

CO-METABOLISM OF DDT BY THE NEWLY ISOLATED BACTERIUM, *PSEUDOXANTHOMONAS* SP. WAX

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ABSTRACT

Microbial degradation of 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) is the most promising way to clean up DDT residues found in the environment. In this paper, a bacterium designated as wax, which was capable of co-metabolizing DDT with other carbon sources, was isolated from a long-term DDT-contaminated soil sample by an enrichment culture technique. The new isolate was identified as a member of the *Pseudoxanthomonas* sp., based on its morphological, physiological and biochemical properties, as well as by 16S rRNA gene analysis. In the presence of 100 mg l⁻¹ glucose, the wax strain could degrade over 95% of the total DDT, at a concentration of 20 mg l⁻¹, in 72 hours, and could degrade over 60% of the total DDT, at a concentration of 100 mg l⁻¹, in 144 hours. The wax strain had the highest degradation efficiency among all of the documented DDT-degrading bacteria. The wax strain could efficiently degrade DDT at temperatures ranging from 20 to 37°C, and with initial pH values ranging from 7 to 9. The bacterium could also simultaneously co-metabolize 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD), 2,2-bis(p-chlorophenyl)-1,1-dichlorethylene (DDE), and other organochlorine compounds. The wax strain could also completely remove 20 mg kg⁻¹ of DDT from both sterile and non-sterile soils in 20 days. This study demonstrates the significant potential use of *Pseudoxanthomonas* sp. wax for the bioremediation of DDT in the environment.

Key words: DDT, Co-metabolism, Bioremediation, *Pseudoxanthomonas* sp.

INTRODUCTION

DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane] has been used extensively as an organochlorine insecticide for the control of insects on agricultural crops and vector-borne diseases, such as typhus and malaria (14). DDT is highly persistent in the environment, with a reported half-life between 2-15 years, and is immobile in most soils. DDT was classified as a priority persistent organic pollutant by several different environmental regulatory agencies because of its toxicity,

hydrophobicity and bioaccumulation (7, 9), and was banned in the 1970s from agricultural use because of its negative impact on wildlife and its ill effects on human health via the food chain. However, DDT continues to be used in some developing countries (primarily tropical countries) to control insect-borne diseases. Due to its extensive use and recalcitrance, numerous sites around the world are contaminated with DDT (1, 9, 19), and many foodstuffs, including processed foods, have been shown to contain high levels of DDT residue (2, 7, 12). Moreover, high levels of DDT and its metabolites have also

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been detected in human adipose tissue, blood plasma, liver, brain, placenta, and breast milk (4, 8, 11, 21). Therefore, the clean-up of DDT pollutants from contaminated sites is of great importance.

Routes of DDT loss from contaminated sites include runoff, volatilization, photolysis and biodegradation. However, microbial degradation of DDT is a promising and cost-effective method for treating DDT contamination. It was difficult for microorganisms to degrade DDT when it was used as the sole carbon source (3), but in the presence of an additional carbon source, DDT could be co-metabolized by many microorganisms under aerobic or anaerobic conditions (3, 13, 24). Under anaerobic conditions, the metabolism of DDT was largely restricted to its alkyl chain (24), but under aerobic conditions, DDT underwent dioxygenation, followed by ring cleavage and the formation of p-chlorobenzoate (16, 17). It was reported that some metabolites, such as 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (DDD) and 2,2-bis(p-chlorophenyl)-1,1-dichlorethylene (DDE), produced during the co-metabolism of DDT, were more recalcitrant and toxic than the parent compound (9). However, few microorganisms have been reported to be capable of degrading DDT and its metabolites (DDD and DDE) simultaneously.

In this study, a bacterial strain, *Pseudoxanthomonas* sp. wax, which is capable of co-metabolizing DDT, DDD and DDE, and even some other organochlorine compounds, was isolated from a long-term DDT-polluted soil sample. The factors that influence the degradation activities of the wax strain were evaluated, and the bioremediation of DDT-contaminated soil by the wax strain was investigated.

MATERIALS AND METHODS

Chemicals and media

Analytical grade DDT (99% pure), purchased from Sigma-Aldrich Chemical, MO, USA, was used as a standard. The technical grade DDT (70% pure), pentachloronitrobenzene (PCNB) (95% pure), cypermethrin (94% pure), cyfluthrin (90% pure), and bifenthrin (95% pure) were provided by the

Yangnong Agricultural & Chemical Co., Ltd, China. Chlorothalonil (98% pure) was purchased from the Jiangyin Suli Fine Chemical Co., Ltd and γ -hexachlorocyclohexane (99% pure) was purchased from the Tianjin Dagu Chemical Factory, China. Acetonitrile, acetone and dichloromethane were purchased from the Shanghai Chemical Reagent Co., Ltd, China. All other reagents used in this study were of analytical grade.

Luria-Bertani (LB) medium and minimal salt medium (MSM) (1.0 g NH_4NO_3 , 1.5 g K_2HPO_4 , 0.5 g KH_2PO_4 , 0.2 g MgSO_4 and 1.0 g NaCl per liter, pH 7.0) were used in this study. MSM, supplemented with 20 mg l^{-1} DDT, was then called DMM, and DMM, supplemented with 100 mg l^{-1} glucose was called GMM. Solid media plates were prepared by adding 1.5% (wt/vol) agar.

Strain isolation and characterization

A soil sample (2.0 g) collected from the long-term DDT polluted soil in Yangzhou, China, was added to 100 ml of GMM, and incubated at 30°C for 7 days in a rotary shaker at 150 rpm. Five milliliters of enriched cultures, showing degradation of DDT, were transferred to 100 ml of fresh GMM. Three subcultures were performed before the final isolation of DDT-degrading strains. The final enriched culture was spread onto GMM plates. The different colonies that formed were isolated, and then tested for their DDT-degrading capabilities. One strain, designated as wax, possessed the highest degradation capacity of DDT, and was selected for further investigation.

The new isolate was identified based on its morphological, physiological and biochemical tests, with reference to *Bergey's Manual of Determinative Bacteriology*, combined with a 16S rRNA gene sequence analysis. The cell morphology was examined by light microscopy (BH-2; Olympus) and transmission electron microscopy (TEM) (H-7650, Hitachi High-Technologies Corporation), using cells from an exponentially growing culture. The total genomic DNA of wax strain was prepared by high-salt precipitation (15). The universal primers, 5'-AGAGTTTGATCCTGGCTCAG-3'

(*Escherichia coli* bases 8-27) and 5'-TACCTTGTTACGACT T-3' (*E. coli* bases 1,507-1,492), were used to amplify the 16S rRNA gene (25). The purified PCR product was sequenced from both directions, using an ABI 377 sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA). The 16S rRNA gene sequence (1,503 bp) of wax strain was deposited in the GenBank database under the accession number FJ796079. Alignment of different 16S rRNA gene sequences from GenBank was performed using Clustal X 1.8.3 (22), with the default settings. The phylogeny was analyzed with the MEGA version 3.0 software, and the distance was calculated using the Kimura 2 parameter distance model. The phylogenetic tree was built using the neighbor-joining method. Each dataset was bootstrapped 1,000 times (18).

Analytical methods

The cell density was monitored spectrophotometrically by measuring the absorbance at 600 nm with a SHIMADZU UV-Vis Recording spectrophotometer (Shimadzu UV-2410, Japan). DDT in the cultures was extracted with five times the volume of dichloromethane, and DDT in the soils was extracted with an equal volume of hexane and acetone (1:1, v/v) by the supersonic extraction method. The organic extract was dried over anhydrous sodium sulfate, and then evaporated using a vacuum rotary evaporator at room temperature. The residual extract was dissolved in 5 ml acetonitrile. Twenty microliters of the resulting solution was analyzed by reverse-phase high performance liquid chromatography (HPLC) (600 Controller, Rheodyne 7725i Manual injector, 2487 Dual I Absorbance Detector; Waters Co., Milford, MA). The separation column (4.6 mm × 250 mm × 5 μm) was filled with Kromasil 100-5C18. The mobile phase was acetonitrile/water (70:30, v/v), with a flow rate of 1.0 ml min⁻¹. The amount of chloride ion released from DDT was measured spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulfate, according to Iwasaki *et al.* (5). The presence of DDD, DDE, PCNB, cypermethrin, cyfluthrin, bifenthrin, chlorothalonil and γ-hexachlorocyclohexane was determined by HPLC, or gas chromatography (GC), as described

previously (10, 23).

Degradation of DDT by wax strain in culture

All aerobic batch cultivations were carried out in 250-ml bottles, containing 100 ml liquid culture, while being shaken at 150 rpm at 30°C. The wax strain was pre-cultured in LB medium for 18 hours, was centrifuged at 5,000 g for 10 min, and the cell pellets were washed three times with fresh MSM. After adjusting the OD_{600nm} of cell density to 1.0, an inoculum (5%, v/v) was inoculated into liquid GMM medium unless otherwise stated. The medium, inoculated with heat-killed wax cells, was maintained as the control. All treatments were performed in triplicate. At different time periods, 2 ml aliquots of the sample were removed for analysis. The degradation efficiency was determined by an estimation of the removal of DDT from the culture.

To investigate the effect of alternative carbon source on DDT biodegradation, 100 mg l⁻¹ of glucose, succinate, starch, dextrin, or maltose, was added to DMM, respectively. To investigate the effect of the initial concentration of DDT on DDT biodegradation, GMM containing 5 mg l⁻¹, 20 mg l⁻¹, 50 mg l⁻¹ and 100 mg l⁻¹ DDT, were used. To investigate the effect of the initial pH value on DDT biodegradation, the pH values of the media were adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, respectively, by changing the potassium-phosphate buffer system. To investigate the effect of temperature on DDT biodegradation, the cultures were incubated at 10°C, 20°C, 25°C, 30°C, 37°C, 40°C, and 45°C, respectively. To investigate the effect of the initial inoculum size on DDT biodegradation, 1%, 5%, and 10% (v/v) of wax cells, respectively, were inoculated into GMM. To investigate the effect of aeration on DDT biodegradation, 250-ml bottles containing 30 ml, 60 ml, 100 ml, 120 ml, 150 ml, and 180 ml of culture were used.

To study whether additional organochlorine compounds could also be degraded by wax strain, MSM media (containing 100 mg l⁻¹ glucose) supplemented with DDD, DDE, PCNB, cypermethrin, cyfluthrin, bifenthrin, chlorothalonil and γ-hexachlorocyclohexane, at final concentrations of 20 mg l⁻¹,

were used.

Degradation of DDT in soil by wax strain

Soil samples, from depths of 0 to 20 cm, were taken of a fine sandy-loam soil in the Jiangsu province, with a pH value of 7.8. The soil samples were treated as described by Jiang *et al* (6). Subsamples (500 g) of sterile and non-sterile soil were amended with 20 mg l⁻¹ DDT, and inoculated with wax cells at concentrations of 10⁸ CFU g⁻¹ soil. The control soil samples were inoculated with heat-killed wax cells that were autoclaved at 121°C for 15 min. The moisture of the soil was adjusted to 40% (wt/wt) during the experiment, and samples were incubated in the dark to preclude photolysis reactions (20). At periodic intervals, 10-g sub-samples were collected and analyzed immediately. All treatments were performed in triplicate.

RESULTS

Isolation and characterization of the DDT-degrading wax strain

A DDT-degrading strain, designated as wax, was isolated from long-term DDT polluted soil by an enrichment culture technique. The wax strain could degrade over 95% of the total DDT, at a concentration of 20 mg l⁻¹, in GMM in 72 hours. The wax strain is a Gram-negative, rod-shaped (0.3-0.5 µm × 1.2-3.0 µm), obligate aerobe that is motile via a polarly localized flagellum (Fig. 1). When growing on LB medium plates, the colonies of wax were yellow and convex with entire margins. The wax strain could grow at temperatures ranging from 10°C to 45°C, and at pH values ranging from 5.0 to 10.0. The optimal temperature and pH value for wax strain growth was 30°C and 7.0, respectively.

Biochemical tests showed that wax strain was oxidase and catalase positive, and urease, arginine dihydrolase, and indole production negative. The wax strain was unable to hydrolyze casein, cellulose, chitin, tween-40, tween-80 and starch; however, the wax strain could hydrolyze aesculin, tween-20 and tyrosine. The predominant isoprenoid quinone of wax

strain was Q-8. Nitrite could not be reduced to nitrous oxide by the wax strain; however, nitrate could be reduced to the end-product of nitrous oxide. The wax strain was resistant to streptomycin, nalidixic acid, kanamycin, ampicillin, gentamicin, fucidin and novobiocin. In addition, the wax strain could use glucose, maltose, starch and dextrin as carbon sources, and use yeast extract, peptone, urea, beef extract, NH₄NO₃, (NH₄)₂SO₄ and KNO₃ as nitrogen sources, and could grow in the presence of 3% NaCl. The phylogenetic tree of the 16S rRNA gene sequences is shown in Fig. 2. The wax strain is related to the *Pseudoxanthomonas* sp. lineage, and is closely clustered with two types of *Pseudoxanthomonas* strains, *Pseudoxanthomonas* suwonensis 4M1^T and *Pseudoxanthomonas* broegbernensis B1616^T, with sequence similarity scores of 98.2% and 98.4%, respectively. The result of the phylogenetic analysis was consistent with the morphological characteristics and the biochemical tests. Therefore, wax strain was determined to be a *Pseudoxanthomonas* sp.

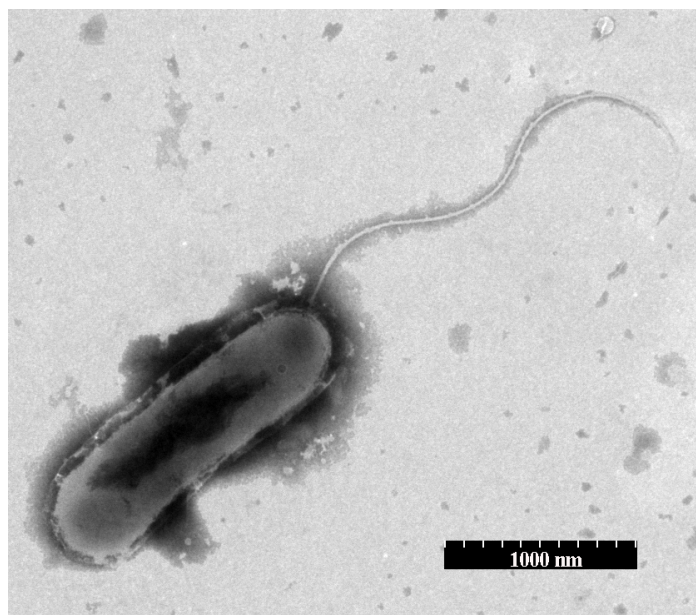


Figure 1. Micrograph of the strain wax under transmission electronic microscope showing its shape, size, and position of flagellum

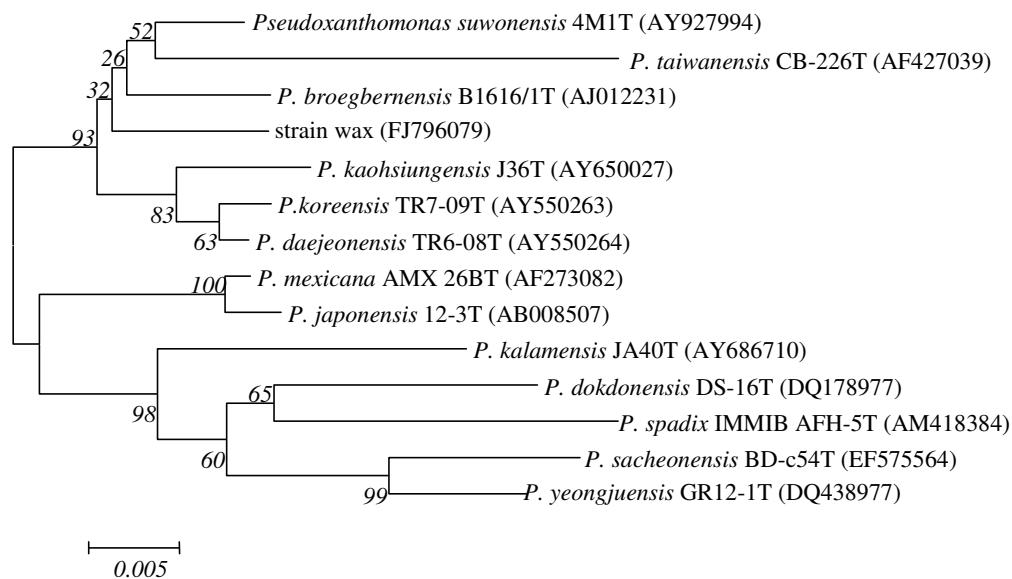


Figure 2. Phylogenetic tree based on the 16S rRNA gene sequences of the wax strain and related species. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after the species name. The scale bar indicates 0.005 substitutions per nucleotide position. Bootstrap values, obtained with 1,000 resamplings, were indicated as percentages at all branches.

Degradation of DDT by the wax strain in culture

In GMM, the wax strain degraded 30.6% of the total DDT, at a concentration of 20 mg l⁻¹ in 24 hours, while over 95% was degraded in 72 hours. However, in MSM, only 10.2% of the total DDT was degraded in 72 hours (Fig. 3). These results demonstrate that the biodegradation of DDT by the wax strain was greatly affected by the alternative carbon source. When the wax strain grew in the presence of glucose as the carbon source, DDT was simultaneously co-metabolized with chloride ion released from DDT. It was found that other carbon sources, such as succinate, starch, dextrin and maltose, could also promote the biodegradation of DDT. Therefore, it is suitable for the wax strain to degrade DDT in naturally contaminated sites, where other carbon sources exist.

The wax strain could degrade DDT at a wide range of concentrations, even at very high concentrations. For instance, over 60% of the total DDT, at a concentration of 100 mg l⁻¹, was degraded in 144 hours, and specifically 50 mg l⁻¹ of DDT was nearly completely degraded in this time (Fig. 4). Even though a high concentration of DDT was toxic to the wax cells, and there was a delay in biodegradation when the initial

concentration of DDT was over 50 mg l⁻¹, the wax strain showed an uncommon tolerance of DDT at high concentrations.

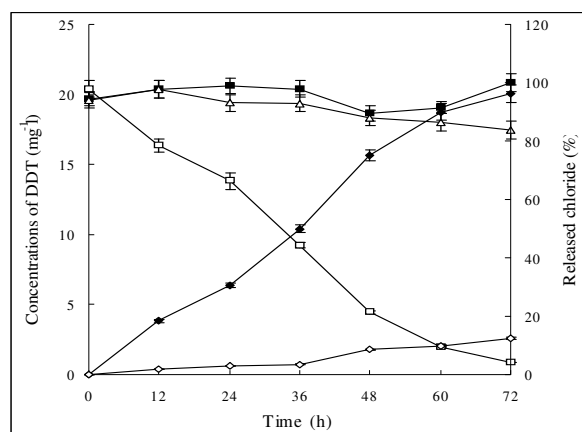


Figure 3. Degradation of DDT by *Pseudoxanthomonas* sp. wax in culture. ■, DDT control; □, DDT biodegradation in DMM medium; □, DDT biodegradation in GMM medium; □, chloride released in DMM medium; □, chloride released in GMM medium. Data are the means of results from triplicate experiments, and error bars indicate standard errors. Error bars that are not visible fall within the data point.

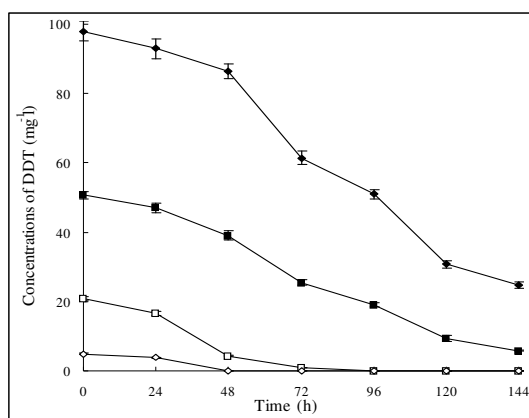


Figure 4. Effect of the initial DDT concentration on biodegradation of DDT by the wax strain in GMM. □, 100 mg l⁻¹; ■, 50 mg l⁻¹; ○, 20 mg l⁻¹; ◇, 5 mg l⁻¹. Data are the means of results from triplicate experiments, and error bars indicate standard errors. Error bars that are not visible fall within the data point.

The wax strain could efficiently degrade DDT at temperatures ranging from 20 to 37°C, with an optimal degradation temperature of 30°C. The biodegradation of DDT was completely inhibited when the temperature was above 45°C or below 10°C. The wax strain could rapidly degrade DDT, at initial pH values ranging from 7 to 9, with an optimal pH value of 7.5; biodegradation of DDT was inhibited when the initial pH value was below 4.0. The degradation of DDT increased with an increase in inoculum size, for instance, when the inoculum size was 10%, 100 mg l⁻¹ of DDT was degraded to a non-detectable level in 144 hours. The effect of aeration on DDT biodegradation was complex. Under significant aerobic conditions (30 ml culture in the 250-ml bottle), growth of the wax strain was optimal, however, the rate of DDT degradation was reduced. On the other hand, the highest rate of DDT degradation was found when the 250-ml bottle contained 100 ml culture, albeit the growth of the wax strain was not the most optimal.

Interestingly, the wax strain had a broad degrading-substrate range of organochlorine compounds. The degradation time course of some organochlorine compounds by the wax strain is presented in Fig. 5. It was found that 20 mg l⁻¹ DDD

and DDE were nearly completely degraded in 144 hours, and that PCNB, cypermethrin, cyfluthrin and bifenthrin were degraded to various extents. However, chlorothalonil and γ -hexachlorocyclohexane could not be degraded, and thus it was deduced that the side chains could also affect the biodegradation of organochlorine compounds.

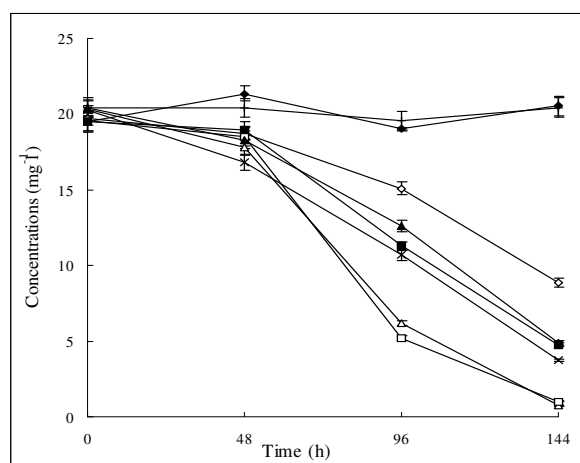


Figure 5. Degradation of different organochlorine compounds by the wax strain in liquid MSM supplemented with 100 mg l⁻¹ glucose. □, cyfluthrin; ▲, bifenthrin; ■, cypermethrin; □, DDD; ○, DDE; ×, PCNB; □, chlorothalonil; □, hexachlorocyclohexane. Data are the means of results from triplicate experiments, and error bars indicate standard errors. Error bars that are not visible fall within the data point.

Degradation of DDT in soil by the wax strain

It was found that 20 mg kg⁻¹ of DDT was completely removed from both sterile and non-sterile soil samples by wax cells, at a concentration of 10⁸ CFU g⁻¹, in 20 days (Fig. 6), while there was nearly no decline in the DDT concentration in the control soil samples, indicating that the wax cells were solely responsible for DDT removal. The high removal efficiency of DDT in non-sterile soil showed that the wax strain is potentially useful for the bioremediation of DDT-contaminated soil, even though there was competition between the indigenous populations and the inoculated strain.

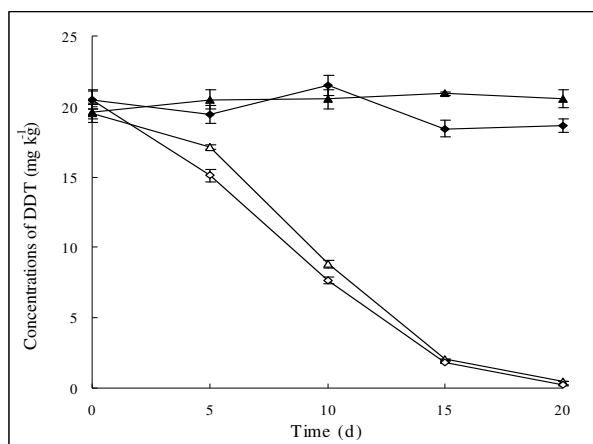


Figure 6. Degradation of DDT in sterile and non-sterile soils by the wax strain at a concentration of 10^8 CFU g^{-1} . ▲, DDT control in sterile soil; □, DDT control in non-sterile soil; ○, DDT degradation in sterile soil; ◇, DDT degradation in non-sterile soil. Data are the means of results from triplicate experiments, and error bars indicate standard errors. Error bars that are not visible fall within the data point.

DISCUSSION

DDT is highly persistent in the environment, and was banned in most of the advanced countries in the 1970s. Numerous sites around the world are contaminated with DDT. The microbial degradation of DDT shows a promising way to clean up the DDT-contaminated sites. Even though isolation of a microorganism capable of utilizing DDT as a sole carbon source is difficult, the co-metabolism of DDT by microorganisms with other carbon sources has been well documented, and the major metabolism products have been identified as DDD and DDE (1). To eliminate these contaminants from the environment, attempts have been made to isolate microorganisms that are capable of simultaneous degradation of DDT, DDD and DDE; however, few microorganisms are reported to have this capability.

In the present study, a DDT degrading strain, designated as wax, was isolated from long-term DDT-contaminated soils. The wax strain could degrade over 95% of total DDT at 20 mg l^{-1} in 72 hours in GMM. Based on the morphological, physiological and biochemical tests, combined with 16S rRNA

gene sequence analysis, the wax strain was identified to be a *Pseudoxanthomonas* sp. of bacteria. Bacteria from the genus *Pseudoxanthomonas* are metabolically versatile, and this plays an important role in the biodegradation of organic xenobiotics. However, to our knowledge, this has been the first isolation of a strain of *Pseudoxanthomonas* species that could degrade DDT efficiently. Moreover, wax strain could not only degrade DDT, but could also degrade DDE, DDD, and other organochlorine compounds, which emphasizes the importance of the wax strain for the bioremediation of sites that are contaminated with multiple organochlorine pesticides. The wax strain could efficiently degrade DDT at temperatures ranging from 20 to 35°C, and at initial pH values ranging from 7 to 9, while the wax strain could even tolerate and degrade high concentrations of DDT (100 mg l^{-1}). To our knowledge, the wax strain had the highest degradation efficiency among all of the documented DDT-degrading bacteria. The inoculation of wax cells at 10^8 CFU g^{-1} into both sterile and non-sterile soils, resulted in the complete removal of 20 mg kg^{-1} of DDT in 20 days. Due to broad substrate specificity, a strong degradation ability and adaptability to temperature variation, the wax strain is a promising candidate for the bioremediation of DDT-contaminated sites; although, the biochemical degradation pathway of DDT by wax strain has not yet been elucidated. Therefore, further research will be needed to clarify the degradation pathway and the properties of the key enzymes involved in the biodegradation of DDT.

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