



Development of a packed bed reactor for the removal of aromatic hydrocarbons from soil using laccase/mediator feeding system

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ABSTRACT

Polyaromatic hydrocarbons (PAH) are persistent pollutants of great concern due to their potential toxicity, mutagenicity and carcinogenicity. A biotechnological approach to remove PAH from soil was evaluated in this work using a laccase mediator system. Initially, laccase was produced by fungal co-cultivation, using kiwi peels as substrate. The produced laccase was applied to PAH contaminated soil to evaluate its efficiency on enzymatic bioremediation. Results showed that laccase mediator system was effective in the degradation of pyrene, fluorene, chrysene and a lower extension anthracene. Mediators improved the PAH degradation and natural mediators (ferulic acid and *p*-coumaric acid) were as effective as the synthetic mediator ABTS. However, the process was not effective in the benzo[a]pyrene degradation, one of the most recalcitrant and toxic PAH. This low degradation rate could be related to the low activity of the laccase mediator system in an environment lacking water. To overcome this issue, a PAH contaminated soil degradation system was developed in packed bed reactor (PBR) fed with laccase/mediator. Continuous flow of laccase/mediator improved the PAH degradation, achieving 74.8 %, 71.9 %, 72.2 %, 81.8 % and 100 % degradation for fluorene, anthracene, phenanthrene, chrysene and pyrene, respectively. This system was able to degrade 96 % benzo[a]pyrene, which was 90 % higher than the degradation in batch system. Results indicated that the produced laccase as well as the fed-batch degradation system developed in PBR could be successfully applied in the degradation of soil PAH pollutants, with the advantage of achieving higher degradation rates than in simple batch, as well as being a faster and simpler process than microorganism bioremediation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are highly toxic organic pollutants, with a structure consisting of two or more fused benzene rings. They are mainly accumulated in sediments and soil due to incomplete combustion or pyrolysis of various organic materials, such as coal, oil, petroleum gas and wood (Li et al., 2019). PAH in the soil can be harmful to people's health through the food chain and therefore, soil pollution by PAH is of great concern (Alexander et al., 2008).

The U.S. Environmental Protection Agency (EPA) has recognized sixteen PAH as priority pollutants due to their high toxicity, mutagenic and carcinogenic potential (Keith, 2015; Xu et al., 2020). As established in many previous studies, 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. For example, benzo[a]pyrene, a five rings molecule of high molecular

weight, is one of the most recalcitrant and toxic PAH (Li et al., 2010).

The degradation of PAH present in the environment includes volatilization, photo-oxidation, chemical oxidation, adsorption on soil particles and leaching (Haritash and Kaushik, 2009; Wang et al., 2018). However, the cost and adverse environmental effects caused by these methods discourage their widespread practice. Bioremediation is an environmentally friendly, economical and efficient method to degrade and transform PAH to non-toxic compound and has been presented as a soil clean-up technique. However, studies have shown that the success of PAH bioremediation has been limited by the failure to remove high-molecular-weight PAH (Ogbonna et al., 2012). Additionally, studies with soil are by far more complicated than with liquid culture, mainly because of the heterogeneity of soil besides many other factors that should be considered such as desorption and bioavailability of PAH (Kadri et al., 2017). Despite the great effort in the development of microbiological technologies to rehabilitate PAH contaminated soil, microbes usually require special conditions, where the toxicity of PAH

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metabolites is of particular concern (Bamforth and Singleton, 2005). At long term, they may restrain the application of bioremediation, especially where there is heavy contamination. Therefore, enzyme bioremediation can be another option that should be considered.

Laccase (EC 1.10.3.2) is a polyphenol oxidase containing four copper ions, which catalyse the oxidation reaction in the presence of oxygen and leading to the decomposition of phenolic compounds (Kirst Tychanowicz et al., 2006; Viswanath et al., 2014). Laccase has strong stability and very low substrate specificity that makes it suitable for PAH degradation (Fernández-Fernández et al., 2013). Comparing with microorganism degradation, enzyme-catalysed reactions are faster and have higher decomposition efficiencies, lower toxicity, a wide application range and constitute a simple operation. However, the relatively low redox potential of laccase is its main limitation, which has been overcome by using redox mediators like ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate)) or 1-hydroxybenzotriazole (HBT), that allow the oxidation of non-phenolic compounds with higher redox potentials (Upadhyay et al., 2016). Laccase mediator system (LMS) was first applied to the biodegradation of lignin. Because of its vast reaction capabilities as well as the broad substrate specificity, it also possesses great biotechnological potential (Kunamneni et al., 2008).

Synthetic mediators, as ABTS and HBT, promote the oxidation of PAH by laccase. However, the most relevant disadvantages of all known effective mediator compounds are the high price, some toxic effects and also the eventual inactivation of laccase during oxidation by radicals mediators (Johannes and Majcherczyk, 2000; Viswanath et al., 2014), which led to the search for alternative mediators. On the other hand, natural compounds such as phenolic ones derived from lignin degradation (acetosyringone, syringaldehyde, vanillin, acetovanillone, ferulic acid and *p*-coumaric acid) can be obtained at low cost or for free because they exist abundantly in nature and do not produce any toxicity in the environment (Vasconcelos et al., 2000). They are capable of promoting similar or even better PAH degradation results through LMS (Bhattacharya et al., 2014; Li et al., 2010).

Many research efforts have been made to find a suitable process for the remediation of PAH contaminated soil. However, most works using laccase were focused on a liquid system (without soil) and using a synthetic mediator, such as ABTS or HBT (Ike et al., 2019; Li et al., 2014a, 2014b; Liu and Hua, 2014; Punnapayak et al., 2009). Additionally, it has been reported that the low PAH degradation rates in the soil are due to the low activity of the laccase-mediator system in an environment lacking water (Li et al., 2010). In the studied cases, and to the best of the authors' knowledge, this is the first time that the PAH degradation in soil through a laccase mediator system has been studied in a packed bed reactor (PBR) in fed-batch mode. Thus, the objective of the present work was to develop a PAH degradation system in soil using PBR with a continuous flow of laccase-mediator to overcome the problem of water limitation in the environment. For that, first, the laccase production by solid-state fermentation (SSF) was performed, using kiwi peels (agroindustrial residue) as a support-substrate for fungal co-cultivation, to be applied in the degradation of six PAH (fluorene, anthracene, phenanthrene, pyrene, chrysene, benzo[a]pyrene) in soil. Furthermore, the effect of natural laccase mediators on PAH degradation, such as ferulic acid and *p*-coumaric acid, was evaluated and compared to the effect of the synthetic mediator ABTS. The efficiency in the PAH degradation in soil using a PBR in fed-batch mode was demonstrated compared to PAH degradation in batch mode.

2. Material and methods

2.1. Fungal strains

Three fungal strains were studied in this work: *Trichoderma viride* (EXF8977), *Penicillium chrysogenum* (EXF1818) and *Irpex lacteus* (MUM 04.98). EXF strains were obtained from Infrastructural Centre Mycosmo, MRIC UL, Slovenia and the MUM strain from Fungal Culture Collection -

Micoteca da Universidade do Minho, Portugal. Stock cultures were maintained on malt extract agar plates, at 4 °C.

2.2. Substrate pretreatment

Kiwi peels were cut to 1 cm² and pretreated with 83 mM of KOH at room temperature (≈ 26 °C) for 20 min, in a ratio of 1:3, to neutralise organic acid (Stredansky and Conti, 1999). After that, they were washed twice with deionised water, dried at 60 °C and autoclaved at 121 °C for 15 min prior to use.

2.3. Enzyme production

2.3.1. Fungal inoculum grown in the liquid medium

The liquid inoculum was prepared by cutting four agar plugs (5 mm x 5 mm) from malt extract agar plates. These were extruded through a syringe into 500 mL Erlenmeyer flasks containing 200 mL sterile malt extract (2 % w/v). Fungi were cultivated at room temperature, with continuous agitation at 120 rpm, for 3 days for *T. viride* and 6 days for *P. chrysogenum* and *I. lacteus*.

2.3.2. SSF in Erlenmeyer flasks

The 500 mL Erlenmeyer flasks were filled with 200 g of kiwi peels, autoclaved at 121 °C for 15 min and 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 centrifuged at 7500 g for 15 min. The recovered supernatant was used for enzymatic assays. SSF was performed in duplicate.

2.3.3. SSF in a packed-bed bioreactor

The horizontal packed-bed bioreactor (PBB) (Fig. 1) consisted of a double jacketed glass column (34 cm length and 3 cm internal diameter) connected to a filtered-air supply. The air was passed through a 0.45 µm cellulose filter and bubbled into distilled water before entering in the column. The airflow was measured and controlled by a flowmeter (Aalborg Instruments & Controls, Inc., USA). The bioreactor and 50 g of kiwi peels as substrate were previously sterilized separately. The substrate was inoculated with 2 mL of fungal inoculum suspension and mixed. After that, the column was filled with the inoculated substrate and maintained at room temperature for 3 weeks. SSFs were performed in duplicate, at different aeration rates of 0.05 L min⁻¹, 0.1 L min⁻¹ and 0.2 L min⁻¹. After SSF, the fermented substrate was transferred to 500 mL Erlenmeyer flasks to proceed with the enzyme extraction, as described previously.

2.4. Laccase and protein determination

Laccase activity was measured using a spectrophotometer (BioTek Synergy HT) at 420 nm by the oxidation of 2,2-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS, Sigma Aldrich) 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. Protein was determined through Bradford's method.

2.5. Soil preparation

Naturaduba Agro soil (Gintegral – Gestão Ambiental, S.A.), presenting 49.1 % organic material, 0.99 % N, 0.20 % P₂O₅, 0.49 % K₂O, 2.17 % Ca, 0.26 % Mg, 0.17 % S, 32.2 % humidity and pH 7.2 was used in this work. The soil was first heated for two days at 60 °C. For pH adjustment to 5.0, 0.43 µl of 1 M HCl was mixed in 10 mL of distilled water and 5 g of soil, vortexed for 2 min, left at room temperature for 30 min, and after that, the pH was confirmed.

Six EPA-priority PAH were used in this study (Table 1). They exist as

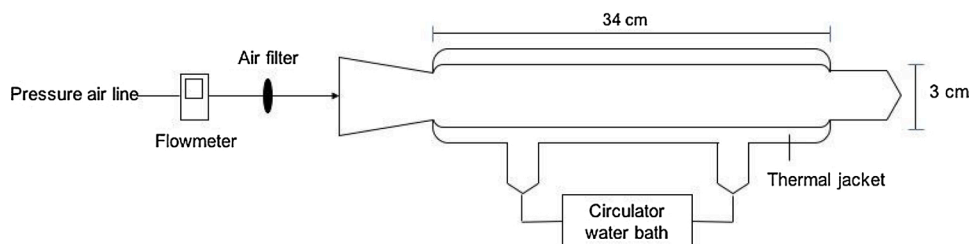








Fig. 1. Horizontal packed-bed bioreactor design for laccase production.

Table 1

Structure, molecular weight and ionization potential (IP) value of different polycyclic aromatic hydrocarbons (PAH).

Compound	Structure	Molecular weight (g/mol)	IP value (eV)*	Purity	Supplier/CAS
Fluorene		166.219	7.89	98 %	Acros Organics, CAS 86-73-7
Phenanthrene		178.229	7.91	97 %	Acros Organics, CAS 85-01-8
Anthracene		178.229	7.43	99%	Acros Organics, CAS 120-12-7
Pyrene		202.251	7.43	98 %	Acros Organics, CAS 129-00-0
Chrysene		228.288	7.59	95 %	Sigma-Aldrich, CAS 218-01-9
Benzo[a]pyrene		252.309	7.12	96 %	Sigma-Aldrich, CAS 50-32-8

* (Levin and Lias, 1982).

crystalline solids at room temperature. Thus, an amount of 1000 mg of each PAH was added to 1000 mL of acetonitrile and used to contaminate soil, giving a desired final concentration mixture.

2.6. Degradation assays

PAH degradation was evaluated using laccase enzyme and LMS in amber laboratory bottle, namely laccase-batch degradation, and in packed bed reactor (PBR), namely laccase-fed degradation.

Laccase batch degradation was set up into 100 mL amber laboratory bottle with 20 g of soil at pH 5.0, containing 2 mL of the PAH mixture appropriately diluted to obtain the concentrations of 100, 300 and 600 ppm of a total mixture of the compound. A laccase load of 2 U/mL (approx. 4 mL) was used for the PAH degradation. The addition of 1 mM (approx. 2 mL) of ABTS, coumaric acid and ferulic acid mediators was also evaluated. The degradation was performed in duplicate at 30 °C, in the dark, for 5 days. Samples were taken on days 0, 3 and 5 for evaluation of PAH degradation by UHPLC. The boiled enzyme was used as a control.

Laccase-fed degradation was set up in PBR (Fig. 2), consisting of a vertical glass column (25 cm length, 3 cm Ø, and 3.5 cm width) filled with contaminated soil (100 g soil spiked with 50 mL of the PAH mixture at 300 ppm of each compound). A load of 2 U/mL of laccase and 1 mM of the mediator were added, in a continuous flow of 0.015 mL/min, using a laboratory peristaltic pump (Masterflex, Cole-Parmer). The laccase flow was refreshed each second day. In order to avoid heterogeneous degradation, laccase solution was equally distributed over the whole contaminated soil through an inner tube with small holes in the middle of the column. This procedure was done in duplicate and performed for 25 days at room temperature. Soil samples were taken each 5 days for

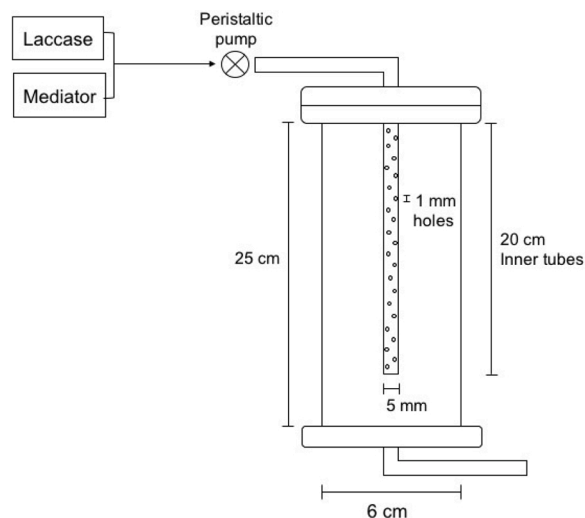


Fig. 2. Packed-bed reactor design for PAH degradation, with an inner tube.

PAH concentration measurement by UHPLC and the assessment of removal efficiency.

2.7. PAH extraction

Acetonitrile was used to extract PAH from the soil, for that 1.5 mL of it was added to 0.5 g of soil sample and extraction was carried out using rotating shaker at 160 rpm for 30 min and another 10 min in an ultrasound bath. The samples were centrifuged at 8000 g, and the supernatant was transferred to 2 mL Eppendorf tubes for UHPLC analyses.

2.8. UHPLC analyses

The quantification of the six selected PAH was performed by ultra-high-performance liquid chromatography, 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-20A) and a system controller (CBM-20A) with built-in software (LabSolutions). For the PAH 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.

3. Results and discussion

3.1. Laccase screening

A screening of laccase producing fungi was performed, aiming to produce high levels of laccase to be applied in PAH degradation. For that, three fungal strains were evaluated through SSF, using kiwi peels as substrate at pH 4.5 and 7.0. SSF mimics the conditions under which fungi grow naturally (Singh Nee Nigam and Pandey, 2009) in lignocellulosic residues. The polysaccharides and lignin present in these materials act as growth and induction substrate, promoting a better fungal growth (Couto and Toca-Herrera, 2007) and the production of extracellular enzymes (Brijwani and Vadlani, 2011), including laccase (Rodríguez Couto and Sanromán, 2005), while making the process more economical (Couto and Toca-Herrera, 2007).

Kiwi peels is an interesting residue from food processing industry due to their high availability and low cost (Rosales et al., 2005). There was 35,410 tons of kiwi fruit produced in Portugal in 2018, representing 1.82 % of world production. However, up to 30 % represent peels. Those contain 10.46 % moisture, 4.02 % ash, 3.84 % protein, 2.10 % lipids, 53.73 % carbohydrates, 6.93 % soluble and 18.92 % insoluble dietary fibres (Soquetta et al., 2016), frees sugar and fatty acid (Dias et al., 2020), and the skin of fruits also contain higher mineral content than their respective edible parts (Soquetta et al., 2016). Lignin content contributes to the insoluble dietary, inducing laccase production by the fungal species.

The higher enzymatic levels were obtained for *Trichoderma viride* (1.45 U/mL) at pH 4.5 for 3 weeks (Table 2). However, the co-cultivation of *Trichoderma viridae* and *Penicillium chrysogenum* showed similar production at pH 4.5 at 2 and 3 weeks, besides better growth (see protein data). The co-cultivation of microorganisms is interesting due to the great diversity of laccase that can be produced, with different properties and mode of action, which could be an advantage in the degradation of different PAH. Besides, higher enzyme levels can be achieved with this approach (Hu et al., 2011). Meehnian et al. (2017) also explored the co-cultivation strategy, using the white-rot fungi *Daedalea flavida* and *Phlebia radiata*, to enhance the production of the ligninolytic enzymes, including laccase, during pretreatment of cotton stalks in SSF. Laccase production increased to 14.19 ± 0.85 IU g⁻¹ solid with the co-culture, which was three- and eight-folds higher than to the

Table 2
Laccase production by fungi.

Strain	pH	Laccase activity (U/mL)			Protein (mg/mL)		
		1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
IL	4.5	0.60 ± 0.01	0.58 ± 0.02	0.93 ± 0.18	1.43 ± 0.04	1.29 ± 0.02	0.87 ± 0.31
		0.53 ± 0.09	0.41 ± 0.07	0.40 ± 0.09	2.08 ± 0.12	1.12 ± 0.09	2.18 ± 0.35
IL + TV	4.5	0.46 ± 0.08	0.42 ± 0.09	0.94 ± 0.03	1.58 ± 0.11	0.88 ± 0.03	1.59 ± 0.17
		0.42 ± 0.22	0.58 ± 0.06	1.10 ± 0.15	2.90 ± 0.03	0.70 ± 0.18	0.79 ± 0.29
PC	4.5	0.45 ± 0.02	0.47 ± 0.01	0.70 ± 0.06	0.70 ± 0.01	2.17 ± 0.12	1.84 ± 0.29
		0.98 ± 0.09	1.16 ± 0.04	1.17 ± 0.09	3.48 ± 0.04	2.34 ± 0.01	1.19 ± 0.20
PC + TV	4.5	1.04 ± 0.07	1.48 ± 0.07	1.39 ± 0.03	3.72 ± 0.17	1.78 ± 0.04	2.80 ± 0.36
		0.53 ± 0.08	0.54 ± 0.03	1.06 ± 0.11	0.87 ± 0.20	1.91 ± 0.03	2.40 ± 0.42
TV	4.5	0.89 ± 0.04	0.70 ± 0.05	1.45 ± 0.01	0.76 ± 0.29	1.19 ± 0.09	1.63 ± 0.51
		0.54 ± 0.01	0.27 ± 0.04	0.52 ± 0.05	2.37 ± 0.36	3.49 ± 0.11	4.08 ± 0.22

IL: *Irpex lacteus*; TV: *Trichoderma viride*; PC: *Penicillium chrysogenum*; IL + TV: co-cultivation of *I. lacteus* and *T. viride*; PC + TV: co-cultivation of *P. chrysogenum* and *T. viride*. The fungi were not able to grow in the IL + PC co-cultivation.

pretreatment by monoculture of *D. flavida* and *P. radiata*, respectively.

Although the pH is not controlled during most cultivations, the initial pH is usually set between 4 and 6 prior inoculum (Brijwani et al., 2010). For example, Thurston (Thurston, 1984) reported higher laccase production when fungi were grown in a medium with pH 5.0. Optimal laccase production from *Trametes pubescens* was obtained between pH 3.0 and 4.5 (Strong, 2011). Thus, the co-cultivation of these two species (*Trichoderma viridae* and *Penicillium chrysogenum*) at pH 4.5 was used in this work.

3.2. Laccase production

Laccase production was performed by SSF using Erlenmeyer flasks and PBR in different aeration rates (Table 3). There are several bioreactor designs used for SSF, but their major limits are heat and mass transfer in solid media (Brijwani et al., 2010). In this work, a horizontal PBR with a double jacketed glass column was used, where laccase production was performed with constant air supply.

The highest values of laccase production (6.32 U/mL) were obtained in PBR at aeration rates 0.2 mL/min, following by 0.1 and 0.05 mL/min with laccase production of 5.45 and 3.79 U/mL, respectively (Table 3). SSF in Erlenmeyer flasks were performed in different temperatures (room temperature, 30 °C and 40 °C), presenting no significant differences among the results of laccase production (2 U/mL). These assays aimed to assess the overall effect of temperature, as well as the overall effect of aeration on laccase production by SSF.

Oxygen supply showed to be one limiting factor for microorganism growth (data not shown), as well as for laccase production, which is frequently reported for aerobic bioprocesses. Another limiting factor in laccase production is temperature, being that the optimal temperature of laccase production diverse from one to another fungal strain. In the presence of light, temperatures of 25 °C is generally accepted as optimum, but in case of dark conditions, the optimum temperature generally is 30 °C (Bamforth and Singleton, 2005; Thurston, 1984), reducing the production when fungi are cultivated at temperatures higher than 30 °C (Lang et al., 2000).

Rodríguez Couto et al. (2003) tested several bioreactor configurations, such as immersion, expanded-bed and tray, with nylon sponge and barley bran as support. The first two were supplied with humidified air in a continuous flow at 0.5 vvm, and the tray reactor was kept at 90 % environmental humidity and passive aeration. Maximum laccase production by *T. versicolor* was reached between 18th and 20th days of cultivation, with 0.23, 0.13 and 0.34 U/mL, respectively. In another work, Rodríguez-Couto (2011) achieved 6 U/mL laccase by *T. pubescens* under SSF in temporary immersion bioreactor, using sunflower-seed shells as substrate and 60 L/h flowrate. Rosales et al. (2007) tested laccase production in a fixed-bed reactor (FBR) and tray reactor on KOH pretreated orange peels by *T. hirsute*. In FBR laccase production reached 3 U/mL on 11th day, and in tray reactor, on day 23rd reached 12.7 U/mL. Rosales et al. (2005) also reported laccase production on pretreated kiwi peels and supplementation by ammonium by *Trametes hirsute*, attaining a value of 5.4 U/mL on the 13th day. Böhmer et al. (2011) have reported laccase production by *T. hirsute* in a modular bioreactor, under SSF on a

Table 3
Laccase production by co-cultivation of *Trichoderma viride* and *Penicillium chrysogenum* on kiwi-peels.

SSF in lab flasks		SSF in packed bed bioreactor*	
Temperature (°C)	Laccase activity (U/mL)	Airflow (L min ⁻¹)	Laccase activity (U/mL)
RT	2.21 ± 0.3	0.05	3.79 ± 0.75
30	2.22 ± 0.3	0.1	5.45 ± 0.06
40	2.21 ± 0.8	0.2	6.32 ± 0.71

* SSF in packed bed bioreactor were performed at room temperature (≈ 26 °C).

mixture of pine wood chips and orange peel, achieving around 0.7 U/mL and 1.1 U/mL in media supplemented with copper sulfate and xyloidine. Another work has also reported the laccase production by white-rot fungi in a bioreactor under submerged fermentation (Hailei et al., 2015).

Optimal temperature and pH of laccase activity are presented in Fig. 3. Maximal activity was reached at pH 5.0 and 30 °C. Similar optimal conditions were reported by Patel and Gupte (Patel and Gupte, 2016) and Chenthamarakshan et al. (2017) for laccase from *Tricholoma giganteum* and *Marasmiellus palmivorus*, respectively, produced under SSF. Regarding the temperature stability, the enzyme showed higher activity in more acid pH, i.e. 3.5–5.0, being significantly reduced at pH 5.5 and 6.0. Regarding the temperature, activity decreased gradually from temperatures higher than 30 °C. The effect of temperature on laccase stability is showed in Table 4. The stability was higher at temperatures from 25 °C to 30 °C, remaining more than 70 % of the initial activity, after 5 days of incubation. At 35 °C, around 64.4 % and 42.6 % of the laccase activity were verified after 3 and 5 days of incubation, respectively. The enzyme showed low stability at 40 °C, presenting 35.2 % and 10.5 % of residual activity after 3 and 5 days of incubation, respectively. Laccase from *T. modesta* was stable at 40 °C for 180 min, with maximum stability at 50 °C (stable for 6 h), but at higher temperatures (60 °C) activity decreased (Nyanhongo et al., 2002). Laccase from wild and mutant *Shiraia* sp. was utterly stable at 35 °C for 30 h, but at 50 °C the stability gradually decreased (Du et al., 2015). It is important to highlight that in the current work, the stability was evaluated for longer incubation time since the PAH degradation studies required prolonged time incubation.

Regarding the pH stability, the laccase showed high stability at pH 5.0, remaining almost 80 % of the activity after 5 days of incubation (Table 5). At pH 4.0 and 6.0 the enzyme still presented ≈ 70 % of activity, while in pH 7.0, around 52 % was verified after 5 days of incubation. However, at pH 3.0 the relative laccase activity was significantly reduced to 27.1 % on day 5. Góralczyk-Binkowska et al. (2020) reported more than 80 % stability for laccase from *Nectriella pironii* at pH from 4.0 to 9.0; however, the stability was evaluated for only 3 h. The pH could affect the enzymatic activity by changing the electrostatic properties of the protein surface and reaction centre, or by influencing the stability of the enzyme (Bonomo et al., 2001). Considering the removal of PAH in contaminated soil, the high stability of the enzyme at room temperature, as well as in pH from 4.0 to 7.0 is very important for its application in this proposal.

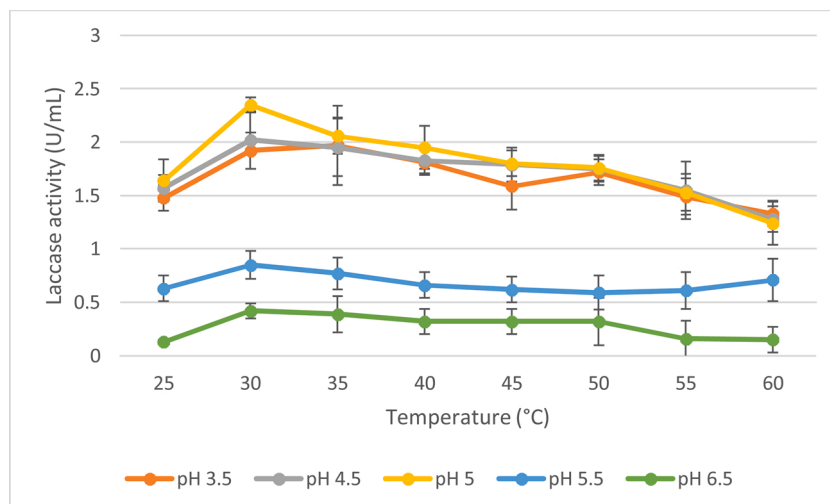


Fig. 3. Laccase activity at different temperatures and pH.

Table 4
Effect of temperature on laccase stability at pH 5.0.

Temperature (°C)	Relative laccase activity (%)		
	0 days	3 days	5 days
25	100	93.25 ± 3.4	72.60 ± 4.6
30	100	89.85 ± 4.6	77.54 ± 3.8
35	100	64.44 ± 4.8	42.59 ± 3.7
40	100	35.15 ± 2.7	10.50 ± 2.4

Table 5
Effect of pH on the laccase stability at 30 °C.

pH	Relative laccase activity (%)		
	0 day	3 days	5 days
3.0	100	91.1 ± 0.4	27.1 ± 0.6
4.0	100	87.5 ± 1.2	66.3 ± 2.2
5.0	100	89.65 ± 0.4	78.5 ± 1.3
6.0	100	72.7 ± 5.6	68.8 ± 6.7
7.0	100	71.1 ± 3.3	52.4 ± 4.5

3.3. PAH degradation in soil

The role of different mediators in the degradation of PAH in soil by laccase was evaluated in batch (Erlenmeyer flasks) and feed-batch in PBR. Laccase was capable of degrading the selected PAH, and the degradation rates increased with the addition of a mediator. Results showed that the laccase obtained from co-cultivation of *Penicillium chrysogenum* and *Trichoderma viride* on kiwi peels with an appropriate mediator promotes the degradation of fluorene, anthracene, pyrene, chrysene and benzo[a]pyrene, in concentrations ranging from 100 ppm till 600 ppm, in soil.

Some of the relevant parameters determining laccase activity in PAH degradation is the mediator and pH, as well as the incubation temperature for maximal laccase activity (Jin et al., 2016). In general, the mediators improve the degradation rates of PAH. Regarding the 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). Thus, the use of room temperature can be interesting, mainly concerning naturally contaminated environments. In this work, a higher degradation rate of PAH in soil was achieved at 30 °C when compared to 40 °C (data not shown). PAH degradation was assessed for 3 and 5 days in Erlenmeyer flasks, being the maximum degradation rates reached with 5 days for all compounds (Fig. 4).

Concerning PAH degradation in soil, it has been reported that the



Fig. 4. PAH degradation from the soil by laccase in batch mode. (A) Pyrene, (B) Fluorene, (C) Chrysene, (D) Anthracene, (E) Benzo[a]pyrene. Degradation was performed at 30 °C, in the dark, using different concentration of PAH in Erlenmeyer flasks. The graphs are presented in a different scale.

distribution of PAH in soil depends mainly on the PAH hydrophobicity and their affinity towards microcompartments of the aggregates (Styrishave et al., 2012). PAH molecules with higher hydrophobicity and more aromatic rings (see Table 1) present stronger sorption affinity (Zhang et al., 2014). Thus, these molecules diffuse into hydrophobic parts (ageing) over time, decreasing their bioavailability to be degraded by microorganisms and/or their enzymes, and consequently hindering their removal (Allard et al., 2000). In the current work artificially contaminated soil was used. So, the desorption rate of PAH in soil is expected to be higher than naturally contaminated soil, as observed by Huesemann et al. (2004) for anthracene degradation in freshly spiked soil (72 % degradation) and aged soil (34 % degradation).

In the current work, pyrene was the most degraded compound, achieving 99 % degradation with laccase mediated by *p*-coumaric acid, in the assay containing 600 ppm of the compound (maximal degradation rate). Regarding the other mediators, 92 % of the compound was degraded with ABTS, 67 % with ferulic acid, and 62 % with only laccase (without mediator). Thus, LMS allowed an increment of more than 35 % in the degradation rate of 600 ppm of the compound. However, for ABTS and ferulic acid as mediators, the maximal degradation rates were

achieved in the assays containing 300 ppm of the compound, being the degradation rate of 94 % and 82 %, respectively. Laccase (without mediator) was able to degrade 66 % of pyrene at 300 ppm in soil (Fig. 4A).

All mediators were effective for fluorene degradation, being the degradation rate lightly higher with *p*-coumaric as mediator. Additionally, maximal degradation was observed in the assay with 600 ppm fluorene, where the degradation rates achieved 82 % with *p*-coumaric, 80 % with ABTS and 79 % with ferulic acid. Laccase (without mediator) was able to degrade 77 % of the pollutant (Fig. 4B). Regarding, chrysene, the maximal degradation rate was also achieved with laccase mediated by *p*-coumaric, at 100 ppm of the compound, achieving 69 % degradation. At higher concentrations, the degradation rate decreased, with maximal removal of 41 % at 600 ppm. Laccase mediated with ferulic acid was able to degrade 62 % of chrysene in all concentration assayed. Maximal degradation with laccase plus ABTS (\approx 61 %) was observed at 600 ppm of the compound, while laccase without mediator degraded 51 % of the compound at 300 ppm (Fig. 4C).

For anthracene degradation, ABTS was the best mediator with 46 % degradation at 100 ppm, while for ferulic acid and *p*-coumaric,

degradation rates of 42 % and 38 %, respectively, were achieved at 300 ppm of the compound. Only 26 % of anthracene was degraded by laccase in the absence of mediator at 300 ppm. At 600 ppm, the degradation rate decreased significantly for laccase, as well as for most of LMS (Fig. 4D). The lowest degradation was observed with Benzo[a]pyrene. Only 5.4 % degradation was achieved with LMS, while laccase without mediator was able to degrade 2.4 % of the compound (Fig. 4E). Li et al. (2010) studied PAH degradation using laccase from *T. versicolor* in batch and verified a higher degradation rate for anthracene (74.6 %) and benzo[a]pyrene (56.7 %) mediated with ABTS, except for pyrene in which only 7.5 % degradation was achieved. It is important highlight that in this work, they used a high enzyme load (100 U laccase) and maintained a high humidity in the medium (70 %).

Regarding PBR trials, a laccase/mediator feeding was performed every 48 h for 25 days aiming to keep laccase activity levels throughout the process and in an attempt to improve the degradation rates. In general, the degradation rate of PAH achieved in PBR was higher than in Erlenmeyer flasks (mainly for benzo[a]pyrene), except for fluorene, where a degradation rate slightly lower (≈ 7 %) was observed in PBR (Fig. 5). This result could be related to the heterogeneity of soil, as commented previously. However, it should be noticed that the higher moisture content of the soil in the end of the PBR trials (86 % in relation to 27 % at the beginning of assay) due to the constant and slow laccase feeding may have helped to improve the degradation rates. PBR has been successful used for degradation of other compounds, such as dyes (Mosleh et al., 2018, 2017, 2016). For example, Mosleh et al. (2018)

used a rotating PBR for degradation of pollutant dyes using photocatalyst compounds, as Cu-based nanoparticles, achieving 90 % or more of degradation. They reported some benefits of using rotating PBR compared to conventional reactors, such as high interfacial surface area caused by the rotational speed, which enhances mass transfer and mixing processes. In the current work, a PBR with a microperforated inner tube was used to allow a homogeneous and efficient degradation process.

The most degraded compound was pyrene, 100 % degradation with *p*-coumaric acid as a mediator, being the degradation rates achieved with laccase mediated by ferulic acid and ABTS of 87 % and 83.5 %, respectively (Fig. 5A). Surprisingly, benzo[a]pyrene, the most carcinogenic, mutagenic and persistent PAH, was almost totally degraded by laccase mediated by ABTS (96 %) in the PBR system, although laccase with ferulic acid was not far behind, with 5.7 % lower degradation rate (Fig. 5E). This represents an increase of more than 90 % compared to the batch degradation. A crucial factor affecting PAH degradation is their molecular weight that also means their bioavailability (Ghosal et al., 2016). PAH with high molecular weight, such as benzo[a]pyrene (see Table 1) were less degradable in batch degradation, probably due to the higher heterogeneity of the soil which is related to the lack of moisture. Li et al. (2010) reported that LMS works actively in the water environment or in soil with a high capacity of water as in slurry. In this work, they achieved 56.7 % of benzo[a]pyrene degradation in soil with laccase plus ABTS, as commented previously. They suggested that the relatively low degradation rate is probably related to the environment lacking

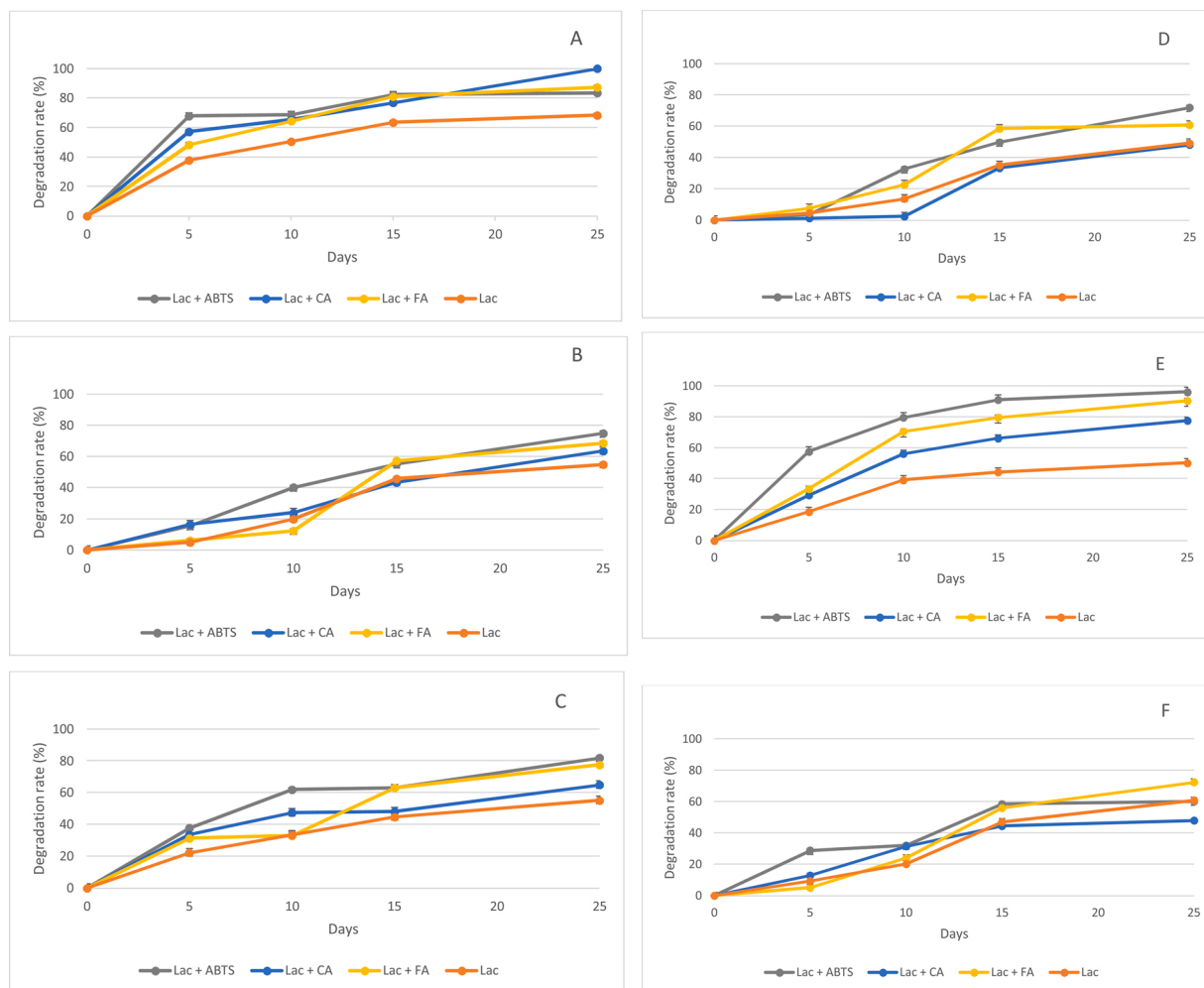


Fig. 5. PAH degradation from the soil in a packed-bed reactor by LMS. (A) Pyrene, (B) Fluorene (C) Chrysene, (D) Anthracene, (E) Benzo[a]pyrene, (F) Phenanthrene. Degradation was performed at room temperature, in the dark, for 25 days, using 300 ppm of PAH.

water. Wu et al. (2008) achieved 60 % of benzo[a]pyrene degradation in soil with laccase.

The degradation rate achieved for chrysene was 81.8 with ABTS and 77.5 % with ferulic acid (Fig. 5C). Similar degradation rates were achieved for fluorene, phenanthrene and anthracene. Decomposition of

fluorene was constant over the entire period of the assay with laccase plus ABTS, achieving 75 % degradation. Degradation rates of 68.5 and 63.5 % were reached with laccase mediated by ferulic acid and *p*-coumaric acid, respectively, at the end of the process (Fig. 5B). Anthracene has started degrading after 5 days with ABTS or ferulic acid as mediators

Table 6

PAH degradation by laccases from different sources.

Laccase source	Reactor	Conditions	Time	Mediator	PAH	Degradation rate	Degradation in soil	Reference
<i>Pycnoporus cinnabarinus</i>	4 mL vials	2 U/mL laccase, 100 mM sodium citrate buffer, pH 5 (containing 1 % Tween 20), 30 °C, in dark	24 h	/	benzo[a]pyrene (50 µM)	12 %	no	Cañas et al., 2007
					pyrene (50 µM)	8%		
					anthracene (50 µM)	60 %		
					benzo[a]pyrene (50 µM)	92 %		
					pyrene (50 µM)	62 %		
<i>Leucoagaricus gongylophorus</i> strain FF-2006	5 mL vials	50 U laccase, 20 mM sodium citrate buffer, pH 6, with 0.25 % v/v Tween 20, 100 rpm	24 h	/	anthracene (50 µM)	95 %	no	Ike et al., 2019
					anthracene (10 ppm)	64.9 %		
					anthracene (10 ppm)	71.8 %		
					fluorene (100 ppm)	41.7 %		
					fluorene (100 ppm)	41.7 %		
<i>Trametes versicolor</i> 951,022	2.5 mL Eppendorf tube	5 U/mL enzyme solution, 0.1 M sodium acetate buffer, pH 5, 40 °C, in horizontal shaker	2 h	/	5 mM ABTS	40 %	no	Han et al., 2004
					5 mM HBT	30 %		
<i>Trametes versicolor</i> (maleic anhydride modified laccase)	HiTrap DEAE FF	1.5 mL of enzyme solution 0.1 g/L dissolved in 20 mM di-sodium hydrogen phosphate-citric acid buffer pH 4.5, 30 °C	72 h	/	1 mM ABTS	50 %	no	Liu and Hua, 2014
					anthracene	36 %		
<i>Trametes versicolor</i>	HiTrap DEAE FF	1.5 mL of enzyme solution 0.1 g/L dissolved in 20 mM di-sodium hydrogen phosphate-citric acid buffer pH 4.5, 30 °C	72 h	/	1 mM ABTS	83 %	no	Liu and Hua, 2014
					anthracene	7%		
<i>Pycnoporus sanguineus</i> KUM60954	Erlenmeyer flasks	30 U/mL laccase, 50 mM sodium citrate buffer, 40 °C, 80 rpm	24 h	/	acenaphthylene	35 %	no	Munusamy et al., 2008
					anthracene	70 %		
<i>Trametes versicolor</i>	15 mL glass tubes	30 U laccase, 0.1 M Britton-Robinson buffer, pH 4, 40 °C	48 h	/	10 ppm mixture: anthracene	89.8 %	no	Li et al., 2010
					phenanthrene	36.8 %		
<i>Trametes versicolor</i>	15 mL glass tubes	100 U laccase, 10 g aged contaminated soil, water content adjust to 70 % moisture and maintain with ultra pure water daily	10 days	/	pyrene	95.7 %	yes	Li et al., 2010
					benzo[a]pyrene	60 %		
Cocultivation of <i>Trichoderma viride</i> and <i>Penicillium chrysogenum</i>	PBR	2 U/mL laccase in a continuous flow of 0.015 mL/min into 100 g of spiked soil	25 days	/	1 mg/L benzo[a]pyrene	98 %	yes	This work
					phenanthrene	15.6 %		
					anthracene	52.7 %		
					pyrene	2.8 %		
					benzo[a]pyrene	40.8 %		
					phenanthrene	32.4 %		
					anthracene	74.6 %		
					pyrene	7.5 %		
					benzo[a]pyrene	56.7 %		
					300 ppm mixture: phenanthrene	60 %		
					anthracene	49 %		
					pyrene	68 %		
1 mM ABTS	benzo[a]pyrene	50 %						
	Chrysene fluorene	55 %						
	300 ppm mixture: phenanthrene	60 %						
	anthracene	72 %						
	pyrene	83.5 %						
	benzo[a]pyrene	96 %						
1 mM <i>p</i> -coumaric acid	benzo[a]pyrene	81.8 %						
	Chrysene fluorene	75 %						
	300 ppm mixture: phenanthrene	48 %						
	anthracene	72 %						
	pyrene	60.8 %						
	anthracene	87 %						
1 mM ferulic acid	Pyrene	87 %						
	benzo[a]pyrene	91.3 %						
	Chrysene fluorene	68.5 %						

but only started degrading after 10 days with *p*-coumaric acid, being together with phenanthrene the less degradable pollutant in 25 days of the experiment. They achieved 72 % degradation mediated by ABTS (Fig. 5D). Phenanthrene also achieved 72 % removal, but mediated by ferulic acid, followed by ABTS and by *p*-coumaric acid with 60 and 48 % degradation, respectively (Fig. 5F).

Therefore, all mediators were effective for PAH degradation, achieving at least 72 % degradation. ABTS was found to be the best mediator for benzo[a]pyrene, chrysene, fluorene and anthracene with degradation rates of 96 %, 82 %, 75 % and 72 %, respectively. Phenanthrene was degraded 72 % with laccase mediated by ferulic acid, and in case of pyrene 100 % degradation was reached with *p*-coumaric acid as mediator. Few works have explored the use of natural mediators for PAH degradation (Cañas et al., 2007; Johannes and Majcherczyk, 2000); however, these works were performed in liquid medium instead of soil. Table 6 presents similar attempts in the area of PAH degradation by laccases and LMS compared to the results obtained in this study. It is also important to note that these degradation rates were achieved with 25 days (maximal degradation time studied). So, a higher degradation rate could be achieved with extended time or possibly with higher laccase loading and mediator.

It has been reported that the specific laccase catalysis is related to the ionization potentials (IPs) of the substrates; therefore, laccase can only oxidize compounds with less than 7.45 eV IP values (Pozdnyakova et al., 2006). Thus, the most degraded compounds should be Benzo[a]pyrene > Pyrene ≥ Anthracene > Chrysene > Fluorene > Phenanthrene (see Table 1). In our work, the highest degradation rates were achieved for Pyrene > Benzo[a]pyrene > Chrysene > Fluorene > Phenanthrene > Anthracene in PBR. Not only IP values but also the solubility among PAH is having an impact on their degradation (Pozdnyakova et al., 2006). The solubility of PAH increase for higher temperatures, which in turn influences the bioavailability of PAH molecules (Ghosal et al., 2016). Longer PAH stays in contact with soil, the more irreversible the sorption is, and lesser is the chemical and biological extractability of the contaminants (Ghosal et al., 2016; Luo et al., 2012; Martin, 2000). Li et al. (2010) also reported this only partially justified theory of IPs. IP of each top degradable PAH (anthracene, benzo[a]pyrene and benzo[a]anthracene) was below 7.45 eV, but the remaining was in a different order. More, the pyrene with 7.43 eV was poorly degraded. Therefore, they suggest further studies for a full elucidation of the catalytic mechanism of laccase. Han et al. (2004) reported that phenanthrene was degraded only in the presence of 5 mM of ABTS or HBT as mediator with 5 U/mL of laccase purified from *T. versicolor* 951022, that was able to oxidize 40 % and 30 % of 20 mg/L of phenanthrene in 2 h, respectively.

Li et al. (2010) observed the highest degradation rate of 1 mg/L benzo[a]pyrene with laccase plus ABTS after 48 h incubation, despite there was no significant difference between 24 and 48 h. The experiment was carried out at 40 °C, pH 4.0, and 30 U of laccase. At the same time, it was tested degradation in soil with 70 % moisture. In 10 days, they observed 40.8 % of degradation with 10 U/g laccase, and 56.7 % degradation by adding 1 mmol/kg ABTS. Wu et al. (2008) studied the degradation of PAH in contaminated soil, with different concentration of laccase, i.e. 1, 3 and 10 U/mL, without a mediator. Regardless of the initial amount of enzyme, its activity gradually declined throughout incubation time till there was no enzyme activity left in any of the soil microcosms after 14 days. Laccase transformed several PAH efficiently after 24 h of incubation, being anthracene and benzo[a]pyrene the most degradable. The total PAH removal by enzyme bioremediation in soil microcosms after 14 days ranged between 67.6 % and 82.4 % (Wu et al., 2008). In the current work, PAH degradation varied between 72 % and 100 % after 25 days of the assay, with 2 U/mL of laccase and 1 mM of the mediator. The constant laccase/mediator feed in the PBR trials throughout the process allowed to work with the same activity levels during all the process, improving the degradation rates of PAH in soil.

4. Conclusions

Data from this study confirm previous observations suggesting the importance of mediators in the oxidation of PAH. The most commonly used mediator, ABTS, is too expensive for practical soil remediation, and the toxic effects from using it for long periods are unknown for now. Fortunately, natural mediators are capable of competing with the synthetic one, and in some cases good results can be obtained without a mediator, which suggests the possibility of other compounds present in the soil or in crude enzymes to enhance enzymatic oxidation. To make this degradation process more competitive, the laccase was produced from agricultural residue and crude laccase was used in all the assays, resulting in efficient degradation (high degradation rates), as well as faster and simpler process, when compared to degradation with microorganisms. The development of a laccase/mediator feeding system through packed bed reactor allowed to increase PAH degradation rate in soil, mainly of the benzo[a]pyrene, the most carcinogenic, mutagenic and persistent PAH, probably due to the higher water availability to the system.

CRedit authorship contribution statement

Ziva Vipotnik: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Michele Michelin:** Conceptualization, Methodology, Validation, Writing - review & editing, Supervision. **Teresa Tavares:** Conceptualization, Supervision, Writing - review & editing, Resources, Funding acquisition.

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