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Original Research

Remediating Chlorpyrifos-Contaminated Soil Using Immobilized Microorganism Technology

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Abstract

Chlorpyrifos, one of the common broad-spectrum insecticides, can damage the human nerve system – even to the point of death under long-term exposure. In addition, chlorpyrifos is hard to be naturally degraded because of its strong combination with soil particles and long half-life. So repairing the polluted soil is urgently needed. In this study, the embedding and crosslinking immobilization techniques were used to determine the degradation of chlorpyrifos in soil. After 16SrDNA analysis, the results showed that LLBD2 is *Bacillus cereus* and LLBD4 is *bacillus* sp., and bacteria immobilized on the degradation of chlorpyrifos were significantly better than the free bacteria. The degradation rate reached 83.28% after LLBD2 being immobilized within 72 h. Furthermore, the environmental factors influenced with LLBD2 showed that immobilized bacteria were more adapted to the changed environment than the free one. Although the initial concentration, pH, and temperature were significantly changed, the degradation rate of chlorpyrifos by immobilized bacteria was stable, suggesting that environmental factors had little influence on the immobilized bacteria.

Keywords: immobilization, *Bacillus cereus*, *Bacillus sp*, embedding-cross-linking, degradation rate

Introduction

O,O-Diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate is one of the most extensively used organophosphate pesticides throughout the world. Chlorpyrifos is stable at room temperature and shows a white granular crystal [1], it is insoluble in water and soluble in most organic solvents, and is easily decomposed in alkaline medium. 3,5,6-trichloro-2-pyridinol (TCP) is a metabolite of chlorpyrifos and chlorpyrifos-methyl, both of which are organophosphorus pesticides [2]. Chlorpyrifos was prepared by the reaction of 3,5,6-trichloro-2-pyridinol with diethyl chlorothiophosphate. Chlorpyrifos is used around the world to control pest insects in agricultural, residential, and commercial settings [3]. The crops with the most use are cotton, corn, almond, and fruit trees, including oranges, bananas, and apples [4]. An annual growth of 10% in the global demand for chlorpyrifos is expected, with more than 200,000 ton by the end of 2015.

As an effective insecticide, chlorpyrifos is widely accepted, but its potential dangers are not negligible for human health and the environment. It is reported

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Table 1. Immobilized microorganism and remediation of contaminated soil.

Subject	Action object	Carrier	Research results	References
Candida tropicalis	Benzopyrene	Polyvinyl alcohol Sodium alginate Activated carbon	The rate of degradation of benzo (a) pyrene was 40.65% by physically embedded IM 219-220-phy	[15]
Fungal oxalate plum ZHJ6	Methamidophos	PET non-woven fabrics	Fourth-day methamidophos degradation rate is more than 90%; sixth-day degradation rate was 100%	[16]
Reverse micelles laccase	DDTs	Diatomaceous earth	The degradation rate of DDTs was 20% higher than that of free laccase	[17]
Serratia	Bensulfuron methyl	Porous charcoal	After 28 d, paddy soil of benzyl its cycling degradation rate was 62%	[18]
Sporolactobacillus, Micrococcus, Pseudomonas	polyacrylamide	Polyvinyl Alcohol + sodium alginate + Additives	The removal rate of polyacrylamide by immobilized particles was up to 79.5%	[19]
Klebsiella sp. *Strain*	Aromatic hydrocarbon	PUF	It has better degradation rate and can be reused 36 times	[20]
Fungal Laccase	Fenarimol	Chitosan	Immobilized enzyme in the pH value of 4.0, the degradation rate of 30%, enzyme activity recovery rate from 58.5% to 58.5%	[21]
esterase B1	Organophosphorus pesticides	Sodium alginate	Degradation rate of methyl parathion in 1,000 mg/L by immobilized enzyme in 3 h >65%	[22]

that chlorpyrifos is suspected to be an environmental endocrine-disrupting substance, with low-dose residues having significant biological toxicity that can be hazardous to the endocrine, respiratory, nervous, or immune systems [5-6]. Chlorpyrifos is used for agricultural production, except as an insecticide, most of them eventually entering the soil environment, degrading slowly in the soil, and the residual period is long, with potential hazards to the soil ecosystem [7-8]. Residual chlorpyrifos in soil also leaches, which can pollute groundwater and surface water through percolation and runoff. With the increase in dosage, chlorpyrifos was detected in soil, water, and other environments in many countries and regions, and the residue and degradation in the environment should be highly valued.

Considering these negative effects, it is necessary to look for effective remedies to protect environmental sustainability. traditional methods are photodegradation, physical degradation, chemical methods [9], heat treatment [10], microbiological degradation [11], etc. Zhang [12] used ultrasonic air purification to clean organophosphorus pesticide chlorpyrifos, and the experiment showed that when bubble intensity was 25 m^3/h, ultrasonic power was 1000 W, clean for 15 min to get the best effect. Xu [13] studied the combined microbial biodegradation of chlorpyrifos, firstly, 3,5,6-trichloro-2-pyridyl (TPC) degraded by *Serratia* sp, then TCP completely mineralized by *Trichosporon* sp. Bioremediation is a widely used method of repair that can improve stability and repair results. Chemical or physical methods are used to disperse biocatalysts (cells or enzymes) into a limited field [14]. Now immobilized microorganism technology, especially using different carriers to immobilize microorganisms, are widely used in soil degradation. The most common methods are embedding and adsorption, but the number of immobilized microorganisms is affected greatly by the carrier and the effect is unstable. Table 1 shows immobilized microorganisms and remediation of contaminated soil. It not only improves the concentration of microbial cells, but also maintains high activity and microbial technology [10, 23]. This technology can greatly enhance the ability to adapt to the environment and increase the degradation of organic microorganisms [24], so as to improve the remediation of pesticide-contaminated soil. An immobilized carrier can enrich the strain [25] and extracellular enzymes, and can also improve contact efficiency between microorganisms and contaminated soil. So immobilized microorganism technology not only enhances the stability of microorganisms, but it also enhances environmental impact resistant ability and increases the number of inoculated microbes and activity, and the reaction initiation rate of degraded pesticides was also accelerated [26].

The objectives of this study were: 1) obtain the dominant degrading bacteria of chlorpyrifos, 2) compare the effect of chlorpyrifos degradation by immobilized bacteria or free bacteria, and 3) compare the adaptability of immobilized bacteria and free bacteria to environmental change.

Experimental

Materials and Methods

The specific dominant degrading bacteria of chlorpyrifos were domesticated and trained in the microbiological laboratory of Shenyang University of Technology. The soil samples were collected from the original factory area of Shenyang Northeast Pharmaceutical Factory, which belongs to the pharmaceutical-contaminated soil. The chlorpyrifos was bought from Shandong Rongbang Pesticide Chemical Co., Ltd (China). Sodium alginate, Polyvinyl alcohol, Na_2SO_4, and $FeCl_3$ were purchased from Tianjin Bo Di Chemical Co., Ltd (China). RTaqDNA polymerase, dNTPs, and T4DNA ligase were purchased from TaKaPa Engineering Company. Dichloromethane was purchased from Tianjin Fuyu Fine Chemical Co., Ltd. (China). Methanol was purchased from Thermo Fisher Scientific.

PCR Amplification instrument (Bio-Rad, American) was used to amplify specific DNA fragments. High-performance liquid chromatography (HPLC, Agilent 1260, Singapore) was used to determine the degradation rate of chlorpyrifos.

Methods

Enrichment of Degrading Bacteria

Two kinds of soil samples collected from wet soil and dry soil were added into distilled water and shocked for 2 h, then supernatant fluid of 150 mL was added to the conical flask. They were labeled as dry and wet samples, and chlorpyrifos was added (at that time the concentration was 16 mg/L). The 2 conical bottles were placed in a constant temperature, shaken, and cultured for 48 h. Enrichment culture of degrading bacteria needed 7 cycles, and the concentration of pesticide was increased appropriately during each transfer process (the specific values of pesticide concentrations are shown in Table 2).

Table 2. Water sample enrichment in each cycle of chlorpyrifos concentration.

Enrichment cycle (d)	Chlorpyrifos concentration (mg/L)
7	16.0
14	32.0
21	34.0
28	64.0
35	76.8
42	96.0
49	128.0

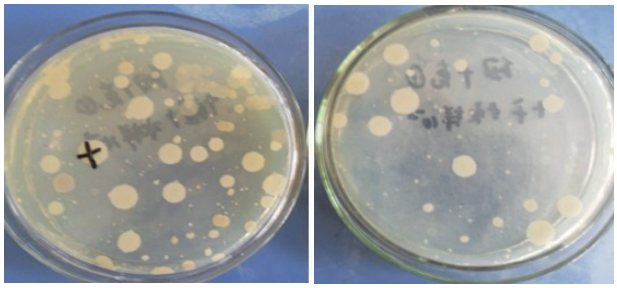

Fig. 1. Bacterial colonies on the culture medium.

Isolating Bacteria

Solid culture medium was placed in a 500 mL erlenmeyer flask and chlorpyrifos was added at 16 mg/L. After sterilization, all instruments and culture medium were quickly taken into a sterile room to 10 prepared tablets and they were marked. When the medium was well solidified, five cycles of water were inoculated with inoculating ring, and all of the tablets had making-on cassion and were cultured for 48 h, with a single colony growing well as shown in Fig. 1.

Purifying Bacteria

After domesticating the strain (in which the healthy colonies were selected and transferred to the corresponding slant medium for purification), 10 strains of chlorpyrifos-degrading bacteria were obtained and marked as LLBD1-10.

Primary Screening of Degrading Bacteria

0.100 g of chlorpyrifos was accurately weighed and put into a 100 mL volumetric flask, then sized down with methanol, 1 mL, 2 mL, 2.5 mL, 3 mL, and 5 mL of solution taken into five 25 mL volumetric flasks, respectively, and constanted volume with methanol. The concentration was diluted to 40, 80, 100, 120, and 200 mg/L, then measured by high-performance liquid chromatography (HPLC), and repeated three times. Chlorpyrifos standard curve was $Y = 1.6735 X - 12.076$, $R^2 = 0.9993$. In a sterile room, 11 doses of chlorpyrifos were prepared in 100 mg/L, and labled as LLBD1-LLB10, and the other was blank control. Then they were placed in a constant temperature shaker and after cultivation for 48h and 72h the samples were performed.

Rescreening Degradation Bacteria

The preliminary screening out of 5 chlorpyrifos dominant-degrading microorganisms were screened again. In a sterile room, 6 doses of chlorpyrifos were prepared in 100 mg/L and marked. Then, the same strains were added to erlenmeyer flask, and one of them was a control.

Water Sample Analysis

200, 120, 100, 80, and 40 mg/L chlorpyrifos were prepared as test water samples, and these samples were extracted by dichloromethane, then the extracted samples were determined by high-performance liquid chromatography (HPLC), and the recovery rate of chlorpyrifos in water samples was calculated by the result of determination. The results suggest that the average recovery rate of chlorpyrifos in water samples was 89.45%, which indicated that the method was feasible.

Small Extraction of Bacterial Genomic DNA

First, a single colony of bacteria was picked in a sterile room and accessed into 5-10 mL LB liquid to cultivate for a night. The bacterial fluid of logarithmic growth phase was split-charged into 1.5 mL centrifuge tubes centrifuged for 2 min at 12,000 r/min, the supernatant fluid was discarded, and bacteria were collected, then washed clean with 1 mL STE. 567 μL of TE buffer was put in a liquid gun and blown repeatedly, and then 30 μL 10% SDS (sodium dodecylsulfate) with 3 μL proteinase K were added, the mixture was evenly inverted and placed in a 37ºC water bath for 1 h. Then 100 μL 5mol/L NaCl solution was put in a container and mixed evenly. 80 μL of CTAB/NaCl solution was mixed well and took to a 65ºC water bath to heat for 20 min. Then equal volume (780) chloroform/isoamyl alcohol (24:1) were added and centrifuged for 15 min at 12,000 r/min. Equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) were put in it and centrifuged for 5 min at 12000 r/min, and the supernatant was discarded. Next, equal volume (780) chloroform/isoamyl alcohol (24:1) was added and it was centrifuged for 15 min at 12000 r/min. Then, equal volume of -20ºC *iso*propyl alcohol was added and mixed until DNA flocculent precipitation was seen, and then it was allowed to stand for 3-5 min. Finally, supernatant was removed, 70% ethanol was added and shaken gently, and then the sample was washed with 100% alcohol and centrifuged for 5 min at 12000 r/min, supernatant was discarded and dried sterilely, and a suitable amount of TE buffer was added to dissolve completely and it was preserved at -20ºC.

Bacteria 16SrDNA Amplified PCR

The forward primer for PCR amplification is 16S-8-F1:5'-AGA GTT TGA TYM TGG CTC AG-3'(Y=C/T, M=A/C) and reverse primer is ITS-23-R:'-CCG GGT TTC CCC ATT CGG-3' [27].

PCR reaction procedures are as follows: the sample was predegenerated for 4 min at 94ºC, degenerated for 1 min, annealed for 50 s at 55ºC, and extended for 2 min at 72ºC. The steps above were repeated 34 times and extended for 10 min at 72ºC. The resulting DNA sequences were entered into GenBank for Blast retrieval, selected and download the corresponding sequence, and the nucleotide sequences obtained by DNASTAR, DNAMAN 4.0, and MEGA 3.1 software were compared and analyzed with the nucleotide sequence of the corresponding group included in the GenBank.

Table 3. 16SrDNA of amplification reaction system in PCR.

Project	volume (μL)
10*PCR Buffer (Mg^{2+})	2.5
dNTPMixture (2.5mmol/L)	2
Primer F (5μmol/L)	2
Primer R (5μmol/L)	2
Template DNA	1
rTaq polymerase (5U/μL)	0.25
Aseptic dd H_2O	15.25
Bulk volume	25

Bacteria Immobilization

10 g polyvinyl alcohol, 0.5 g alginate, and 5 g activated carbon were accurately taken and put in a 100 mL beaker containing 60 mL distilled water. The next day it was sterilized for 30 min at 110ºC and cooled to about 40ºC. Then, 20 mL bacterial fluid was predetermined volume added to 100 mL with aquae sterilisata, and fed into a granulator to be crosslinked by crosslinking agent for 24 h after being mixed evenly. The cross-linking agent was saturated boric acid solution (300 mL), and a small amount of calcium chloride and ferric chloride was made into a spherical carrier with a diameter of 2.5 mm. To ensure the strength of the immobilized ball, pellets were placed in a sodium sulfate solution of a mass ratio of 10% to stabilize 24 h. Then it washed and soaked by water after immobilization, and proliferated in the multiplication medium and labled as H1.

Preparing Chlorpyrifos-Contaminated Soil

First, a soil sample was collected and filtered through 20 mesh at room temperature, and chlorpyrifos was accurately weighed and dissolved into 500 mL volumetric flask containing methanol. For each test, 15 mL chlorpyrifos solution with 30 g soil was placed in a 250 mL conical flask, and at that moment the mass concentration of chlorpyrifos in soil was 100 mg/L. Next, 90 mL bacterial culture solution was added to the sample and made into slurry (soil water ratio was 3:1) after the soil has dried naturally, and then it was put into an autoclave sterilizer. Each sample was made of 3 parallel samples.

Determining Degradation Rate

1.00 mL slurry-containing pesticide with 10.00 mL methylene chloride was put into a glass centrifuge tube. Next, it was oscillated for 10 min at 300 r/min and placed

in an intermittent ultrasonic bath for 2 h. Then it was centrifuged for 5 min at 5000 r/min, and constanted volume with methanol to 25 mL in a volumetric flask after the supernatant was left and dried. Finally, 1 mL sample was absorbed by a syringe with a 0.45 μm organic filter and moved into the sample bottle for high-performance liquid chromatography (HPLC).

The Conditions of HPLC

The mobile phase was prepared with methyl alcohol and distilled water (proportion 78:22). UV wavelength was set to 280 nm at room temperature. The sample size was 10 uL at a rate of 1.2 mL/min and the retention time was 11.3 min.

The removal ratio of chlorpyrifos in soils was calculated as follows:

$$\text{Removal ratio (\%)} = 100\times(C_0-Cx)/C_0 \qquad (1)$$

…where C_0 and Cx are the peak area of chlorpyrifos before and after degradation, respectively. An experiment was also performed using free bacteria of an equal weight under the same conditions.

Data Processing Tools

Microsoft Excel was applied to process all the experimental data. SPSS 10.0 was applied for statistical analysis, Duncan's multiple comparison correction, on the different experimental conditions, which were used to evaluate the degradation efficiency of chlorpyrifos, and $p<0.05$ was considered a significant difference.

Results and Discussion

Effect of Degrading Strain on Degradation of Chlorpyrifos

As shown in Fig. 2, after primary screening experiments the results showed that LLBD2, LLBD3, LLBD4, LLBD6, and LLBD7 had better effects on the degradation of chlorpyrifos. After 48 h, their degradation rates respectively reached 73.64%, 51.12%, 64.56%, 58.31%, 47.85%, and at 72 h the degradation rates were 94.74%, 76.53%, 93.29%, 77.81%, and 80.34%. Compared with the other 5 strains, degradation efficiency of chlorpyrifos was improved and has obvious advantages. In addition, some strains reached the desired degradation effect after 72 h.

As shown in Fig. 3, the results showed that the degradation efficiency of chlorpyrifos was the same as that of the primary screening, among them the optimal degradation efficiencies of the 2 strains were LLBD2 and LLBD4. The degradation rate was 70.6% and 61.5% after 48 h, respectively, and after 72 h the degradation rates were 92.3% and 91.4%. The data was the average

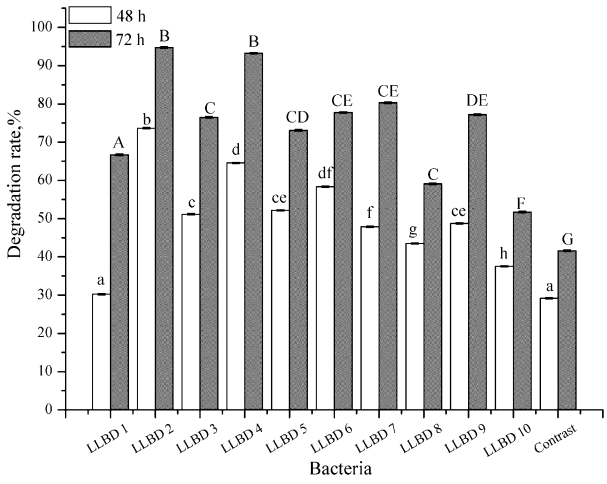

Fig. 2. Screening chlorpyrifos-degrading bacteria.

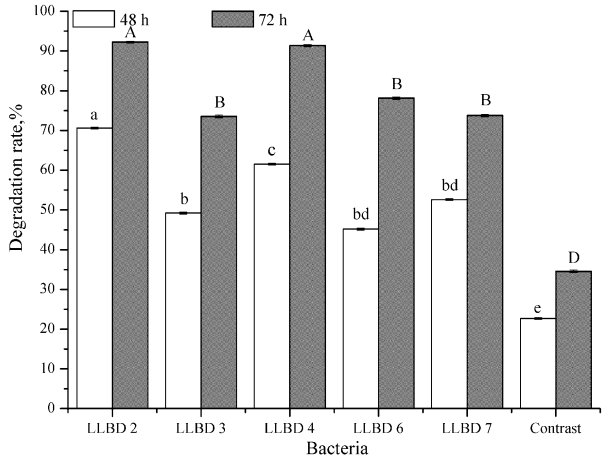

Fig. 3. Rescreening chlorpyrifos-degrading bacteria.

± standard error (n = 3), different letters indicating a significant difference in the rate of degradation strains ($p<0.05$), while the same letter indicates it had no significant difference.

16SrDNA Identification of Bacteria

In the degradation experiment of chlorpyrifos, LLBD2 and LLBD4 were the dominant bacteria, and their sequence results of 16SrDNA were compared with GenBank data by Blast program. The results showed that the homology between LLBD2 and *Bacillus cereus* strain BDU8 was 99%, while the homology between LLBD4 and *Bacillus* sp. A57 was 99%. Preliminary identification results showed that LLBD2 was *Bacillus cereus*, LLBD4 was *Bacillus* sp. *Bacillus cereus* and *Bacillus* sp were observed under the microscope at 1000 times, as shown in Fig. 4 and Fig. 5.

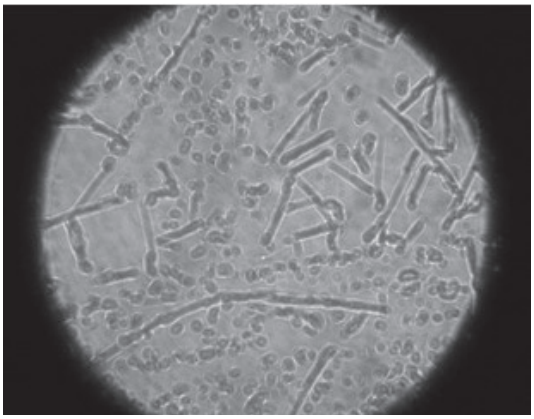

Fig. 4 Microphoto for *Bacillus cereus*.

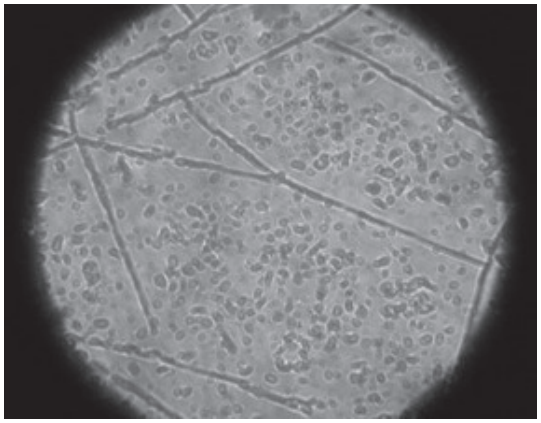

Fig. 5 Microphoto for *Bacillus* sp.

Effect of Immobilized Nicroorganisms on Degradation of Chlorpyrifos

As can be seen in Fig. 6, the degradation rate of chlorpyrifos obviously improved after the free bacteria were immobilized. Indeed, some studies have shown that the removal rate of chlorpyrifos using immobilized

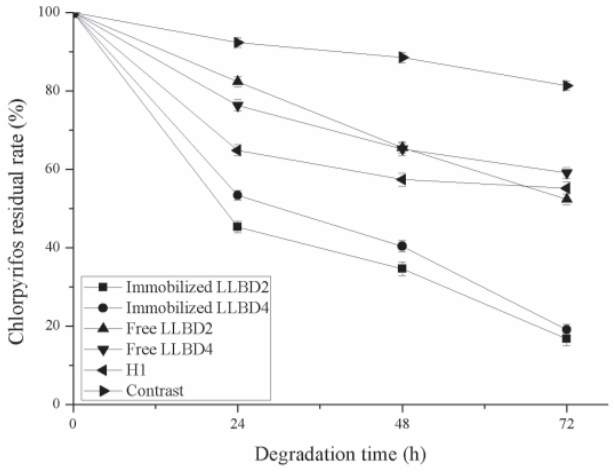

Fig. 6. Degradation of chlorpyrifos by immobilized strain in H1.

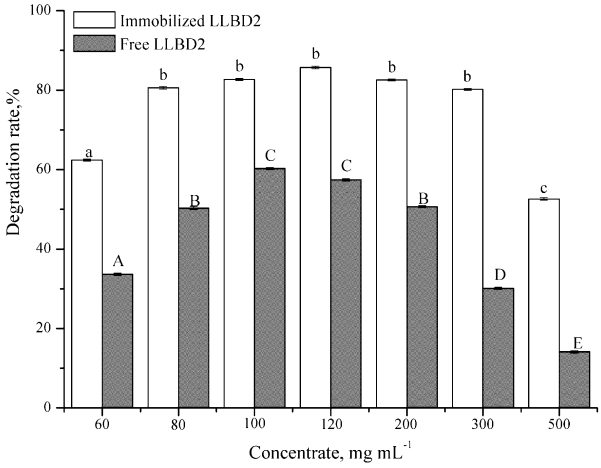

Fig. 7. The influence of initial concentration to immobilized bacteria degradation of chlorpyrifos.

bacteria was much higher than free bacteria [28], which should be attributed to the protection of immobilized pellets. After 24 h, 48 h, and 72 h, the residual rates of immobilized LLBD2 for chlorpyrifos were 45.29%, 34.63%, and 16.72%, respectively, while residual rates of free LLBD2 were 82.33%, 65.51%, and 52.33%, respectively. In this experiment, whether it was immobilized or not, the degradation rate of chlorpyrifos by LLBD2 strain was slightly higher than LLBD4, so the LLBD2 strain was used for subsequent experiments.

Removal Ratios of Initial Concentration on Chlorpyrifos

For immobilized bacteria, when the initial concentration was 100 mg/L, the highest removal ratio within 72 hours was 84.39%, the initial concentration of chlorpyrifos increased from 60.0 to 100.0 mg/L, and the removal ratio of chlorpyrifos increased from 60.10% to 84.39%. As the initial concentration of chlorpyrifos continued to rise, the degradation rate of immobilized bacteria remained unchanged, the concentration reached 300 mg/L, and the degradation rate began to decrease obviously. The optimal range of immobilized LLBD2 on the degradation of the initial concentration of chlorpyrifos was 80-300 mg/L. The degradation ratio of chlorpyrifos under different initial chlorpyrifos concentrations is shown in Fig. 7.

LLBD2, without being immobilized, was greatly affected by the initial concentration of chlorpyrifos. For free bacteria, when the initial concentration of chlorpyrifos was increased from 100 to 500 mg/L, degradation rate of chlorpyrifos could be reduced from 60.01% to 14.13% within 72 h. This showed that the range of tolerance of immobilized bacteria to chlorpyrifos was much wider than that in the free state. A high concentration of chlorpyrifos inhibited the growth of the strain and caused the degradation rate to decrease. Previous studies [29] showed that the degradation rate

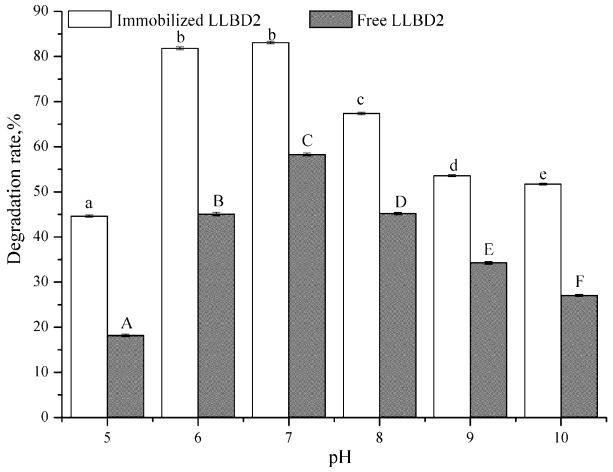

Fig. 8. The influence of pH on immobilized bacteria degradation of chlorpyrifos.

of chlorpyrifos by immobilized bacteria was higher than that of free bacteria, but the tolerance of immobilized bacteria to chlorpyrifos has a certain limitation.

Removal Ratio of pH Value on Chlorpyrifos

Under the state of different pH values, the removal ratio of chlorpyrifos was shown in Fig. 8. The immobilized bacteria had a wider pH range than the free one. When the pH value was 6.0-7.0, the degradation effect was better. The growth was best when the pH value was 7 and the degradation rate was 83.10%. Alkaline conditions were easier to survive than acidic conditions, but too much acid or alkali would affect growth. And the pH of free LLBD2 strain has a narrow adaptation ability, when pH was lower than 7 or higher than 7, the degradation rate fluctuated more widely. This showed that the immobilized carrier can protect the degrading bacteria and shield the harmful effects of the outside to a certain extent. Li Ying [30] showed that after degrading bacteria were immobilized, its tolerance to acid and alkali was significantly raised, but too acid or too alkaline also had an adverse effect on the biodegradation of chlorpyrifos ($p<0.05$). Compared with free bacteria, the sensitivity of immobilized bacteria to pH was decreased.

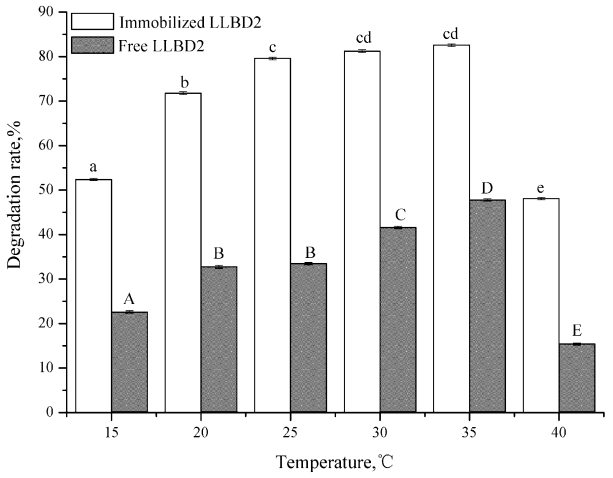

Fig. 9. Influence of temperature on immobilized bacteria degradation of chlorpyrifos.

Removal Ratio of Temperature on Chlorpyrifos

Temperature could affect the physiological metabolism and adsorption thermodynamics of bacteria and thus influence the degradation rate of chlorpyrifos in soils. For immobilized bacteria, the results in Fig. 9 showed that 35ºC was the best temperature for the degradation of chlorpyrifos in soils, and the removal rate was 82.57%. However, an extreme temperature also can cause a bad effect, and excess temperature could reduce the degradation rate. The experimental results were similar to Yen [31] et al., and 37ºC was the best temperature for the degradation of chlorpyrifos in soils, and the removal ratio was 89.37%. This might be mainly due to moderately increased soil temperature leading to the half-life of the cicada being shortened significantly, and also helping to improve enzyme activity.

Conclusion

In the experiment, the bacteria were immobilized by embedding and cross-linking, the results showing that the bacteria immobilized on chlorpyrifos degradation of pollutants was better than the free bacteria, and that half-life was also greatly shortened. After 72 h, more than 83.28% of chlorpyrifos could be removed by immobilized LLBD2 which was higher than LLBD4. And compared with the free LLBD2, the immobilized LLBD2 has a wide range of environmental adaptation.

The effect of degradation could be better when the initial chlorpyrifos concentration was 80-300 mg/L, pH value was 6.0-7.0, and temperature was 25-35ºC. When the initial pollutant concentration, pH, and temperature change greatly, immobilized bacteria were more adapted to environmental changes than free bacteria, and immobilized microorganisms were less affected by environmental factors and have strong adaptability, so their degradation rate was relatively stable.

The application of immobilized microorganism technology provides a new way for the remediating pesticide-contaminated soil. The direction of future research can be analyzed from three aspects:

1) The carrier is an important part of immobilization technology and is crucial to further developing an immobilized carrier model with good performance, improve the activity and concentration of the immobilized microorganism, and improve treatment effect and performance.
2) The actual pollutant is a complex system. It is difficult to meet the requirements for a single species

generally, so whether use of mixed bacteria or single bacteria classification needs further research.

3) The scope of the current application of immobilization technology is still relatively small, combining genetic engineering bacteria and the immobilization technology can expand the types of pollutants for processing, so the idea that the immobilization technique can be widely used should be the focus of future research. In conclusion, immobilization technology is an important means for improving the ecological environment. Predictably, along with the continuous research and development of immobilization technology, the application of this technology in soil pollution control will be taken as a practical step.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

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