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RESEARCH ARTICLE

In situ enhanced bioremediation of dichlorvos by a phyllosphere *Flavobacterium* strain

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Abstract A bacterium capable of degrading dichlorvos was isolated from the rape phyllosphere and designated YD4. The strain was identified as *Flavobacterium* sp., based on its phenotypic features and 16S rRNA gene sequence. Strain YD4 was able to utilize dichlorvos as the sole source of phosphorus. *In situ* enhanced bioremediation of dichlorvos by YD4 was hereafter studied. Chlorpyrifos and phoxim could also be degraded by this strain as the sole phosphorus source. A higher degradation rate of dichlorvos was observed after spraying YD4 onto the surface of rape leaves when compared to the sterilized-YD4 and water-treated samples. The results indicated that pesticide-degrading epiphytic bacterium could become a new way for *in situ* phyllosphere bioremediation where the hostile niche is unsuitable for other pesticide-degrading bacteria isolated from soil and water.

Keywords enhanced bioremediation, organophosphorus pesticides, phyllosphere, *Flavobacterium* sp.

1 Introduction

Organophosphorus pesticides are increasingly used in agriculture to control pests [1]. Without the use of pesticides, as much as 45% of the world's crops would be destroyed by plant pests and diseases [2]. Dichlorvos, as a relatively low toxic organophosphate pesticide, has been a longstanding recommendation for the control of various pests in agriculture. However, due to a lack of training and education regarding appropriate pesticide use and widespread misuse of pesticides in developing countries, as well as the high demand for farm produce and low perception of the toxic effects of residual pesticides in food [2,3], some farmers do not wait long enough for the residues to be cleaned up after spraying before harvesting. The undesirable accumulation of such pesticides in food products may ultimately result in adverse effects in biologic systems and human health. Accordingly, it is essential to develop safe, convenient, and economically feasible methods for pesticide detoxification [4,5].

Biodegradation has been proven to be a reliable and cost-effective technique for pesticide removal, and successfully applied to bioremediate soil contaminated with organophosphorus pesticides [6–8]. After spraying, a considerable amount of pesticides remain on plant leaves. Although some enhanced bioremediation of pesticides by using microbes from soil and from recombinant technology was reported, on arrival, new immigrants are challenged with the harsh conditions of the leaf environment including highly fluctuating water availability, exposure to ultra violet (UV) radiation from sunlight, and limited access to nutrient resources. Therefore, pesticide-degrading epiphytic bacterium may become a new way for *in situ* phyllosphere bioremediation. However, enhanced bioremediation of pesticides on plant leaves *in situ* by using a phyllosphere microbe has been seldom reported to date besides bioremediation of pollutants by phyllosphere microbe [9].

The large collective surface area of the leaves of a terrestrial plant, which is known as the phyllosphere, provides a habitat for a large and complex microbial population. Our previous study in the view of microbial ecology indicated that the phyllosphere microbial population had a substantial contribution to the degradation of dichlorvos [10]. Here, a bacterial strain YD4 capable of biodegrading dichlorvos was screened directly from the rape phyllosphere for *in situ* enhanced bioremediation of pesticides. The biodegradation capacity of dichlorvos by YD4 was evaluated by spraying the cultured strain onto the rape phyllosphere. The ability of this organism to degrade other organophosphorus pesticides was also tested. The

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isolated strain YD4 is expected to be commercialized and provide much better safeguards for large-scale food production.

2 Materials and methods

2.1 Enrichment and isolation of dichlorvos-degrading bacteria

Oil seed rape (*Brassica napus* L.) was planted on March 1, 2009 in a greenhouse located in Dongbeiwang, Beijing, China. Bacteria were eluted from 10 g leaf samples with 0.1 mol·L^{-1} sodium phosphate buffer (pH 7.5), after which they were sonicated for 7 min in an ultrasonic cleaning bath (40 kHz) as described by Zhang et al. [11]. The cells were then harvested by centrifugation at 8000 g for 15 min at 4°C, and the pellet was then resuspended in the same buffer. Enrichment was conducted by adding the cells to 100 mL of basal medium (MM) containing 200 mg·L^{-1} dichlorvos and then incubating the samples at 25°C for five days. The basal medium contained (g·L^{-1}) glucose (5.0), $(NH_4)_2SO_4$ (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), KH_2PO_4 (0.5), K_2HPO_4 (1.0), $FeSO_4 \cdot 7H_2O$ (0.002), $CaCl_2$ (0.1), yeast extract (5), at pH 7.2. After five days of incubation, different dilutions of medium were spread over MM agar plates (1.5% agar) in which the phosphorus or carbon sources had been replaced with dichlorvos. Dichlorvos was filter sterilized through sterile filter units (0.2 μm), after which it was added to the medium at a concentration of 200 mg·L^{-1}. The resulting colonies were repeatedly subcultured in MM containing 400 mg·L^{-1} of dichlorvos to confirm their ability to utilize dichlorvos. Visually distinct colonies obtained by serial dilution of the developed consortia on basal agar medium containing dichlorvos were selected and purified. The isolates were then screened for dichlorvos degrading ability in liquid medium by high-performance liquid chromatography (HPLC). HPLC studies were conducted at room temperature using a C18 column (C18-ODS, 250 × 4.6 mm, Agilent, USA) and methanol: water in the ratio of 80:20 as the mobile phase at a flow rate of 0.6 mL·min^{-1}. Detection of dichlorvos was performed at 215 nm and an injection volume was 20 μL. The retention time for dichlorvos was 5.2 min in the above conditions. Strain YD4 was selected from several isolated strains for further evaluation because it demonstrated the highest degrading efficiency. The isolated dichlorvos-degrading bacterium YD4 are available upon request from the corresponding author.

2.2 Identification of strain YD4

Strain YD4 was identified according to Bergey's manual of determinative bacteriology [12]. Bacterial DNA for Polymerase Chain Reaction (PCR) was extracted using the AxyPrep™ Bacterial Genomic DNA Purification Kit (AP-MN-BT-GDNA-50, Axygen, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR with the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) [13]. The conditions for PCR were as follows: 5 min of denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 52°C for 30 s, 72°C for 1.5 min, and a final extension at 72°C for 10 min. PCR fragments were purified by agarose gel electrophoresis, then ligated into the pGEM-T Easy Vector (A1360, Progema, USA) and then transformed into competent *Escherichia coli* DH5α cells. The 16S rRNA gene sequence was obtained using an automatic sequencer (model 3730, Applied Biosystems,USA) and deposited in GenBank under accession number GU458295. The determined sequence was compared with those available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program. Multiple alignments were conducted using CLUSTALX 1.83 (http://www.clustal.org/), and phylogenesis was analyzed using MEGA version 3.1 software packages (http://www.megasoftware.net/) [14]. A phylogenetic tree was constructed using neighbor-joining analysis with 1000 replicates of bootstrap analysis.

2.3 Biodegradation studies

The isolated strain YD4 was grown in MM liquid medium plus dichlorvos as described above. At 0 h and 48 h, samples were collected from the growing cultures, after which the concentrations of the dichlorvos were determined by HPLC following the protocol described above. Briefly, 1 mL of sample was taken from liquid cultures and centrifuged at 10000 g for 10 min and the supernatant was diluted with HPLC grade methanol and filtered. An aliquot of the solution (20 μL) was injected into an HPLC system for analysis. Uninoculated medium with the same concentration of dichlorvos was used as a control. The ability of the isolate to degrade dichlorvos was assayed by HPLC. To determine if the dichlorvos-degrading isolate could use dichlorvos as the sole source of carbon or phosphorus, or both of these compounds, the inorganic phosphorus sources (KH_2PO_4 and K_2HPO_4) in MM were replaced with dichlorvos (400 mg·L^{-1}) to study the utilization of dichlorvos as sole phosphorus source. Glucose and yeast extract in MM were replaced with dichlorvos to study the utilization of dichlorvos as the sole carbon source. The inorganic phosphorus sources (KH_2PO_4 and K_2HPO_4) and carbon source (glucose and yeast extract) in MM were replaced with dichlorvos to study the utilization of dichlorvos as sole carbon and phosphorus source. The growth of the isolate was also studied in MM when dichlorvos (400 mg·L^{-1}) was added as an additional carbon and phosphorus source.

To evaluate the effect of pesticide concentration on the degrading ability of the strain, aliquots of MM (50 mL)

were prepared containing dichlorvos at five different concentrations: 50, 100, 200, 400, and 800 mg·L^{-1}. To evaluate the effect of temperature, the strain YD4 was incubated at 20°C, 25°C, 30°C, 35°C, 40°C in the MM plus dichlorvos (400 mg·L^{-1}) media. The dissipation of dichlorvos at each concentration was also measured in uninoculated controls. To evaluate the effect of pH, the degrading ability of YD4 was determined in MM at pH values ranging from 4.0 to 8.0. Uninoculated controls with various pH values were also prepared. All cultures were incubated and the degradation of dichlorvos was determined as before.

2.4 Degradation of other organophosphorus pesticides by strain YD4

Chlorpyrifos, phoxim, profenofos, and dimethoate were selected as substrates. Strain YD4 was grown in 250 mL Erlenmeyer flasks containing 50 mL of Luria–Bertani medium. After incubation at 30°C and 180 r·min^{-1}, the bacterial culture was harvested in the late-exponential growth phase by centrifugation at 6000 g for 5 min, and then washed twice with MM medium. After the OD_{600} was adjusted to 1.0, an inoculum (2%, v/v) was added to 50 mL of MM medium containing the selected pesticide (200 mg·L^{-1}) as the sole phosphate source. The cultures were then incubated on a rotary shaker at 30°C and 180 r·min^{-1}. At 0 h and 48 h, samples were collected from the growing cultures and the concentrations of the selected pesticides were determined by HPLC. Three replicates of each treatment were conducted and a control experiment without microorganisms was carried out under the same conditions.

2.5 *In situ* degradation ability of dichlorvos by strain YD4

Rape was planted in a greenhouse as described above and the plants were watered and fertilized in accordance with local grower practices. After seven weeks, the plants were treated as described below. The organism had been grown in Luria–Bertani medium and harvested during the log phase and resuspended in basal medium, after which a part of the medium was used as the inoculum at a cell concentration of 7×10^8 cells·mL^{-1}, and another part was autoclaved to prepare sterilized-YD4. The rape plants were separated into the following three groups and then sprayed with 1 L of the indicated treatment: Groups 1 was sprayed with isolated YD4 cells, Group 2 was sprayed with sterilized-YD4, and Group 3 without inoculation was served as a control. Next, the three groups were sprayed with an identical amount of dichlorvos. At different time points between 0 and 7 days, aliquots of 50 g rape leaves were collected into sterile valve bags, cooled to 4°C, and sent to the Beijing Center for Physical and Chemical Analysis for analysis of the dichlorvos residues.

Dissipation data was subjected to regression equation for computing residual half-life and theoretical days of reaching MRL (t_i).

3 Results

3.1 Isolation, identification, and characterization of dichlorvos-degrading strain YD4

Microorganisms capable of degrading dichlorvos were screened by the enrichment technique and several bacterial strains were isolated from the rape phyllosphere in the presence of dichlorvos as the sole phosphorus source in MM and glucose as the carbon source. Strain YD4 was selected because it had a relatively higher degradation ability for dichlorvos than the other isolates. The phylogenetic tree based on a comparison of the sequences is shown in Fig. 1. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after the species name. Bootstrap values expressed as a percentage of 1000 replications are shown at the branch points.

Isolated strain YD4 was found to be Gram-negative and strictly aerobic. The cells of YD4 were short, non-motile and rod-shaped. The morphological and biochemical characteristics of the strain YD4 are listed in Table 1.

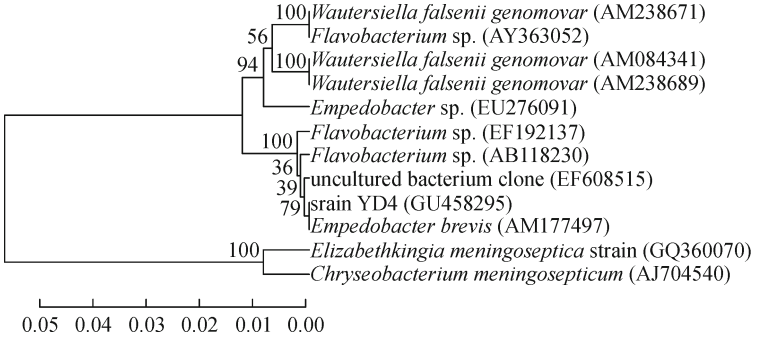

Fig. 1 Unweighted Pair Group Method with Arithmetic (UPGAM) phylogenetic tree based on the 16S rRNA gene sequences of strain YD4. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. GenBank accession numbers are given in brackets

Table 1 Morphological and biochemical characteristics of dichlorvos-degrading phyllosphere bacterium YD4

characteristics	isolate
colony morphology	smooth circular, semitransparent raised colony
gram strain	– ve bacilli
fluorescence on King'B medium	–
malonic acid utilization	+
citric acid utilization	–
swarming	–
biochemical tests	
catalase test	+
keto lactose test	–
methylred reaction test	+
glucose fermentation	
presumptive identification	*Flavobacterium* sp.

Notes: +, positive reaction; –, negative reaction

Strain YD4 was determined to be *Flavobacterium* sp. based on its 16S rRNA gene sequence and its morphological and biochemical characteristics. The partial 16S rRNA gene fragment was 99% similar to that of *Flavobacterium* sp. ANU301 (EF192137) and *Empedobacter brevis* strain LMG 4011T (AM177497).

3.2 Biodegradation studies

The degradation rate of dichlorvos by YD4 cultured in MM that did not contain another source of phosphorus was 60.89%. When the dichlorvos was used as an additional phosphorus source, the growth pattern was similar to that in MM without another phosphorus source, and the degradation rate of dichlorvos was 69.03%. The results revealed that strain YD4 was able to degrade dichlorvos and use it as the sole source of phosphorus (Fig. 2); however, growth was inhibited when dichlorvos was used as the sole carbon source.

The degradation of dichlorvos by strain YD4 at a range of dichlorvos concentrations, temperatures, and initial pH values are also studied (Fig. 3). The concentration of dichlorvos had an effect on the degradation rate with the most rapid degradation being obtained at 400 $mg \cdot L^{-1}$ (Fig. 3(a)). Dichlorvos concentrations higher than 800 $mg \cdot L^{-1}$ were toxic to the isolate, while lower dichlorvos concentration did not support the growth, and even led to restricted growth. The optimum degradation was observed at 30°C, while the degradation rate was somewhat slower at 25°C and 35°C (Fig. 3(b)). Strain YD4 degraded dichlorvos at a relatively slow rate at 20°C, with

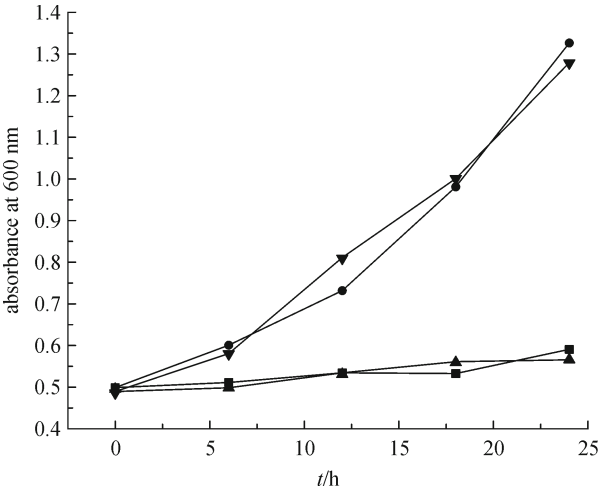

Fig. 2 Growth kinetics of strain YD4 in MM plus dichlorvos (▼), MM containing dichlorvos as a sole source of phosphorus (●), MM containing dichlorvos as a source of carbon (▲), and MM containing dichlorvos as sources of carbon and phosphorus (■) at 30°C

less than 45% degradation of the pesticide being observed in the culture at 48 h after inoculation. Dichlorvos degradation by strain YD4 was also slower at 40°C, with only about 60% of the initial concentrations being degraded after 48 h, and there was 19.6% of dissipation in the corresponding uninoculated culture at that temperature. Dichlorvos was degraded rapidly at pH values ranging from 5.5 to 7.0, with an optimal pH of 6.0 being observed (Fig. 3(c)). The degradation rate was somewhat reduced when the pH of the medium was 5.0. Conversely, the degrading ability of the isolate was markedly reduced when the pH of the medium was 4.0, with less than 30% of the initial concentration of dichlorvos being degraded at 48 h after inoculation. The degradation of dichlorvos was negligible and no bacterial growth was observed in any of the uninoculated cultures, regardless of pH.

3.3 Degradation of other organophosphorus pesticides by strain YD4

To evaluate the ability of the isolated strain to degrade other organophosphorus pesticides, four pesticides (chlorpyrifos, phoxim, profenofos, and dimethoate) commonly used in China were subjected to further analysis. The HPLC results indicated that chlorpyrifos and phoxim could be degraded by strain YD4 when provided as a sole phosphorus source, and the degradation rates at 48 h after inoculation were 57.8% and 34.7%, respectively (Table 2).

Table 2 Degradation rate of other organophosphorus pesticides by the isolated YD4

organosphorous pesticides	chlorpyrifos	phoxim	profenofos	dimethoate
degradation rate	57.8%	34.7%	–	–

Note: –, no detectable degradation

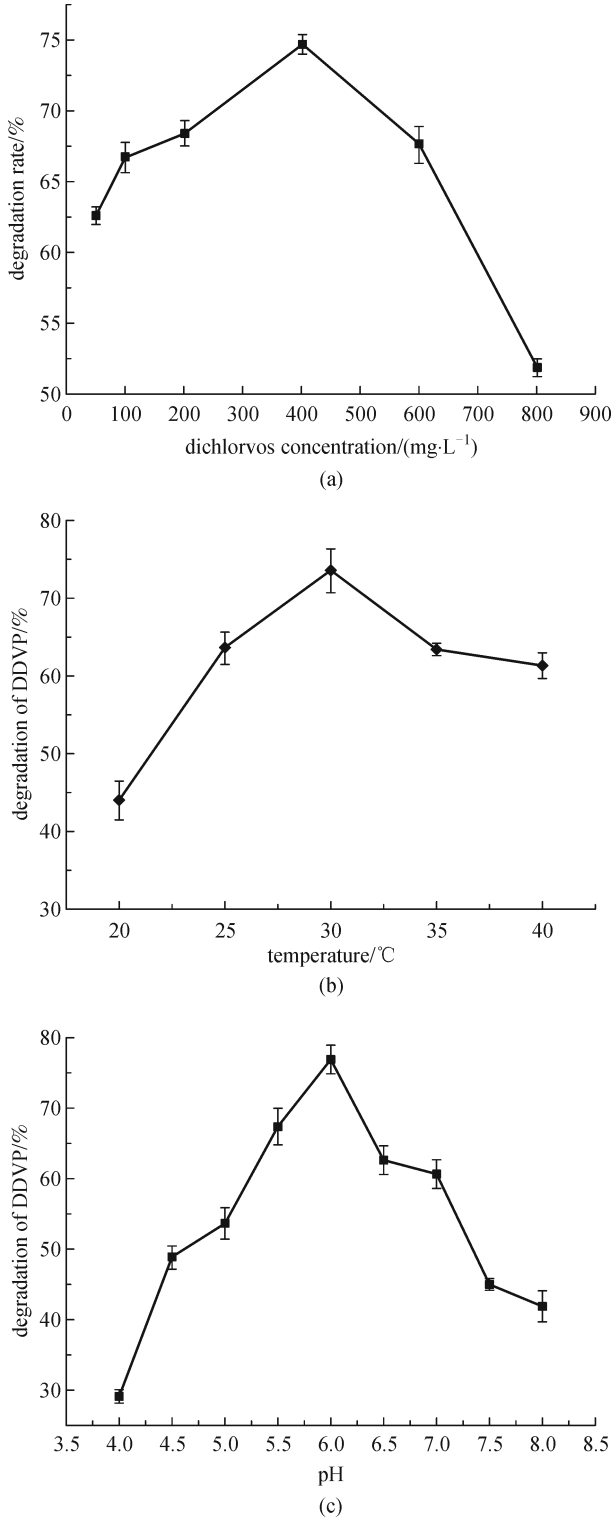

Fig. 3 Effects of dichlorvos concentration (a), different temperatures (b), and initial pH (c) on dichlorvos degradation by strain YD4

3.4 *In situ* degradation ability of dichlorvos by strain YD4

Residue, half-life values and the regression equation of dichlorvos for water, sterilized-YD4 and YD4-treated samples are presented in Table 3. For these three treatments, the correlation coefficients (R^2) were 0.995, 0.997, and 0.964, respectively, indicating a very good data fit. The half-life values calculated from the fit lines of the residual concentrations versus time period, follow first order reaction kinetics in the dissipation of dichlorvos residue. The average initial residues for the three treatments were found to be 5.10, 5.05, and 5.15 mg·kg^{-1}, respectively, at almost the same concentration. The level of dichlorvos residue after two days was 0.54 mg·kg^{-1} for both the water control sample and sterilized-YD4 treated sample. In contrast, the level of dichlorvos residue was 0.14 mg·kg^{-1} when strain YD4 was sprayed onto the rape leaves, which was below the Maximum Residue Level (MRL) of China (0.2 mg·kg^{-1}, GB2763-2005). The calculated theoretical days of reaching MRL were 2.50 for YD4-treated samples, 3.40 for water-treated samples, and 3.12 for sterilized-YD4 treated samples, respectively. Obviously, the spraying of strain YD4 onto rape leaves resulted in a more rapid degradation of dichlorvos than occurred in response to the indigenous microflora.

4 Discussion

The accumulation of organophosphorus pesticides in agricultural products is of considerable concern because plants act as intermediates in the transport of contaminants from soil, water, and air to humans and fauna [15]. Considering that most of the sprayed pesticides remain on the plant leaves, *in situ* bioremediation of residual pesticide is crucial to avoiding the injury of non-target species including humans, and contamination of ecological environment. Numerous microorganisms have been reported to harbor the ability to degrade organophosphorus pesticide, including *Pseudomonas*, *Azospirillum*, *Flavobacterium*, and *Agrobacterium* [16–18]. Indeed, the strain *Roseomonas* sp. was thought to have the degradation efficiency of 69.0% in biodegrading dichlorvos [19]. However, most of these strains were isolated from soil, which may be unsuitable for *in situ* phyllosphere dichlorvos bioremediation. Here, the strain YD4 capable of biodegrading dichlorvos was isolated using enrichment culture technique from the dichlorvos-treated rape phyllosphere, and was subsequently identified as *Flavobacterium* sp. *In situ* biodegradative activity of the strain was further evaluated by spraying YD4 onto the surface of rape phyllosphere. Compared to the treatment with sterilized-YD4 and water, a higher degradation rate of dichlorvos was observed after treatment of the surface of rape leaves with YD4. After two days, the level of dichlorvos residue was 0.14 mg·kg^{-1}, which was below the MRL of China (0.2 mg·kg^{-1}). When using dichlorvos, the safe intervals should be more than 5 days in summer (or 7 days in winter) according to the "guideline of safe handling of pesticide"

Table 3 Dichlorvos residue on rape leaves sprayed with water, sterilized-YD4, and YD4

		dichlorvos residue/(mg·kg^{-1})		
		water	sterilized-YD4	YD4
experimental results	0 d	5.10	5.05	5.15
	2 d	0.54	0.54	0.14*
	5 d	0.052	0.027	0.016
	7 d	0.007	0.005	0.002
calculated	regression equation	$y = 4.399e^{-0.91x}$	$y = 4.426e^{-0.99x}$	$y = 2.831e^{-1.06x}$
	R^2	0.995	0.997	0.964
	t_i /d	3.40	3.12	2.50
	half-life ($t_{1/2}$)/d	0.76	0.70	0.65

Notes: * MRL of dichlorvos in China was 0.2 mg·kg^{-1} (GB2763-2005); t_i represents the theoretical days of reaching MRL

of China (GB/T 4285-1989). The results provide the direct evidence that YD4 has the potential for use in enhanced bioremediation of dichlorvos *in situ* and acceleration of the harvest of the planted rape after treatment with dichlorvos.

Besides dichlorvos, the isolate YD4 can also degrade chlorpyrifos and phoxim, which actually have a phosphorothionate bond similar to the phosphoester bond present in dichlorvos. This feature may explain why YD4 was able to degrade these two compounds. It is most likely that strain YD4 harbors a versatile gene or enzyme system that enables the remediation of diverse toxic organophosphate nerve agents. The degradation mechanism of organophosphorus pesticides with phosphoester bond by microorganisms is presently well known [20,21]. Although speculative for YD4, one easily presumes that a similar or same strategy to degrade dichlorvos might be employed by this strain. The versatility of YD4 in degrading organophosphorus pesticides implicates its extensive application prospects for sustainable agricultural development and human health maintenance.

Taken together, this work and the previously conducted research suggest that further study of this genus may contribute to our greater understanding of the ecological diversity of these organisms and their usefulness as a biodegradation bacterium. Additional data are also needed to track the more detailed degradation pathway of organophosphorus insecticides in the plant phyllosphere. The isolated strain YD4 could be commercialized and provide much better safeguards for the production of green foods. The observed higher biodegradation capacity of residual dichlorvos by strain YD4 has demonstrated that isolating the phyllosphere microbes is an effective strategy for *in situ* enhanced bioremediation of residual pesticides. This same approach could be further extended to discover more microbial species that could biodegrade organophosphorus pesticide residues.

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