



Immobilization of laccase on grape seed-derived hydrochar and biochar as sustainable biocatalysts for efficient pharmaceutical degradation in real wastewater

Helena Sá^{a,*}, Michele Michelin^{a,b,**}, Teresa Tavares^{a,b}, Maria Angeles Sanroman^c, Emilio Rosales^c, Isabel C. Neves^{a,d}, Bruna Silva^{a,b}

^a CEB – Centre of Biological Engineering, University of Minho, Braga, 4710-057, Portugal

^b LABBELS – Associate Laboratory, Braga, Guimarães, Portugal

^c CINTECX - Grupo de Bioingeniería y Procesos Sostenibles, Departamento de Ingeniería Química, Universidade de Vigo, Vigo 36310, Spain

^d CQUM - Centre of Chemistry, University of Minho, Braga, 4710-057, Portugal

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ABSTRACT

Pharmaceutical active compounds pose a serious global threat due to their continuous release into aquatic systems. This study explores an innovative approach for the degradation of acetaminophen (APAP), sulfamethoxazole (SMX) and tetracycline (TC) by the covalent immobilization of crude laccase from *Lentinus sajor-caju* on grape seed-derived biochar (GS-BC-Lac) and hydrochar (GS-HC-Lac). Characterization through Scanning Electron Microscopy (SEM) and X-ray Photoelectron Spectroscopy (XPS) confirmed the successful covalent immobilization of laccase. The immobilized enzyme retained nearly 90 % of its activity after 30 days and exhibited high degradation efficiency toward pharmaceutical compounds. Complete removal of APAP was achieved in just 30 min using GS-HC-Lac and 90 min with GS-BC-Lac, both assisted by syringaldehyde (SYR) as a redox mediator at pH 5.0. Under the same conditions, SMX was efficiently degraded within 30 min, whereas TC required 5 h to reach comparable degradation levels. The biocatalysts maintained high SMX degradation efficiency over eight reuse cycles and demonstrated consistent performance even in wastewater matrices. Additionally, degradation pathways involving different transformation products were proposed, highlighting key oxidative and coupling reactions. Hence, covalently immobilized laccase on GS-BC and GS-HC proved to be efficient biocatalysts for the degradation of pharmaceuticals in wastewater, offering promising potential for environmental remediation and industrial-scale applications.

1. Introduction

Pharmaceutical active compounds (PhAC), recognized as significant emerging contaminants, have become a serious global problem due to their continuous release into aquatic systems [1]. Most PhAC present high persistence and bioaccumulation, low biodegradability into non-toxic byproducts, and potential toxicity even at very low concentrations [2]. Therefore, these biologically active compounds pose a threat to human and animal health, as well as to ecosystems and environmental sustainability [3,4]. The worldwide occurrence and distribution of these compounds have been reported in various environmental

matrices, including drinking water, wastewater, surface water, groundwater and sediments [5]. For example, the analgesic and antipyretic paracetamol, which is also known as acetaminophen (APAP), is one of the most frequently detected PhAC in wastewater at concentrations ranging from 100 to 300 $\mu\text{g}\cdot\text{L}^{-1}$ [6,7]. Likewise, antibiotics such as sulfamethoxazole (SMX) and tetracycline (TC), found in concentrations of $\text{ng}\cdot\text{L}^{-1}$ to $\mu\text{g}\cdot\text{L}^{-1}$, contribute to the development of antibiotic-resistant bacteria and present toxicity risks to aquatic organisms [8–10].

The removal of PhAC in conventional wastewater treatment plants (WWTP) is neither complete nor accurate, thus making the finding of alternative approaches mandatory. Current research has reported

* Corresponding author.

** Corresponding author at: CEB – Centre of Biological Engineering, University of Minho, Braga, 4710-057, Portugal.

E-mail addresses: helena.sa@ceb.uminho.pt (H. Sá), mimichelin@ceb.uminho.pt (M. Michelin), ttavares@deb.uminho.pt (T. Tavares), sanroman@uvigo.es (M.A. Sanroman), emiliiorv@uvigo.es (E. Rosales), ineves@quimica.uminho.pt (I.C. Neves), bsilva@ceb.uminho.pt (B. Silva).

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several strategies for remediating contaminants. Examples include physical processes (such as adsorption [11]), biological treatments (such as activated sludge and biotransformation [12,13]) and chemical processes (such as photocatalytic oxidation [14,15], catalytic ozone processes [16] and the Fenton-like process [17]).

Bioremediation, especially enzyme-based oxidation processes, has gained attention for its efficiency, biodegradability and high selectivity under mild conditions [18,19]. Fungal laccase is one of the most investigated biocatalysts. This oxidoreductase catalyzes the degradation of phenolic compounds, reducing oxygen to water and converting them into more bioavailable or less harmful metabolites [20,21]. However, treatment with a free form of laccase faces some limitations that result in its impractical use on a large scale, namely low thermal and pH stability, high costs and difficulty in recovery [22].

Enzyme immobilization offers a reliable approach to enhance enzymatic activity, improve reusability across multiple cycles, and maintain stability under harsh conditions and long storage periods [23]. Immobilization methods are broadly categorized into physical processes, such as adsorption and encapsulation, and chemical methods, such as covalent bonding and crosslinking [24]. The covalent bond provides strong enzyme-support interactions due to the reactions between the activated chemical groups on the support surface and the nucleophilic groups on the enzyme, mainly $-NH_2$, $-SH$ and $-OH$ [25,26].

To ensure optimal performance in remediation processes, the choice of an immobilization support should prioritize non-toxicity, biocompatibility, cost-benefit ratio, eco-friendliness and high substrate affinity [27]. Carbon-based materials, such as biochar (BC) and hydrochar (HC), are considered ideal solid supports due to their advantageous physico-chemical properties, including a large surface area, especially for BC, a porous structure and the presence of aromatic and functional groups [28].

BC is produced through the pyrolysis of lignocellulosic and other biomass in the absence of oxygen [29], while HC is created by converting biomass under hydrothermal conditions [30]. Recent studies have focused on the synthesis of BC from agricultural residues for use as a sustainable support for the immobilization of laccase [31–33]. In contrast, no studies have been reported on laccase immobilization on HC or its application in pharmaceutical degradation. For example, Silva et al. [34] treated avocado seed BC with citric acid and glutaraldehyde (GA) to enhance its surface area and introduce carbonyl groups. This resulted in an immobilized laccase that achieved 99 % degradation of APAP. García-Delgado et al. [35] covalently immobilized laccase on holm oak BC using aminopropyltriethoxysilane (APTES) and GA as pre-treatment. The authors achieved complete TC removal using 2,2-azobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a mediator, and approximately 40 % degradation of sulfonamides with syringaldehyde (SYR). Lonappan et al. [36] demonstrated almost complete removal of diclofenac using crude laccase adsorbed in citric acid-activated BC from different feedstocks.

While the aforementioned studies indicate the potential of laccase immobilized on BC for pharmaceutical degradation, further investigations are needed to validate its application in real-world wastewater treatment. This study investigates the immobilization of crude laccase from *Lentinus sajor-caju* on BC and HC derived from grape seed (GS) residue, a widely available but underutilized resource. Using GS-BC and GS-HC in their original form, without grinding, supports bioremediation and simplifies the separation process. The system was tested using SYR as a redox mediator to degrade micropollutants such as SMX, TC and APAP in aqueous solutions and real WWTP wastewater. The reusability of the system for SMX degradation was also assessed, and the degradation products and potential pathways were proposed.

2. Materials and methods

2.1. Materials

GS was provided by a local wine producer in Marín, Spain. ABTS ($\geq 98\%$), SYR (98%), SMX ($\geq 98\%$), TC ($\geq 98\%$), APAP (98%), GA (50% in H_2O) and APTES were purchased from Sigma-Aldrich. Laccase enzyme (*Lentinus sajor-caju*, CCB 020) was produced through solid-state fermentation with brewer's spent grains and mineral salt solution (67% w/v moisture), as described previously [37]. Stock solutions of SMX, TC and APAP were prepared in distilled water and stored at $4\text{ }^\circ\text{C}$.

2.2. HC and BC preparation

HC was synthesized in a 100 mL cylindrical hydrothermal reactor with a PTFE inner chamber under autogenic pressure. A 1:3 (w/v) ratio of GS to distilled water was processed at $220\text{ }^\circ\text{C}$ for 2 h. The resulting HC was dried overnight at $50\text{ }^\circ\text{C}$ and stored for further use. BC was obtained by pyrolysis of GS at $750\text{ }^\circ\text{C}$ for 2 h under a nitrogen atmosphere ($2\text{ L}\cdot\text{min}^{-1}$ flow rate) in a Carbolite TF1–120 Thermoline, with a heating rate of $12.5\text{ }^\circ\text{C}\cdot\text{min}^{-1}$.

2.3. Surface modification and enzyme immobilization

HC and BC were functionalized with APTES (8% v/v in ethanol) by stirring 1 g of material at 170 rpm, $30\text{ }^\circ\text{C}$ for 24 h. The materials were rinsed with ethanol and subsequently dried at $60\text{ }^\circ\text{C}$. Activation was performed with 3% (v/v) GA stock solution in $50\text{ mmol}\cdot\text{L}^{-1}$ acetate buffer, pH 6.0, under gentle stirring for 2 h, followed by washing and drying at $60\text{ }^\circ\text{C}$.

For enzyme immobilization, the functionalized supports were mixed, in a ratio of $8\text{ mg}\cdot\text{mL}^{-1}$, with laccase solution ($1.8\text{ mg}\cdot\text{mL}^{-1}$ protein in $50\text{ mmol}\cdot\text{L}^{-1}$ acetate buffer, pH 5.0) and shaken at $30\text{ }^\circ\text{C}$ for 2 h. The supports with immobilized laccase, GS-HC-Lac and GS-BC-Lac, were subsequently filtered, washed with buffer, dried and stored at $4\text{ }^\circ\text{C}$.

2.4. Materials characterization

The materials were characterized by Fourier-Transform Infrared Spectroscopy (FTIR, Bruker Alpha II) equipped with a Platinum ATR/Transmission accessory with a spectral range of $400\text{--}4000\text{ cm}^{-1}$ and Scanning Electron Microscopy (SEM, FEI Nova 200) equipped with Energy-Dispersive X-ray Spectroscopy (EDS). Samples for SEM analysis were coated with gold.

The materials were also analyzed using X-ray Photoelectron Spectroscopy (XPS) on a Kratos Axis-Supra instrument (Thermo Fisher Scientific). The excitation monochromatic X-ray source was $Mg\text{ K}\alpha$ ($h\nu = 1253.6\text{ eV}$). Measurements were conducted in Constant Analyzer Energy (CAE) mode, including a survey spectrum with a pass energy of 150 eV and high-resolution spectra with a pass energy of 40 eV . Data analysis and atomic quantification were determined from the XPS peak areas using the ESCAPE software by the manufacturer Kratos Analytical.

The analysis of GS-BC and GS-HC surface charge as a function of pH was performed by determining the zero charge point (pH_{zpc}), following the methodology established by Gogoi et al. [38].

2.5. Enzymatic activity and protein content

Laccase activity was determined spectrophotometrically by monitoring the oxidation of ABTS ($1.5\text{ mmol}\cdot\text{L}^{-1}$) in $100\text{ mmol}\cdot\text{L}^{-1}$ citrate-phosphate buffer (pH 4.0) at 420 nm ($\epsilon = 36\text{ mM}^{-1}\cdot\text{cm}^{-1}$). One unit of activity (U) was defined as the amount of enzyme required to oxidize $1\text{ }\mu\text{mol}$ of ABTS per minute. For immobilized enzymes, activity was calculated by Eq. 1:

$$\text{Immobilized Lac activity}(\text{U}\cdot\text{g}^{-1}) = \frac{\Delta A \times DF \times V}{\epsilon \times t \times m} \# \quad (1)$$

where ΔA represents the change in absorbance, DF is the dilution factor, V is the reaction volume (mL), ϵ is the molar extinction coefficient, t is the reaction time (min), and m is the mass of immobilized enzyme (g).

The protein concentration was quantified using the Bradford method [39]. Immobilization efficiency was evaluated in terms of immobilization yield and loading amount, as described in Eqs. 2 and 3, respectively:

$$\text{Immobilization yield}(\%) = \frac{A_i - A_f}{A_i} \times 100 \# \quad (2)$$

$$\text{Loading amount}(\text{mg}\cdot\text{g}^{-1}) = \frac{P_i - P_f}{\text{Support mass}} \# \quad (3)$$

where A_i and P_i are the initial laccase activity and protein content, respectively, and A_f and P_f are the residual laccase activity and protein content after immobilization.

2.6. Stability and reusability

The pH stability of free and immobilized laccase was evaluated by incubating the enzymes in 100 mmol·L⁻¹ citrate-phosphate buffer at pH 3.0, 5.0, and 8.0 for 24 h at 25 °C. Thermal stability was assessed by incubating the enzyme at temperatures ranging from 25 to 65 °C for 1 h. Storage stability was studied over 30 days at 4 °C, with residual activities measured at regular intervals. Enzyme activity was expressed as a percentage of the maximum observed activity, set at 100 %.

2.7. Degradation of pharmaceuticals

Degradation experiments were conducted in 20 mL of citrate-phosphate buffer (100 mmol·L⁻¹, pH 5.0) with 5 mg·L⁻¹ of SMX, TC or APAP with and without 0.5 mmol·L⁻¹ SYR. A total of 0.4 g of GS-HC-Lac and GS-BC-Lac was used in the reactions at 25 °C under continuous stirring at 150 rpm. Controls included GS-HC and GS-BC without Lac. SMX degradation was evaluated over eight cycles (2, 4 and 24 h) using GS-HC-Lac and GS-BC-Lac. Samples were collected at defined intervals, and degradation efficiency was assessed.

First-order degradation rate constants were calculated using Eq. 4:

$$C_t = C_0 + C_i e^{-kt} \# \quad (4)$$

Where C_t is the concentration at time t ; C_0 is the offset; C_i is the initial concentration; k is the first-order rate constant (h⁻¹).

2.8. Quantification and transformation products

Pharmaceutical degradation was assessed using Ultra-High-Performance Liquid Chromatography (UHPLC, Shimadzu Nexera X2) with Diode Array Detection (DAD) at 268 nm, 270 nm and 244 nm for SMX, TC and APAP, respectively. Separation was achieved on a Kinetex C18 column (2.1 × 100 mm, 1.7 μm), using an isocratic mobile phase of 80:20 (v/v) acetonitrile and 0.1 % formic acid in water, at a flow rate of 0.2 mL·min⁻¹, with a 5 μL injection volume.

Transformation products (TP) were identified using HPLC coupled with a trapped ion-mobility spectrometry time-of-flight mass spectrometer (TIMS-TOF-MS, Bruker Daltonics). Separation was performed on a Zorbax Eclipse XDB-C18 column (2.1 × 100 mm, 1.8 μm) at 30 °C with a gradient mobile phase, at a flow rate of 0.5 mL·min⁻¹, comprising acetonitrile and water containing 0.1 % (v/v) formic acid. Mass spectra were acquired in positive electrospray ionization (ESI) mode (300–1300 m/z). Data acquisition and analysis were performed using Bruker Otof Control and Data Analysis Software version 5.1.

2.9. Degradation in real wastewater

A treated wastewater sample was obtained from the effluent of the municipal wastewater treatment plant operated by Águas do Norte S.A. (Esposende, Portugal) and filtered through a 0.45 μm membrane to remove particulates and microorganisms. The wastewater (pH 7.9) was spiked with 5 mg·L⁻¹ of each pharmaceutical (SMX, TC and APAP) and 0.5 mmol·L⁻¹ SYR, without pH modification. Batch treatments were conducted in 40 mL stirred reactors at 25 °C for 24 h, using 0.8 g GS-HC-Lac or GS-BC-Lac.

2.10. Data analysis

All experiments were conducted in triplicate, and GraphPad Prism® software (version 8.0; GraphPad Software, Inc., San Diego, CA, USA) was used for linear regression analysis and data visualization.

3. Results and discussion

3.1. Covalent immobilization of crude laccase on GS-BC and GS-HC

GS-BC was produced through a dry carbonization process (pyrolysis) of carbonaceous organic biomass in an oxygen-limited environment [40]. On the other hand, GS-HC was obtained via hydrothermal carbonization, where the organic residue is converted into a solid product with high carbon content [41]. As a result, the two materials exhibit distinct physical and chemical properties (Fig. 1). GS-HC is characterized by a high lignin content, with cellulose and hemicellulose partially or fully degraded, and various chemical groups present in the material. In contrast, GS-BC has typically lower lignin content due to the decomposition of lignocellulosic biomass at around 600 °C [41,42]. As observed by FTIR analysis, the carbonization process at high temperatures resulted in a significant decrease in the chemical groups present in the material.

The laccase used in this study was produced via solid-state fermentation of the fungus *L. sajor-caju*, using brewer's spent grain as substrate. This process yielded a high laccase activity of approximately 15000 U·L⁻¹.

Chemical modifications were applied to the support materials to achieve efficient laccase immobilization on GS-BC and GS-HC (Fig. 2). These materials were used without grinding or processing, maintaining their original spherical shape derived from GS (around 4–5 mm of diameter). As far as we know, this is the first study exploring this approach. First, amino groups were introduced onto the surface of the carbon materials using APTES. These groups were then activated with

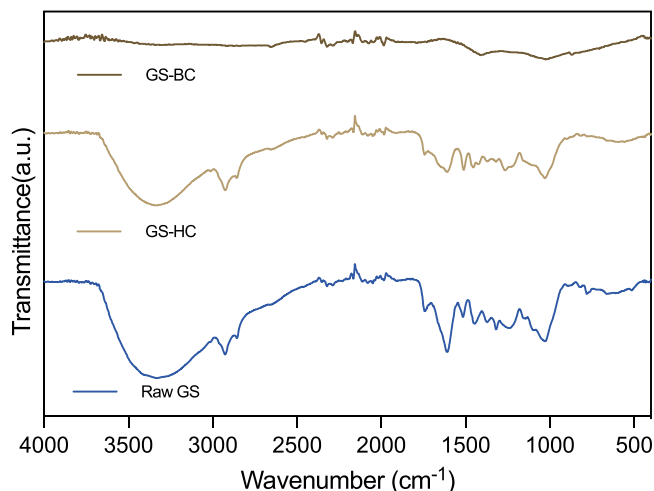


Fig. 1. FTIR spectra of raw GS and derived GS-HC and GS-BC.

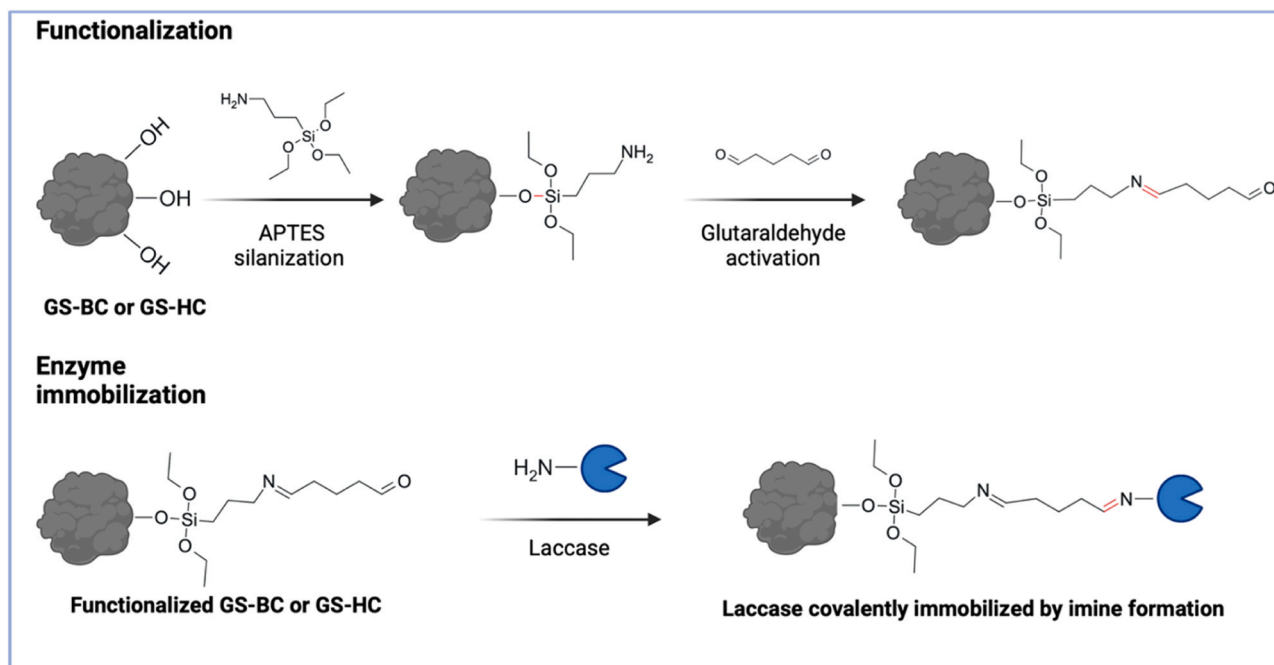


Fig. 2. Mechanism of functionalization and immobilization of laccase on GS-BC and GS-HC.

the coupling agent GA to promote covalent enzyme immobilization and crosslinking [43].

Although both materials were functionalized with APTES and GA for covalent enzyme immobilization, the initial surface charge (pH_{zpc} of 9.77 for GS-BC and 3.61 for GS-HC) likely influenced the density and orientation of silane grafting, as well as the accessibility and reactivity of

surface hydroxyl groups involved in the condensation reaction with APTES for enzyme anchoring.

Laccase immobilization on GS-BC achieved a yield of $10.7 \pm 0.3 \%$ and a loading efficiency of $39.54 \pm 0.07 \%$, corresponding to a loading capacity of $85.5 \pm 0.4 \text{ mg} \cdot \text{g}^{-1}$. Similarly, GS-HC exhibited an immobilization yield of $10 \pm 1 \%$ and a loading efficiency of $34.3 \pm 0.5 \%$,

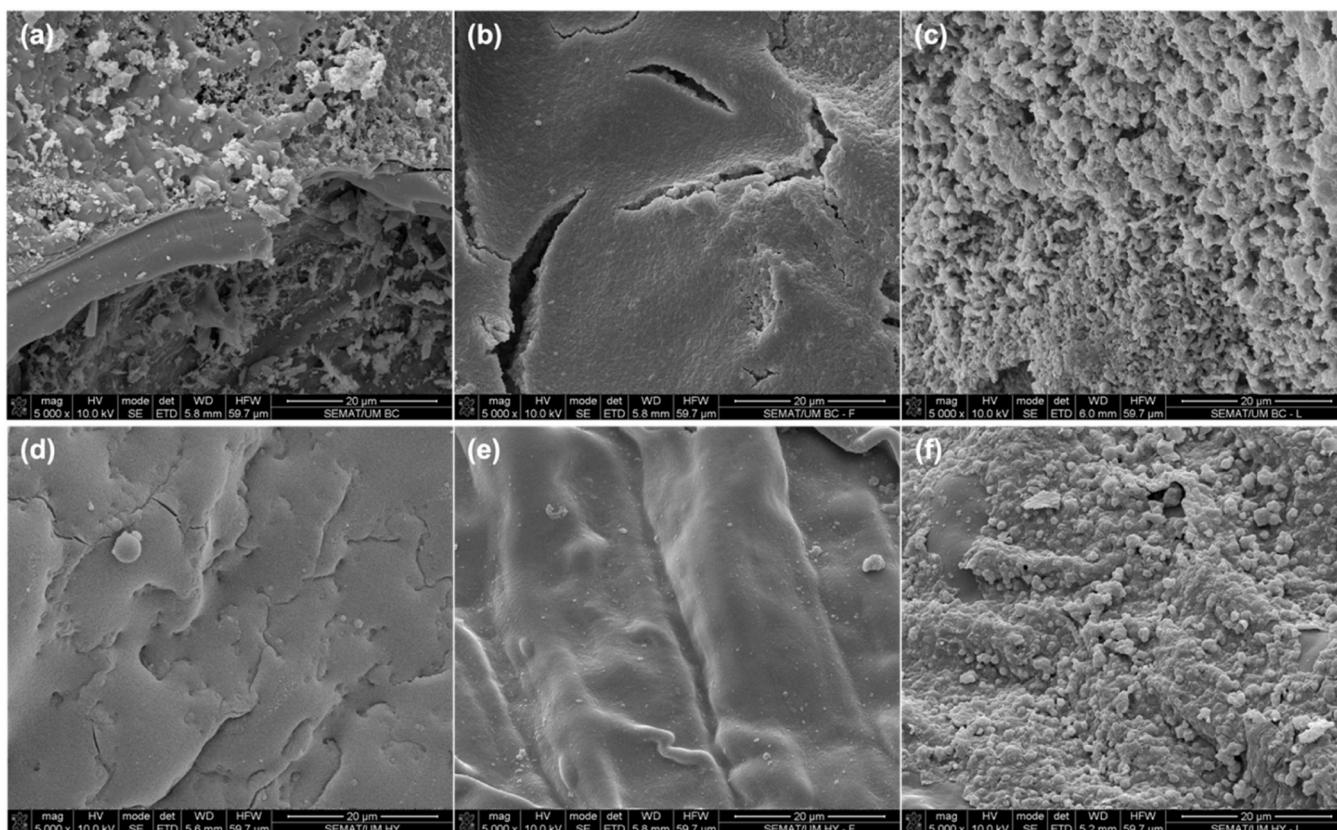


Fig. 3. SEM micrographs of raw GS-BC (a), functionalized GS-BC (b), GS-BC-Lac (c), raw GS-HC (d), functionalized GS-HC (e) and GS-HC-Lac (f).

corresponding to a loading capacity of $74.7 \pm 0.9 \text{ mg} \cdot \text{g}^{-1}$. Moreover, the specific activity of GS-BC-Lac and GS-HC-Lac was $607 \pm 48 \text{ g}^{-1}$ and $546 \pm 45 \text{ g}^{-1}$, respectively. These differences likely stem from the distinct structural and surface properties of the support materials, which result from their respective carbonization processes, in which GS-BC results in more developed porous structure, higher surface area, and favorable surface charge.

For comparison, Silva et al. [34] achieved a similar yield of 5.5 % for the covalent immobilization of laccase on powdered BC treated with citric acid and functionalized with GA. However, using BC and HC derived from GS in their original particle size offers a significant advantage over conventional powdered carbons for enzyme immobilization. Their larger size eliminates the necessity for complex separation steps in remediation media, reducing material loss and simplifying recovery and reuse.

3.2. Characterization of GS-BC-Lac and GS-HC-Lac

Fig. 3 presents SEM images of GS-BC and GS-HC at three stages: raw (Figs. 3a and 3d), after surface functionalization (Figs. 3b and 3e), and after laccase immobilization (Figs. 3c and 3f).

Raw GS-BC (Fig. 3a) exhibited a porous irregular and rough surface structure, while raw GS-HC showed a comparatively smoother surface, with fewer visible pores or irregularities (Fig. 3d). These morphological differences may be attributed to the distinct carbonization processes, with pyrolysis (used for GS-BC) producing a higher surface area, distinct pores and structural shrinkage [44].

Following the surface functionalization process with APTES and GA (Fig. 3b and e), both materials exhibit noticeable changes in surface texture. The formation of a coating layer, more noticeable in GS-BC, resulted in a smoother surface due to silane deposition. This functionalization made the surface potentially more reactive, enhancing the potential for covalent and crosslinking interactions during enzyme immobilization [33,43].

After enzyme immobilization (Fig. 3c and f), a distinct granular topography becomes apparent on the surface of both materials. This morphological feature can be attributed to enzyme clumping, consistent with findings from other studies [34,45].

These morphological changes observed via SEM are further supported by EDX analysis (Table 1). The appearance of nitrogen (N) after immobilization indicates the presence of protein, confirming successful enzyme anchoring [31]. Additionally, the detection of silicon (Si) after functionalization supports the incorporation of the APTES-derived silane layer.

XPS analysis was performed on GS-HC and GS-BC samples before and after functionalization with APTES and GA, followed by laccase immobilization, to investigate modifications in elemental composition and surface chemical structure. The XPS survey spectra confirm the presence of C, O, Si, and N elements on the surface (see Figure S1, supplementary materials). The deconvolution of the high-resolution spectra for the C 1 s, O 1 s, N 1 s, and Si 2p regions is presented in Fig. 4, and provides insights into the chemical modifications induced by the functionalization process. Table 2 summarizes the binding energies (BE) and weight percentages (wt%) of the elements detected on the sample surfaces.

Table 1

Weight percentage of the elements carbon (C), oxygen (O), K, Si and N in the GS-HC and GS-BC samples with and without laccase determined by EDX.

Element (wt%)	GS-HC	GS-HC-Lac	GS-BC	GS-BC-Lac
C	71 ± 1	58 ± 1	77 ± 1	52 ± 1
O	29 ± 3	24 ± 3	14 ± 3	21 ± 3
K	ND	ND	ND	5 ± 4
Si	ND	10 ± 2	ND	7 ± 2
N	ND	14 ± 7	ND	12 ± 8

ND: not detected.

The C 1 s spectra of the raw GS-BC and GS-HC samples (Fig. 4a and b) are dominated by the C–C peak at approximately 285 eV, consistent with the characteristics of carbonaceous materials [46]. After functionalization and laccase immobilization (Fig. 4c and d), surface modifications were evidenced by a shift in the positions of the C–O and the C=O peaks and an increase in their content (wt%), while the C–C bond amount decreased. Particularly, the C–O peak at 286 eV potentially corresponds to the integration of R-CH₂*-NH(CO)- functional groups, whereas the slight shift in the C=O peak from 290 eV in GS-BC to 288 eV in GS-BC-Lac, and from 289 eV in GS-HC to 288 eV in GS-HC-Lac, suggests the integration of R-CH₂-NH(C*O)- groups [47]. These discrepancies are assigned to chemical groups characteristic of the enzyme and can confirm the effective immobilization of laccase on the functionalized surfaces [48].

The O 1 s deconvoluted spectra (Fig. 4e–h) further corroborate these findings, showing a pronounced C=O peak and an increase in its amount, which can also be attributed to the introduction of aldehyde groups by GA during the functionalization process.

The N 1 s signal was detected exclusively in the XPS spectra of the laccase-immobilized samples, with peaks at 400 eV and 401 eV (Fig. 4i and j), which correspond to N atoms in C–N and C=N bonds, respectively [49]. These results agree with values reported in the literature and confirm the incorporation of N-containing groups from APTES and laccase.

Additionally, the Si 2p signal observed at 102 eV (Fig. 4k and l), along with the peak at 534 eV in GS-HC-Lac (Fig. 4g), aligns with the presence of Si–O–C bonds [50]. This indicates that silane groups of APTES undergo condensation with hydroxyl groups on the char surface during functionalization, as corroborated by the SEM/EDX results.

3.3. Stability of GS-BC-Lac and GS-HC-Lac

One of the main challenges of applying free laccase in the industry is its limited operational stability. Consequently, the primary objective of the enzyme immobilization is to enhance enzymatic robustness under varying pH, temperature and storage conditions. After the successful immobilization of laccase on GS-BC and GS-HC, the acid-base and thermal stabilities of the free and immobilized laccase were evaluated at three different pH media (3.0, 5.0 and 8.0) and temperatures (25–65 °C).

As depicted in Fig. 5a, immobilization improved the pH stability of the enzyme. However, both free and immobilized laccase (GS-BC-Lac and GS-HC-Lac) remained stable at pH 8.0, retaining nearly 100 % activity. The enzyme's tolerance to more alkaline pH is particularly relevant for wastewater treatment applications, where the pH typically ranges from 6.0 to 8.0. Otherwise, most studies report reduced stability at pH values above 6.0. For example, Yang et al. [49] found that laccase immobilized on amino-modified magnetic pine BC retained less than 20 % activity above pH 6.0, while Zhou et al. [32] observed less than 60 % relative activity between pH 6.0 and pH 8.0. Similarly, Rasheed et al. [51] reported a decline in stability at pH 7.0 and 8.0 for Fe-MOF-laccase-magnetic BC compared to free laccase.

In this study, GS-HC-Lac also demonstrated high stability at pH 5.0 (100 % residual activity), which could result from the enhanced chemical stability of the support and its interaction with laccase. Furthermore, GS-BC-Lac and free laccase activities remained above 74 % and 48 %, respectively. At pH 3.0 the enzyme was not stable, with a maximal residual activity of 23 % verified for GS-BC-Lac. These results demonstrate the superior pH resilience and broader application range of immobilized laccase. Overall, enzyme immobilization effectively minimized structural conformational changes through intermolecular interactions between the amino acid side chains of laccase and the GA functional groups on the char [52].

Thermal stability is another important parameter, and the corresponding results are presented in Fig. 5b. Free and immobilized laccase exhibited comparable relative activities (> 90 % of residual activity) within the temperature range of 25–45 °C. At higher temperatures,

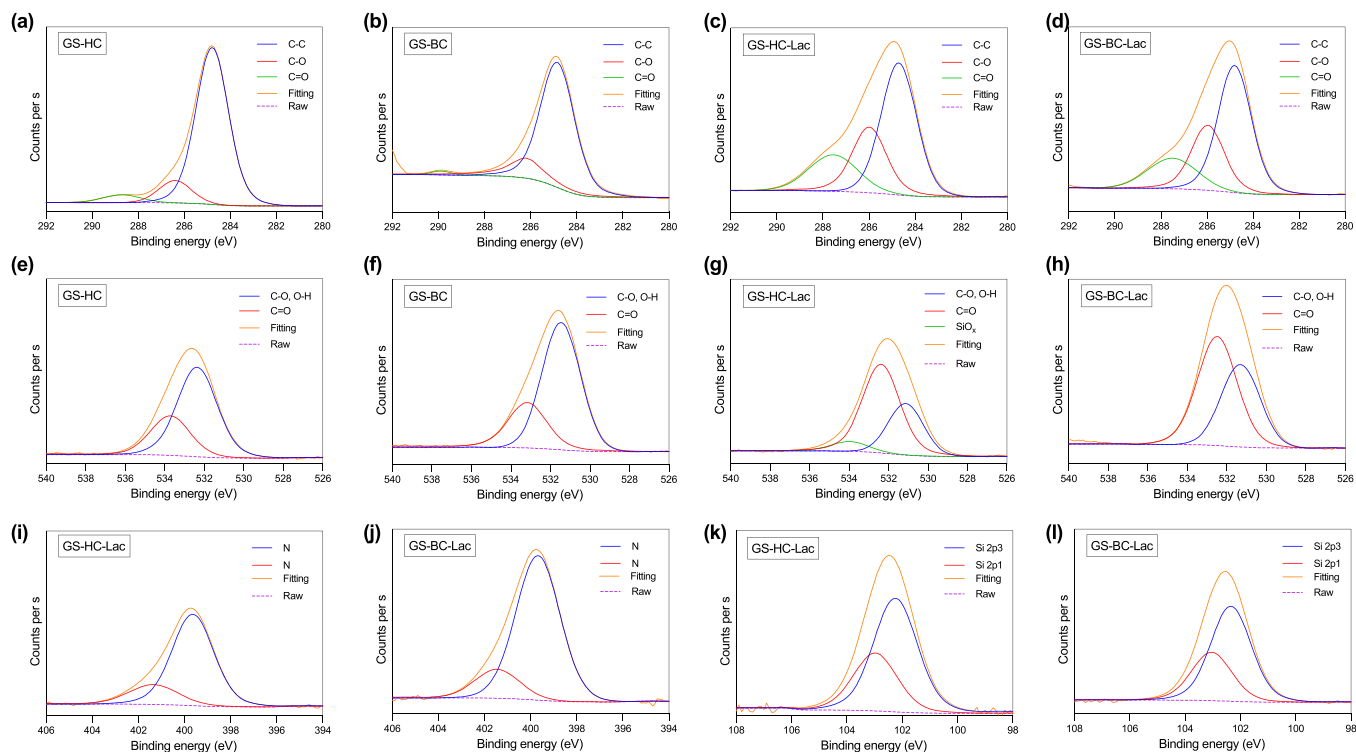


Fig. 4. High-resolution spectra of elements: C (a–d) and O (e–h) for GS-HC, GS-BC, GS-HC-Lac and GS-BC-Lac; N (i, j) and Si (k, l) for GS-HC-Lac and GS-BC-Lac.

Table 2

Binding energies (BE) and relative surface element compositions (wt%) of raw GS-BC and GS-HC samples, and after laccase immobilization, as determined by XPS.

Element	GS-BC		GS-BC-Lac		GS-HC		GS-HC-Lac	
	BE (eV)	wt%	BE (eV)	wt%	BE (eV)	wt%	BE (eV)	wt%
C1 (C-C)	284.83	32.77	284.8	31.1	284.78	69.70	284.81	33.55
C2 (C-O)	286.19	6.73	285.96	16.2	286.4	9.72	286.09	17.43
C3 (C=O)	289.88	0.54	287.5	10.6	288.7	3.92	287.65	12.76
O1 (C-O, O-H)	531.45	21.61	531.31	11.1	532.36	8.52	531.22	8.04
O2 (C=O)	533.16	8.44	532.49	15.2	533.72	3.92	532.47	15.6
O3 (Si-O)	ND	ND	ND	ND	ND	ND	534.06	1.64
Si	ND	ND	102.34	3.21	ND	ND	102.34	2.84
N1 (C-N)	ND	ND	399.68	5.95	ND	0.97	399.74	5.85
N2 (C=N)	ND	ND	401.45	1.21	ND	ND	401.45	1.50

ND: not detected.

immobilized laccase demonstrated greater thermal stability, maintaining 84 % and 88 % of its relative activity for GS-HC and GS-BC, respectively, at 55 °C, and 84 % (GS-HC) and 58 % (GS-BC) at 65 °C. In contrast, free laccase exhibited lower thermal tolerance, retaining only 36 % activity at the same temperature. The immobilization of laccase on GS-HC and GS-BC provided tertiary structure protection, preventing protein denaturation at higher temperatures [53]. Among these, GS-HC once again demonstrated enhanced performance.

The storage stability of free and immobilized laccase was assessed by incubating them at 4 °C for 30 days, with activity measured at regular intervals, as presented in Fig. 5c. The immobilized laccase exhibited slightly higher storage stability than the free laccase. After the storage period, the immobilized enzyme maintained 86 % (GS-BC) and 91 % (GS-HC) activity, compared with 79 % for free laccase. The immobilization process improved the long-term effectiveness of laccase, consistent with similar enhancements reported for enzyme immobilization on BC in other studies [32,49,54].

Nevertheless, this study highlights that the laccase produced by *Lentinus sajor-caju* presented superior resistance to storage conditions in its free form, contrasting with other studies that reported lower stability for free laccase stored for the same time [32,51,53]. Overall, the enzyme

stability studies have proven the success of the immobilization procedure and emphasized the potential of GS-BC-Lac and GS-HC-Lac as cost-effective solutions for large-scale environmental and industrial bioremediation applications.

3.4. Pharmaceutical degradation by GS-HC-Lac and GS-BC-Lac

3.4.1. Biodegradation of antibiotics

The degradation of the SMX and TC antibiotics by GS-HC-Lac and GS-BC-Lac was limited or slow in the absence of a redox mediator. As shown in Fig. 6, less than 15 % of SMX and approximately 35 % TC were degraded after 72 h of reaction. Laccase is a multicopper oxidase that oxidizes various substrates into free radicals using molecular oxygen as an electron acceptor. However, the low redox potential of the laccase T1Cu site (0.43–0.79 V) restricts the oxidation of non-phenolic compounds, such as recalcitrant pollutants, often requiring mediators to enhance the reaction [55]. Similarly, TC may not enter the active sites of laccase due to steric hindrances, which may also explain the very slow reaction [56].

Thus, to enhance the bioremediation of SMX and TC, this study investigated their degradation using GS-HC-Lac and GS-BC-Lac in the

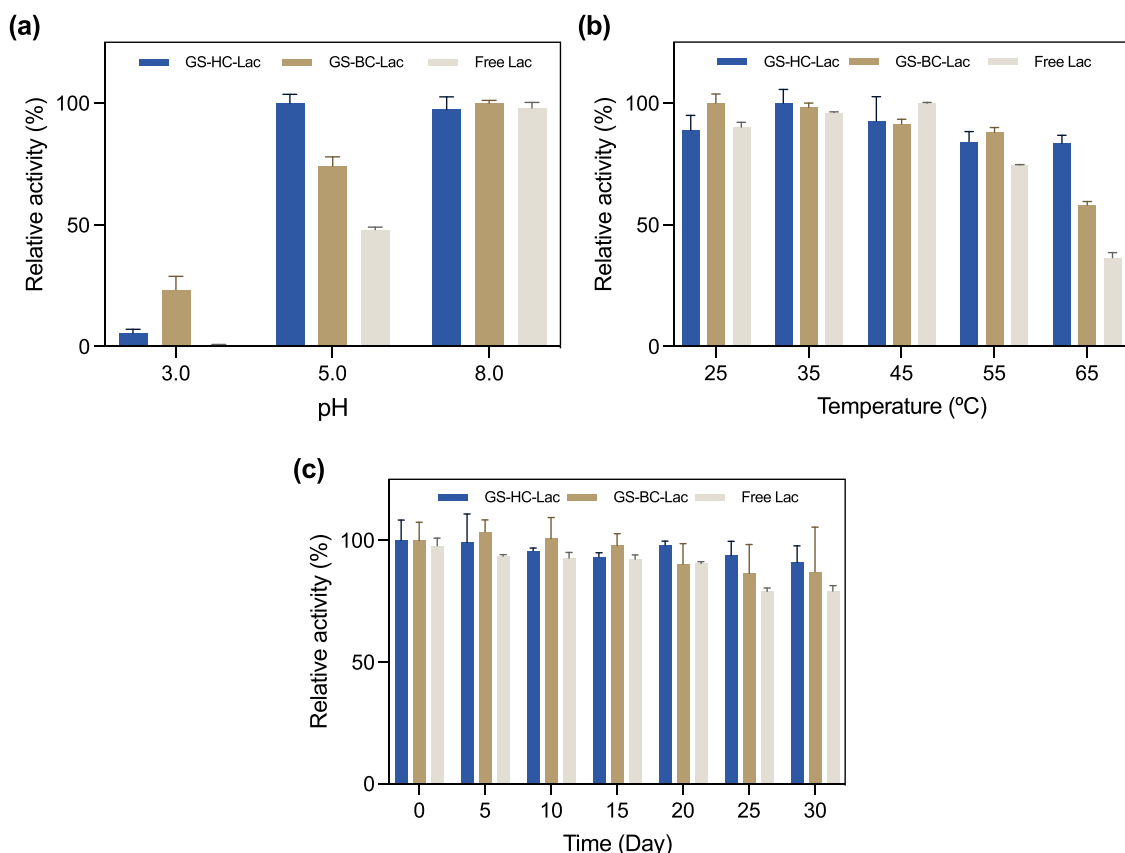


Fig. 5. Stability of free and immobilized laccase on GS-BC and GS-HC under varying conditions: pH (a), temperature (b) and storage time (c).

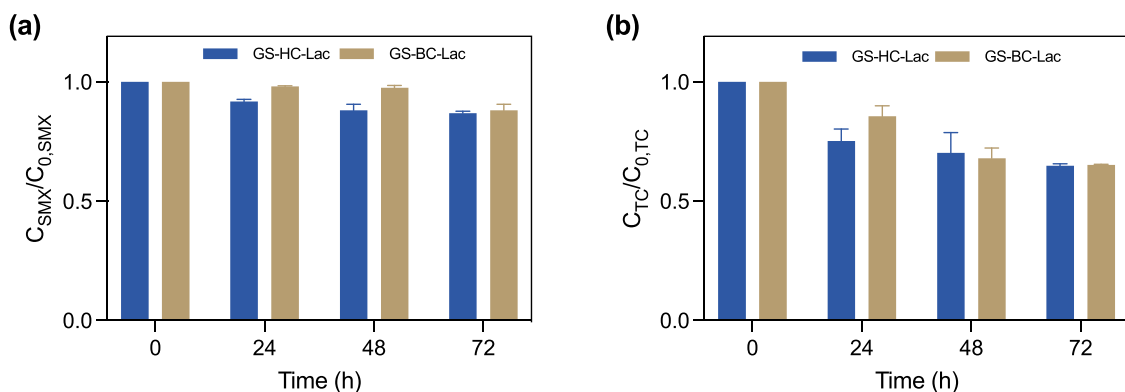


Fig. 6. Degradation efficacy of 5 mg·L⁻¹ SMX (a) and TC (b) by GS-HC-Lac and GS-BC-Lac. Incubation conditions: 0.4 g of char, pH 5.0 and 25 °C.

presence of the natural redox mediator SYR. This redox mediator is oxidized by laccase into phenoxy radicals via the hydrogen atom transfer (HAT) mechanism, which then mediates the oxidation of the target compounds [51]. The degradation results and predicted kinetic parameters are presented in Fig. 7 and Table 3. Likewise, to assess any adsorption contribution of the supports, control experiments were conducted in the absence of laccase.

SMX was completely degraded ($C/C_0 < 0.1$) within approximately 25–30 min of reaction using both GS-HC-Lac and GS-BC-Lac at pH 5.0 and 25 °C. However, the reaction was faster with GS-BC-Lac ($k = 6.099 \text{ h}^{-1}$) compared to GS-HC-Lac ($k = 3.995 \text{ h}^{-1}$). In the case of TC, complete degradation ($C/C_0 < 0.1$) was achieved within 5 h reaction. GS-HC-Lac enabled a slightly quicker reaction ($k = 0.509 \text{ h}^{-1}$) compared to GS-BC-Lac ($k = 0.318 \text{ h}^{-1}$). No adsorption was observed, confirming that the process involved oxidative degradation. These

findings demonstrate that redox mediators significantly enhance enzymatic degradation, in agreement with other studies on immobilized laccase systems. For instance, Chen et al. [57] verified 57 % SMX degradation using laccase immobilized on chitin with SYR, while Kadam et al. [58] achieved 69 % degradation using guaiacol as a mediator. In comparison, the system developed in this study enabled complete SMX degradation in less than 30 min, highlighting its higher efficiency.

Similarly, while laccase immobilized on activated carbon achieved 79 % TC degradation in 6 h and 99.5 % in 24 h using 0.5 mmol L⁻¹ SYR [56], the GS-HC-Lac-SYR system developed in this study reached almost complete degradation within 5 h, demonstrating competitive performance with an alternative immobilization strategy. Furthermore, Shen et al. [59] observed a synergistic effect between metal-organic framework adsorption and laccase biocatalysis using the synthetic mediator ABTS, achieving 98 % removal within 1 h. It is important to highlight

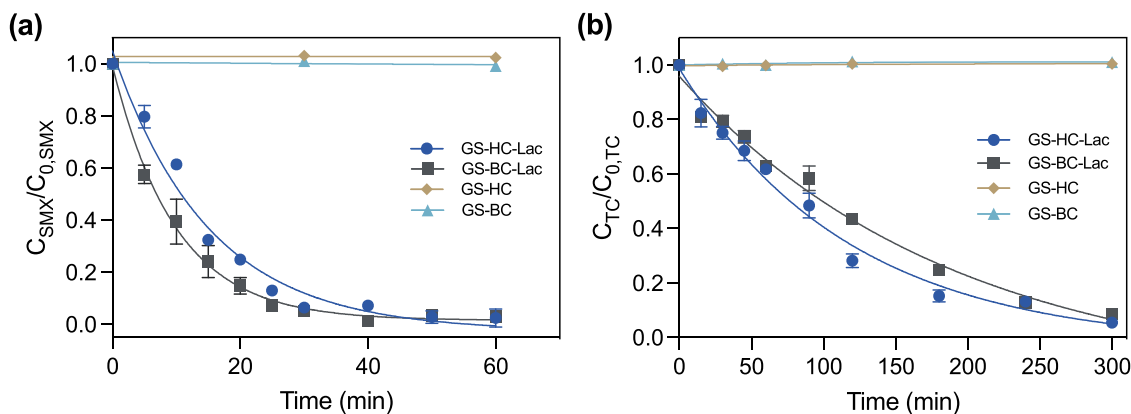


Fig. 7. Degradation of 5 mg·L⁻¹ SMX (a) and TC (b) by GS-HC-Lac and GS-BC-Lac. Controls: GS-HC and GS-BC. Reaction conditions: 0.4 g of char, 0.5 mmol·L⁻¹ SYR, pH 5.0, and 25 °C.

Table 3

Kinetic parameters predicted by the pseudo-first-order kinetic model for SMX and TC degradation using GS-HC-Lac and GS-BC-Lac and the mediator SYR.

Compound	Condition	k (h ⁻¹)	$t_{1/2}$ (h)	R^2
SMX	GS-HC-Lac-SYR	3.995 ± 0.551	0.174 ± 0.024	0.9899 ± 0.0291
	GS-BC-Lac-SYR	6.099 ± 0.303	0.114 ± 0.006	0.9971 ± 0.1034
	GS-HC-Lac	0.509 ± 0.072	1.361 ± 0.193	0.9822 ± 0.0110
	GS-BC-Lac	0.318 ± 0.069	2.180 ± 0.471	0.9900 ± 0.0090
TC	GS-HC-Lac-SYR	3.995 ± 0.551	0.174 ± 0.024	0.9899 ± 0.0291
	GS-BC-Lac-SYR	6.099 ± 0.303	0.114 ± 0.006	0.9971 ± 0.1034
	GS-HC-Lac	0.509 ± 0.072	1.361 ± 0.193	0.9822 ± 0.0110
	GS-BC-Lac	0.318 ± 0.069	2.180 ± 0.471	0.9900 ± 0.0090

that although our system achieved complete TC degradation over a longer timeframe, this study suggests that BC and HC-based materials provide effective support for enzymatic oxidation without requiring additional adsorption mechanisms.

3.4.2. Biodegradation of analgesic and antipyretic

The APAP degradation performance was investigated using GS-HC-Lac and GS-BC-Lac, with and without SYR redox mediator. GS-HC-Lac and GS-BC-Lac achieved degradation efficiencies of 54 % and 71 %, respectively, within 24 h (Fig. 8a). As shown in Table 4, GS-BC-Lac demonstrated superior catalytic efficiency, with a higher degradation rate constant ($k = 0.125$ h⁻¹) compared to GS-HC-Lac ($k = 0.077$ h⁻¹). This difference may be attributed to variations in APAP diffusion within the support pores and its interactions with laccase. In contrast, APAP adsorption by the chars without laccase remained below 10 %,

Table 4

Kinetic parameters predicted by the pseudo-first-order kinetic model for APAP degradation using GS-HC-Lac and GS-BC-Lac.

Condition	k (h ⁻¹)	$t_{1/2}$ (h)	R^2
GS-HC-Lac	0.077 ± 0.017	8.997 ± 2.529	0.9872 ± 0.0293
GS-BC-Lac	0.125 ± 0.022	5.530 ± 1.099	0.9899 ± 0.0241
GS-HC-Lac-SYR	7.492 ± 0.336	0.091 ± 0.004	0.9991 ± 0.0533
GS-BC-Lac-SYR	1.185 ± 0.238	0.585 ± 0.118	0.9865 ± 0.0228

emphasizing the enzymatic contribution to the process.

The extent of APAP degradation was consistent with findings from other studies, although differences in degradation efficiency can be observed due to variations in experimental conditions. For instance, Hachi et al. [60] achieved 70 % APAP degradation after 24 h using immobilized laccase in a binary system with carbamazepine, whereas only 30 % degradation was observed in a single system with APAP. Similarly, Silva et al. [34] also investigated the degradation of APAP using laccase immobilized on BC powder and reported a degradation efficiency of 99 % within 24 h.

Furthermore, the presence of the SYR mediator significantly enhanced the degradation rate in both GS-HC-Lac and GS-BC-Lac systems. Under these conditions, complete APAP degradation ($C/C_0 < 0.1$) was achieved within 30 min for GS-HC-Lac ($k = 7.492$ h⁻¹) and 90 min for GS-BC-Lac ($k = 1.185$ h⁻¹). This confirms the crucial role of the mediator in enhancing the catalytic efficiency. In comparison, Bao et al. [61] demonstrated that adding ABTS significantly enhanced APAP degradation, achieving 100 % removal within 12 h.

These results highlight GS-HC-Lac and GS-BC-Lac as promising

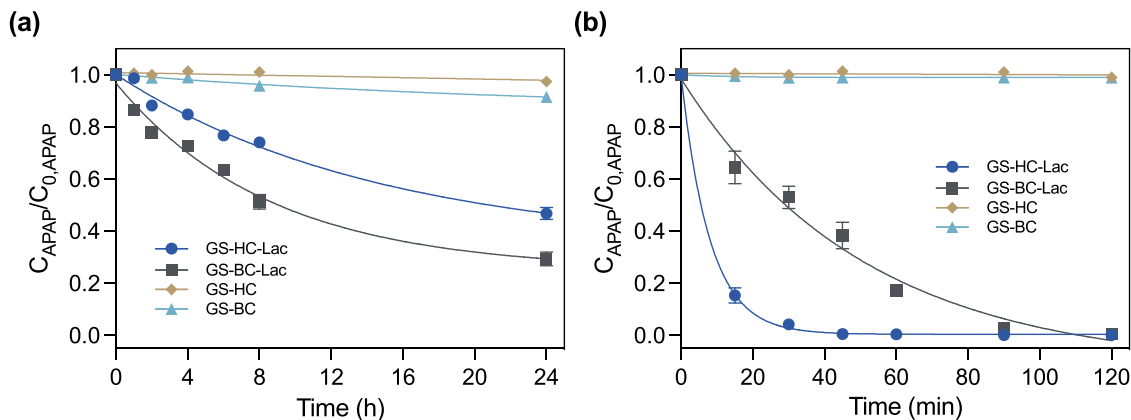


Fig. 8. Degradation of 5 mg·L⁻¹ APAP by GS-HC-Lac and GS-BC-Lac in the absence (a) and presence (b) of mediator SYR. Controls: GS-HC and GS-BC. Reaction: 0.4 g char, pH 5.0, 25 °C.

biocatalysts for pharmaceutical removal from water, with their efficiency significantly enhanced by the redox mediator SYR.

3.4.3. Reusability of GS-HC-Lac and GS-BC-Lac

The pharmaceutical compound SMX was chosen as a model compound for the reusability study. Therefore, to assess the practical application of enzyme immobilization, the reusability of GS-HC-Lac and GS-BC-Lac was evaluated over eight consecutive degradation cycles of 2, 4 and 24 h. The results (Fig. 9) showed that the degradation efficiency of GS-HC-Lac and GS-BC-Lac gradually decreased over the reuse cycles. However, the immobilized laccase still maintained significant efficiency, particularly in longer reaction times. The enzyme inactivation and the gradual leakage of enzyme molecules may explain this phenomenon [53].

GS-HC-Lac presented nearly 100 % degradation efficiency for two cycles. Degradation efficiencies of 35 %, 39 % and 100 % were verified after 2, 4 and 24 h, respectively, in the fourth cycle. With eight reuse cycles, GS-HC-Lac reached only 47 % SMX degradation. On the other hand, GS-BC-Lac showed a higher potential for reuse. Although at least 4 h were necessary for complete degradation in the second cycle, after four reuse cycles, GS-BC-Lac showed approximately 56 %, 59 % and 100 % degradation efficiency after 2, 4 and 24 h, respectively. With eight reuse cycles, GS-BC-Lac maintained a high degradation value of 66 %. These results suggested that both immobilized enzymes demonstrated reusability, though GS-BC-Lac provided greater long-term stability, particularly under shorter reaction times.

The enhanced performance of GS-BC-Lac may be attributed to increased laccase stability through covalent bonding, which helps preserve the enzyme structure, preventing denaturation and minimizing chain coiling. Similarly, in a study conducted by Rasheed et al. [48], laccase was immobilized on NH_2 -activated magnetic BC via covalent bonding, resulting in 87 % removal of aflatoxin B1 after five reuse cycles due to the enhanced enzyme stability provided by the support material. Similarly, Zou et al. [54] reported a decrease in the degradation efficiency of norfloxacin, enrofloxacin, and moxifloxacin from 85 %, 63 % and 73–49 %, 27 % and 35 %, respectively, after seven reuse cycles of laccase immobilized on magnetically modified BC. Nevertheless, both GS-BC and GS-HC-Lac systems demonstrate the operational stability necessary for real wastewater treatment applications.

3.5. Transformation products and degradation pathways

The degraded intermediates of SMX, TC and APAP were identified using HPLC-TIMS-TOF-MS. SMX and TC were incubated with immobilized laccase at pH 5.0, in the presence of $0.5 \text{ mmol} \cdot \text{L}^{-1}$ SYR, at 25°C , while APAP was transformed using only immobilized laccase. The results were identical for degradation with GS-BC-Lac and GS-HC-Lac. The TP was established by comparing spectra from the initial to final treatment stages. The molecular structures of the parent compounds and

their TP were proposed based on the accurate mass-to-charge ratios (m/z) (Table 5). Details of the MS chromatograms and TP are presented in the supplementary materials (see Figure S2–S4).

The proposed transformation pathway for SMX is shown in Fig. 10a. GS-BC-Lac and GS-HC-Lac oxidized the redox mediator SYR into 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ, m/z 169.0590) [62]. In line with other reported works [37,63], this study demonstrated that this phenolic species can further undergo a C-O coupling reaction with the parent compound (SMX, m/z 254.0603), leading to the formation of a cross-coupled product (TP-S2, m/z 404.0911). In another study, the oxidation of SMX by the laccase-SYR system resulted in the same product, and the authors observed that the growth inhibition of the green algae *Pseudokirchneriella subcapitata* was reduced to 61 % compared to untreated SMX, indicating lower toxicity [64]. Similar findings were also reported in our previous study, which used *Vibrio fischeri* as a biosensor [37].

In the case of TC degradation, six possible TP were identified, and two degradation pathways are proposed (Fig. 10b). In addition to the peak corresponding to the parent compound, a second peak with a shorter retention time was detected at the initial reaction stage, corresponding to reversible epimerization at the tertiary amine of the TC [65]. In the first pathway, TC is converted to TP-T1 (m/z 405.1278) via demethylation [66]. This intermediate then lost a carbonyl and an amine group, forming TP-T2 (m/z 370.0908) [65,67]. Finally, further

Table 5

Characteristics of the parent compound and the main TP from GS-HC-Lac and GS-BC-Lac degradation of SMX and TC (with SYR) and APAP (without mediator).

Compound	Retention time (min)	Measured mass (m/z) [$\text{M}+\text{H}$] ⁺	Elemental composition
SMX	5.6	254.0603	$\text{C}_{10}\text{H}_{12}\text{N}_3\text{O}_3\text{S}$
DMBQ	2.7	169.0490	$\text{C}_8\text{H}_9\text{O}_4$
TP-S1	9.9	406.1067	$\text{C}_{18}\text{H}_{20}\text{N}_3\text{O}_6\text{S}$
TP-S2	10.2	404.0911	$\text{C}_{18}\text{H}_{18}\text{N}_3\text{O}_6\text{S}$
TP-S3	10	216.1017	$\text{C}_{13}\text{H}_{14}\text{NO}_2$
TC	3.9	445.1606	$\text{C}_{22}\text{H}_{25}\text{N}_2\text{O}_8$
TC epimer	2.8	445.1606	$\text{C}_{22}\text{H}_{25}\text{N}_2\text{O}_8$
TP-T1	10.2	405.1278	$\text{C}_{19}\text{H}_{21}\text{N}_2\text{O}_8$
TP-T2	10.2	370.0908	$\text{C}_{19}\text{H}_{16}\text{NO}_7$
TP-T3	11.1	319.0818	$\text{C}_{16}\text{H}_{15}\text{O}_7$
TP-T4	6.2	414.1189	$\text{C}_{21}\text{H}_{20}\text{NO}_8$
TP-T5	10.6	377.1218	$\text{C}_{19}\text{H}_{21}\text{O}_8$
TP-T6	1.9	323.0768	$\text{C}_{15}\text{H}_{15}\text{O}_8$
APAP	7.4	152.0700	$\text{C}_8\text{H}_{10}\text{NO}_2$
TP-A1	12.6	301.1177	$\text{C}_{16}\text{H}_{17}\text{N}_2\text{O}_4$
TP-A2	5.1	166.0861	$\text{C}_9\text{H}_{12}\text{NO}_2$
TP-A3	5.1	120.0805	$\text{C}_8\text{H}_{10}\text{N}$
TP-A4	1.6	236.1188	$\text{C}_{10}\text{H}_{22}\text{NO}_5$
TP-A5	2.7	220.1540	$\text{C}_{10}\text{H}_{22}\text{NO}_4$
TP-A6	7.9	384.1138	$\text{C}_{13}\text{H}_{22}\text{NO}_{12}$

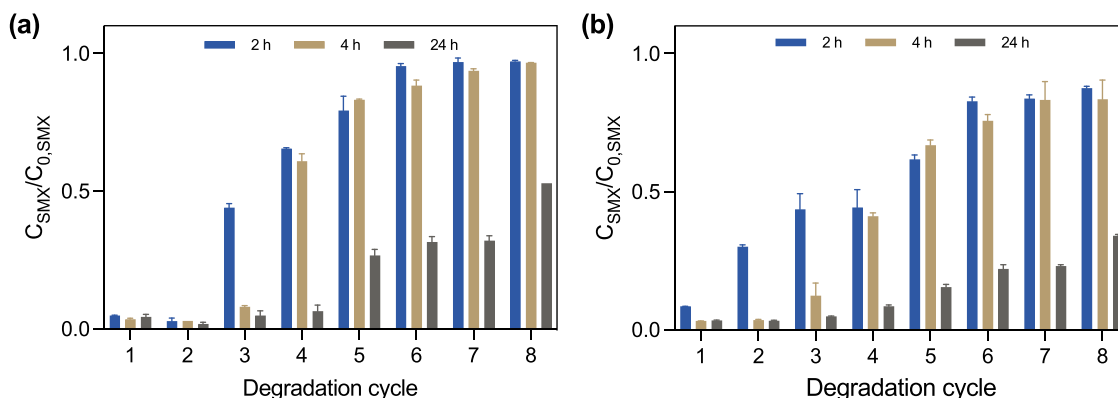


Fig. 9. Reusability of GS-HC-Lac (a) and GS-BC-Lac (b) for SMX degradation at reaction times of 2, 4 and 24 h.

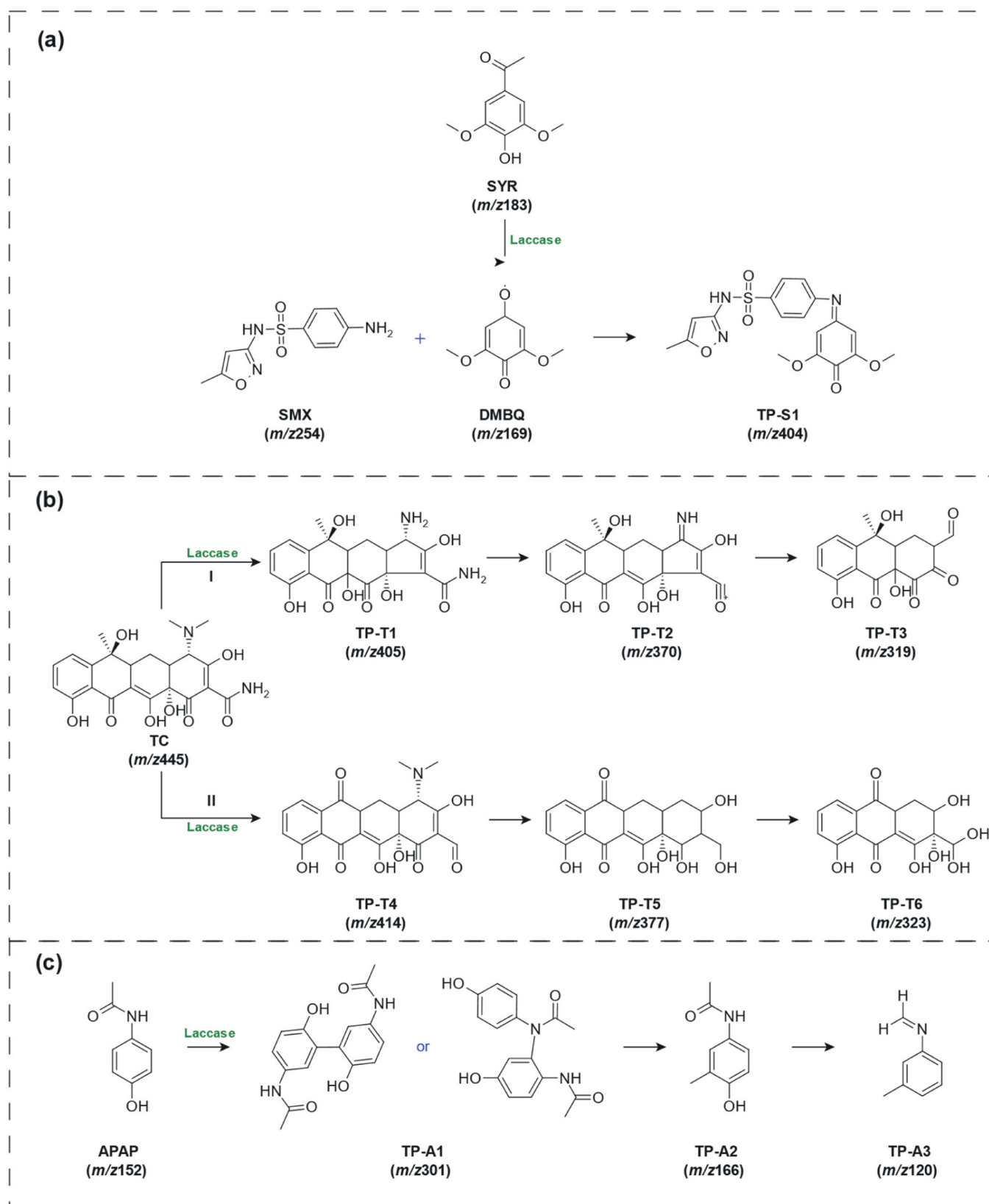


Fig. 10. Proposed metabolic pathways for the transformation of SMX (a) and TC (b) using GS-BC and GS-HC Lac-SYR systems, and APAP (c) using the GS-BC and GS-HC Lac systems.

deamination and oxidative cleavage resulted in the production of TP-S3 (m/z 319.0818) [68]. Other biotransformation studies identified the compound with m/z 370.09 as the main TP. Remarkably, Liu et al. [65] reported lower cytotoxicity and antimicrobial potency compared to the parent compound against *Bacillus subtilis* RH33 and *Escherichia coli* DH5 α . Similarly, Leng et al. [67] observed a reduction in antimicrobial potency in *E. coli* disk diffusion tests following TC treatment.

The second pathway for TC degradation was a series of demethylation and deamination reactions, leading to the formation of TP-T4 (m/z 414.1189), followed by TP-T5 (m/z 377.1218) and finally TP-T6 (m/z 323.0768).

Furthermore, the proposed degradation pathway of APAP along with its intermediates is presented in Fig. 10c. The APAP compound (m/z 152.0700) was oxidized by laccase, generating the APAP phenoxyl radical (APAP $^{\bullet}$, not detected), which subsequently underwent radical coupling. This interaction can occur at the ortho position via radical recombination, forming the APAP dimer (TP-A1, m/z 301.1177). Alternatively, coupling may occur at the amide group by forming a nitrogen-centered radical, resulting in another APAP dimer. These radical coupling reactions have also been reported in other studies involving laccase-mediated oxidation and other treatments, such as photodegradation [69,70]. Furthermore, a second major product, TP-A2 (m/z 166.0861), is observed and may have resulted from the oxidation of the APAP dimer, which led to a modified aromatic structure. This structural modification then facilitated the formation of the compound TP-A3 (m/z 120.0805) through the loss of a hydroxyl and a carbonyl group.

3.6. Enzymatic degradation of pharmaceuticals in real wastewater

To further investigate the potential practical application of GS-HC-

Lac and GS-BC-Lac, the biodegradation of SMX, TC and APAP was evaluated in a water matrix collected from a WWTP before discharge (see Table S1 supplementary materials). The wastewater presented moderate levels of chemical oxygen demand (COD, 56 mg·L $^{-1}$) and suspended solids (15 mg·L $^{-1}$), factors that may affect enzymatic performance due to increased matrix complexity and potential competition between substrates.

Enzymatic degradation in the presence of SYR mediator was conducted in a batch-stirred reactor using wastewater effluent (pH 7.9) spiked with 5 mg·L $^{-1}$ of each compound. As shown in Fig. 11, the maximum degradation of SMX and APAP in wastewater ($C/C_0 < 0.1$) required a longer time than in buffer medium (Sections 3.4.1 and 3.4.2), whereas the same was not observed for TC. This behavior is further

Table 6

Kinetic parameters predicted by the pseudo-first-order kinetic model for SMX, TC and APAP degradation using GS-HC-Lac and GS-BC-Lac, and SYR in wastewater.

Compound	Condition	k (h $^{-1}$)	$t_{1/2}$ (h)	R^2
SMX	GS-HC-Lac-	2.762	0.247	0.9983
	SYR	± 0.115	± 0.010	± 0.0362
	GS-BC-Lac-	0.569	1.219	0.9921
	SYR	± 0.076	± 0.162	± 0.0215
TC	GS-HC-Lac-	1.610	0.480	0.9722
	SYR	± 0.321	± 0.107	± 0.0131
	GS-BC-Lac-	4.138	0.185	0.9581
	SYR	± 1.233	± 0.061	± 0.0543
APAP	GS-HC-Lac-	0.887	0.782	0.9963
	SYR	± 0.063	± 0.056	± 0.1329
	GS-BC-Lac-	0.243	2.850	0.9933
	SYR	± 0.017	± 0.197	± 0.0321

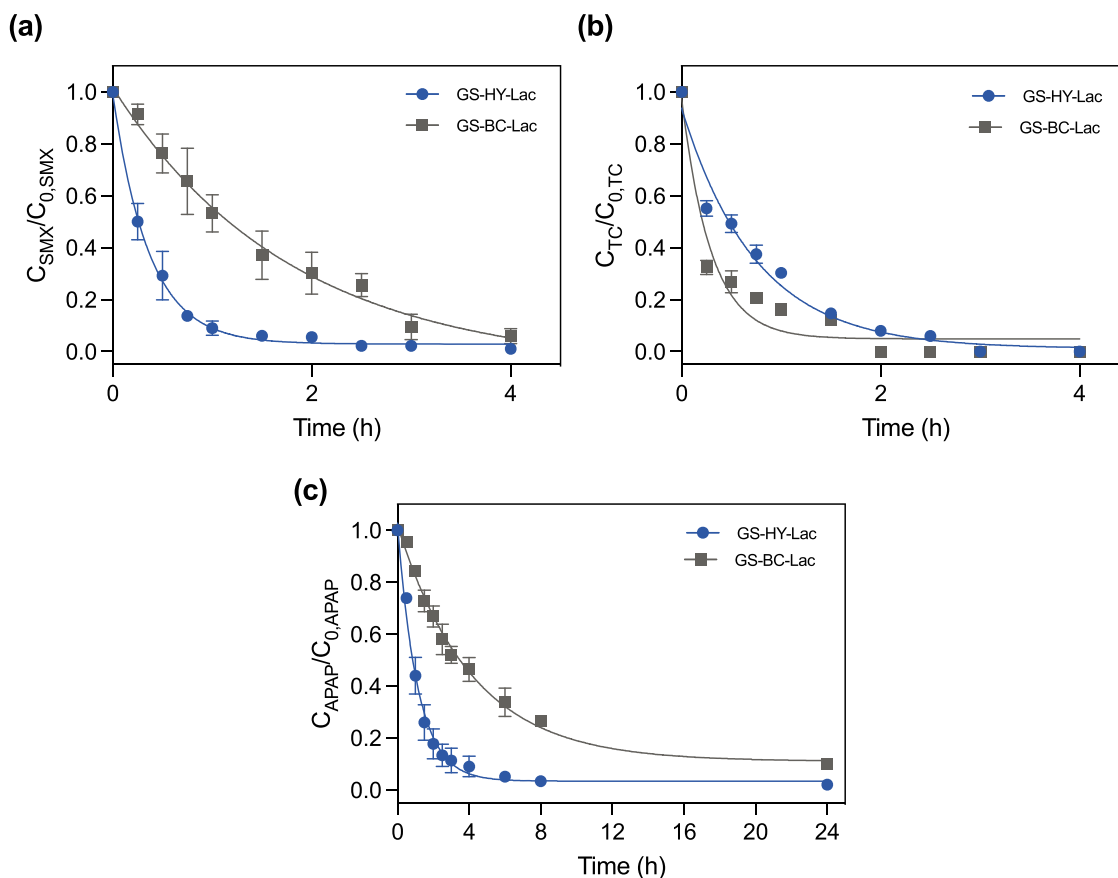


Fig. 11. Degradation of 5 mg·L $^{-1}$ SMX, TC and APAP in wastewater using GS-HC-Lac and GS-BC-Lac. Reaction conditions: 0.8 g char, 40 mL volume, 0.5 mmol·L $^{-1}$ SYR, pH 7.9, and 25 °C.

supported by the degradation rate constants derived from the pseudo-first-order model (Table 6). Despite the variations in degradation kinetics, the prepared catalysts effectively degraded pharmaceuticals from real wastewater, with GS-HC-Lac and GS-BC-Lac demonstrating satisfactory performance under these conditions.

The lower degradation efficiency may be related to the characteristics of the wastewater, including pH and the presence of organic and inorganic constituents. For example, various organic matter compounds in wastewater can be more reactive with laccase than the target pollutants, potentially hindering remediation [71]. On the other hand, the increased degradation efficiency observed for TC may result from the potential mediator effect of other compounds (SMX and APAP) present in the solution, as already reported [60].

Nevertheless, the results of this study highlight an innovative technology for pharmaceutical degradation in wastewater effluents and thus demonstrate its potential as an alternative process for WWTP. Although both GS-derived chars proved to be effective supports, with only slight differences in degradation performance and enzymatic stability, their production processes differ significantly. GS-HC may represent a more cost-effective option, requiring lower processing temperatures and yielding higher material recovery.

4. Conclusion

In this study, laccase from *Lentinus sajor-caju* was successfully immobilized onto GS-BC and GS-HC, two carbon-based materials derived from GS and processed through different methods. SEM-EDX and XPS analyses confirmed the condensation of silane groups from APTES with hydroxyl groups on the char surface, as well as the successful laccase immobilization by covalent bonding. Both GS-HC-Lac and GS-BC-Lac demonstrated enhanced pH, thermal and storage stability, ensuring improved long-term performance. GS-HC-Lac achieved complete APAP degradation in 30 min, while GS-BC-Lac required 90 min, both with SYR as a mediator. Similarly, SMX was fully degraded within 30 min, whereas TC required 5 h to reach the same level of degradation. Both immobilized laccase systems maintained high degradation efficiency over multiple reuse cycles. Even in wastewater, successful degradation was demonstrated, reinforcing their potential for practical applications.

Furthermore, this study elucidates the transformation pathways of SMX, TC and APAP, emphasizing key oxidative and coupling reactions that led to the formation of various TP. These findings highlight the potential of covalently immobilized laccase on GS-BC and GS-HC to enhance enzyme stability and reusability, thereby overcoming limitations in enzymatic treatment and enabling large-scale continuous biocatalytic applications in wastewater treatment.

CRedit authorship contribution statement

Emilio Rosales: Validation, Resources. **Maria Angeles Sanroman:** Validation, Resources. **Bruna Silva:** Writing – review & editing, Supervision. **Isabel C. Neves:** Validation, Resources. **Michele Michelin:** Writing – review & editing, Supervision. **Helena Sá:** Writing – original draft, Methodology, Investigation, Conceptualization. **Teresa Tavares:** Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.118237.

Data availability

The authors do not have permission to share data.

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