

# Degradation of Carbofuran in Contaminated Soil by Immobilized Laccase

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## Abstract

The present study used the method of embedding-adsorption to immobilize fungal laccase and to determine the suitable conditions of immobilization by measuring various activities of immobilized laccase. In addition, the immobilized laccase was further employed to repair the carbofuran-contaminated soil, and then the degradation rates of carbofuran were measured under different conditions. The experimental results showed that the appropriate conditions of embedding-adsorption were presented as follows: the weight of powdered active carbon (PAC) was 0.4 g, the concentration of CaCl<sub>2</sub> was 1.5%, the volume of crude laccase was 80 ml, and the immobilized time was 6 h. After 48 h, the degradation rate of carbofuran in soil could reach almost 86% by the immobilized laccase. In fact, the artificially polluted soil and polluted soils in the environment have many differences, so the coming experiment will be concentrated directly on contaminated soil based on laboratory studies in order to investigate the influence of various factors on *in-situ* remediation.

**Keywords:** fungal laccase, carbofuran, immobilization, degradation rate

## Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a broad spectrum carbamate pesticide that has been used worldwide to control insects, nematodes, and acarids in vegetables, fruits, and forest crops [1]. In soil, carbofuran is moderately persistent with a half-life of up to 110 days. Its good water solubility (700 mg/L at 25°C) and low adsorption (mean K<sub>oc</sub> of 29.4) result in the contamination of soil [2-6]. Carbofuran was first

used by the American FMC company in 1968, and China began to register this compound in 1986. The pollution areas of carbofuran in China are found to the northeast, in the Yangtze River, and the coastal areas of the vast plain farming area. These areas are the main producing regions of spring wheat, soybean, corn, cotton, rice, grain, and other crops. Carbofuran has only rarely been applied in western China.

Carbofuran can be efficiently degraded through a biodegradation process, and several metabolites also have been identified [7-9]. As a kind of oxidoreductive enzyme, the laccase can catalyze the oxidation of a broad range of organic compounds such as polyphenols,

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2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), aromatic compounds, etc. [10-12]. Laccase (EC.1.10.3.2), namely hydroquinone oxidase, is a class of Cu-containing polyphenol oxidase. It is a type of protein found in *Rhus vernicifera* by Yoshida in 1883, which can catalyze immobilized paint [13]. More than a decade later, Bertrand also found this enzyme in the fungi and named it laccase [14]. Laccases are widely distributed in wood-rotting fungi, and some have been derived from plants, bacteria, and insects [15]. Compared to other kinds of laccase, fungal laccase has received much attention for its intensive applications [16]. Previous studies have proved that laccase has strong stability and broad substrate specificity, and many studies have shown the process of laccase catalysis [16].

With the rapid development of immobilized enzyme technology, contemporary immobilized enzyme technology has been applied widely in the degradation of pollutants [17-18], biofuel cells [19-20], residue detection [21-22], and other relevant areas. Ma et al. [23] extracted crude enzymes from HB-5 and immobilized enzyme made from crude enzyme on sodium alginate, and the two methods were introduced to atrazine-polluted soils to evaluate the degrading ability for practical use. Results showed, at 144 h, that only about 10% of the initially applied amount of atrazine was left in the two soils. Although crude and immobilized enzyme could be used, the immobilized enzyme is preferred for its stability. *Rhodococcus* sp. and *Pseudomonas* sp. bioremediation experiments were carried out using free and immobilized cells on natural carrier material (corn cob powder) in order to evaluate the feasibility of its use in the bioremediation of hydrocarbon-contaminated soils. When the cells were properly adsorbed on the porous matrix, immobilization became a suitable bioremediation strategy [24]. In reactive industrial dyes, *Trametes pubescens* and *Pleurotus ostreatus* were immobilized on polyurethane foam cubes in bioreactors, then used to decolorize three industrial and model dyes at concentrations of 200, 1,000, and 2,000 ppm. Both fungi were able to efficiently decolorize all dyes, even at the highest concentration, and the duckweed test showed a significant reduction ( $p \leq 0.05$ ) of toxicity after the decolorization treatment [25].

Wasi et al. immobilized the *Pseudomonas fluorescens* SM1 strain in calcium alginate beads for remediation of the major toxicants in Indian water bodies. From the results, a roughly 80% reduction in the concentration of phenols was observed by immobilized SM1 cells compared with 60% by the free cells. Also, in the case of the bioremediation of heavy metals, immobilized SM1 cells were found to be more efficient compared with the free cells [26]. But at the same time, the single immobilized method always has its own disadvantages and the activity of the immobilized enzyme is influenced by various environmental factors, leading to a limited range of application to some extent. The adsorption method can be divided into physical adsorption and ionic adsorption. This method uses the carriers with high adsorption capacity and the free enzyme will be adsorbed to the surface of the carriers (the common

carriers for this method are silica gel, activated carbon, zeolite, porous glass, quartz sand, and cellulose). The adsorption is a relatively simple and inexpensive method, the carriers can be reused, and the reaction conditions are mild. But the soundness of absorption is poor; if the contact force changes suddenly, the enzyme will easily fall off the carriers [27]. Entrapment could be defined as the physical retention of enzymes in a porous solid matrix. This method will not change the structure of the enzyme, and it can keep the highest activity of the enzyme. However, the immobilized enzyme would lose activity easily because the combination is not very strong. Hence it has always been used together with other immobilized methods [28, 29].

This study combined two kinds of immobilized methods: adsorption and entrapment, improving the activity of immobilized laccase and enhancing the stability of immobilized laccase. The objectives of this study were: 1) to compare the activities of immobilized laccase and non-immobilized laccase, 2) to explore the factors affecting the process of immobilized laccase, and 3) to evaluate the efficiency of immobilized laccase applications on soil remediation for carbofuran contamination.

## Materials and Methods

### Chemicals and Equipment

The specific white-rot fungi were domesticated and trained in the microbiological laboratory of Shenyang University of Technology. The soils without carbofuran were obtained from the campus. The carbofuran was acquired from Zhenjiang Jiansu Pesticide Chemical Co., Ltd (China). Sodium alginate was purchased from Tianjin Bodi Chemical Co., Ltd (China). 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Ruibio (Germany). Glutaraldehyde (AR) was purchased from Sinopharm Chemical Reagent Co., Ltd, China. Ultraviolet-visible spectrophotometer (EOK UV721, China) was used to determine laccase activity. High-performance liquid chromatography (HPLC, Agilent 1260, Singapore) was used to determine the carbofuran degradation rate.

### Preparation of Crude Laccase

A certain quantity of straws (each about 3 cm long) was weighed into a conical flask, then water was added to soak for one day and the wet straws were sterilized at high temperature and high pressure for 30 min. After cooling, the straws were used as raw material of producing enzyme culture medium. We grafted white-rot fungi (*Lenzites betulinus*) into this culture medium and fostered it in an incubator at 26°C, then added 100 ml aseptic abstract liquid and soaking-draw under 26°C and 120 revolutions per minute (rpm) for 24 h. For removing the impurity, the mixture was centrifuged for 15 min at 4,000 rpm and the supernatant was collected as crude laccase.

### Immobilization Procedure

We weighed 0.4 g sodium alginate into 20 ml distilled water, which was then heated at 60°C until the compounds became colloidal solution. PAC was then taken into colloidal solution. After mixing, the mixture was dropped into CaCl<sub>2</sub> by a special granulator and put into the oscillator at 120 rpm and 25°C for 4 h. Later, the carriers were taken out and washed by distilled water repeatedly until the residual CaCl<sub>2</sub> was cleaned. Finally, the carriers were put in 250 ml free laccase for immobilization under 120 rpm at 25°C. After immobilization, the residual free laccase on the surface of carriers was cleaned, and the activity of immobilized laccase was determined.

### Determining Free Laccase Activity

ABTS was used to determine the activity of free laccase with 2 ml of reaction solution containing 200 µL of 500 mM C<sub>3</sub>H<sub>4</sub>O<sub>4</sub>-C<sub>3</sub>H<sub>2</sub>O<sub>4</sub>Na<sub>2</sub> (pH = 4.5), 100 µL of 20 mM ABTS, 1,500 µL of H<sub>2</sub>O, and 0.2 ml of laccase liquid. We took the reaction solution into HPLC and started the reaction at 28°C, then increased the absorbance to 420 nm during 3 min. The measurement data were used to calculate the free laccase activity. One unit (*U*) of laccase activity was defined as the amount of laccase used for catalytic oxidation of 1 µM ABTS after 1 min.

The free laccase activity was calculated using the following formula:

$$U = \frac{\Delta A \times V \times 1000 \times V_1}{\Delta t \times \epsilon_{420} \times V_0 \times l \times m_s} \quad (1)$$

...where  $\Delta A$  was the increasing absorbance during 3 min,  $V$  was reaction solution,  $V = 2$  ml and  $V_1$  was the sterile leach liquor,  $t$  was 3 min,  $\epsilon_{420} = 3.6 \times 10^3 \text{ L} \cdot \mu\text{mol}^{-1} \cdot \text{cm}^{-1}$ ,  $V_0$  was laccase liquid,  $V_0 = 0.2$  ml,  $l$  was the inner diameter of cuvette,  $l = 1$  cm, and  $m_s$  was the quality of the medium.

### Determining Immobilized Laccase Activity

To determine the activity of immobilized laccase, the study used the same method --- ABTS, weighing 0.1 g immobilized laccase into beaker, 1.8 ml of reaction solution containing 200 µL of 500 mM C<sub>3</sub>H<sub>4</sub>O<sub>4</sub>-C<sub>3</sub>H<sub>2</sub>O<sub>4</sub>Na<sub>2</sub> (pH = 4.5), 100 µL of 20 mM ABTS, and 1,500 µL of H<sub>2</sub>O. We mixed the reaction solution for 2 min at 28°C, then the supernatant was taken into HPLC and started the reaction, as well as determining free laccase activity and increasing the absorbance to 420 nm during 3 min. Measurement data was used to calculate the immobilized laccase activity. The activity was calculated by the following formula:

$$U_i = \frac{\Delta A \times V \times 1000}{\Delta t \times \epsilon_{420} \times M_0 \times 1} \quad (2)$$

...where  $\Delta A$  was increment of absorbance during 3 min,  $V$  was 2 ml of reaction solution,  $t$  was 3 min,  $M_0$  was 0.1 g immobilized laccase, and  $U_i$  was the activity of immobilized laccase.

### Preparation of Carbofuran-Contaminated Soil

After sampling, the soil was filtered through 20 mesh under room temperature. Carbofuran was weighed into a volumetric flask (500 ml), making up to the mark with water and shaking thoroughly. Then this solution was taken into 300 g soil and water was added for fully mixing to prepare the artificial carbofuran-contaminated soil. After drying, 15 g soil was taken out and made into 1:3 of slurry with 45 ml water.

### Degradation of Carbofuran by Immobilized Laccase

We weighed 15 g contaminated soil into 45 ml water for making the contaminated slurry, then put some immobilized laccase in the slurry and set the degradation conditions of 25°C and 120 rpm, and the degradation rates of carbofuran were measured every 8 h.

### Conditions of HPLC

The mobile phase was prepared with methyl alcohol and distilled water (78:22, v/v). UV wavelength was set to 280 nm at room temperature. The sample size was 10 µm at a rate of 1.2 ml/min and the retention time was 11 min.

### Data Processing

Microsoft Excel was applied to process all the experimental data. SPSS 17.0 (SPSS Inc., Chicago, IL) was applied for statistical analysis, and  $p < 0.05$  was considered a significant difference.

## Results and Discussion

### The factors affecting laccase immobilization

#### *The Influence of Weight of Powder Activated Carbon (PAC) on the Immobilized Laccase*

According to Fig. 1, the weight of PAC increased with the activity of immobilized laccase, which could be attributable to the adsorption ability of PAC for laccase. At the same time, the weight of PAC (data not shown) also affected the formation of carriers. With the rising weight of PAC, the viscosity of the mixed solution was also increasing, and the high viscosity would make the formation of carriers become hard. When the weight of PAC reached 0.8 g, the mixed solution was difficult to drop from the granulator because of the high viscosity. It could be concluded that the added PAC did not dissolve in the mixed solution – they mainly existed in the form

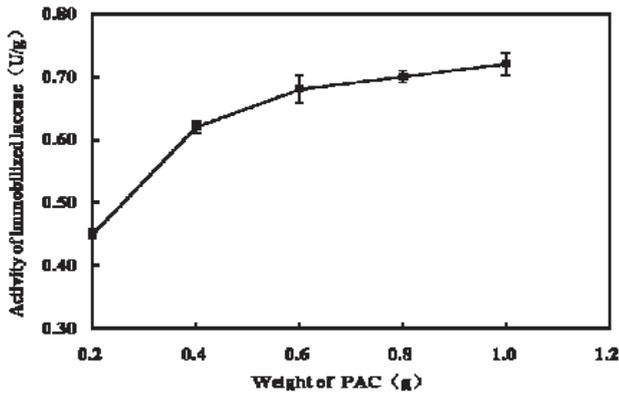


Fig. 1. The influence of PAC weight on immobilized laccase activity.

of tiny powder, and too much tiny powder might lead to “accumulation” in the mixed solution. To comprehensively consider the forming conditions of carriers and the activity of immobilized laccase, it was determined that the appropriate weight of PAC was 0.6 g. Hydrophilic nanoporous PMMA/O-MMT composite microfibrus membrane was used in laccase immobilization, and the immobilized laccase showed better resistance to pH and temperature changes than that of the free-form laccase, and after 10 successive runs of repeated use, the immobilized laccase still retained 30% of its initial activity [30].

#### The Influence of CaCl<sub>2</sub> Concentrations on Immobilized Laccase

In the process of preparing the carrier, CaCl<sub>2</sub> was used to consolidate the sodium alginate-activated carbon colloids into carriers. If the concentration of CaCl<sub>2</sub> was too low (below 1.0%), the intensity of carriers would be small and the activity of immobilize laccase would be low. But if the concentration was too high (above 2%), the surface of carriers would be full of Ca<sup>2+</sup> and it could interact with laccase easily. This reaction would reduce the activity of immobilized laccase. As can be seen from Fig. 2, the activity of immobilized laccase was significantly higher

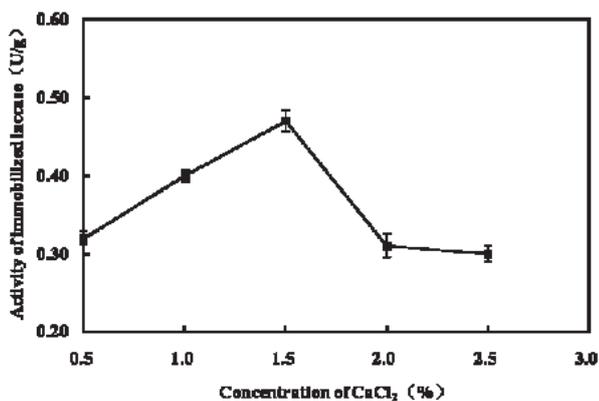


Fig. 2. The influence of CaCl<sub>2</sub> concentration on immobilized laccase activity.

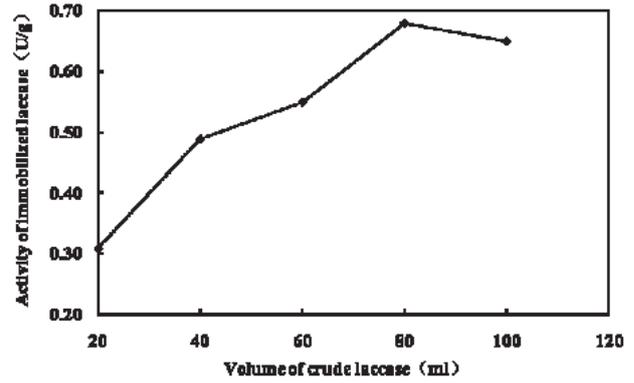


Fig. 3. The influence of crude laccase volume on immobilized laccase activity.

than others when the concentration of CaCl<sub>2</sub> was 1.5%. M. S. Mohy Eldin et al. [31] immobilized Glucoamylase onto p-benzoquinone-activated alginate beads by affinity covalent, and investigated the effect of CaCl<sub>2</sub> concentrations that resulted in maximum activity being obtained with beads cross-linked at 3% CaCl<sub>2</sub> solution. Indeed, the activity of the covalently immobilized enzyme is almost double its entrapped counterpart.

#### The Influence of Crude Laccase Volume on Immobilized Laccase

As shown in Fig. 3, the activity of immobilized laccase changed with different volumes of crude laccase. With the increase of the volume of crude laccase, the immobilized laccase activity showed a rising trend. When the volume was more than 80 ml, the carriers may have reached “saturation” and the activity of immobilized laccase tended to stabilize with a slight decline. So 80 ml was chosen as the appropriate volume of crude laccase. Bai et al. [32] studied the effect of the glucoamylase amount added on the activity recovery of covalently immobilized enzyme onto hydrophilic bead carriers containing epoxy groups. They found that the maximum activity recovery and retained activity percentage could reach 78% when

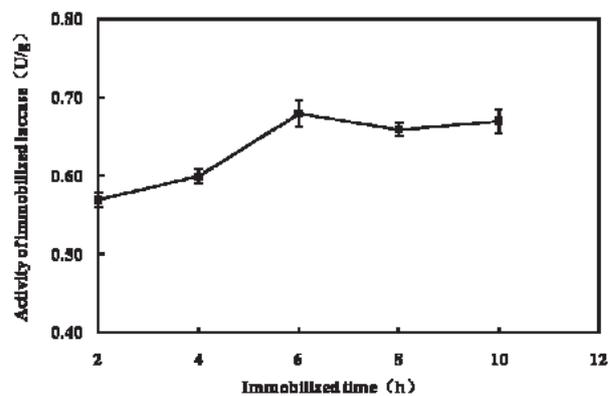


Fig. 4. The influence of immobilized time on immobilized laccase activity.

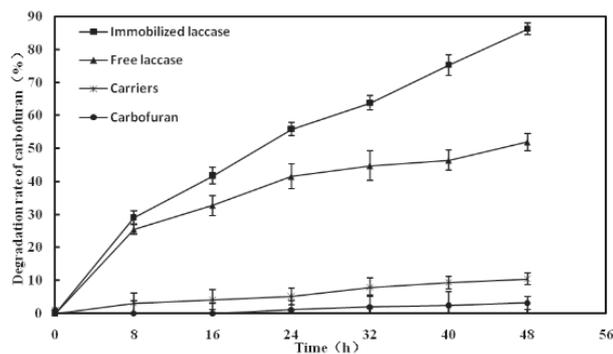


Fig. 5. The influence of immobilized laccase on carbofuran degradation.

the enzyme-adding amount was 0.5 mg with 50 mg of the carrier.

### The Influence of Immobilized Time on the Immobilized Laccase

With the extension of immobilized time, the activity of immobilized laccase showed an increasing trend, but this tendency was indistinctive (Fig. 4). After training for 6 h, the activity did not increase with the time gone. It could be inferred that there is not a close relationship between the activity of immobilized laccase and immobilized time. Previous studies [31] show that the change of cross-linking time had a significant effect on the activity of immobilized enzyme. It is clear that the activity of covalently immobilized enzyme increases by about 30% with time increase from 30 to 180 min, while its entrapped counterpart does not respond effectively under the same conditions. Accordingly, the percentage of activity increment of covalently immobilized enzyme to an entrapped one ranged from 58% to 98% at 30 to 180 min cross-linking time.

### The Efficacy of Immobilized Laccase for Remediation of Carbofuran-Polluted Soils

#### The Influence of Immobilized Laccase on the Degradation of Carbofuran

Fig. 5 shows that laccase degradation ability was improved significantly ( $p < 0.05$ ) by immobilization, and the carriers also had a certain degree of adsorption. After 48 h, the degradation rates under different conditions are detailed as below: the degradation rates of carbofuran by immobilized laccase, free laccase, and carrier were 83.2%, 52.0%, and 10.4% respectively. In addition, the carbofuran sample was 3.2%.

#### The Influence of Environmental Parameters on the Degradation Rate of Carbofuran

As in Fig. 6, the degradations of immobilized laccase and free laccase were different ( $p < 0.05$ ) under

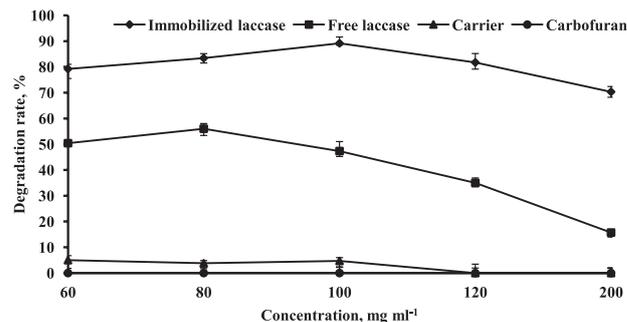


Fig. 6. The influence of initial concentration on the rate of carbofuran degradation.

the experimental range, but the degradations have the same changing trend and increased with the rising concentration. When the concentration was set to 120 mg/ml, the degradation rate declined slightly and showed that a high initial concentration above 200 mg/ml might inhibit the catalysis activity of laccase. In the last century, many studies [33] have used immobilized methods to degrade organic compounds in the environment. Nawaz et al. [34] immobilized cells of a *Pseudomonas* sp.- and *Xanthomonas maltophilia*-degraded acrylamide. Wiesel et al. [35] immobilized mixed bacterial culture-degraded polycyclic aromatic hydrocarbons (PAH). With the development of immobilization technology and genetic engineering, the repair methods of immobilized cells will be more and more extensive.

The results of degradation rates affected by pH are shown in Fig. 7. The degradation rates were all over 60% under the range of pH, but the degradation rate of  $pH < 7$  was obviously higher in others, showing us that the used laccase was more suitable for catalytic reaction in an acidic environment. Based on SPSS analysis, the influences of immobilized laccase and free laccase on pH had significant differences ( $p < 0.05$ ). Similarly, Palvannan et al. [36] used bacterial and fungal laccase to degrade the high concentrations of micropollutants, determining that the laccase showed highest activities under acidic conditions.

As shown in Fig. 8, when the temperature was set to 15°C, 20°C, 25°C, 30°C, and 35°C the immobilized laccase had strong degradation ability. After immobilization,

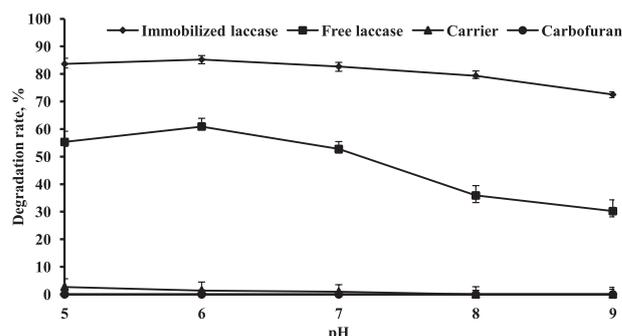


Fig. 7. The influence of pH on the degradation rate of carbofuran.

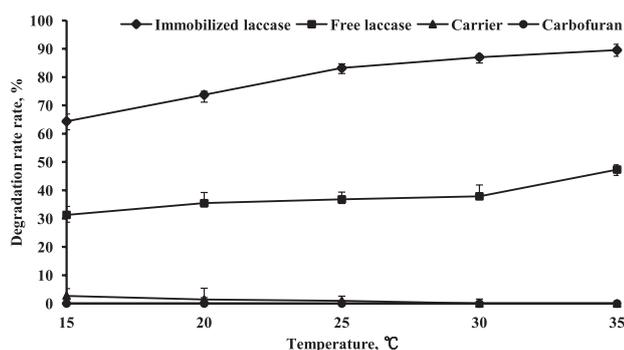


Fig. 8. The influence of temperature on the degradation rate of carbofuran.

the laccase could adapt to higher temperature, so the immobilized laccase could be used under a broad temperature range. Besides the advantages of immobilization at different temperatures, for example, we saw higher enzyme-support reactivity [37], more possibilities of getting an intense multipoint covalent attachment [38], and more rapid immobilization [37]. But there might be some problems to be considered, for example stability of enzyme, stability of the active groups on support, and even stability of the support itself. So different enzymes should be immobilized by their activity to choose the appropriate temperature.

For different kinds of laccase, the training conditions were also different. However, it could not be ignored that only crude laccases were acquired and determined in the training process, while the purifications were not implemented in the experiment. Jaiswal et al. [39] studied the purification of laccase from papaya leaves by a two-step procedure, including heat treatment and con-aaffinity chromatography, to obtain some information about the characteristics of the purified laccase, such as the purified laccase exhibiting acidic and alkaline pH optima of 6.0 and 8.0 with the non-phenolic substrate (ABTS) and phenolic substrate (catechol), respectively.

The immobilized method adopted by this study was the embedding-adsorption method. Previous studies [28, 29, 38-40] have shown that different immobilized methods have different processes, and the immobilized process was influenced by different factors. Palvannan et al. [41] immobilized the laccase on the zein polyurethane nanofiber, and studied the application in phenyl urea herbicide chloroxuron. They also got the result that the immobilized laccase has better pH and thermal stability than the free laccase.

This study used artificially polluted soil for remediation (*ex-situ* remediation). In fact, the artificially polluted soil and polluted soils in the environment have many differences, for example some other natural microbial enzymes in the soil may have some impact on the activity of immobilized enzyme, temperature, humidity, PH, and other environmental factors. The next experiment will be done directly on contaminated soil based on laboratory studies (*in-situ* remediation). Investigating the influence

of various factors on *in-situ* remediation should be the focus of future research.

## Conclusions

In the present study, the immobilized laccase was used to degrade the carbofuran and the appropriate degradation conditions, including the initial concentration of 100 mg/ml, pH of 6, and temperature of 30°C. Bioremediation for its high efficiency, safety, low cost, no two pollution has great potential and prospects for development, but the microbes are living organisms that need to have appropriate living conditions, and the bioremediation process is usually better than the physical and chemical repair process, which is slow, and the difference in different regions is large, meaning that repair has restricted the application of the field of bioremediation. Bioaugmentation can integrate the advantages of various immobilized microbial methods into one body, which has incomparable advantages compared with other single immobilized technologies. In this study, the laccase system has the characteristics of high microbial activity, high density of laccase in unit space, and tight cross-linking, but stability is not good and is vulnerable to environmental impact. While embedding into the protective structure can be a good solution to this problem, not only can we put the enzyme into an adverse environment to maintain activity, but we can put the enzyme in the process of drying and storage invariance.

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