 ± 0.44	53.34 ± 0.402	65.36 ± 0.03	3.12 ± 0.31	8.43 ± 0.21	10.75 ± 0.74
L + 2 mM CA	51.07 ± 0.42	57.20 ± 0.42	58.60 ± 0.03	7.11 ± 0.71	8.04 ± 0.41	10.60 ± 0.71

L – laccase.

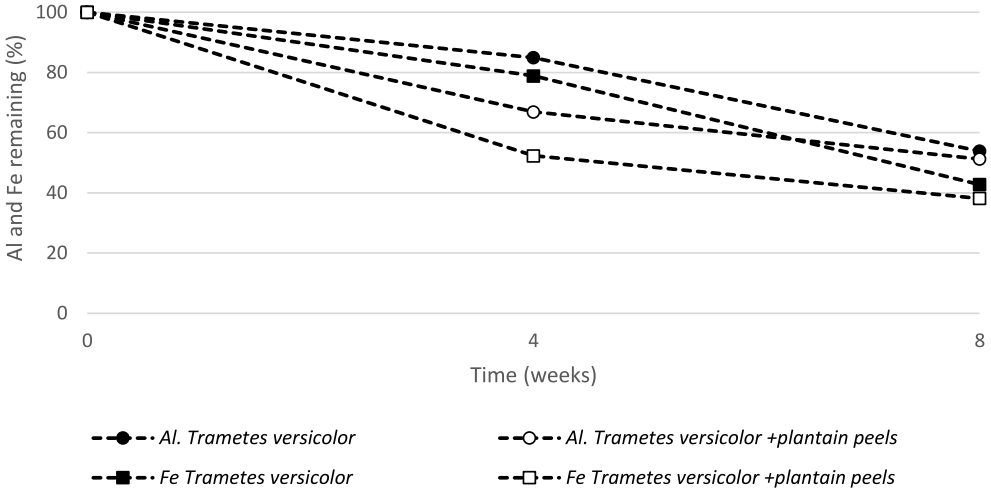

Fig. 3. Iron and aluminium biosorption in batch by *T. versicolor* with and without plantain peels as support at room temperature for 8 weeks in triplicate. Plotted values are the mean ± SD.

degradation rate achieved for chrysene was 67.4% using ABTS as a mediator. It was 11.83% higher than only laccase, being this improvement on degradation of 9.6% and 9.8% with ferulic acid and coumaric acid, respectively.

Benzo[a]pyrene was degradable for 8.7% with only laccase and by adding 0.5 mM of ABTS and ferulic acid, degradation increased by 4.9 and 1.6%, respectively. For coumaric acid, the lowest degradation ratio was observed with 0.5 mM of this mediator, yet with 1 and 2 mM there was no significant difference in the benzo[a]pyrene degradation, this being 2.1% and 1.9% higher than only laccase. Such low degradation rate is a consequence of benzo[a]pyrene being one of the most persistent PAH, which increases with aging. Moreover, the LMS is less efficient in system lacking water.

Some of the relevant parameters determining laccase activity in PAH degradation are the mediator and pH, as well as the incubation temperature for maximal laccase activity (Jin et al., 2016). Regarding temperature, a higher temperature is preferable for laccase catalysis, but it also leads to a faster loss of activity (Aktaş and Tanyolaç, 2003; Zhang et al., 2008). In general, mediators improve the degradation rates of PAH. Li et al. (2010b) also reported that LMS works actively in water environment or in soil with high capacity of water as in slurry. In this reported work, experiments were carried out with 10 U/g of laccase and soil with 70% of moisture content. After 10 days, 40.8% degradation was confirmed, which increased to 56.7% by adding 1 mmol/kg ABTS.

Regarding the removal of metals, around 84.9% of iron was removed with an enzymatic load of 2 U/g, and 73.3% with 6 U/g laccase (**Fig S3-C**). Aluminium concentration in soil was reduced in 98.8% with 2 U/g laccase, and 95.1% with a 6 U/g laccase load (**Fig. S3-D**). This is the first report about the enzymatic bioremediation of metals in naturally contaminated soil by laccases. However, the mechanism by which laccase would be able to reduce the amount of metals is not clearly understood. It have been reported that heavy metals can be biologically transformed by enzymes (e.g., by oxidation, reduction and methylation) to other harmless metal forms (Saravanan et al., 2021). Ahmadi Khozani et al. (2021) have reported the heavy metals removal and precipitation by a fungal laccase using tannin as a natural mediator. According to the authors, the radical intermediate of the tannin oxidation generated by laccase could react and precipitate the metal. Thus, tannin would be helping in the metal oxidation by enzyme, while it is reduced as mediator. Furthermore, they suggest that tannin (mediator) could react with the metal to form bioactive mineral complex, such as the fulvic acid.

Nathan et al. (2018) have reported the use of laccases for the paper pulp deinking process. During enzymatic deinking, there are possibilities for the release of heavy metals from the ink particles; however, they verified that metals like Fe, Pb and Zn were not detected in the enzyme assisted deinking effluent sample, and that there was a reduction in heavy metal concentration in the paper pulp compared to the untreated pulp after the enzyme treatment. Thus, oxidative and reductive enzymes play a crucial role in transforming metals, being one of the emerging techniques for pollution-free remediation methods (Saravanan et al., 2021).

3.4.3. Laccase treatment in PBR (fed-batch)

As in batch set-up, also in PBR laccase starts transforming PAH immediately upon entering in contact with the soil (Fig. 4), even if the mixture of soil and laccase differs. It is possible to prepare a slurry in batch, in which column assays aim the mimicking of a microcosms in field conditions. Therefore, 300 g soil in a fixed bed were daily fed with 21.6 mL of crude laccase: with and without mediator in ration. Degradation was rapid in the first 10 days for chrysene and benzo[a]pyrene (the presence of other PAH and organic pollutants in soil was not monitored), and then started slowing down, despite fresh laccase was used every 5 days.

Laccase removed 68.5% of chrysene in the first 10 days and, in total, 75.8% till the end of the experiment. Despite the fact that laccase with ABTS was capable of removing more chrysene, 87.9%, by the end of the assay, the process was slower

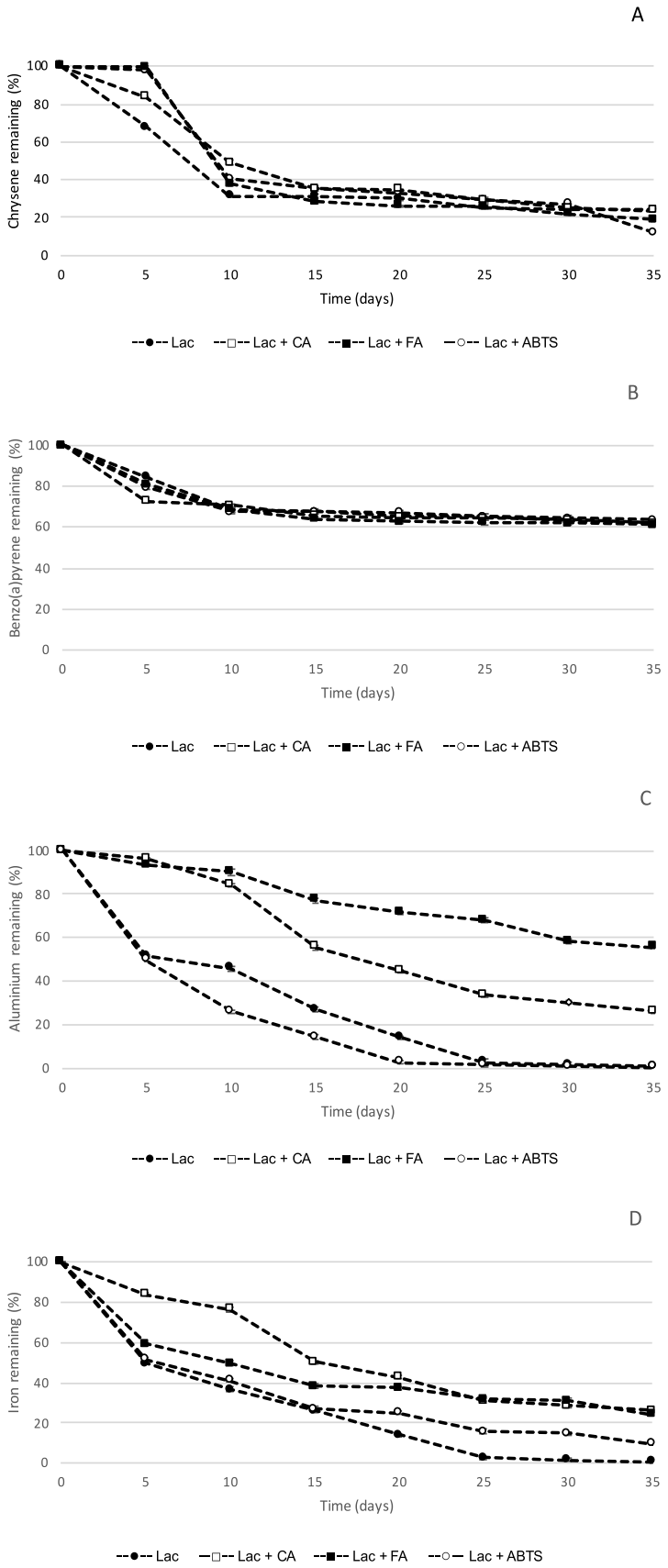

Fig. 4. Degradation of chrysene (A), benzo[a]pyrene (B), and removal of aluminium (C) and iron (D) in soil by laccase and laccase-mediator system in PBR in triplicate. Plotted values are the mean ± SD.

during the whole period. By adding ferulic acid, 81.1% of chrysene was removed and with coumaric acid 76.3% removal was reached (Fig. 4A). The degradation of benzo[a]pyrene was similar with and without mediator. Laccase degraded 35.6% of this molecule, while with ferulic acid, coumaric acid and ABTS as mediators the degradation changed to 38.8%, 37.9% and 36.5%, respectively (Fig. 4B), showing a limitation in benzo[a]pyrene degradation by laccase and LMS, one of the most recalcitrant and toxic PAH. Moreover, soil used in this assay was heavily contaminated, so it is possible that other compounds might compete in the degradation pathway or even inhibit the laccase activity.

Laccase achieved, *per se,* the highest removal rates for iron. In 35 days, 99.2% of iron was removed by laccase, reducing to 90.2% removal when mediated with ABTS, 76.1% with coumaric acid and 74.2% with ferulic acid (Fig. 4C). Regarding aluminium, more than 99% was removed, remaining in soil 0.4% when the laccase treatment was mediated with ABTS. Laccase with ferulic acid removed 74.1% of aluminium and with coumaric acid 44.5% of removal was achieved (Fig. 4D). Zhou et al. (2017) studied the effect of metals on commercial laccase from *T. versicolor* in buffer. Metal cation, K^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, and Cu^{2+} and the anion $SO_4{}^{2-}$ had almost no effect on laccase activity during the initial stage of the catalytic reactions, inhibitory effect was shown at 30 mM of each compound. High concentration of Mn^{2+} only showed weak inhibition on laccase, Fe^{2+} had no direct effect on the binding of laccase to its substrate, but strongly retarded the progress of the catalytic reaction by reducing the intermediate free radicals.

In a previous study (Vipotnik et al., 2021), a commercial soil was spiked with 300 ppm of 6 PAH and within 25 days, 81.8% of chrysene and 96% of benzo[a]pyrene were removed with a load of 2 U/mL laccase from cocultivation of *Penicillium chrysogenum* and *Trichoderma viride* and 1 mM ABTS, but the moisture content of soil increased to 59% by the end of the experiment, indicating that the LMS is not able to work effectively in an environment lacking water. On the other side in current study, moisture content at the end of assay was 31 $\pm$ 0.082%, therefore LMS was less successful. Moreover, in the current study naturally contaminated soil was used, with different aged organic and inorganic pollutant, and other microorganisms present. Therefore, the different incubation conditions and compositions of the reaction mixtures make it difficult to compare the ability of laccases from different fungal species to degrade PAH. Jones et al. (2014) reported competitive inhibition of PAH degradation when a soil contains a mixture of contaminants, and more than one substrate is metabolized by the same enzymes. Therefore, despite the same amount of replicated, using naturally non-sterilized contaminated soil cannot be compared or standardized as the artificial spiked soil.

4. Conclusion

In the present study, an efficient degradation of PAH in soil was achieved without redox mediators, which indicates that some compounds present in the soil may have acted as mediator in the enzymatic oxidation. Enzymatic treatment of contaminated soil revealed to be an alternative or a supplement to microbial bioremediation. However, production of laccase in large scale still need to be optimized as well as stability and usage in field. An overview of PAH degradation rates and heavy metals removal achieved using the different strategies (microbial and enzymatic remediation) is provided in Fig. S4 of supplementary material. Similar PAH degradation was achieved by *T. versicolor* (strategy I) and laccase/LMS in fed-batch mode in PBR (strategy II); however, the removal of heavy metals was higher by laccase/LMS (strategy II), both in batch and fed-batch, than by fungus, showing a possible metal toxicity in *T. versicolor*. Although some metal ions can act as cofactors to assist cell growth with even trace level, they can become toxic in excess to most living systems.

CRediT authorship contribution statement

Ziva Vipotnik: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft. **Michele Michelin:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – review & editing. **Teresa Tavares:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.eti.2022.102737.

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