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Isolation and characterization of a chlorpyrifos and 3,5,6-trichloro-2-pyridinol degrading bacterium

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Abstract

A bacterium, isolated from contaminated soils around a chemical factory and named strain DSP3 was capable of biodegrading both chlorpyrifos and 3,5,6-trichloro-2-pyridinol. Based on the results of phenotypic features, phylogenetic similarity of 16S rRNA gene sequences, DNA G + C content, and DNA homology between strain DSP3 and reference strains, strain DSP3 was identified as *Alcaligenes faecalis*. Chlorpyrifos was utilized as the sole source of carbon and phosphorus by strain DSP3. We examined the role of strain DSP3 in the degradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol under different culture conditions. Parathion and diazinon could also be degraded by strain DSP3 when provided as the sole sources of carbon and phosphorus. An addition of strain DSP3 (10^8 cells g^{-1}) to soil with chlorpyrifos (100 mg kg^{-1}) resulted in a higher degradation rate than the one obtained from non-inoculated soils. Different degradation rates of chlorpyrifos in six types of treated soils suggested that soils used for cabbage growing in combination with inoculation of strain DSP3 showed enhanced microbial degradation of chlorpyrifos.
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1. Introduction

Organophosphorus insecticides are increasingly used in agriculture as a substitute for organochlorine and carbamate insecticides because of their high efficiency and lower persistence in the environment. Chlorpyrifos (CP), *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate, a phosphorothioate insecticide, has been commercially used since the 1960s, particularly for the control of foliar insects on cotton, paddy fields, pasture and vegetable crops. When it is applied to plants or mixed with soil, it produces hazardous effects to the environment [1]. Leaching of the applied pesticide may pollute the surface/ground water, ultimately resulting in adverse effects on the biological systems [2].

In general, pesticide degradation in soil can be influenced by both biotic and abiotic factors, which act in tandem and complement one another in the microenvironment [3]. Microbial activity has been deemed the most influential and significant cause of organophosphorus pesticide removal. Therefore, biodegradation is considered to be a reliable and cost-effective technique for pesticide abatement and a major factor determining the fate of organophosphorus pesticides in the environment [4]. There is an increasing need to develop new methods to detect, isolate and characterize the strains

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playing a part in these degradation processes [5]. Larger microbial populations can exist in rhizosphere soil than in bulk soil; these larger populations have been shown to increase the degradation of organic chemicals including pesticides [6–9].

CP is characterized by a P–O–C linkage as are other organophosphate pesticides, such as diazinon, parathion, methyl parathion and fenitrothion. Most reports on the fate of CP in soil and compost fail to examine the fate of its primary degradation: 3,5,6-trichloro-2-pyridinol (TCP) [10]. TCP, the main transformation product of CP, is a charged molecule at neutral pH and more leachable into groundwater and surface water than the parent molecule [11]. It is moderately mobile due to its greater water solubility, which causes the widespread contamination in soils and in the aquatic environment. Attempts to isolate CP-degrading bacteria from CP-treated soil have not been successful until recently when Singh et al. isolated six CP-degrading bacteria from an Australian soil [12]. CP has been shown to be degraded co-metabolically in liquid media by bacteria [13–16]. Although degradation of TCP by a *Pseudomonas* sp. has been reported [17], no isolates capable of degrading both CP and TCP were obtained.

Hence, a study was undertaken to look for strains that were able to degrade CP and TCP. For the present work, the authors studied the effect of strain DSP3 on CP and TCP in liquid culture and in soils, characterized strain DSP3, and studied ecological factors that govern chlorpyrifos degradation in soil.

2. Materials and methods

2.1. Chemical

All reagents used in the present study were of analytical grade and were used without further purification. Samples of CP, TCP, parathion, diazinon and coumaphos (97.5% pure analytical grade) were obtained from the Pesticide and Environmental Toxicology Research Institute, Zhejiang University, China. All glassware used in the experiment was washed three times, rinsed three times with distilled de-ionized water, rinsed three times with reagent grade acetone and heated to remove acetone, and rinsed with distilled de-ionized water just prior to use.

2.2. Enrichment of CP and TCP degrading bacteria

A mineral salt medium (MSM) [18] was used in both enrichment culture of pretreated soils and liquid culture of isolated bacteria. CP was dissolved in chloroform as a stock solution and was rationed into MSM. When the chloroform had evaporated completely, it provided a wanted pesticide concentration in MSM. Soil samples (5 g) from the contaminated soils around a chemical factory were used to inoculate baffled Erlenmeyer flasks containing 50 ml MSM with CP (50 mg l^{-1}) as the sole carbon sources. The properties of the soil were pH 7.2 and composed of 15.8% sand, 39.3% silt, 35.0% clay and 9.9% organic matter. It has been three years since the factory started to produce the CP; therefore, the soil had been contaminated with CP for three years. Flasks were incubated at 28 °C on a shaker at 280 rpm in the dark for 72 h. The bacteria then were washed and inoculated in MSM with CP (100 mg l^{-1}) as the sole carbon-source for another 72 h. Samples from these cultures were spread-plated on MSM agar containing 100 mg CP. 38 bacteria isolates and no fungi were obtained. The isolates were tested for the ability to degrade TCP and five positive isolates were obtained. Strain DSP3, which degraded CP and TCP rapidly, was selected for further analysis.

2.3. Inoculum preparation for degradation studies

Strain DSP3 was pre-cultured in baffled Erlenmeyer flasks containing MSM with 100 mg l^{-1} chlopyrifos and TCP, respectively. Flasks were incubated overnight at 28 °C on a shaker at 280 rpm in the dark. The contents of the inoculated flasks containing medium and CP/TCP were centrifuged and the cell pellet was washed four times with fresh medium and quantified by the dilution plate count technique. For all experiments, 10^8 cells ml^{-1} were used and samples were incubated at 28 °C on a shaker at 280 rpm unless otherwise stated.

2.4. Growth and degradation of CP and TCP

A total of 100 μl of the bacterial culture suspension (10^8 cells ml^{-1}) was inoculated into MSM with CP/TCP (100 mg l^{-1}). At periodic intervals, an individual flask was sacrificed and its contents were used to determine growth and degradation of CP and TCP. Cultures were run in triplicate to ensure accuracy. Growth was monitored by spectrophotometer at 600 nm (Shimadzu, Japan). Uninoculated medium with CP/TCP (100 mg l^{-1}) was used as a control. The degradation ability of the isolate was assayed with GC-FID [10]. In order to investigate if the CP-degradation isolates could use CP as the sole source of carbon and phosphorus, the medium was modified as described by Karpouzas and Walter [19].

2.5. Identification of isolates

The bacterial strain obtained above was further classified by using standard methods [20] combined with 16SrDNA sequence analysis. Chromosomal DNA was isolated from bacterial cells grown overnight using the method of Marmur [21]. The G + C content of DNA and DNA–DNA hybridization was measured with a

SHIMADZU UV-2550 spectrophotometer (Japan) by the thermal denaturation method [22] and by the initial renaturation rate method [23], respectively. The reference strains: *Alcaligenes faecalis* ATCC 8750T and *A. faecalis* CICC AS1.767 came from the American Type Culture Collection and from the Institute of Industrial Microbiology, Shanghai, China, respectively.

Bacterial DNA for PCR was extracted using the Promega Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified by PCR using the universal primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (16S rRNA gene position 8–27 of *E. coli*) and 5′-AAGGAGGTGATCCAGCCGCA-3′ (16S rRNA gene position 1522–1541 of *E. coli*) [24]. The purified PCR product was sequenced, on both strands, using an ABI 377 sequencer and a Taq FS Dye Terminator Sequencing Kit (ABI, USA). The determined sequence was compared with those available in the GenBank/EMBL database using the BLAST program [25]. Multiple alignments of sequences, construction of a neighbor-joining phylogenetic tree [26] with the Kimura 2-parameter model, and a bootstrap analysis for evaluation of the phylogenetic topology [27] were operated by the CLUSTAL X program [28]. The nucleotide sequence coding for 16S rRNA of *A. faecalis* strain DSP3 (404 bp) was deposited in the GenBank database with accession number AY748468.

2.6. The effect on the protein profiles of the isolate caused by CP and TCP

The isolate was grown in Luria broth at 30 °C overnight, and the controls were grown in MSM with CP and MSM with TCP, respectively. The cells were pelleted by centrifugation at 5000 *g* for 5 min. Pellets were washed and re-suspended in 100 μl of phosphate buffer (0.02 M, pH 7.4). Two samples from each treatment were stored at −70 °C. One sample from each treatment was mixed with 50 μl of Nu–PAGE sodium dodecyl sulfate (SDS) loading buffer. Samples were boiled for 5 min and then centrifuged at 13,000 *g* for 2 min. SDS–PAGE was performed at 100 mV for the first 30 min and then at 150 mV for about 4 h until the tracer dye had reached the bottom on the gel. Gels were stained with 0.25% (w/v) Comassie brilliant blue in 50% methanol plus 10% acetic acid for approximately 1 h and destained with methanol–water–acetic acid [29].

2.7. Substrate range

Crossed-feeding studies with other organophosphorus insecticides were also performed. The liquid medium was supplemented with diazinon or parathion at 100 mg l^{-1}. The pesticide residues were measured by GC-FID.

2.8. Degradation of CP in soil

Soil from a vegetable plot on the Huajiachi campus of Zhejiang University, Hangzhou, China, was used for this study. The soil had been used to grow cabbage and CP had been used for half-a-year.

The soil (pH 6.89) was composed of 25.8% sand, 34.3% silt, 37.2% clay and 2.7% organic matter. Soil samples (5 kg) were sterilized as described by Singh et al. [12]. Subsamples (350 g) of the fumigated and nonfumigated soil were treated under aseptic condition with CP (100 mg kg^{-1}), respectively. Three sets of fumigated soil, nonfumigated soil, fumigated soil planted with cabbage, and nonfumigated soil planted with cabbage were inoculated with CP-degrading bacterium (10^8 cells g^{-1}). Another set without inoculation was kept as a control. The inoculum was thoroughly mixed into the soils under sterile condition. The soil moisture was adjusted by the addition of distilled water to 50% of its water-holding capacity [30]. The soils were incubated at 25 °C in the lab. CP and TCP was extracted and determined by GC-FID [10].

3. Results and discussion

3.1. Isolation, selection and characterization of strains

From the soil samples, five bacterial strains were isolated using CP and TCP as the sole carbon and energy source. One of them was named strain DSP3; it degraded both CP and TCP rapidly and was selected for further study.

Strain DSP3 formed the same colonies as *A. faecalis* on LB agar plates; the strain was gram negative, rod shaped, and did not have a flagellum. It was catalase and oxidase positive. Further study revealed additional similarities between strain DSP3 and *A. faecalis*. Utilization of mannitol, glucose, xylose, lactose, sucrose maltose, and nitrate reduction were negative, and positive results were observed in the utilization of nitrite reduction and simmon's citrate. The DNA G + C content of strain DSP3 was 56.8 mol%, which corresponded to the ones of *A. faecalis* ATCC 8750^T (54.8–59.2%) and *A. faecalis* CICC AS1.767 (55.3%). The DNA homology among these three strains is shown in Table 1, which indicates that strain DSP3 belonged to the *A. faecalis* group.

Table 1
DNA homology between strain DSP3 and reference strains and DNA G + C content of strain DSP3 and the reference strains

Strain	DNA (G + C) content (mol%)	DNA homology with strain DSP3 (%)
DSP3	56.8	100
A. faecalis ATCC 8750^T	54.8–59.2	85
A. faecalis CICC AS1.767	55.3	93

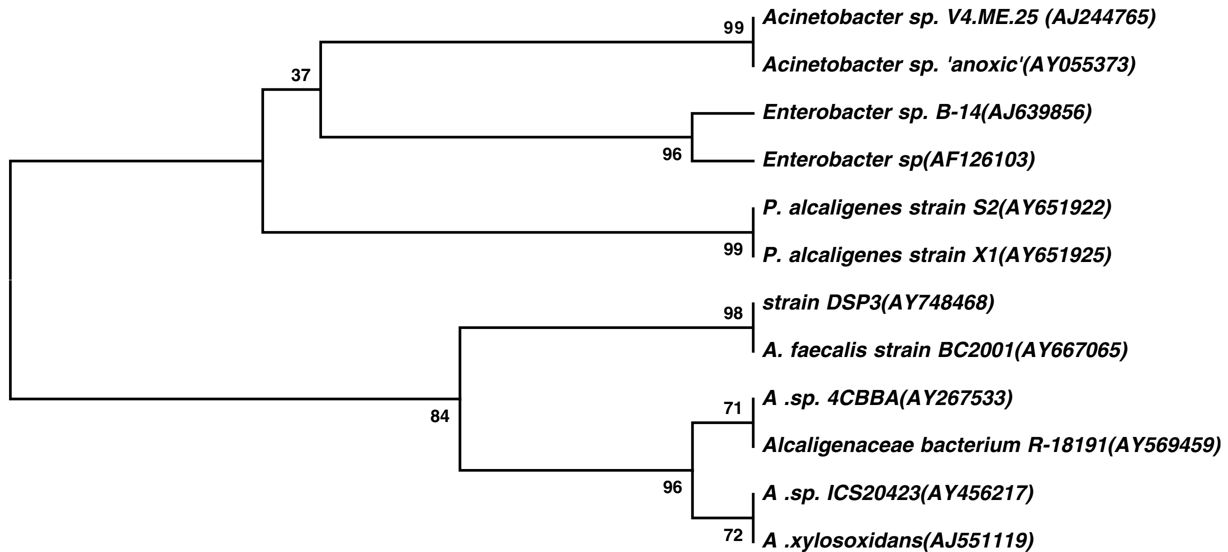

Fig. 1. UPGAM phylogenetic tree based on the 16S rRNA gene sequences of strain DSP3 Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches. GeneBank accession numbers are given in brackets.

The phylogenetic tree (Fig. 1) depicts the position of strain DSP3 within the genus *Alcaligenes*. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after the species name. The bar represents 0.02 substitutions per base position. Bootstrap values expressed as percentages of 1000 replications are shown at the branch points.

Strain DSP3 was shown to be an *A. faecalis* strain as indicated by its morphological, physiological, and chemo-taxonomical properties as well as by comparison of the 16S rRNA gene sequence. A few strains (sequences of the 16S rRNA gene were not available) of *A. faecalis* with potential values for bioremediation have been reported in the literature. Bastos et al. [31] isolated *A. faecalis* CCT 7145 with the capability to degrade phenol and Iwata et al. [32] studied the enzymatic degradation and adsorption on poly [(*R*)-3-hydroxybutyrate] single crystals with two types of extracellular PHB depolymerases from *Comamonas acidovorans* YM 1609 and *A. faecalis* T1. However, this is the first report of a strain of *Alcaligenes* species degrading both CP and TCP. These articles suggest that further study of this genus may help to understand the ecological diversity of these organisms and their usefulness as a biodegradation bacterium.

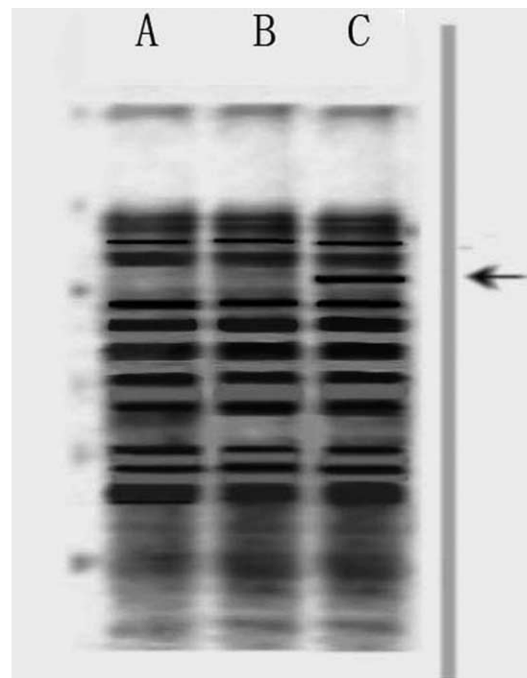

Fig. 2. Whole cell protein SDS–PAGE profiles of strain DSP3: (A) strain DSP3 grown in MSM without CP and TCP; (B) strain DSP3 grown in MSM with TCP and (C) strain DSP3 grown in MSM with CP.

3.2. Whole cell protein profiles of strain DSP3

When protein profiles of strain DSP3 grown under different conditions were compared, all of them were nearly identical (Fig. 2). However, close inspection of Fig. 2 indicates that when strain DSP3 was grown in MSM with CP, a new band appeared pointed out by the arrow that was not present in the other two profiles. We conclude that the new band may correspond to an enzyme involved in degrading CP. That is to say, some of the enzymes degrading CP were induced by CP, while the enzymes degrading TCP were constitutive.

3.3. Cell growth and degradation in liquid culture

Strain DSP3 was able to degrade CP and TCP, and to use them as the sole source of carbon. CP and TCP were

almost completely degraded within 10 days (Fig. 3). During the first two days, strain DSP3 exhibited an initial rapid degradation of TCP and CP, of approximately 37 mg TCP and 20 mg CP l^{-1} d^{-1}. The rate then slowed to 8 mg TCP and 7 mg CP l^{-1} d^{-1}, eventually attaining 100% and 93.5% degradation of TCP and CP, respectively. A higher bacterial population of DSP3 was observed in MSM with TCP than with CP and TCP was degraded faster. This suggested that CP and TCP degradation was accompanied by microbial growth.

The most rapid degradation was observed at 30 °C, and the slowest one was observed at the lower temperature limit (5 °C). The concentration of both TCP and CP had an effect on the degradation rate. The most rapid degradation was obtained at 500 mg l^{-1} of CP, and 800 mg l^{-1} of TCP. Degradation of CP and TCP increased with an increase in the concentration of TCP (from 10 to 800 mg l^{-1}), and CP (from 10 to 500 mg l^{-1}). The optimal pH was 8. The degradation rate was similar at pH 7 and 9, and the slowest was observed at the two pH limits (6 and 11).

All organophosphorus insecticides tested in the cross-feeding experiment were degraded by strain DSP3 (Fig. 4). All tested organophosphorus insecticides have diethyl phosphorothionate side chains, which may explain the reason for their degradation. There was a phosphotriester bond in all compounds except TCP, thus suggesting that the compounds were hydrolyzed at a phosphotriester bond. Due to its broad specificity against a range of organophosphorus compounds, the isolated *A. faecalis* strain possesses a great potential to provide a versatile gene or enzyme system that may be used for the remediation of highly toxic organophosphate nerve agents.

3.4. Degradation of CP in soils

The addition of DSP3 in cabbage field soils resulted in a more rapid degradation of CP than that by indigenous microflora. A significant difference in the degradation of CP was observed in soil with different treatments ($p < 0.01$, $n = 18$). The efficiency of degradation in the nonfumigated soil was generally better than in the fumigated soil, suggesting that microorganisms in the soils may play a role in the degradation of CP (Fig. 5). The best degradation of CP resulted from the non-fumigated soil planted with cabbage with the addition of the strain DSP3. Generally, microorganisms in the rhizosphere grew more rapidly than those in the non-rhizosphere [33]. The efficiency of degradation of CP in the non-fumigated soil was generally better than in the fumigated soil, and the differences were also significant between the cabbage soil and in no-cabbage soil

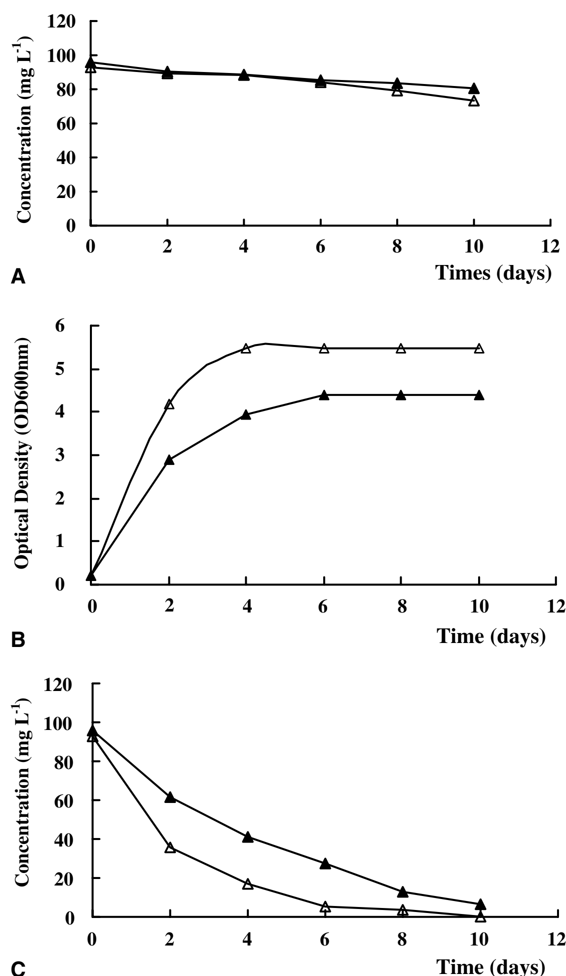

Fig. 3. Degradation of CP and TCP by strain DSP3: (A) non-inoculated medium; (B) optical density of the growth of strain DSP3 on CP (▲) and TCP (△) and (C) degradation of TCP (△) and CP (▲) by strain DSP3 . The standard errors were within 5% of the mean.

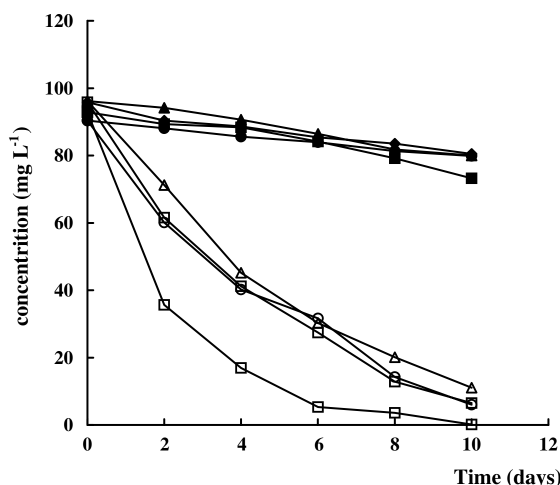

Fig. 4. Degradation of different organophosphate insecticides by strain DSP3. (■) TCP control; (□) TCP inoculated; (◆) CP control; (◇) CP inoculated; (▲) diazinon control; (△) diazinon inoculated; (●) parathion control; (○) parathion inoculated. The standard errors were within 5% of the mean.

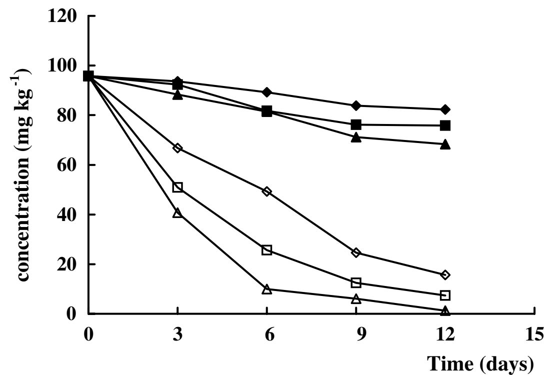

Fig. 5. Degradation of CP in soils inoculated with strain DSP3 at (10^8 cells g^{-1}): (◆) fumigated soil, uninoculated; (■) nonfumigated soil, uninoculated; (▲) nonfumigated soil with growing cabbage plant, uninoculated; (◇) fumigated soil inoculated; (□) nofumigated soil inoculated; (△) nonfumigated soil with growing cabbage plant inoculated. The standard errors were within 5% of the mean.

($p < 0.01$, $n = 12$). The best degradation occurred in the native cabbage planted soil with strain DSP3 added. These results indicate that the degradation of CP was related to the strain employed and to the quality and amount of competitive species inhabiting the soil.

Plants and actively growing roots released organic compounds into the rhizosphere, which supported growth of the microbial community in the rhizosphere. This resulted not only in an increased population density, but also created a community structure distinct from that in the bulk soil [33–37]. A higher degradation of CP in cabbage-planted soils might be due to a higher population of CP degradation bacteria in the rhizosphere. However, the possibility of the uptake of CP in soils by cabbage through its root system cannot be ruled out. TCP was not observed to accumulate in soil samples.

Degradation of different pesticides at high concentrations by isolated microorganisms has been reported previously [19,38]. However, the inhibitory effects of chlorpyrifos on indigenous non-adapted soil microflora at a concentration of less than 30 mg kg^{-1} as reported by Racke and Coats [39] are in contrast to the tolerance shown by the chlorpyrifos-degrading strain DSP3. One of the important reasons cited for the lack of isolation of chlorpyrifos-utilizing microorganisms is antimicrobial activity of TCP at high concentrations [40]. Growth of the isolated *Alcaligenes* species and degradation of TCP was not affected by TCP, even at concentrations of more than 800 mg l^{-1}.

In this study, one CP- and TCP-degrading isolate was obtained from soil taken from outside of a chemical factory. We studied the role of microorganisms, especially *A. faecalis*, in the degradation of CP and TCP in liquid culture and in the soil. Microorganisms are a major component of the ecosystem and play a considerable role in the degradation of insecticides. Significantly more work is needed to better understand the interactions among the plant varieties, the rhizosphere bacterial communities and the population dynamics of the CP and TCP degrading bacterium. Further investigations are needed to improve our understanding of the fate of organophosphorus insecticides in aquatic environments and in soils.

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