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Original Research

In-situ Remediation of Carbofuran-Contaminated Soil by Immobilized White-Rot Fungi

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Abstract

The widespread use of carbofuran in agriculture and concomitant pollution of soil has caused health issues in virtue of its toxic effect and residue. Most bacteria degrade carbofuran incompletely, as phenolic metabolites are unable to break down. White-rot fungi are potent degraders of carbofuran owing to their extracellular ligninolytic enzyme systems. Here we studied the catabolism of carbofuran by *Phlebia sp.* (C strain), *Lenzites betulinus* (Y strain), and *IIrpex lacteus* (W strain). Corn stover, wheat straw, peanut shells, wood chips, and corn cobs were supplied as carriers to immobilize strains. The results showed that *Phlebia.sp* and *Iopex lacteus* were fit for the degradation of strains while corn stover and wheat straw are suitable as carriers. The strains grew better on the carrier at 28ºC under acidic conditions. The degradation rate of carbofuran was up to 69.83% after *Iopex lacteus* within 5 days by corn stover. The orthogonal experiment proved that temperature and pH primarily affected degradation rate.

Keywords: *in-situ* remediation, carbofuran, soil, immobilized, white rot fungi

Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a common and efficient carbamate pesticide that has been used as insecticide and acaricide [1-3]. It is used extensively for the control of pests, for example white grubs, corn rootworm, and mosquitoes [4, 5]. Carbofuran has instant reaction against both nymphs and adults, killing them within 20 min [6]. However, it has strong toxicity, does great harm to the environment, and becomes a serious problem in environmental protection. In soil, carbofuran is very persistent with a half-life of about 110 days [7]. Air, soil, and food containing carbofuran have become a serious problem because of adverse impacts that pose threats not only to humans, but also animals, wildlife, and fish [8, 9]. With strong contact and stomach toxicity, the compound can inhibit the production of cholinesterase [10-12]. Even worse, the process of the combination with cholinesterase is irreversible. Recent surveys suggest that carbofuran has significant negative influence on the liver and kidney functions and on certain blood parameters [13, 14]. As a result, it is important to find a proper way to remove carbofuran in the environment.

Different approaches were used to degrade carbofuran in soil, for example photodegradation [15], adsorption [16,17], bioremediation [18,19], solid-phase extraction (SPE), and solid-phase microextraction (SPME) [20]. In recent years, immobilized technology has been applied to this field, with the advantage

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of high-capacity organisms and strong tolerance to adverse environments [21, 22]. Immobilization is defined as limiting the mobility of the microbial cells or their enzymes with a simultaneous preservation of their viability and catalytic functions [23-26]. The immobilized technology not only improves the activity of microbes, but also realizes the recycling of microbes [27, 28]. Adsorption is one of the most common methods of immobilization [29, 30]. It is through the action of electrostatic, surface tension and adhesion between the charged microorganism cell and the carrier to make the microorganism immobilized on the surface and inside of the carrier and form the biofilm. The most obvious advantage of adsorption is that the operation is simple and easy to operate [31]. At the same time, because of the mild reaction condition, it will not cause the mutated inactivation of the microorganism. The materials of the carrier have the following characteristics: inexpensive and available, simple preparation and high mechanical strength [21].

White-rot fungi are efficient in degradation of recalcitrant compounds like xenobiotics and lignin by their extracellular ligninolytic enzyme system [32, 33]. Compared with bacteria, white-rot fungi have a distinctive superiority. The ligninolytic enzyme system comprises lignin peroxidase (LiP), Mn-dependent peroxidase (MnP), and laccase. The extracellular degradation system is activated by its own H_2O_2, triggering a series of free radical chain reactions by the enzyme to achieve no specific oxidative degradation of the substrate [34]. Recent studies have shown that immobilized technology can promote the growth and production of enzymes and produce enzymes in advance [35]. White-rot fungi do not need to precondition to particular pollutants, i.e., they can degrade certain low concentration substances in the environment to a level that is not detected. Moreover, they produce OH free radicals to change the pH value of the environment, which antagonizes the invasion of microbes.

The objectives of this study were to: 1) select the most suitable degrading strain and optimum carrier of white-rot fung, 2) reveal the factors affecting the process of degradation, and 3) evaluate the efficiency of the immobilized white-rot fungi applications on the soil remediation for carbofuran contaminations.

Materials and Methods

Reagents

The specific white-rot fungi were domesticated and trained in the Shenyang Institute of Applied Ecology, Chinese Academy of Sciences (Shenyang, China). The testing soil, free from pollution, were obtained from Shenyang University of Technology. Carbofuran (purity of 98%) used in the experiment was purchased from Zhenjiang Jiansu Pesticide Chemical Company L.T.D (Zhenjiang, China).

Activation of Strains

The strain was inoculated into a solid agar medium in which malt extract powder was used as a nitrogen source for the cellulose decomposing microorganisms to grow, and fostered in an incubator at 28ºC for 72 h. White-rot fungi was transferred to a new solid medium of the same ingredient when it spread all over the agar plates. The operations above were repeated 3 times.

Immobilization Procedure

The carrier materials in the experiment contain wheat straw, corn stover, corn cob, wood chip, and peanut shells, which were cut into 3 mm pieces. 1 g carrier materials were placed into 10 ml distilled water and heated at 121ºC, 1.01325×10^5Pa in high pressure steam sterilizer pot for 30 min. After activation, accurately weighted strains of the same size were put with carriers under aseptic conditions. Then white-rot fungi was fostered in an incubator sealing with breathable films.

Selecting Suitable Degrading Strain

White-rot fungi were cultured at their best growth situation (30ºC, slightly acidic conditions) after being linked to carriers. Then the contaminated soil was degraded by different strains and carriers (Table 1) in order to obtain suitable degrading strain and degrading time.

Preparing Carbofuran-Contaminated Soil

1.0 g carbofuran was accurately measured and dissolved in 500 ml water in a conical flask by ultrasound. A 15 ml sample was poured into the beaker containing 300 g soil and stirred by glass rods to make the water and soil mix evenly. The mass concentration of carbofuran was 100 mg/kg.

Table 1. Treatment of experimental design.

No.	Materials
S1	Blank
S2	Wheat straw
S3	Corn stover
S4	Wheat straw+Immobilized C strain
S5	Wheat straw+Immobilized Y strain
S6	Wheat straw+Immobilized W strain
S7	Corn stover+Immobilized C strain
S8	Corn stover+Immobilized Y strain
S9	Corn stover+Immobilized W strain

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Table 2. Factor level of immobilized white rot fungi degradation; four factors: A: initial concentration (mg/kg); B: temperature (ºC); C: pH value; D: dosage of white rot fungi (g), same as below table.

Factor level	A	B	C	D
1	80	25	6	0.8
2	100	30	7	1.0
3	120	35	8	1.2

Degradation of Carbofuran by White-Rot Fungi

30 g soil was taken into a culture vessel after drying, and then 1.0 g immobilized white rot fungus was added. The degradation condition was set to 30ºC and neutral pH. The degradation rates of carbofuran were determined every 8 h.

In the first group of experiments, the influence of a single factor on degradation rate was discussed. A total of four elements were contained: initial concentration of carbofuran, pH value, temperature and dosage of white rot fungi. Each experiment changed one factor. The initial concentrations of carbofuran were 60, 80, 100, 120 and 200 (mg/kg). pH of soils were 5, 6, 7, 8 and 9. The temperatures of incubators were 15, 20, 25, 30 and 35ºC. The dosages of white rot fungi were 0.4, 0.8, 1.0, 1.5 and 2.0 g, respectively.

In the orthogonal experiment, each factor was given three factor levels in Table 2. High degradation rates were achieved under these levels according to the single-factor experiment. Based upon Taguchi's L_9 (3^4) fractional orthogonal array, four factor levels were combined as experiment plan, which are shown in Table 3.

Sample Pretreatment

1 g soil was placed in a centrifuge tube and immersed with 10 ml dichloromethane solution. Then the solution had an intermittent ultrasonic water bath at low temperature for 2 h and was centrifuged for 5 min at 4500 r/min in a refrigerated centrifuge. The upper extract liquid was transferred to a petri plate and methanol was added to 10 ml after evaporation. At the end, the mixed solution was into an HPLC sampling bottle through a syringe of organic microporous membrane in 0.5 ml volume for carbofuran measurement.

Table 3. Orthogonal analysis of immobilized white rot fungi degradation factors.

No.	factor & level
1	$A_1B_1C_1D_1$
2	$A_1B_2C_2D_2$
3	$A_1B_3C_3D_3$
4	$A_2B_1C_2D_3$
5	$A_2B_2C_3D_1$
6	$A_2B_3C_1D_2$
7	$A_3B_1C_3D_2$
8	$A_3B_2C_1D_3$
9	$A_3B_3C_2D_1$

HPLC Conditions

The mobile phase was prepared with methyl alcohol and distilled water (55:45 volume ratio). An alkyl silica gel column was used. The UV wavelength was set to 280 nm at room temperature. The sample size was 10 μL at a rate of 1.0 ml/min and the retention time was 10 min.

Data Processing

Microsoft Excel software was used to process all the experimental data. SPSS Statistics 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and $p<0.05$ was considered as a significant difference.

Results and Discussion

Selection of Immobilized Carriers and Degrading Strains

Selection of Immobilized Carriers

Strain C, Y and W in different carriers were put into an incubator at 30ºC for 20 days. Strains did not grow in the wood chip and grew poorly in the peanut shell and corn cob. In the wheat straw and corn stover, strains developed rapidly. Therefore, wheat straw and corn stover were selected as carriers in the next experiment.

According to the result, not every material is suitable for immobilization. Different support materials affect the result of strain growth. The adsorption capacity of various carriers is different, which lead to the difference of the amount of cells fixed. Some adsorption carriers may be toxic to microbial cells. The binding degree between various carriers and microbial cells is different, and some cells are easy to fall off.

Selection of Degrading Strains

Fig. 1 compared the degradation of carbofuran by immobilized white-rot fungi, blank carriers and carbofuran blank. The immobilized C and W strains had better degradation efficiency than Y strain. The curve rose rapidly in the first 5 days and then slowed down after 5 days of incubation. A small amount of carbofuran can be absorbed by two carrier materials as well as in illumination. The degradation rates were 66.35%, 55.46%, and 65.48% with C, Y, and W strains

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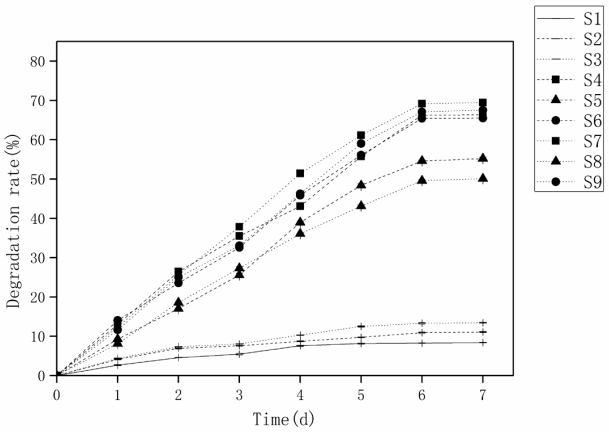

Fig. 1. Influence of immobilized strains on degradation of carbofuran.

by wheat straw and 69.14%, 49.86% and 67.12% by corn stover, respectively. The blank wheat straw was 11.14%, blank corn stover 13.47%, carbofuran blank 8.35%. As a result, immobilized C and W strains were selected to degrade carbofuran in the single factor experiment, while corn stover was chosen as the carrier in the orthogonal experiment.

Factors Affecting Carbofuran Degradation

Influence of Initial Concentration on the Degradation Rate of Carbofuran

As in Fig. 2, immobilized white-rot fungi have good degradation effect of carbofuran under five concentration conditions. However, different strains and carrier materials obviously result in discrepant (p<0.05). At first, the degradation ability increased with the rising concentration. The degradation rate reached its maximum at initial carbofuran concentration of 100 mg/kg. When the initial concentration was up to 120 mg/kg, the degradation rate of the pollutants had a slight decline as well as significantly decreasing at 200 mg/kg. This would be ascribable to the fact that a high concentration of carbofuran could inhibit the metabolism of white-rot fungi to a certain extent.

Therefore, the 100 mg/kg soil was the best initial concentration for remediation of carbofuran-contaminated soil in this experiment. The degradation rates were 66.42% and 60.38%, with C and W strains by wheat straw as well as 72.84% and 74.35% by corn stover, respectively.

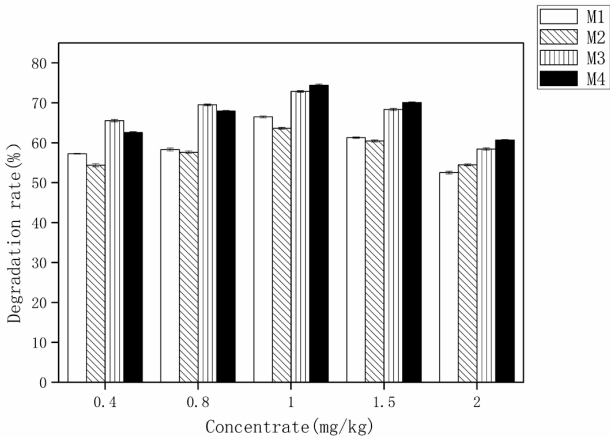

Fig. 2. Influence of initial concentration on immobilized strains degradation of carbofuran, M1: selected wheat straw+Immobilized C strain, M2: wheat straw+Immobilized W strain, M3: corn stover+Immobilized C strain, M4: corn stover+Immobilized W strain, same as below figure.

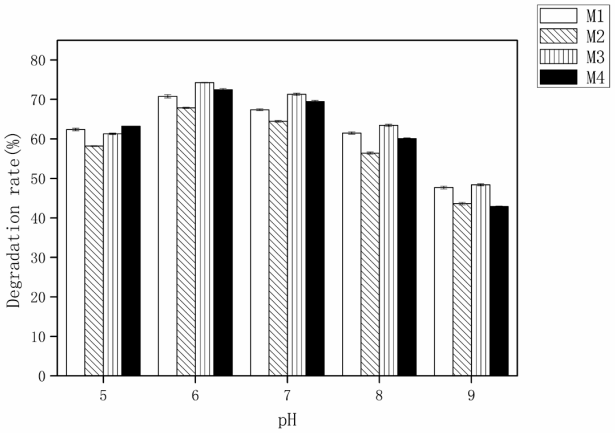

Fig. 3. Influence of pH value on immobilized strains degradation of carbofuran.

Influence of pH on the Degradation Rate of Carbofuran

The results of degradation rates affected by pH are shown in Fig. 3. Based on statistical analysis, the influence of pH on C and W strains had significant differences (p<0.05). More than 50% of carbofuran was removed in different pH conditions. The degradation

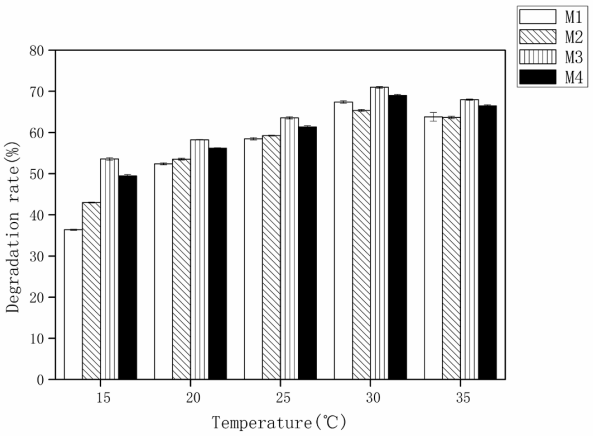

Fig. 4. Influence of temperature on immobilized strains degradation of carbofuran.

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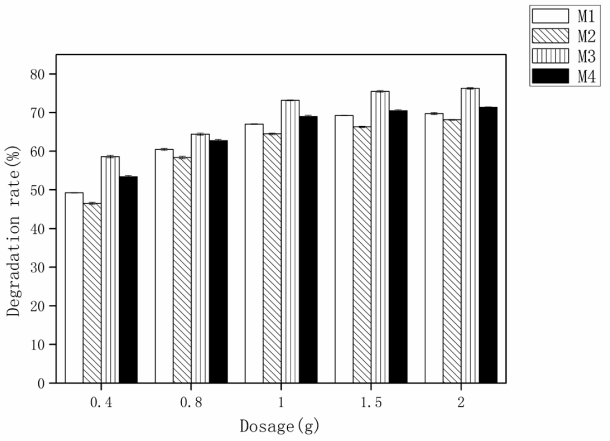

Fig. 5. Influence of dosage on immobilized strains degradation of carbofuran.

rates of pH = 6 was the highest as the secretion process of ligninolytic enzymes was promoted in a slight acidic environment. The degradation rates were up to 70.84% and 67.92% with C and Y strains by wheat straw and 74.39% and 72.42% by corn stover, respectively.

Influence of Temperature on Carbofuran Degradation Rate

After immobilization, white-rot fungi could adapt to higher temperature than before, so immobilized strains would be applied in a more broad temperature range than free strains. As shown in Fig. 4, the degradation rates were low at 15ºC and 20ºC, which may be related to the fact that the growth of white-rot fungi was restricted and produced secondary metabolites at low temperatures. The degradation rate was highest at 30ºC, in accordance with optimum growth temperature of white-rot fungi. The result indicated that temperature had a major influence on the production of laccase in secondary metabolism of white-rot fungi (p<0.05). At 30ºC, the degradation rates were 67.39% and 65.28% with C and Y strains by wheat straw as well as 70.92% and 68.94% by corn stover, respectively.

Influence of Dosage on the Degradation Rate of Carbofuran

The influence of the dosage of white-rot fungi on the degradation rate of carbofuran was shown in Fig. 5. The degradation effect was enhanced significantly with increasing dosage (p<0.05). When the dosage was set to 1.0 g, the degradation effect improved little due to the limitation of carbofuran degradation in soil by white-rot fungi. The best initial dosage was set to 1.0 g and the degradation rates were 67.14% and 64.61% with C and Y strains by wheat straw, as well as 73.28% and 69.17% by corn stover, respectively.

Analysis of the Orthogonal Experimental Results

According to test results of orthogonal experiment (Table 4), the efficiency of immobilized strain was the highest (68.32%) in the 6th experiment and lowest (49.65%) in the 7th experiment. As in Table 5, the temperature was the key factor to degrade carbofuran, pH was the important factor, and dosage and initial concentrate were the general factor.

Catalytic efficiency was affected by each parameter as below. The degradation ability of strains was the best at 30ºC, followed by 35ºC and 25ºC. The increasing order of degradation rate was pH8<pH7<pH6. The optimum initial carbofuran concentration was 120 mg/kg, and the next ones were 100 mg/kg and 80 mg/kg. The activity of strains was significantly highest when the dosage of white-rot fungi was 1.0 g than that of 1.2 g and 0.8 g.

Above all, the optimum condition to degrade carbofuran was as follows: initial concentration of carbofuran – 80 mg/kg, temperature– 30ºC, pH 6 and dosage of white-rot fungi –1.0 g, the combination of $A_1B_2C_1D_2$. However, this condition was not included in the orthogonal experiment plan. Another experiment was performed according to the combination and the degradation rates were up to 71.69%.

Table 4. Test results of orthogonal experiment.

No.	Degradation rate
1	55.31%
2	67.43%
3	59.84%
4	53.56%
5	57.19%
6	68.32%
7	49.65%
8	66.32%
9	61.29%

Table 5. Analysis of carbofuran degradation orthogonal experimental results, k_1,k_2 and k_3 are the average of each experiment result for certain factor at level 1, 2 and 3; R are the extreme deviation of k for certain factors.

Factor	A	B	C	D
k_1	60.86	52.84	63.32	57.93
k_2	59.69	63.65	60.76	61.80
k_3	59.09	63.15	55.56	59.91
R	1.77	10.81	7.76	3.87

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Conclusions

In this study, wheat straw and corn stover were selected as carriers of immobilized white-rot fungi. C and W strains were effective on carbofuran within 5 days and the highest degradation rate reached 69.14% and 67.12%, respectively. The appropriate degradation conditions included the initial concentration of 100~200 mg/L, pH of 6~8 and temperature of 25~35ºC. Subsequent orthogonal experiments illustrated that reaction temperature and pH value had a bigger impact on degradation effect than initial carbofuran concentration and dosage of white-rot fungi. The best situation was the initial concentration of 80 mg/kg, pH of 6, temperature of 30ºC, and dosage of white-rot fungi of 1.0 g. The data acquired from the experiment could be used for future research on pesticide degradation.

Acknowledgements

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Conflict of Interest

None of the authors have any conflicts of interest to declare.

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