Environmental Technology & Innovation 38 (2025) 104093

Contents lists available at ScienceDirect

Environmental Technology & Innovation

journal homepage: www.elsevier.com/locate/eti

Optimization and stability of a reusable laccase-polymer hybrid film for the removal of bisphenol A in water

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ARTICLE INFO

Keywords:
Acrylic polymer
Biodegradation
Bisphenol A
Laccase
Myceliophthora thermophila
Trametes versicolor

ABSTRACT

Laccases are a group of versatile and green biocatalysts with high efficiency for the degradation of a wide variety of environmental pollutants. However, the feasibility of free laccase applications is hampered by enzyme inactivation due to exposure to denaturing conditions and difficult recovery, which limits reuse and continuous process design, resulting in high costs. Enzyme immobilization technology is a promising way of overcoming these drawbacks. In this work, laccases from two fungal sources, *Myceliophthora thermophila* and *Trametes versicolor*, were immobilized for the first time by covalent interaction (azo-linkages) on a film-shaped polyacrylic material. Optimal immobilization parameters were 10 U/mL enzyme load, 1 h and 25 ºC. The resultant immobilized laccases were characterized as a function of pH, temperature, and substrate concentrations (kinetic parameters) and compared with the free enzyme counterpart. The laccase-polymer hybrid (*M. thermophila* laccase) showed excellent operational and storage stability, retaining 89.8 % of the initial activity after 15 cycles of reuse and 10 days of storage at 4 ºC. Finally, the laccase-polymer films exhibited > 90 % depletion of bisphenol A in water by a dual adsorption-catalysis mechanism. The adsorption capacity ranged from 61.4 % to 30.5 %. GC-MS analysis revealed the degradation products 5,5′-bis-[1-(4-(4-hydroxyphenyl)-1-methyl-ethyl]-biphenyl-2,2′-diol and 3- or 2-methyl-2,3-ditrobenzofuran, as adsorbed compounds, as well as non-degraded bisphenol A. The significance of this research lies in the demonstrated catalytic efficiency of a new, reusable and cost-effective hybrid material for the removal of phenolic contaminants in water. The findings suggest that laccase-polymer hybrids could be a very useful tool for sustainable wastewater treatment.

1. Introduction

The treatment of phenol contamination of various water sources has gained great interest due to its harmful effects, but this process remains challenging because phenolic compounds are highly stable and soluble in water (Hubab et al., 2025). Various methods have been developed and applied for phenol contamination, such as adsorption, liquid-liquid extraction, photo-degradation, advanced oxidation, membrane separation and bioremediation (microorganisms, enzymes) (Ahmaruzzaman et al., 2024; Mamman et al., 2024;

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https://doi.org/10.1016/j.eti.2025.104093
Received 12 December 2024; Received in revised form 4 February 2025; Accepted 18 February 2025
Available online 19 February 2025

Tian et al., 2017). The enzymatic degradation approach has several advantages over other water treatment techniques in terms of high pollutant specificity, catalytic efficiency under mild conditions and low toxicity of degradation by-products (Xing et al., 2022; Ji et al., 2017).

Fungal laccases are enzymes of great interest in environmental biotechnology because they can degrade a broad range of xenobiotics, including phenols and derivatives, dyes, polycyclic aromatic hydrocarbons, pharmaceutically active compounds, personal care products, pesticides, plasticizers and others (Dong et al., 2023; Jeyabalan et al., 2023; Sodhi et al., 2024). These multi-copper oxidases do not require any additional/expensive substrate or cofactor, only molecular oxygen, which makes them very attractive compared to other enzyme systems (Sekretaryova et al., 2019).

The efficiency of laccases for removing emerging organic contaminants from aqueous solutions has been demonstrated at a laboratory scale (Cen et al., 2022; Lou et al., 2023). However, the cost of the enzymes limits their large-scale application due to their lack of reusability and rapid denaturation when applied in free form. This problem can be mitigated by immobilizing the enzyme in/on different supports, such as hydrogels, nanoparticles, mesoporous silica, membranes, nanofibers, and polymeric materials (Bević et al., 2020b; Kyomuhimbo and Brink, 2023; Maryskova et al., 2022; Rodríguez-Couto, 2023). Numerous immobilization methods have been investigated and developed, including physical (adsorption, entrapment, ionic bonding) and chemical (covalent bonding, cross-linking) methods or combinations of both (Abdelhamid et al., 2024; Jiang et al., 2024). Among these, chemical immobilization offers superior stability compared to physical methods due to the formation of stronger bonds between the enzyme and the support material (Prabhakar et al., 2025).

For protein immobilization, synthetic polymers can offer some advantages such as molecular tailorability, inertness to microbial attack, biocompatibility, excellent processability and enormous available aggregation forms that can be achieved through different processing and self-assembling methods (Rodriguez-Abetxuco et al., 2020). In this regard, several laccase-polymer systems have been developed yielding diverse outcomes (Ariste et al., 2021; Kolak et al., 2023; Rybarczyk et al., 2023; Zdarta et al., 2022). Recently, we have reported the synthesis of a highly manageable polyacrylic material for enzyme immobilization (Vallejo-García et al., 2023). This polymer prepared in a film shape has shown excellent reusability in both continuous and batch processes, offering significant practical advantages for biotechnological applications.

Bisphenol A (2, 2-bis(4-hydroxyphenyl)propane), is a widely manufactured chemical, largely used in polycarbonate plastics and epoxy resins for food packaging. This xenobiotic compound is an endocrine disruptor continuously released into the environment and has been declared a social, environmental and global issue. As a result, a great deal of work has been done over the last few years using immobilized laccases for their application in the degradation of this pollutant present in water and industrial effluents (Du et al., 2025; Sun et al., 2023; Yu et al., 2024; Zayed et al., 2024). However, the development of a stable and cost-effective biocatalyst with the possibility of recovery and reuse remains one of the major challenges for facing environmental biocatalysis, such as the treatment of wastewater containing BPA.

In this context, the present study aims to investigate the immobilization of two commercially available fungal laccases, from the thermophilic fungus *Myceliophthora thermophila* and the wood-rotting fungus *Trametes versicolor,* through covalent interaction with the new film-shaped acrylic polymer for stabilization and efficient reuse. To our knowledge, this approach has not been reported before. Optimization of the immobilization process and comparative characterization of the two fungal immobilized laccases in terms of optimum pH and temperature, kinetic parameters, and operational and storage stability were studied. Eventually, the efficacy of laccase-polymer hybrids in the degradation of the emerging pollutant BPA was investigated.

2. Material and methods

2.1. Materials

1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99 %), methylmethacrylate (MMA) (Merck, 99 %), 4-aminostyrene (SNH_2) (TCI, 98 %), ethylene glycol dimethacrylate (E) (Aldrich, 98 %), ethanol (VWR, 99.96 %), methanol (VWR-Prolabo, 99.9 %), hydrochloric acid (VWR, 37 %) dimethylsulfoxide-d_6 (VWR, 99.8 %), citric acid (VWR, ≥99.5 %), disodium hydrogen phosphate anhydrous (VWR, 99 %), sodium carbonate (Sigma-Aldrich, 99.9 %), sodium azide (Alfa Aesar, 99 %), sodium nitrite (Alfa Aesar, 98 %), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich, ≥98 %), bisphenol A (BPA) (Aldrich, 99 +%). Commercial laccases were selected from two fungal sources, a laccase from the thermophilic ascomycete fungus *Myceliophthora thermophila* expressed in *Aspergillus oryzae* (≥1000 U/g) and another from the basidiomycete fungus *Trametes versicolor* (≥0.5 U/mg). These enzymes have different characteristics (e.g. kinetics, redox potentials) that are of interest for comparative studies and future applications. The enzymes were purchased from Sigma-Aldrich. All the other reagents were analytical grade and used without further purification unless stated otherwise.

2.2. Synthesis of laccase-polyacrylic film

The acrylic polymer designed for laccase immobilization has previously been employed as a support for the immobilization of β-galactosidase (Vallejo-García et al., 2023). This formulation has been developed over several years through our research and publications, incorporating various monomers that provide the properties required for its intended application (García et al., 2022). For instance, the material must work in aqueous environments, needing the inclusion of a water-compatible (hydrophilic) monomer, such as VP (vinylpyrrolidone). However, this alone is insufficient; the material must also be manageable, as the potential user will handle it manually or with tweezers. To meet this requirement, a monomer that imparts suitable mechanical properties and rigidity,

such as MMA (methyl methacrylate), is incorporated. Additionally, the material may be exposed to organic solvents during certain washing cycles, requiring the inclusion of a crosslinking agent for structural integrity. Lastly, the material must possess anchoring points to allow enzyme immobilization, achieved by incorporating SNH_2. The resulting material is notable for its exceptional resistance to breakage, making it highly versatile and suitable for a wide range of applications without the need for delicate handling.

This acrylic polymer (F_{NH2}) is synthesized by bulk radical polymerization at 60 ºC overnight following the experimental procedure described by Vallejo-García et al. (2023). After the preparation of F_{NH2}, three solid phase reactions were carried out (Fig. 1), briefly: (i) Formation of benzene diazonium salts through the NH_2 pendant groups, resulting in the material F_{N2+}, (ii) Anchoring of the laccases into F_{N2+} through the formation of diazo bonds, and (iii) blocking of the leftover free N_2^+ groups with sodium azide, resulting in the laccase-polymer hybrid (FLac).

Laccase immobilization by azo coupling, the second step, was carried out in a 2.5 mL Eppendorf with 1 disc of F_{N2+} and 0.75 mL of enzyme solution, in an orbital shaker at 25 ºC. Different enzyme dosages of *M. thermophila* laccase in water (1, 5, 10, 20, 50, 100, and 150 U/mL) and treatment times (0.5, 1, 2, 6 and 24 h) were used to obtain the optimal immobilization conditions. It should be noted that the same enzyme solution could be used for a total of 6 discs with minimal reduction of laccase activity. The surface morphology and element distribution were visualized using scanning electron microscopy and energy disperse-ray spectroscopy (SEM-EDS, GemminiSEM560, ZEISS). Films were dried, freeze fratured, and gold coated in vacuum to ensure the electrical conductivity of the films.

2.3. Laccase activity and protein content assays

The activity of laccase was determined spectrophotometrically using ABTS as substrate. Specifically, 0.1 mL of laccase solution or 1 FLac disc (6 mm diameter), 0.2 mL of 4 mM ABTS, 0.1 M NaOAc buffer at pH 4.0, in a total reaction volume of 2.0 mL, were incubated at 30 ºC for 10 min in an orbital shaker at 130 rpm. The reaction was stopped by adding 0.1 mL of 50 % (w/v) TCA. Oxidation of ABTS was monitored in a microplate reader (PowerWave XS2, BioTek) with 96-well plates at 420 nm (ε=3.6 $\times 10^4$ $M^{-1}cm^{-1}$). Laccase activity was expressed as μg $ABTS^{\bullet+}$/min·mL enzyme or μg $ABTS^{\bullet+}$/min·cm^2 for the free and the immobilized enzyme, respectively. All assays were performed in triplicate including substrate blanks.

Control samples of the polymer without the enzyme confirmed that the support was inert and did not react with the substrate.

Protein content was determined using the bicinchoninic acid (BCA) assay, a reliable and suitable method for quantifying surface-bound protein (Goddard et al., 2007). The immobilized enzyme (1 FLac disc of 6 mm diameter) was submerged in 1 mL of BCA reagent and 0.1 mL of distilled water and incubated for 30 min at 60 ºC in an orbital shaker (130 rpm). Absorbance was read at 562 nm and the protein content was determined by comparison to a bovine albumin standard curve.

Specific activity was determined as μg $ABTS^{\bullet+}$/min·mg protein.

In addition, a protein leaching study was carried out with the laccase-polymer hybrids for 30 h at different times (30 min, 1, 6, 20 and 30 h) in aqueous solution with orbital shaking (180 rpm) at 30 ºC, and the protein content of the solution was measured according to the Bradford method (Bradford, 1976).

2.4. Biochemical characterization of free and immobilized laccase

2.4.1. Determination of kinetic parameters

To determine the kinetic parameters of free and immobilized laccases, enzymatic activity was measured for concentrations of ABTS ranging from 0.05 to 4 mM at 30 ºC, according to the protocol described previously in Section 2.3.

The Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) were calculated from Hanes-Woolf plot equation.

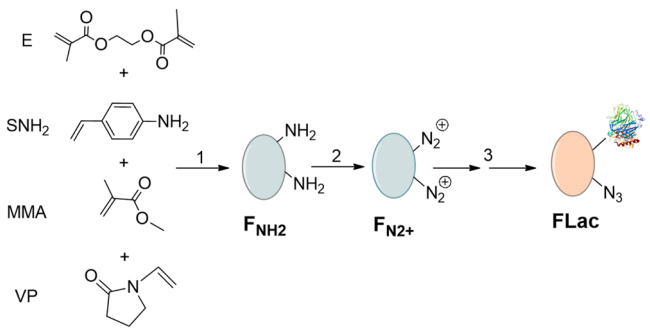

Fig. 1. A simplified schematic representation of the synthesis of laccase-polyacrylic film. The material is shown at different stages of the process: (1) Polymerization, F_{NH2} (initial film with lateral aminostyrene groups), (2) formation of benzene diazonium salt, F_{N2+} (film with lateral benzene diazonium groups), (3) azo coupling, FLac (film with anchored enzyme and free N_2^+ groups blocked with azide). VP (vinylpyrrolidone), MMA (methyl methacrylate), SNH_2 (aminostyrene), E (ethylene glycol dimethacrylate).

2.4.2. Optimal pH and temperature

To evaluate the optimum pH and temperature, the enzyme activity was determined at a pH range (2.0–7.0) of citrate-phosphate buffer and a wide temperature range (30–80 ºC, 10 ºC intervals). The relative activity of the free and immobilized laccases was calculated by considering the maximum activity as 100 %.

2.5. Operational/storage stability

The stability of the immobilized laccases was evaluated in successive batch cycles of reuse and storage at 4 ºC (2, 10, and 40 days) between batches. The experiment was performed in quintuplicate by incubating 1 FLac disc in 2 mL of 4 mM ABTS in acetate buffer (pH 4). Each cycle was performed at 30 ºC for 10 min with orbital shaking. At the end of each cycle, the discs were washed three times with 4 mL of distilled water by vigorous vortexing, and maintaining them in water for 5 min between washes. In each batch, the FLac discs were reused in 5 consecutive cycles and then stored moist at 4 ºC until reuse.

2.6. Determination of BPA removal efficiency

FLac discs (5 discs) were added to an aqueous solution of 80 mg/L BPA (5 mL) with orbital shaking (180 rpm) at 30 ºC. Aliquots of 0.6 mL were taken to determine the BPA content after 0.5, 1, and 2 h of treatment. The discs were then separated from the reaction medium and washed with absolute ethanol for 30 min at 180 rpm to determine the adsorption of the phenolic compound on the immobilization support. Control samples with the polymer without the enzyme were also included to demonstrated that the polymer did not degrade the xenobiotic compound.

The content of BPA in the different samples was determined using a colorimetric assay for phenols under alkaline conditions, according to Modaressi et al. (2005). Reagents were added in the following order: 0.6 mL of BPA solution, 1.4 mL 0.1 M phosphate buffer at pH 8, and 0.1 mL 4-AAP (0.1 M). Potassium ferricyanide (0.1 M) was then added (0.1 mL) and allowed to stand for 10–15 min at room temperature. The absorbance was monitored at 506 nm, and the BPA concentration was determined from a standard curve (Sadeghzadeh et al., 2020).

The removal efficiency was calculated by applying the following equation:

$$\textit{Removal efficiency} = (C_{BPA0}\text{-}C_{BPA})\ x100/C_{BPA0}$$

where C_{BPA0} y C_{BPA} are the BPA concentrations (mg/L) before and after the treatment with the immobilized laccases, respectively.

Identification of biodegradation products of BPA was performed by GC-MS (6545 Q-TOF, Agilent) using a positive/negative electrospray ionization (ESI) source and 0.1 % formic acid/methanol (30:70, v/v) as the mobile phase.

2.7. Statistical analysis

Experimental data were expressed as the mean ± standar deviation for at least three replicates. The statistical analysis was performed using GraphPad Prism v. 8.0.2. Data were first analyzed for normality and homoscedasticity. When both assumptions were fulfilled, a one-way ANOVA was conducted, followed by Tukey's multiple comparisons test ($p < 0.05$). When the normality assumption was not fulfilled, a non-parametric Kruskal–Wallis test ($p < 0.05$) was performed.

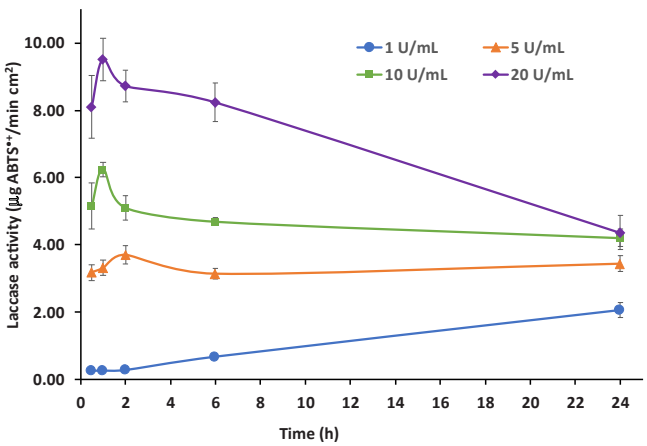

Fig. 2. Effect of the enzyme concentration and immobilization time at 25 ºC on the activity of laccase-polymer hybrid (FLac). U, units of commercial laccase from *M. thermophila*. Data are the arithmetic mean ± standard deviation of three independent replicates (standard deviations for 1 U/mL samples and reaction times of 0.5, 1, 2 and 6 h are between ± 0.010 and ± 0.032).

3. Results

3.1. Development of laccase-polyacrylic film (FLac) under optimized process parameters

To explore the optimal immobilization conditions, the effect of enzyme dosage (*M. thermophila* laccase) and treatment time on laccase activity in FLac were studied (Fig. 2). According to the results obtained, when the lowest enzyme concentration (1 U/mL) was used, the enzyme activity increased over time, but did not reach the activity levels of the other concentrations even after 24 h of treatment. At enzyme dosages of 5, 10, and 20 U/mL, laccase activity in the film increased up to 1–2 h and then decreased with immobilization time. In fact, the highest levels of active enzyme were observed during the first hour at a concentration of 10 and 20 U/mL, with a significant decrease in laccase activity of 32.5 and 54.1 %, respectively, after 24 h. In particular, no differences were observed in the laccase activity of FLac obtained at enzyme concentrations of 20 or 10 U/mL when the immobilization time was 24 h. The results confirmed the assumption that the anchoring of the laccase to the support was a rather fast process. Therefore, it was decided to fix the immobilization time at one hour, also due to the fact that an overall shorter method could be developed.

To evaluate the efficiency of immobilization in terms of specific activity, the laccase activity and the amount of protein were determined in FLac obtained at increasing dosages of enzyme and for 1 h of treatment at 25 ºC (Fig. 3). The results showed a progressive increase in enzyme activity and protein load on the films up to 20 U/mL. However, at higher enzyme concentrations (50, 100, and 150 U/mL), a significant decrease in laccase activity was observed with minimal changes in protein loading. These results showed that additional doses of enzyme could not bind to the support because all available functional groups were already occupied by proteins, leaving no free sites (Amari et al., 2021). Thus, the immobilization efficiency was reduced. The highest specific activities, 7.5, 7.8, and 6.3 μg $ABTS^{\bullet+}$/min·mg protein, were obtained at 5, 10 and 20 U/mL, respectively.

Based on the levels of active enzyme and the specific activity results in FLac, the enzyme dosages of 10 and 20 U/mL and 1 h of treatment were chosen to study the effect of the temperature on the immobilization process. According to the data obtained, increasing the temperature from 25 to 50 ºC reduced the immobilized laccase activity by 32.7 and 35.8 % for 10 and 20 U/mL, respectively.

According to the above, the optimal immobilization parameters for laccase immobilization on the acrylic polymer were 10 U/mL enzyme concentration, 1 h treatment, and 25 ºC. These conditions were also used to immobilize the *T. versicolor* laccase in the comparative study of the two fungal laccases.

Laccase-polymer hybrids obtained with *T. versicolor* laccase under the optimized conditions, showed a 50.8 % reduction in enzyme activity (3.161 ± 0.627 μg $ABTS^{\bullet+}$/min·cm^2 *versus* 6.234 ± 0.218 μg $ABTS^{\bullet+}$/min·cm^2), and lower protein content (0.327 ± 0.056 mg/cm^2 *versus* 0.730 ± 0.031 mg/cm^2) compared to FLac from *M. thermophila* laccase.

On the other hand, it was observed that the laccase-polyacrylic film became more intense in color as the amount of protein anchored increased. To verify the relationship between color and protein concentration, we used an optical technique (digital image correlation) (Gaona-Ruiz et al., 2024; Guirado-Moreno et al., 2023). FLac discs were photographed with a smartphone after dipping the F_{N2+} discs in *M. thermophila* laccase solutions with concentrations ranging from 1 to 150 U/mL. The G parameter extracted from the digital images was represented against the protein concentration in the film. The trend was linear over this concentration range, as shown in Fig. 4. Specifically, the protein content of FLac (*T. versicolor* laccase) by resolving the value of the G parameter in the calibration equation (Fig. 4) was 0.362 ± 0.013 mg/cm^2.

Immobilization of laccase on the polymeric material was characterized by SEM and EDS (Supplementary Fig. S1). The image of the F_{N2+} material showed a dense structure, in contrast to FLac, which showed a slightly rough surface due to the enzyme immobilization (Vallejo-García et al., 2023). Elemental analysis revealed the presence of chlorine in F_{N2+} due to diazonium salts and sodium in FLac due to azide treatment, the other elements being very similar.

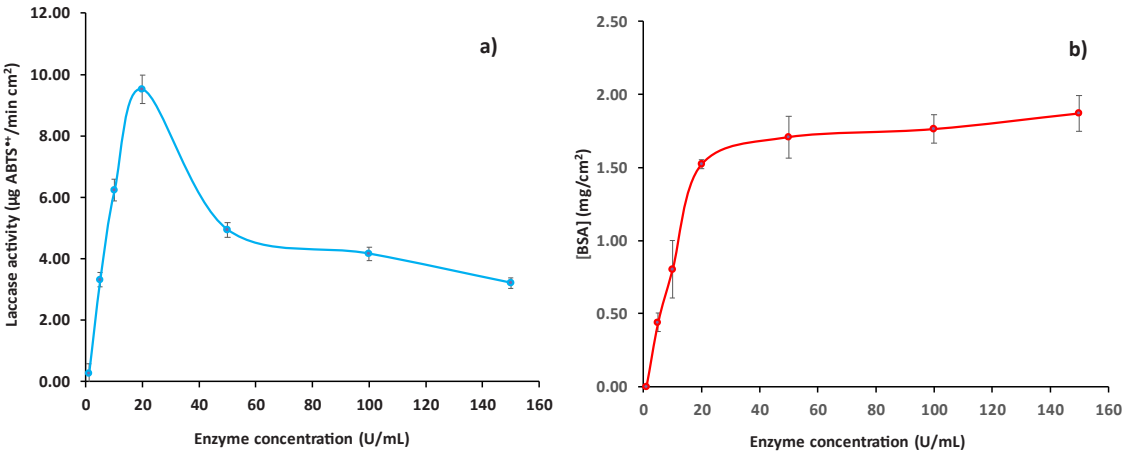

Fig. 3. Effect of the enzyme concentration on the laccase activity (a) and protein content (b) in the laccase-polymer hybrid (FLac). Immobilization time 1 h at 25 ºC. U, units of commercial laccase from *M. thermophila.* Data are the arithmetic mean ± standard deviation of three independent replicates.

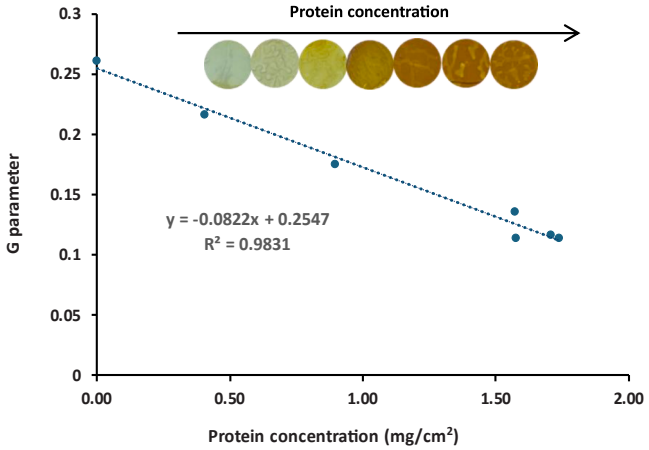

Fig. 4. Graphical representation of the G parameter from the photographed discs of laccase-polymer hybrid (*M. thermophila* laccase) *versus* protein concentration. At the top of the figure are the real images (photographs) of the discs, visually showing the color differences depending on the amount of protein anchored to the polymer.

No protein leaching into the aqueous medium was detected during the 30 h of incubation of the laccase-polymer hybrids, confirming the strong binding of the enzyme to this polymer matrix. Similar results were obtained in previous studies with the enzyme β-galactosidase (Vallejo-García et al., 2023).

3.2. Biochemical characterization of fungal laccases immobilized on the acrylic polymer

A comparative study between the free and the immobilized enzyme for the laccases from the fungi *M. thermophila* and *T. versicolor*, was provided in terms of activity dependence on pH and temperature and kinetic behavior.

3.2.1. Determination of kinetic parameters

Soluble and immobilized laccases followed the Michaelis-Menten kinetic model but with different kinetic parameters. Table 1 shows comparative data for K_m and V_{max}, estimated by Hanes-Woolf plot using ABTS as substrate, for free and immobilized laccases. Although both fungal laccases showed similar affinity for the substrate (K_m), the free enzyme from *M. thermophila* was catalytically more effective against the ABTS substrate. The *T. versicolor* laccase immobilized on the film-shaped acrylic polymer showed a slightly higher K_m than the free counterpart, whereas no changes in K_m were observed for the *M. thermophila* laccase. For both FLac, the value of apparent V_{max} decreased compared to the soluble enzyme. In addition, immobilization increased the differences in catalytic efficiency between the fungal laccases, whereas the free *M. thermophila* laccase was 1.2 times more efficient than *T. versicolor* laccase in the immobilized form the efficiency increased by a factor of 2.3.

3.2.2. Effect of pH and temperature on laccase activity

The effect of pH on the activity of the free and immobilized laccases was determined in the pH range of 2–7 (Fig. 5). In general, fungal laccases are acidic proteins and are quite active at pH 2–5 (Baldrian, 2006). As shown in Fig. 5, the two laccases were highly active in a narrow pH range between 2 and 4, with an optimal pH of 3 and 4 for laccase from *T. versicolor* and *M. thermophila*, respectively. Nevertheless, the pH profiles of the laccases were different, while the *M. thermophila* laccase had a bell-shaped profile, the *T. versicolor* laccase showed an ascending profile towards acidic pH values. It can also be observed that the pH profile of the immobilized laccase (*M. thermophila*) was slightly shifted towards acidic pH, with the optimum at pH 3 and retaining more than 40 % of its maximum activity at pH 2, whereas the free enzyme retained only 14 %. The shift of the pH activity optimum of immobilized laccase towards a more acidic region is not unusual and can also be found in the literature (Bević et al., 2020a; Xia et al., 2016). The immobilized laccase (*T. versicolor*) showed a similar pH/activity curve to its free counterpart, with an optimum pH of 2. However, the immobilized laccase retained 37.7 and 25.1 % of the activity at pH 6 and 7, respectively, while the free laccase showed about 21.5 (pH

Table 1
Kinetic parameters for free and immobilized laccases (FLac) from *M. thermophila*[a] and *T. versicolor*[b].

Biocatalyst	V_{max} (μg $ABTS^{\bullet+}$ min^{-1})	K_m (mM)	Efficiency (V_{max}/K_m)	R^2
Free enzyme[a]	74.07 ± 0.56	0.10	740.70	0.958
FLac[a]	6.00 ± 0.67	0.10	60.00	0.957
Free enzyme[b]	43.86 ± 0.10	0.07	626.57	0.953
FLac[b]	2.76 ± 0.28	0.11	25.09	0.950

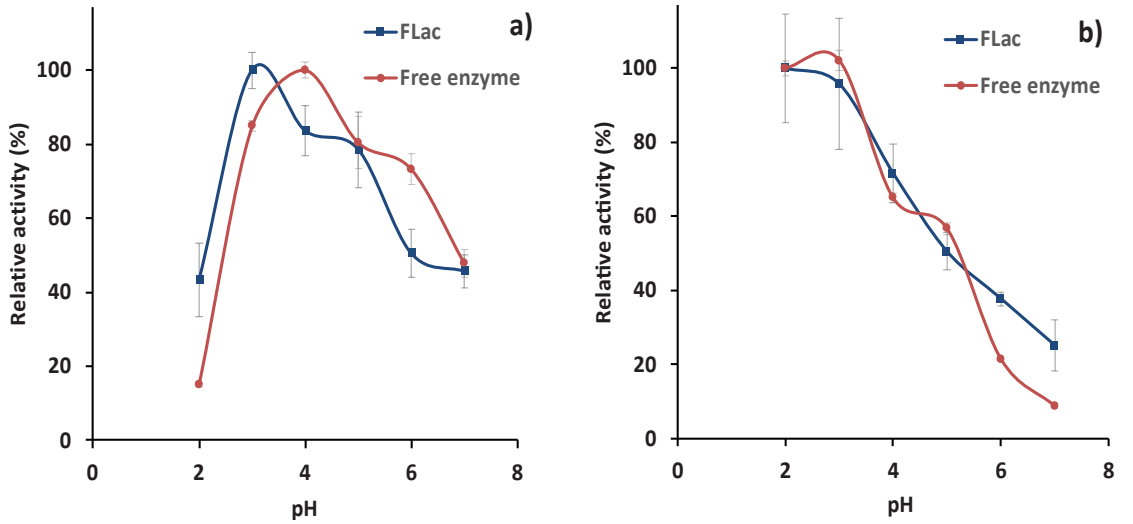

Fig. 5. Effect of pH on the free and immobilized laccase (FLac) from *M. thermophila* (a) and *T. versicolor* (b). Data are the arithmetic mean ± standard deviation of three independent replicates.

6) and 8.9 % (pH 7). In this regard, *M. thermophila* laccase showed greater stability at these pH values, with retention above 45 % for both the free and the immobilized enzyme.

To investigate the effect of temperature on the free and immobilized enzyme, laccase activity was assayed at different temperatures from 20 to 80 ºC (Fig. 6). The two fungal laccases, free or immobilized, maintained high relative activity (>60 %) over a wide temperature range (40–80 ºC). The optimum temperature for *T. versicolor* and *M. thermophila* laccase was 60 ºC and 80 ºC, respectively. The immobilized laccases showed no significant differences in the activity temperature curves compared to their free counterparts.

3.3. Operational/storage stability

Several consecutive reaction cycles were carried out in batch experiments using the standard substrate ABTS to evaluate the operational stability of the immobilized laccases. The reusability of FLac was studied for up to five consecutive cycles before the samples were stored moist at 4 ºC for subsequent recycling. FLac retained a residual activity of 100 % (*M. thermophila* laccase*)* and 92.1 % (*T. versicolor* laccase), after the first 5 cycles of reuse (Fig. 7). For the industrial application of immobilized enzymes, it is also useful to have a better knowledge of their storage stability. As shown in Fig. 7, immobilized laccase from *M. thermophila* and *T versicolor*

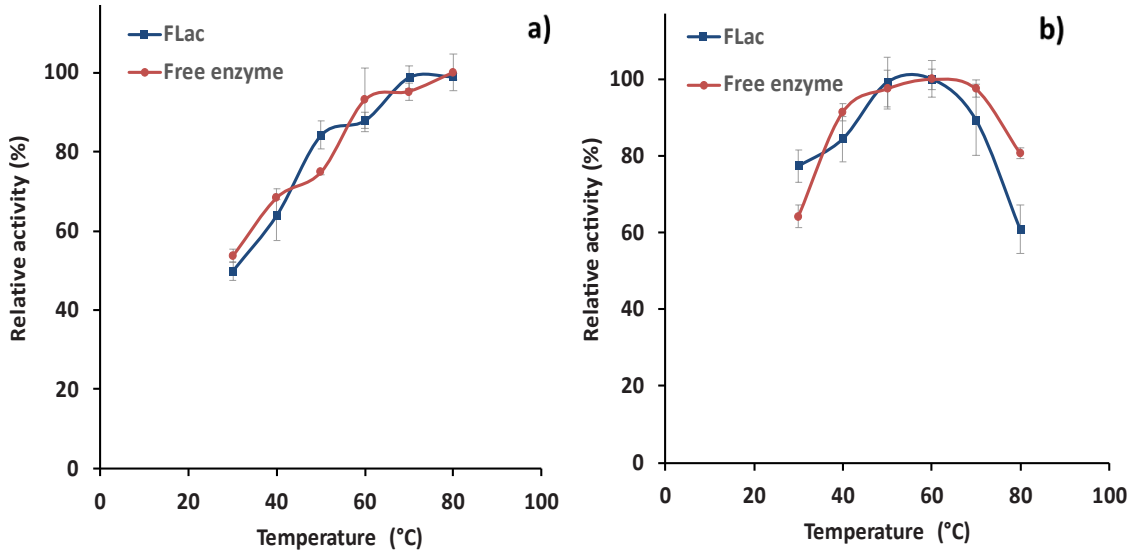

Fig. 6. Effect of temperature on the free and immobilized laccase (FLac) from *M. thermophila* (a) and *T. versicolor* (b). Data are the arithmetic mean ± standard deviation of three independent replicates.

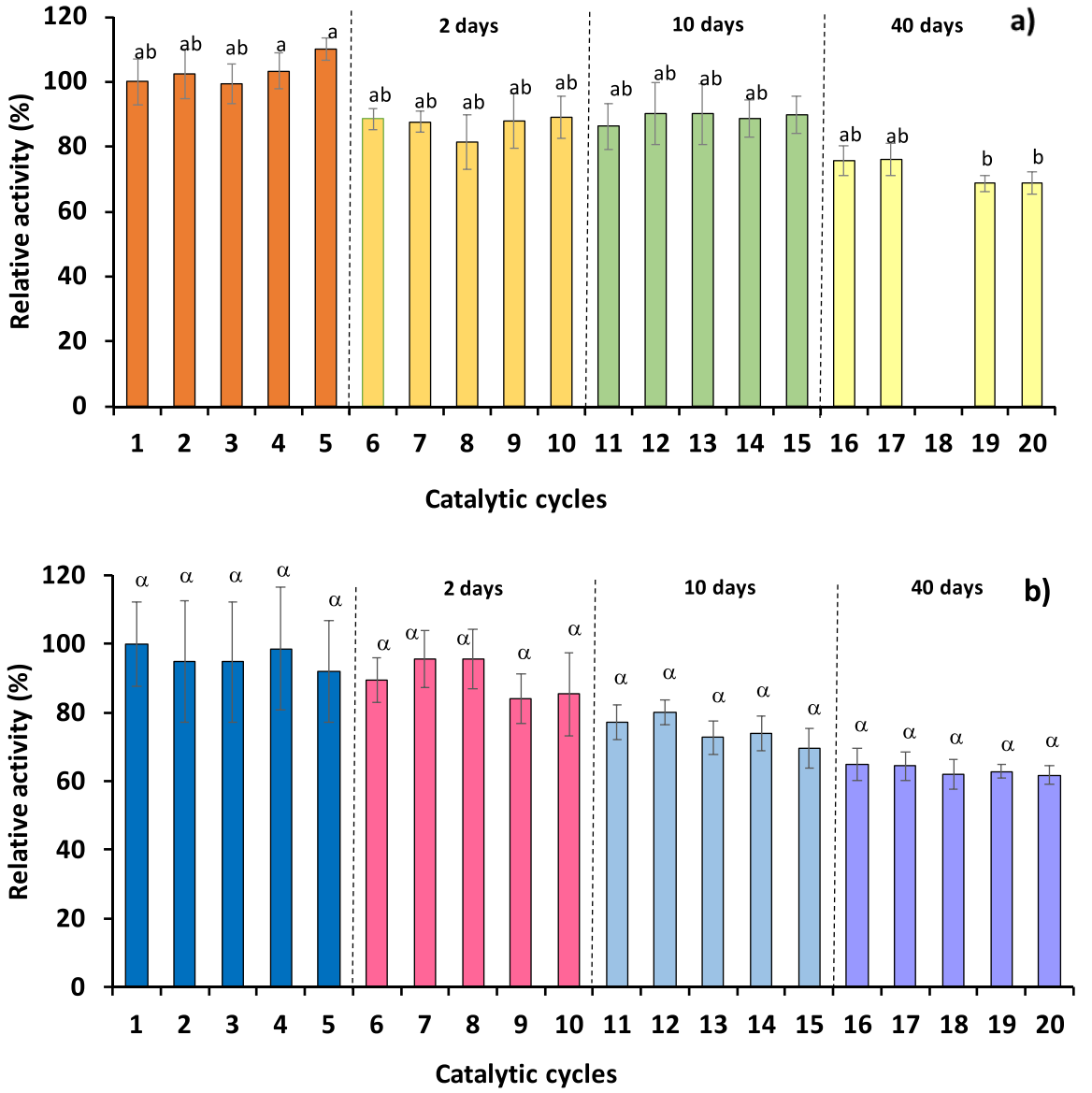

Fig. 7. Reusability and storage stability of laccase-polymer hybrid (FLac), (a) *M. thermophila* laccase, and (b) *T. versicolor* laccase. The reusability of FLac was studied for up to five consecutive cycles before the samples were stored moist at 4 ºC (0, 2, 10, and 40 days) for subsequent recycling. The FLac activity in the first cycle was taken as a reference value of 100 %. Values represent the mean and vertical bars the standard deviation for at least three replicates. Different letters (Latin letters) indicate significant differences between the reuse cycles data of (a) ($p < 0.05$, one-way ANOVA followed by Tukey's multiple comparisons test). No significant differences (Greek letter) were observed in the data of (b) (Kruskal–Wallis test, $p < 0.05$).

retained 89.9 % and 69.5 % of activity, respectively, after 10 days of storage at 4 ºC and 15 cycles of reuse. After 40 days of storage and 20 reuse cycles, the FLac lost only between 31.2 % and 38.3 % of their activity.

3.4. Application of FLac for BPA removal

A gradual depletion of BPA by FLac was observed with increasing treatment time (Fig. 8). The removal efficiencies were between 34.0 % and 37.9 % after 30 min, 56–71 % after 1 h, and 91.6–93.1 % after 2 h. Therefore, both immobilized fungal laccases were shown to be effective in removing BPA. In addition, CG-MS analysis of the reaction media identified 3- or 2-methyl-2,3-dithtdrobenzofuran, as a degradation product of BPA by laccase action. No degradation products were detected in the control samples with the polymer without the enzyme.

To know the adsorption capacity of the immobilization support, the FLac discs used for BPA removal were washed with absolute

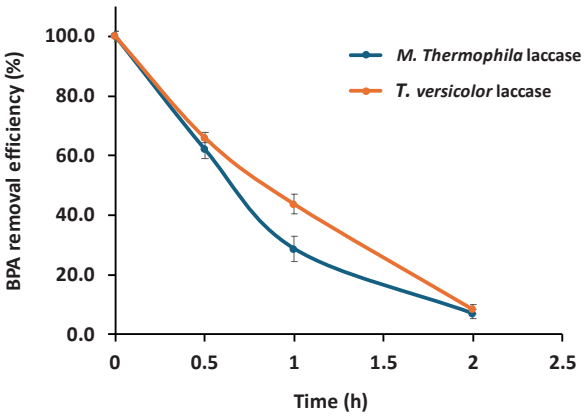

Fig. 8. Removal of BPA (80 mg L^{-1} BPA in deionized water, 25 ºC) by laccase-polymer hybrid (FLac) *versus* time. Error bars are related to standard deviation of triplicate test.

alcohol (30 min at 180 rpm) and the concentration of phenolic compounds in the washes was evaluated. The adsorption percentage varied from 61.4 ± 2.7 % (FLac discs with *M. thermophila* laccase) to 30.5 ± 0.2 % (FLac discs with *T. versicolor* laccase). The phenolic compound 5,5´-bis-[1-(4-hydroxyphenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol, a degradation product of BPA, was identified in all ethanol washes together with 3- or 2-methyl-2,3-dithtdrobenzofuran. In addition, BPA was also detected in the washes when FLac obtained with *M. thermophila* laccase was used. Interestingly, the immobilized laccases retained more than 93 % of their initial activity after washing with absolute ethanol.

4. Discussion

In this research, two fungal laccases were covalently immobilized on a film-shaped acrylic polymer through azo linkages. In order to optimize the immobilization process, the effect of enzyme concentration and immobilization time, parameters directly related to the final cost, were studied. It was observed that at the lowest enzyme dosages, all the protein offered was bound to the film, so that laccase activity increased with time to a maximum. In this sense, the loss of activity at higher enzyme concentrations could be explained by considering that as the treatment time increased, too many enzymes on the support surface could lead to a lack of space and steric hindrance and interactions between the protein structures, resulting in limited mass transfer and a reduction in catalytic efficiency (Wang et al., 2021). On the other hand, the functional groups on the surface of this film are limited, so that if there is an excess of enzyme, it cannot be anchored to the support by covalent bonding. Therefore, above the optimal enzyme dosage, the protein content of the laccase-polymer hybrid did not show significant changes. The decrease in laccase activity observed at high enzyme concentrations is a result of a polymer oversaturation which would lead to clustering and multilayer configuration and eventually drastic enzyme conformational changes and substrate diffusion limitations. (Abdel-Mageed et al., 2019; Demarche et al., 2012). Similar observations have been reported by several authors where the decrease in the immobilized enzyme activity occurs beyond the optimal enzyme concentration (Smith et al., 2009; Zhou, 2010). The specific activity of the immobilized enzyme depends on its conformational flexibility, which can be altered at higher protein loads (Bević et al., 2020b), which is in line with the higher specific activity obtained at enzyme concentrations of 10 U/mL as compared to 20 U/mL.

On the other hand, the color observed in the FLac discs was due to the azocoupling reactions with the phenol groups of the enzymatic protein, producing a compound with a strong yellow-orange color (an azo dye). In this research, a linear relationship between the color parameter "G" and the concentration of protein in the discs was described and compared with the BCA assay for the quantification of protein in a surface. In fact, this method and the bicinchoninic acid assay gave very similar results for the protein content anchored to the polymer. Both methods represent an interesting alternative for the direct quantification of protein immobilized on the polymeric support compared to the usual indirect determination, which requires the measurement of the protein content in the supernatant and the wash samples obtained in the immobilization process. In addition, the relationship found between the color parameter G and the protein content bound to the polyacrylic polymer could be used in another line of research, where the polymer would be adapted for use as an analytical tool for the quantitative determination of proteins in different samples.

Regarding the effect of temperature in the immobilization process, when an aryl diazonium salt is heated in the presence of water, the diazo group can be replaced via a phenyl cation (Wu and Glaser, 2004). This could explain the lower enzyme activity observed in the laccase-polymer hybrid obtained at 50 ºC, as the coupling reactions with the aromatic functional groups of the protein were reduced.

Under the optimized immobilization conditions, FLac obtained with *M. thermophila* laccase showed higher catalytic activity than FLac obtained with *T. versicolor* laccase. These results can be related to the structural differences of laccases depending on their microbial source. Laccases from different sources share similar oxidation mechanisms and conserved copper-binding motifs, but show low sequence similarity and a broad oxidative spectrum (Sekretaryova et al., 2019). Similar results have been widely reported in the literature for laccases and many other enzymes (Ortega et al., 2022; Rostami et al., 2022; Timur et al., 2003). In a previously reported

study, this polyacrylic film material also proved to be an efficient support for the immobilization of β-galactosidase from *Aspergillus orzyae* (Vallejo-García et al., 2023). However, this is the first time that this film-shaped material has been used to immobilize laccases.

Covalent immobilization techniques provide strong, stable enzyme attachment with reduced leaching and deactivation rates. However, during covalent immobilization, the native structure of the enzyme is usually affected due to the multipoint attachments and the enzyme activity is often reduced due to the rigorous treatment with toxic coupling reagents (Kyomuhimbo et al., 2023). Information about the possible interactions between the enzyme and its immobilization support can often be figured out by comparing the kinetic parameters of the immobilized enzyme and its free counterpart. The slow down of the reaction rate (V_{max}) of FLac indicated that the immobilization process could lead to conformational changes in the enzyme, restrictions on the substrate transport, and steric constraints for biomolecular reactions (Dong et al., 2023; Jankowska et al., 2020). These effects appear to be more pronounced for *T. versicolor* laccase, which showed a significant decrease in activity after immobilization. Similar outcomes of lower V_{max} and catalytic efficacy after immobilization of laccases have been published (Lou et al., 2023; Nicolucci et al., 2011; Rybarczyk et al., 2023; Zou et al., 2023). The K_m values for the immobilized and free laccases were in the range of 0.07–0.11 mM, demonstrating that the binding affinity between the laccase-polymer hybrids and the substrate was similar to that of free laccase. The same behavior has also been reported by Vallejo-García et al. (2023) using this acrylic polymer to immobilize β-galactosidase and by other authors using different immobilization methods with laccases (Arsenault et al., 2011; Özdemir and Yalçinkaya, 2023). On the contrary, some studies have reported higher and lower K_m values of immobilized laccases (Bilal et al., 2021; Zou et al., 2023). Thus, the affinity of laccase to catalyze ABTS oxidation may o may not be altered by the immobilization procedure, depending on the specific characteristics of the enzyme and the immobilization method and support used.

It is well known that pH and temperature are important factors affecting the activity and stability of enzymes. The pH and temperature optima of laccases are very substrate-dependent and can vary considerably due to changes in the reaction caused by the oxygen, substrate, or the enzyme itself and its origin. The pH optima obtained in this study are consistent with the pH range of 3–5 reported in the literature for laccases using ABTS as a substrate (Kolak et al., 2023; Zayed et al., 2024). The bi-phasic pH/activity profile of *M. thermophila* laccase could reflect the possible combinatory contribution from the opposing effects of the pH-induced redox potential change (on both the T1 center and substrate) and the hydroxide anion inhibition (on the T2/T3 copper sites) (Xu, 1997). In the case of *T. versicolor* laccase, the monotonic pH profile, when increasing the pH, could result from the involvement of hydroxide anion inhibition (Xu, 1997). In addition, possible changes in the dissociation and ionization state of the enzymes during the immobilization process could also explain the differences observed in the pH/activity curves (Wang et al., 2021).

On the other hand, the optimum temperature reported for laccases is in the range of 50–70 ºC (Amari et al., 2021; Sampaio et al., 2016; Xu et al., 2016), which is also consistent with the results of this research. In general, the immobilized enzymes showed the same trend as their free counterparts. Thus, the differences observed in the temperature/activity curves between the two laccases would be related to their fungal origin and not to the immobilization process. This behavior fits with the results reported by other researchers (Zhu et al., 2020).

Operational and storage stability are significant advantages of immobilized enzymes from the point of view of industrial application. In this respect, satisfactory findings were obtained for the laccase-polymer hybrids. The acrylic polymer, as a protective support for laccases, maintained long-term recyclability and catalytic stability. The decrease in activity observed in FLac over successive cycles of discontinuous operation could be attributed to the partial inactivation of the enzyme due to mechanical damage during the handling of the immobilizates (Jankowska et al., 2020; Sadeghzadeh et al., 2020). This acrylic polymer was previously used as a support for the immobilization of β-galactosidase with similar results, showing a relative activity $>$ 99 % after 10 cycles of reuse (Vallejo-García et al., 2023). Numerous studies have investigated the operational stability and reusability of laccase immobilized on a wide variety of supports, with very different results. For example, Sathishkumar et al. (2014) immobilized laccase on cellulose nanofibers and observed 33 % activity loss after 10 ABTS oxidation cycles. In a similar work, Zhao et al. (2023) immobilized laccase on flexible ceramic nanofibrous membranes and reported 80 % residual activity after 5 cycles. Kolak et al. (2023) also described a residual activity of 33.2 % of laccase immobilized in an electrospun fiber material after 10 repeated cycles. In terms of storage stability, Zayed et al. (2024) described 71–83 % of the original activity of a laccase immobilized in novel polymeric support material after 30 days of storage, and Maryskova et al. (2022) showed that laccase immobilized in a polyamide-nanofiber membrane preserved 50 % of the activity after 30 days at 4 ºC.

It is worth noting that most of the research described in the literature, including that mentioned above, does not simultaneously investigate reusability and storage stability as in the present work.

BPA, a hazardous pollutant commonly found in wastewater, was selected as a model for a proof-of-concept experiment on the applicability of laccase-polymer hybrids in bioremediation. The results obtained are excellent compared to recent research reported in the literature. For example, Xing et al. (2022), using a membrane-enclosed laccase, reported 40.7 % degradation of BPA in 4 h at 40 ºC with an initial concentration of 0.1 mM BPA. Maryskova et al. (2022) were able to remove 90 % of BPA in 20 h using laccase immobilized onto laminated poly(acrylic acid) nanofibers, and Lugo-Bueno et al. (2022) reported 76 % removal of BPA in 6 h using immobilized laccases assisted by electrochemical processes.

The biotransformation products identified in this study correspond to some of the compounds described in the literature for laccase-catalyzed BPA removal. For example, Xing et al. (2022) proposed a possible degradation pathway of BPA by *T. versicolor* laccase, in which 3- or 2-methyl-2,3-dithtdrobenzofuran were the result of electron loss and alkylation of 4-(2-hydroxypropan 2-yl) phenol, one of the first degradation products of BPA. In addition, an oxidative condensation pathway of BPA has also been proposed to form oligomers with C-C bonds, such as 5,5´-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol BPA dimer (De Freitas et al., 2017).

The results showed that the catalytic action of laccase-polymer hybrids, rather than its adsorptive action, played a leading role in the degradation process of BPA. Nevertheless, the combined action of efficient adsorption and enzymatic reaction improved the

degradation efficiency of the pollutant. It should also be noted that the immobilization support, due to its adsorption capacity, also removed some of the BPA degradation products from the reaction medium. A proposed mechanism for this behavior assumes that BPA is adsorbed on free sites of the polymer, and after initiation of biodegradation by laccase, the occupied sites are liberated, and the sorption-biodegradation cycle can start again (Naghdi et al., 2017). Other researchers have also reported this dual behavior of adsorption and degradation. For example, Yang et al. (2023) showed a total degradation efficiency of 67.6 % for Lac@HOFs/hydrogel composite, in which the adsorption contribution of the support was 54.4 %. Sadeghzadeh et al. (2020) described adsorption of 7.5 ppm of the initial 60 ppm by magnetic cross-linked laccase aggregates from *T. hirsuta*, and Naghdi et al. (2017) reported a biodegradation contribution of more than 45 %, while adsorption accounted for < 30 % of the total removal.

5. Conclusions

In this study, a durable, film-shaped, and easy-to-handle polymer was produced and successfully used as support for the covalent immobilization of two fungal laccases while preserving their biocatalytic activity. Analysis of the biochemical properties of the laccase-polymer hybrids (FLac) demonstrated their potential application over a wide range of pH (2−7) and temperature (30–80 ºC), depending on the microbial source of the enzyme. This suggests that these biocatalytic films could be selected for application in a wide range of environmental conditions.

On the other hand, the FLac system achieved very efficient removal of bisphenol A (removal rates all above 90 % in 2 h) through an adsorption-catalysis process, with an initial concentration of 80 ppm.

To evaluate the benefits of a biocatalytic system, its operational and storage stability are important factors in determining the processing costs. In particular, this immobilization procedure ensured both repeated use, with over 89–70 % of initial activity retained after 15 ABTS catalytic cycles and storage stability at 4 ºC (at least 40 days after repeated use). Taking this into account, the cost of 1 g of FLac (1 g of material corresponds to 400 discs) was estimated at 0.07 € (*M. thermophila* laccase) or 0.60 € (*T. versicolor* laccase), demonstrating its cost-effectiveness, especially when using the *M. thermophila* enzyme.

We have designed the process with industrial applicability in mind. All the monomers used are commercially available and widely accessible, and enzyme anchoring is performed through immersion or bath processes, which are well-established procedures in the industry. These aspects ensure that the methodology can be implemented without the need for specialized infrastructure or significant modifications to existing production lines. Although the proposed method is feasible for industrial applications, its implementation may face challenges such as the stability of enzyme anchoring under extreme conditions, specific handling and storage requirements to preserve enzymatic activity, and potential variations in reproducibility when scaling the process. While these limitations are manageable, they highlight areas where future adjustments could further optimize its application in real-world scenarios. Future studies should evaluate the performance of the laccase-polymer hybrids with wastewater, under industrial conditions and in continuous mode operation to know the real applicability of laccases for decontamination of aqueous systems.

In conclusion, the present findings suggest that these laccase-polymer hybrids are a potentially useful tool in processes for the removal of BPA. Additionally, the high versatility of laccases implies that their application could be extended to other organic pollutants (e.g. dyes, drugs, pesticides…). Therefore, this work can be considered as a new step in the knowledge and application of the bioremediation in the elimination of emerging organic contaminants in aqueous solutions.

CRediT authorship contribution statement

Vallejo J. Lucas: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Vallejos Saul:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Trigo-López Miriam:** Validation, Methodology, Investigation, Formal analysis. **García José M.:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Busto Maria D.:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Conceptualization.

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.

Acknowledgements

We gratefully acknowledge the financial support provided by all funders. The financial support provided by Fondo Europeo de Desarrollo Regional-European Regional Development Fund (FEDER, ERDF) and Regional Government of Castilla y León -Consejería de Educación, Junta de Castilla y León - (BU025P23) is gratefully acknowledged. We also acknowledge grant PID2023–147301OB-I00 funded by MICIU/AEI /10.13039/501100011033 and FEDER, EU. This work was supported by the Regional Government of Castilla y León (Junta de Castilla y León) and the Ministry of Science and Innovation MICIN and the European Union NextGeneration EU PRTR. Author Saul Vallejos received grant BG22/00086 funded by the Spanish Ministerio de Universidades.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2025.104093.

Data availability

Data will be made available on request.

References

Abdelhamid, M.A.A., Khalifa, H.O., Yoon, H.J., Ki, M.R., Pack, S.P., 2024. Microbial immobilized enzyme biocatalysts for multipollutant mitigation: harnessing Nature's toolkit for environmental sustainability. Int. J. Mol. Sci. 25, 8616. https://doi.org/10.3390/ijms25168616.

Abdel-Mageed, H.M., Radwan, R.A., AbuelEzz, N.Z., Nasser, H.A., El Shamy, A.A., Abdelnaby, R.N., El Gohary, N.A., 2019. Bioconjugation as a smart immobilization approach for-amylase enzyme using stimuliresponsive Eudragit-L100 polymer: a robust biocatalyst for applications in pharmaceutical industry. Artif. Cells Nanomed. Biotechnol. 47 (1), 2361–2368. https://doi.org/10.1080/21691401.2019.1626414.

Ahmaruzzaman, M., Mishra, S.R., Gadore, V., Yadav, G., Roy, Bhattacharjee, B., Bhuyan, A., Hazarika, B., Darabdhara, J., Kumari, K., 2024. Phenolic compounds in water: from toxicity and source to sustainable solutions – an integrated review of removal methods, advances technologies, cost analysis, and future prospects. J. Environ. Chem. Eng. 12 (3), 112964. https://doi.org/10.1016/j.jece.2024.112964.

Amari, A., Alzahrani, F.M., Alsaiari, N.S., Katubi, K.M., Rebah, F.B., Tahoon, M.A., 2021. Magnetic metal organic framework immobilized laccase for wasterwater decolorization. Process 9, 774. https://doi.org/10.3390/pr9050774.

Ariste, A.F., Haroune, L., Saibi, S., Cabana, H., 2021. Enzyme polymer engineered structure strategy to enhance cross-linked enzyme aggregate stability: a step forwad in laccase explotation for cannabidiol removal from wasterwater. Environ. Sci. Pollut. Res. 28, 44051–44063. https://doi.org/10.1007/s11356-021-13746-4.

Arsenault, A., Cabana, H., Jones, J.P., 2011. Laccase-based CLEAs: chitosan as a novel cross-linking agent. Enzym. Res 211, 376015. http://10.406/2011/376015.

Baldrian, P., 2006. Fungal laccases-ocurrence and properties. FEMS Microbiol. Rev. 30 (2), 215–242. http://10.1111/j.1574-4976.2005.00010.x.

Bević, J., Banjanac, K., Corović, M., Milovojević, A., Simović, M., Marinković, A., Bezbradica, D., 2020a. Immobilization of laccase from *Myceliophthora thermophila* on functionalized silica nanoparticles: optimization and application in lindane degradation. Chin. J. Chem. Eng. 28 (4), 1136–1144. http://10.1016/j.cjche.2019.12.025.

Bević, J., Banjanac, K., Rusmirović, J., Corović, M., Milivojević, A., Simović, M., Marinković, A., Bezbradica, D., 2020b. Amino-modified kraft lignin microspheres as a support for enzyme immobilization. Rsc. Adv. 10, 21495. http://10.1039/d0ra03439h.

Bilal, M., Noreen, S., Asgher, M., Parveen, S., 2021. Development and characterization of coss-linked laccase aggregates (Lac-CLEAs) from *Tramete versicolor* IBL-04 as eco-friendly biocatalysts for degradation of dye-based environmental pollutants. Environ. Technol. Inno. 21, 101364. http://10.1016/j.eti.2021.101364.

Bradford, M.M., 1976. A rapid and sensitive method for the quatification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. http://10.1016/0003-2697(76)90527-3.

Cen, Q., Wu, X., Cao, L., Lu, Y., Lu, X., Chen, J., Fu, G., Liu, Y., Ruan, R., 2022. Green production of a yellow laccase by *Coloriolopsis gallic*a for phenolic pollutants removal. AMB Express 12, 96. http://10.1186/s13568-022-01434-6.

De Freitas, E.N., Bubna, G.A., Brugnari, T., Kato, C.G., Nolli, M., Rauen, Th.G., Peralta, R.F., Peralta, R.A., Bracht, A., de Souza, C.G.M., Peralta, R.M., 2017. Removal of bisphenol A by laccases form *Pleurotus ostreatus* and *Pleurotus pulmonatius* and evaluation of ecotoxicity of degradation products. Chem. Eng. J. 330, 1361–1369. http://10.1016/j.eti.2017.08.051.

Demarche, P., Junghanns, Ch, Mazy, N., Agathos, S.N., 2012. Design-of-experiment strategie for the formulation of laccase biocatalysts and their application to degrade bisphenol A, 30 (1), 96–103. http://10.1016/j.nbt.2012.05.023.

Dong, Ch, Tiwari, A., Anisan, G.S., Chen, Ch-W., Sing, A., Haldar, D., Patel, A., Singhania, R.R., 2023. Laccase: a potential biocatalyst for pollutant degradation. Environ. Pollut. 319, 120999. http://10.1016/j.envpol.2023.120999.

Du, M., Liu, J., Wang, Q., Wang, F., Bi, L., Ma, Ch, Song, M., Jiang, G., 2025. Immobilization of laccase on magnetic PEGDA-CS inverse opal hydrogen for enhancement of bisphenol A degradation in aqueous solutions. J. Environ. Sci. 147, 74–82. https://doi.org/10.1016/j.jes.2023.10.017.

Gaona-Ruiz, M., Vallejo-García, J.L., Arnaiz, A., Sedano-Labrador, C., Trigo-López, M., Rodríguez, A., Carrillo, C., Vallejos, S., 2024. Smart polymers and smartphones for Betalain measurement in cooked beetroots. Food Chem. 459, 140358. https://doi.org/10.1016/j.foodchem.2024.140358.

García, J.M., García, F.C., Reglero, J.A., Vallejos, S., Trigo-López, M. 2022. Smart Polymers. Principles and Applications, De Gruyter, Boston. http://10.1515/9781501522468.

Goddard, J.M., Talbert, J.N., Hotchkiss, J.H., 2007. Covalent attachment of lactase to low-density polyethylene films. J. Food Sci. 72, E36–E41. https://doi.org/10.1111/j.1750-3841.2006.00203.x.

Guirado-Moreno, J.C., Carreira-Barral, I., Ibeas, S., Garcí, J.M., Granès, D., Marcher, N., Vallejos, S., 2023. Democratization of copper analysis in grape must following a polymer-based lab-a-chip approach. ACS Appl. Mater. Interfaces 15 (12), 16055–16062. https://doi.org/10.1021/acsami.3c00395.

Hubab, M., Gilani, I.E., Al-Ghouti, M.A., 2025. Metal-organic frameworks: a promising solution for addressing phenol pollution and promoting environmental sustainability. Environ. Technol. Innov. 37, 104004. https://doi.org/10.1016/j.eti.2024.104004.

Jankowska, K., Zdarta, J., Grywaczyk, A., Kijeńska-Garońska, E., Biadasz, A., Jesionowski, T., 2020. Elestrospun poly(methylmetathacrylate)/polyaniline fibres as a support for laccase immobilisation and use in dye decolourisation. Environ. Res. 184, 109332. https://doi.org/10.1016/j.envres.2020.109332.

Jeyabalan, J., Veluchamy, A., Priyan, V., Kumar, A., 2023. A review on the laccase assisted decolourisation of dyes: recent trends and research progress. J. Taiwan Inst. Chem. Eng. 151, 105081. https://doi.org/10.1016/j.jtice.2023.105081.

Ji, C., Nguyen, I.N., Hou, J.W., Hai, F.I., Chen, V., 2017. Direct immobilization of laccase on titania nanoparticles from crude enzyme extracts of *P. ostreatus* culture for micro-pollutant degradation. Sep. Purif. Technol. 178, 215–223. https://doi.org/10.1016/j.seppur.2017.01.043.

Jiang, Y., Zheng, J., Wang, M., Xu, W., Wang, Y., Wen, L., Dong, J., 2024. Pros and Cons in Various immobilization techniques and carriers for enzymes. Appl. Biochem. Biotechnol. 196, 5633–5655. https://doi.org/10.1007/s12010-023-04838-7.

Kolak, S., Birnali, E., Boran, F., Bakar, B., Ulu, A., Yesilada, O., Ates, B., 2023. Tailor-made novel electrospun polycaprolactone/polyethyleneimine fiber membranes for laccase immobilization: an all-in-one material to biodegrade textile dyes and phenolic compounds. Chemosphere 313, 137478. https://doi.org/10.1016/j.chemosphere.2022.137478.

Kyomuhimbo, D., Brink, H.G., 2023. Applications and immobilization strategies of the copper-centred laccase enzyme; a review. Heliyon 9, e13156. https://doi.org/10.1016/j.heliyon.2023.e13156.

Lou, X., Zhi, F., Sun, X., Wang, F., Hou, X., Lv, Ch, Hu, Q., 2023. Construction of co-immobilized laccase and mediator based on MOFs membrane for enhancing organic pollutants removal. Chem. Eng. J. 451, 138080. https://doi.org/10.1016/j.cej.2022.138080.

Lugo-Bueno, S.F., García-Morales, R., Coronel, R., Aguilar-Hernández, I., Becerril-Bravo, J.E., Barrios-Pérez, J.A., Mahlknecht, J., Cano-Quiroz, A., Ornelas-Soto, N., 2022. Biocatalysis assisted by electrochemical processes for the removal of bisphenol A and triclosan in wastewater. Environ. Technol. Inno. 28, 102921. https://doi.org/10.1016/j.eti.2022.102921.

Mamman, S., Abdullahi, S.S., Birniwa, A.H., Opaluwa, O.D., Mohammad, R.E.A., Okiemute, O., Suleiman, S.B., Jagaba, A.H., 2024. Influence of adsorption parameters on phenolic compounds removal from aqueous solutions: a mini-review. Desalin. Water Treat. 320, 100631. https://doi.org/10.1016/j.dwt.2024.100631.
Maryskova, M., Vrsanska, M., Sevcu, A., Novotny, V., Blahutova, A., Vobercova, S., 2022. Laminated PAA nanofibers as a practical support for crude laccase: a new perspective for biocatalytic treatment of micropollutants in wastewaters. Environ. Tecnol. Inno. 26, 102316. https://doi.org/10.1016/j.eti.2022.102316.
Modaressi, K., Taylor, K.E., Betra, J.K., Biswas, N., 2005. Laccase-catalyzed removal of bisphenol-A from water: protective effect of PEG on enzyme activity. Water Res 39 (18), 4309–4316. https://doi.org/10.1016/j.watres.2005.08.005.
Naghdi, M., Taheran, M., Brar, S.K., Kermanshahi-pour, A., Verma, M., Surampalli, R.Y., 2017. Immobilized laccase on oxygen functionalized nanobiocars through mineral acids treatment for removal of carbamazepine. Sci. Total Environ. 584-585, 393–401. https://doi.org/10.1016/j.scitotenv.2017.01.021.
Nicolucci, C., Rossi, Menale, C., Godjevargova, T., Ivanov, Y., Bianco, M., Mita, L., Bencivenga, U., Mita, D.G., Diano, N., 2011. Biodegradation of bisphenols with immobilized laccase or tyrosinase on polyacrylonitrilo beads. Biodeg 22, 673–683. https://doi.org/10.1007/s10532-010-9440-2.
Ortega, N., Sáez, L., Palacios, D., Busto, M.D., 2022. Kinetic modeling, thermodynamic approach and molecular dynamics simulation of thermal inactivation of lipases from *Burkholderia cepacia* and *Rizomucor miehei*. Int. J. Mol. Sci. 23, 6828. https://doi.org/10.3390/ijms23126828.
Özdemir, F., Yalçinkaya, Z., 2023. Examination of the immobilization and kinetics of the laccase enzyme on various clay minerals. Mas. Jpn. 8 (2), 286–306. https://doi.org/10.5281/zenodo.7956783.
Prabhakar, T., Giaretta, J., Zulli, R., Rath, R.J., Farajikhah, S., Talebian, S., Dehghani, F., 2025. Covalent immobilization: a review from an enzyme perspective. Chem. Eng. J. 503, 158054. https://doi.org/10.1016/j.cej.2024.158054.
Rodriguez-Abetxuco, A., Sánchez-de Alcázar, D., Muñumer, P., Beloqui, A., 2020. Tunable polymeric scaffolds for enzyme immobilization. Front Bioeng. Biotechnol. 8, 830. https://doi.org/10.3389/fbioe.2020.00830.
Rodríguez-Couto, S., 2023. Immobilized-laccase bioreactors for wastewater treatment. Biotechnol. J. 19, 2300354. https://doi.org/10.1002/biot.202300354.
Rostami, A., Abdelrasoul, A., Shokri, Z., Shirvandi, Z., 2022. Applications and mechanisms of free and immobilized laccase in detoxification of phenolic compounds-a review. Korean J. Chem. Eng. 39, 821–832.
Rybarczyk, A., Smulek, W., Grzywaczyk, A., Kaczorek, E., Jesionowski, T., Nghiem, L.D., Zdarta, J., 2023. 3D printed polylactide scaffolding for laccase immobilization to improve enzyme stability and estrogen removal from wastewater. Bioresour. Technol. 381, 129144. https://doi.org/10.1016/j.biotechh.2023.129144.
Sadeghzadeh, S., Nejad, Z.G., Ghasemi, Sh, Khafaji, M., Borghei, S.M., 2020. Removal of bisphenol A in aqueous solution using magnetic cross-linked laccase aggregates from *Trametes hirsuta*. Bioresour. Technol. 306, 123169. https://doi.org/10.1016/j.biortech.2020.123169.
Sampaio, L.M.P., Padrao, J., Faria, J., Silva, J., Silva, C.J., Dourado, F., Zille, A., 2016. Laccase immobilization on bacterial nanocellulose membranes: antimicrobial, kinetic and stability properties. Carbohydr. Polym. 145, 1–12. https://doi.org/10.1016/j.carbpol.2016.03.009.
Sathishkumar, P., Kamal-Kaman, S., Cho, M., Kim, J.S., Hadibarata, T., Salim, M.R., Byun-Taek, O., 2014. Laccase immobilization on cellulose nanofiber: the catalytic efficiency and recycling application for simulated dye effluent treatment. J. Mol. Catal. B Enzym 100, 111–120. http://10.1016./j.molcatb.2013.12.008.
Sekretaryova, A., Jones, S.M., Solomon, E.I., 2019. O_2 Reduction to water by high potential multicopper oxidases: contribution of the T1 copper site potential and the local environment of the trinuclear copper cluster. J. Am. Chem. Soc. 141 (28), 11304–11314. https://doi.org/10.1021/jacs.9b05230.
Smith, E., Zhang, Q., Shen, J., Schroeder, M., Silva, C.J., 2009. Modification of esperase (R) by covalent bonding to Eudragit (R) polymers L100 and S100 for wool fiber surface treatment. Biocat. Biotrans. 26 (5), 391–398. https://doi.org/10.1080/10242420802249521.
Sodhi, A.S., Bhatia, S., Batra, N., 2024. Laccase: sustainable production strategies, heterologous expression and potential biotechnological applications. Int. J. Biol. Macromol. 280, 135745. https://doi.org/10.1016/j.ijbiomac.2024.135745.
Sun, H., Yuan, F., Jia, S., Zang, X., Xing, W., 2023. Laccase encapsulation immobilized in mesoporous ZIF-8 for enhancement bisphenol A degradation. J. Hazard. Mater. 445, 130460. http://10.1016/j.jhazmat.2022.13460.
Tian, M., Du, D., Zhou, W., Zeng, W., Cheng, G., 2017. Phenol degradation and genotypic analysis of dioxygenase genes in bacteria isolated from sediments. Braz. J. Microbiol. 48 (2), 305–313. https://doi.org/10.1016/j.bjm.2016.12.002.
Timur, S., Pazarlioğlu, N., Pilloton, R., Telefoncu, A., 2003. Thick film sensors based on laccases from different sources immobilized in polyaniline matriz. Sens. Actuators B Chem. 97 (1), 132–136. https://doi.org/10.1016/j.snb.2003.07.018.
Vallejo-García, J., Arnaiz, A., Busto, M.D., García, J.M., Vallejos, S., 2023. Film-shaped reusable smart polymer to produce lactose-free milk by simple immersion. Eur. Polym. J. 200, 112495. http://10.1016/j.europolymj.2023.112495.
Wang, Z., Ren, D., Jiang, Sh, Yu, H., Cheng, Y., Zhang, Sh, Zhang, X., Chen, W., 2021. The study of laccase immobilization optimization and stability improvement on CTAB-KOH modified biochar. BMC Biotechnol. 21, 47. http://10.1186/s12896-021-00709-3.
Wu, Z., Glaser, R., 2004. Ab initio study of the S_N1Ar and S_N2Ar reactions of benzenediazonium ion with water. On the conception of unimolecular dediazoniation in solvolysis reactions. J. Am. Chem. Soc. 126 (34), 10632–10639. http://10.1021/ja047620a.
Xia, T., Liu, C., Hu, J., Guo, C., 2016. Improved performance on immobilized laccase on amine-functioned magnetic Fe_3O_4 nanoparticles modified with polyethylenimine. Chem. Eng. J. 295, 201–206. http://10.1016/j.cej.2016.03.044.
Xing, H., Zhang, J., Tan, L., Li, R., Chen, J., Lan, J., Zhang, J., Liu, X., Li, Z., 2022. Degradation of bisphenol A enhanced by dialysis membrane enclosed laccase catalysis. Environ. Technol. Inno. 27, 102435. http://10.1016/j.eti/2022.102435.
Xu, F., 1997. Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. J. Biol. Chem. 272 (2), 924–928. http://10.1074/jbc.272.2.924.
Xu, X., Feng, L., Han, Z., Luo, S., Wu, A., Xie, J., 2016. Selection of high laccase-production *Coriolopsis gallica* strain T906: mutation breeding strain characterization, and features of the extracellular laccases. J. Microbiol. Biotechnol. 26, 1570–1578. http://10.4014/jmb.1604.04011.
Yang, X., Shi, F., Su, X., Cavaco-Paulo, A., Wang, H., Su, J., 2023. In-situ encapsulation and construction of Lac@HOFs/hidrogel composite for enhancing laccase stability and azo dyes decolorization efficiency. Carbohydr. Polym. 320, 121157. http://10.1016/j.carbpol.2023.121157.
Yu, H., Feng, L., Abbas, M., Liang, X., Zhang, T., Yang, G., Liu, Y., Xu, M., An, Y., Yang, W., 2024. Enhancing enzymatic catalysis efficiency: Immobilizing laccase on HHSS for synergistic bisphenol A adsorption and biodegradation through optimized external surface utilization. Int. J. Biol. Macromol. 278, 134586. http://10.1016/j.ijbiomac.2024.134586.
Zayed, M.E.M., Obaid, A.Y., Almulaiky, Y.Q., El-Shishtawy, R.M., 2024. Enhancing the sustainable immobilization of lactase by amino-functionalized PMMA-reinforced graphene nanomaterials. J. Environ. Manag. 351, 119503. http://10.1016/j.jenvman.2023.119503.
Zdarta, J., Jankowska, K., Strybel, U., Marczak, L., Nguyen, L.N., Oleskowicz-Popiel, P., Jesionowski, T., 2022. Bioremoval of estrogens by laccase immobilized onto polyacrylonitrile/polyethersulfone material: effect of inhibitors and mediators, process characterization and catalytic pathways determination. J. Hazard. Mat. 432, 128688. http://10.1016/j.jhazmat.2022.128688.
Zhao, D., Leth, M.L., Hachem, M.A., Aziz, I., Jančič, N., Luxbacher, Th, Hélix-Nielsen, C., Zhang, W., 2023. Facile fabrication of flexible ceramic nanofibrous membranes for enzyme immobilization and transformation of emerging pollutants. Chem. Eng. J. 451, 138902. http://10.1016/j.cej.2022.138902.
Zhou, J., 2010. Immobilization of cellulase on a reversibly soluble-insoluble support: properties and application. J. Agric. Food Chem. 58 (11), 6741–6746. https://doi.org/10.1021/jf100759c.
Zhu, Y., Qiu, F., Rong, J., Zhang, T., Mao, K., Yang, D., 2020. Covalent laccase immobilization on the surface of poly(vinylidene fluoride) polymer membrane for enhanced biocatalytic removal of dyes pollutants from aqueous environment. Colloids Surf. B Biointerfaces 191, 111025. https://doi.org/10.1016/j.colsurfb.2020.111025.
Zou, M., Tian, W., Chu, M., Lu, Zh, Liu, B., Xu, D., 2023. Magnetically separable laccase-biochar composite enable highly efficient adsorption-degradation of quinolone antibiotics: Immobilization, removal performance and mechanisms. Sci. Total Environ. 879, 163057. https://doi.org/10.1016/j.scitotenv.2023.163057.