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Characterization of a bacterial strain capable of degrading DDT congeners and its use in bioremediation of contaminated soil

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ABSTRACT

A bacterial strain DDT-6 (D6) capable of utilizing dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethane (DDD), and dichlorodiphenyldichloroethylene (DDE) (DDTs) as its sole carbon and energy source was isolated and identified as *Sphingobacterium* sp. The degradation of DDTs by strain D6 in mineral salt medium and in field soil was investigated. The half-lives of the degradation of DDTs increased with increasing concentration ranging from 1 to 50 mg L^{-1}. Favorable degradation conditions for DDTs by strain D6 were found to be pH 7.0 and 30 °C. The degradation of DDTs by strain D6 was found to be statistically significantly enhanced ($p \leq 0.05$) by the addition of glucose. Based on the metabolites detected, a pathway was proposed for DDT degradation in which it undergoes dechlorination, hydrogenation, dioxygenation, decarboxylation, hydroxylation, and phenyl ring-cleavage reactions to complete the mineralization process. The addition of strain D6 into the contaminated soils was found to statistically significantly enhance ($p \leq 0.05$) the degradation of DDTs. The results indicate that the isolate D6 can be used successfully for the removal or detoxification of residues of DDTs in contaminated soil.

1. Introduction

1,1,1-Trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) has been used extensively since the 1940s as an insecticide, both for agricultural purposes and as an agent of public health against mosquito-borne diseases such as malaria and dengue [1]. Numerous studies have reported that DDT is readily converted to two major metabolites, 1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene (DDE) and 1,1-dichloro-2,2-bis(4-chlorophenyl) ethane (DDD), by microbial action, chemical reactions or photochemical reactions [2]. Due to their great persistence and toxicity, the US Environmental Protection Agency has classified DDT, DDD and DDE (DDTs) as priority pollutants. Although the use of DDT has been banned in the United States since 1972 and in China since 1983, residual levels of DDTs have still been detected at high levels in water, soil, air, and foodstuffs from some areas of China [3]. As strongly lipophilic pollutants and endocrine disrupters, they tend to accumulate in food sources and adipose tissues of organisms, resulting in problems such as breast cancer and reproductive faults even in humans [4]. Therefore, it is necessary to develop a remediation method for the cleanup of residues of DDTs.

Some studies have found that rapid degradation of DDTs may occur in some bacteria and fungi by mineralization or co-metabolism under aerobic and anaerobic conditions [2,5]. Thus, microbial detoxification has been considered as a cost-effective, safe and promising method for the removal of residues of DDTs from the environment [6,7]. So far, a few strains capable of degrading DDT have been documented, such as *Alcaligenes eutrophus* A5 [8], *Serratia marcescens* DT-1P [9], *Pseudomonas fluorescens* [10], and twelve species of brown-rot fungi [11]. Juhasz and Naidu [12] reported that 21% of DDT was degraded in potato-dextrose broth within 12 days after inoculation of *Cladosporium* sp. AJR[3] 18501. Kantachote et al. [13] found that the bacterial strain *Bacillus* sp. BHD-4 could remove 51% of DDT from liquid culture after incubation for 7 days. Hay and Focht [14] demonstrated the ability of *Ralstonia eutropha* A5 to degrade DDD under aerobic condition. The co-metabolism of DDE by *Pseudomonas acidovorans* M3GY or *Terrabacter* sp. DDE-1 has been revealed in pure cultures when induced by biphenyl [15,16]. However, little information is available on the removal or detoxification of DDT and its metabolic products DDE and DDD in mineral salt media and in field soil.

In the present study, a new bacterial strain, DDT-6 (D6), has been found to be capable of utilizing DDTs as its sole carbon and energy source. Strain D6 was isolated from soil contaminated by DDTs, and the isolate was used as an inoculant in all experiments to examine its degradation ability for DDTs in pure cultures and in field soil. The objectives of this study were: (1) to characterize the ability of strain D6 to degrade DDTs in mineral salts media; (2) to explore degradation pathways induced by strain D6 in pure cultures; and (3) to examine the possibility of applica-

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tion of strain D6 for *in situ* bioremediation of soil contaminated by DDTs.

2. Materials and methods

2.1. Chemicals

Standard samples of *p*,*p*′-DDT (purity ≥99.5%), *o*,*p*′-DDT (purity ≥ 98%), *p*,*p*′-DDD (purity ≥ 98%), and *p*,*p*′-DDE (purity ≥96.0%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acetone, *n*-hexane, and ethyl acetate of analytical grade were purchased from Dafang Chemical Co., Hangzhou, China.

2.2. Isolation of microorganisms

Soil samples were collected from vegetable field located in Cixi, Zhejiang, China, and then homogenized and divided into three subsamples (10 g). Each subsample was mixed well with 100 mL of sterilized water in 250-mL Erlenmeyer flask. A 1 mL aliquot of soil supernatants was transferred as inoculant into a 100-mL Erlenmeyer flask containing 20 mL of sterile mineral salts medium ($MgSO_4{\cdot}7H_2O$, 0.40 g; $FeSO_4{\cdot}7H_2O$, 0.002 g; K_2HPO_4, 0.20 g; $(NH_4)_2SO_4$, 0.20 g; $CaSO_4$, 0.08 g; H_2O, 1000 mL; pH 7.2) supplemented with 10 mg L^{-1} of DDTs as the sole source of carbon and energy. After incubation for 7 days at 30 °C and 150 rpm on a rotary shaker, the culture was inoculated into 20 mL of sterile mineral salts medium containing DDTs (20 mg L^{-1}) and incubated for another 7 days under the same conditions. The cultures were gradually acclimated to increasing concentration of DDTs ranging from 20 to 50 mg L^{-1} at weekly intervals. After 6–8 transfers, a mixed microbial population was appropriately diluted and then cultivated onto Luria–Bertoni (LB) agar plates (yeast extract, 10 g; peptone, 5 g; NaCl, 5 g; agar, 20 g; DDTs, 0.01 g; distilled water, 1000 mL; pH 7.2) and then incubated for 1 day at 30 °C. A bacterial strain D6 was isolated from colonies formed on the plates.

2.3. Identification of microorganisms

The isolate D6 was purified by repetitive subculturing on LB plates containing 10 mg L^{-1} of DDTs. Subsequently, the purified bacterial strain D6 was identified based on its morphological characteristics and 16S rDNA sequencing. The colony morphology of the isolate D6 was monitored on agar plates after incubation for 1 day at 30 °C, and the cell morphology was observed by transmission electron microscope (Hitachi H-7650, Japan). The 16S rDNA sequencing from the isolate D6 was accomplished by Invitrogen Biotechnology Limited Company, Shanghai, China. Similarity analyses of the 16S rDNA sequences were conducted using the Blast function of NCBI GenBank.

2.4. Inoculum preparation

The isolate D6 was cultured in 250-mL Erlenmeyer flasks containing 150 mL of LB medium at 30 °C and 150 rpm on a rotary shaker. At the exponential phase (1 day), the cell pellets were harvested by centrifugation (10,000 × *g*, 10 min), and then immediately washed 3 times with 20 mL of NaH_2PO_4-Na_2HPO_4 buffer (0.1 mol L^{-1}, pH 7.0), and finally suspended in the same phosphate buffer as inoculant.

2.5. Degradation of DDTs by the isolate D6 in pure culture

In order to study the biodegradation of DDTs by a microorganism, the mineral salt medium was supplemented with DDTs as sole carbon and energy source. Each flask was inoculated with a suspension of the microorganism up to a biomass level of OD_{610} = 0.4. All flasks were incubated in a shaker at 150 rpm in the dark. The whole culture was sampled for an assay of DDTs immediately after inoculation and after 3, 7, 14, and 21 days. Each treatment was performed in triplicate, and the control experiment without microorganism was performed under the same conditions.

To determine the effect of the concentration of the DDTs themselves on biodegradation, the mineral salt medium was fortified with DDTs at levels of 1, 10, and 50 mg L^{-1}. The medium was prepared with buffers at pH 5.0, 7.0, and 9.0 for the measurement of pH effect on the degradation. To confirm the effect of temperature on degradation, the medium was incubated at 20, 30, and 40 °C. To measure the effect of additional carbon sources on the degradation, the medium was fortified with 50 mg L^{-1} of glucose, yeast extract, sucrose, and fructose, respectively.

2.6. Field trials and sampling

To assess the feasibility of using strain D6 to remove residual DDTs in soil, a field-scale bioremediation study was conducted in a vegetable field (160 m^2, 10 m^2/plot) located in Cixi, Zhejiang, China. The field soils had been heavily polluted due to the extensive and indiscriminate use of DDT prior to its banning in China, and the field is currently used to grow pumpkin (*Cucurbita moschata* Duch.). In this study, the bioremediation experiments were designed as four treatments: control, pumpkin vegetation, D6 inoculation, and pumpkin vegetation with D6 inoculation. The suspension of strain D6 (6 L) with 0.5% glucose was sprayed with water using a knapsack sprayer at the dose of 150 L ha^{-1}, and the inoculation level of strain D6 reached approximately 1.0×10^6 colony-forming units (CFU) g^{-1} in the upper 10 cm of soil. The plots receiving the same amount of water without strain D6 were used as the controls. At the beginning and after 90 days, soil samples were collected from each plot using a stainless-steel manual auger, and each sample consisted of five cores and composited by manual blending, followed by intensive homogenization, sieving (2 mm), and storage at −20 °C until the determination of DDTs residues. All treatments were quadruplicated.

2.7. Extraction of DDTs from mineral salt medium and soil

To determine the concentration of DDTs in the mineral salt medium, the culture was transferred into a 250-mL separating funnel, and then extracted 3 times with 50 mL of *n*-hexane. The organic phase was dried through anhydrous sodium sulfate and collected in a 250-mL flat-bottom flask. It was subsequently concentrated to about 1 mL on a vacuum rotary evaporator and immediately concentrated to almost dryness under a gentle nitrogen flow. More *n*-hexane was added to redissolve the DDTs and to augment the volume to 10 mL prior to determination.

A soil sample (20 g dry weight) was weighed into a 250-mL Erlenmeyer flask. 10 mL of distilled water and 50 mL of acetone were successively added, and the mixture was shaken in the dark for 2 h at room temperature and 150 rpm on a rotary shaker. The soil suspension was filtered through a 7-cm Buchner funnel, and the filter cake was washed 3 times with 20 mL of acetone. All filtrates were collected in a 250-mL Erlenmeyer flask and evaporated on a vacuum rotary evaporator to remove acetone, and then transferred into a 250-mL separating funnel and extracted by the procedure described above.

2.8. Analysis of DDTs and their metabolites

Analyses of DDTs were performed using an Agilent 6890N gas chromatography (Agilent Technologies, USA) equipped with an electron capture detector (μ-ECD) and a DB-1701 silica capillary

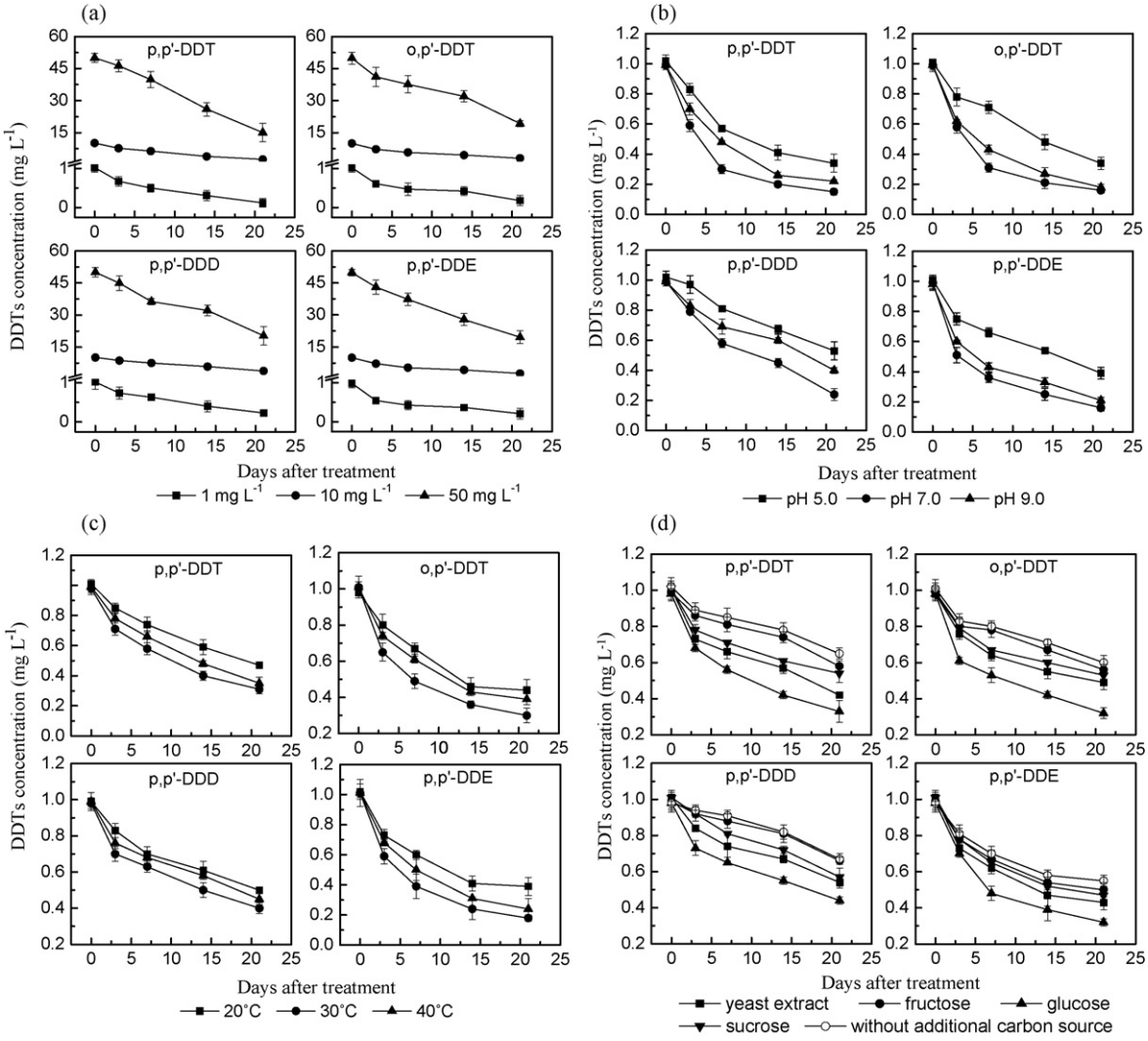

Fig. 1. Effect of concentration (a), pH (b), temperature (c), and additional carbon sources (d) on the biodegradation of DDTs by strain D6 in mineral salt medium.

column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies, USA), as described by Fang et al. [17].

Experiments exploring the pathway of DDT degradation by strain D6 were conducted in mineral salt medium supplemented with 20 mg L^{-1} of DDT at pH 7.0 and 30 °C. After 21 days of incubation, the degradation products of DDT were extracted with *n*-hexane and ethyl acetate according to the extraction procedure described above. DDT metabolites were identified using an Agilent 6890N/5975B inert XL MSD (Agilent Technologies, USA) equipped with a HP-5 silica capillary column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies, USA). The oven temperature was initially 80 °C, and was then raised to 200 °C at 15 °C min^{-1}, which was held for 10 min. The injector and detector temperatures were maintained at 250 and 280 °C, respectively. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}.

3. Results and discussion

3.1. Identification of the isolate D6

A bacterial strain D6 capable of utilizing DDTs as its sole source of carbon and energy was isolated from the DDTs-contaminated soils. The isolate D6 is a gram-negative, aerobic, and rod shaped bacterium. Its colony morphology on plain agar plate is observed to be yellow, round, smooth, wet, and slightly raised. The 16S rDNA sequences from the isolate D6 have been deposited in the NCBI GenBank database under the accession number EU927288. After alignment with other 16S rDNA sequences in GenBank database, it had a high degree of similarity (99%) to the members of *Sphingobacterium* sp. Therefore, the isolate D6 was identified as *Sphingobacterium* sp. based on its morphological characteristics and 16S rDNA sequences.

3.2. Evaluation of recovery

To confirm the validity of the extraction method of DDTs, recovery studies were carried out at spiking levels of 0.01, 0.1, 1, and 50 mg L^{-1} in mineral salt medium and 0.01, 0.1, and 1 mg L^{-1} in soil. The recovery of DDTs ranged from 91.2% to 103.8% with relative standard deviation (RSD) ≤ 6.9% in mineral salt medium and 82.1–105.3% with RSD ≤ 6.8% in soil. These data indicate that the extraction method is satisfactory for the analysis of DDTs residues.

3.3. Effect of DDTs concentration itself on biodegradation

The degradation of DDTs at various levels was examined in mineral salt media at pH 7.0 and 30 °C (Fig. 1a). In all controls, the hydrolysis percentages of the DDTs were less than 5%. After

Table 1
Kinetic data of DDTs degradation by strain D6 at different concentrations, pH, and temperature.

DDTs	pH	Temperature (°C)	Level (mg L^{-1})	Total degradation rate (mg L^{-1} day^{-1})	Kinetic function	$t_{1/2}$[a] (days)	r^2
Effect of DDTs concentration							
p,*p*′-DDT	7.0	30	1	0.043	$C = 0.99e^{-0.0955 \times t}$	7.26a	0.9801
p,*p*′-DDT	7.0	30	10	0.362	$C = 9.82e^{-0.0652 \times t}$	10.63b	0.9965
p,*p*′-DDT	7.0	30	50	1.665	$C = 54.67e^{-0.0578 \times t}$	11.99b	0.9739
o,*p*′-DDT	7.0	30	1	0.040	$C = 0.88e^{-0.0711 \times t}$	9.75a	0.9150
o,*p*′-DDT	7.0	30	10	0.330	$C = 9.03e^{-0.0527 \times t}$	13.15b	0.9739
o,*p*′-DDT	7.0	30	50	1.461	$C = 49.70e^{-0.0411 \times t}$	16.86c	0.9412
p,*p*′-DDD	7.0	30	1	0.038	$C = 0.98e^{-0.0678 \times t}$	10.22a	0.9910
p,*p*′-DDD	7.0	30	10	0.300	$C = 10.21e^{-0.0445 \times t}$	15.57b	0.9870
p,*p*′-DDD	7.0	30	50	1.417	$C = 50.56e^{-0.0406 \times t}$	17.07b	0.9605
p,*p*′-DDE	7.0	30	1	0.037	$C = 0.79e^{-0.0632 \times t}$	10.97a	0.9033
p,*p*′-DDE	7.0	30	10	0.351	$C = 9.05e^{-0.0587 \times t}$	11.81a	0.9694
p,*p*′-DDE	7.0	30	50	1.443	$C = 50.03e^{-0.0437 \times t}$	15.86b	0.9970
Effect of pH							
p,*p*′-DDT	5.0	30	1	0.032	$C = 0.94e^{-0.0529 \times t}$	13.10a	0.9514
p,*p*′-DDT	7.0	30	1	0.040	$C = 0.76e^{-0.0867 \times t}$	7.99b	0.9054
p,*p*′-DDT	9.0	30	1	0.037	$C = 0.87e^{-0.0731 \times t}$	9.48c	0.9491
o,*p*′-DDT	5.0	30	1	0.032	$C = 0.97e^{-0.0501 \times t}$	13.83a	0.9895
o,*p*′-DDT	7.0	30	1	0.039	$C = 0.76e^{-0.0831 \times t}$	8.34b	0.9011
o,*p*′-DDT	9.0	30	1	0.038	$C = 0.84e^{-0.0775 \times t}$	8.94b	0.9676
p,*p*′-DDD	5.0	30	1	0.023	$C = 1.03e^{-0.0317 \times t}$	21.86a	0.9940
p,*p*′-DDD	7.0	30	1	0.036	$C = 0.97e^{-0.0639 \times t}$	10.85b	0.9801
p,*p*′-DDD	9.0	30	1	0.028	$C = 0.96e^{-0.0400 \times t}$	17.33c	0.9718
p,*p*′-DDE	5.0	30	1	0.029	$C = 0.92e^{-0.0411 \times t}$	16.86a	0.9626
p,*p*′-DDE	7.0	30	1	0.039	$C = 0.76e^{-0.0784 \times t}$	8.84b	0.9254
p,*p*′-DDE	9.0	30	1	0.037	$C = 0.81e^{-0.0667 \times t}$	10.39c	0.9431
Effect of temperature							
p,*p*′-DDT	7.0	20	1	0.026	$C = 0.97e^{-0.0352 \times t}$	19.69a	0.9913
p,*p*′-DDT	7.0	30	1	0.032	$C = 0.88e^{-0.0528 \times t}$	13.13c	0.9716
p,*p*′-DDT	7.0	40	1	0.031	$C = 0.94e^{-0.0478 \times t}$	14.50b	0.9922
o,*p*′-DDT	7.0	20	1	0.026	$C = 0.91e^{-0.0394 \times t}$	17.59a	0.9301
o,*p*′-DDT	7.0	30	1	0.034	$C = 0.83e^{-0.0539 \times t}$	12.86c	0.9061
o,*p*′-DDT	7.0	40	1	0.030	$C = 0.89e^{-0.0442 \times t}$	15.68b	0.9251
p,*p*′-DDD	7.0	20	1	0.023	$C = 0.93e^{-0.0306 \times t}$	22.65a	0.9651
p,*p*′-DDD	7.0	30	1	0.028	$C = 0.87e^{-0.0387 \times t}$	17.91c	0.9383
p,*p*′-DDD	7.0	40	1	0.026	$C = 0.91e^{-0.0338 \times t}$	20.50b	0.9518
p,*p*′-DDE	7.0	20	1	0.030	$C = 0.88e^{-0.0449 \times t}$	15.43a	0.9013
p,*p*′-DDE	7.0	30	1	0.040	$C = 0.81e^{-0.0785 \times t}$	8.83c	0.9356
p,*p*′-DDE	7.0	40	1	0.037	$C = 0.88e^{-0.0668 \times t}$	10.37b	0.9598

[a] Data followed by a different letter in same column are significantly different ($p \leq 0.05$).

incubation for 21 days, The degradation rates of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, *p*,*p*′-DDE were found to be 0.043, 0.040, 0.038, and 0.037 mg L^{-1} day^{-1} at the level of 1 mg L^{-1}, 0.362, 0.330, 0.300, and 0.351 mg L^{-1} day^{-1} at the level of 10 mg L^{-1}, and 1.665, 1.461, 1.417, and 1.443 mg L^{-1} day^{-1} at the level of 50 mg L^{-1}, respectively. The degradation rates were proportional to the concentrations of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, *p*,*p*′-DDE ranging from 1 to 50 mg L^{-1} with R^2 values of 0.9998, 0.9996, 0.9999, and 0.9983, respectively, suggesting that the degradation is subjected to pseudo-first-order kinetics ($C_t = C_0 \times e^{-k \times t}$). The degradation half-lives of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, *p*,*p*′-DDE, obtained from the first-order function, were measured to be 7.26, 9.75, 10.22, and 10.97 days at the concentration of 1 mg L^{-1}, 10.63, 13.15, 15.57, and 11.81 days at the concentration of 10 mg L^{-1}, and 11.99, 16.86, 17.07, and 15.86 days at the concentration of 50 mg L^{-1}, respectively (Table 1).

As shown in Fig. 1a and Table 1, the ability of strain D6 to degrade DDTs was somewhat inhibited by DDTs at the level as high as 50 mg L^{-1}. This may be due to that DDTs at high concentration is toxic to strain D6 and inhibits the degradation. A similar phenomenon was observed by Bidlan and Manonmani [9] in which the degradation rate of DDT by *S. marcescens* DT-1P in basal mineral medium was inhibited at DDT concentration of 50 mg L^{-1}. The results obtained in this study showed that the degradation rate of DDTs decreased with increasing concentrations. In agreement with our results, Sharma et al. [18] found that the mixed bacteria (*Bacillus* sp., *Micrococcus* sp., *Pseudomonas* sp., and *Flavobacterium* sp.) from activated sludge showed a decreased degradation with increasing concentrations of DDT ranging from 50 to 500 mg L^{-1} after incubation for 1 month. Li et al. [19] also reported that 69.0% and 30.7% of DDT at concentrations of 40 and 60 mg L^{-1}, respectively, were removed by *Stenotrophomonas* sp. D-1 in liquid medium, which indicated that the degradation of DDT obviously decreased with increasing concentrations.

3.4. Effect of pH on DDTs biodegradation

The degradation of DDTs at the concentration of 1 mg L^{-1} in mineral salt media of pH 5.0, 7.0, and 9.0 is shown in Fig. 1b. Throughout the whole experiment, the hydrolysis percentages of DDTs were less than 5% in all controls without strain D6, except at pH 9.0 (12.8%). As observed in Table 1, the half-lives of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, and *p*,*p*′-DDE were measured to be 13.10, 13.83, 21.86, and 16.86 days at pH 5.0, 7.99, 8.34, 10.85, and 8.84 days at pH 7.0, and 9.48, 8.94, 17.33, and 10.39 days at pH 9.0, respectively. The ANOVA analysis showed that the half-lives for degradation of DDTs at pH 7.0 were statistically significantly shorter ($p \leq 0.05$) than those at pH 5.0 and 9.0.

The results indicated that neutral condition is favorable for the degradation of DDTs by strain D6, whereas higher or lower pH inhibits degradation. Bidlan and Manonmani [9] also reported that near-neutral pH was most favorable for degradation of DDT by *S. marcescens* DT-1P in basal mineral medium. However, a different

result was observed by Gu et al. [20], who found that the highest degradation rate of DDT by the strain *Brevunmdimonas* sp. W-1 was detected at pH 8.0.

3.5. Effect of temperature on DDTs biodegradation

The effect of temperature (20, 30, and 40 °C) on biodegradation of 1 mg L^{-1} of DDTs in mineral salt media at pH 7.0 is shown in Fig. 1c. In all controls, the hydrolysis percentages of DDTs were less than 5%. As shown in Table 1, the degradation half-lives of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, and *p*,*p*′-DDE at the level of 1 mg L^{-1} were measured to be 19.69, 17.59, 22.65, and 15.43 days at 20 °C, 13.13, 12.86, 17.91, and 8.83 days at 30 °C, and 14.50, 15.68, 20.50, and 10.37 days at 40 °C, respectively. The ANOVA analysis showed that the degradation half-lives of DDTs at 30 °C were statistically significantly shorter ($p \leq 0.05$) than those at 20 °C and 40 °C.

The results indicated that the optimum temperature for the biodegradation of DDTs by strain D6 in pure culture is 30 °C. In agreement with our results, Gu et al. [20] reported that the highest degradation rate of DDT by *Brevunmdimonas* sp. W-1 was observed at 30 °C. Bidlan and Manonmani [9] also found that maximum degradation of DDT by *S. marcescens* DT-1P in basal mineral medium occurred at 30 °C.

3.6. Effect of additional carbon sources on DDTs biodegradation

The effects of various carbon sources on 1 mg L^{-1} of DDTs biodegradation in mineral salt media at pH 7.0 are presented in Fig. 1d. In all controls without strain D6, the hydrolysis percentages of DDTs were less than 5%. As observed in Table 2, the degradation half-lives of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, and *p*,*p*′-DDE at the level of 1 mg L^{-1} were examined to be 36.09, 31.50, 39.83, and 25.96 days in the treatment without additional carbon source, 14.38, 14.78,

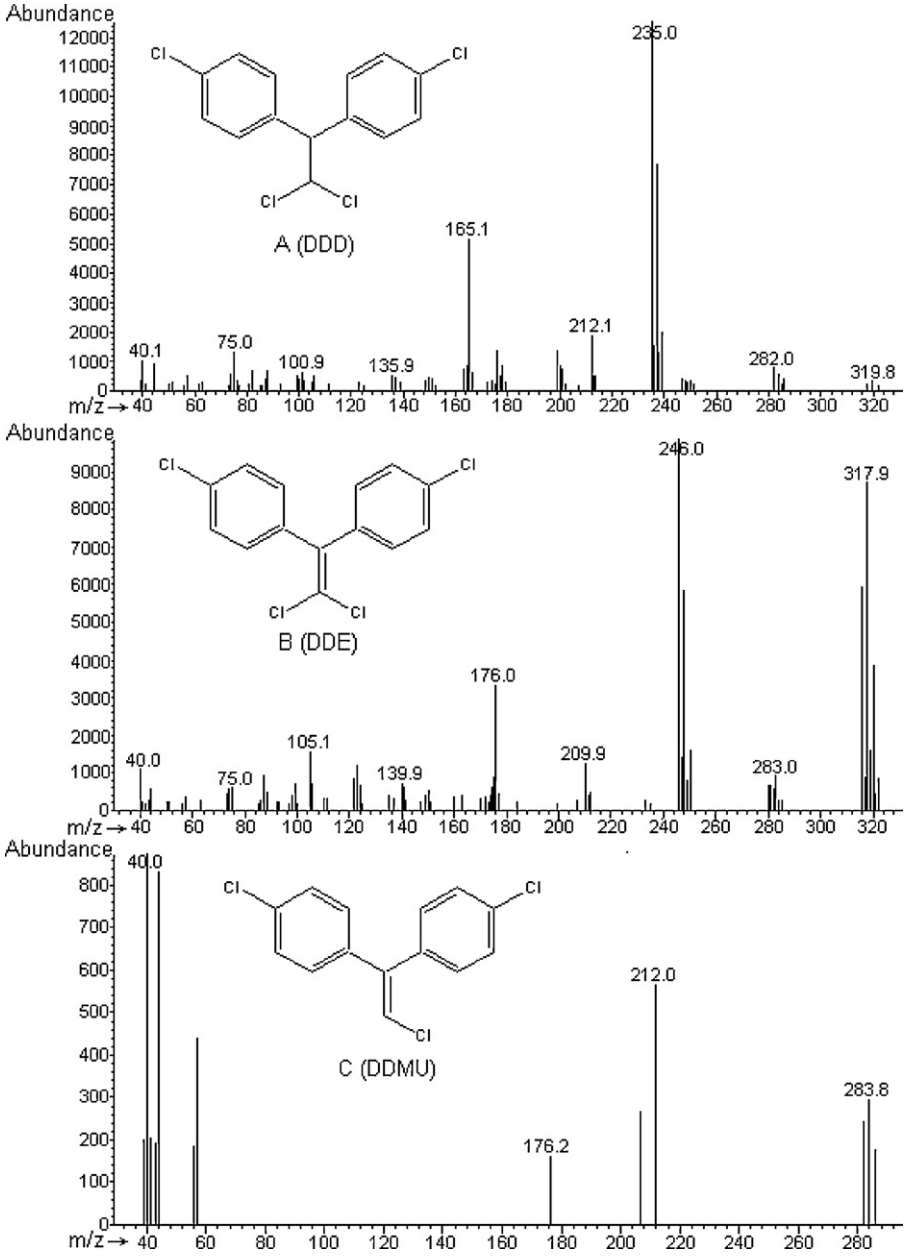

Fig. 2. Mass spectrogram and proposed structures of DDT metabolites A (DDD), B (DDE), C (DDMU), D (DDNS), E (DDA), F (DBP), and G [3-hydroxyl-2-(*p*-chlorophenyl) propionic acid].

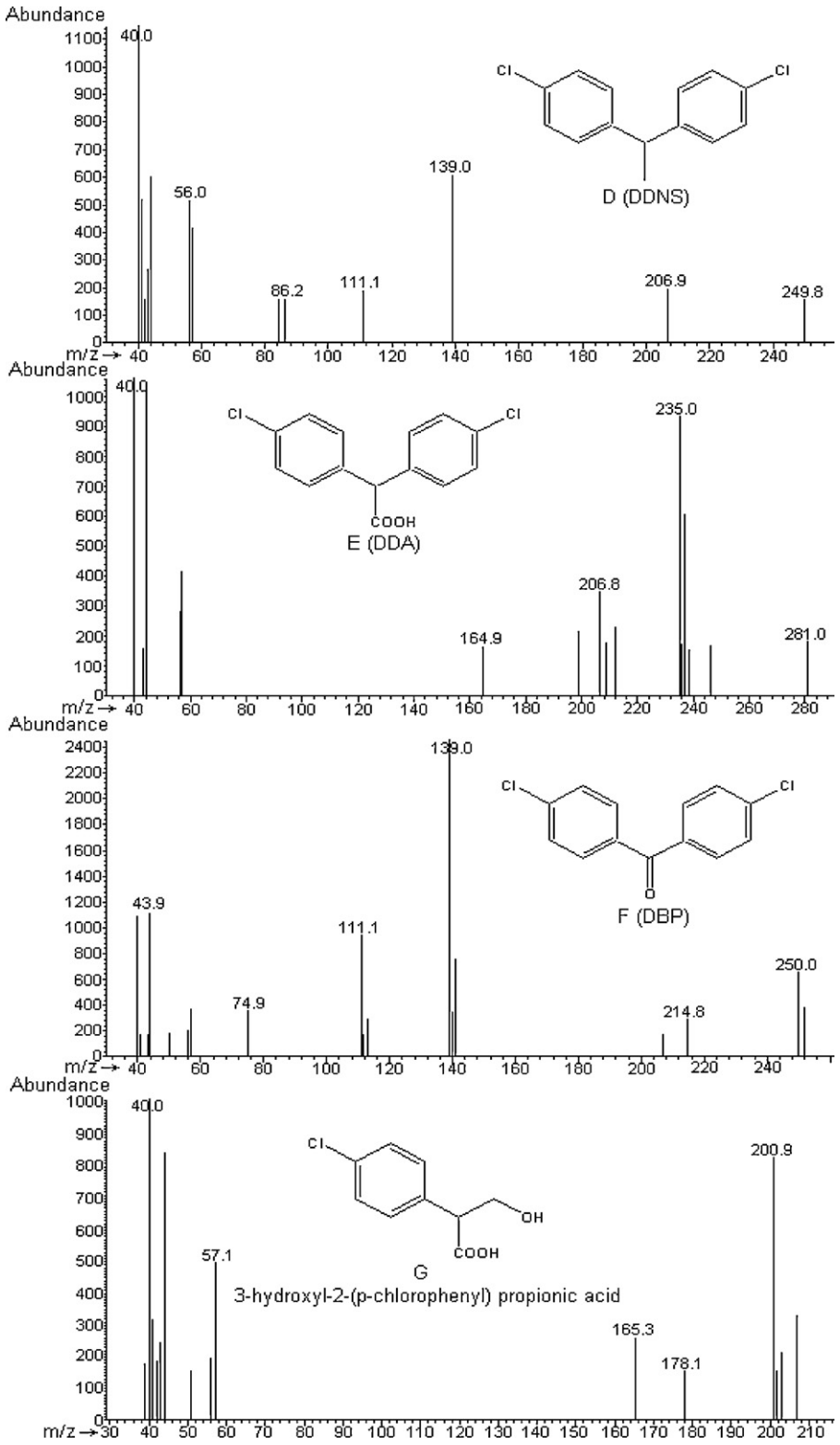

Fig. 2. (*Continued*)

20.26, and 13.78 days in the presence of glucose, 18.99, 23.02, 25.67, and 18.05 days in the presence of yeast extract, 25.86, 25.86, 26.55, and 19.97 days in the presence of sucrose, and 30.94, 28.52, 38.72, and 22.28 days in the presence of fructose, respectively. The ANOVA analysis showed that the degradation half-lives of DDTs in the presence of glucose, yeast extract, sucrose, and fructose were statistically significantly shorter ($p \leq 0.05$) than those in the treatment without an additional carbon source, and the presence of glucose showed the fastest degradation of DDTs.

The results obtained showed that the presence of any of four carbon sources could enhance biodegradation of DDTs following the order of glucose > yeast extract > sucrose > fructose. Similarly, Gu et al. [20] reported that the addition of 10 mg L^{-1} of glucose to culture medium could enhance the degradation of DDT by *Brevunmdimonas*

Table 2
Kinetic data of DDTs degradation by strain D6 in the presence of additional carbon sources.

DDTs	Type of carbon source	Total degradation rate (mg L^{-1} day^{-1})	Kinetic function	$t_{1/2}$[a] (days)	r^2
p,p′-DDT	Glucose	0.031	$C = 0.85e^{-0.0482 \times t}$	14.38a	0.9467
p,p′-DDT	Yeast extract	0.028	$C = 0.90e^{-0.0365 \times t}$	18.99b	0.9299
p,p′-DDT	Sucrose	0.020	$C = 0.91e^{-0.0268 \times t}$	25.86c	0.9037
p,p′-DDT	Fructose	0.019	$C = 0.96e^{-0.0224 \times t}$	30.94d	0.9333
p,p′-DDT	Without additional carbon source	0.018	$C = 0.99e^{-0.0192 \times t}$	36.09e	0.9577
o,p′-DDT	Glucose	0.030	$C = 0.81e^{-0.0469 \times t}$	14.78a	0.9022
o,p′-DDT	Yeast extract	0.023	$C = 0.87e^{-0.0301 \times t}$	23.02b	0.9043
o,p′-DDT	Sucrose	0.021	$C = 0.89e^{-0.0268 \times t}$	25.86b	0.9031
o,p′-DDT	Fructose	0.020	$C = 0.93e^{-0.0243 \times t}$	28.52c	0.9440
o,p′-DDT	Without additional carbon source	0.019	$C = 0.95e^{-0.0220 \times t}$	31.50d	0.9388
p,p′-DDD	Glucose	0.026	$C = 0.88e^{-0.0342 \times t}$	20.26a	0.9353
p,p′-DDD	Yeast extract	0.022	$C = 0.95e^{-0.0270 \times t}$	25.67b	0.9548
p,p′-DDD	Sucrose	0.021	$C = 1.00e^{-0.0261 \times t}$	26.55b	0.9876
p,p′-DDD	Fructose	0.016	$C = 0.99e^{-0.0179 \times t}$	38.72c	0.9608
p,p′-DDD	Without additional carbon source	0.015	$C = 1.00e^{-0.0174 \times t}$	39.83c	0.9513
p,p′-DDE	Glucose	0.032	$C = 0.83e^{-0.0503 \times t}$	13.78a	0.9023
p,p′-DDE	Yeast extract	0.028	$C = 0.88e^{-0.0384 \times t}$	18.05b	0.9014
p,p′-DDE	Sucrose	0.026	$C = 0.90e^{-0.0347 \times t}$	19.97b	0.9166
p,p′-DDE	Fructose	0.023	$C = 0.89e^{-0.0311 \times t}$	22.28c	0.9117
p,p′-DDE	Without additional carbon source	0.020	$C = 0.90e^{-0.0267 \times t}$	25.96d	0.9077

[a] Each value is a mean of three replicates. Data followed by a different letter in the same column are statistically significantly different ($p \leq 0.05$).

sp. W-1. Bidlan and Manonmani [9] found that the degradation rate of DDT (10 mg L^{-1}) by *S. marcescens* DT-1P in basal mineral medium with 0.5% yeast extract was 1.33 times higher than the control without any additional carbon source after an incubation period of 72 h. However, a contrary result was observed that the presence of 0.5% glucose and sucrose in basal mineral medium inhibited the degradation of DDT (10 mg L^{-1}) by *S. marcescens* DT-1P [9].

3.7. Degradation pathway of DDT by strain D6

As shown in Fig. 2, seven metabolites were identified as A (DDD) at *m/z* 319.8, B (DDE) at *m/z* 317.9, C [1-chloro-2,2-bis(*p*-chlorophenyl) ethylene, DDMU] at *m/z* 283.8, D [2,2-bis(*p*-chlorophenyl) ethane, DDNS] at *m/z* 250, E [2,2-bis(*p*-chlorophenyl) acetate, DDA] at *m/z* 249.8, F [4,4′-dichlorobenzophenone DBP] at *m/z* 281, and G [3-hydroxyl-2-(*p*-chlorophenyl) propionic acid] at *m/z* 200.9, respectively.

The metabolites A (DDD), B (DDE), and F (DBP) had been previously detected as metabolic products of DDT by *Thanatephorus cucumeris* [21] and *Gloeophyllum trabeum* [11]. DDD and DDE had been found to be more persistent and toxic than the parent compound DDT. In particular, DDE was previously considered a dead-end metabolite [1]. Nevertheless, a few studies have demonstrated that DDD can be transformed by *R. eutropha* A5 via a *meta* fission pathway, and DDE can be mineralized by *P. acidovorans* M3GY via dioxygenation and subsequent *meta* cleavage [14,15] and by the wood-rotting basidiomycete *Phanerochaete chrysosporium* via DBP [5]. In our study, accumulation of DDT metabolites was not observed at any time by GC determination.

Based on the identification of DDT metabolites, a proposed degradation pathway is presented in Fig. 3. DDT appears to be initially dechlorinated at the trichloromethyl group to form DDD and DDE, and then to undergo dechlorination to form DDMU, followed by dechlorination and hydrogenation to DDNS. This undergoes dioxygenation to form DDA, and then decarboxylates and hydroxylates to form DBP, and subsequently undergoes cleavage of one of the aromatic rings to form 3-hydroxyl-2-(*p*-chlorophenyl) propionic acid. It ultimately presumably oxidizes to carbon dioxide as a mineralization process. A similar pathway has been reported by Purnomo et al. [11].

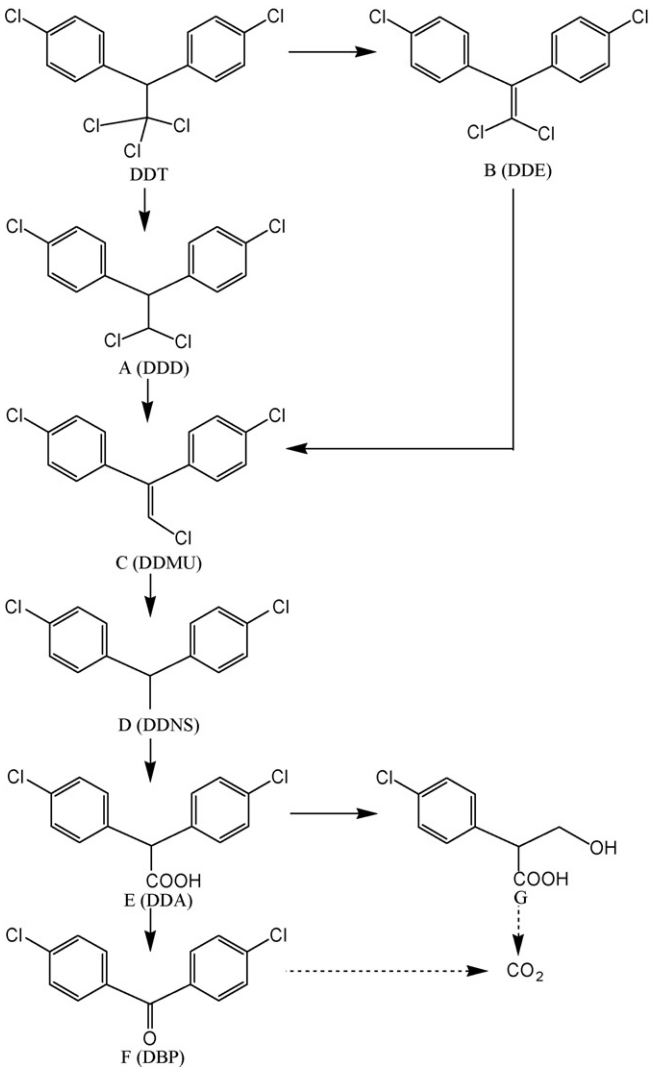

Fig. 3. Proposed pathway for the metabolism of DDT by strain D6.

Table 3
Bioremediation of DDTs contamination by strain D6 in field soils.

Treatment	Time (days)	*p*,*p*′-DDT (mg kg^{-1})	*o*,*p*′-DDT (mg kg^{-1})	*p*,*p*′-DDD (mg kg^{-1})	*p*,*p*′-DDE (mg kg^{-1})
Control	0	0.033 ± 0.013[d]a	0.029 ± 0.016a	0.010 ± 0.001a	0.058 ± 0.013a
	90	0.030 ± 0.047a	0.021 ± 0.017a	0.009 ± 0.003a	0.055 ± 0.014a
PV[a]	0	0.031 ± 0.004a	0.032 ± 0.002a	0.012 ± 0.004a	0.061 ± 0.016a
	90	0.027 ± 0.002a	0.007 ± 0.005b	0.002 ± 0.001b	0.043 ± 0.014a
DI[b]	0	0.035 ± 0.004a	0.028 ± 0.006a	0.013 ± 0.001a	0.063 ± 0.023a
	90	0.028 ± 0.010a	0.014 ± 0.003b	0.007 ± 0.002b	0.053 ± 0.022a
PVDI[c]	0	0.036 ± 0.006a	0.035 ± 0.008a	0.011 ± 0.001a	0.060 ± 0.023a
	90	0.026 ± 0.005b	0.006 ± 0.001b	0.002 ± 0.001b	0.041 ± 0.012b

[a] PV: pumpkin vegetation.
[b] DI: D6 inoculation.
[c] PVDI: pumpkin vegetation with D6 inoculation.
[d] All data are mean ± SD of four replicates.

3.8. *In situ* bioremediation of DDTs by strain D6 in field soils

Concentrations of DDTs residues in field soils after different treatments are shown in Table 3. In all controls, DDTs concentrations barely decreased throughout the experiments. After treatment for 90 days, the removal percentages of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, and *p*,*p*′-DDE were measured to be 12.9%, 78.1%, 83.3%, and 29.5% in the treatment of pumpkin vegetation, 20.0%, 50.0%, 46.2%, and 15.9% in the treatment of D6 inoculation, and 27.8%, 82.9%, 81.8%, and 31.7% in the treatment of pumpkin vegetation with D6 inoculation. The ANOVA analysis indicated that the removal percentages of *o*,*p*′-DDT and *p*,*p*′-DDE in the treatments of pumpkin vegetation and D6 inoculation were statistically significantly higher ($p \leq 0.05$), as were those of *p*,*p*′-DDT, *o*,*p*′-DDT, *p*,*p*′-DDD, and *p*,*p*′-DDE in the treatment of pumpkin vegetation with D6 inoculation, compared with those in the controls.

The results show that the incorporation of strain D6 into the field soils causes an enhancement in the degradation of DDTs, indicating that strain D6 has the potential to reduce significantly DDTs concentrations in a cost-effective manner and is thereby considered as a promising candidate for the bioremediation of sites contaminated by DDTs. A similar study by Mitra et al. [7] reported that genetically improved recombinants of the soil fungus *Fusarium solani* possessed superior degradation quality for DDT in bioremediation. Bioaugmentation may be carried out by adding either pure culture or microbial consortium. Generally, the introduction of microbial consortium has the advantage over pure culture because the consortium can resist wide variations in natural environment. Some studies also indicated that it may be useful to introduce mixed populations of anaerobic bacteria that degrade DDTs by reductive dechlorination, or ligninolytic fungi or aerobic bacteria with ring-cleavage activity [4,16,21]. However, the isolation and maintenance of microbial consortium is difficult. In our study, *Sphingobacterium* sp. D6 capable of utilizing DDTs as its sole source of carbon and energy was isolated and purified. Therefore, all experiments were conducted in pure-culture systems, which simplifying the experimental processes and facilitating to reveal the degradation mechanism.

In this study, the biodegradation efficiency of DDTs in pure culture was much higher as compared with agricultural soil. This may be attributed to be that soil is a complex environment containing mixed populations of microorganisms with synergistic and antagonistic activities, and its properties and prevailing environmental conditions will influence the behavior of both microflora and DDTs. Thus, the conclusions from pure-culture studies may not accurately reflect the degradation potential of DDTs in contaminated soil. In addition, DDD and DDE, two main products of DDT in soil under anaerobic and aerobic conditions, are also persistent and toxic pollutants with chemical properties similar to their parent compound. Thus, like as this study, it is important to address the fate and potential accumulation of these products in bioremediation processes.

Considerable studies showed that the presence of large numbers of introduced strains in contaminated site is certainly key to successful bioremediation. Therefore, inoculum density and homogeneity, survival, colonization, and physical diffusion capacity play a crucial role in bioremediation of DDT contamination [7,9,22]. Additionally, the bioremediation potential of strain D6 may be affected by many other factors, such as interspecies competition, microbial and protozoal predators, soil characterization (aeration, pH, cation exchange capacity, organic matter content, clay content, salinity etc.), prevailing environmental conditions (redox potential, temperature, humidity, dissolved oxygen, solar irradiation, rainfall etc.), nutriment levels (N, P), surfactants, electron acceptors, other toxic chemicals, and concentrations of DDTs [2,23–25]. In some cases, a successful bioremediation of contaminants may be attributed to their high bioavailability in field soil. Nevertheless, soil aging over a long period leads to a reduction in the bioavailability of DDTs due to sorption by the soil organic matter [6]. Thus, the bioavailability of DDTs in field soil containing high organic matter may become a significant limited factor affecting the efficiency of bioremediation [1,25]. In a word, considering and understanding how these factors affect DDTs biodegradation would enhance the bioremediation efficiency of strain D6 in soils.

4. Conclusions

The inoculation of strain D6 into mineral salt media and field soil could enhance the degradation of DDTs. The pathway for DDT biodegradation was proposed to be a multistep process involving dechlorination, hydrogenation, dioxygenation, decarboxylation, hydroxylation, and phenyl ring-cleavage reactions. The data from this study indicate that strain D6 might be a promising candidate in a bioremediation project of water and soils contaminated by DDTs and their analogs. However, further studies on survival, colonization, and population levels of strain D6 in soil need to be conducted carefully in order to develop more effective bioremediation.

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