Enhanced biocatalysis of phenanthrene in aqueous phase by novel CA-Ca-SBE-laccase biocatalyst: Performance and mechanism

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Abstract

Enzymes-mediated biocatalysis is believed to be a promising strategy for the degradation of polycyclic aromatic hydrocarbon (e.g., phenanthrene) in aqueous phase, due to its high efficiency and environmental harmfulness. However, the reusability of enzymes-mediated biocatalysis and its requirement for redox mediators limited its application. In this paper, enhanced phenanthrene removal was achieved by using a novel biocatalyst which coencapsulating laccase and natural redox mediator soybean meal extract on calcium modified chitosan-alginate (CA-Ca-SBE-laccase) beads with a network pore structure. High degradation efficiency of 94.4 % was achieved after treating aqueous solutions of 0.5 ppm phenanthrene for 20 min with the CA-Ca-SBE-laccase beads, which was 20–30 % higher than that of free laccase and ordinary immobilized laccase beads. Both the radical pathway of oxygen-containing stable free radicals and the nonradical laccase-SBE combined substrates with higher redox potential generated in CA-Ca-SBE-laccase beads contributed to the degradation of phenanthrene, and achieved the maximum double synergy of mediator and laccase. Benefiting from redox-mediating encapsulated SBE and from Ca modification of chitosan, the biocatalyst maintained fairly stable catalytic activities over a wide range of pH values, and considerable repeatability and storage stability were obtained. This study put forward an efficient laccase, redox mediator and immobilized support cofunctionalization technology, and would provide a new perspective for the increasing application scope of enzymes-mediated biocatalysis.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) refer to a group of compounds with two or more benzene rings that are linked to form large conjugated systems with high fat solubility and relatively low water solubility [1]. PAHs have attracted worldwide attention due to their high toxicity, serious carcinogenic properties and refractory degradation [2]. The US Environmental Protection Agency (EPA) recognized 16 PAHs as priority pollutants [1]. Biochemical treatment is regarded as one of the most promising technologies to remove PAHs in aqueous environment due to its low operating cost, however, PAHs tend to retain their conjugated ring systems when they react because of their stable chemical properties and low bioavailability [3,4]. Thus, advanced oxidation processes (AOPs), one of the most efficient approaches for the *in situ* degradation of PAHs in aqueous environments, are usually used together with biochemical treatment [5–7]. Nonetheless, due to irreversible catalytic deactivation, secondary pollution and expensive treatment, the application of AOPs in practical fields is limited [8,9]. Therefore, it is of great significance to develop high efficiency and ecofriendly PAHs wastewater treatment method.

The enzymatic system has been verified to be a practicable solution in the textile, food and paper industry [10], since enzymes can catalyze specific reactions under moderate operational conditions. As opposed to other chemically catalyzed processes, enzymatic treatment consumes less water, chemicals and energy and produces less waste, although the removal efficiencies of refractory organic contaminants depend on the substrate and operational environment [11]. This system has great potential to be used as an alternative for PAHs treatment [12]. Specifically, laccase (EC: 1.10.3.2), the simplest copper-containing (T1, T2 and binuclear T3 copper atom) polyphenol oxidase, which could oxidize a wide range of aromatic and nonaromatic compounds via continuous single-electron oxidation processes stimulated by the unique redox ability of copper ions [13]. However, there are two main problems in the catalytic processes of laccase: the first one is the low redox potential of the T1 copper atom as a direct electron acceptor, which limits its catalytic activities for many refractory organic compounds [14]; the other one is the reusability of laccase, which leads to large amount of reagents waste [10].

To overcome these two challenges, laccase-redox mediator systems and laccase immobilization technology were developed. To date, many researchers have been conducted focusing on suitable redox mediators to enlarge the catalytic range of laccase [15]. Among them, the laccase oxidizes the synthetic redox mediators, such as 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) [16], 1-hydroxybenzotriazole (HBT) [17] and violuric acid (VLA) [18], forming reactive radicals that can further react with the target pollutants, which has been reported to have better catalytic ability. However, due to the fact the oxidation mechanism of these synthetic mediators by laccase is that the enzymatic removal of an electron followed by release of a proton, the laccase activity will inevitably decrease in the long-term reaction process [19]. More importantly, little attention was paid to the dissipation and toxicity caused by free redox mediators in practical applications [20,21]. As suggested by Weng et al. [22], complete elimination of target pollutants could be realized in laccase-mediator solutions within 30 min, however, the toxicity of the solution varied with the mediator used. Thus, considering the one-way oxidation mechanism and ecological effects of synthetic redox mediators, increasing attention has been paid to the study of natural redox mediators [23,24]. The feasibility and effectiveness of natural phenolic redox mediators as laccase mediators depends not only on the formation of long-living radicals but also on stable secondary species which can act as mediators [19]. One such

study by Camarero et al. [25] showed that the mediating ability of sinapic acid as a natural mediator oxidized by laccase associated with the generation of both phenoxyl radicals and phenolic dimeric products capable to act as redox mediators. Liang et al. [26] employed soybean meal extract (SBE) containing apocynin, vanillin, sinapic acid and daidzein simultaneously as a laccase natural mediator to degrade sulfadimethoxazole with comparable degradation efficiency, which exceeded the catalytic range of a simple natural redox mediator. However, understanding on the complex catalysis mechanism of SBE is still lacking, and the dispersion of SBE also can cause some environmental burdens in operational conditions. At present, although the operational stability of laccase could be improved by the development of laccase immobilization strategies, such as cross-linked laccase aggregates [27], laccase bound to magnetic nanoparticles [28], and laccase-grafted membranes [29], there is little research on how to immobilize dispersed redox mediators. Therefore, there is urgent need to study the coimmobilization technology which could effectively merge the advantages of the laccase-redox mediator system and laccase immobilization technology.

Coimmobilization technology has been widely used in medicine and the food industry [30,31]. This technology enables enzyme-enzyme, enzyme-coenzyme, enzyme-cell and cell-cell immobilization on the same carrier, enables participation in more than two steps of the enzymatic catalysis, and represents a possible alternative for the combination of laccase-redox mediator system. Qiu et al. [32] co-immobilized laccase and synthetic mediator ABTS on magnetic chitosan nanoparticles modified with amino-functionalized ionic liquid, and achieved high 2, 4-dichlorophenol removal rate of 100.0 %. In Gao et al.'s [33] study, glutaraldehyde crosslinking was used to coimmobilize the synthetic mediator TEMPO and laccase on amino-functionalized Fe_3O_4 nanoparticles. Acidic fuchsin was decolorized by these coimmobilized nanoparticles at a maximum rate of 77.4 %. However, the mentioned studies focused only on the most commonly used synthetic redox mediators, which results in high cost and a decrease in laccase activity of the coimmobilization system. What's more important is that the research on immobilization materials of laccase and redox mediators from a single perspective has not achieved the maximum synergy between laccase, redox mediators and immobilized supports. Therefore, the efficient cofunctionalization of immobilized supports, mediators and laccase is still a great challenge.

In this study, a novel Ca-modified chitosan-alginate system was constructed to coencapsulate laccase and the natural redox mediator SBE (CA-Ca-SBE-laccase) and was subsequently examined for phenanthrene degradation. Gas chromatograph-mass spectrometry (GC-MS, Shimadzu QP2020) and low-temperature excitation electron spin resonance (ESR, JES-X320) measurement were conducted to investigate its degradation mechanism.

2. Materials and methods

2.1. Fabrication of the CA-Ca-SBE-laccase biocatalyst

SBE was prepared by shaking 10 g of soybean meal with 300 mL of deionized water at 160 rpm for 4 days. The extract was then filtered through a 0.45 mm cellulose acetate membrane and stored at 4 °C for subsequent use [26]. A modified chitosan-alginate encapsulation method was used in this study. Firstly, to coencapsulate laccase and SBE, 2.5 % (w/v) sodium alginate was dissolved in 20 mL of natural mediator SBE solution and 20 mL of deionized water. The alginate-SBE solution and the alginate solution were shaken for 24 h at 120 rpm and 60 °C, and then mixed with 0.05 g, 0.1 g, 0.3 g and 0.5 g of laccase from *Aspergillus*
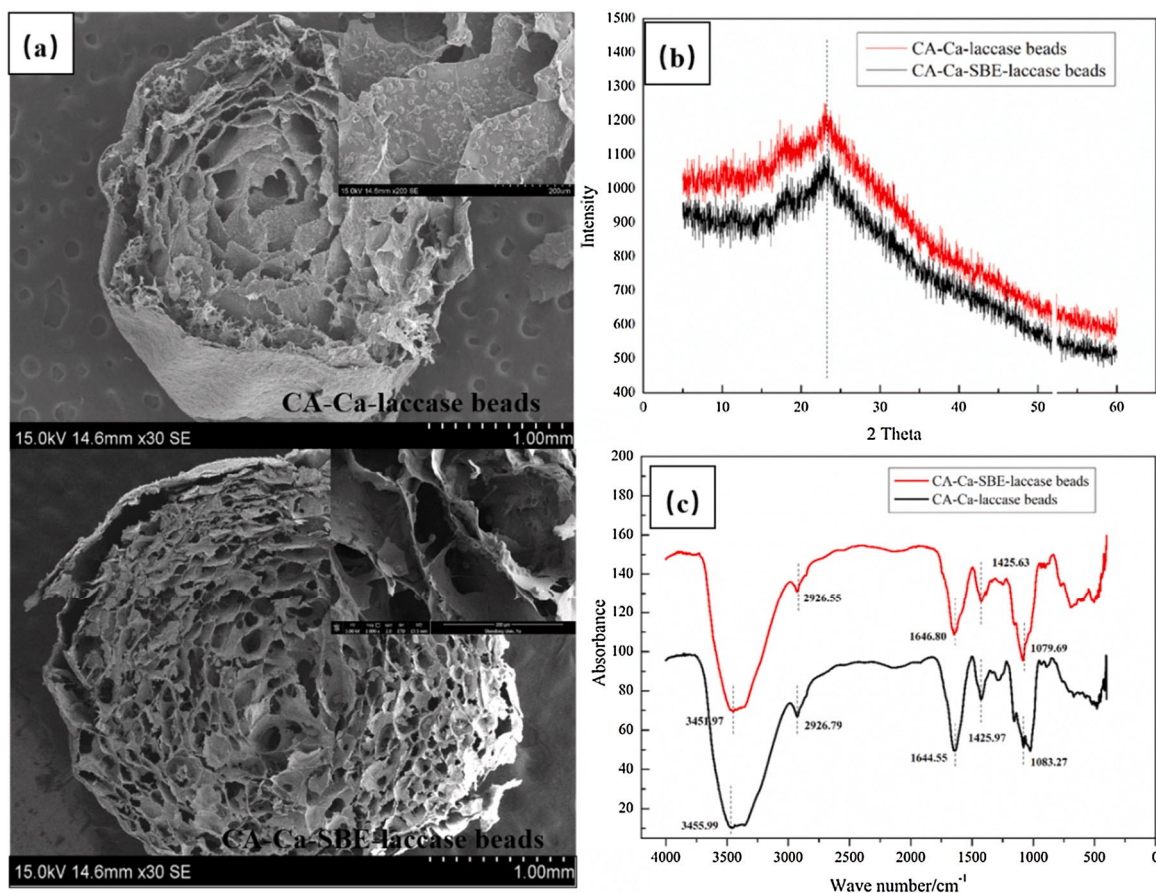
Fig. 1. SEM (a)、XRD (b)、FT-IR (c) images of CA-Ca-laccase beads and CA-Ca-SBE-laccase beads.

sp., respectively. Secondly, chitosan modified with approximately 2.0 % (w/v) chitosan was dispersed in 5.0 % (w/v) acetic acid solution by ultrasound for 30 min, followed by addition of 2.0 % (w/v) $CaCl_2$ solution. Finally, the alginate-SBE solution and the alginate solution containing different quantities of laccase were slowly added to 50 mL chitosan-$CaCl_2$ solution through a syringe needle to form spherical beads with diameter around 4 mm. The newly formed CA-Ca-SBE-laccase biocatalytic beads were hardened at room temperature for 6 h. To determine the laccase activity, experiments were conducted according to the method proposed by Bourbonnais et al. [34]. The calculation of the enzyme immobilization yield was based on the formula proposed by Wehaidy et al. [35].

2.2. Characterization of the CA-Ca-SBE-laccase biocatalyst

The morphologies of CA-Ca/SBE-laccase beads and their cross-section were characterized by scanning electron microscopy (SEM, FEI Quanta 250 FEG) after gold plating. X-ray diffraction (XRD, D8 Advance) was used to explore the crystal structure of CA-Ca/SBE-laccase beads. Fourier transform infrared (FT-IR) spectra were recorded in the range of 400−4000 cm^{-1} by an FT-IR spectrometer (Nicolet iS10) in the attenuated total reflection (ATR) mode at 4 cm^{-1} resolution. Stable electron-transfer radical signals on CA-Ca-SBE-laccase beads were detected by using low-temperature excitation electron spin resonance (ESR, JES-X320) instrument.

2.3. Phenanthrene degradation tests of CA-Ca-SBE-laccase biocatalyst

Unless specified, all phenanthrene degradation tests were conducted by mixing the catalysts, taken from the same batch, and 0.5 ppm phenanthrene in 125 mL Erlenmeyer flask. After the reaction was initiated for 24 h, 40 mL of sample was taken and filtered using a 0.22-μm filter, and then eluted with 10 mL dichloromethane by solid phase extraction (SPE, Thermo Fisher Scientific). The residual phenanthrene and its metabolites were determined by gas chromatography-mass spectrometry (GC-MS, Shimadzu QP2020) with a C-18 column. Phenanthrene degradation was evaluated at different free laccase quantities (0.05−0.5 g), CA-laccase (0.05−0.5 g) beads, CA-Ca/SBE-laccase (0.05−0.5 g) beads, and pH conditions (4−9). To test the operational and storage stability, CA-Ca-SBE-laccase beads and CA-Ca-SBE-laccase beads stored at 4 ℃ for 50 days were subjected to 10 cycles of phenanthrene catalytic treatment [36].

2.4. Statistical analyses

All tests were conducted in triplicate. The measured data were presented using the mean±standard deviation (SD). Statistical significance was determined with a one-way analysis of variance (ANOVA) using SPSS 13.0. Correlations at p < 0.05 were considered to be significant. In all tests, Excel was used to calculate the standard deviation.

3. Results and discussion

3.1. Morphology and properties of CA-Ca-SBE-laccase

The CA-Ca-SBE-laccase beads were regular spheroids with diameters of approximately 4 mm, as shown by the SEM images in Fig. 1(a). It was obvious that SBE encapsulated in CA-Ca-laccase beads formed many cavities with network pore structures capable of transporting the substrate molecules. Moreover, after freeze-drying, the surface of the CA-Ca-laccase beads was smoother than that of the CA-Ca-SBE-laccase beads, due to the presence of a higher number of cavities. In addition,
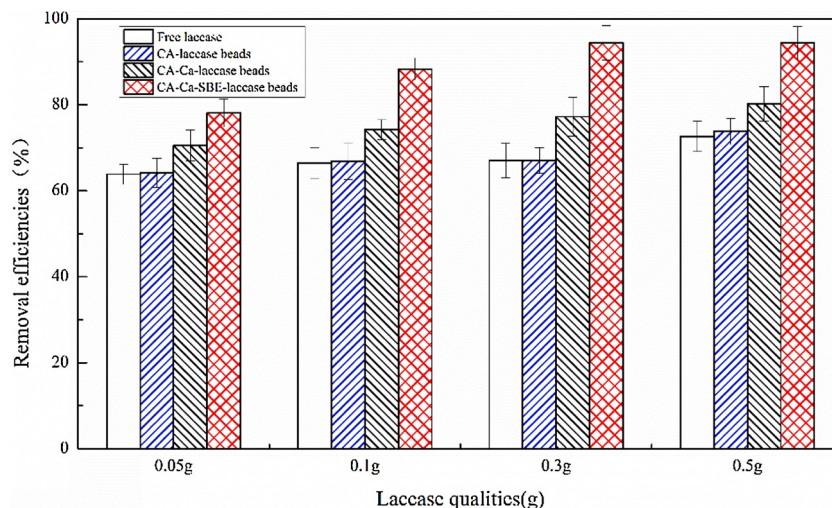
Fig. 2. Phenanthrene degradation by different laccase amounts of free laccase and CA-Ca/SBE-laccase beads.

the surface of the CA-Ca-SBE-laccase beads indicated a distinctly modified exterior surface with a notable extent of amorphous aggregates as a result of the combination of SBE molecules on the CA-Ca-laccase bead surface. By further enlarging the cross-section of the beads, it was found that the surface of the cavities formed by the CA-Ca-SBE-laccase beads were smoother compared with the surface of CA-Ca-laccase beads, indicating that SBE was an efficient carrier for laccase immobilization.

To investigate the crystalline structure of CA-Ca/SBE-laccase beads, the XRD patterns are shown in Fig. 1 (b). Modification with Ca or addition of SBE did not change the 2 theta value. The crystallinity of CA-Ca-SBE-laccase beads was lower than that of CA-Ca-laccase beads, indicating that the active components of CA-Ca-SBE-laccase beads were better dispersed. According to Wang et al. [37], free chitosan films have two diffraction peaks near 2 theta= 10.5° and 19.9°. The peak near 10.5° is caused by the generation of intramolecular hydrogen bonds between hydroxyl groups and NH^{3+}, which inhibits the movement of the chitosan chains [38], and the peak at approximately 19.9° is attributed to the dissolution of chitosan in the acetic acid solution [39]. The pure sodium alginate film had a characteristically broad crystalline peak at approximately 2 theta 21.5° [39]. The XRD pattern of the chitosan-alginate film displayed that two diffraction peaks of chitosan disappeared near 2 theta= 10.5° and 19.9°, and a maximum of broadening and diffusion appeared near 2 theta= 23.2°. This result suggested that sodium alginate molecules affect the crystalline features of the chitosan film in view of the electrostatic interactions and hydrogen bonding interactions between the positively charged chitosan film and the negatively charged sodium alginate film.

The structure and composition of CA-Ca/SBE-laccase beads were further investigated by functional group characterization. Fig. 1 (c) displays the FTIR spectra of CA-Ca-SBE-laccase beads. The characteristic wide band at 3455.99 cm^{-1} was due to the stretching vibrations of polymeric hydrogen-bonded OH [40]. The peak at 1644.55 cm^{-1} was designated to ν(CO)α-hydroxyl diaryl ketone groups or hydrogen bonded carbonyl (ν (CO···H—O—H)) [41]. The bands at 1425.97 cm^{-1} and 1083.27 cm^{-1} were attributed to the stretching vibration of C—O—C of the glycosidic linkage of the polymer and scissoring vibrations of NH₂ groups [42]. FTIR spectra of CA-Ca-SBE-laccase beads showed a narrow, higher-intensity band near 3451.97 cm^{-1}. In addition, the peaks near 1425.97 cm^{-1} and 1083.27 cm^{-1} were shifted to 1425.63 cm^{-1} and 1079.63 cm^{-1}, respectively. FTIR spectra indicated that there were more microinteractions in CA-Ca-SBE-laccase beads than in CA-Ca-laccase beads, without obvious covalent bond formation.
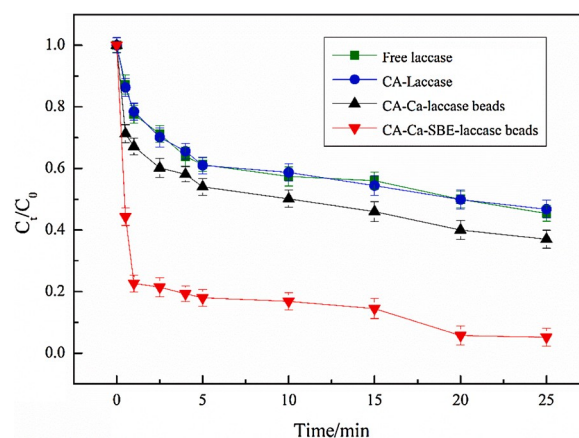

Fig. 3. Phenanthrene removal rates under CA-Ca-SBE-0.3 g laccase beads.

3.2. Phenanthrene catalytic performance of CA-Ca-SBE-laccase beads

Results of phenanthrene degradation tests are shown in Figs. 2 and 3. SBE encapsulated into CA-Ca-SBE-laccase beads performed critical functions in enhancing the biocatalytic activities of laccase for phenanthrene. The results showed that CA-Ca-SBE-laccase beads had the highest degradation efficiency (94.4 %) and removal rate k (0.4 mol/L min for phenanthrene in water, and significant differences (p < 0.05) in the phenanthrene removal efficiency were observed among the four treatment groups. The average effluent phenanthrene concentrations were 0.110 ppm, 0.058 ppm, 0.0281 ppm, and 0.0283 ppm among the different laccase quantity treatments (p < 0.05). It was inferred that 0.3 g laccase may be sufficient for the biocatalytic degradation of phenanthrene by laccase-SBE substrates. In addition, the degradation efficiency of phenanthrene was only 67.0 % by CA-laccase beads prepared using a single CA under the same conditions. This was mainly because the binding of CA gel was limited by the diffusion of chitosan in the alginate network. The results of immobilization efficiency experiments showed that laccase and SBE immobilization by coencapsulation in Ca-modified chitosan-alginate beads had an immobilization yield of 83 %, which indicated a high recovery efficiency of SBE. The average recoveries acquired from GC–MS analysis of the aqueous samples ranged from 98.8 % to 110.0 %, indicating that it could accurately quantify phenanthrene concentrations in aqueous samples.
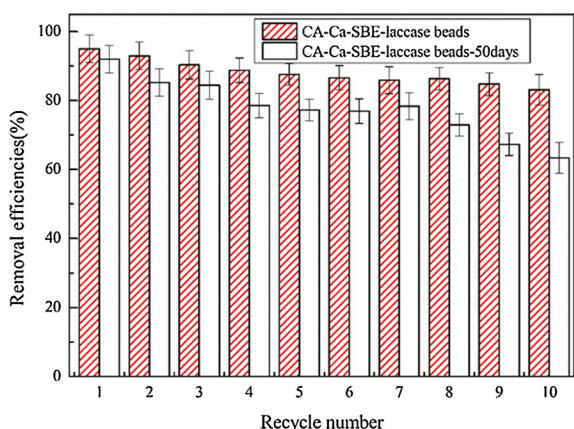
(a)
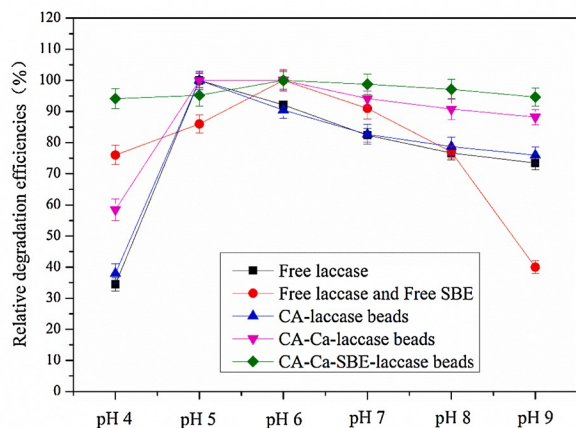
(b)

Fig. 4. (a) Effect of pH on phenanthrene degradation by free laccase 、 free laccase and SBE and CA-Ca/SBE-laccase beads; (b) reusability and storage stability of CA-Ca-SBE-laccase beads for phenanthrene degradation.

3.3. Reusability and stability of CA-Ca-SBE-laccase beads

pH has significant impact on the activity and structure of the coencapsulated enzyme. As shown in Fig. 4(a) significant differences (p < 0.05) in the relative phenanthrene removal efficiency by CA-Ca-SBE-laccase beads were observed in the pH range of 4–9, which was significantly higher than that of other biocatalysts [43]. The appropriate pH range of free laccase, CA-laccase beads and CA-Ca-laccase beads was significantly lower than that of CA-Ca-SBE-laccase beads, indicating that CA-Ca-SBE-laccase beads exhibited strong acid-base tolerance. From two aspects of molecular dynamics simulation and quantum chemistry, it was found that the combination of laccase and SBE leads to deprotonation, which plays a key role in the coordinated transfer of phenolic hydroxyl protons [44]. Bertrand et al. [45] studied the effect of laccase on substrate binding and catalytic oxidation, indicating that the activity of laccase at pH=6.0 was higher than that at pH=3.0, which may be due to the deprotonation of laccase at pH=6.0 and the stabilization of protons removed from the substrate by negatively charged laccase. This deprotonated laccase assisted electron-proton synergistic transfer process has an important influence on the catalytic oxidation process in kinetics and thermodynamics [8,46]. Therefore, CA-Ca-SBE-laccase
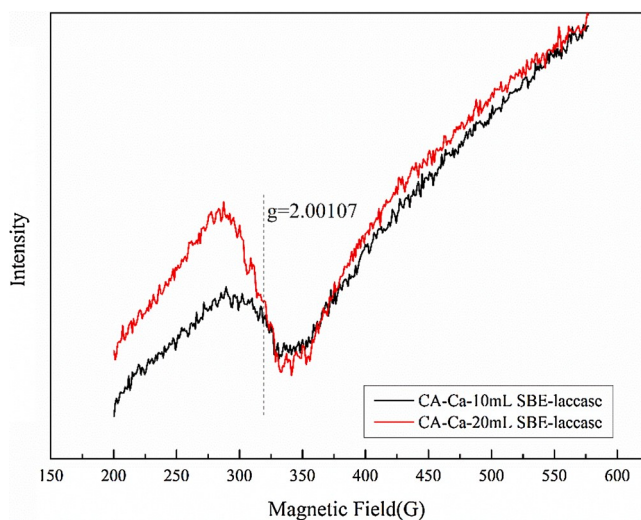

Fig. 5. ESR spectra of CA-Ca-SBE-laccase beads.

beads can be employed as biocatalyst for degrading in a wide pH range.

The reusability of immobilized enzymes is one of the crucial properties for economically practicable wastewater treatment processes. Phenanthrene at 0.5 ppm was treated with the same batch of CA-Ca-SBE-laccase beads for 10 consecutive cycles. The degradation efficiency of phenanthrene in each cycle is shown in Fig. 4(b). The degradation efficiency of phenanthrene was 87.6 % after 5 continuous cycles and 83.1 % at the end of 10 cycles. In Gao et al.'s [25] study, the co-immobilized nanoparticles retained only 50 % residual laccase activity after the particles were used 8 times repeatedly. CA-Ca-SBE-laccase beads were stored at 4 ℃ for 50 days and then treated with 0.5 ppm phenanthrene for 10 consecutive cycles to test its storage stability. As shown in Fig. 4 (b), the catalytic degradation efficiency of phenanthrene was 92.0 % after 50 days of storage. Luo et al. [17] reported that the functional groups produced by laccase and redox mediators were strong electron-pushing functional groups, which can increase the electron density of free radicals. It can promote electron transfer of laccase and stabilize the free radical structure, resulting in high laccase reusability. In addition, the immobilized laccase-natural redox mediator system can degrade and release the adsorbed phenanthrene, and the preadsorption of laccase and SBE on the catalyst beads can be used for the *in-situ* regeneration of biocatalysts to stabilize the system performance. However, after 50 days, it was directly observed that the catalyst had reduced in size and adopted a yellow color, and the catalytic activity was reduced accordingly. Systematic research is needed to further improve the stability of this catalyst.

3.4. Phenanthrene degradation mechanism of CA-Ca-SBE-laccase beads

Stable electron transfer radical signals of CA-Ca-SBE-laccase beads were monitored by the ESR. As shown in Fig. 5, strong stable free radical signals were observed in the complex system of CA-Ca-SBE-laccase beads, and their intensity generally increased with increasing SBE content. The different behaviors of stable free radicals can be further elucidated by variation in the g value of the first derivative signal of the ESR spectrum. The g value is a constant of the purified compound, arising from the limited magnetic moment and angular momentum of electrons in a particular chemical environment [47]. Petrakis et al. [48] classified the g values of many stable free radicals to obtain general information about the different kinds of free radicals in biocatalytic reactions. Oxygen-containing free radicals (σ-type) ranging from 2.0008 to 2.0014 were generated by SBE oxidation via an electron-transfer route in CA-Ca-SBE-laccase beads. It is generally accepted that oxygen-containing free radicals can oxidize organic compounds in the
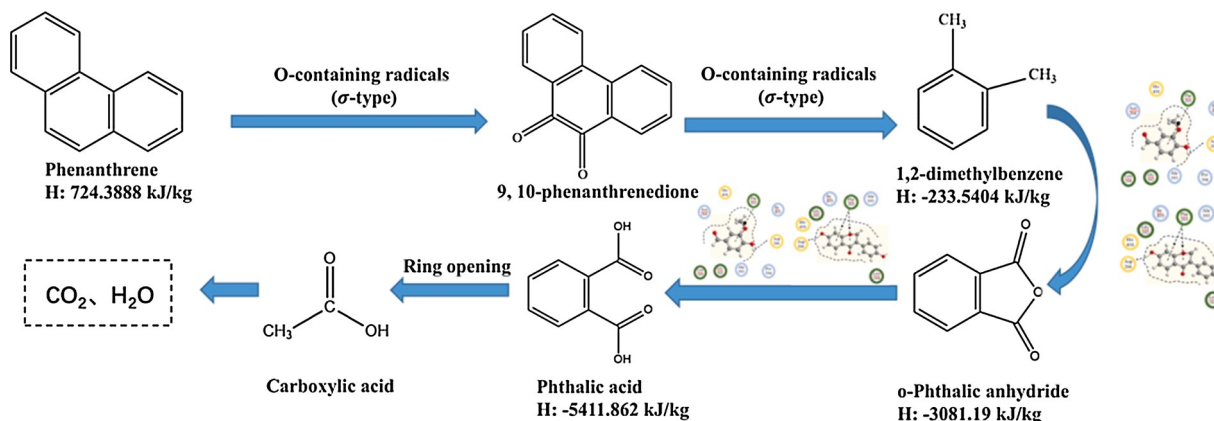
Fig. 6. Proposed pathways for the biodegradation of phenanthrene by CA-Ca-SBE-laccase beads.
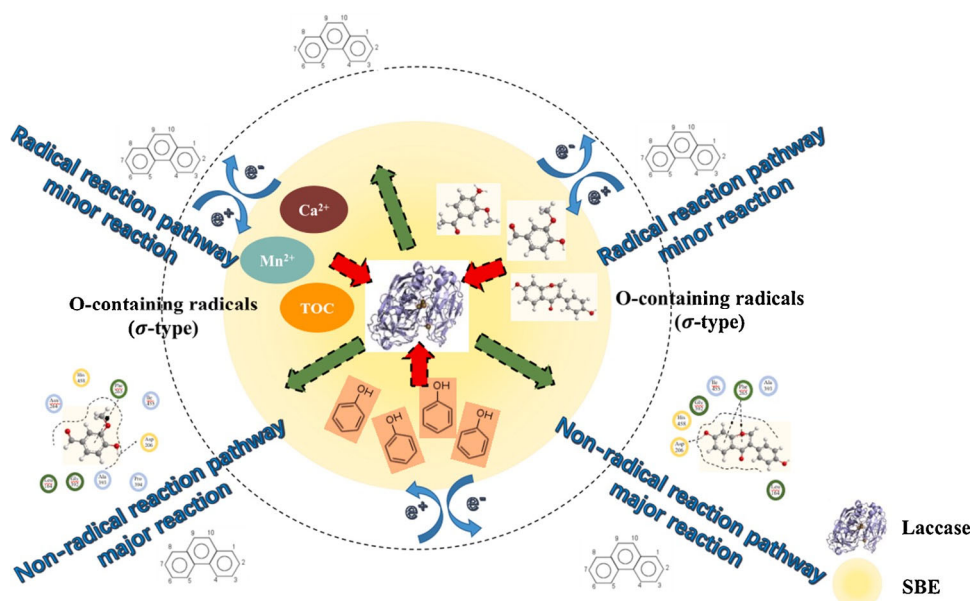

Fig. 7. The schematic diagram of biocatalytic mechanism for phenanthrene degradation by CA-Ca-SBE-laccase beads.

environment [49].

GC–MS analysis was carried out to identify the degradation intermediates of phenanthrene in the CA-Ca-SBE-laccase system after 0, 1, 2, 3, 4, 5, 10 and 15 min of reaction. The right-angled structure of phenanthrene confines π-electrons to the 9, 10 bonds to a large extent, and the chemical properties of the 9, 10 bonds resembled those of olefin bonds. The presence of small-molecular phenolic lignins (vanillin, apolipoprotein, daidzein) in SBE resulted in significant stronger nonpolar interactions with laccase, leading to formation of binding intermediates with high redox potential and stable structures, which constituted a nonradical pathway in the catalytic degradation of phenanthrene. The degradation pathway was proposed based on the detected degradation intermediates and the analysis results discussed above, as shown in Fig. 6. The cleavage of two carbonyl groups in phenanthrene's 9, 10 bonds occurred due to the attack of oxygen-containing free radicals (σ-type), and the formation of 9, 10-phenanthrenequionone intermediate. Methylation of 9, 10-phenanthrenequinone on the susceptible benzene ring due to the attack of oxygen-containing free radicals (σ-type) resulted in the formation of 1,2-dimethylbenzene intermediates. The intermediates of o-phthalic anhydride and phthalic acid were obtained after the conjugation of laccase-SBE intermediates to the two methyl groups. Observation of carboxylic acid further proved the possibility of the complete degradation of phenanthrene.

Based on the abovementioned results, it can be concluded that both the radical pathway of oxygen-containing stable free radicals and the nonradical laccase-SBE combined substrates with higher redox potential generated in CA-Ca-SBE-laccase beads were the crucial mechanisms for phenanthrene degradation, while oxygen-containing free radicals (σ-type) only showed a limited contribution in the CA-Ca-SBE-laccase system. The electron-transfer reactions among laccase-SBE combined substrates played a significant role in the biocatalysis of phenanthrene (Fig. 7). By calculating and balancing the relative proportion of degradation products based on GC–MS qualitative results, the contributions of the free radical pathway and nonradical pathway to phenanthrene were 12.5 % and 87.5 %, respectively, and achieved the maximum double synergy of mediator and laccase. In addition, SBE is a protein-based organic nitrogen fertilizer that could deteriorate into nitroso compounds [50], which have been suggested to have excellent removal efficiencies in the laccase catalysis of problematic organic pollutants [51]. SBE is particularly high in calcium, magnesium, and total organic carbon, which can act as mediators when oxidized by enzymes [52,53]. The content of organic matter in SBE solution can supply more natural chelating agents with metal complexation, thus promoting its enzymatic catalysis [53]. The coexistence of various mediators could result in cooperative functions in the laccase-SBE system.

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4. Conclusions

Cofunctionalized laccase biocatalyst is a promising tool for achieving economically competent biotechnological processes in removal of persistent organic pollutants. In this study, high-efficiency removal of phenanthrene in the aqueous phase was obtained by using a novel CA-Ca-SBE-laccase biocatalyst. The key to achieve the maximum double synergy of mediator and laccase was to generate both the radical pathway of oxygen-containing stable free radicals and the nonradical laccase-SBE combined substrates with higher redox potential in CA-Ca- SBE-laccase beads. Intermediate products were identified, which proved that phenanthrene ring-opening occurred and resulted in the production of CO_2 and H_2O in aqueous environments via CA-Ca-SBE-laccase bead catalysis. CA-Ca-SBE-laccase beads exhibited fairly stable catalytic activities over a wide range of pH values, and the catalytic activities remained stable during 10 cyclic runs and after 50 days of storage. Therefore, the application of laccase-mediated biocatalysis on sustainable operational conditions could be pushed forward by the successful cofunctionalization of laccase, environmentally-friendly mediators and immobilized supports.

CRediT authorship contribution statement

Xinhan Chen: Conceptualization, Methodology, Writing - original draft, Data curation, Visualization. **Zhen Hu:** Methodology, Formal analysis, Writing - review & editing, Supervision. **Huijun Xie:** Methodology. **Huu Hao Ngo:** Project administration. **Wenshan Guo:** Project administration. **Jian Zhang:** Supervision, Project administration, 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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