Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *81* (10) 1215–1224 (2016)
JSCS–4920
UDC 504.53.054:631.466+542.92:
577.152.1–034.3
Original scientific paper

Degradation of chlorpyrifos in contaminated soil by immobilized laccase

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(Received 28 January, revised 28 May, accepted 9 June 2016)

Abstract: In the present study, the embedding-crosslinking method was used to immobilize fungal laccase, and to determine the suitable conditions for immobilization by measuring various activities of the immobilized laccase. In addition, the immobilized laccase was further employed to repair chlorpyrifos-contaminated soil, and then the degradation rates of chlorpyrifos were measured under different conditions. Based on the results, the appropriate conditions for the method of embedding-crosslinking were found to be as follows: concentration of sodium alginate – 3 %, concentration of glutaraldehyde – 1 %, crosslinking time – 6h, volume of crude laccase – 60 ml, and immobilization time – 4 h. After 48 h, the degradation rate of chlorpyrifos in soil could reach at least 70 % by the use of immobilized laccase that was prepared by the method of embedding-crosslinking under different environmental conditions.

Keywords: fungal; immobilization; embedding-crosslinking; degradation rate.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) is a multicopper oxidase that was originally obtained from the juice of *Rhus verniciflua*. Subsequently, it has been found in some insects,[1,2] certain bacteria,[3] plants[4] and various fungi.[5,6] Details related to laccase industrial applications are given in Supplementary material to this paper. However, there are still some limitations in the applications of laccase in environmental engineering, *e.g.*, the conditions of the applications are limited, the enzyme activity is easy to lose, and the operating parameters are changeable.[7]

In the recent years, immobilization methods have become available for the partition of the enzyme catalyst from the reaction mixture without difficulty thus reducing the cost of the enzymes significantly. Different immobilization proce-

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doi: 10.2298/JSC160128066W

dures are considered in the Supplementary material. The immobilization of the enzyme might cause certain alterations in its physical and chemical properties; therefore, it is very important to choose a suitable immobilization technology. The entrapment technique could be defined as physical entrapment within a polymer matrix support. This method does not change the structure of the enzyme, but it can keep its highest activity.[8–12] Crosslinking is similar to covalent bonding, and the immobilization by this method is based on the crosslinking between the functional groups on the carrier surface and the enzyme, At the same time, this method is different from covalent bonding and the carriers used for the crosslinking are non-water-soluble.[13–18]

Although the entrapment is widely used for immobilization of enzymes and has the advantages of an inexpensive and simple operation, the combination between the enzyme and the carriers, obtained by this method is not very strong, which would cause the immobilized enzyme to lose its activity easily.[8] In the process of crosslinking, the combination between the enzyme and the carriers is strong, the immobilized enzyme has high stability, but the crosslinking process is complex, and the violent reaction inhibits the activity of the enzyme.[19]

Chlorpyrifos (*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) (CPF) is one of the most extensively used organophosphate pesticides in the world (for details see Supplementary material).[20–22]

In this study, the entrapment and crosslinking were combined to achieve the best laccase immobilization effect; the fungal laccase was immobilized on sodium alginate, and the glutaraldehyde was employed as crosslinking agent to improve the immobilization effect and CPF removal efficiency from the soil by laccase.

The objectives are: 1) to compare the activities of immobilized laccase and non-immobilized laccase; 2) to explore the factors affecting the process of laccase immobilization; 3) to evaluate the efficiency of the immobilized laccase applications on the soil remediation for CPF contaminations.

EXPERIMENTAL

Materials and methods

Details related to chemicals and equipment used are given in the Supplementary material.

Methods

Immobilization procedure. Firstly, the weighted sodium alginate was placed into 20 ml distilled water, and heated at 60 °C until the compounds form a colloidal solution. Then the cold colloidal solution was dropped into 1.5 % $CaCl_2$ by a special granulator, and they were placed in an oscillator at 120 revolutions per min, 25 °C for 4 h. Later, the carriers were taken out and washed by distilled water repeatedly, until the residual $CaCl_2$ was washed clean. Finally, the immobilization was carried out by placing the carriers in 250 ml free laccase at 120 rpm and 25 °C. After the immobilization (Fig. S-1 of the Supplementary material), the residual free laccase was washed away from the surface of the carriers, and the activity of the immobilized laccase was determined.

Preparation of crude laccase. Straw with certain quality (length of about 3 cm) was weighted into conical flask, and then some water was added to soak it for a day. It was sterilized at high temperature and high pressure for 30 min, and used as raw material for producing enzyme culture medium until cooling. White-rot fungi (*Lenzites betulinus*) were grafted into the above culture medium, and fostering was conducted in an incubator at a temperature of 26 °C. After that, 100ml aseptic abstract liquid was added and soaking-draw was conducted for 24 h at 26 °C and 120 rpm. It was centrifuged for 15min at 4000 rpm to remove the rope and solid impurities. The supernatant was collected as crude laccase concentration liquid.

Determination of laccase activity

Determination of free laccase activity. ABTS was used to determine the activity of free laccase. For this experiment reaction solution with volume of 2 ml containing 200 μl of 500 mM $C_3H_4O_4$–$C_3H_2O_4Na_2$ (pH 4.5), 100 μL of 20 mM ABTS, 1500 μl of H_2O, and 0.2 ml of laccase liquid were used. The reaction solution was introduced into the HPLC system. The reaction was conducted at 28 °C for 3 min and the increase in the absorbance at 420 nm was measured. The measured value was 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 by the following formula:

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

where ΔA was the increase in the absorbance for the duration of the experiment – 3 min, V was the volume of the reaction solution – 2 ml, V_1 was the volume of the sterile leach liquor, Δt was 3 min, $\varepsilon_{420} = 3.6\times10^3$ L·μmol^{-1}·cm^{-1}, V_0 was the volume of laccase liquid – 0.2 ml, l was the inner diameter of the cuvette (l = 1 cm) and m_s was the quality of the medium.

Degradation of chlorpyrifos by immobilized laccase

A slurry was prepared by mixing 15 g soil containing chlorpyrifos with 45 ml water and then the immobilized laccase was added. The degradation conditions were set to 25 °C and 120 rpm. The degradation rate of chlorpyrifos was determined every 8 h.

The conditions of HPLC

Mobile phase was prepared with methyl alcohol and distilled water (9:1 volume ratio). The UV wavelength was set to 300 nm at room temperature. The sample size was 10 μm at a rate of 1.0 ml/min and the retention time was 6 min.

Data processing

The Microsoft Excel software was used to process all the experimental data. The SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis, and $p < 0.05$ was considered as significant difference.

RESULTS AND DISCUSSION

Influence of sodium alginate and glutaraldehyde concentration on the activity of the immobilized laccase

The results presented in Fig. 1 showed that an increase in the concentration of sodium alginate led to increase in the activity of the immobilized laccase until

a concentration of 3 % was reached. The highest activity was recorded at concentration of 3 %. Further increase in the concentration of the sodium alginate resulted in decrease in the activity of the immobilized laccase. A possible reason for this was that when the concentration was lower than 3 %, the immobilized laccase was insecure, and the free laccase was easy to escape from the carriers. However, concentrations higher than 3 % led to denseness in the membrane structure of the carrier surface, thus decreasing the activity of the immobilized laccase in this case. Therefore, the most suitable concentration of sodium alginate was found to be 3 %.

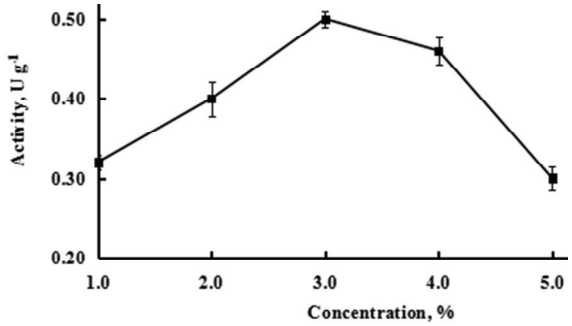

Fig. 1. Influence of sodium alginate concentration on the activity of immobilized laccase.

As shown in Fig. 2, the activity of the immobilized laccase increased when the concentration of glutaraldehyde increased from 0.1 to 1.0 %. Further increase in the concentration led to decline in the activity of the immobilized laccase, This could be explained with the fact that, at concentrations of the crosslinking agent lower than 1.0 %, the crosslinking process was not complete, which led to low activity of the immobilized laccase. However, if the concentration was higher than 1.0 %, it was easy to form a tight network on the carrier surface, which hindered the reaction between the laccase and the substrate. Therefore, the optimal concentration of glutaraldehyde was determined to be 1.0 %.

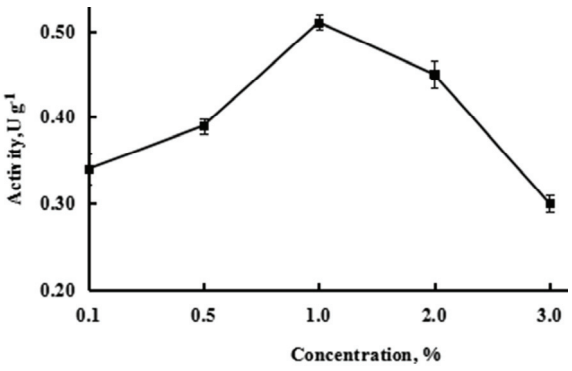

Fig. 2. Influence of glutaraldehyde concentration on the activity of immobilized laccase.

Influence of crosslinking time and immobilization time on the activity of the immobilized laccase

Like the concentration of the crosslinking agent, the crosslinking time also affected the activity of the immobilized laccase to some extent. The results presented in Fig. 3 showed that with increasing of the crosslinking time from 2 to 6 h, the activity of the immobilized laccase increased. The highest activity was reached at crosslinking time of 6 h when the space structure of the carrier was dense. Therefore a crosslinking time of 6 h was found to be the optimal one.

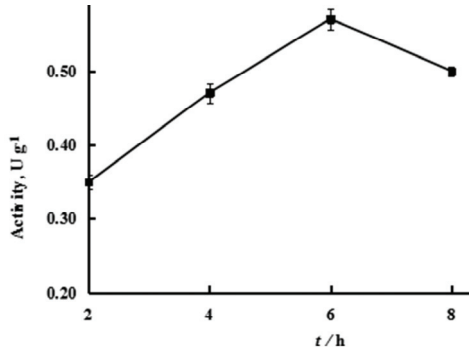

Fig. 3. Influence of corsslinking time on the activity of immobilized laccase.

The combination between free laccase and carrier was somehow related to the immobilization time. Theoretically, the longer immobilization time leads to better activity of the immobilized enzyme. During the period of the measurement, the change in the activity with the immobilization time was relatively flat. However, as shown in Fig. 4, the activity of the immobilized enzyme reached high levels after 2 and 4 h. Through comprehensive consideration of other factors, 4 h was accepted as the optimal immobilization time.

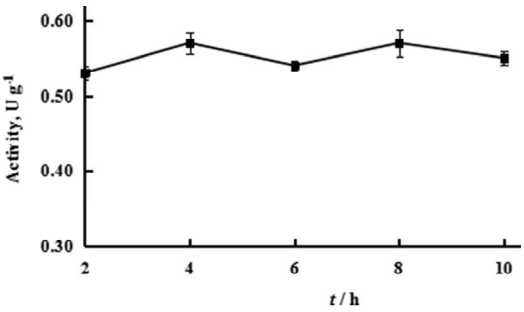

Fig. 4. Influence of immobilization time on the activity of immobilized laccase.

Influence of volume of crude laccase on the activity of the immobilized laccase

As shown in Fig. 5, the activity of the immobilized laccase changed with the change in the volume of crude laccase. With increase of the volume of crude laccase, the activity of the immobilized laccase showed a growing trend, but when

the volume exceeded 60 ml, the activity of the immobilized laccase had slight decline. Therefore, 60 ml was found to be the optimal volume of crude laccase.

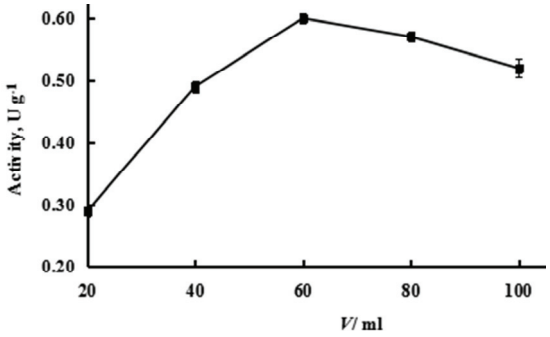

Fig. 5. Influence of volume of crude laccase on the activity of immobilized laccase.

Influence of immobilized laccase on the degradation of chlorpyrifos

The efficiency of immobilized laccase was tested on the soil remediation, and it showed a good treatment effect. After immobilization, the degradation ability of laccase was significantly improved. When chlorpyrifos-contaminated soil was treated with immobilized laccase for up to 48 h or more, the degradation rate of chlorpyrifos reached 70 %. As a control, experiments of degradation of contaminated soil by using free laccase and blank carrier were also conducted. The free laccase had good degradation ability only at the beginning of the degradation. Over time, the activity of free laccase decreased and after 48 h the degradation rate of chlorpyrifos reached only 46 %. However, through statistical analysis ($p > 0.05$), in the case of temperature as the test treatment, the difference between the immobilized laccase and free laccase was not significant. The results showed that the degradation rate of chlorpyrifos was very low without laccase (Fig. 6). This was demonstrated through determination of the chlorpyrifos residue.

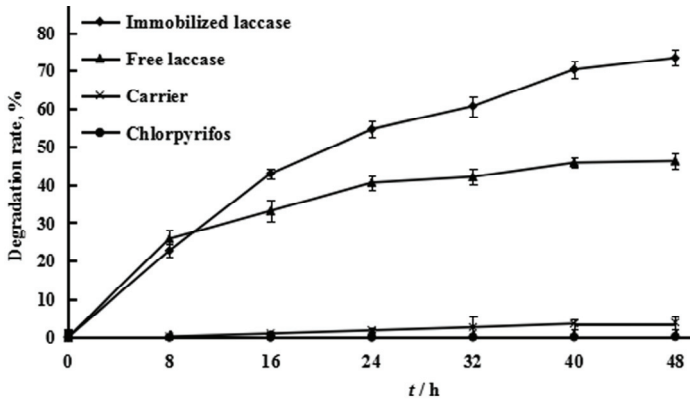

Fig. 6. Influence of immobilized laccase on the degradation of chlorpyrifos.

Influence of initial concentration on the degradation rate of chlorpyrifos

The influence of the initial concentration on the degradation rate of chlorpyrifos is shown in Fig. 7. The results showed that at different initial concentrations of chlorpyrifos, the immobilized laccase and free laccase have different degradation abilities ($p < 0.05$). When the initial concentration of chlorpyrifos was less than 100 mg/ml, the increase in the concentration of the contaminants led to increase in the degradation ability of the immobilized laccase. The degradation rate reached its maximum at initial chlorpyrifos concentration of 100 mg/ml. When the initial concentration reached 120 mg/ml, the degradation rate of the contaminants had a small decline, and the degradation rate significantly decreased at concentration of 200 mg/ml. This could be attributed to the fact that the high concentrations of chlorpyrifos would inhibit the catalytic function of the laccase to a certain extent.

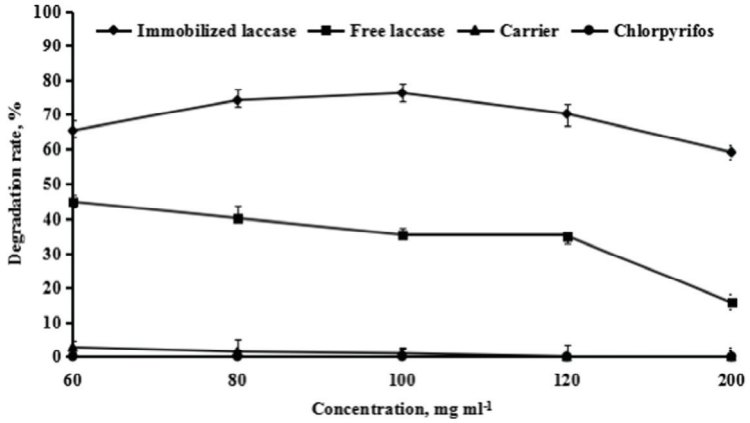

Fig. 7. Influence of initial concentration on the degradation rate of chlorpyrifos.

Influence of pH on the degradation rate of chlorpyrifos

As shown in Fig. 8, the degradation rate of chlorpyrifos when free laccase was used decreased sharply with the variation of pH. However, the value of pH had little influence on the degradation ability of the immobilized laccase, leading to small change in the degradation rate of chlorpyrifos.

Based upon the statistical analysis, the influence of immobilized laccase and free laccase on pH had significant difference ($p < 0.05$). It is possible that the immobilization process had a protecting effect on the laccase. The results showed that the optimal degradation rate was reached at pH 7. Therefore, it could be deduced that, when compared to the acidic and basic conditions, the neutral conditions were the ideal ones for degradation of chlorpyrifos.

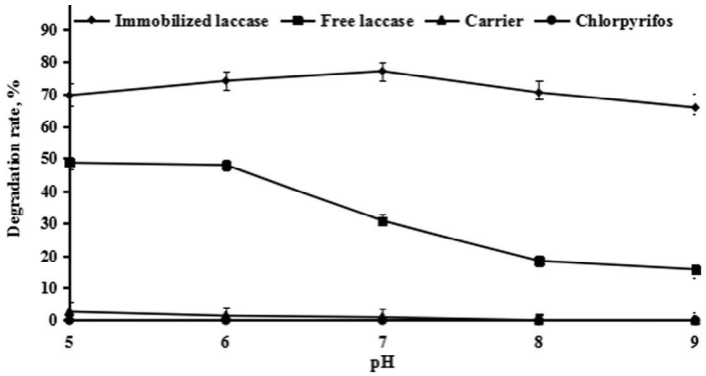

Fig. 8. Influence of pH on the degradation rate of chlorpyrifos.

Influence of temperature on the degradation rate of chlorpyrifos

With increase in the temperature, the degradation rate of chlorpyrifos by using both immobilized laccase and free laccase showed an increasing trend within the range of determination ($p < 0.05$). The reviewing of the experimental data shown in Fig. 9 led to the conclusion that a high degradation rate of chlorpyrifos was reached at temperature of 30 °C, which showed that the normal outdoor environment was suitable for chlorpyrifos degradation.

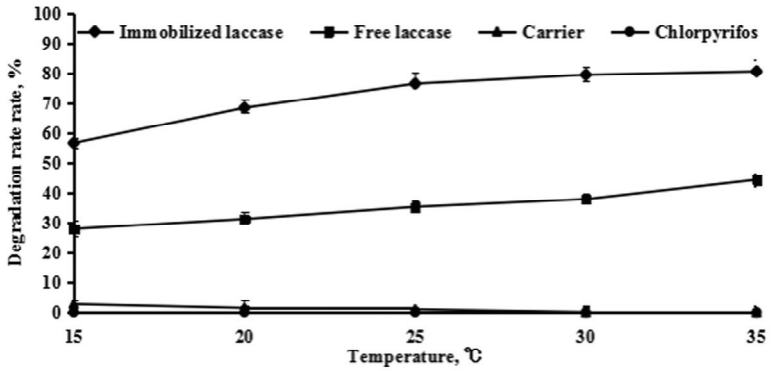

Fig. 9. Influence of temperature on the degradation rate of chlorpyrifos.

Comparison with other research

Previous studies[13,23] have shown that different immobilization methods could be used, and the immobilization process was influenced by different factors. The immobilization method that was used in this study was the embedding– cross-linking method, and the appropriate conditions of this method were found to be as follows: concentration of sodium alginate – 3 %, concentration of glutaraldehyde – 1 %, corsslinking time 6 h volume of crude laccase – 60 ml, and immobil-

ization time – 4 h. Yang *et al.*[24] isolated an *Alcaligenes faecalis* DSP3, capable of degrading both chlorpyrifos and its primary metabolite 3,5,6-trichloro-2-pyridinol (TCP). Thengodkar and Sivakami[25] showed that TCP could be degraded by using alkaline phosphatase obtained from the cyanobacterium *Spirulina platensis*. When assayed at different pH values, four peaks for the optimum rate of the enzymatic reaction were observed. The maximum activity was observed with a sharp peak at pH 10.5. The results reported in this study showed that the optimal pH is 7.0. This difference presumably was due to the method of immobilization making the enzyme more active in neutral conditions. In the recent years, the applications of plant-microbe for degradation of chlorpyrifos have been considered as a biological strengthening technology.[26,27] Although the microbial degradation of chlorpyrifos has been investigated, little is known about the degradation of chlorpyrifos by using immobilized enzymes.

CONCLUSIONS

In this study, the appropriate conditions for degradation of chlorprifos were obtained as follows: initial concentration of chlorpyrifos – 100 mg/ml, pH 7, and temperature – 30 °C. Using laccase for biodegradation appeared to be an inexpensive and ideal option for decreasing the levels of chlorprifos contaminations in the soil environment. Besides using an immobilized laccase on sodium alginate could enhance the activity of the enzyme thus improving the efficiency of the degradation process. Consequently, the results presented in this study provided data, which could be used for future research on the pesticide degradation. In addition, the bio-augmentation technologies for remediation of pesticide-contaminated soils would be still warranted in future.

SUPPLEMENTARY MATERIAL

Details about laccase and chlorpyrifos and laccase immobilization, as well as additional experimental details are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

Acknowledgements. This research was funded by the National Nature Science Foundation of China (No.21507092).

И З В О Д

РАЗГРАДЊА ХЛОРПИРИФОСА У КОНТАМИНИРАНОМ ЗЕМЉИШТУ ИМОБИЛИЗОВАНОМ ЛАКАЗОМ

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У овом раду је методом наношења/умрежавања имобилизована фунгална лаказа, а затим су одређивани погодни услови за имобилизацију мерењем активности имобилизоване лаказе. Поред тога, имобилизована лаказа је даље употребљена за санацију земљишта контаминираног хлорпирифосом, а затим су брзине разградње хлорпирифоса

одређиване под различитим условима. Према резултатима, погодни услови за методу наношења/умрежавања су били следећи: концентрација натријум-алгината 3 %, концентрација глутаралдехида 1 %, време умрежавања 6 h, запремина сирове лактазе 60 mL, а време имобилизације 4 h. Након 48 h степен разградње хлорпирифоса у земљишту имобилизованом лактазом припремљеном под различитим условима је достизао најмање 70 %.

(Примљено 28. јануара, ревидирано 28. маја, прихваћено 9. јуна 2016)

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