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# The remediation of chlorpyrifos-contaminated soil by immobilized white-rot fungi

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*Abstract*: This research focused on the degradation of chlorpyrifos *via* immobilized white rot fungi in soil, with the aim to select excellent degrading strains and an optimal carrier of white rot fungi. Immobilization of white rot fungi was assessed on corn stover, wheat straw, peanut shells, wood chip, and corn cobs. *Phlebia* sp., *Lenzites betulinus* and *Irpex lacteus* were grown in defined nutrient media for the remediation of pesticide-contaminated soils. The carrier of the biomass was determined by observing the growth of white rot fungi. The results showed that corn stover and wheat straw are suitable carriers of the immobilized white rot fungi and that *Phlebia* sp. and *Lenzites betulinus* have a positive effect on the degradation of chlorpyrifos. At 30 °C and neutral pH, the degradation rate of chlorpyrifos was 74.35 %, *Phlebia* sp. being immobilized by corn stover in 7 days, which was the best result compared to other combinations of strains and carriers. The orthogonal experiment showed that the pH value and temperature affected the pollutant degradability more than the initial concentration and the biomass dosage.

*Keywords*: fungal; carrier; immobilization; degradation; pesticide.

## INTRODUCTION

Chlorpyrifos (*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) (CPF) is one of the most widely used organophosphorus pesticides,[1,2] with registrations in about 90 countries. This would inhibit the activity of acetylcholinesterase enzymes and butyrylcholinesterases in the nerve of an insect,[3] and cause a series of toxic symptoms to eliminate parasites. However, the metabolites of chlorpyrifos after decomposition have a great influence on the development of the human brain, which even leads to behavioural variation in humans. In addition, chronic exposure to chlorpyrifos can have adverse effects on non-target organisms, posing a potential threat to the ecological balance.[4–6] In 2000, the United

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States Environmental Protection Agency (USEPA) declared a ban on the residential use of chlorpyrifos across the country.[7] In China, the law provides for a maximum residue of 1mg/kg in vegetables, well above the limit set by the Codex Alimentarius Commission. Furthermore, China has completely banned the production and processing of five highly toxic pesticides since 2008, increasing the production and use of chlorpyrifos. In general, with a steady increase in its use, chlorpyrifos has become one of the largest varieties of pesticides produced and sold worldwide.

Given its potential threat to agricultural safety and human health, some effective remediation methods should be developed. Recently, all kinds of chemical and biological methods have been studied to remove chlorpyrifos from contaminated soil.[8,9] With the superiority of microbial metabolism, biological remediation has been considered as an abundant, permanent and non-invasive alternative for the removal of contaminants.[10,11] The biodegradation of pesticides is mainly carried out by microorganisms, which secrete a large number of enzymes. Enzymatic sanitation is a fast and highly effective technique for removing pesticide residues from the environment.[12,13] Some enzymes are able to oxidize organic and inorganic substrates.[14] For example, laccase immobilized from white rot fungi has been able to transform carbofuran effectively.[15]

White rot fungi are considered to be one of the most effective microorganisms for the biodegradation of refractory compounds such as xenobiotics and lignin.[16,17] The mushrooms are named to promote the decomposition of the wood into a white rot mass of pale sponges. In the secondary metabolism stage, white rot fungi form a variety of enzymes when nitrogen, carbon, sulfur, and other essential nutrients are limited, to trigger a series of chain reaction processes based on free radicals. Their extracellular ligninolytic enzyme systems contain lignin peroxidase, manganese peroxidase and laccase, which can attack a wide variety of complex compounds. In recent decades has appeared evidence that white rot fungi have good effects on the removal of many environmental pollutants, including dyed wastewaters,[18] herbicide[19] and TNT,[20] toxic organic compound[21], *etc*. In comparison with bacteria, white rot fungi have unique advantages.[22] For example, certain types of white rot fungi do not require preconditioning of particular pollutants, *i.e.*, low-level pollutants can be degraded to undetectable levels. To grow well and reproduce well, the hydroxyl radical (•OH) oxidizes the proteins and DNA of other microorganisms to antagonize invading microbes. The cell body undergoes less damage in the degradation process when the reactions are extracellular. The degradation by white rot fungi corresponds to pseudo-first order kinetics and the Michaelis constant ($K_m$) of the enzyme reaction is useless, because pollutants were completely eliminated.

Microbial immobilization technology consists mainly of physically or chemically fixing dispersant and free micro-organisms in a small space in order to inc-

rease the concentration of microbial cells and maintain their high biological activity. In the beginning, this technology was used to increase the microbial secretion of amino acids, organic acids, antibiotics, and other useful substances or as biocatalysts in bioreactors.[23–26] Since the 1980s, this technology has been applied to the treatment of industrial wastewater, petroleum pollution and various types of organic pollution in the environmental media, all of which have been successful.[27–29] The immobilization of white rot fungi accelerates the reaction and improves the effect of degradation of pollutants. Iqbal and Asgher immobilized the *Ganoderma lucidum* strain by the sol–gel matrix entrapment in order to treat different textile effluents.[30] After 4 h of reaction, the industrial effluents were discoloured to a maximum of 99.2 %. Formaldehyde and nitroamines were analyzed in the maximum discoloured effluent and the results showed that the toxicity parameters were below the allowable limits. However, the research on the immobilization of white rot fungi has been relatively limited in the area of soil remediation. In addition, recent studies have shown little interest in straw biomass, which is common in China and is considered an agricultural residue and a sensitive environmental issue. With straw burning being one of the main causes of smog in northern China, researchers are actively exploring ways to improve the rate of straw utilization. In this study, white rot fungi were immobilized on carriers by the adsorption method, and optimal conditions were determined to obtain the highest degradation rate of chlorpyrifos.

The objectives of this study were to: 1) select the most appropriate degrading strain and the optimal carrier of immobilized white rot fungi, 2) explore factors affecting the degradation process and 3) evaluate the efficacy of applications of the immobilized white-rot fungi on soil remediation in case of contamination by chlorpyrifos.

## EXPERIMENTAL

### *Materials*

Specific white rot fungi (*Phlebia* sp., *Lenzites betulinus*, and *Irpex lacteus*) were provided by the Microbiology Laboratory of the Shenyang Institute of Applied Ecology of the Chinese Academy of Sciences (Shenyang, China). Test soils without chlorpyrifos were obtained from the Shenyang University of Technology. The soil is of agricultural type with organic matter content of 14 mg/g. Chlorpyrifos (purity 95.9 %) was purchased from Shandong Rongbang Chemical Co., Ltd. (China). The malt extract powder was purchased in Beijing, Aoboxing Biotechnology Co., Ltd. (China). Methanol was purchased from Thermo Fisher Scientific (USA). High-performance liquid chromatography (HPLC, Agilent 1260, Singapore) was used to determine the degradation rate of chlorpyrifos.

### *Activation of strains*

Two successive inoculations were performed in a solid 1.5 % malt extract/agar medium and stimulated in an incubator at 28 °C.

*Immobilization procedure*

The prepared support materials used in the experiment contained wheat straw, corn stover, corn cobs, wood chips and peanut shells, which were cut into 3 mm fragments. The 1 g carrier materials were placed in 10 ml of distilled water and heated at 121 °C in a high-pressure steam sterilizer pot for 30 min. The culture dish including malt extract provides nitrogen source for the growth of strains. Each culture dish contains 10 ml malt extract solution and 1.0 mg chlorpyrifos. After activation, the strains of the same size, which were accurately weighted, were placed with carriers for 20 days in the incubator at 30 °C. Then white rot fungi were favoured in a sealing incubator with breathable films. The carrier of the biomass was determined by observing the growth of white rot fungi.

*Preparation of chlorpyrifos-contaminated soil*

1.0 g of chlorpyrifos was dissolved in 30 ml methanol with a 500 ml volumetric flask, adjusted to the mark with water and stirred vigorously. An aliquot of this solution was added to 300 g of aseptic soil and 30 ml water was added, for the complete mixing to prepare the artificial soil contaminated with chlorpyrifos. The final mass concentration of chlorpyrifos in soil was in the range of 60–200 mg/kg.

*Selection of suitable degrading strain*

A perforator was used to punch the white rot fungi culture medium, and the inoculation ring was used to pick it up to the soil surface. About 1 g of fungi were grown with carriers and introduced into the soil at 30 °C. Then, chlorpyrifos was degraded by different strains and carriers (Table I), in order to obtain an appropriate strain and degradation time. After several days, the mushrooms grow hyphae in the soil. The sample was homogenized. Each unit experience was performed in triplicate.

TABLE I. Treatment of experimental design

| No. | Materials |
|---|---|
| S1 | blank |
| S2 | Wheat straw |
| S3 | Corn stover |
| S4 | Wheat straw+Immobilized *Phlebia*.sp |
| S5 | Wheat straw+Immobilized *Lenzites betulinus* |
| S6 | Wheat straw+Immobilized *Irpex lacteus* |
| S7 | Corn stover+Immobilized *Phlebia*.sp |
| S8 | Corn stover+Immobilized *Lenzites betulinus* |
| S9 | Corn stover+Immobilized *Irpex lacteus* |

*Degradation of chlorpyrifos by white-rot fungi*

30 g of chlorpyrifos contaminated soil (dry weight) was added to a culture vessel and 1.0 g of immobilized white rot fungi (mycelium mass) was added to the soil surface of the solid plate. The degradation rates of chlorpyrifos were determined every 24 h.

In the first group of experiments, the influence of a single factor on the rate of degradation was discussed. A total of four elements were contained: initial concentration of chlorpyrifos, pH value, temperature, and dosage of white rot fungi. Every experience has changed by one factor. Initial concentrations of chlorpyrifos were 60, 80, 100, 120 and 200 mg/kg. Soil pH was 5, 6, 7, 8 and 9. Incubation temperatures were 15, 20, 25, 30 and 35 °C. The doses of white rot fungi were 0.4, 0.8, 1.0, 1.5 and 2.0 g, respectively.

In the orthogonal experiment, each factor received three-factor levels based on the outcome of the single-factor experiment. High degradation rates have been achieved below, these levels. The initial concentration of chlorpyrifos (*A*), the temperature (*B*), the pH value (*C*) and the dosage of white rot fungi (*D*) were four factors in this survey and their level is shown in Table II. Based on Taguchi's L9 ($3^4$) fractional orthogonal array, the four-factor levels were combined as experimental design as follows: $A_1B_1C_1D_1$, $A_1B_2C_2D_2$, $A_1B_3C_3D_3$, $A_2B_1C_2D_3$, $A_2B_2C_3D_2$, $A_3B_1C_3D_2$, $A_3B_2C_1D_3$, $A_3B_3C_2D_1$, $A_3B_3C_2D_1$.

TABLE II. The factor level of chlorpyrifos degradation by immobilized white rot fungi. Four factors: *A*: initial concentration; *B*: temperature; *C*: pH value; *D*: dosage of white rot fungi

| Level | *A* / mg kg$^{-1}$ | *B* / °C | *C* | *D* / g |
|---|---|---|---|---|
| 1 | 80 ($A_1$) | 25 ($B_1$) | 6 ($C_1$) | 0.8 ($D_1$) |
| 2 | 100 ($A_2$) | 30 ($B_2$) | 7 ($C_2$) | 1.0 ($D_2$) |
| 3 | 120 ($A_3$) | 35 ($B_3$) | 8 ($C_3$) | 1.2 ($D_3$) |

*Sample pretreatment*

After the degradation process, the soil (weight of 1 g) was placed in a centrifuge tube and immersed in 10 ml of acetone-phosphoric acid solution (99.5:0.5). Then, the solution was placed into an intermittent ultrasonic water bath at a low temperature for 2 h and was centrifuged for 5 min. The upper extract liquid was transferred to a separating funnel, supplemented with 15 % sodium chloride. The centrifuge tube was washed with 10 ml of acetone twice and the wash solution was poured into the separating funnel. The solution was extracted with 10 ml ethyl acetate twice and the mixture was stratified into two liquid phases at rest. The upper layer of ethyl acetate was transferred to a culture vessel and then 10 ml of methanol was added after evaporation of ethyl acetate. Finally, the mixed solution was introduced into an HPLC sampling bottle through a syringe of the organic microporous membrane in a volume of 0.5 ml for the measurement of chlorpyrifos.

*The conditions of HPLC*

The mobile phase was prepared with methyl alcohol and distilled water (9:1 volume ratio). An alkyl silica gel column was used. The UV wavelength was set at 300 nm at room temperature. The sample size was 10 μL at a flow rate of 1.0 ml/min and the retention time 6 min.

*Data processing*

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

## RESULTS AND DISCUSSION

### *Selection of immobilized carriers and degrading strains*

*Selection of immobilized carriers.* After incubation for 20 days at 30 °C, *Phlebia* sp., *Lenzites betulinus* and *Irpex lacteus* did not grow in wood chips and developed poorly in a peanut shell and corn cob. In the wheat straw and corn stover, the strains grew rapidly. As a result, wheat straw and corn stover were chosen and used as carriers in the next experiment.

*Selection of degrading strains.* Fig. 1 compared the degradation rates of chlorpyrifos under immobilized white rot fungi, blank carriers and chlorpyrifos blank. As can be seen in the figure, the curve S1 showed that the chlorpyrifos itself underwent photodecomposition. The S2 and S3 curves almost coincide, indicating that wheat straw and corn stover without carriers have similar efficiencies to remove chlorpyrifos.

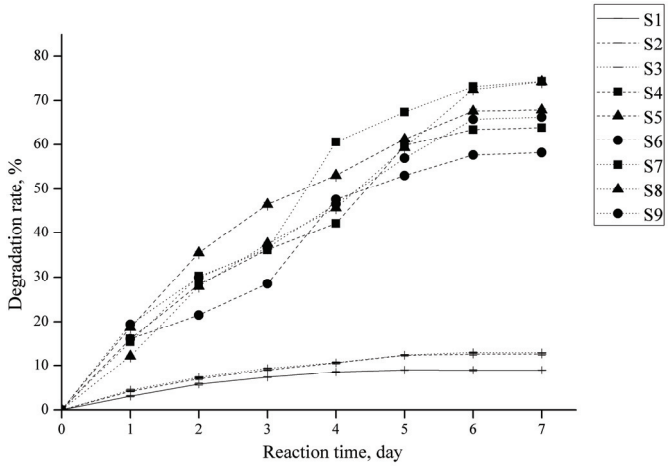


Fig. 1. The influence of immobilized strains on the degradation of 100 mg/kg chlorpyrifos

The immobilized *Phlebia* sp. and *Lenzites betulinus* exhibited better degradation efficacy than *Irpex lacteus*, which may be related to different types and quantities of enzymes in the metabolic process. The curve rose rapidly during the first 6 days and then slowed down. The degradation rates were 64.08, 67.75 and 58.15 %, with more than three strains by wheat straw; 74.35, 74.29 and 66.34 % per corn stover, respectively. The blank wheat straw was 12.55 %, blank corn stover 12.90 %, blank chlorpyrifos 8.92 %. As a result, the immobilized *Phlebia* sp. and *Lenzites betulinus* were selected to degrade chlorpyrifos in the single-factor experiment, while the corn stover was selected as a carrier in the orthogonal experiment.

*The factors affecting the degradation of chlorpyrifos*

*Influence of initial concentration on the degradation rate of chlorpyrifos.* As in Fig. 2, the degradation rates with combination of different carriers and strains were obviously discordant ($p < 0.05$), but the rate of degradation had the same tendency to change and was greater than 50 % in the range of the experimental concentration. At first, the capacity for degradation increased with concentration. The degradation rate peaked at the initial chlorpyrifos concentration of 100 mg/kg. When the initial concentration reached 120 mg/kg, the rate of degradation

of pollutants decreased slightly and significantly to 200 mg/kg. This is due to the fact that a high concentration of chlorpyrifos could inhibit the metabolism of white rot fungi to some extent. Similar inhibitory effects of chlorpyrifos have been reported in *Synechocystis* sp. strain PUPCCC 64.[31] The strain PUPCCC 64 could survive below the 15 mg/L of chlorpyrifos and its growth was inhibited to varying degrees at different concentrations.

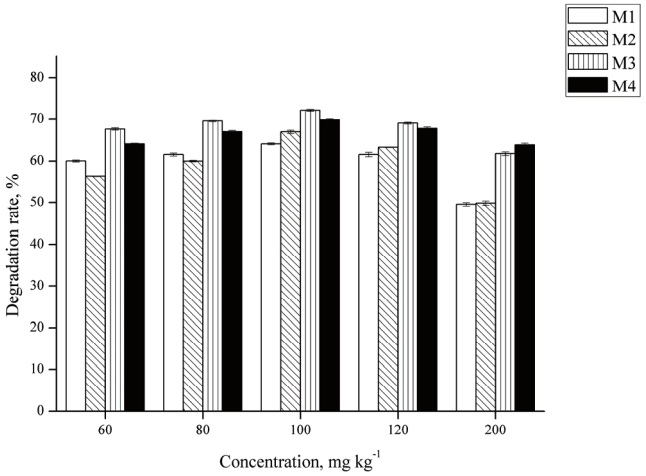


Fig. 2. The influence of initial concentration on chlorpyrifos degraded by immobilization white-rot fungi, M1: wheat straw+immobilized *Phlebia* sp.; M2: wheat straw+immobilized *Lenzites betulinus*; M3: corn stover+immobilized *Phlebia* sp.; M4: corn stover+immobilized *Lenzites betulinus*.

Therefore, the initial concentration of 100 mg/kg soil was most effectively removed for the remediation of chlorpyrifos-contaminated soils in this experiment. Degradation rates were 64.02 and 66.94 % with *Phlebia* sp. and *Lenzites betulinus* with wheat straw, as well as 71.98 and 69.83 % with a corn stover, respectively.

*Influence of pH on the degradation rate of chlorpyrifos.* The results of the degradation rate affected by pH are shown in Fig. 3. On the basis of statistical analysis, the influence of pH on *Phlebia* sp. and *Lenzites betulinus* showed significant differences ($p < 0.05$). The immobilized strains had a great effect of degradation in slightly acidic, neutral or slightly basic conditions. The degradation rates reached their maximum at pH 6, *i.e.*, 67.43 and 66.82 % with more than two strains with wheat straw, as well as 78.21 and 76.87 % with corn stover, respectively. Silambarasan and Abraham reported that a new fungus, *Aspergillus terreus* JAS1, rapidly degraded chlorpyrifos in the mineral environment at pH 6.8, which was similar to our current work.[32]

White rot fungi may be able to adapt to higher temperatures after immobilization, so immobilized strains would operate in a wider temperature range than free strains. As shown in Fig. 4, the immobilized strains exhibited a high rate of degradation at most temperatures except at 15 °C.

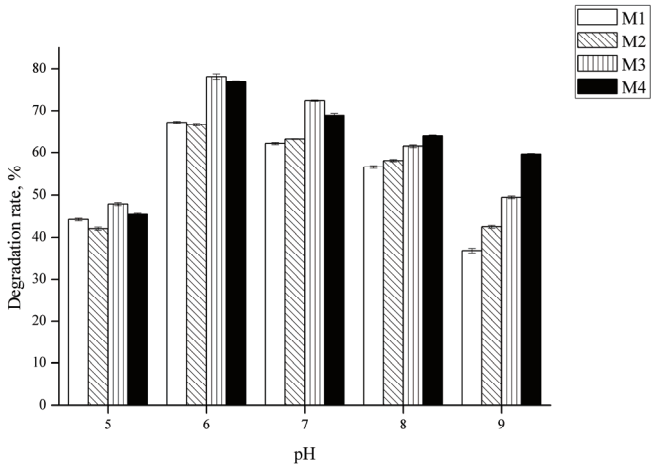


Fig. 3. The influence of pH value on immobilized strains degradation of chlorpyrifos.

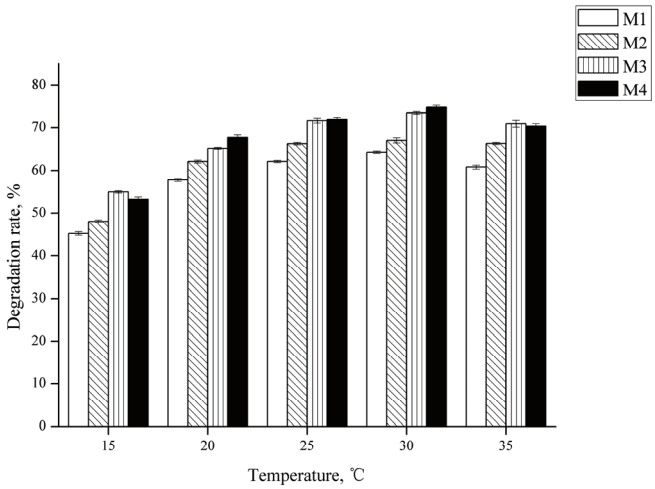


Fig. 4. The influence of temperature on immobilized strains degradation of chlorpyrifos.

*Influence of temperature on the degradation rate of chlorpyrifos.* The results showed that at different temperatures different strains and carriers had different degradation capabilities. ($p < 0.05$). The degradation rates were highest at 30 °C, *i.e.*, 64.18 and 66.49 % with *Phlebia* sp. and *Lenzites betulinus* by wheat straw, as well as 73.29 and 74.87 % with a corn stover, respectively. Our observations

are in agreement with the maximum chlorpyrifos removal by the mixed culture of the bacterium *Serratia* sp. and the fungus *Trichosporon* sp. at 30 °C.[33]

*Influence of dosage on the degradation rate of chlorpyrifos.* With an increase in the dosage of white-rot fungi, the rate of degradation of chlorpyrifos showed an upward trend in the determination range (Fig. 5). When the dose was fixed at 1.5 g, the degradation effect improved slightly due to the limitation of the degradation of chlorpyrifos in the soil by white rot fungi. Compared to other situations, the best dose was 1.0 g, while the degradation rates were 64.18 and 67.29 % with *Phlebia* sp. and *Lenzites betulinus* by wheat straw, and 72.13 and 69.32 % by corn stover, respectively.

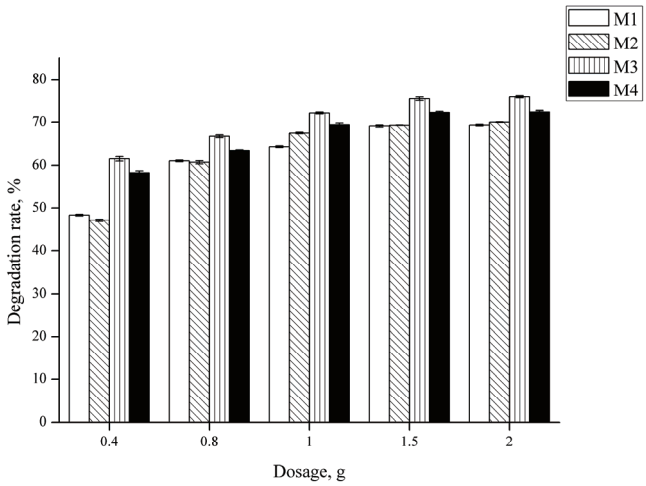


Fig. 5. The influence of dosage on immobilized strains degradation of chlorpyrifos.

*Analysis of the orthogonal experimental results*

The result of the orthogonal experiment is shown in Table III. The highest degradation rate was 78.53 % in the eighth experiment, while the lowest rate was 53.15 % in the seventh experiment. Based on the range analysis (Table IV), the

TABLE III. Degradation rates obtained in orthogonal experiment

| No. | Factor and level | Degradation rate, % |
|---|---|---|
| 1 | $A_1B_1C_1D_1$ | 56.21 |
| 2 | $A_1B_2C_2D_2$ | 68.72 |
| 3 | $A_1B_3C_3D_3$ | 61.39 |
| 4 | $A_2B_1C_2D_3$ | 57.35 |
| 5 | $A_2B_2C_3D_1$ | 56.31 |
| 6 | $A_2B_3C_1D_2$ | 75.64 |
| 7 | $A_3B_1C_3D_2$ | 53.15 |
| 8 | $A_3B_2C_1D_3$ | 78.53 |
| 9 | $A_3B_3C_2D_1$ | 73.53 |

reaction temperature and pH value were the key factors influencing the degradation rate of chlorpyrifos, while the initial concentration of chlorpyrifos and the dosage of white-rot fungi were general factors.

TABLE IV. The analysis of cross 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 factor

| Factor | *A* / % | *B* / % | *C* / % | *D* / % |
|---|---|---|---|---|
| $k_1$ | 62.11 | 55.57 | 70.13 | 62.02 |
| $k_2$ | 63.10 | 67.85 | 66.53 | 65.84 |
| $k_3$ | 68.40 | 70.19 | 56.95 | 65.76 |
| $R$ | 6.29 | 14.62 | 13.18 | 3.82 |

The efficiency of chlorpyrifos removal was affected by each parameter as shown in Table III. The degradation capacity of the strains was best at 35 °C, then at 30 and 25 °C. The increased order of degradation rate was pH 8 < < pH 7 < pH 6. The initial optimal concentration of chlorpyrifos was 120 mg/kg, followed by 100 and 80 mg/kg. Strain activity was signifycantly higher when the white rot fungus dose was 1.0 g higher than that of 1.2 and 0.8 g.

Therefore, the optimal conditions for degradation of chlorpyrifos by immobilized white rot fungi were determined as follows: the initial concentration of chlorpyrifos – 120 mg/kg, the temperature – 35 °C, pH – 6 and determination of white rot fungi – 1.0 g, the combination of $A_3B_3C_1D_2$. However, this condition was not included in the orthogonal experiment plan. According to this situation, the degradation rate reaches 76.34 % in the experiment. The interactions between various factors in orthogonal experiments result in differences in the optimal conditions of single-factor experiments. After careful examination, the combination of $A_3B_3C_1D_2$ has a better degradation efficiency.

## CONCLUSION

In this study, wheat straw and corn stover were selected as carriers for the immobilized white rot fungi. *Phlebia* sp. and *Lenzites betulinus* were effective against chlorpyrifos within 5 days, with the highest degradation rates of 74.35 and 74.29 %, respectively. Suitable degradation conditions include an initial concentration of 100–120 mg/kg, a pH of 6–7, and a temperature of 25–35 °C. The subsequent orthogonal experiments showed that the effect of reaction temperature and pH on the degradation effect was greater than the initial chlorpyrifos concentration and the number of white rot fungi. The best case is an initial concentration of 120 mg/kg, a pH of 6, a temperature of 35 °C, and a white rot fungus (mycelium) of 1.0 g. The use of agricultural waste as a carrier of immobilized microorganisms will provide an economical and environmentally friendly method for in situ remediations of pesticide-contaminated soil. Therefore, the

conditions obtained from the experiments can be used for future pesticide degradation research.

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ИЗВОД

## РЕМЕДИЈАЦИЈА ЗЕМЉИШТА ЗАГАЂЕНОГ ПИРОФОСОМ ПРИМЕНОМ ИМОБИЛИЗОВАНИХ ГЉИВА БЕЛЕ ТРУЛЕЖИ

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У овом раду је испитан процес разлагања пирофоса у земљи применом имобилизованих гљива беле трулежи, а у циљу селекције најбољих сојева и оптималних носача. Имобилизација гљива је изведена на кукурузној слами и клипу, пшеничној слами, љускама кикирикија и пиљевини дрвета. Гљиве *Phlebia* sp., *Lenzites betulinus* и *Irpex lacteus* су гајене у дефинисаним хранљивим медијумима и коришћене су за ремедијацију земљишта загађених пирофосом. Оптималан носач биомасе је одређен праћењем раста гљива беле трулежи. Резултати су показали да су кукурузна и пшенична слама погодни носачи гљива, а сојеви *Phlebia* sp. и *Lenzites betulinus* су испољили позитиван ефекат на разлагање пирофоса. Најбољи резултат је постигнут на 30 °C и неутралном pH, када је брзина разлагања пирофоса била 74,35 %, уз имобилизовану гљиву *Phlebia* sp. на кукурузној слами. Показано је да pH и температура више утичу на разградњу загађивача него почетна концентрација и количина биомасе.

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