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Functionalized Chitosan and Alginate Composite Hydrogel-Immobilized Laccase with Sustainable Biocatalysts for the Effective Removal of Organic Pollutant Bisphenol A

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Abstract: The immobilization of enzymes is an important strategy to improve their stability and reusability. Enzyme immobilization technology has broad application prospects in biotechnology, biochemistry, environmental remediation, and other fields. In this study, composites of chitosan (CS) and sodium alginate (SA) with Cu^{2+} forming a double-network crosslinked structure of hydrogels were prepared and used for the immobilization of laccase. Fourier infrared spectroscopy, scanning electron microscopy, and X-ray photoelectron spectroscopy tests revealed that laccase molecules were immobilized on the composite hydrogel surface by a covalent bonding method. Compared to free laccase, the pH, temperature, and storage stability of the immobilized laccase were markedly improved. In addition, the immobilized laccase could be easily separated from the reaction system and reused, and it maintained 81.6% of its initial viability after six cycles of use. Bisphenol A (BPA) in polluted water was efficiently degraded using immobilized laccase, and the factors affecting the degradation efficiency were analyzed. Under the optimal conditions, the BPA removal was greater than 82%, and the addition of a small amount of ABTS had a significant effect on BPA degradation, with a removal rate of up to 99.1%. Experimental results indicated that immobilized laccases had enormous potential in actual industrial applications.

Keywords: laccase immobilization; hydrogel composites; chitosan; alginate; bisphenol A degradation

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1. Introduction

Bisphenol A, scientifically known as 2,2-bis (4-hydroxyphenyl) propane, is a trace environmental pollutant with estrogenic activity, and its pollution of the environment is a growing concern worldwide. Its hormonal effects on the endocrine system can disrupt physiological functions; it is highly toxic and difficult to biodegrade. Studies have shown that even sub-nanogram levels per liter can affect human hormone levels, reduce sperm concentration in males and reduce the likelihood of pregnancy in females, increase the risk of diabetes, high blood pressure, and heart disease, cause obesity and asthma, impact liver and thyroid function, etc., which can have a serious impact on human health [1,2]. There are a wide range of sources of BPA in life, the most important of which is industrial wastewater effluent from wastewater treatment plants. Therefore, it is particularly important to effectively remove BPA in the treatment process prior to discharging wastewater. Various methods have been reported for the removal of BPA from industrial wastewater, which include activated carbon adsorption, resin adsorption, extraction, chemical oxidation, wet oxidation, photo-oxidation, and biodegradation [3–5]. Biodegradation is a widely used wastewater treatment technology. Bio-enzymatic treatment has the advantages of environmental protection and high efficiency, no harsh conditions required, non-toxic products,

a fast degradation rate, etc., and it can completely eliminate trace amounts of BPA in the environment, so it has received more and more attention.

Laccase (EC 1.10.3.2) is a copper-containing oxidoreductase widely found in fungi, plants, and bacteria. It is an extracellular copper enzyme belonging to the group of copper-blue oxidases, also known as phenolases, polyphenol oxidases, laccase oxidases, etc. [6]. The process of laccase degradation is the catalytic oxidation of phenolics under the condition that molecular oxygen is reduced as an oxidant, which has high catalytic efficiency and does not produce other harmful by-products in the process of the reaction. As a biocatalyst, laccase also catalyzes the oxidation of a very wide range of substrates and has successfully degraded and treated a wide variety of environmental contaminants, including phenols and aromatic amine organic compounds [7,8], and thus has attracted much attention in environmental management [9,10]. However, all free enzymes have some drawbacks in removing pollutants from aqueous solutions, such as low stability, non-recovery, and non-recyclability, and their enzyme activity expression and stability performance are susceptible to interference by environmental factors (temperature, pH, ionic strength, etc.), thus limiting their application in wastewater treatment [11]. Therefore, the development of a method that can overcome the disadvantages of free enzyme while retaining the original activity of the enzyme, laccase immobilized on solid carriers, can make the enzyme more stable in different reaction environments, improve customer service and the disadvantages of free enzyme, make it easy to store, and allow its reusability [12]. The immobilization effects of laccase are highly dependent on the structure of the enzyme, the nature of the carrier materials, and the immobilization methods. The immobilization methods of enzymes include adsorption, embedding, cross-linking, and covalent bonding, among which covalent bonding refers to the immobilization of the enzyme by using the formation of covalent bonds between the groups in the enzyme (the amino group, carboxyl group, complex amino group, and aromatic ring of histidine) and the organic groups on the carrier. The covalent binding method is relatively well established and has the advantages of a strong connection between the enzyme and the carrier, better stability, and the enzyme being immobilized and not leaking from the carrier [13,14]. Therefore, the covalent binding method was used for the immobilization of laccase in the next work.

In recent years, the development of high-value-added biomass functional materials using renewable resource biomass-based polymers has attracted the attention of researchers, and chitosan has been demonstrated to be a biodegradable, nontoxic, nonantigenic, and biocompatible polysaccharide polymer with excellent antimicrobial properties. It is also one of the most advanced and environmentally friendly biomaterials in the world at present [15]. Chitosan is a biocompatible natural material obtained by deacetylation of natural chitin prepared from shrimp and crab shells. The surface of chitosan consists mainly of -OH and -NH_2 functional groups, which can be bound to the enzyme with direct and weak interactions. Improved chemical modification interactions resulted in a stable, covalently immobilized enzyme that was not easily dislodged [16]. Enzyme immobilization on chitosan hydrogel soft carriers has the potential to improve the physical and chemical properties of the enzymes and product yields, and it has been reported to be used as a carrier for immobilized enzymes including laccase, trypsin, and lipase; thus, this green and effective method has attracted the interest of a large number of researchers [17–19]. In the previous literature, the most used strategies for the improvement of composite-formed, graft-copolymerized, and chitosan-formed hydrogel beads to enhance their catalytic stability and recyclability are currently available [20,21]. Polymer hydrogels constitute cross-linked, three-dimensional hydrophilic networks that have the ability to retain large amounts of water and biofluids within the polymer chains, thus providing a suitable physiological environment. The aqueous environment of biopolymer-based hydrogels minimizes enzyme denaturation and contributes to the catalytic function of enzymes [22]. In addition, the retention of enzyme activity was greatly enhanced after immobilization into a polymeric hydrogel matrix. Sodium alginate (SA) is another natural linear polymer with outstanding characteristics such as non-toxicity, biocompatibility, and

biodegradability [23]. Such compounds encountering divalent cations such as Ca^{2+}, Mg^{2+}, and Cu^{2+} can form spherical shapes through crosslinked structures. SA hydrogel beads have attracted extensive attention and in-depth study by researchers due to their low cost, good stability, and non-toxic safety in organic pollutant removal. [24,25]. Although hydrogels have important applications in biotechnology, sometimes conventional hydrogels do not meet the criteria for specific applications, such as low solubility, poor mechanical properties, and poor stability. Therefore, mixing chitosan with sodium alginate solution by electrostatic interaction and hydrogen bonding usually better combines the advantages of prepolymers and newly synthesized prepolymers with good mechanical properties and stability, which can be solved by the mixing method of further cross-linking the polymer components to form hybrid composites.

Inspired by the above, we developed a reusable composite hydrogel material consisting of chitosan and sodium alginate for use as laccase immobilization. First, we applied electrostatic charge hydrogen bonding to mix chitosan with sodium alginate to make a composite material, and then we formed hydrogel beads by chelating and cross-linking with Cu^{2+}. Laccase was immobilized on composite hydrogel beads using the covalent binding method, and the composite formed by laccase with chitosan and sodium alginate was covalently bound via amide bonds. The properties of the composite hydrogel materials and the laccase after immobilization on the carriers were studied, respectively. In addition, the role of free and immobilized laccase in the removal of bisphenol A pollutants was investigated for a comparative study. The results showed that immobilized laccase was effective in removing bisphenol A, providing a new technology for the removal of polyphenols from wastewater in industry and increasing the feasibility of practical application.

2. Results and Discussion

2.1. Characterization of CS-SA Beads and Lac/CS-SA Beads

To obtain functional CS-SA carriers for immobilization of laccase, chitosan was mixed with sodium alginate to form a composite solution. CS-SA hydrogel beads were obtained by dropping the CS-SA composite solution into a 4% $CuCl_2$ solution using a syringe. Laccase was covalently bound to CS-SA by hydrogen bonding and immobilized on the prepared hydrogel beads. Bisphenol A (BPA) was efficiently degraded using immobilized laccase, and the synthesis route and principle of the immobilized carrier are shown in Figure 1.

The molecular structure and chemical bonding of the synthetic products were verified using FTIR-ATR (Figure 2). The FTIR results showed that for CS, the peak near 3260 cm^{-1} was formed by the overlap of the absorption peaks of the O and N–H bonds, 2870 cm^{-1} was caused by the C–H bond stretching vibration, the absorption peak near 1652 cm^{-1} was the amide I peak of the acetamido group, there was a bending vibration of the N–H (amide II) at 1560 cm^{-1}, and 1065 cm^{-1} was the absorption peak of the C–C backbone stretching vibration. This indicated that chitosan was not fully deacetylated. The FT-IR spectra of SA at 1600 and 1406 cm^{-1} showed symmetric and asymmetric stretching vibrations of the –COO– group, respectively. Comparing the spectra of CS, SA, and CS-SA, the stretching vibration of the –COO– group of SA was observed near 1600 cm^{-1} for CS-SA, and the absorption peak of the C–C skeleton of CS was observed at 1065 cm^{-1} for CS-SA, which contained the characteristic peaks of each of the CS and SA, and there were no new peaks, suggesting that the CS-SA was formed by the physical bonding of the three substances with each other. The significant enhancement of the stretching vibration peaks of the O–H and N–H bonds in the spectrum at 3260 cm^{-1} might be due to the hydrogen bonding interactions between CS and SA, which further indicated that the bonding of the two substances occurred. The peaks at 1560 and 1406 cm^{-1} corresponded, respectively, to the stretching vibration of N–H (amide II) and the out-of-plane bending vibration of the –COO– group. It was observed that the main vibration of the benzene ring and the tensile vibration of the amide bond in CS-SA were weakened because the absorption was possibly masked by the cross-linking action of Cu^{2+}. However, from the contrast before and after immobilization of the carrier material, the peaks at 1406 cm^{-1} and 1560 cm^{-1} form an

intense contrast, indirectly confirming that the laccase successfully cross-linked on the surface of the CS-SA aggregation beads, thereby enhancing absorption [26].

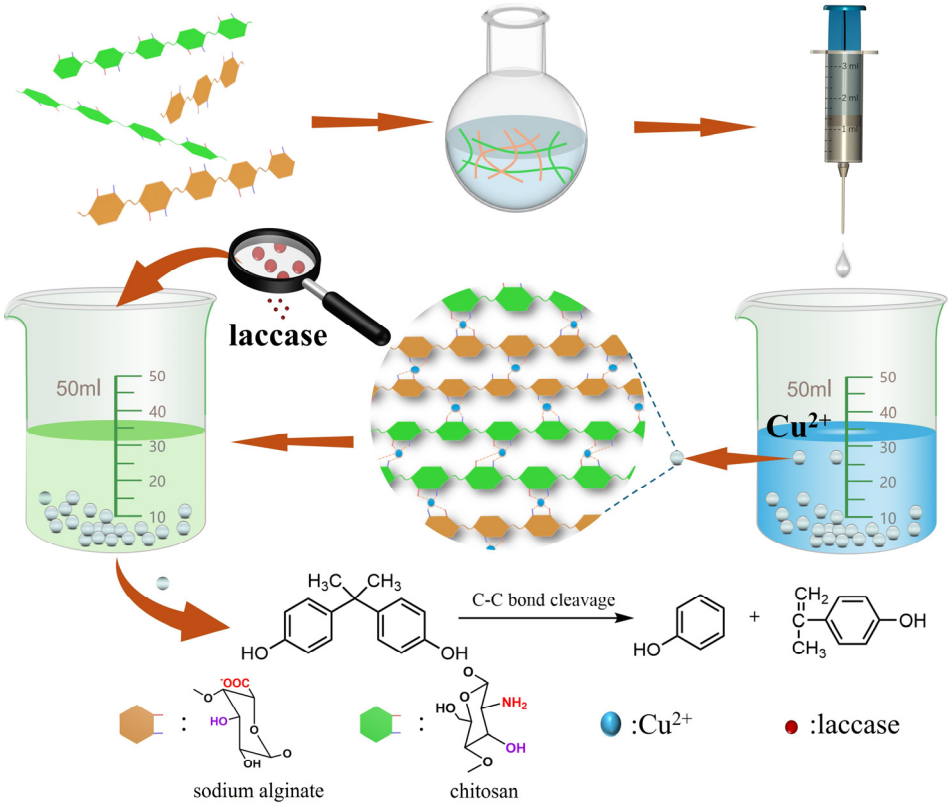

Figure 1. Diagram for the preparation of functionalized chitosan/sodium alginate composite hydrogel beads and schematic diagram of laccase immobilization for the effective removal of organic pollutants bisphenol A.

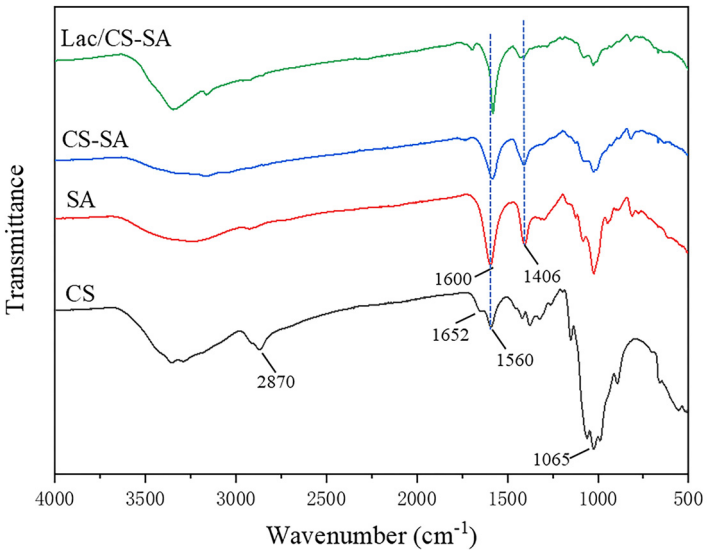

Figure 2. The FT-IR spectra of purified chitosan (95% deacetylation), purified sodium alginate, CS-SA beads, and Lac/CS-SA beads.

The surface of the prepared CS-SA hydrogel beads and Lac/CS-SA hydrogel beads showed that the prepared hybridized hydrogel beads were about 2 mm in diameter, non-adhesive to each other, and easy to separate from the substrate after taking part in the catalytic reaction (Figure 3e,f). Surface morphology analysis of the CS-SA hydrogel beads

and Lac/CS-SA hydrogel beads also verified the enzyme binding to the carrier. Figure 3a,b shows the structure of CS-SA. The overall morphology of the material has a fibrous appearance and an interconnected network structure; clustered or protruding shapes appear on the surface of functionalized shell polysaccharide fibers, and there is the formation of inter- or intramolecular linkages between the chitosan chains and the sodium ions of copper alginate. These connections could manifest as bridges or linkages between different fibers or within individual fibers. As shown in Figure 3c,d, the Lac/CS-SA condensate beads also had a fibrous appearance and interconnected network structure, intertwined with the lamellar enzyme aggregates.

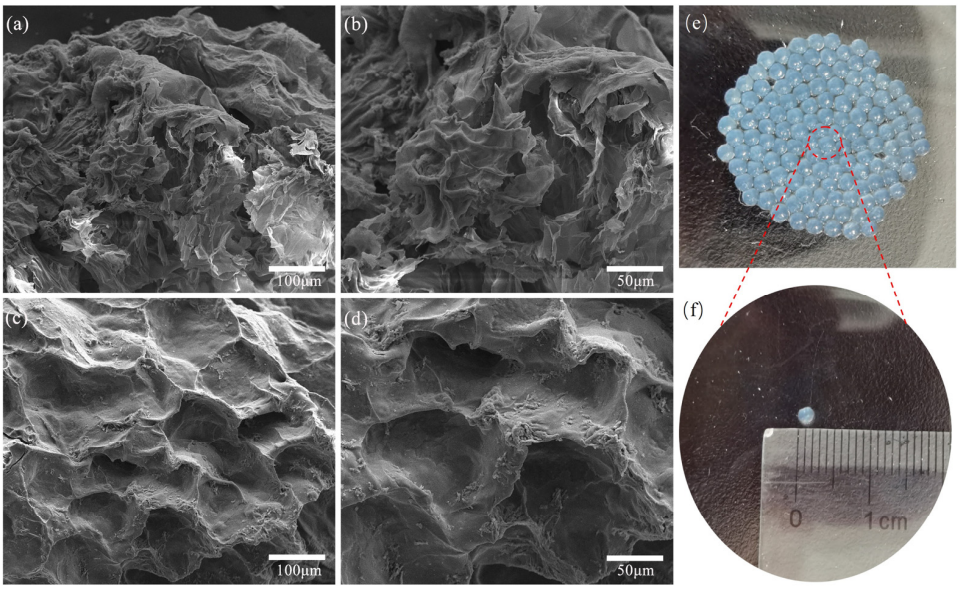

Figure 3. SEM photographs of (**a**,**b**) CS-SA beads and (**c**,**d**) Lac/CS-SA beads; (**e**) digital photo of many CS-SA beads in real life; and (**f**) digital photo of one CS-SA bead in real life.

The elemental C, O, and Cu on the surface of CS-SA and Lac/CS-SA were determined by XPS analysis and compared. As shown in Figure 4, we can observe that the peak of the Cu element of Lac/CS-SA was enhanced compared with that of CS-SA, which indicates that the structure of the carrier becomes stronger and the crystallinity is elevated, and it can also indicate that the laccase molecules are successfully immobilized on the surface of CS-SA. However, both the C and O elemental peaks were diminished, probably because the carrier surface was adhered to by laccase molecules, which prevented the electrons from being excited.

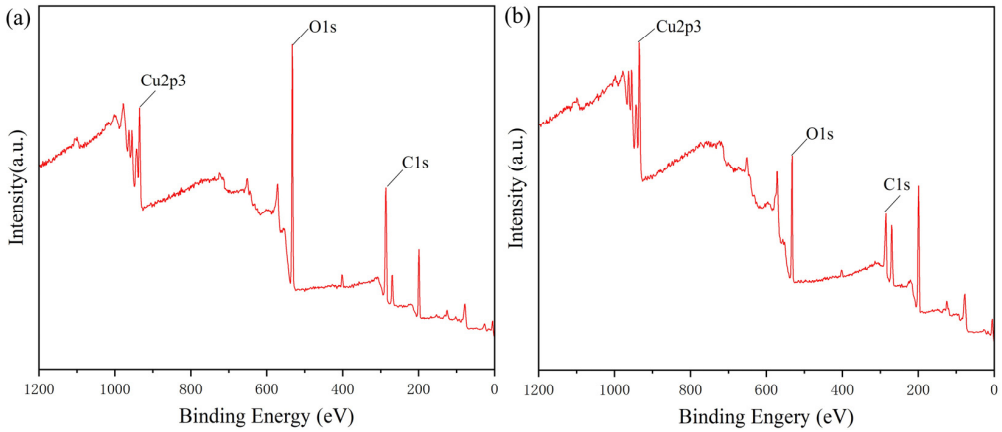

Figure 4. XPS wide spectrum scanning of (**a**) CS-SA beads and (**b**) Lac/CS-SA beads.

2.2. Effect of Initial Concentration of Enzyme on Laccase Immobilization Process

Laccase was immobilized on CS-SA beads by the covalent binding method, which was chosen for the immobilization of laccase because the covalent binding was strong and stable and the enzyme was not easy to leak from the carrier. The initial concentration of the enzyme and the pH of the buffer solution during the immobilization process had a very great influence on the immobilization and were the key factors affecting the immobilization effect. pH > 5.0, the functional groups of sodium alginate mainly existed in the form of acid, which was conducive to the amidation reaction of the enzyme to form covalent immobilization. Considering that laccase is easily inactivated in an alkaline environment (pH > 7.0), the optimum pH was 4.5–6.5, and phosphate buffer (50 mM) with pH 6.0 was used for the immobilization of laccase in all of the experiments [27].

Figure 5 depicts the effect of the initial concentration of laccase on enzyme activity after immobilization. As the concentration of laccase was increased to 3 mg·mL^{-1}, the protein loading increased significantly to about 9.3 mg·g^{-1}. With a further increase in concentration, sex did not lead to a further increase in loading and reached a plateau. This could be because, at higher enzyme concentrations, the binding sites of CS-SA might become crowded, inhibiting spatial sites and inhibiting contact between the laccase and the substrate [28]. Thus, the optimal loading of the laccase enzyme was about 8.2 mg in CS-SA beads with a protein content per gram of wet bead similar to laccase immobilized on nanofiber membranes [29] and magnetic nanoparticles [30], with 96.1% moisture content. The amount of immobilized enzyme protein on the CS-SA beads was 9.3 mg/g, demonstrating that laccase immobilization by covalent binding was highly efficient and stable. As can be observed in Figure 5, the highest enzyme activity of the wet hydrogel beads was about 492 U/g^{-1} and was not at the highest laccase concentration. As the laccase concentration increases, the enzyme activity decreases and gradually enters a plateau phase with little change. The substrates for laccase oxidation are usually compounds such as polyphenols and aromatic amines, and too high laccase concentrations may cause the internal enzyme molecule transfer process to be restricted, leading to a decrease in both enzyme activity and enzyme immobilization efficiency.

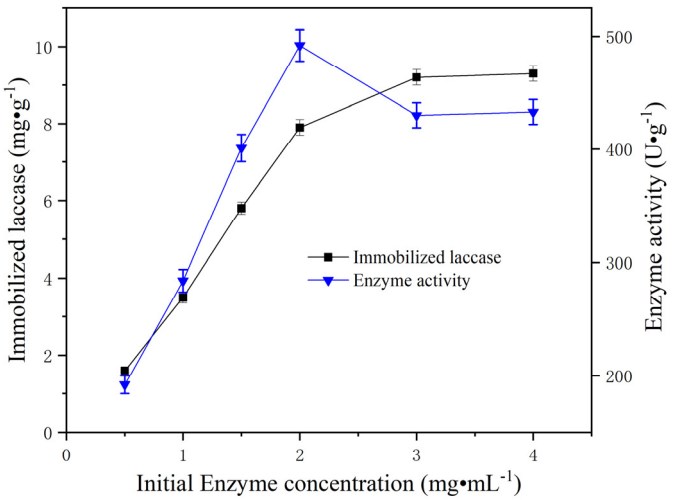

Figure 5. Effect of enzyme concentration and enzyme activity on laccase immobilization. Conditions: pH 6.0 of laccase solution prepared with phosphate−buffered solution.

2.3. Michaelis–Menten Kinetics Analysis

Michaelis–Menten kinetic studies were shown in Figure 6, which could provide valuable insights into the substrate affinity and reaction rate of immobilized laccase compared to free laccase. The kinetic parameters of the two forms of laccase are listed in Table 1. The reaction rate constant K_m of immobilized laccase was smaller than that of free laccase, and

the catalytic constant k_{cat} of the immobilized enzyme was less than that of free laccase. Kinetic parameters indicated a rise in affinity for the substrate by the immobilized laccase.

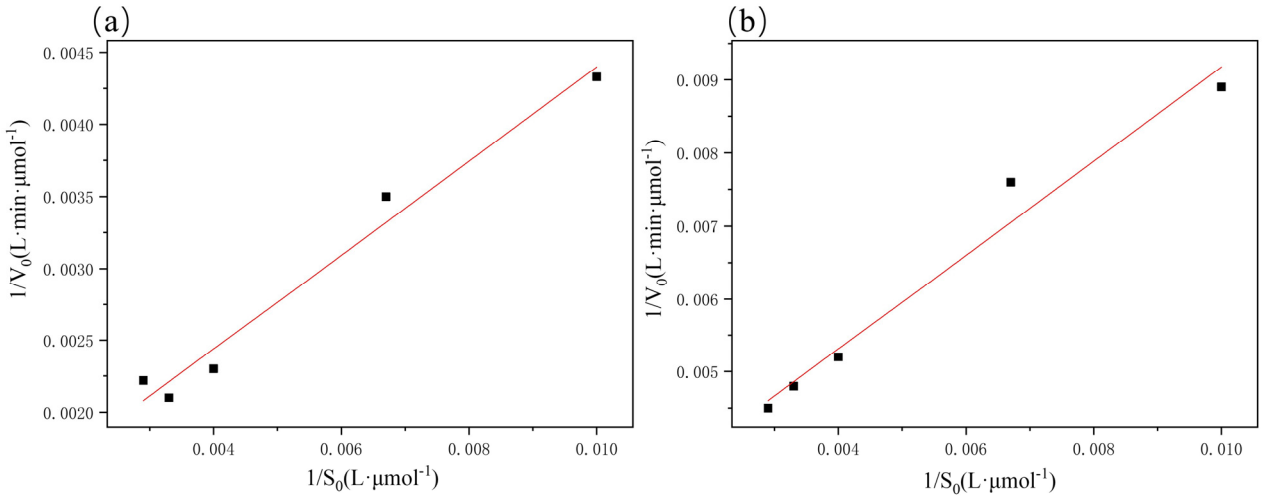

Figure 6. Lineweaver−Burk plot of (**a**) free laccase and (**b**) immobilized laccase.

Table 1. Parameters obtained from fitting the Lineweaver–Burk plot.

Samples	R^2	k_{cat} (s^{-1})	K_m (μmol/L)
Free laccase	0.9697	0.589	288.85
Immobilized laccase	0.9643	0.243	234.92

2.4. Stability and Reusability of Immobilized Laccase

The stability of enzyme catalysts is one of their important characteristics in biotechnological applications. In order to comprehensively evaluate the performance of immobilized laccase on CS-SA beads, the pH and thermal stability of free and immobilized laccase were comparatively studied. The effect of pH in the range of 3.5–6.0 on the relative activities of the two laccases was studied. As shown in Figure 7a, the free laccase enzyme activity reached a maximum at pH 4.5, while the enzyme activity of the immobilized laccase reached a maximum at pH 4.0, indicating that the optimal pH for the immobilized laccase has shifted toward acidic conditions. The reason might be that immobilizing the laccase on CS-SA beads by the covalent bonding method could significantly increase the laccase's tolerance to acidic environments. This might be attributed to the chemical stability of the carrier and the interaction of the enzyme with the surface of the CS-SA beads, including electrostatic interactions and hydrogen bonding [30]. More interestingly, compared with free laccase, immobilized laccase maintained more than 90% of its relative activity between pH 4.0 and 5.5, indicating that the optimum pH range of immobilized laccase was wider. Experimental results showed that laccase was covalently immobilized on the CS-SA carrier, making the laccase more resistant to changes in pH acidity.

The effect of reaction temperature on the activity of free and immobilized laccases is shown in Figure 7b. Free laccase exhibited optimal catalytic activity at 55 °C. Laccase decreased its optimal reaction temperature through immobilization, and immobilized laccase activity reached its peak at 50 °C. The maximum activities of free and immobilized laccase were 49.6% and 74.2% at 40 °C, respectively. We could observe that immobilized laccase demonstrated higher relative activity and a wider range of suitable temperatures for substrate oxidation (i.e., 40 °C to 65 °C). With increasing temperature, the relative activity of free laccase decreased rapidly and was essentially inactivated at 80 °C, whereas the immobilized laccase still maintained 42.1% of its maximum activity. After immobilization, the suitable temperature for immobilized laccase became more broadly defined, a phenomenon that was the same as that reported by others [31].

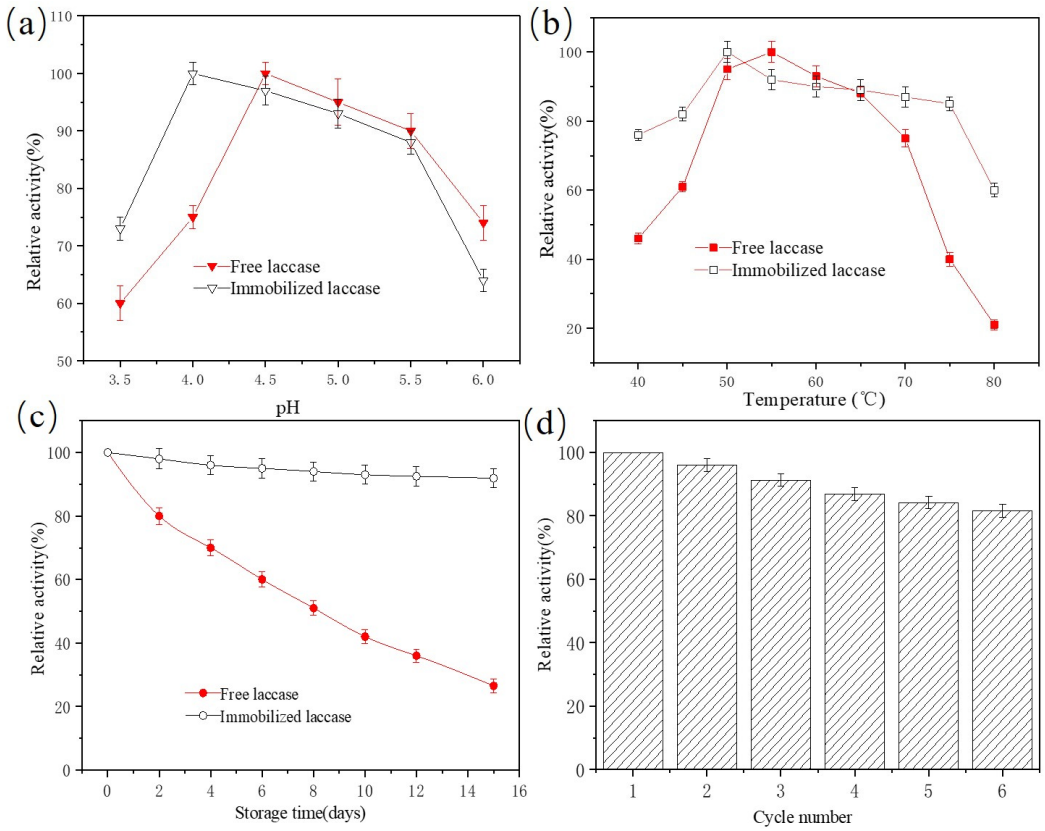

Figure 7. Effects of (**a**) pH, (**b**) temperature on free and immobilized laccase activity, (**c**) storage stability of free and immobilized laccase at 4 °C, and (**d**) reusability of immobilized laccase. Reaction condition: the standard reaction was carried out in a 20 mL round-bottom flask containing free laccase 13 mg or immobilized laccase 40 mg (protein content: 9.3 mg), 1 mmol ABTS, and 5 mL of 50 mM sodium acetate buffer (pH 4.5 or 4.0). The resulting mixture was shaken at different temperatures (55 or 45 °C) at 180 rpm.

The immobilized enzyme had high storage stability and reusability. As shown in Figure 7c, under the same storage conditions, the relative activity of free laccase declined faster than that of immobilized enzymes. In addition, the relative activity of immobilized laccase could maintain 91.9% of its initial state after 15 days of storage, while that of the free laccase was only 26.5%. Apparently, immobilized laccase had better storage stability. The decrease in water-soluble laccase stability might be due to the twisting action of the aqueous medium on the free laccase active site. However, when immobilizing laccases, the catalytically active site of the enzyme could usually be protected by a carrier, which would help its stability during storage [32].

Unlike free enzymes, immobilized enzymes could be easily separated from their substrates and used repeatedly, which greatly reduces the actual cost of using enzymes as biocatalysts and increases their viability in industrial applications. Figure 7d illustrates the cyclical utility of immobilized laccases. After six cycles of filtration separation and reuse, the activity of the immobilized laccase was maintained at 81.6% of its initial activity. In particular, there was almost no loss of relative activity of the immobilized laccase in the first three cycles. The sides reconfirmed that covalent bonding bonds were formed between the laccase and CS-SA beads and that the laccase did not leak from the carrier, maintaining relatively better enzyme activity. In conclusion, the incredible efficacy of the covalent bonding enzyme in catalyzing substrate oxidation over six operating cycles demonstrated its great practical potential as an ideal biocatalyst in bioprocessing technology [33].

2.5. Effects of pH, Temperature, and Initial Concentration on BPA Removal

In order to evaluate the catalytic performance of immobilized laccase and free laccase as biocatalysts, the degradation rates of BPA were determined separately. High-performance liquid chromatography (HPLC) was used to analyze the content of BPA and other oxides. The liquid chromatography analysis revealed that the UV absorption peak appearing at 3.80 ± 0.2 min was BPA. The removal efficiency of BPA by free and immobilized laccase in this study is shown in Figure 8. Under the optimal reaction conditions (pH 6.0, 50 °C, initial BPA concentration of 40 mg/L, CS-SA beads 1.0 g), the removal of BPA within 12 h was as high as 86%, with much higher BPA removal by immobilized laccase than currently reported for similar materials.

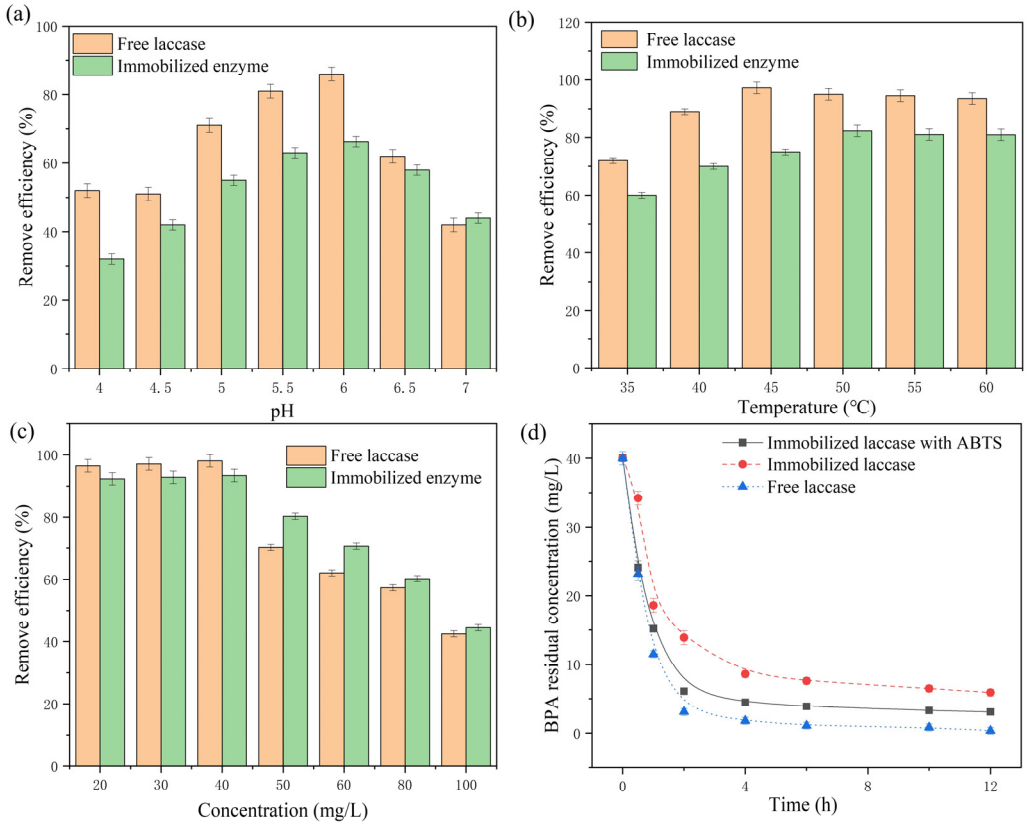

Figure 8. Effect of (**a**) pH, (**b**) temperature, (**c**) initial substrate concentration on the efficiency of BPA removal by free and immobilized laccase, and (**d**) degradation profiles of BPA by free and immobilized laccase. Reaction condition: the standard reaction was carried out with 25 mL of BPA solution and 1.0 g (wet weight) of immobilized laccase or an appropriate amount of free laccase reacted in a 50 mL round bottom reaction flask. pH (4.0–7.0), temperature (35–60 °C), BPA concentration (20–100 mg/L), and reaction time (1–16 h) influenced the BPA removal factors.

The pH stability of two forms of laccase was studied with the use of ABTS as the substrate for the oxidation. The immobilized laccase was observed to maintain more than 90% of the relative activity in the pH range of 4.0 to 5.5 (Figure 7a). A near-neutral pH (pH 7.0), on the other hand, was more conducive to non-enzymatic reactions, which allows further polymerization of intermediates generated by enzyme-catalyzed reactions into insoluble polymers. Therefore, the effect of pH in the range of pH 4.0–7.0 on BPA degradation was investigated. As shown in Figure 8a, the highest activity of both free and immobilized laccases appeared at pH 6.0, with degradation rates of 86.0% and 66.3% for BPA, respectively. Compared to free laccase, the degradation rate of immobilized laccase for BPA decreased in the pH range of 4.0–6.5, possibly as a result of increased diffusion and spatial site resistance of the enzyme molecules after immobilization. The removal

efficiency of immobilized laccase for BPA did not change much in the range of pH 5.0–7.0. Considering that the pH of paper mill industrial wastewater was in the range of 5.0–9.0, immobilized laccase could effectively remove BPA from industrial wastewater, especially paper mill industrial wastewater.

Figure 8b displays the BPA removal efficiencies of free and immobilized laccases in the temperature range of 35–60 °C, respectively. 97.2% of the free laccase removal efficiency for BPA was highest at an optimum temperature of around 45 °C. The optimum temperature for the immobilized laccase was 50 °C, and the removal rate of BPA was 82.3%. Experimental results indicated that the degradation and removal rate of BPA increase with increasing temperature. At the same time, in the temperature range of 50–60 °C with a high ratio, the degradation rate of immobilized laccase for BPA could be maintained at around 80%. Considering that the temperature of industrial wastewater discharged from factories must generally be below 60 °C, immobilized laccase could be applied for BPA removal in industrial wastewater.

Figure 8c depicts the effect of initial BPA concentration on the removal efficiency of both forms of laccase. When the initial concentration of BPA was in the range of 20–100 mg/L, both free and immobilized laccase had high BPA removal rates. From Figure 8c, we observed that the BPA removal rate by free laccase decreased from 98.1% to 42.5%, while the BPA removal rate by immobilized laccase decreased from 93.4% to 44.5%. However, the immobilized laccase showed better removal efficiency than the free laccase at higher initial BPA concentrations of 80 and 100 mg/L. This could be the lower removal efficiency of BPA caused by the inactivation of the free enzyme at high concentrations.

Figure 8d shows the degradation curves of free and immobilized laccases for BPA. The removal rates of both free and immobilized laccases increased with increasing time, with the removal rate of BPA by 12 h of free laccase reaching 98.1% and that of immobilized laccase within 12 h at 93.4%. The removal of BPA by immobilized laccase catalyzed the reaction system by adding 2 mg ABTS as a substrate for transferring electrons, reaching 99.1% in 12 h.

In immobilized enzyme systems, recyclability is one of the important indicators. In this experiment, the recyclability of immobilized laccase in removing BPA was investigated. In Figure 9, we could observe that the immobilized laccase still maintains 82.3% of its relative activity after six cycles of use. This recyclability of immobilized enzymes not only improves enzyme utilization but also significantly enhances economic efficiency. Overall, immobilized enzymes have shown great application potential and value in several fields due to their excellent recyclability.

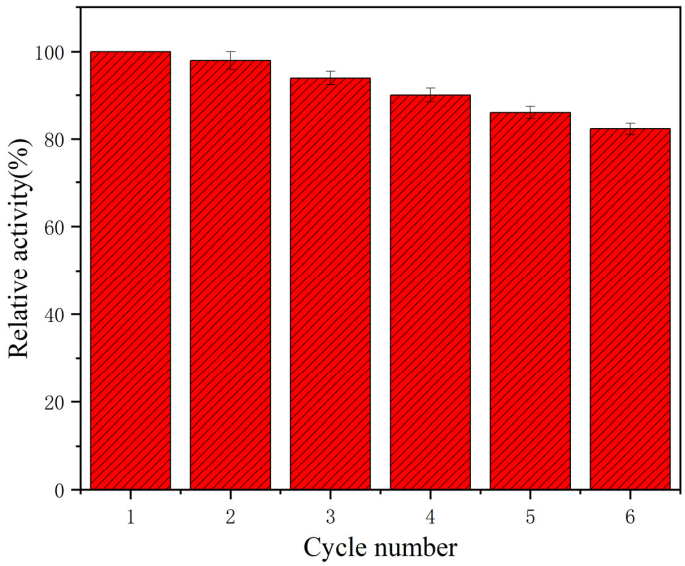

Figure 9. Recyclability of immobilized laccase for BPA degradation.

3. Materials and Methods

3.1. Materials and Reagents

Laccase (≥0.5 U/mg) from Aspergillus and Chitosan (CS, degree of deacetylation ≥ 95%, viscosity: 100–200 mpa.s) from crab shells were purchased from Sigma-Aldrich China Co. (Beijing, China). 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), bisphenol A (BPA) were obtained by Aladdin Reagent (Shanghai) Co., Ltd. (Shanghai, China). Cupric chloride was obtained by J&K Scientific Co., Ltd. (Beijing, China). Other reagents of reagent grade and desiccants were purchased from Shanghai Chemical Reagent Company (Shanghai, China). All reagents were used as received without further purification, and all solutions were prepared with distilled water.

3.2. Preparation of Lac/CS-SA Hydrogel Beads

Sodium alginate was ultrasonically stirred and fully dissolved in deionized water to obtain a 2% (*w*/*v*) solution of sodium alginate. Chitosan (degree of deacetylation ≥ 95) was ultrasonically stirred and dispersed homogeneously to obtain a 2% (*w*/*v*) chitosan solution. The chitosan solution was dropped into the sodium alginate solution during ultrasonication and stirred well, which was named CS-SA. At the end of the reaction, the prepared mixed solution was carefully dropped into a 4% $CuCl_2$ solution using a 5 mL syringe, and a small amount was added to each drop to obtain hydrogel beads. After 6 h of cross-linking in $CuCl_2$ solution, the solution was filtered and washed three times with deionized water. Laccase (U/mL) diluted to saturation with PBS buffer solution at pH 5 was mixed 1:1 (*v*/*v*) with the above CS-SA hydrogel beads, and the mixture was shaken at 200 rpm. After a 4 h reaction, the solution was filtered, washed three times with deionized water, and named Lac/CS-SA hydrogel beads.

3.3. Characterization of CS-SA Hydrogel Beads and Lac/CS-SA Hydrogel Beads

All prepared samples were lyophilized by a BÜCHI-L200 lyophilizer (BÜCHI Labortechnik AG, Flawil, Switzerland). The surfaces of CS-SA hydrogel beads and Lac/CS-SA hydrogel beads were characterized by Fourier transform infrared spectroscopy (PerkinElmer FTIR Spectrometer Spectrum Two, Waltham, MA, USA). A scanning electron microscope (JEOL-JSM-6700F, Tokyo, Japan) was used to observe the morphological characteristics of CS-SA hydrogel beads and Lac/CS-SA hydrogel beads. XPS analysis (Thermo SCIENTIFIC ESCALAB 250 Xi, Waltham, MA, USA) showed the basic composition of the hydrogel bead elements.

3.4. Measurements of Free and Immobilized Enzyme Activities and Kinetic Parameters

ABTS was used as the substrate to determine the activities of free and immobilized laccases [34]. Laccase could decompose the substrate ABTS to produce ABTS radicals, and the product had a characteristic absorption peak at 420 nm. The activity of laccase could be characterized by a change in absorbance value. 1 mM ABTS and appropriate amounts of free or immobilized laccase were added to the phosphate-buffered solution (50 mM, pH 6.0), and the reaction time was 4 min. The rate of oxidation of ABTS by laccase to produce $ABTS^+$ was monitored using a U-3900 HITACHI UV–Vis spectrophotometer (Tokyo, Japan) at 420 nm, and approximately 1 μmol of ABTS was converted to one laccase unit (U) in 1 min, i.e., the absorbance of the product $ABTS^+$ radical concentration increased from A_1 to A_2. One unit of laccase activity (1 U) is defined as the amount of enzyme required to oxidize one micromole of substrate per minute. The maximum value of enzyme activity is expressed as 100%, and laccase activity is calculated as follows:

$$\text{Enzymeactivity} = \frac{1000 \times 0.1844 \times (A_2 - A_1) \times 60 \times N}{t} \quad (1)$$

Specific enzyme activity is the ratio of the apparent activity of immobilized laccase to the laccase protein content per gram of immobilized enzyme. Enzyme activity units are $\mu mol \cdot L^{-1} \cdot min^{-1}$ (U/mL or U/mg). A_1 represents the initial absorbance of the $ABTS^+$ radical concentration, and A_2 represents the absorbance of the $ABTS^+$ radical concentration

at a reaction time of t. t is the time for the absorbance value to go from A_1 to A_2. N stands for the number of dilutions.

The kinetic parameters of free and immobilized laccase are determined by ABTS oxidation monitoring. The reaction rates of free and immobilized laccase were determined under optimal reaction conditions. The reaction conditions of free laccase were as follows: concentration 0.5 mg·mL^{-1}, reaction temperature 55 °C, solution pH 4.5, and immobilized laccase: reaction temperature 50 °C, solution pH 4.0. The concentration of ABTS in phosphate buffer varied between 0.2 and 5 mM. The Michaelis–Menten (Equation (2)) and Lineweaver–Burk (Equation (3)) were used to fit the data [34].

$$v = \frac{v_{max} \cdot [S]}{K_m + [S]} \quad (2)$$

where v (μmol/(L·min)) represents the initial reaction speed, v_{max} (μmol/(L·min)) represents the maximum reaction speed, [S] (μmol/L) represents the substrate concentration, and K_m (μmol/L) represents the Michaelis constant. The reciprocal treatment of the above equation is as follows:

$$\frac{1}{v} = \frac{K_m}{v_{max}} \cdot \frac{1}{[S]} + \frac{1}{v_{max}} \quad (3)$$

Let us draw a straight line from $\frac{1}{v}$ to $\frac{1}{[S]}$, the slope is $\frac{K_m}{v_{max}}$, the cross intercept is $-\frac{1}{K_m}$, the vertical intercept is $\frac{1}{v_{max}}$, we know the value of v_{max} and K_m

3.5. pH, Thermal and Storage Stability, and Reusability of Free and Immobilized Laccases

The effects of pH on the activities of free and immobilized laccase were investigated at pH 3.5–6.0 and 40 °C, and the optimum pH was screened. Since reaction temperature greatly affected enzyme activity, we evaluated 6 cycles of immobilized laccase for cyclability by measuring the relative enzyme activity of free and immobilized laccases at 40–80 °C and pH 4.5. At the end of each cycle-use reaction, the immobilized laccase was separated from the mixed reactant solution, washed with PBS buffer (pH 4.5, 50 mM) to remove residual reactant, and the immobilized laccase was placed as a catalyst in a new reactant for a new round of use. Storage stability of free laccase and immobilized laccase under temperature maintained at 4 °C was compared by periodically measuring the residual activity of free and immobilized laccases.

3.6. Removal of Environmental Pollutant Bisphenol A

First, 25 mL of BPA solution and 1.0 g (wet weight) of immobilized laccase or an appropriate amount of free laccase were reacted in a 50 mL round bottom reaction flask. pH (4.0–7.0), temperature (35–60 °C), BPA concentration (20–100 mg/L), and reaction time (1–16 h) influenced the BPA removal factors. All flasks were then sealed and placed on a thermostatic oscillator, which was shaken at a rate of 200 rpm.

A high-performance liquid chromatograph Shimadzu LC-20A (Kyoto, Japan) with a differential detector, a C18 column (Shim-pack GIST C 18, 5 μm), and a UV detector lamp (254 nm) were used for the quantitative analysis of BPA residue concentrations. The injection volume was 10 μL, and the UV absorption was detected at 278 nm. The mobile phase was methanol and water (75:25 *v*/*v*) at a flow rate of 1 mL/min and operated in isocratic mode. The standard solution of BPA was prepared, and the standard working curve was obtained.

3.7. Data Analysis

Based on the first-order model (Equations (4)–(6)), linear regression was used to calculate the first-order reaction rate constant (k) to obtain the time required to degrade 50% BPA ($t_{1/2}$) and the removal rate (RE).

$C_t = C_0 \exp(-kt)$ formula deformation:

$$-\ln\frac{C_t}{C_0} = kt \tag{4}$$

$$t_{1/2} = \frac{\ln 2}{K} \tag{5}$$

$$RE = \frac{C_0 - C_t}{C_0} \times 100\% \tag{6}$$

where C_0 represents the initial concentration of BPA, C_t represents the concentration of BPA at time t, t represents the reaction time, and k represents the rate constant for the first-order reaction.

A linear fit of Equation (4) with t as the horizontal coordinate and $-\ln\frac{C_t}{C_0}$ as the vertical coordinate, and the slope is the value of k. The two efficiency factors $t_{1/2}$ and the removal rate of BPA can be calculated from Equations (5) and (6), where $t_{1/2}$ represents the half-life, which is the time it takes for the level of a particular substance to be reduced to half its initial value by a certain reaction [35].

4. Conclusions

In this study, chitosan (CS) functionalized sodium alginate (SA) composite hydrogel was prepared, and CS and SA formed a network cross-linking structure with Cu^{2+}, respectively. This hydrogel carrier material with a dual network cross-linking structure was more stable after covalent binding with laccase, which was better for the reuse of immobilized laccase. Immobilized Lac/CS-SA hydrogel beads exhibited wider pH coverage, thermal stability, and storage stability compared to free laccase. In addition, bead biocatalysts were used to remove BPA from water. The removal of immobilized laccase was higher than that of free laccase at higher initial concentrations of BPA. The addition of ABTS greatly improved the catalytic reaction rate of the immobilized enzyme and the BPA removal efficiency. The kinetic distribution of the BPA degradation reaction fitted the first-order reaction kinetics. The catalytic performance of immobilized laccases in the BPA degradation reaction was better, which might be mainly attributed to the catalytic degradation by the enzyme. Thus, our study revealed the potential application of Lac/CS-SA beads as biocatalysts in industrial wastewater treatment.

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