ORIGINAL PAPER

Characterization of a strain of *Pseudomonas putida* isolated from agricultural soil that degrades cadusafos (an organophosphorus pesticide)

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Abstract Bacteria capable of degrading the pesticide, cadusafos, were isolated from agricultural soil using an enrichment method. In this way, five distinct cadusafos-degrading strains of *Pseudomonas putidia* were isolated, and were characterized using morphological and biochemical analysis, as well as 16S rRNA sequencing. Strain PC1 exhibited the greatest cadusafos degradation rate and was consequently selected for further investigation. Degradation of cadusafos by strain PC1 was rapid at 20 and 37°C, but was greatly reduced ($\sim$1.5-fold) by the presence of carbon sources. Strain PC1 was able to effectively degrade cadusafos in sterilized soil using low inoculum levels. The maximum degradation rate of cadusafos (V_{max}) was calculated as 1.1 mg l^{-1} day^{-1}, and its saturation constant (K_s) was determined as 2.5 mg l^{-1}. Bacteria such as strain PC1, that use cadusafos as a carbon source, could be employed for the bioremediation of sites contaminated with pesticides.

Keywords Biodegradation · Cadusafos · 16S rRNA · *Pseudomonas putida* · Bioremediation

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Introduction

Pesticides, considered as some of the most serious environmental pollutants, are frequently used in the control of agricultural and domestic pests. Although they are economically important, pesticides can be harmful to the health of humans and animals, and they can have a detrimental environmental impact (e.g. the extermination of beneficial insects, particularly bees). In addition, resistant strains of some pest species are emerging and this reduces the usefulness of certain pesticides.

Organophosphorus pesticides are widely used as a mean of controlling nematodes and insects in agriculture (Tomlin 2003). Cadusafos, *O*-ethyl *S, S*-bis (1-methylpropyl) phosphorodithioate, is an organophosphorus pesticide with demonstrated success against a broad spectrum of both nematode and insect pests of several important crops. However, use of such pesticides presents environmental problems associated with surface and ground water contamination. Some pesticides are transformed by biological and physicochemical processes within soils, generating products which have toxicity that can be greater or less than that of the original compound (Bavcon et al. 2003; Karpouzas and Singh 2006). Remediation of contaminated ecosystems is important in the reduction of the detrimental side effects caused by pesticides. Degradation of organophosphorus pesticides by bacteria is of particular interest because of their extensive use and high toxicity for mammals. Organophosphorus pesticides are degraded by many bacteria isolated from soil. For example, a *Brevibacterium* sp., *Microbacterium esteraromaticum* and other bacteria, were able to hydrolyse the P-O-C bond of fenamiphos (ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate) (Megharaj et al. 2003; Caceres et al. 2009; Cabrera et al. 2010). *Rhodococcus* and *Bacillus* strains were also able to rapidly

degrade methyl isothiocyanate (Warton et al. 2001). In addition, a *Rhodococcus* strain and *Serratia marcescens* DI101 were found to be competent in the rapid degradation of 1,3-dichloropropene and diazinon (*O,O*-diethyl *O*-2-isopropyl-6-methylpyrimidin-4-yl phosphorothioate), respectively (Ou et al. 2001; Abo-Amer 2011). Although, a *Pseudomonas* strain was able to degrade rapidly isofenphos (*O*-ethyl *O*-2-isopropoxycarbonylphenyl isopropylphosphoramidothioate), it was unable to degrade other organophosphorus pesticides (Racke and Coats 1988), indicating that degradation is compound specific. *Flavobacterium* ATCC 27551 and *Pseudomonas diminuta* GM, isolated from soils in the Philippines and USA, respectively, had the ability to degrade parathion (*O, O*-diethyl *O*-[4-nitrophenyl] phosphorothioate; Serdar et al. 1982; Sethunathan and Yoshida 1973). Also, *Pseudomonas aeruginosa* AA112 isolated from Egyptian soil could degrade malathion (diethyl [dimethoxythiophosphorylthio]succinate) (Abo-Amer 2007). A *Flavobacterium* sp. and *Sphingomonas paucimobilis* degraded cadusafos and ethoprophos (*O*-ethyl *S*, *S*-dipropyl phosphorodithioate) (Karpouzas et al. 2005). A bacterial consortium which degrades tetrachlorvinphos ([*Z*]-2-chloro-1[2, 4, 5-trichlorophenyl] vinyl dimethyl phosphate) was isolated from agricultural soil (Ortiz-Hernandez and Sanchez-Salinas 2010).

However, use of pesticide-degrading microbial systems for removal of pollutants from contaminated systems requires an understanding of the ecological, physiological and biochemical requirements of the degrading strains. Biodegradation is a common method for the removal of organic pollutants because of its low cost (Qiu et al. 2006; Liu et al. 2007). Degradation of pesticides in soil and water can occur by biotic and abiotic pathways, but the main mechanism of degradation and detoxification is by microorganisms. Such microorganisms may have a major effect on the persistence of pesticides in soil (Surekha et al. 2008) although their biodegradation activity would be affected by various environmental and biotic factors (Ramadan et al. 1990).

Although cadusafos has been used widely in agricultural practices for several years, up to date, there has been no report concerning the isolation and characterization of bacteria able to metabolize cadusafos rapidly. Moreover, cadusafos is thought to cause various deleterious effects on human health by inhibition of acetylcholinesterase activity (Chambers 1992). So, it may be important to find suitable bacteria that can degrade this (and other) organophosphorus pesticides in the environment to enable bioremediation of contaminated soils.

Therefore, the aim of this work was to isolation and characterize cadusafos-degrading bacteria from soil, and then to study the influence of relevant environmental factors on their cadusafos biodegradation activity.

Materials and methods

Pesticides and soil collection

Pesticides used in this study were technical grade cadusafos (97.1%), ethoprophos (97%), fenamiphos (98%), isazofos (95%) and isofenphos (98%) from FMC, USA and Promochem, UK. Stock solutions of cadusafos, fenamiphos and ethoprophos were prepared in distilled water and autoclaved at 120°C for 15 min. Isazofos and isofenphos were dissolved in methanol and sterilized by filtration (Millipore filter, 0.45 μm). The pesticides were used at 10 mg l^{-1}, unless otherwise stated. Soil samples were randomly collected at 10 cm depth from different farms in Southern Taif, Saudi Arabia. Samples were kept in clean plastic bags packed in an ice box and transported to the laboratory.

Media preparation

Minimal salts media (MSM) were used for isolation and characterization of cadusafos-degrading bacteria. The MSM comprised of: 5.97 g $Na_2HPO_4.12H_2O$, 2.27 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4.7H_2O$, 0.02 g $MnSO_4$, $4H_2O$, 0.01 g $CaCl_2.2H_2O$, 0.025 g $FeSO_4$, 1 l distilled H_2O, pH 6.9 (Roberts et al. 1993). Mineral salts medium agar (MSMA) was prepared by adding 15 g l^{-1} agar to MSM. Nutrient broth (NB) and nutrient agar (NA) was prepared according to the manufacturer's instructions (Difco). All media were sterilized by autoclaving at 121°C for 15 min. Chemicals were purchased from Sigma, USA or Merck, Germany; unless otherwise stated.

Isolation, screening and identification of cadusafos-degrading bacteria

Bacteria capable of utilizing cadusafos as a source of carbon were isolated from soil by the enrichment method (Abo-Amer 2007) using MSM containing cadusafos at 30°C. At the final stage of the enrichment method, serial dilutions of the enrichment cultures were spread out on MSMA containing 10 mM cadusafos and incubated at 30°C for 48 h. Single colonies were randomly selected and purified by streaking onto the same medium. The purified isolates were screened for their degradation of cadusafos. Gas liquid chromatograph (GLC) was used for detecting the remaining cadusafos, as described previously (Karpouzas et al. 2004). Bacterial isolates showing ability to degrade cadusafos were initially identified according to *Bergey's Manual of Systematic Bacteriology* (Holt 1994) and maintained in 10% glycerol at −70°C.

Molecular characterization of isolated cadusafos-degrading bacteria

Extraction of chromosomal DNA and 16S rRNA gene PCR amplification

Chromosomal DNA was extracted from cadusafos-degrading bacterial isolates using a Qiagen DNA extraction kit according to manufacturer's instructions. The DNA was suspended in 50 ml of TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and stored at −20°C until used in PCR amplification.

For molecular characterization of cadusafos-degrading isolates, a ∼433 bp fragment of the corresponding 16S rRNA genes was amplified from each isolate using universal primers (Devereux and Willis 1995). Forward and reverse primers were 5′-aacgcgaagaaccttac-3′ and 5′-cggt gtgtacaagaccc-3′, respectively. The PCR reactions (50 μl) were carried out in 0.5 ml Eppendorf tubes containing 1x Pfu buffer, 2 U of Pfu DNA polymerase (Promega), 0.5 μM of each primer, 200 μM of each nucleotide (Promega), 1 μg of chromosomal DNA, and adjusted to 50 μl with sterilized ddH$_2$O. The PCR was programmed as follows: 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1.5 min. After amplification, 5 μl of each reaction mixture was analysed by agarose gel electrophoresis to confirm the size and validity of PCR products. PCR products were then purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions.

DNA sequencing and phylogenetic analyses

PCR products of 16S rRNA genes were used for DNA sequencing. Sequence analysis of the DNA fragments was performed by the Taq dye-deoxy terminator method and an automated 373A DNA sequencer (Applied Biosystems). Selected sequences of other microorganisms with greatest similarity to the 16S rRNA sequences of the cadusafos-degardaing isolates (PC1, PC3, PC5, PC7 and PC10) were obtained from the nucleotide sequence databases and aligned using the MacClade program. Using the packages DNADIST and Bootstrap, the sequences were compared and distance trees were generated using phylograms from within the PAUP (Ver. 4.0b10) suite of packages (Swofford 2002). Different trees were compared and found to provide essentially the same results.

The 16S rRNA gene sequences of the cadusafos-degradaing isolates reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB541363 (PC1 strain), AB541364 (PC3 strain), AB541365 (PC5 strain), AB541365 (PC7 strain), and AB541367 (PC10 strain).

Preparation of inocula

To prepare inocula, cadusafos-degrading bacteria were grown in 50 ml MSM at 30°C overnight and at 200 rev min^{-1}. Bacterial cultures were centrifuged at 8,000 g for 5 min. Bacterial cells were washed twice with 25 ml of sterile sodium phosphate buffer (0.012 M, pH 7.2). The colony-forming units (c.f.u.) for the inocula were determined by plate counting. Inocula densities of 2.1×10^6 c.f.u. ml^{-1}, and incubation at 30°C and at 200 rpm, were used in all experiments unless otherwise stated. All following experiments were carried out in triplicate.

Degradation of cadusafos during bacterial growth

To investigate the degradation of cadusafos during bacterial growth curve, 250 ml conical flasks containing 50 ml MSM supplemented with cadusafos were inoculated with strain PC1. Other conical flasks containing 50 ml medium were used as non-inoculated controls. Growth of the isolates and degradation of cadusafos were determined at 0, 2, 4, 5, 6, 8 and 10 days following inoculation. Bacterial growth was quantified by optical density at 600 nm (OD$_{600 \text{ nm}}$) using a Spekol 11 spectrophotometer.

Effect of temperature and carbon sources on biodegradation of cadusafos

To study the effect of temperature on cadusafos degradation, conical flasks containing 50 ml MSM amended with cadusafos were inoculated with PC1. Further non-inoculated controls were incorporated. Cultures were incubated at 10, 20, 37 or 45°C and 200 rpm. Samples were withdrawn at 0, 2, 4, 5, 6, 8 and 10 days post-inoculation for detection of residual cadusafos. In order to investigate the effect of other carbon sources on degradation of cadusafos, 250 ml flasks containing 50 ml of MSM and MSM amended with 0.1% glucose or succinate were inoculated with strain PC1 and incubated (non-inoculated controls were included). At 0, 2, 4, 5, 6 and 8 days post-inoculation, samples were taken for cadusafos analysis.

Effect of pesticide concentration and phosphate on biodegradation of cadusafos

To test the effect of pesticide concentration on cadusafos biodegradation, 250 ml flasks containing 50 ml MSM amended with cadusafos at different concentrations (5, 10, 20 and 40 mg l^{-1}) were prepared. The conical flasks were inoculated strain and incubated, along with non-inoculated controls. Samples were analysed for residual cadusafos at 0, 2, 4, 5, 6 and 8 days post-inoculation. To study the effect of phosphorus source on cadusafos degradation, MSM was

prepared without KH_2PO_4 and Na_2HPO_4 and replaced by K_2SO_4 and NaCl, respectively. Cadusafos-containing MSM (50 ml), or MSM minus phosphorus sources (50 ml), were inoculated with PC1 and incubated as above, along with non-inoculated controls. For detection of remaining cadusafos, samples were taken at 0, 2, 4, 5, 6 and 8 post-inoculation.

Biodegradation of cadusafos in soil

Degradation of cadusafos was studied in soil from which strain PC1 was isolated. The degradation was carried out in non-sterilized (natural) and sterilized soils. Sterilized soil samples were obtained by autoclaving twice at 121°C for 30 min. All soil samples were kept at 4°C until use. Samples of non-sterilized and sterilized soils were treated with 10 mg cadusafos kg^{-1} and inoculated with 2.1×10^6 c.f.u. g^{-1} of the isolate PC1. Other sterilized and non-sterilized samples supplemented with cadusafos were inoculated with 2.1×10^3 c.f.u. g^{-1} of PC1. Also, samples of sterilized and non-sterilized soil containing added cadusafos were employed as non-inoculated controls. The samples were incubated at 30°C. At regular interval times, samples of soil were taken for detection of cadusafos residue.

Biodegradation of other organophosphorus pesticides by strain PC1

To study the role of strain PC1 in degradation of organophosphorus pesticides other than cadusafos, 250 ml flasks containing 50 ml of MSM supplemented with different pesticides (ethoprophos, fenamiphos, isazofos and isofenphos) were inoculated with PC1 and incubated, along with uninoculated controls. The degradation of these pesticides over 26 days was measured as reported previously (Karpouzas and Walker 2000).

Data analysis

Correlation between the degradation rate of cadusafos and its concentration was calculated using a Michaelis-Menton kinetic equation, as follows: $v = V_{max}(S)/K_s + (S)$ where S is the concentration of cadusafos (mg l^{-1}), V_{max} is the degradation rate of cadusafos (mg l^{-1} day^{-1}), and K_s is the cadusafos concentration at half the maximum degrading rate. The degradation rate of cadusafos was determined by plotting ln C (concentration of cadusafos) against the time and the slope of the linear regression represents the degradation rate. The data were evaluated by one-way analysis of variance (ANOVA) using the SPSS 11.0. statistical analysis program. The statistical analyses were performed using triplicate results obtained from each experiment.

Results and discussion

Isolation and characterization of cadusafos-degrading bacteria

Approximately 60 bacterial isolates were initially selected as potential cadusafos-degrading bacteria. These were obtained by propagation of soil-inoculated cadusafos–containing MSM cultures followed by plating out of diluted cultures onto cadusafos-containing MSM agar. However, of these 60 isolates, only ten appeared to degrade cadusafos when cultures were assayed for residual pesticide by GLC (see "Methods"); these ten strains were designated PC1, PC2, etc. up to PC10. Isolates PC1 and PC7 completely degraded 10 mg of cadusafos l^{-1} within 5 and 6 days with degradation rates of 2.3 and 1.5 mg l^{-1} day^{-1}, respectively; isolate PC3 required slightly longer (8 days) for complete degradation and exhibited a somewhat slower degradation rate of 1.1 mg l^{-1} day^{-1}. However, isolates PC5 and PC10 displayed a far slower degradation rate; complete degradation took more than 15 days and the degradation rate was 0.2 mg l^{-1} day^{-1}. The other isolates (PC2, PC4, PC6, PC8 and PC9) gave even poorer cadusafos degradation times and rates.

The five isolates capable of degrading cadusafos at significant rates were characterized morphologically and biochemically (Table 1). The results revealed that all five isolates share the same characteristics and consequently, these isolates were identified as members of the genus *Pseudomonas* according to *Bergey's Manual of Systematic Bacteriology*.

For further characterization, part of the 16S rRNA-encoding genes of the five cadusafos-degrading isolates (PC1, PC3, PC5, PC7 and PC10) were PCR-amplified and sequenced. The resulting nucleotide sequences were compared to available sequences in the databases. A dendrogram illustrating the results of 16S rRNA analysis is presented in Fig. 1. The results indicate greatest similarity to members of the *Pseudomonas* group, which matches the conclusions of the morphological and biochemical analysis. As illustrated, the 16S rRNA sequences of the five isolates are most closely related to *Pseudomonas putida*. The 16S rRNA gene of PC1 shares 97.9% similarity with that of *P. putida* ATCC 17484, *P. putida* ATCC 17522 and *Pseudomonas* sp. 35L. Moreover, the isolate PC1 scored 96.6, 96.8, 93.8 and 94.3% similarities with that of PC3, PC5, PC7 and PC10, respectively. These results suggest that the cadusafos-degrading isolates (PC1, PC3, PC5, PC7 and PC10) are new isolates of the bacterium *P. putida*. Because of its high ability to degrade cadusafos, the strain PC1 was selected for further analysis.

Bacteria belonging to the genus *Pseudomonas* are metabolically adaptable, and able to degrade aromatic

Table 1 Morphological and biochemical characterization of bacterial isolates

Characteristics	PC1	PC3	PC5	PC7	PC10
Gram stain	−ve	−ve	−ve	−ve	−ve
Cell shape	Short rod	Short rod	Short rod	Short rod	Short rod
Motility	Motile	Motile	Motile	Motile	Motile
Gelatin liquefaction	+ve	+ve	+ve	+ve	+ve
Starch hydrolysis	−ve	−ve	−ve	−ve	−ve
Nitrate reduction	+ve	+ve	+ve	+ve	+ve
Indole production	−ve	−ve	−ve	−ve	−ve
Oxidase test	+ve	+ve	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve	+ve	+ve
M-R test	−ve	−ve	−ve	−ve	−ve
V-P test	−ve	−ve	−ve	−ve	−ve
Citrate utilization	+ve	+ve	+ve	+ve	+ve
Acid from glucose	+ve	+ve	+ve	+ve	+ve
Acid from lactose	−ve	−ve	−ve	−ve	−ve
Acid from mannitol	−ve	−ve	−ve	−ve	−ve
Acid from sucrose	−ve	−ve	−ve	−ve	−ve

+ve positive reaction, −ve negative reaction
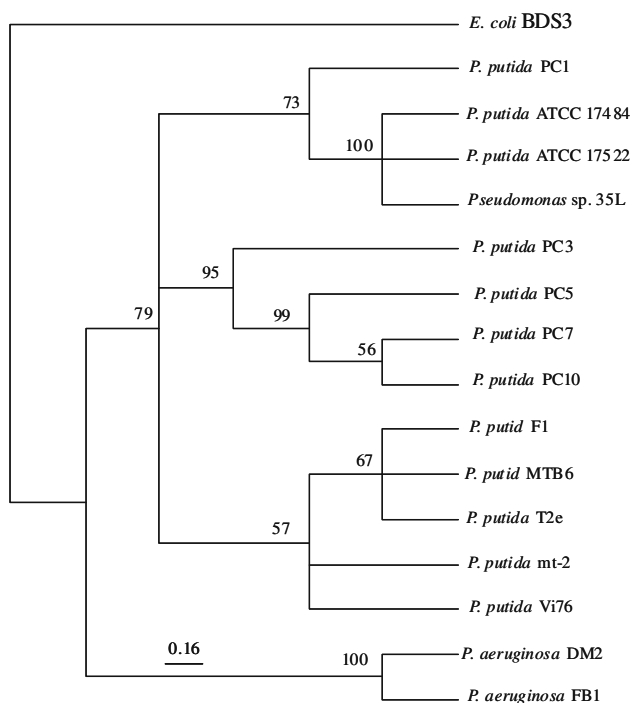
Fig. 1 Dendrogram illustrating the similarity of nucleotide sequences of the 16S rRNA genes of cadusafos-degrading bacteria to that of other *Pseudomonas* sp. Phylogenetic tree of *Pseudomonas* strains based on the nucleotide sequences of the partial 16S rRNA genes. The tree was constructed by using the neighbor-joining method, and genetics distances were computed by the Jukes-Cantor model. The *scale bar* indicates the genetic distance. The *number shown next to each node* indicates the percentage bootstrap value of 100 replicates. The sequence from *E. coli* was treated as the out-group

hydrocarbons, oil, petroleum products and pesticides (Otenio et al. 2005; Abo-amer 2007; Kalyani et al. 2008; Agarry et al. 2008; Cycon et al. 2009; Gautam and Sumathi 2009; Sarkar et al. 2009; Toyama et al. 2010). Pseudomonads are a diverse group of bacteria that occur in large numbers in the soil, where they are active in the mineralization of organic matter. Most species can grow well in simple minimal medium with a single organic compound as carbon and energy sources (Palleroni 1986). Moreover, pseudomonads possess a variety of diverse catabolic pathways that enable them to metabolize a diverse range of low-molecular-weight compounds, including chlorinated aliphatic hydrocarbons such as phenoxyalkanoic acid herbicides (Lynch and Hobbie 1988). In addition *Pseudomonas* is known for its capacity to degrade phenolic compounds (Hughes and Cooper 1996) and other aromatic substances, and therefore it is an ideal choice for use in degradative biotechnologies. The catabolic diversity of this genus is illustrated by the capacity of *P. cepacia* to utilize more than 100 different substrates as sole C, N, or S source (Dagley 1986).

Cadusafos degradation during PC1 growth

Cadusafos degradation and growth of isolate PC1 in cadusafos-containing MSM are shown in Fig. 2. The degradation rate (0.81 mg l^{-1} day^{-1}) of cadusafos by PC1 was significantly ($P < 0.005$) higher than that occurring in the absence of PC1 (0.002 mg l^{-1} day^{-1}). For the first day
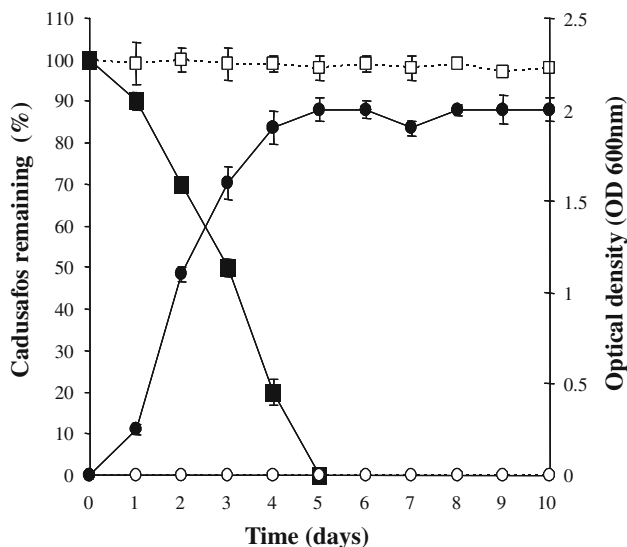
Fig. 2 Cadusafos degradation (*filled square*) during bacterial cell growth (*filled circle*) in MSM inoculated with *P. putida* isolate PC1. *Dotted lines* and *opened symbols* represent non-inoculated cultures. *Data* represent the means of triplicate experiments and *error bars* indicate the standard deviation of the mean

after inoculation, cadusafos degradation was relatively slow with just ~10% degradation achieved (Fig. 2). The rate of degradation increased to 20% per day for days 2 and 3, and reached a maximum (30%) between days 3 and 4, and was complete by day 5. The gradual cadusafos consumption was accompanied by a consistent increase in culture density, which reached a maximum ($OD_{600 \ nm}$ 2.0) once cadusafos had been completely consumed (Fig. 2). In non-inoculated samples, no bacterial growth was reported and consequently cadusafos degradation was insignificant ($P < 0.005$). Degradation of cadusafos by PC1 in MSM matched bacterial growth well, suggesting that PC1 growth is driven by cadusafos catabolism. This result is similar to those reported by Zhaoa et al. (2009).

Effect of temperature and carbon source on cadusafos degradation

Cadusafos degradation by PC1 at different of temperatures is presented in Fig. 3a. The degradation rate of cadusafos was significantly ($P < 0.005$) affected by temperature. Degradation rates of cadusafos were 0.3, 0.6, 0.8 and 0.04 mg l^{-1} day^{-1} at 10, 20, 37 and 45°C, respectively. Cadusafos degradation was fastest at 20 and 37°C, where it was complete within 6 and 5 days, respectively. Degradation was twofold slower at 10 than at 20°C and it took 10 days to completely degrade the pesticide. Cadusafos degradation by PC1 was very much inhibited at 45°C, indeed only 35% degradation occurred over 10 days. Thus, at temperatures between 10 and 37°C, cadusafos degradation by PC1 was relatively rapid, while at 45°C degradation was weak. This result presumably reflects the inability of *Pseudomonas* species to propagate at 45°C (Palleroni 1986). Recent results reported that the methanotrophic bacterium, *Methylosinus trichosporium* OB3b, is able to degrade methane most effectively at 30°C (Rodrigues et al. 2009), and phenol degradation by *Ewingella Americana* (Khleifat 2006) and phenanthrene degradation by *Pseudomonas stutzeri* ZP2 (Zhaoa et al. 2009) were optimally achieved at 37°C. Thus, other pollutant-degrading bacteria likewise show temperature optima similar to that observed here.

Cadusafos degradation by PC1 in MSM amended with succinate or glucose is displayed in Fig. 3b. Cadusafos degradation by PC1 was slightly slower in the presence of

Fig. 3 Effect of temperature (**a**) and carbon source (**b**) on the biodegradation of cadusafos. **a** Isolate PC1 was incubated at 10°C (*filled diamond*), 20°C (*filled square*), 37°C (*filled triangle*), and 45°C (*filled circle*). **b** Isolate PC1 was grown in MSM (*filled diamond*), MSM plus glucose (*filled square*) or MSM plus succinate (*filled triangle*). *Dotted lines* and *opened symbols* represent non-inoculated cultures. *Data* represent the means of triplicate experiments and *error bars* indicate the standard deviation of the mean
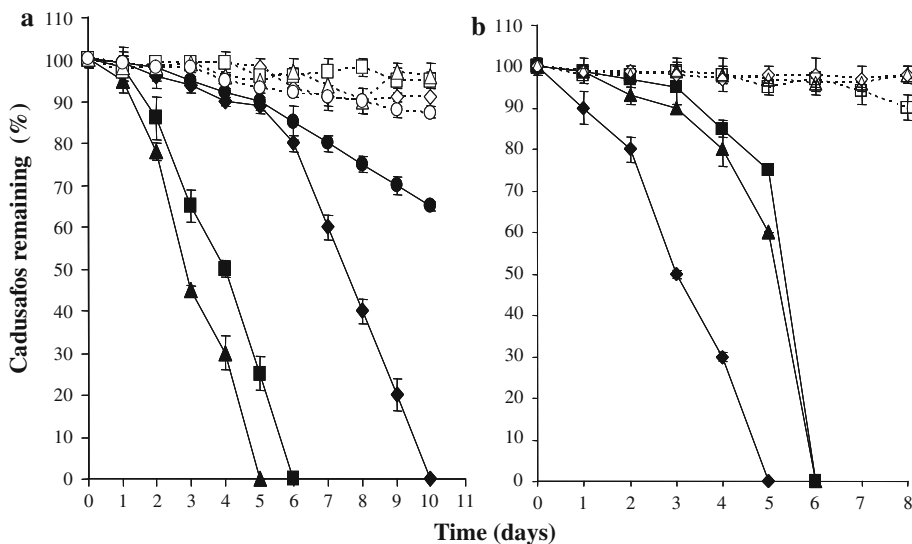
succinate or glucose. In 5 days, PC1 completely degraded cadusafos in MSM, but with succinate or glucose more than 60 or 65% of the initial concentration of cadusafos remained at this time, respectively. The time required for complete degradation of cadusafos in MSM supplemented with succinate or glucose was 6 days (Fig. 6b) and degradation rates were reduced from 0.77 to 0.52 and 0.53 mg l^{-1} day^{-1}, respectively. These differences are significant ($P < 0.005$). Thus, the presence of other carbon sources reduced the cadusafos-degradation capacity of PC1, possibly because of utilization of succinate or glucose as carbon sources in place of cadusafos. Indeed, the rates of cadusafos-degradation with carbon or succinate were relatively slow initially (day 0–4) but were relatively rapid from day 4 which suggests that fast consumption of cadusafos required depletion of these carbon sources (Fig. 3b). This ability to utilize cadusafos when other carbon sources are not available could provide PC1 with a competitive advantage in soils containing significant levels of this insecticide. Similar findings to those above have been reported for *Enterobacter* B-14 (Singh et al. 2004) and *Pseudomonas putida* epII (Karpouzas and Walker 2000) where the degradation of chlorpyrifos and ethroprophos (respectively) in MSMN was inhibited by succinate or glucose. In contrast, a recent report pointed out that pyruvate does not inhibit naphthalene biodegradation but instead can be used as an additional carbon source to stimulate the growth of *P. putida* G7 which boosts the degradation of polynuclear aromatic hydrocarbons (Lee et al. 2003). In other studies, the addition of sodium acetate or glucose did not affect the degradation of pentachlorophenol by *Pseudomonas mendocina* NSYSU (Kao et al. 2005), neither did supplementation with glucose improve degradation of phenanthrene (Zhaoa et al. 2009). Thus, inclusion of other carbon sources may or may not affect the biodegradation of pollutants by bacteria.

Degradation of cadusafos at different concentrations and the role of phosphate

As shown in Fig. 4a, isolate PC1 completely degraded cadusafos at an initial concentrations of 5 and 10 mg l^{-1} in MSM at degradation rates of 1.07 and 0.81 mg l^{-1} day^{-1} within 4 and 5 days, respectively. With an initial concentration of 20 and 40 mg l^{-1}, there was complete degradation by isolate PC1 in 6 days with degradation rates of 0.66 and 0.63 mg l^{-1} day^{-1}, respectively. These results indicate that the concentration of the pesticide had a significant effect on degradation rate ($P < 0.005$). Importantly, isolate PC1 was able to rapidly degrade concentrations of cadusafos as high as 40 mg l^{-1}.

The correlation between the degradation rate and concentration of cadusafos was analyzed using Michaelis–Menten kinetics. Degradation of cadusafos was found to follow a first-order reaction when the concentration of cdausafos was less than 5 mg l^{-1} (i.e. at low concentrations). However, when the concentrations of cadusafos was between 5 and 40 mg l^{-1} (at high concentrations), the degradation rate followed a zero-order reaction and reached a maximum value that was not affected by further increasing the concentration of cadusafos. The values of this kinetic reaction were V_{max} equals 1.1 mg l^{-1} day^{-1}, and K_s equals 2.5 mg l^{-1}.

As presented in Fig. 4b, degradation patterns of cadusafos by isolate PC1 in MSM with and without a phosphorus source were slightly different ($P < 0.005$). Cadusafos was degraded completely in 5 and 6 days with degradation rates of 0.87 and 0.71 mg l^{-1} day^{-1} when PC1 was grown in MSM with and without phosphorus, respectively. Thus, degradation was faster in the MSM with phosphorus than without it, where cadusafos acted as a single source of carbon and phosphorus. This might be attributed to a more rapid assimilation of the inorganic phosphorus found in


Fig. 4 Effect of cadusafos concentration (**a**) and phosphorus source (**b**) on the biodegradation of cadusafos. **a** Isolate PC1 was grown on different concentrations of cadusafos: 5 mg ml^{-1} (*filled diamond*), 10 mg ml^{-1} (*filled square*), 20 mg ml^{-1} (*filled triangle*) and 40 mg ml^{-1} (*filled circle*). **b** Isolate PC1 was grown in MSM (*filled triangle*), MSM minus phosphate (*filled square*). *Dotted lines* represent non-inoculated cultures. *Data* represent the means of triplicate experiments and *error bars* indicate the standard deviation of the mean
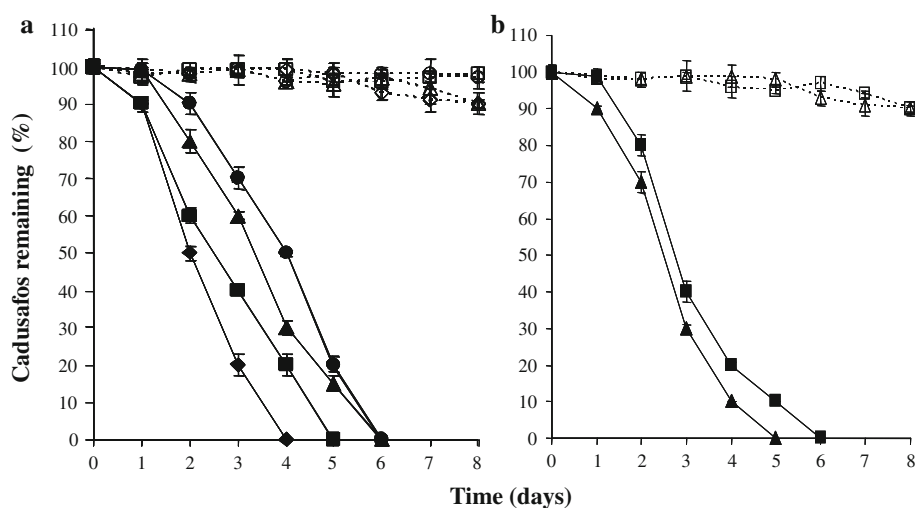
MSM by PC1 than that provided in the form of cadusafos. However, an important finding is that cadusafos can be used as a sole source of phosphorus by PC1. This suggests that isolate PC1 has the necessary metabolic capacity to liberate phosphorus from cadusafos. Thus, there results indicate that PC1 can utilize cadusafos both as sole source of carbon and phosphorus. There are a few reports showing that organophosphate compounds can used for the supply of both carbon and phosphorus for a single species. For instance, *Enterobacter* B-14 can utilize chlorpyrifos as a source of phosphorus and carbon (Singh et al. 2004) whereas a *Flavobacterium* species could use parathion as a source of phosphorus but not carbon, but could use diazinon as a carbon source only (Sethunathan and Yoshida 1973).

Biodegradation of cadusafos in soil by strain PC1

Although the above results clearly demonstrate the ability of PC1 to degrade cadusafos rapidly and to employ it as sole carbon, energy and phosphorous source in pure culture, the ability of this organism to degrade soil contaminated with cadusafos remains unclear. To test this ability, sterilized and non-sterilized soil containing cadusafos was inoculated with PC1 (at high and low dosage) and cadusafos degradation measured over time (Fig. 5). The cadusafos-degradation rates were significantly different for the different inoculation levels used in non-sterile soil ($P < 0.005$). Degradation rates
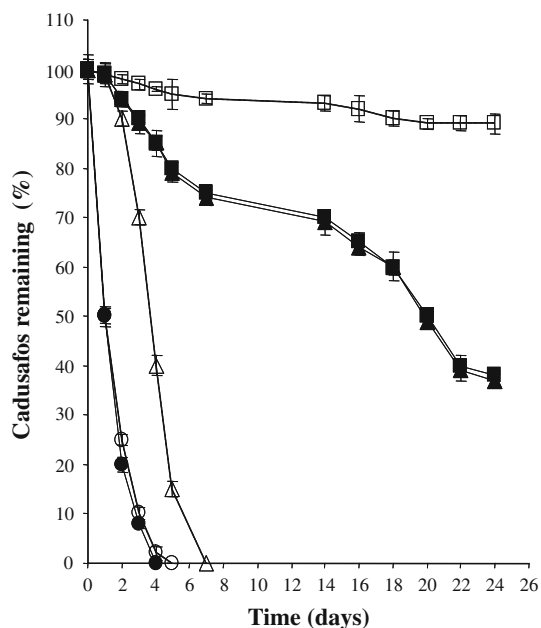

Fig. 5 Cadusafos degradation in sterilized (*open symbols*) and non-sterilized (*closed symbols*) samples inoculated with PC1. Samples were inoculated with 2.1×10^3 (*filled triangle*) or 2.1×10^6 c.f.u. g^{-1} soil (*filled circle*), or remained non-inoculated controls (*filled square*). *Data* represent the means of triplicate experiments and *error bars* indicate the standard deviation of the mean

of cadusafos in sterilized and non-sterilized soil inoculated with 2.1×10^6 c.f.u. g^{-1} of isolate PC1 were similar at 0.96 and 1.1 mg l^{-1} day^{-1}, respectively, and no cadusafos remained 5 and 4 days, respectively, after inoculation. The cadusafos degradation rate was slightly slower (0.66 mg l^{-1} day^{-1}) in the sterilized samples inoculated with 2.1×10^3 c.f.u. g^{-1} of PC1 and complete degradation took 7 days. However, the degradation rate was very much slower (0.08 mg l^{-1} day^{-1}) in the non-sterilized samples inoculated at low density ($2. \times 10^3$ c.f.u. g^{-1}). The degradation rate of cadusafos was insignificant in the non-inoculated controls. Thus, the PC1 inoculum density of 2.1×10^6 c.f.u. g^{-1} allowed efficient degradation of cadusafos in both sterilized and non-sterilized soils. In contrast, the lower inocula density only allowed rapid degradation in the sterilized soil, not in the non-sterile soil (Fig. 5). This effect probably relates to competition between resident soil bacteria and inoculated PC1 for nutrients and bio-space which is overcome when PC1 is provided in high does, but not in low dose. A major effect of inoculum density on the efficiency of bioremediation has been reported by previous studies (Karpouzas et al. 2005; Ramadan et al. 1990; Karpouzas and Walker 2000; Duquenne et al. 1996).

Biodegradation of other organophosphorus nematicides by strain PC1

The degradation of the nematicides—cadusafos, ethoprophos, fenamiphos, isofenphos and isazofos—is shown in Fig. 6. Cadusafos and ethoprophos were completely degraded in 5 and 11 days, respectively, by isolate PC1. However, the other organophosphorus nematicides (fenamiphos, isofenphos and isazofos) were not completely degraded until day 17, 20 and 26, respectively. These results suggest the ability of isolate PC1 to utilize multiple organophosphorus nematicides. Several studies report the involvement of microbial consortia in the degradation of pesticides in soil (Ou et al. 2001; Karpouzas et al. 2005; Roberts et al. 1993). The ability of PC1 to degrade ethoprophos as well as cadusafos could be attributed to the similarity of their chemical structure (they differ by the absence/presence of the methyl groups on each of the propyl moieties). The degradation of fenamiphos, isofenphos and isazofos molecules by isolate PC1 could be attributed to the shared presence of *O*-ethyl lateral chains, as found in cadusafos and ethoprophos, while the presence of the aromatic rings in the these three molecules (absent in cadusafos and ethoprophos) might result in the considerably slower degradation. The ability of isolate PC1 to utilize cadusafos as a source of carbon and phosphorus indicates that this isolate contains enzymes able to break the P-O-C and P-S-C bonds present in this compound. This new PC1 strain of *P. putida* may prove useful in the
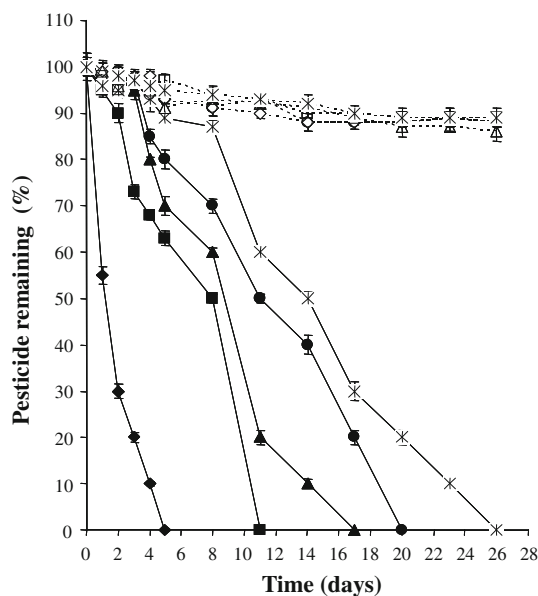
Fig. 6 Degradation of cadusafos (*filled diamond*), ethoprophos (*filled square*), fenamiphos (*filled triangle*), isofenphos (*filled circle*) and isazofos (*asterisk*) in MSM inoculated with 2.1×10^8 c.f.u. ml^{-1} of isolate PC1. *Dotted lines* represent non-inoculated cultures. *Data* represent the means of triplicate experiments and *error bars* indicate the standard deviation of the mean

removal of pesticides, such as cadusafos, from contaminated environments.

Conclusions

A new cadusafos-degrading strain, identified as *P. putida* PC1, was obtained from agricultural soil. This is the first isolation and characterization of a bacterium able to degrade cadusafos. PC1 also has the ability to use cadusafos as a source of carbon, energy and phosphorus, and can additionally utilize other organophosphorus nematicides. PC1 maintained its cadusafos-degrading ability under a range of temperatures. Therefore, this organism might prove useful in pesticide biodegradation under variable and various environmental conditions.

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