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Immobilization of laccase on hollow mesoporous carbon nanospheres: Noteworthy immobilization, excellent stability and efficacious for antibiotic contaminants removal

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ABSTRACT

In this study, the hollow mesoporous carbon spheres (HMCs) were synthesized and modified for laccase (Lac) immobilization, and the structural characteristics of HMCs materials were determined by FESEM, TEM and FTIR etc. The maximum loading of Lac on the HMCs materials could reach 835 mg/g, meanwhile, the immobilized Lac exhibited excellent thermo-stability, pH stability, storage stability and reusability. The antibiotics removal experiments indicated that the immobilized Lac possess efficient removal efficiency for both tetracycline hydrochloride (TCH) and ciprofloxacin hydrochloride (CPH) in the presence of redox mediator. The synergy of the adsorption by HMCs and the degradation by Lac could be the reasons for the high removal of antibiotics. Meanwhile, for investigating degradation mechanism, the degradation product analysis and molecular docking method had been introduced to this study. According to the degradation products, dehydroxylation and demethylation are major degradation reactions for TCH degradation, and the oxidation of the piperazinyl substituent and hydroxylation are the major degradation for CPH degradation. The docking results showed that some important residues played the key role in the degradation process. This study indicated that the immobilization of Lac on HMCs could be potentially applied in environmental remediation of antibiotics.

1. Introduction

In recent decades, the overdose and incomplete metabolism of antibiotics have made they are frequently detected in natural water, wastewater and sediment [1], which could cause environmental pollution and threaten human health [2,3]. Many methods were applied to eliminate antibiotic contaminants, including adsorption, advanced oxidation, biodegradation, enzyme degradation etc. [1,4–6]. Among these methods, enzyme degradation could be an efficient and environmentally friendly approach, such as laccase (Lac) degradation.

Lac (EC 1.10.3.2) is one of the multi-copper oxidase. It belongs to extracellular enzyme, and was first discovered in the secretions of Sumac [4,7]. In recent years, the application of Lac in bioremediation has won great attention due to the serious environmental pollution. Many studies showed that Lac can degrade a variety of pollutants due to the broad substrate specificity and high catalytic activity, especially for phenolic pollutants [8–10]. However, the complex natural conditions limits the use of free Lac on account of the low stability, poor reusability and high cost [11].

To overcome the deficiency, immobilization enzyme has become a perfect choice in increasing the stability, operability, durability and economy of Lac [12–15]. Three immobilization methods are frequently applied, which including, physical adsorption immobilization, covalent bonding and cross-linking aggregate. For each immobilization method, the advantages and disadvantages exist simultaneously [13,14]. Up to now, various materials have been used as the carrier for Lac immobilization, such as metal nanoparticles [2,12], carbon nanotube [16], nano-fibrous membrane [9,17] and mesoporous materials [11,18] etc. Among these materials, the hollow mesoporous carbon spheres (HMCs) should be a very promising immobilization carrier due to the typical the hollow core-mesoporous shell structure [19]. Particularly, the large hollow volume and inner surface are very useful for the storage of enzyme, which can increase the immobilization quantity of enzyme. Meanwhile, the shortened mesoporous shell is beneficial to the

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diffusion of enzyme in the immobilization process, and reduce the steric effects [19,20]. Moreover, the superior conductivity, biocompatibility and corrosion resistance of HMCs make them especially suitable for the application in enzyme immobilization [14,19,20].

Molecular docking is a method to predict the interaction of protein-ligands [21], generally, it is useful in computer drug design, but now frequently used in biodegradation analysis for revealing the binding modes between pollutants and enzymes due to the conveniences [22]. Although the substrates/Lac interactions could be revealed through experimental techniques, it's very inefficient and the atomic details of interaction can't be displayed. However, molecular docking can operate on the computer and predict the binding mode between ligand and receptor, which is time-saving and cost less [23]. Therefore, we could investigate the degradation mechanism of antibiotics by Lac and the stimulation effects of redox mediators in the degradation process, which were benefit to find out the degradation strategy [23].

In current experiment, the HMCs were prepared by one-pot and surfactant free synthesis firstly, and the morphology and structure properties were explored by several characterization techniques. Following, the immobilized Lac were prepared by physical adsorption immobilization or covalent bonding immobilization. Furthermore, the stability of immobilized Lac was also investigated. Then, two common antibiotics (namely TCH and CPH) were selected for studying the antibiotic removal ability of immobilization Lac on HMCs materials, and the effects of redox mediator concentration and type in the degradation process were also evaluated. Finally, to reveal the mechanism of antibiotic degradation by Lac, the degradation product analysis and molecular docking method had been introduced. Interestingly, the removal of antibiotic contaminations by the immobilized Lac on HMCs materials provided novel method for organic pollutants removal. Moreover, the degradation mechanism analysis according to the Lac combine experimental method and molecular docking method displayed interesting results.

2. Materials and methods

2.1. Materials

Lac (canary yellow powder) produced by *Trametes versicolor* was purchased from J&K Scientific (Massachusetts, USA); Tetrapropyl orthosilicate (TPOS, purity > 97%) was purchased from Aladdin industrial corporation (Shanghai, China); TCH and CPH were purchased from Bomei biological technology Co, Ltd (Hefei, China); Syringaldehyde (SA), 1-hydroxybenzotriazole (HBT) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), glutaraldehyde (GTA) were purchased from Dibo reagent corporation (Shanghai, China); Some parameters were listed in Table S.1 and the structures were showed in Fig. S.1. All other chemicals were analytical grade and used as received. The ultrapure water was used throughout the experiment with resistivity was 18.25 MΩ/cm.

2.2. Synthesis of HMCs, HMCs-NH_2

The synthesis steps of HMCs was described in previous report [24], the amino-functionalization of HMCs was referred to [25], and the details were showed in Supporting information (SI).

2.3. Immobilization of Lac

Two immobilization methods, namely, physical adsorption immobilization and covalent binding immobilization had been performed in this study, and the details were described in SI. Meanwhile, for investigating the properties of Lac immobilized on HMCs materials, the effects of immobilization time (0–120 min) and initial Lac concentration (0.2–1.8 mg/mL) have been studied. The immobilization quantity of Lac was calculated according to Bradford method [26].

2.4. The stability of free and immobilized Lac

The stability analysis of free and immobilized Lac were performed as described in SI. The activity of free and immobilized Lac was monitored according to the previous study [18,27], and the details were showed in SI.

2.5. Removal of antibiotics

The removal of antibiotics (TCH and CPH) was performed at 30 °C and 150 rpm for 3 h, and the samples were prepared in triplicate, the details were described in SI.

2.6. Structural characterizations

The morphology and structure of the samples were characterized by field emission scanning electron microscope (FESEM, FEI, Quanta-F250) and transmission electron microscope (TEM, FEI, Tecnai-G2 20), operating at 20 kV and 200 kV, respectively. The functional groups were measured through Fourier transform infrared spectrum (FTIR, NICOLET, 5700) in KBr pellet at room temperature. The typical element was analyzed by energy dispersive spectrometer (EDS, EDAX, Genesis). N_2 adsorption–desorption isotherms were measured on Quadrasorb SI analyzer at 77 K. The specific surface area and the total pore volume were calculated based on Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) analyses, respectively.

2.7. Molecular docking

The three dimensional (3D) structures of Lac (PDBID: 1GYC [28]) was downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do), while the 3D structures of antibiotics and redox mediators were downloaded from Pub Chem Compound database (https://pubchem.ncbi.nlm.nih.gov), the some details information was shown in Table S.1. MVD, a graphical-automatic docking software with high accuracy (about 87%) and versatility, was used to dock antibiotics or redox mediators into the binding pockets of Lac [29]. LigPlot$^+$ was chosen was the optimal pose for the following interactional profile analysis, which is a program to generate principle diagrams of protein-ligand interactions [30]. It needs to indicate that only hydrophobic interactions and hydrogen bonds can be shown by LigPlot$^+$. The parameters were set as described in the SI.

3. Results and discussion

3.1. Morphology and structure of HMCs

As shown in SEM images (Fig. 1A and B), the HMCs were uniform sphere, and many open entrances distributed on the surface of the HMCs. Therefore, the Lac could enter the HMCs by these entrances and immobilize inner surface, which is beneficial to the immobilization and the stability of Lac. The hollow morphology and the porous shell are indicated by the TEM image, and the pore channels in the shells were disordered (Fig. 1D), which is a sharp contrast to the carbon material before etching (Fig. 1C). According to measurement by Nano Measurer software, the average diameter of HMCs was about 323 nm (the standard deviation was about 12.3 nm), and the average thickness of shell was about 57 nm (the standard deviation was about 3.7 nm). Such a large cavity provided a huge storage space for Lac. Furthermore, the mesoporous character was verified by the N_2 adsorption-desorption isotherms (Fig. S.2A). These figures revealed clearly that both pristine HMCs and HMCs-NH_2 followed the type-IV isotherm with a H1 hysteresis loop according to IUPAC classification, which represented the material was mesoporous material. Additionally, the pore diameter distribution showed the pore diameter of pristine HMCs and HMCs-NH_2 mainly range at 2–40 nm and 2–30 nm, respectively. The average pore

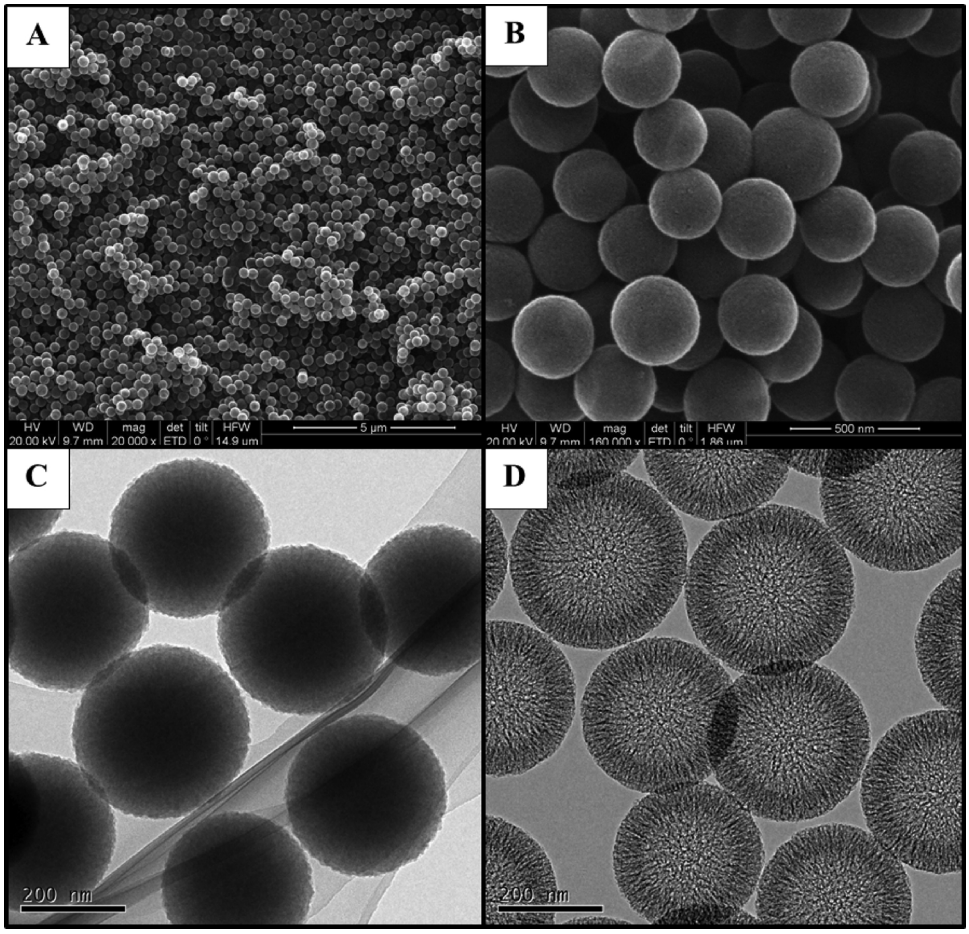

Fig. 1. A and B are SEM images of HMCs; C (before etching) and D (after etching) are TEM images of HMCs.

Table 1
Pore structure parameters of HMCs and HMCs-NH_2.

Sample	BET surface area (m^2/g)	Average pore diameter (nm)	Pore volume (cm^3/g)
HMCs	484.7	11.8	1.43
HMCs-NH_2	1090.9	13.5	3.69

diameter (PD) was calculated based on the N_2 adsorption branch, and the value was 11.8 nm and 13.5 nm, respectively. Therefore, such pore channel size was larger than the size of Lac (about 6.5 × 5.5 × 4.5 nm [28]), which was very beneficial to the immobilization of Lac in HMCs materials. Meanwhile, the BET surface area (BET) and pore volume (PV) were also calculated (Table 1), the values were 484.7 m^2/g and 1.43 cm^3/g for pristine HMCs, respectively. However, after the amino-functionalization of pristine HMCs (HMCs-NH_2), the BET and PV became greater, namely 1090.9 m^2/g and 3.69 cm^3/g, respectively. These results indicated that the performance of amino-functionalization was conducive to the structure properties of HMCs. The high PD, BET and PV could both facilitate the adsorption of Lac and antibiotics in these HMCs materials. Furthermore, several typical elements and the functional groups on HMCs materials were analyzed by EDS and FTIR, respectively. The results indicated that C, N and O were the main elements in HMCs materials, and abundant functional groups distributed on the surface HMCs materials, and the details were described in SI.

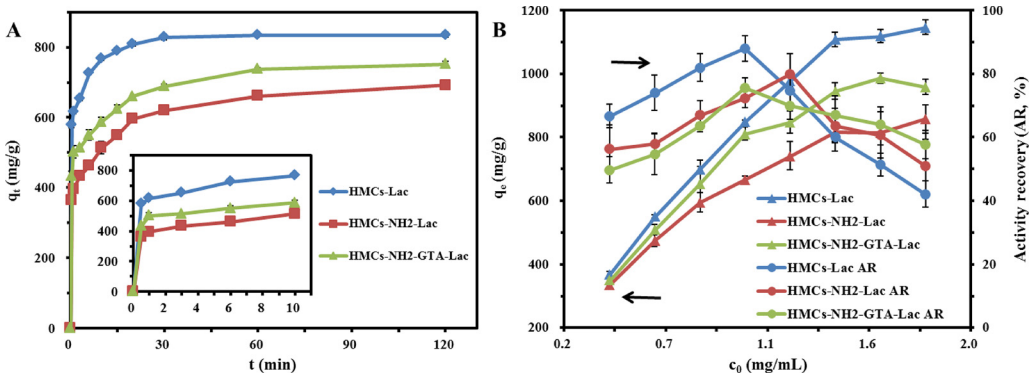

Fig. 2. The change of Lac loading on HMCs materials over time (A); The change of Lac loading and activity recovery on HMCs materials with the increase of initial Lac concentration (B).

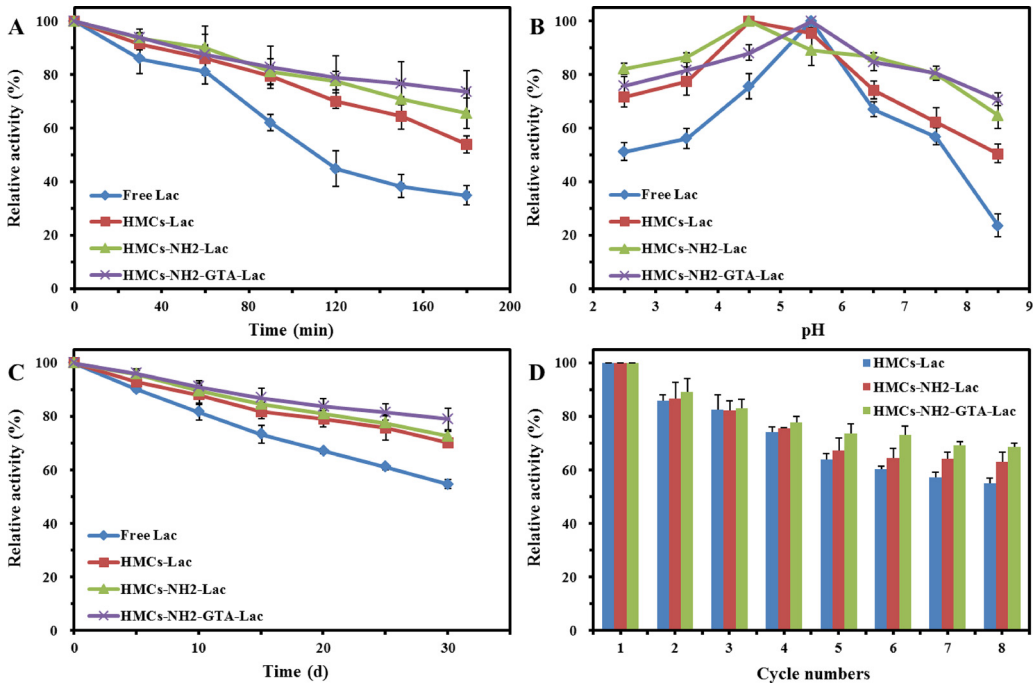

Fig. 3. The thermal stability (A), pH stability (B), storage stability (C) and reusability (D) of free Lac and immobilized Lac.

3.2. Immobilization of Lac on HMCs

The immobilization of Lac on HMCs has been carried out through physical absorption and covalent binding. The immobilization behavior of Lac on pristine and modified HMCs have been investigated and compared. Firstly, the Lac loading varies with time on three carriers (namely pristine HMCs, HMCs-NH_2 and HMCs-NH_2-GTA) was shown in Fig. 2A. The maximum loading of Lac on pristine HMCs, HMCs-NH_2 and HMCs-NH_2-GTA was 835, 692 and 752 mg/g, respectively. The immobilization capacity of Lac by HMCs materials was far more than that reported in the previous studies [16,18,31,32], and the comparison was listed in Table S.2. The excellent loading by HMCs might be due to the high BET, PD, PV (Table 1) and the special mesoporous shell-hollow cavity structure. Meanwhile, rich functional groups on HMCs materials might be another reason. However, according to the results, the loading of Lac on HMCs-NH_2 and HMCs-NH_2-GTA was less than that on pristine HMCs, the order was HMCs-Lac > HMCs-NH_2-GTA-Lac > HMCs-NH_2-Lac, which might be due to the changes of the types and quantities of functional groups on HMCs after modifying, as shown in Fig. S.2B. When the pristine HMCs was modified by $NH_3{\cdot}H_2O$ (HMCs-NH_2), the −OH group reduced obviously but the $-NH_2$ group became more abundant, which was not conducive to the immobilization of Lac on HMCs by adsorption, thus the loading of Lac on HMCs-NH_2 decreased [13]. However, when GTA was grafted onto the HMCs-NH_2 (HMCs-NH_2-GTA), the Lac could be immobilized on the HMCs material by covalent binding, which was a stable method to reduce the loss of immobilized Lac [13,14], therefore the Lac loading on HMCs-NH_2-GTA was higher than that on HMCs-NH_2. Meanwhile, the Lac aggregation appeared easily when Lac immobilized on the HMCs material by covalent binding, which could block the small channel on shell and reduce the diffusion of Lac into the hollow cavity [14], therefore the decreasing Lac loading on HMCs-NH_2-GTA occurred in comparison to that on pristine HMCs. Furthermore, the effects of initial Lac concentration and the activity recovery of Lac immobilized on HMCs materials have been investigated. As shown in Fig. 2B, the loading of Lac on HMCs materials increased rapidly when the concentration was less than 1.4 mg/mL, but increased a little after 1.4 mg/mL. The reason might be that the binding sites distributed on HMCs material were sufficient for Lac when the concentration was less than 1.4 mg/mL; however, the binding sites became scarce when Lac concentration was high. Some similar results have been reported previously [16,18]. Additionally, the activity recovery of HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac has been compared (Fig. 2B). The activity recovery increased firstly, and then decreased. The HMCs-Lac gave the highest activity recovery, namely 88.0% when the Lac concentration was l.0 mg/mL. However, the activity recovery of HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac was lower than that of the HMCs-Lac, namely 80.0% at l.2 mg/mL Lac and 75.7% at l.0 mg/mL Lac, respectively. The reason might be that covalent binding could cause irreversible deactivation of the enzyme by chemical modification or change of conformation, which could suppress Lac activity in some degree [14]. However, the suppression effect would not happen when immobilized Lac on carriers by adsorption, therefore, the HMCs-NH_2-GTA-Lac showed the lowest activity recovery [13,14]. Compared to the activity recovery of HMCs-Lac, the HMCs-NH_2-Lac showed lower activity recovery. The reason might be that the Lac loading on HMCs was higher than that on HMCs-NH_2, thus more active site would be exposed to substrate molecule (namely ABTS), which would exhibit higher activity recovery [33].

In this study, two adsorption kinetic models (i.e. pseudo-second-order (PSO) (Fig. S.4A) and intraparticle diffusion (ID) kinetic models (Fig. S.4B)) and two adsorption isotherm models (i.e. Langmuir (Fig. S.4C) and Freundlich (Fig. S.4D) isotherm models) have been introduced to elucidate the immobilization process of Lac on HMCs materials. According to the simulations, the PSO model and Langmuir isotherms were more suitable for the immobilization of Lac, and the details were described in the SI.

3.3. The stability of free Lac and immobilized Lac

After the successful immobilization of Lac on three HMCs materials, the stabilities of immobilized Lac were investigated, which were important and prerequisite when applied the biocatalyst in practical industrial. As shown in Fig. 3A, the three immobilized Lac have shown more excellent thermal stability compared to free Lac. The relative enzyme activity of free Lac only retained 35% after 180 min at 60 °C.

However, the highest relative activity of HMCs-NH_2-GTA-Lac was up to 74%. The influence of pH on the activity of free and immobilized Lac were evaluated in the pH range from 2.5 to 8.5 (Fig. 3B). As displayed in the graph, the pH stability of the three immobilized Lac were superior to that of the free Lac obviously. The maximal activity of the free Lac was presented at pH 5.5. Too acid (pH < 3.5) and too alkali (pH > 7.5) were both not conducive to Lac activity. Nevertheless, the activity of immobilized Lac could maintain at a high level over a broad pH range (2.5–8.5). Fig. 3C showed the residual activity of immobilized Lac and free Lac. Almost half of the initial activity has been lost for free Lac, and the activity only remained 54%. However, the higher activity was preserved when immobilized the enzyme on carriers, and the retained activity were 70%, 73% and 79% for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively. Except for the excellent thermostability, pH stability and storage stability, outstanding reusability is also needed for the practical application of immobilized Lac. The reusability capacity of the three immobilized Lac after eight cycle experiments were displayed in Fig. 3D, the HMCs-NH_2-GTA-Lac had the optimal reusability. These results indicated that the HMCs materials are very suitable for the immobilization of Lac, which could significantly improve the stability of Lac.

3.4. Effective removal of antibiotics by immobilized Lac

Many previous studies have shown that mesoporous carbon can effective removal various pollutants by adsorption, such as dyes, heavy metals and phenols etc. [34–36]. Firstly, the adsorption capacity of the inactivated immobilized Lac for the antibiotics had been studied, and the results were showed in Figs. 4 and 5A. As shown in these graphs, the inactivated HMCs-Lac composite have shown an excellent adsorption capacity for antibiotics. The maximum removal efficiency reached to 55% and 77% for TCH (Fig. 4A) and CPH (Fig. 5A) by inactivated HMCs-Lac, respectively. However, the inactivated HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac possessed slightly lower adsorption capacity than inactivated HMCs-Lac for TCH and CPH removal. The reason might be that the amino-functionalization have changed some properties of pristine HMCs. Less functional groups, such as −OH group, existed in modified HMCs materials (Fig. S.2B), which lowered the adsorption of antibiotics by HMCs. Additionally, previous study reported that tetracycline and quinolone antibiotics were easily adsorbed due to their high K_d values [1].

The excellent adsorption capability of HMCs materials can not only remove antibiotics from aqueous phase quickly, but also be convenient for the degradation of antibiotics by immobilized Lac. In addition, the removal of antibiotics by normal immobilized Lac was investigated further. As shown in Figs. 4B and 5 B, the increased removal efficiency was not obvious, only increase by 3.5%, 7.9%, 12.1% for TCH removal and 4.1%, 3.7%, 6.3% for CPH removal by HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac respectively, compared to the corresponding inactivated immobilized Lac. These results indicated the unconspicuous degradation of TCH and CPH by Lac alone. The reason might be that the redox potentials of antibiotics were higher than that of Lac, therefore Lac couldn't oxidize them effectively [37,38]. Similar results have been reported previously [1,39].

To improve the oxidative ability of Lac and increase the degradation of substances, generally, some low molecular weight mediators, such as SA and HBT, were added to the Lac-oxidation system [1,40]. The main mechanism was that the Lac could oxidize mediators to high-activity radicals, which could oxidize compound efficiently. Meanwhile, the mediator act as "electrons shuttles" between the enzyme and target contaminants, which enhanced the oxidation of contaminants that did not enter the active sites of the enzyme due to steric hindrances [37,40]. As shown in Fig. 4C and D, when mediators were added to the immobilized Lac-TCH system, the removal efficiency of TCH increased obviously along with the increase of mediator concentration. In the presence of SA (Fig. 4C), the maximum removal efficiency of TCH was 98.1%, 99.4%, and 99.2% for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively with 3 mmol/L SA. Compared to the SA, HBT showed a smaller stimulation for TCH removal (Fig. 4D). The maximum removal efficiency reached 93.8%, 97.6%, and 99.1% for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively, with 3 mmol/L HBT. These results indicated that the superiority of SA in accelerating TCH removal in the Lac-oxidation system. Similarly, some previous studies also indicated that SA mediator had a remarkable

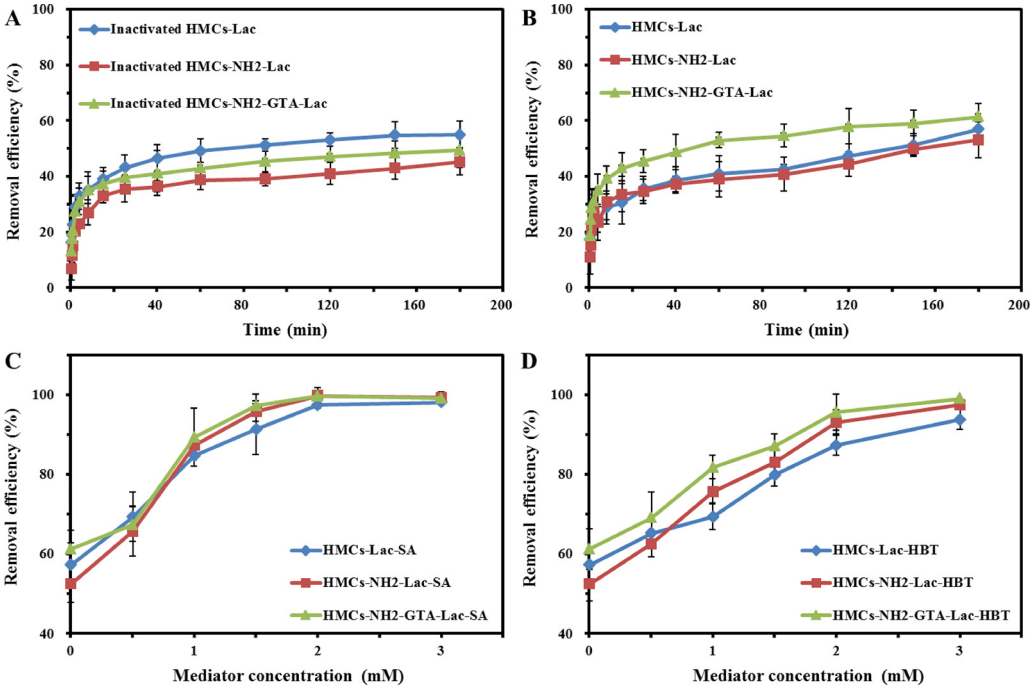

Fig. 4. The removal of TCH by inactivated immobilized Lac (A) and normal immobilized Lac without redox mediators (B); The removal of TCH by normal immobilized Lac in the presence of SA (C) and HBT (D).

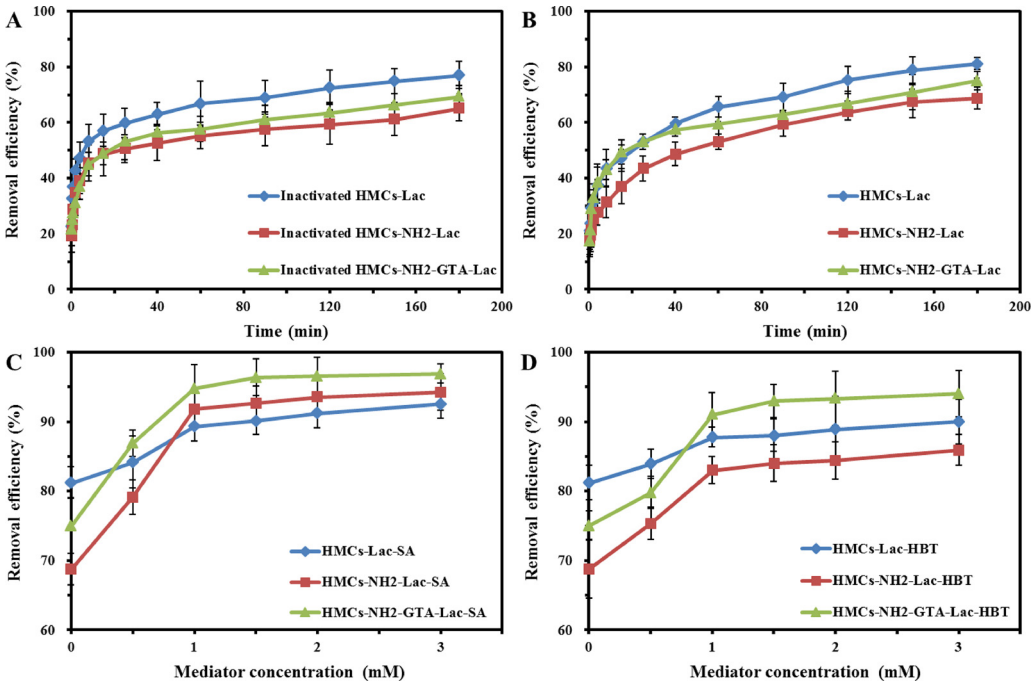

Fig. 5. The removal of CPH by inactivated immobilized Lac (A) and normal immobilized Lac without redox mediators (B); The removal of CPH by normal immobilized Lac in the presence of SA (C) and HBT (D).

promotion effect in Lac-tetracycline antibiotics system [1,41]. Meanwhile, the different promotion effects of SA and HBT could be due to the different structure (Fig. S.1), the different affinity and the different mechanism of mediators in Lac-TCH system [1,2,38,41]. However, the increase of CPH removal efficiency was slightly lower when the mediators were added to the Lac-oxidation system (Fig. 5C and D). Compared to no HBT, the increased removal efficiency of CPH was 8.8%, 17.1%, and 19.0% for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively, at 3 mmol/L HBT (Fig. 5C). With addition of 3 mmol/L SA, the removal efficiency was increased by 11.4%, 25.5%, and 21.9% for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively (Fig. 5D). The final removal efficiency achieved to 90.0%, 85.9% and 94.0% in the presence of HBT, and 92.55%, 94.2% and 96.9% in the presence of SA, for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively. The different degradation efficiency of TCH and CPH in Lac-oxidation systems might be due to their different structures. Namely, tetracycline antibiotics had the phenol structure, which were the main structures that tended to be oxidized by Lac. However, these structures were inexistent in quinolone antibiotics (Fig. S.1) [1]. These results indicated that the high removal efficiency of antibiotics by HMCs-Lac composite have obtained due to the synergy of the adsorption removal of HMCs and the Lac degradation. Meanwhile, the different mediators could accelerate the degradation of antibiotics by Lac in different degree and the acceleration of mediator in Lac-antibiotics degradation system did not always happened.

The reusability is an important parameter for evaluating the practical application of immobilized enzyme. To investigate the reusability of HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, the TCH removal experiments of these immobilized enzymes were performed for five cycles in the presence of 3 mmol/L SA. After each cycle, the immobilized enzymes were recycled by centrifugation for next run. As showed in Fig. S.5A, the TCH removal efficiencies were reduced by 23.95%, 19.33% and 12.89% for HMCs-Lac, HMCs-NH_2-Lac and HMCs-NH_2-GTA-Lac, respectively, after five cyclic experiments. It was obvious that the HMCs-NH_2-GTA-Lac showed the highest reusability, these results were consistent with the above experiment results. The FTIR image (Fig. S.5B) manifested that a new absorption band at 2366 cm^{-1} could be ascribed to ketone structures after the immobilized enzymes were used, which could be due to the adsorption of TCH or the degradation products [42,43]. Meanwhile, the types of functional groups on immobilized enzymes almost had no significant change, but there were some changes of the absorption bands of these functional groups, it could be that the loss and/or inactivation of Lac after the cyclic experiments. Furthermore, the FESEM images (Fig. S.6) displayed that the morphology of the immobilized enzymes were unchanged. However, some HMCs carriers were broken, which could be caused by centrifuging for many times. These results verified that the immobilized enzymes performed an magnificent reusability and had great potential in practical application.

3.5. Degradation products and the proposed transformation pathway

In this study, some major degradation products are listed in Tables S.5 and S.6. As shown in Table S.5, five degradation products, namely TP431, TP415, TP401, TP371 and TP344 were produced in the degradation process of TCH antibiotic. In some previous studies, the same or similar degradation products had been identified in the TCH-Lac degradation system [38,44,45]. According to these degradation products, two possible degradation pathways were proposed as shown in Fig. 6. (a) TCH could be transformed to anhydrotetracycline (TP415) by dehydroxylation [44,45], then the TP415 transformed to TP401 by bi-demethylation [38,44], subsequently, the loss of amino group and oxidation resulted in TP327; (b) TCH could be oxidized to the corresponding ketone (oxytetracycline), and then the TP431 could be formed by bi-demethylation [38,44]. For CPH antibiotic, four degradation products, including TP348, TP306, TP280 and TP263 had been identified (Table S.6), the results are similar to previous studies [46–49], and the possible degradation pathways were proposed as shown in Fig. 6. In detail, the net loss of C_2H_2 at the piperazinyl ring of CPH resulted in the formation of TP306, then the further oxidation of the piperazinyl ring with a loss of a C_2H_5N fragment producing TP263 [46–48]. Or, the CPH could transform to the TP348 after hydroxylation, and then the piperazinyl ring was substituted by hydroxyl group to form TP280 [47,49].

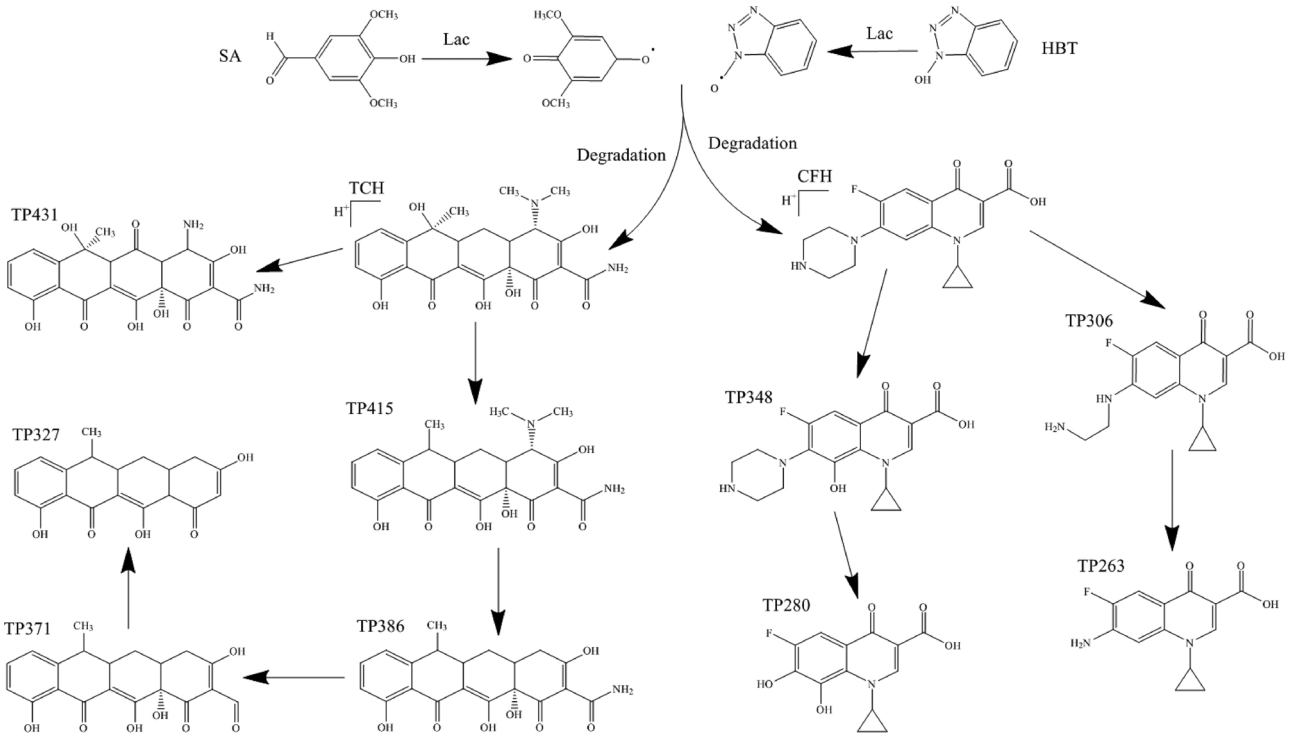

Fig. 6. The proposed degradation pathway of TCH (left) and CPH (right) by Lac.

3.6. Results of molecular docking

To explore the interactive mechanisms between Lac and antibiotics or redox mediators, molecular docking had been used. The docking results were showed in Fig. 7, Fig. S.7 and S.8, Table 2. According to Fig. S.7, the cavity volume of optimal binding pocket was 96.8 Å, it was like an along tunnel-shaped cavity and was located on near the surface of Lac, which is conducive to the interactions between Lac and substrates and improve the degradation of substrates. Further, the binding modes between Lac and two antibiotics were shown in Fig. 7. As we can see, the binding modes between Lac-TCH and Lac-CPH were radically different (Fig. 7A, B). The binding between TCH and Lac happened in front of the active pocket, which is closer to the surface of Lac (Fig. 7C), however, the binding between CPH and Lac was embedded into the active sites (Fig. 7D). The different conformations and binding orientations could represent the biodegradation possibility of antibiotics by Lac, the binding happened in the surface of Lac would more likely to be degraded, which was consistent with the results of the experiments, namely, TCH was more easily to be degraded by Lac compared with CPH. Meanwhile, the binding details between Lac and antibiotics were analyzed based on the scoring function (i.e. MolDock score and Re-Rank score), and the results were shown in Table 2. The MolDock score values of the most favorable conformations for two complexes, i.e. Lac-TCH and Lac-CPH, were −93.1 and −131.5 kcal/mol respectively. According to Re-Rank scoring function, the values were −61.1 and −100.1 kcal/mol for Lac-TCH and Lac-CPH respectively. The conformation with lower score could represent better binding affinity and robustness between Lac and antibiotic, but it is not conducive to the degradation of antibiotic by Lac [21,23]. However, two scoring function both showed that Lac-TCH conformation possess higher score compared with Lac-CPH, namely, TCH is more easily to be degraded by Lac than CPH, which verified the reliability of the experimental results again. Some others values, such as interaction and docking score, also had shown the similar tendency with MolDock score and Re-Rank score, namely, the values of Lac-TCH were higher than those of Lac-CPH, however, the HBond value of Lac-TCH was slightly lower than Lac-CPH. For understanding the degradation mechanism of antibiotic by Lac more clearly, the key amino acid residues involved in the docking process were analyzed by using the software LigPlot$^+$ (VersionV.1.4.5), the results were showed in Fig. 7E and F clearly. As shown in Fig. 7E, there was 11 important residues shown in Lac-TCH interaction diagrams, among of them, residues Glu460, Pro346, Arg157, Leu58, Ala80, Leu112, Phe81 and Glu496 were participated in hydrophobic interactions, and residues Tyr491, Gln499 and Ser113 were important for hydrogen bonding. However, in Lac-CPH interaction diagrams, the hydrophobic interactions involved residues Glu460, Pro346, Leu112, Ser113, Ala80, Phe450, Leu459 and Phe344, hydrogen bonding involved only residue His111 was displayed. As we can know that for different antibiotic the different residues in Lac will participate in the degradation process, meanwhile, the same residue could play a different role in different antibiotic degradation process.

According to Fig. S.8, the docking results between Lac and redox mediators also were showed intuitively. The binding modes for Lac-SA and Lac-HBT had a strong resemblance (Fig. S.8A, B), but differently, the conformation of Lac-SA was close to the surface of Lac, however, conformation Lac-HBT was buried in Lac (Fig. S.8C, D). That might be one reason that SA gave a better promoting effect in the antibiotic degradation by Lac than HBT. Further, the MolDock score values of Lac-SA and Lac-HBT were -77.5 and -91.2 kcal/mol respectively, the Re-Rank score values were -63.0 and -74.1 kcal/mol respectively, the values of Lac-SA higher than Lac-HBT under two scoring function. The higher score verified that SA could be easily oxidized by Lac than HBT, and more free radicals are produced to promote the degradation of antibiotics. Meanwhile, the values of interaction, HBond and docking score also were showed in Table 2. As we can see from the Table 2, the values of Lac-redox mediators conformation were most higher than Lac-antibiotics conformation for each index, which might be the reason that redox mediators could be a “promoter”. Finally, the key amino acids between Lac and redox mediators were showed in Fig. S.8E, F. The hydrophobic interactions were the main interaction between Lac and HBT, involving residues Ala80, Leu112, Pro79, Ser113, Pro346, Glu460, Phe344, Leu459 and Phe450. For Lac-SA, residues Ala80, Leu112, Pro79, Ser113, Phe81, Pro346, Glu460, Phe344, Leu459 and Phe450 were participated in hydrophobic interactions, and residue

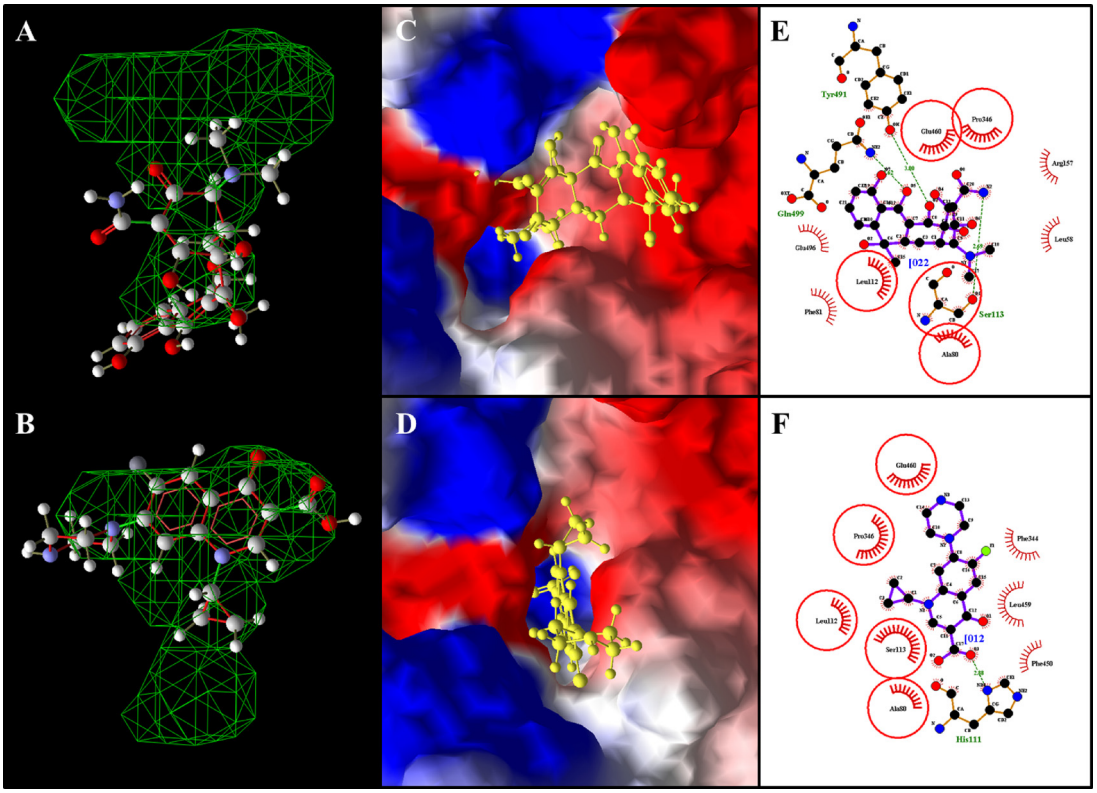

Fig. 7. Binding modes between Lac and two antibiotics. A and B display the binding orientations of TCH and CPH in binding pockets, respectively. C and D display the close-up view of TCH and CPH binding in Lac. E and F display the detailed interactions of Lac-TCH and Lac-CPH complex, respectively. The antibiotics are clearly showed in ball-stick model. The green dashed lines represent the hydrogen bonds between Lac residues and antibiotics, and the numbers on the lines show the length (Å) of the hydrogen bonds. The red spoked arcs represent the hydrophobic interactions. The black and green numbers represent the residues name. The residue in red circle represent the same residue occur in Lac-TCH and Lac-CPH complex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The docking information between laccase and ligands.

Complexes	ReRank score (kcal/mol)	MolDock score (kcal/mol)	Interaction (kcal/mol)	HBond (kcal/mol)	Docking score (kcal/mol)
Lac-TCH	−61.1	−93.1	−102.9	−7.9	−82.1
Lac-CPH	−100.1	−131.5	−130.3	−7.1	−118.2
Lac-SA	−63.0	−77.5	−78.4	−10.4	−74.8
Lac-HBT	−74.1	−91.2	−92.2	−6.9	−84.7

Notes: "Interaction" represents the total interaction energy between the pose and enzymes; "HBond" represents Hydrogen bonding energy; "Docking score" represents the score assigned to the pose during the docking.

His111 was important for hydrogen bonding. The results indicated that hydrophobic interactions were dominant in the interaction between Lac and redox mediators. According to molecular docking study, we could know that the discriminating degradation of antibiotics by Lac not only due to the different properties of antibiotics, but the key amino acids play an important role in the degradation process. This study was helpful in understanding the antibiotics biodegradation mechanisms by Lac and the stimulation effects of redox mediators in the degradation process.

4. Conclusions

In summary, the HMCs were successfully prepared and modified for Lac immobilization. The maximum loading of Lac on HMCs materials reached 835 mg/g. The stability analysis indicated that the immobilized Lac possess excellent thermo-stability, pH stability, storage stability and reusability. Furthermore, the immobilized Lac exhibited 99.4% and 96.9% highest removal efficiency for TCH and CPH, respectively, in the presence of 3 mM SA redox mediator. The synergy effect of HMCs adsorption and Lac degradation should be responsible for the high antibiotic removal efficiency by immobilized Lac. Finally, the degradation product analysis and molecular docking method were employed for studying the degradation mechanism. The results indicated that dehydroxylation and demethylation are the major degradation reactions in TCH degradation, and the piperazinyl substituent oxidation and hydroxylation are the major degradation reactions in CPH degradation. Meanwhile, some key residues played a significant role in the degradation process, especially for hydrophobic interaction and hydrogen bonding. This study indicated that the immobilization of Lac on HMCs have shown a great application potential in environmental remediation.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.08.069.

References

[1] H. Ding, Y. Wu, B. Zou, Q. Lou, W. Zhang, J. Zhong, L. Lu, G. Dai, Simultaneous removal and degradation characteristics of sulfonamide, tetracycline, and quinolone antibiotics by laccase-mediated oxidation coupled with soil adsorption, J. Hazard. Mater. 307 (2016) 350–358.

[2] L. Shi, F. Ma, Y. Han, X. Zhang, H. Yu, Removal of sulfonamide antibiotics by oriented immobilized laccase on Fe3O4 nanoparticles with natural mediators, J. Hazard. Mater. 279 (2014) 203.

[3] Y. Liu, G. Zeng, H. Zhong, Z. Wang, Z. Liu, M. Cheng, G. Liu, X. Yang, S. Liu, Effect of rhamnolipid solubilization on hexadecane bioavailability: enhancement or reduction? J. Hazard. Mater. 322 (2017) 394.

[4] R. Sarma, M.S. Islam, A.-F. Miller, D. Bhattacharyya, Layer-by-layer-assembled laccase enzyme on stimuli-responsive membranes for chloro-organics degradation, ACS Appl. Mater. Interfaces 9 (2017) 14858–14867.

[5] S. Zargari, R. Rahimi, A. Ghaffarinejad, A. Morsali, Enhanced visible light photocurrent response and photodegradation efficiency over TiO2-graphene nanocomposite pillared with tin porphyrin, J. Colloid Interface Sci. 466 (2016) 310–321.

[6] R. Rahimi, S. Zargari, A. Yousefi, M.Y. Berijani, A. Ghaffarinejad, A. Morsali, Visible light photocatalytic disinfection of E. coli with TiO2-graphene nanocomposite sensitized with tetrakis(4-carboxyphenyl)porphyrin, Appl. Surf. Sci. 355 (2015) 1098–1106.

[7] H. Liang, F. Lin, Z. Zhang, B. Liu, S. Jiang, Q. Yuan, J. Liu, Multi-copper laccase mimicking nanozymes with nucleotides as ligands, ACS Appl. Mater. Interfaces 9 (2017) 1352.

[8] B. Shao, Z. Liu, H. Zhong, G. Zeng, G. Liu, M. Yu, Y. Liu, X. Yang, Z. Li, Z. Fang, J. Zhang, C. Zhao, Effects of rhamnolipids on microorganism characteristics and applications in composting: a review, Microbiol. Res. 200 (2017) 33–44.

[9] R. Xu, C. Chi, F. Li, B. Zhang, Laccase–polyacrylonitrile nanofibrous membrane: highly immobilized, stable, reusable, and efficacious for 2,4,6-trichlorophenol removal, Appl. Mater. Interfaces 5 (2013) 12554–12560.

[10] M. Naghdi, M. Taheran, S.K. Brar, A. Kermanshahi-Pour, M. Verma, R.Y. Surampalli, Immobilized laccase on oxygen functionalized nanobiochars through mineral acids treatment for removal of carbamazepine, Sci. Total Environ. 584 (2017).

[11] Y. Zhu, S. Kaskel, J. Shi, T. Wage, K.H.V. Pée, Immobilization of trametes versicolor laccase on magnetically separable mesoporous silica spheres, Chem. Mater. 19 (2007) 6408–6413.

[12] T.T. Xia, C.Z. Liu, J.H. Hu, C. Guo, Improved performance of immobilized laccase on amine-functioned magnetic Fe3O4 nanoparticles modified with polyethylenimine, Chem. Eng. J. 295 (2016) 201–206.

[13] F.F. María, S.M. Ángeles, M. Diego, Recent developments and applications of immobilized laccase, Biotechnol. Adv. 31 (2013) 1808–1825.

[14] Z. Zhou, M. Hartmann, Progress in enzyme immobilization in ordered mesoporous materials and related applications, Chem. Soc. Rev. 42 (2013) 3894–3912.

[15] J. Zdarta, K. Antecka, R. Frankowski, A. Zgołagrześkowiak, H. Ehrlich, T. Jesionowski, The effect of operational parameters on the biodegradation of bisphenols by trametes versicolor laccase immobilized on hippospongia communis spongin scaffolds, Sci. Total Environ. 615 (2017) 784.

[16] A.A. Kadam, J. Jang, D.S. Lee, Supermagnetically tuned halloysite nanotubes functionalized with aminosilane for covalent laccase immobilization, ACS Appl. Mater. Interfaces 9 (2017) 15492–15501.

[17] M. Taheran, M. Naghdi, S.K. Brar, E.J. Knystautas, M. Verma, R.Y. Surampalli, Degradation of chlortetracycline using immobilized laccase on polyacrylonitrile-biochar composite nanofibrous membrane, Sci. Total Environ. 605–606 (2017) 315.

[18] Y. Liu, Z. Zeng, G. Zeng, L. Tang, Y. Pang, Z. Li, C. Liu, X. Lei, M. Wu, P. Ren, Immobilization of laccase on magnetic bimodal mesoporous carbon and the application in the removal of phenolic compounds, Bioresour. Technol. 115 (2012) 21–26.

[19] G. Prieto, H. Tüysüz, N. Duyckaerts, J. Knossalla, G.H. Wang, F. Schüth, Hollow nano- and microstructures as catalysts, Chem. Rev. 116 (2016) 14056.

[20] Y. Li, J. Shi, Hollow-structured mesoporous materials: chemical synthesis, functionalization and applications, Adv. Mater. 26 (2014) 3176.

[21] Y. Zhang, Z. Zeng, G. Zeng, X. Liu, Z. Liu, M. Chen, L. Liu, J. Li, G. Xie, Effect of triton X-100 on the removal of aqueous phenol by laccase analyzed with a combined approach of experiments and molecular docking, Colloids Surf. B 97 (2012) 7–12.

[22] M. Chen, G. Zeng, P. Xu, Y. Zhang, D. Jiang, S. Zhou, Understanding enzymatic degradation of single-walled carbon nanotubes triggered by functionalization using molecular dynamics simulation, Environ. Sci. Nano 4 (2017).

[23] M. Chen, G. Zeng, Z. Tan, M. Jiang, H. Li, L. Liu, Y. Zhu, Z. Yu, Z. Wei, Y. Liu, Understanding lignin-degrading reactions of ligninolytic enzymes: binding affinity and interactional profile, PLoS One 6 (2011) e25647.

[24] H. Zhang, O. Noonan, X. Huang, Y. Yang, C. Xu, L. Zhou, C. Yu, Surfactant-Free assembly of mesoporous carbon hollow spheres with large tunable pore sizes, Acs Nano 10 (2016) 4579.

[25] C. Zhang, R. Hao, H. Liao, Y. Hou, Synthesis of amino-functionalized graphene as metal-free catalyst and exploration of the roles of various nitrogen states in oxygen reduction reaction, Nano Energy 2 (2013) 88–97.

[26] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[27] J.A. Buswell, Y. Cai, S.-t. Chang, Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by Lentinula (Lentinus) edodes, FEMS Microbiol. Lett. 128 (1995) 81–87.

[28] K. Piontek, M. Antorini, T. Choinowski, Crystal structure of a laccase from the fungusTrametes versicolor at 1.90-Å resolution containing a full complement of coppers, J. Biol. Chem. 277 (2002) 37663–37669.

[29] R. Thomsen, M.H. Christensen, MolDock: a new technique for high-accuracy molecular docking, J. Med. Chem. 49 (2006) 3315–3321.

[30] A.C. Wallace, R.A. Laskowski, J.M. Thornton, LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions, Protein Eng. Des. Sel. 8 (1995) 127–134.

[31] T.A. Nguyen, C.-C. Fu, R.-S. Juang, Effective removal of sulfur dyes from water by biosorption and subsequent immobilized laccase degradation on crosslinked chitosan beads, Chem. Eng. J. 304 (2016) 313–324.

[32] C. Chao, J. Liu, J. Wang, Y. Zhang, B. Zhang, Y. Zhang, X. Xiang, R. Chen, Surface modification of halloysite nanotubes with dopamine for enzyme immobilization, ACS Appl. Mater. Interfaces 5 (2013) 10559.

[33] B. Branchi, C. Galli, P. Gentili, Kinetics of oxidation of benzyl alcohols by the dication and radical cation of ABTS. Comparison with laccase-ABTS oxidations: an apparent paradox, Org. Biomol. Chem. 3 (2005) 2604–2614.

[34] Y. Kim, J. Bae, H. Park, J.K. Suh, Y.W. You, H. Choi, Adsorption dynamics of methyl violet onto granulated mesoporous carbon: facile synthesis and adsorption kinetics, Water Res. 101 (2016) 187–194.

[35] D. Saha, S. Barakat, S.V. Bramer, K.A. Nelson, D.K. Hensley, J. Chen, Non-competitive and competitive adsorption of heavy metals in sulfur-functionalized ordered mesoporous carbon, ACS Appl. Mater. Interfaces 8 (2016) 34132.

[36] T. Yamamoto, S.I. Kim, J. Chaichanawong, E.U. Apiluck, T. Ohmori, Removal of aqueous organic pollutants by adsorption-catalytic process using mesoporous carbon beads loaded with metal oxides, Appl. Catal. B: Environ. 88 (2009) 455–461.

[37] F. D'Acunzo, Mechanistic and steric issues in the oxidation of phenolic and non-phenolic compounds by laccase or laccase-mediator systems. The case of bifunctional substrates, New J. Chem. 30 (2006) 583–591.

[38] J. Yang, Y. Lin, X. Yang, T.B. Ng, X. Ye, J. Lin, Degradation of tetracycline by immobilized laccase and the proposed transformation pathway, J. Hazard. Mater. 322 (2017) 525–531.

[39] L. Migliore, M. Fiori, A. Spadoni, E. Galli, Biodegradation of oxytetracycline by Pleurotus ostreatus mycelium: a mycoremediation technique, J. Hazard. Mater 215–216 (2012) 227.

[40] A.I. Cañas, S. Camarero, Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes, Biotechnol. Adv. 28 (2010) 694.

[41] T. Suda, T. Hata, S. Kawai, H. Okamura, T. Nishida, Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole, Bioresour. Technol. 103 (2012) 498–501.

[42] L. Zhang, X. Song, X. Liu, L. Yang, F. Pan, J. Lv, Studies on the removal of tetracycline by multi-walled carbon nanotubes, Chem. Eng. J. 178 (2011) 26–33.

[43] S. Álvarez-Torrellas, A. Rodríguez, G. Ovejero, J. García, Comparative adsorption performance of ibuprofen and tetracycline from aqueous solution by carbonaceous materials, Chem. Eng. J. 283 (2016) 936–947.

[44] M. Llorca, S. Rodríguez-Mozaz, O. Couillerot, K. Panigoni, J.D. Gunzburg, S. Bayer, R. Czaja, D. Barceló, Identification of new transformation products during enzymatic treatment of tetracycline and erythromycin antibiotics at laboratory scale by an on-line turbulent flow liquid-chromatography coupled to a high resolution mass spectrometer LTQ-orbitrap, Chemosphere 119 (2015) 90–98.

[45] M.D. Cazes, M.P. Belleville, E. Petit, M. Llorca, S. Rodríguez-Mozaz, J.D. Gunzburg, D. Barceló, J. Sanchez-Marcano, Design and optimization of an enzymatic membrane reactor for tetracycline degradation, Catal. Today 236 (2014) 146–152.

[46] L.J. Pan, J. Li, C.X. Li, X.D. Tang, G.W. Yu, Y. Wang, Study of ciprofloxacin biodegradation by a Thermus sp. isolated from pharmaceutical sludge, J. Hazard. Mater. 343 (2017) 59.

[47] A. Prieto, M. Möder, R. Rodil, L. Adrian, E. Marco-Urrea, Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products, Bioresour. Technol. 102 (2011) 10987–10995.

[48] R.S. Sutar, V.K. Rathod, Ultrasound assisted laccase catalyzed degradation of Ciprofloxacin hydrochloride, J. Ind. Eng. Chem. 31 (2015) 276–282.

[49] H.G. Wetzstein, M. Stadler, H.V. Tichy, A. Dalhoff, W. Karl, Degradation of ciprofloxacin by basidiomycetes and identification of metabolites generated by the brown rot fungus Gloeophyllum striatum, Appl. Environ. Microbiol. 65 (1999) 1556–1563.