

Research Article

Immobilization of laccase on Cu-MOF-alginate composite for degradation of BHPF: Characterization, optimization, and application

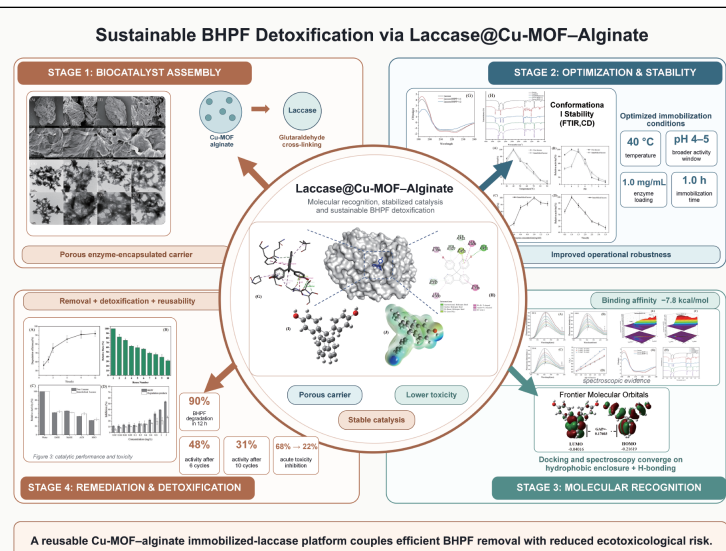
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Abstract: Growing food-safety and environmental concerns associated with fluorene-9-bisphenol (BHPF) highlight the need for sustainable remediation strategies. Herein, we report a rationally designed Cu-MOF-alginate composite for efficient laccase immobilization via glutaraldehyde cross-linking, yielding markedly enhanced operational stability and biodegradability. Comprehensive morphological characterization revealed a porous, enzyme-encapsulated architecture. Systematic optimization identified the optimal immobilization conditions as 40 °C, pH 4–5, 1.0 mg/mL enzyme loading, and 1.0 h immobilization duration. Laccase@Cu-MOF-alginate composite achieved 90% BHPF degradation within 12 h, with kinetic profiles indicating substrate-dependent turnover. Multi-spectroscopic analysis and molecular docking were used to characterize the interactions between BHPF and laccase and to identify the proposed binding mode. Notably, acute toxicity assays using *Photobacterium phosphoreum* demonstrated substantial detoxification of degradation intermediates (inhibition reduced from 68% to 22%). Immobilized laccase retained 48% of its initial activity after six cycles and 31% after ten cycles. These findings establish the Cu-MOF-alginate platform as a sustainable strategy for laccase immobilization, with practical advantages in reusability, operational stability, and detoxification of BHPF.



Keywords: laccase@Cu-MOF-alginate; degradation; composite immobilized; fluorene-9-bisphenol; molecular docking

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1 Introduction

Endocrine-disrupting chemicals (EDCs) pose significant threats to both human health and the environment^[1]. In humans, EDCs have been linked to conditions such as diabetes, obesity, infertility, thyroid dysfunction, and neurological disorders^[2]. In the environment, EDCs contaminate aquatic ecosystems and wildlife, disrupting the endocrine systems of aquatic organisms and leading to population declines. Their persistence in the environment and

ability to bioaccumulate in adipose tissue further exacerbate their long-term impacts^[3]. Fluorene-9-bisphenol, also known as 9,9-bis(4-hydroxyphenyl)-fluorene or 4,4'-(9-fluorenylidene) diphenol (BHPF), is a fluorene derivative with excellent thermal stability^[4]. Notably, BHPF-containing materials are extensively used in the electronics, aerospace, and automotive industries for manufacturing products such as protective coatings, semiconductor encapsulations, composite matrices, molded products, insulation materials, solder-resistant materials, epoxy

floor coatings, and structural adhesives^[5]. However, its potential use in food-contact materials, such as milk bottles, children's bottles, and sippy cups, and the extent of human exposure to BHPF remain unclear. Several studies have reported the toxicity of BHPF, particularly its interaction with estrogen receptors, acting as a xenoestrogen^[6]. Notably, biodegradation techniques, such as enzyme-based catalysis and microbial degradation, offer promising solutions for the remediation of BHPF and other persistent organic pollutants, providing a sustainable alternative to conventional chemical treatments.

Laccase (EC 1.10.3.2), a copper-containing polyphenol oxidase primarily sourced from prokaryotes and fungi, has garnered significant attention in biotechnological applications due to its ability to oxidize a wide range of organic pollutants, including phenols, polyphenols, and azo dyes^[7]. This enzyme is particularly valuable for environmental remediation, as it can effectively degrade pollutants such as BPA and other persistent organic compounds, offering a sustainable alternative to traditional chemical treatments^[8-10]. However, free laccase exhibits limitations in stability, recyclability, and operational robustness, which hinder its large-scale industrial application^[11,12]. To address these challenges, enzyme immobilization techniques have been widely explored. Immobilizing laccase on porous supports not only enhances its stability and reusability but also combines biodegradation with adsorption, thereby improving the efficiency of pollutant removal^[13]. This approach has proven effective in degrading a variety of pollutants, including microplastics and pharmaceuticals, making laccase-based biodegradation a promising and environmentally friendly method for addressing environmental contamination.

Recent studies have increasingly explored the use of micro/nanomaterials for laccase immobilization, with metal-organic frameworks (MOFs) emerging as the most promising candidates due to their exceptional physicochemical properties^[14]. MOFs are a novel class of porous crystalline materials synthesized through the self-assembly of metal ions and organic bridging ligands, resulting in highly ordered, periodic porous structures^[15,16]. These materials are distinguished by their remarkable specific surface areas, which can reach up to 7,000 m²/g, along with tunable pore sizes and exceptional modifiability^[17,18]. The diverse range of MOFs developed to date has demonstrated their inherent advantages, including high thermal stability and adjustable pore properties, making them versatile platforms for various applications^[19]. Notably, laccase immobilization onto MOFs can be achieved through multiple strategies, such as physical adsorption, covalent binding, self-assembly, and co-precipitation^[20]. These approaches not only provide robust support for laccase molecules but also enhance the stability and applicability of the immobilized enzyme. Additionally, MOFs serve as efficient adsorbents for pollutants, further expanding their utility in environmental remediation and biocatalysis. This integration of MOF properties with laccase immobilization strategies underscores their significant potential for advancing biotechnological applications.

In this study, a Cu-MOF-alginate composite prepared by a solvothermal method was used to immobilize laccase through glutaraldehyde-mediated cross-linking. Laccase@Cu-MOF-alginate was subsequently applied for the removal of BHPF. The resulting laccase@Cu-MOF-alginate composite was characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Furthermore, the effects of temperature, pH, enzyme concentration, and immobilization time on laccase activity were systematically evaluated. The stability of free and

immobilized laccase in various organic solvents was also assessed. Additionally, the interaction between laccase and BHPF was investigated using multispectroscopic analysis and molecular docking. Finally, the efficiency of immobilized laccase in degrading BHPF was quantified, and the activity of the reused laccase@Cu-MOF-alginate was evaluated. Through this integrated approach, we aim to provide both a mechanistic understanding of laccase-BHPF recognition and a scalable platform for the remediation of emerging BHPF pollutants.

2 Materials and methods

2.1 Materials

Laccase and ABTS were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Copper nitrate trihydrate, sodium alginate, 1,3,5-benzenetricarboxylic acid, glutaraldehyde, and sodium acetate/acetic acid reagents were obtained from Shanghai Goybio Industries Co., Ltd. (Shanghai, China). All of the other materials were of analytical grade.

2.2 Preparation of laccase@Cu-MOF-alginate composites

The laccase@Cu-MOF-alginate composites were prepared according to previously reported method with some modifications^[21]. Hydrogel material was prepared by adding sodium alginate solution (10 mg/mL) dropwise to copper nitrate trihydrate solution (30 mg/mL), followed by stirring for 18 h. Material was then left overnight and washed twice with water and twice with ethanol to remove excess Cu²⁺. The resulting Cu²⁺-cross-linked hydrogel material were transferred to 15 mL of 1,3,5-benzenetricarboxylic acid (TMA) solution in ethanol (30 mg/mL) and incubated at 85 °C for 10 h to promote MOF growth. The obtained Cu-MOF-alginate composites were washed three times with ethanol and collected by filtration. Prior to enzyme immobilization, glutaraldehyde was dissolved by heating and stirring at 90 °C to obtain a 120 mg/mL solution. The Cu-MOF-alginate carrier was added to a 5% (v/v) glutaraldehyde solution and stirred for 10 h. After cross-linking, the carrier was washed with deionized water until neutral. Activated carrier was then incubated with laccase solution (5 mg/mL) in a thermostatic shaker at 37 °C for 4 h. The supernatant was collected and stored at 4 °C for subsequent determination of enzyme loading. The solid composite was washed with deionized water to remove residual glutaraldehyde and unbound enzyme, collected by suction filtration.

2.3 Morphological characterization

TEM patterns of the MOF-alginate composites were obtained via TEM (T-120, Philips, Netherlands). SEM images were collected on a scanning electron microscope (S-4800, Hitachi Limited, Japan). Samples were dehydrated, critical-point dried, frozen in liquid nitrogen, and coated with a 5 nm platinum layer before imaging.

2.4 Conformational characterization

Fourier-transform infrared (FTIR) spectra were recorded using a 670-IR + 610-IR spectrometer. The specific experimental conditions are as follows: Freeze-dry the immobilized laccase samples for 24 h in advance, grind them with an agate mortar and mix them fully, and scan them with the reflection method. For each spectral measurement, the data of pure and immobilized

laccase were collected under identical conditions from 4,000 to 400 cm^{-1} at room temperature.

The CD spectrum of the pure and immobilized laccase (1×10^{-6} mol/L) was scanned using a J-810CD spectropolarimeter (JASCO Company, Japan). The scan speed was 60 nm min^{-1} , the response time was 1 s, and each sample was scanned three times in a quartz cuvette with a diameter of 1 cm. CD data were expressed as mean residue ellipticity (MRE, $\text{deg cm}^2 \text{dmol}^{-1}$) and analyzed using K2D software. The α -helical and β -sheet percent contents of the proteins were calculated using the CD from 190 to 250 nm as extracted from the CD spectra.

2.5 Free and immobilized enzymatic properties assessment

The activity of free and immobilized laccase was assayed according to the previous method^[22]. Generally, the activity of the free and immobilized laccase was determined spectrophotometrically in a reaction medium containing ABTS at a final concentration of 1.0 mmol/L as substrate in buffer solution at 30 °C for 5 min. The UV-2000 spectrophotometer (Shanghai, China) measured the increase in the absorbance of the oxidation product of ABTS by laccase, ABTS^+ , at 450 nm during the process. The calculation formula of immobilized laccase activity is as follows:

$$\text{Immobilized laccase activity (U/g)} = (\Delta A \times V_{\text{total}} \times 10^3) / (t \times m \times \epsilon)$$

where, ΔA -absorbance change within reaction time; t -reaction time, unit: min; V_{total} -total volume of solution, unit: mL; m -mass of immobilized laccase added, unit: g; ϵ -molar extinction coefficient of ABTS^+ , unit: $360 \text{ M}^{-1} \text{cm}^{-1}$.

The effects of pH (3.0–9.0), temperature (30–90 °C), laccase concentration (1–7 mg/mL), and immobilization time (0.5–3.5 h) on enzyme activity were evaluated. Effects of temperature and pH conditions on free and immobilized laccase activity were determined as the relative activity under a variety of pH and temperature.

2.6 Percentage immobilization of laccase

The equation for the percentage immobilization of the immobilized laccase is calculated according to the formula:

$$\text{Percentage immobilization} = (V_{\text{total}} - V_{\text{sus}}) / (V_{\text{total}}) \times 100\%$$

where, V_{total} = activity of free enzyme used for immobilization (U); V_{sus} = supernatant enzyme activity (U).

2.7 Assessment of organic solvent stability

To evaluate the stability of free and immobilized laccase in organic solvents, a specified amount of free laccase and laccase@Cu-MOF-alginate was immersed in ultrapure water, anhydrous ethanol, methanol, acetonitrile, and petroleum ether for 30 min^[23]. The enzyme activities of free and immobilized laccase in ultrapure water were designated as 100%, and residual activity was calculated accordingly. Each solvent was tested in triplicate to ensure reliability.

2.8 Reusability experiments

To evaluate the reusability of laccase@Cu-MOF-alginate, experiments were conducted under optimal conditions^[11]. Specifically, 100 mg of immobilized laccase was immersed in

50 mg/L BHPF solution (pH 5.0). Degradation was carried out at 30 °C, 120 r/min for 15 min, the laccase@Cu-MOF-alginate was retrieved and rinsed. Initial enzyme activity was set as 100%, and the activity of the immobilized laccase was measured after degradation cycles.

2.9 Removal of BHPF by laccase@Cu-MOF-alginate

The remaining concentration of BHPF in the treated samples was quantified by a high-performance liquid chromatography system (HPLC, Agilent, USA), equipped with a fluorescence detector (Shimadzu LC-10 AT, Shimadzu, Tokyo, Japan). Prior to analysis, the quenched samples were filtered through 0.2 μm polyvinylidene fluoride membrane filters. A detection wavelength of 274 nm was used. The retention time of BHPF and the injection volume were 3.80 min and 20 μL , respectively. The mobile phase, with an isocratic elution rate of 1 mL/min, consisted of acetonitrile and deionized water (70:30, v/v). Using the peak area and the slope of an external calibration curve, the removal rate and extent of BHPF in the presence and the absence of RL were determined.

2.10 Acute toxicity assay

The acute toxicity of the BHPF degradation products generated by laccase@Cu-MOF-alginate was evaluated using *Photobacterium phosphoreum*. Luminescence intensity was measured using a photodetector (Model DXY-3, China). Bacterial cultivation and toxicity assessment procedures are described in our previous research. All acute toxicity assays were performed in triplicate.

2.11 Multi-spectroscopic measurements

Fluorescence spectra were recorded using an F-320 fluorescence spectrophotometer according to a previously reported method^[24]. The excitation wavelength was set to 280 nm, and emission spectra were collected from 280 to 500 nm at different temperatures. Three-dimensional (3D) fluorescence measurements and FTIR of free laccase and BHPF-laccase complex were recorded at room temperature. The 3D spectrum of the emission wavelength in the range of 250–500 nm was measured at excitation wavelength of 280 nm.

2.12 Density functional theory (DFT) calculation

An *ab initio* method was used to optimize the molecules^[25]. Geometry optimization and molecular orbital calculations were performed using Gaussian 16 and GaussView 6 at the B3LYP/6-311G(d) level. The highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), and electrostatic potential (ESP) maps were calculated from the optimized geometry.

2.13 Molecular docking

The structures of laccase-BHPF complexes were predicted by molecular docking. The crystalline structure of the laccase (PDB ID: 9RCK, laccase/multicopper oxidase from *Pediococcus pentosaceus* 4618) was downloaded from the RCSB PDB database^[26]. The possible conformations of the complex were generated using the Lamarckian genetic algorithm (LGA) method. Potential binding sites and binding energies were investigated using AutoDockTools 1.5.6 by setting the grid size to 70, 70, and 70 along the X, Y, and Z axes with a grid spacing of 0.375 Å. The conformation with the lowest binding energy was selected and visualized using PyMOL.

2.14 Statistical analysis

All experiments were performed in triplicate ($n = 3$), and results are expressed as mean $\pm$ SD. Statistical comparisons were performed using one-way ANOVA with Tukey's post-hoc test for multiple-group comparisons, and student's *t*-test for two-group comparisons. Significance was set at $P < 0.05$.

3 Results and discussion

3.1 Characterization of immobilized laccase-Cu-MOF material

As shown in Figs. 1 (A–H), the morphology of the Cu-MOF-alginate composite and the immobilized laccase-Cu-MOF-alginate material was characterized by SEM and TEM. Cu-MOF-alginate composite material exhibited a compact and complex fibrillar morphology, which is favorable for substrate contact and fixation. Following immobilization, the surface of the laccase@Cu-MOF-alginate material appeared rough and dense, which formed through intermolecular interactions. Such surface roughness increases the specific surface area available for enzyme-substrate interactions, potentially enhancing the overall degradation efficiency of the immobilized system. Additionally, the formation of enzyme aggregates may facilitate cooperative effects among

adjacent laccase molecules, contributing to sustained enzymatic activity. These morphological features suggest successful immobilization of laccase onto the Cu-MOF-alginate surface *via* covalent coupling.

TEM analysis further revealed structural differences between the Cu-MOF-alginate composite and the immobilized laccase-Cu-MOF-alginate material. The immobilized laccase-Cu-MOF material displayed a higher pore density and a more compact architecture compared to the Cu-MOF-alginate composite material. These structural enhancements are attributed to the crosslinking effect of glutaraldehyde on the carrier matrix, which facilitated efficient immobilization of laccase. The increased pore density likely improves mass transfer of substrates and products within the composite material, thereby reducing diffusion limitations that often hinder immobilized enzyme performance. Furthermore, the compact architecture may provide enhanced mechanical stability, enabling the immobilized biocatalyst to withstand repeated handling and prolonged operation. Collectively, the SEM and TEM observations indicate effective covalent binding between laccase and the Cu-MOF support.

3.2 Optimization of immobilization conditions

The catalytic performance of immobilized enzymes is governed by the interplay between intrinsic enzymatic properties and

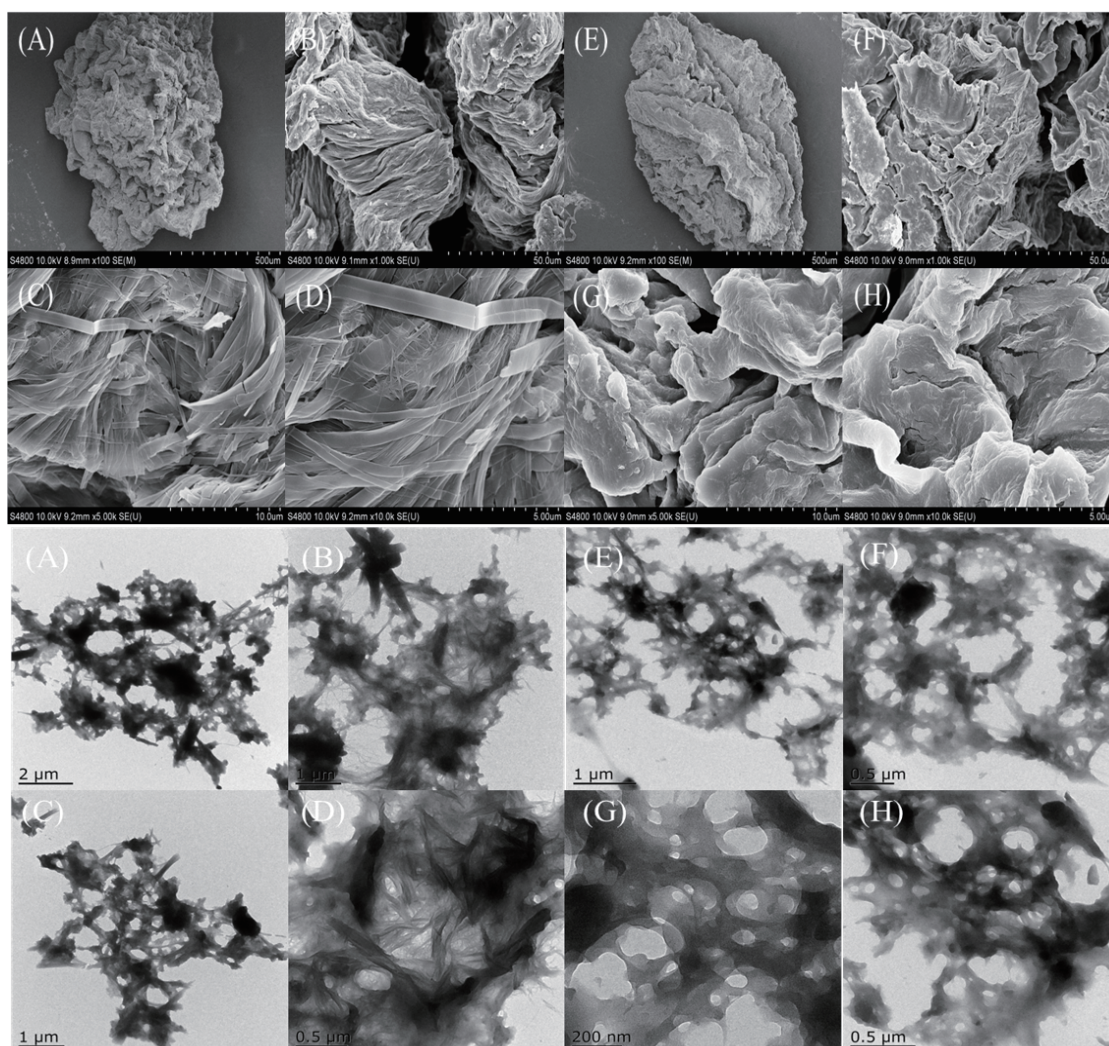


Figure 1 Morphological characterization of Cu-MOF-alginate and laccase@Cu-MOF-alginate composites. Upper panel: SEM images of Cu-MOF-alginate (A–D) and laccase@Cu-MOF-alginate (E–H). Lower panel: TEM images of Cu-MOF-alginate (A–D) and laccase@Cu-MOF-alginate (E–H).

extrinsic matrix constraints^[27]. A systematic evaluation of temperature, pH, enzyme loading, and immobilization duration was conducted to delineate the operational window of the laccase@Cu-MOF-alginate composite^[28].

3.2.1 Temperature dependence

The thermal profiles of free and immobilized laccase are presented in Fig. 2A. Both preparations exhibited optimal activity at 40 °C, with the immobilized enzyme retaining comparable catalytic efficiency across the entire temperature range. The similar thermal profiles of free and immobilized laccase suggest that the Cu-MOF-alginate matrix did not impose substantial diffusional limitations or steric hindrance under the tested conditions. At suboptimal temperatures, both enzyme forms displayed diminished activity (65%), reflecting the reduced kinetic energy available for substrate-enzyme collisions. Beyond 40 °C, thermal denaturation precipitated a precipitous decline, with residual activity falling below 10% at 90 °C. The absence of substantial thermal stabilization *via* immobilization suggests that the alginate hydrogel and Cu-MOF components do not confer additional rigidity to the tertiary structure of enzyme.

3.2.2 pH sensitivity

Fig. 2B illustrates the pH-activity relationships of free and immobilized laccase. The free enzyme exhibited a sharp optimum at pH 4, with activity plummeting markedly under alkaline conditions, and retaining merely 12% activity at pH 9. In contrast, the immobilized preparation displayed a broader, albeit slightly shifted, pH optimum spanning pH 4–5 and, critically, maintained superior activity in the neutral to mildly alkaline range. At pH 7, the immobilized laccase retained approximately 60% of maximal

activity versus 20% for the free enzyme. This attenuated pH sensitivity is attributable to the buffering capacity of the alginate matrix and the proton-donating or -withdrawing microenvironment established by the Cu-MOF surface functionalities. Such expansion of the operational pH window is particularly advantageous for practical applications, where wastewater pH often fluctuates beyond the narrow acidic range optimal for free fungal laccases. This enhanced robustness underscores the practical viability of the developed composite for environmental remediation.

3.2.3 Enzyme loading optimization

The influence of laccase concentration during immobilization on activity recovery is depicted in Fig. 2C. Activity increased sigmoidally with enzyme loading, plateauing at 1.0 mg/mL with near-complete activity recovery. This saturation profile indicates that the Cu-MOF-alginate composite possesses finite binding sites, including coordination sites on Cu-MOF nodes and carboxyl moieties within the alginate polymer. The steep ascent between 0.4 and 0.8 mg/mL suggests cooperative binding or matrix swelling effects that enhance enzyme accessibility. Notably, the marginal decline observed at 1.2–1.4 mg/mL may reflect protein-protein interactions or aggregation, which induced steric congestion at excessive loading densities. This trade-off between enzyme loading and activity recovery highlights the importance of optimizing immobilization stoichiometry to balance catalytic output with material economy.

3.2.4 Immobilization duration

Fig. 2D presents the time-dependent evolution of immobilized laccase activity. Maximal activity was attained after 1.0 h, with a

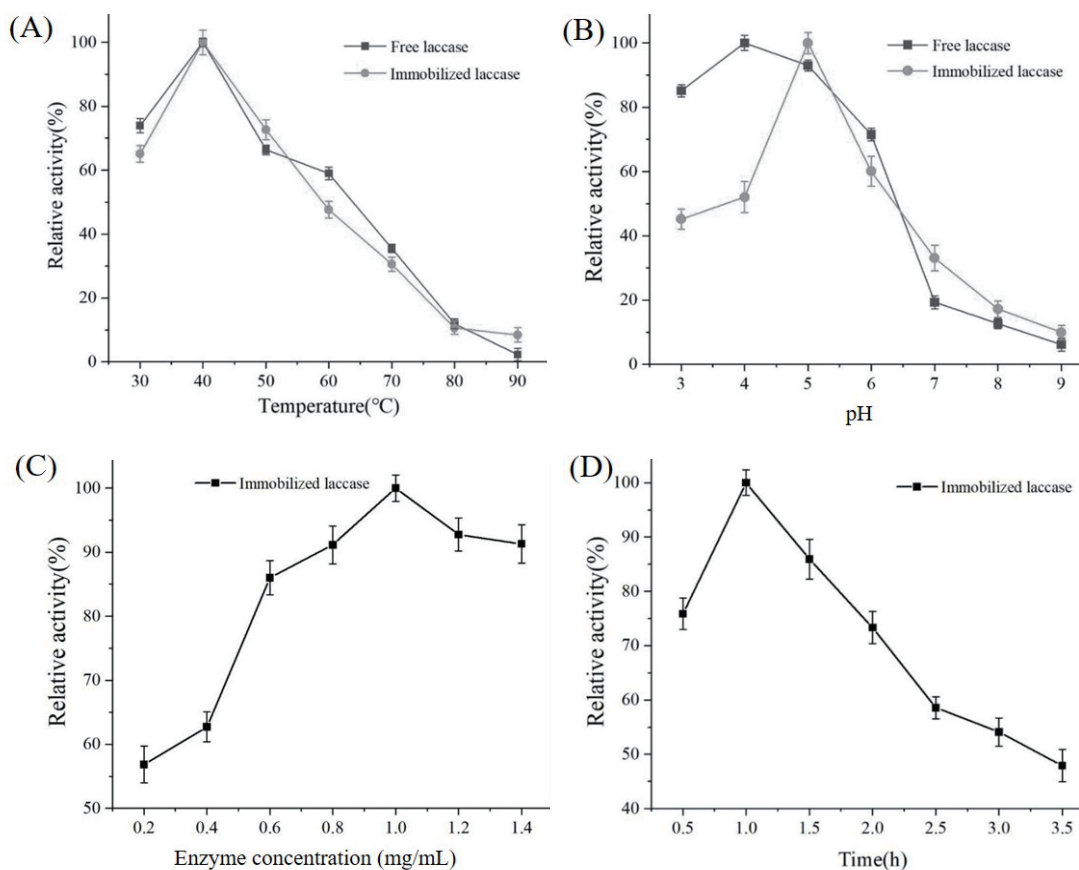


Figure 2 Effect of different factors on the enzymatic activity of free and immobilized laccase. (A) Temperature. (B) pH. (C) Enzyme concentration. (D) Time.

pronounced decline evident at extended durations. The initial ascending phase corresponds to progressive enzyme diffusion into the porous Cu-MOF-alginate network and the establishment of stabilizing electrostatic and hydrophobic interactions. The subsequent deterioration beyond 1.0 h likely stems from enzyme denaturation under prolonged exposure to cross-linking agents or physical shear forces during the immobilization procedure. These temporal optimum underscores the need to control the immobilization time precisely to prevent over-processing, a factor that is often overlooked in batch immobilization protocols. Optimizing this window is essential for achieving high enzyme loading while preserving the native catalytic conformation. These results suggest that immobilization performance depends on a balance between enzyme loading and catalytic accessibility. Excessive loading or prolonged immobilization may reduce activity by increasing steric crowding or conformational restriction.

3.3 Degradation performance and stability evaluation

The practical utility of the laccase@Cu-MOF-alginate composite was assessed through degradation kinetics, reusability, solvent tolerance, and ecotoxicity of transformation products.

3.3.1 Degradation kinetics

The time-dependent degradation of BHPF by the immobilized laccase is presented in Fig. 3A. The degradation efficiency increased progressively with reaction time, reaching equilibrium at approximately 12 h, with a final removal rate of 90%. The initial rapid phase within the first 6 h likely reflects the high substrate

availability and unrestricted access to the active sites of surface-immobilized enzyme molecules. The subsequent plateau suggests either substrate depletion or the establishment of a steady-state equilibrium between adsorption and degradation. Notably, the absence of a lag phase indicates that the immobilization procedure preserved the catalytic integrity of laccase without imposing diffusional barriers that typically delay substrate access in heterogeneous biocatalytic systems. This kinetic profile is consistent with our previous observations on $\text{Fe}_3\text{O}_4@\text{Al}^{3+}$ -immobilized laccase for diethylstilbestrol degradation, where equilibrium was similarly reached within 12 h with a comparable removal efficiency^[28].

3.3.2 Reusability

The operational stability of the immobilized laccase was evaluated over ten consecutive degradation cycles (Fig. 3B). Relative activity declined progressively with increasing cycle number, retaining 48% of the initial activity after six cycles and 31% after ten cycles. This gradual decay is attributable to cumulative enzyme denaturation under repeated reaction conditions, as well as potential enzyme leaching from the Cu-MOF-alginate matrix during washing steps. Despite this decline, the residual activity after ten cycles remains comparable to that reported for NiFe_2O_4 -immobilized laccase (34% after eight cycles) and $\text{Fe}_3\text{O}_4@\text{Al}^{3+}$ -immobilized laccase (31% after ten cycles), suggesting that the Cu-MOF-alginate composite offers competitive operational stability among magnetic nanoparticle-based immobilization systems. The sustained activity over multiple cycles underscores the potential of this composite for

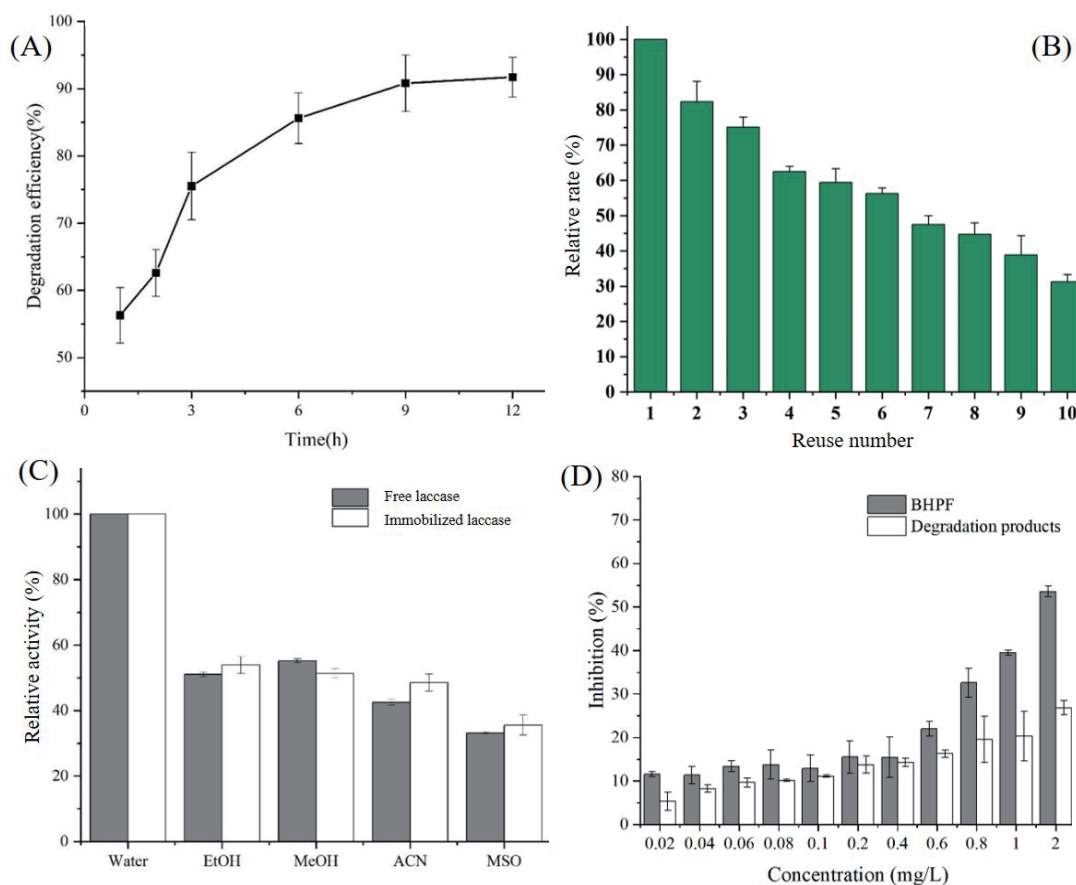


Figure 3 Catalytic performance and toxicity assessment of laccase@Cu-MOF-Alginate. (A) BHPF degradation kinetics. (B) Reusability over ten cycles. (C) Solvent tolerance of free and immobilized laccase. (D) Acute toxicity of BHPF and degradation products. Error bars indicate standard deviations ($n = 3$).

cost-effective, repeated use in continuous treatment processes. At the same time, the gradual activity loss indicates that matrix protection is incomplete during repeated operation. Enzyme leaching and structural changes may therefore remain important factors limiting long-term reuse.

3.3.3 Organic solvent tolerance

The stability of free and immobilized laccase in various organic solvents is illustrated in Fig. 3C. For both preparations, enzyme activity decreased across all tested organic solvents relative to the aqueous control, a phenomenon attributed to disruption of the essential hydration layer surrounding the enzyme molecule by polar organic solvents. Notably, the immobilized laccase consistently exhibited higher residual activity than the free enzyme in all organic solvent systems, with particularly pronounced differences in ethanol and methanol. This enhanced tolerance likely results from the protective microenvironment provided by the alginate hydrogel and the Cu-MOF matrix, which restricts direct solvent-enzyme contact and preserves the native conformation of the immobilized laccase. Such robustness is advantageous for applications involving organic co-solvents or the treatment of industrial effluents containing organic contaminants.

3.3.4 Acute toxicity of degradation products

The acute toxicity of BHPF and its degradation intermediates was assessed using the luminescent bacterium *Photobacterium phosphoreum* (Fig. 3D). The untreated BHPF solution exhibited significant toxicity, inhibiting approximately 68% of bacterial luminescence. Following degradation by laccase@Cu-MOF-alginate, the inhibition rate decreased markedly to 22% after 60 min, indicating substantial detoxification of the reaction mixture. This reduction in toxicity is consistent with the formation of less hazardous intermediates, as previously documented for laccase-mediated degradation of bisphenolic compounds. The observed detoxification pattern suggests that the immobilized laccase system not only removes the parent compound but also effectively mitigates the ecotoxicological risk associated with BHPF contamination. Importantly, the decrease in BHPF concentration was accompanied by a reduction in acute toxicity. This result highlights the value of evaluating detoxification together with removal efficiency, although direct identification of the degradation intermediates is still needed.

3.4 Spectroscopic investigation of laccase-BHPF interactions

The molecular recognition events underpinning laccase-mediated BHPF degradation were elucidated through multi-spectroscopic analyses, probing conformational dynamics, binding thermodynamics, and secondary structural perturbations^[29,30].

3.4.1 Fluorescence quenching mechanism and binding thermodynamics

As depicted in Figs. 4A–C, incremental addition of BHPF elicited concentration-dependent fluorescence attenuation at 300, 310, and 320 K, with red shifts of the emission maxima from 360 nm to approximately 371 nm. This red-shift suggests that BHPF binding alters the microenvironment surrounding tryptophan residues, which increasing local polarity and solvent exposure. Stern-Volmer analysis (Fig. 4D, Table 1) revealed linear relationships between relative fluorescence intensity and quencher concentration across all temperatures. Notably, the derived

Stern-Volmer constants exhibited direct temperature dependence—escalating from $9.114,8 \times 10^4$ to $5.336,5 \times 10^5$ L·mol⁻¹ as temperature ascended from 300 to 320 K. This thermodynamic signature indicates collisional encounters between BHPF and laccase, rather than pre-associative complex formation in the ground state. The calculated bimolecular quenching rate constants substantially exceed the maximum diffusion-controlled rate, further corroborating the dynamic nature of the quenching process.

Binding constants on the order of 10^6 L·mol⁻¹, with values diminishing from $6.354,7 \times 10^6$ to $2.930,2 \times 10^6$ L·mol⁻¹ with increasing temperature. The binding site numbers suggest multiple binding interactions between BHPF and laccase. The negative Gibbs free energy changes corroborate spontaneous association, with the temperature-dependent decrease in K_a implying enthalpically favorable binding dominated by hydrogen bonding and van der Waals interactions.

3.4.2 3D fluorescence spectroscopic fingerprints

3D fluorescence spectroscopy was employed to resolve spatially distinct fluorophore populations and their differential responses to BHPF binding. The 3D fluorescence fingerprints (Fig. 4E, molar ratio 1:0) exhibited two prominent peaks: Peak 1 ($\lambda_{em}/\lambda_{ex} \approx 280/340$ nm) attributable to tryptophan/tyrosine residues, and Peak 2 ($\lambda_{em}/\lambda_{ex} \approx 230/340$ nm) corresponding to polypeptide backbone fluorescence. Upon BHPF titration to 1:2 molar ratio (Fig. 4F), Peak 1 intensity diminished markedly with marginal spectral shifting, whereas Peak 2 underwent both intensity quenching and excitation wavelength perturbation. The differential quenching pattern indicated specific BHPF docking near the enzyme's hydrophobic pockets rather than nonspecific surface adsorption. The emergence of a weak, long-wavelength emission feature in the ligand-bound spectrum may reflect exciplex formation between BHPF and aromatic residues, indicative of intimate molecular proximity required for subsequent electron transfer.

3.4.3 Secondary structural study

Circular dichroism spectroscopy was utilized to quantify BHPF-induced conformational remodeling of laccase. The native laccase displayed characteristic dichroic bands: A broad negative extremum at 208 nm ($\pi \rightarrow \pi^*$ transition) and a shoulder at 222 nm ($n \rightarrow \pi^*$ transition), diagnostic of substantial α -helical content with appreciable β -sheet and random coil contributions. Progressive BHPF addition engendered dose-dependent ellipticity diminution across the 190–240 nm region (Fig. 4G), with the 1:2 complexes exhibiting reduced mean residue ellipticity at 208 nm relative to apo-laccase. Notably, the spectral contour retained its characteristic double-minimum shape, indicating that BHPF binding does not obliterate the native fold but rather modulates local dynamics essential for electron transfer to the T1 copper center. The observed structural plasticity aligns with the conformational selection mechanism proposed for laccase-substrate encounters, wherein pre-existing conformational substates are stabilized upon ligand binding.

3.4.4 FTIR analysis

FTIR spectroscopy provided complementary verification of immobilization-induced and substrate-binding-related structural modifications (Fig. 4H). For immobilized laccase@Cu-MOF-alginate, the amide I band (1,600–1,700 cm⁻¹), sensitive to protein backbone hydrogen bonding exhibited subtle narrowing relative

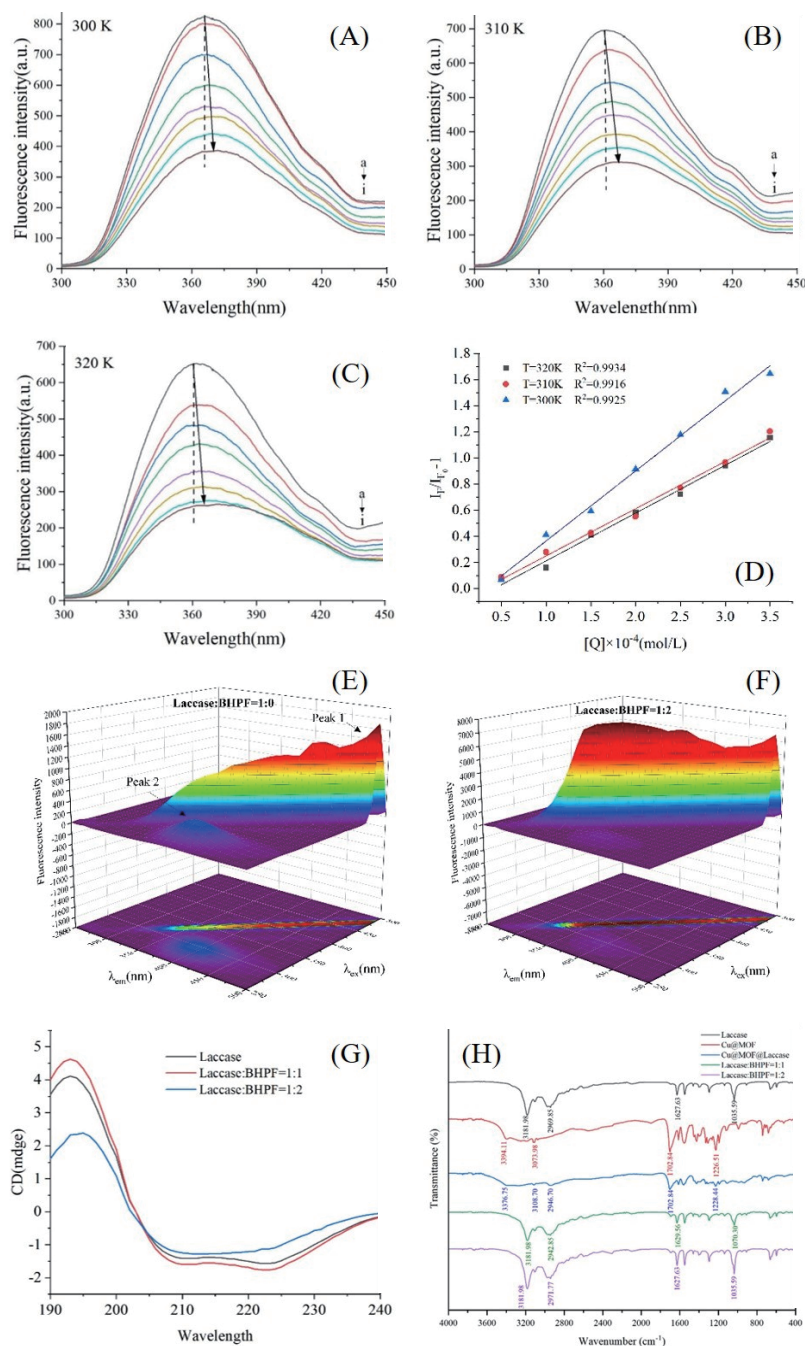


Figure 4 Multi-spectroscopic analysis of laccase-BHPF interactions. (A-C) fluorescence spectra of laccase in the presence of increasing BHPF concentrations at 300, 310, and 320 K, respectively. (D) Stern-Volmer plots with linear regression fits. (E) 3D fluorescence contour map of free laccase. (F) 3D fluorescence contour map of the laccase-BHPF complex. (G) CD spectra of free laccase and laccase-BHPF complexes; and (H) FTIR spectra of laccase, laccase@Cu-MOF-alginate, and the laccase-BHPF complex.

Table 1 Fluorescence quenching constant and linear correlation coefficient of BHPF and laccase complex

T/K	K_{sv}	K_q	R^2
300	$9.114,8 \times 10^4$	$9.114,8 \times 10^{12}$	0.995,3
310	$3.543,4 \times 10^5$	$3.543,4 \times 10^{13}$	0.992,5
320	$5.336,5 \times 10^5$	$5.336,5 \times 10^{13}$	0.993,2
T/K	$K_a(L \cdot mol^{-1})$	n	Fitting equation
300	$6.354,7 \times 10^6$	1.509,9	$y = 1.373,9x + 6.803,1$ (0.993,5)
310	$5.547,5 \times 10^6$	1.292,1	$y = 1.292,1x + 6.744,1$ (0.991,5)
320	$2.930,2 \times 10^6$	1.369,7	$y = 1.396,7x + 7.466,9$ (0.992,8)

to free laccase, indicative of constrained conformational freedom within the composite matrix. The amide II band ($\sim 1,540\text{ cm}^{-1}$) remained largely invariant, suggesting preservation of overall secondary structure content upon immobilization. In the laccase-BHPF complex spectrum, distinctive perturbations emerged in the $1,400\text{--}1,500\text{ cm}^{-1}$ region, which BHPF engagement at the enzyme active site *via* aromatic ring vibrations and C–N stretching modes. The emergence of absorption features near $1,230\text{ cm}^{-1}$ and $1,035\text{ cm}^{-1}$ corroborates substrate binding prior to oxidation. Furthermore, the O–H stretching region ($3,200\text{--}3,400\text{ cm}^{-1}$) displayed bandwidth reduction and frequency shifts, implying dehydration of the binding pocket and altered hydrogen bonding networks upon substrate entry. These spectral signatures collectively substantiate the formation of a well-defined enzyme-substrate complex.

3.5 Frontier molecular orbital (FMO) analysis

Density functional theory calculations were performed to assess the electronic structure prerequisites for BHPF oxidation by laccase. The HOMO of BHPF exhibited pronounced π -electron density over the phenolic rings (Fig. 5). The calculated HOMO energy and estimated ionization potential position BHPF as a kinetically accessible substrate for single-electron oxidation by the T1 Cu(II) center. The thermodynamic driving force corroborates the rapid initial degradation kinetics observed experimentally, while the absence of catalytic inhibition at elevated concentrations suggests that electron transfer is not rate-limiting under the investigated conditions. The spatial distribution of the LUMO across the fluorene backbone, with nodal planes intersecting the phenolic substituents, indicates that electron acceptance into this orbital would require substantial geometric reorganization. This orbital topology implies that initial electron abstraction occurs preferentially from the phenolic π -system, generating a phenoxy radical intermediate that subsequently delocalizes into the extended conjugated framework. The moderate HOMO-LUMO energy gap reflects the polarizability of the fluorene scaffold, which may facilitate charge stabilization during the catalytic cycle. These electronic structure features collectively rationalize the efficient turnover of BHPF by laccase, wherein the substrate's frontier orbital architecture complements the geometric and electronic demands of the active site.

3.6 Binding behavior study

To delineate the atomic-level recognition events, molecular

docking were performed, complemented by ESP surface mapping. Molecular docking of BHPF into the laccase substrate pocket converged upon a cluster of energetically favorable conformations, with the top-ranked pose exhibiting a binding affinity of -7.8 kcal/mol . As illustrated in Fig. 6, BHPF adopts an extended orientation within the hydrophobic cavity. The binding pocket surface, visualized through solvent-accessible surface representation, reveals a bipartite architecture: A deep hydrophobic cleft accommodating the central fluorene moiety, and a shallower polar rim engaging the terminal phenolic groups. This geometric complementarity rationalizes the substrate recognition ability of laccase toward biphenolic architectures while tolerating variable bridging substituents. Binding free energy decomposition dissected the molecular recognition forces into constituent contributions (Figs. 6A–F)^[31]. Van der Waals interactions dominated the association, with Phe239, Pro394, and Leu300 establishing extensive hydrophobic contacts with the fluorene rings. Hydrogen bonding, though modest in net energetic contribution, exhibited precise geometric constraints: Ser410 and Gly411 donate backbone amide protons to the phenolic oxygens, while Gln237 engages in hydrogen bonding with the opposing hydroxyl. These polar interactions likely enforce substrate orientation, restricting rotational freedom and pre-organizing the phenolic rings for optimal approach to the center. The MEP surface of BHPF (Fig. 6f) displays marked polarity asymmetry, particular negative potential over the phenolic regions and neutral-to-positive potential over the fluorene bridge. This dipolar distribution is consistent with the electrostatic environment of the laccase pocket, where the T1 copper vicinity is electron-deficient while the pocket periphery bears negative potential. Such complementarity may facilitate substrate steering into the binding cleft, followed by short-range hydrogen bonding and hydrophobic interactions. The positive potential near the copper may aid stabilization of the deprotonated phenolate, which could lower the barrier for oxidation, consistent with the observed pH optimum, as alkaline conditions would disfavor the protonated phenolic form required for these interactions. Together with the spectroscopic results, the docking analysis suggests that BHPF recognition involves both hydrophobic contacts and hydrogen bonding. These interactions may help maintain a favorable substrate orientation near the T1 copper region.

4 Conclusion

This study establishes Cu-MOF-alginate as a high-performance

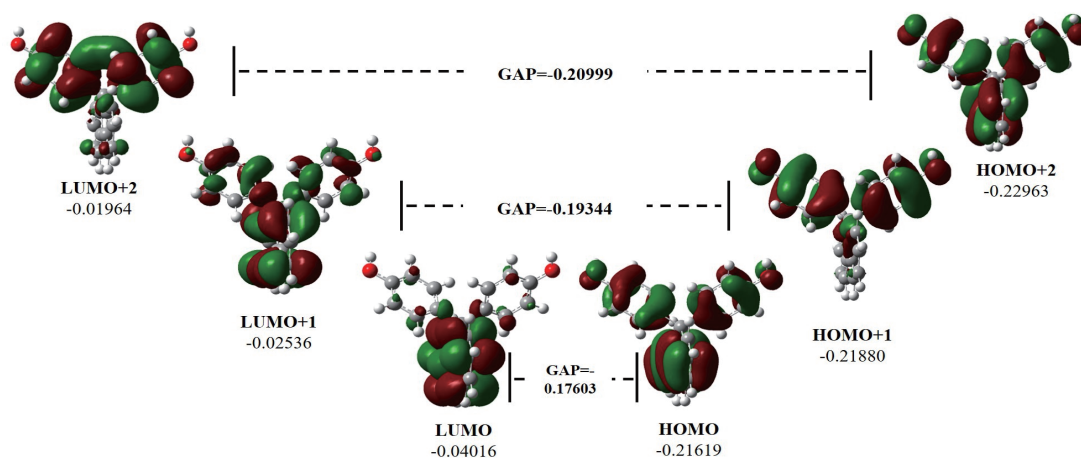


Figure 5 Frontier molecular orbital calculations of BHPF.

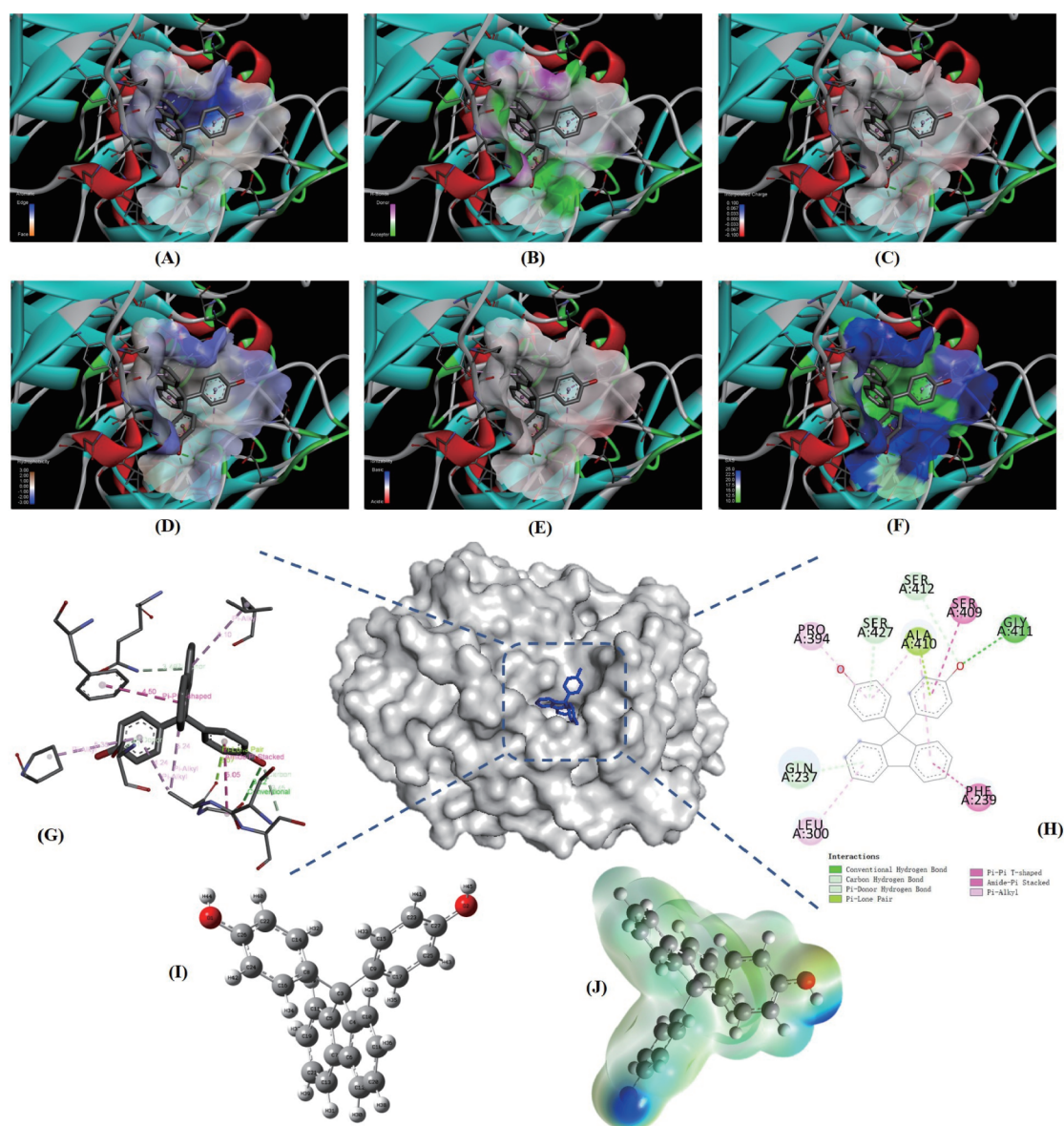


Figure 6 Molecular docking and electrostatic characterization of the laccase-BHPF complex. (A-F) Representative docking poses and interaction-surface visualizations. (G) 3D interaction map of the docked complex. (H) 2D interaction diagram. (I) optimized molecular structure of BHPF; and (J) ESP-mapped surface of BHPF.

carrier for laccase immobilization, addressing critical limitations of free enzyme deployment in environmental remediation. Morphology of Laccase@Cu-MOF-alginate indicated that porous matrix facilitates both enzyme stabilization and substrate accessibility. Optimization of immobilization parameters achieved 90% BHPF degradation within 12 h, which demonstrated the practical potential of the immobilized system. Multi-spectroscopic and computational analysis convergently demonstrate that BHPF engages the laccase pocket through a combination of hydrophobic enclosure. The marked reduction in the acute toxicity of the degradation products, together with the retained activity across ten reuse cycles, further supports the practical potential. The integration of MOF-derived materials offers a generalizable strategy for advancing sustainable enzymatic technologies in food and environmental biotechnology. We acknowledge the absence of real-matrix validation and quantitative leaching data. Future work will address these through optimized cross-linking and food-simulant testing, toward practical deployment of this enzymatic platform.

Data availability

All data needed to support the conclusions in the paper are presented in the manuscript. Additional data related to this paper may be requested from the corresponding author upon request.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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Author contribution statement

Tian-Zhu Guan: Investigation, formal analysis, visualization, data curation, writing – original draft. Ming-Yue Fu: Formal

analysis, writing – review & editing. Yi-Ning Feng: Resources, writing – review & editing. Wei-Guo Zong: Resources, writing – review & editing. Yu-Qing Wang: Formal analysis, writing – review & editing. Ayyoob Ujalaa: Formal analysis, writing – review & editing. Siddiki-Md-Robin Hossain: Conceptualization. Fu-Qing Wang: Supervision, writing – review & editing.

Informed consent

Not applicable.

Ethics statement

Not applicable.

Use of AI statement

None.

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