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Isolation and Identification of a DDVP-degrading Bacterial Strain from Soil*

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ABSTRACT Objective: Organophosphorus pesticides and pesticide widely used in a large number of domestic and foreign production, its number has more than 100 kinds. Heavily used organophosphorus pesticides can increase agricultural production, but also caused incalculable environmental pollution. To research a microorganisms which can degrade DDVP for exploring microbial agents which can decrease the residues of DDVP and restore the pesticides-polluted soils. **Methods:** A bacterium strain which could degrade one kind of organophosphate pesticides, DDVP (dimethyl-dichloro-vinyl-phosphate), was isolated from soil samples collected from greenhouses planting vegetables in Zibo, Shandong Province. The bacterium strain was identified as *Pseudomonas fluorescens* strain P according to its morphological, physiological, biochemical characteristics and 16S rDNA sequence blast. **Results:** The optimum growth temperature for this strain was 27 ℃, and the optimum initial pH was 7.0. *P. fluorescens* strain P could degrade DDVP with an efficiency of 61.24% in four days. **Conclusion:** This experiment screened a strain capable of efficient degradation of dichlorvos strains of Pseudomonas fluorescence through the identification. This study will lay a foundation for exploring microbial agents which can decrease the residues of DDVP and restore the pesticides-polluted soil.

Key words: Bacterium; DDVP; Degradation; Identification

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Introduction

Pesticides are a class of biocides and have been extensively used for controlling pests in agriculture, which have contributed much to the agricultural yield. Pesticides can be divided into several types according to their molecular structures such as organophosphate pesticides, carbamate pesticides, and organochlorine insecticides, among which organophosphate pesticides accounted for 80 % of the total pesticides production[1]. Organophosphates pesticides are a class of organic phosphorus esters, which affect the nervous system of animals by disrupting the enzyme that regulates the synthesis of acetylcholine in nerve system, and have characteristics of high efficacy, low cost, rapid degradation in the environment and low residue [2]. However, with the large-scale use of organophosphate pesticides, excessive pesticides entry into soil and water, which causes serious environmental pollution.

Microbial degradation of organophosphate pesticides is considered as an important means to control pesticide pollution. The most significant step for microorganisms to detoxify organophosphate pesticides is hydrolysis which makes the pesticides more easier to be further degraded[3]. The enzyme responsible for catalyzing this reaction is referred as an esterase or phosphotriesterase[4]. Therefore, it is essential to understand species of microorganisms that degrade organophosphate pesticides in the environment. In this study, soil samples greenhouses, through a medium containing dichlorvos screened strains isolated strain can degrade dichlorvos, and detected with a spectrophotometer degradation rate of strain. According to analysis of morphological characteristics of bacteria, as well as physiological and biochemical characteristics of 16S rDNA sequence analysis, the strain was identified as Pseudomonas fluorescens, and to detect the strain optimum growth temperature and pH. This study isolated and identified a bacterial strain which can degrade one kind of organophosphate pesticides, dimethyl-dichloro-vinyl-phosphate (DDVP), from greenhouse planting vegetables in order to utilize it in controlling pesticides residues in the future.

1 Materials and methods

1.1 Materials

Soil samples were collected from greenhouses planting vegetables from Zibo, Shandong province, where DDVP were widely used. The soil samples were preserved in sterilized beaker, and the sampling time and sampling locations were marked. DDVP was purchased from Nantong Jiangshan Agrochemical & Chemicals Co., LTD.

1.2 Experimental methods

1.2.1 Isolation bacteria degrading DDVP Five grams greenhouses soil was immersed in 50 ml of sterilized water to obtain bacterial suspension, and the bacterial suspension was made ten-fold serial dilutions using sterilized water, then a volume of 0.1

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ml of the appropriately diluted suspension were coated onto the nutrient agar plates incubated at 30 ℃ for 2d. Morphology of colonies such as color, size and convexity were observed. Single colonies were picked out and streaked on nutrient agar plates supplemented with 450 mg/L DDVP and cultured for 3 d at 30 ℃, colonies growing well on pesticides plates were selected and used for further screening and identification.

1.2.2 Identification of bacterial strain degrading DDVP The bacterial isolate degrading was identified by morphology, physiological and biochemical properties, 16S rDNA sequencing and phylogenetic analysis. The shape observing, Gram staining, and flagella staining tests of bacterial cells were performed for preliminary identification. Physiological and biochemical tests such as catalase, hydrolysis of starch, grease, gelatin and urea, citrate utilization, nitrate reduction, ammonia test were carried out according to the method published previously[5].

Genomic DNA of strain P was extracted according to the method published previously[6]. Two uinversal primers: 5'-AGAGT-TTGATCCTGGCTCAG-3', and 5'-AGTAAGGAGGTAGTCCAACCGC-3' were designed, and 16S rDNA fragment was amplified by PCR using the genomic DNA as template according to the program: 95 ℃ for 1 min, 55 ℃ for 1 min and 72 ℃ for 1.5 min, and total 35 cycles were performed. PCR products were cloned into pMD-18T (TaKaRa) and sequenced by Beijing Genomics Technologies. The phylogenic tree was constructed based on the 16S rDNA sequence using the neighbor-joining method.

1.2.3 Determination of DDVP concentration DDVP concentrations were determined by the method described by Liu (2008)[7]. Briefly, OD290 values for DDVP of different concentrations at a range of 200-450 mg/L in chloroform were measured using a UV-spectrophotometer using chloroform as control, and a standard curve was created with DDVP concentrations as X-axis and OD290 as Y-axis. DDVP in LB (Luria-Bertani) medium was firstly extracted with chloroform, and then assayed according to the method described above using chloroform extract of LB medium as control. All tests were performed three times.

1.2.4 Determination of DDVP degradation rate In A, B, C flasks containing 50 mL LB medium, flask A was inoculated with Strain P of 1% inoculum, DDVP was added into flask B to a final concentration of 450 mg/L, flask C was inoculated with strain P of 1% inoculum and supplemented with 450 mg/L DDVP. The flasks were incubated in a shaker at 30 ℃ for 7 days, and then DDVP concentrations in flask B and C were assayed every 24 h, respectively. The test was performed three times. Degradation curve of DDVP was drawn with OD_{290} as Y-axis and time as X-axis.

1.2.5 Measurement of the optimum temperature and pH for culturing Strain P Strain P was cultured in LB medium with initial pH 7.0 at 15 ℃, 20 ℃, 25 ℃, 27 ℃, 29 ℃, 30 ℃, 35 ℃, 40 ℃ for 7 d in a shaker, respectively. Then OD_{600} of the culture was measured everyday and growth plot was drawn. To investigate effect of the pH values on the growth of isolate strain P, the initial pH of LB medium was adjusted to pH4, pH5, pH6, pH7, pH8, pH9 with 0.1 mol/L HCl or NaOH, strain P was inoculated into the medium and shaking-cultured at 30 ℃ for 7 d. Then OD_{600} of the culture was measured everyday and growth plot was drawn.

2 Results

2.1 Isolation and identification of a bacterial isolate degrading DDVP

Three strains designated as L, B, and P were screened out on the DDVP containing plates from soil samples collected in greenhouses plating vegetables. Culturing tests of the three strains in liquid LB medium containing DDVP indicated that only strain P could degrade DDVP. Therefore, isolate strain B was selected for further investigation. Colonies of strain P was pale yellow and radial, bacterial cells were rod-shaped, Gram-negative (Fig.1A) with end flagella (Fig.1B), no endospore could be observed, the test results of gelatin hydrolysis, catalase hydrolysis, starch hydrolysis, grease hydrolysis, citrate utilization, ammonia test, urea hydrolysis and KNO_3 utilization are positive in Table 1.

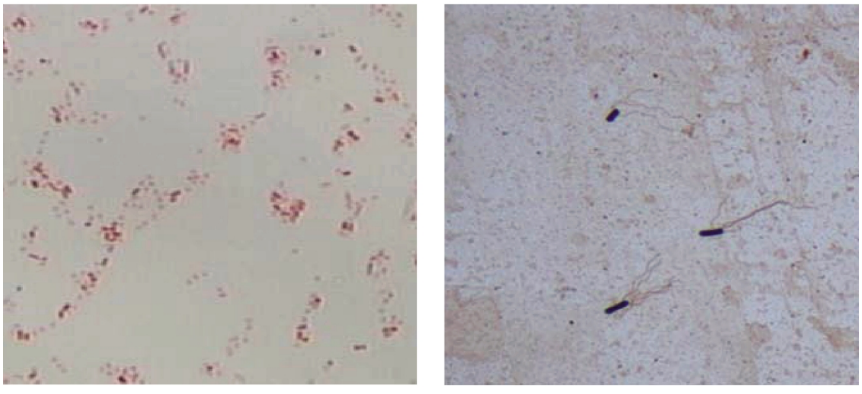

Fig.1 Gram staining (A) and flagella observation (B) of strain P

Besides the routine morphological identification, 16S rDNA fragment of 1501 bp was also amplified from strain P and sequenced. The sequence blast results indicated that 16S rDNA fragment of isolate strain P was homologous to that of Pseudomonas

fluorescens HQ876462 with a similarity of 99.3% (Fig.3). Therefore, based on the 16S rDNA sequence, morphological and physiological characteristics, strain P was identified as *Pseudomonas fluorescens* strain P.

Table 1 Physiological and biochemical characteristics of the strain P

Items	Strain P	Items	Strain P
Gelatin hydrolysis	+	Citrate utilization	+
Catalase hydrolysis	+	Ammonia test	+
Starch hydrolysis	+	Urea hydrolysis	+
Grease hydrolysis	+	KNO3 utilization	+

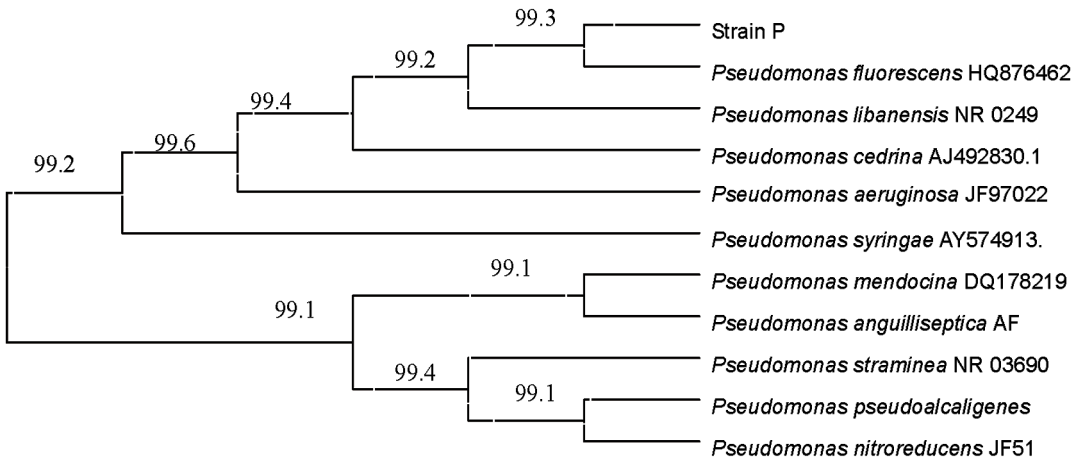

Fig.3 Phylogenic relationship of strain P and some related species based on 16S rDNA sequences

2.2 Preparation of DDVP standard curve

DDVP in chloroform has obvious UV absorbance at 290nm, there was a good linear relation between the OD_{290} and DDVP concentrations at the range of 200~450 mg/L (Fig.4), and r^2 is 0.9979. The method of UV absorbance was suitable for quantitative determination of DDVP.

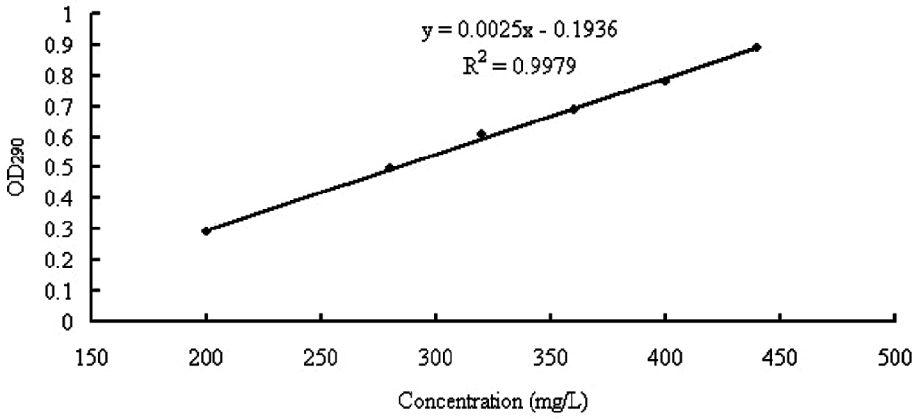

Fig.4 Standard curve for quantitative determination of DDVP

2.3 Biodegradation of DDVP by *P. fluorescens* strain P

To evaluate the degradation effect of DDVP by *P. fluorescens strain* P, *P. fluorescens* strain P was inoculated into LB medium containing 450 mg/L DDVP and cultured for a period of time. The result showed that DDVP decreased quickly in the first four days, and more than 60% in this period, then DDVP degraded slowly probably due to the limitation of nutrient in the medium (Fig.5).

2.4 Optimal conditions for culturing *P. fluorescens* strain P

To select the optimal culturing conditions, the culturing temperature and initial pH of LB medium were investigated. As shown in Fig.6, *P. fluorescens* strain P could grow well between 25 ℃ and 30 ℃, with optimal temperature of 27 ℃ (Fig.6A), and the optimal initial pH of medium was 7.0 (Fig.6B).

3 Discussion

DDVP is an organophosphate pesticide, which is colorless to pale brown liquid and soluble in water at room temperature. With the wide use of DDVP in controlling agricultural pests, its toxic effects on human beings and large amount of DDVP residues in environment have gained more and more concerns recently. Pollu-

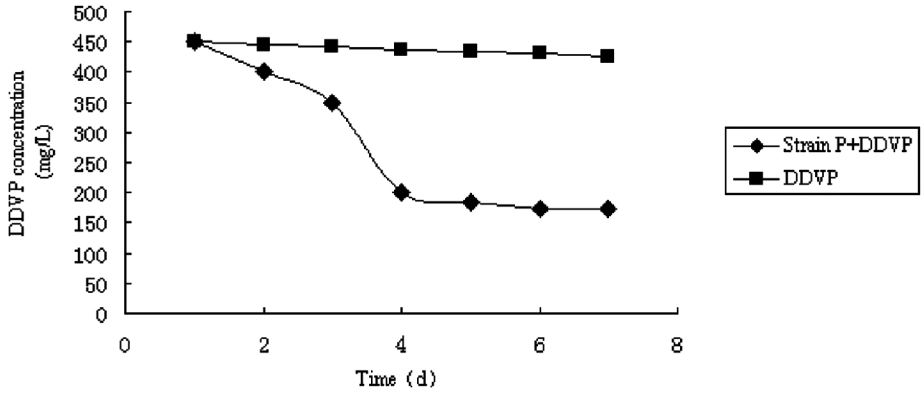

Fig.5 Time course of DDVP degradation by *P. fluorescens* strain P

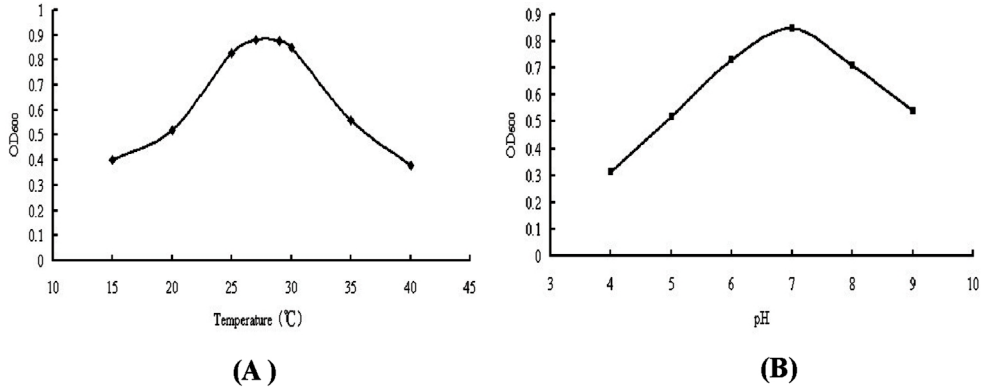

Fig.6 The optimal temperature (A)and initial pH of medium (B)for culturing *P. fluorescens* strain P

tion of organophosphate pesticides could be controlled by microbial degradation, and supplementation of microorganisms in soil could enhance the degradation of organophosphate pesticides[8]. The microorganisms degrading organophosphate pesticides which have been reported included *Arthrobacter sp.*[8], *Pseudomonas sp.* and Azospirillum sp.[9], *Streptomycetes sp.*[10] and *Trichoderma sp.* [11].

Microbial degradation of its features is the most important factor. Different types of microorganisms, metabolic activity is not same, there are also differences adaptability, and different strains of the same species respond to the same organic substrates are different [12-15]. Temperature plays an important role in affecting microorganism, with high temperature can kill microorganism and low temperature can inhibit their activity, while pH values may cause similar effects [16]. Liu's [17] and Ruan's [18] researches stated clearly that microorganism or enzymes produces own optimum temperature and pH values in degradation of pesticide. And their results are similar with Thomas's[19] and Donna's[20] reports.

In this paper, a bacterial strain degrading DDVP was isolated from soil where DDVP was used frequently and identified as *Pseudomonas fluorescens* strain P. The strain could grow well in a wide range of temperatures and initial pH values and degrade DDVP in a relatively high efficiency, which indicated that it might be used for restoring soils polluted by DDVP or eliminating DDVP residues left on crops. The mechanism of degrading DDVP by *Pseudomonas fluorescens* strain P is still undergoing.

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一株降解 O,O- 二甲基 -O-(2,2- 二氯乙烯基)磷酸酯的土壤微生物的分离与鉴定 *

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摘要 目的：有机磷农药和杀虫剂广泛应用于众多国内和国外生产的，其数量已超过 100 种。大量使用的有机磷农药会增加农业生产，而且还造成了不可估量的环境污染。研究降解敌敌畏的微生物，为微生物以降低产品敌敌畏农药残留，恢复敌敌畏污染土壤中的研究奠定基础。**方法：**本文从种植蔬菜的温室大棚的土壤中分离了一株降解 O,O- 二甲基 -O-(2,2- 二氯乙烯基)磷酸酯(敌敌畏)的细菌，根据该菌的形态学、生理生化特征及 16S rDNA 序列比对。**结果：**该菌鉴定为荧光假单胞菌（菌株 P）。该菌的最适生长温度为 27 ℃，其培养基的最适初始 pH 为 7.0，4 天内该菌可将培养液中 61.24%的降解。**结论：**本实验从蔬菜大棚的土样中筛选出一株能降解敌敌畏的菌株，并鉴定为荧光假单胞菌。本研究将为基于微生物以降低产品敌敌畏农药残留，恢复敌敌畏污染土壤中的研究打下基础。

关键词：细菌；O,O- 二甲基 -O-(2,2- 二氯乙烯基)磷酸酯；降解；鉴定

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