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Full Length Research Paper

Isolation and application of a chlorpyrifos-degrading *Bacillus licheniformis* ZHU-1

Jiangwei Zhu[1], Yan Zhao[2] and Jiangping Qiu[1]*

[1]School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, PR China.
[2]Shanghai Institute of Quality Inspection and Technical Research, Shanghai 200233, PR China.

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A strain ZHU-1 capable of utilizing chlorpyrifos as the sole carbon sources and energy was isolated from soil. ZHU-1 was identified as *Bacillus licheniformis* based on analysis of morphology, physiological and biochemical characters and 16S rRNA. The addition of ZHU-1 to soil treated with chlorpyrifos resulted in a higher degradation rate than noninoculated soils, the degradation rate of chlorpyrifos (100 mg kg^{-1}) could reach 99% or above after 14 days. The microbial manure added by strain ZHU-1 can be applied not only as fertilizer, but also in degrading chlorpyrifos residue in soil. This study may provide basis for prevention and control of pesticides pollution.

Key words: Chlorpyrifos, degradation, *Bacillus licheniformis*, microbial manure.

INTRODUCTION

Chlorpyrifos was a broad-spectrum moderately toxic organophosphate insecticide, and was widely used in the prevention of both agriculture pests and urban public health pests (Fang et al., 2006). It has large blights on public health and environment resulting from its long residual period in soil and water (Mohan et al., 2004). Therefore its contamination and degradation were paid close attention (Racke et al., 1988; Yang et al., 2005). The microbial degradation was considered the most important degradation method because it was the main factor impacting degradation of chlorpyrifos residue (Munnecke et al., 1974; Racke et al., 1996). The effective measure to deal with pollution of chlorpyrifos or bio-remedy of chlorpyrifos-contaminated environment was isolation and screening of microbe strains which was able to degrade chlorpyrifos with high performance. Some chlorpyrifos-degrading bacteria had been isolated in lab, but they were applied seldom to commercial purpose.

Singh et al. (2004) separated *Enterobacter* B-14, a strain could degrade chlorpyrifos. Yang et al. (2006) and Li et al. (2007) separated *Stenotrophomonas sp*. and *Sphingomonas sp*. respectively, which could utilize chlorpyrifos as the only source of carbon and phosphorus. Singh et al. (2006) also studied the influence of different environmental conditions on bioremediation of chlorpyrifos pollution in soil and water to study the bioremedial potential of isolated strains for chlorpyrifos.

A lot of studies on chlorpyrifos degradation have been accomplished, but it was still a problem that how to eliminate chlorpyrifos residue in soil (Thomas et al., 2002). Thereby, we hoped to develop a new-type microbial manure, it was applied not only as fertilizer, but also could degrade pesticide residue. In this study, the *Bacillus licheniformis* ZHU-1, a strain that could degrade chlorpyrifos was isolated from soil. A new microbial manure was developed by adding strain ZHU-1 into the Yihai^{TM} microbial manure (it was commercially available).

The result of experimentation showed that new microbial manure contained strain ZHU-1 could be applied not only as fertilizer, but also could degrade chlorpyrifos residue in soil.

ISOLATION AND IDENTIFICATION OF CHLORPYRIFOS-DEGRADING BACTERIA

Soil samples were obtained from Wuqi Farm in Shanghai, China. The soil had been exposed to chlorpyrifos for more than 10 years. 5 g soil samples were added into 100 ml nutrient broth medium containing 100 mg L^{-1} chlorpyrifos (as enrichment medium). The suspension was incubated at 30°C with shaking at 120 rpm for

*Corresponding author. E-mail: qiujp06@gmail.com.

48 h, then it was spread on the plate (mineral salt medium containing 100 mg L^{-1} chlorpyrifos, as isolation medium). After inversion incubation at 30℃ for 3 days, pick vigorous growing colony on the plate for streaking and purified by repeated streaking. Lastly, isolates were inoculated onto enrichment plate for preservation. Isolates would be identified by using Biolog™ Microbial Identification System and reference to its morphology, physiological and biochemical characters.

BIODEGRADATION OF CHLORPYRIFOS BY STRAIN ZHU-1

Inoculum preparation

Seed culture of strain ZHU-1 was grown in nutrient broth medium, harvested by centrifugation at 5000 rpm for 5 min, washed with sterile saline twice and re-suspended in sterile saline to set an OD_{600} of 1.0. Colony forming units (CFU mL^{-1}) of this suspension were quantified by the dilution plate count technique. This suspension was used as inoculum for chlorpyrifos biodegradation studies until otherwise mentioned.

Degradation of chlorpyrifos in soil

Fresh soil from farms was taken and divided into four groups. (1) sterilization soil (121℃ sterilization for 30 min); (2) fresh soil; (3) sterilization soil added with strain ZHU-1: each 100 g sterilization soil was added with 10 ml inoculum; (4) fresh soil added with strain ZHU-1: each 100 g fresh soil was added with 10 ml inoculum. Each group was added with sterile chlorpyrifos that made the concentration 100 mg kg^{-1}. They were incubated at 35℃ in the incubator at last. During the incubation the moisture of soil was adjusted to 40% water-holding capability and residual chlorpyrifos in soil was determined every 48 h for 14 days so as to evaluate the degradation effect of strain ZHU-1 on the chlorpyrifos in soil. The residual chlorpyrifos was analyzed by GC as described in details previously (Yu et al., 2006).

Preparation of microbial manure containing strain ZHU-1

The inoculum and sterilization nutrient broth medium were mixed (1:1, v/v) and the mixture liquid were incubated for 12 h on a rotary shaker (120 rpm, 35℃). In the end, Yihai™ microbial manure (containing *Bacillus laterosporus, Bacillus subtilis* and so on) was used as carrier and each 100 g carrier was mixed with 20 ml ready-made mixture liquid. After antigrading the mixed manure was put in the 35℃ incubator for 12 h during which agitation once was needed, then put the manure containing strain ZHU-1 in the sterile glass bottle for preservation at -4℃.

Test on application of the microbial manure containing strain ZHU-1

Preparation of soil

Chlorpyrifos was added into fresh soil and the concentration was 100 mg kg^{-1}. Then put soil into several 20 × 30 cm boxes making 10 cm depth each so as to make sure each box contained the same weight of soil.

Degradation of chlorpyrifos in soil by microbial manure containing strain ZHU-1

15 boxes of soil were divided into 3 groups, so each group contained 5 boxes. Group I, Yihai™ microbial manure (not containing ZHU-1) was added into soil: 0.5 g manure was mixed into 30 ml sterile water and then sprinkled on the soil in box. Group II, the microbial manure containing strain ZHU-1 was added into soil (the method was same to group I). Group III as the control group, 30ml sterile water was sprinkled. Then the three groups were put in the 25℃ incubator, agitated every 12 h and supplied water properly (each of the 15 boxes was under same water supply). The chlorpyrifos concentration in soil was determined every 48 h for 14 days.

RESULTS AND DISCUSSION

Identification and characterization of isolated chlorpyrifos-degrading bacteria: A strain capable of utilizing chlorpyrifos as the sole carbon sources and energy was isolated from soil samples and named it ZHU-1. ZHU-1 was identified as *Bacillus licheniformis* by Biolog™ Microbial Identification System combined with analysis of the morphological, physiological and biochemical property, which had concordance with the result of 16S rRNA analysis made by TaKaRa Biotechnology (Dalian) Co., Ltd. The strain ZHU-1 was straight or curvulate bacillus, 0.7 - 0.8 × 2.0 - 2.5 um in size, Gram-positive, motile, facultative anaerobe, central spore or subterminal spore, and forms opaque and rough colonies on nutrient broth plates. It was positive in tests for catalase, Voges-Proskauer (V.P. test), gelatin liquefaction and starch hydrolysis, but negative for indole test.

The optimal temperature and pH for the growth of ZHU-1 were 35℃ and pH 7.5 respectively. To our knowledge, this is the first report for biodegradation of chlorpyrifos by Gram-positive bacterium.

The degradation rate of chlorpyrifos in soil added with strain ZHU-1 was apparently higher than those in soil without ZHU-1 (Figure 1), the degradation rate of chlorpyrifos(100 mg kg^{-1}) could reach 99% or above after 14 days' incubation, abutment to complete degradation. While the degradation rate in soil without ZHU-1 could only reach 36% (in fresh soil) and 19% (in sterilized soil), hence it was considered that the strain ZHU-1 possessed well viability and could degrade chlorpyrifos effectively in soil. These could also indicate that it is available to degrade chlorpyrifos in soil by adding strain ZHU-1. On the side, it was also showed in Figure 1 that the degradation rate of chlorpyrifos in fresh soil was higher than those in sterilized soil, but just a little.

It was a reason possibly that some natural microorganism in fresh soil possessed ability to degrade chlorpyrifos. The amount of chlorpyrifos decreased by 19% after 14 days in sterilized soil without microorganism (Figure 1, curve (1)), chlorpyrifos reduction with time might be caused by hydrolysis, photodecomposition or other reason.

It could be known from Figure 2 that degradation of chlorpyrifos in soil could be highly enhanced by adding the microbial manure, especially by adding the microbial manure containing strain ZHU-1. The degradation rate of

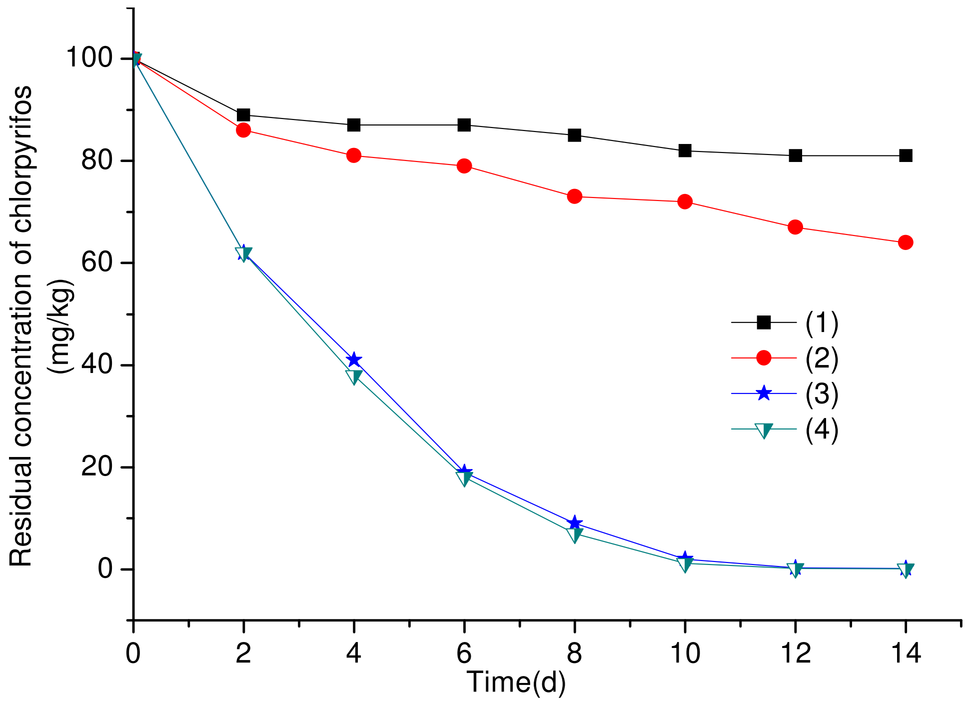

Figure 1. The curve on chlorpyrifos degradation in the different treated soils with strain ZHU-1 [curve (1)-Sterilized soil, curve (2)-Fresh soil, curve (3)-Sterilized soil added with ZHU-1; curve (4)-Fresh soil added with ZHU-1].

chlorpyrifos (100 mg kg^{-1}) could reach 99.8% by the microbial manure containing ZHU-1 after 14 days, which resulted from chlorpyrifos-degrading strains or assistance strains in the microbial manure. It was also considered that some microorganism in the Yihai™ microbial manure could degrade chlorpyrifos or assist degradation, but their ability of degradation was much lower than that of strain ZHU-1. The amount of chlorpyrifos in control group decreased by 32% after 14 days (Figure 2), which might be caused by some chlorpyrifos-degrading strains in natural soil or other physical, chemical factor. These chlorpyrifos-degrading strains or other assistance strains by co-metabolism must be stable and active in soil, which is an essential requirement for any *in situ* xenobiotic degradation studies (Vidya et al., 2008).

The strain ZHU-1 was conjectured to survive well in soil or microbial manure according to experiment results, because it could degrade chlorpyrifos effectively. Lastly, it was considered that the microbial manure added by specified strains was able to degrade pesticides residue while in fertilization but some details remained improvement.

The chlorpyrifos utilization ability of strain ZHU-1 was checked under various culture conditions, all test results indicated that the strain ZHU-1 had effective chlorpyrifos degradation ability. The degradation ability of ZHU-1 might have relation with the long-time usage of chlorpyrifos in the soil that isolated this bacterium as the record had shown the time of chlorpyrifos usage was above ten years. Generally, soil microorganism that repeatedly or continuously encounter synthetic chemicals were likely to develop capabilities to degrade such chemicals, and such microorganism with newly evolved traits have been implicated in the rapid inactivation of pesticides in problem soil (Felsot et al., 1989). The application research of the microbial manure containing ZHU-1, however, has shown that there was no apparent adverse effect on agricultural production which initially indicated the safety of the strains. While the mutual effect and proper match between degradation strains and microbial manure strains was waiting for further study, especially the complex mutual effect such as symbiosis, antagonism and competition should be noticed. Some kinds of strains might be able to degrade several types of pesticides effectively and might have harmful effect on the normal usage of pesticides such as pharmacodynamic time or efficacy decrement, which should be noticed.

Environmental factors such as physical and chemical characteristics of the substrate, nutrients status, pH, temperature and biotic factors such inoculum density interfered the accomplishment of any bioremediation process (Ramadan et al., 1990; Singh et al., 2003; Samina Anwar et al., 2009). Hence, a lot of elements should be further improved, and it was expected that strain ZHU-1 could be utilized for developing agricultural

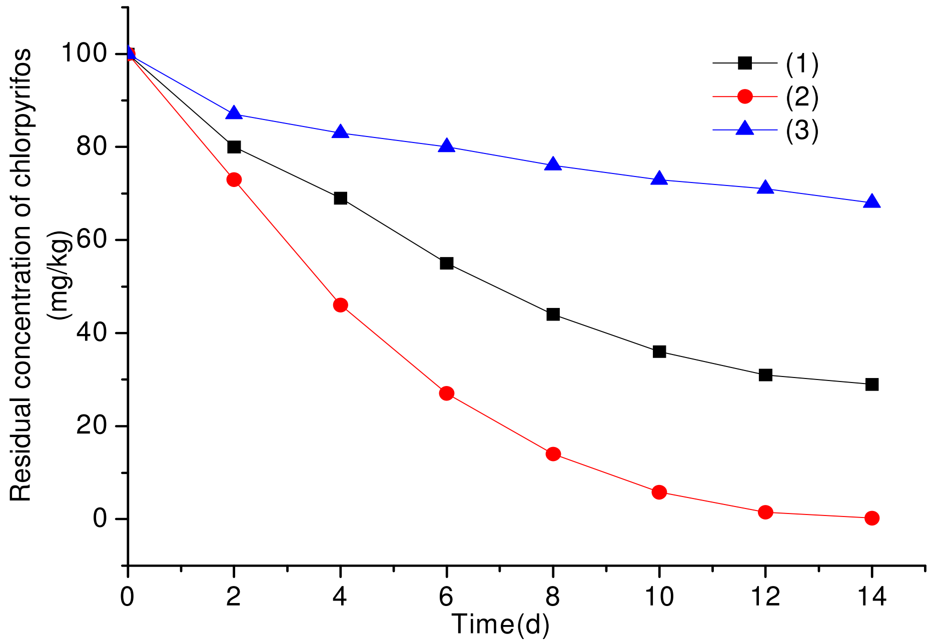

Figure 2. Degradation of chlorpyrifos by the microbial manure (containing strain ZHU-1 or not) in soil containing 100 mg kg $^{-1}$ chlorpyrifos [curve (1)-microbial manure (Yihai^{TM}. no strain ZHU-1), curve (2)-microbial manure contained strain ZHU-1, curve (3)-control].

microorganism preparation used in crop disease prevention, pesticides residue degradation and farmland soil improvement, etc.

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