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Biodegradation of Organophosphorus Insecticides, Chlorpyrifos, by *Pseudomonas putida* CP-1

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ABSTRACT

Synthetic organophosphorous pesticides (OPs) are highly toxic compounds used widely, amounting to about 38% of the world-wide pesticides. The enormous and inadequate use of OPs is a continuous threat for the whole ecosystem due to their persistence and toxicity. This study was designed to isolate chlorpyrifos degrading bacteria from indigenous soil. After successive enrichment process and sub-culturing on phosphate free medium, a single bacterial strain has been isolated from contaminated soil, designated as CP-1. The strain CP-1 was identified on the basis of sequence homology of 16S rRNA gene and found to be *Pseudomonas putida*. It can grow efficiently on phosphorus free media while utilizing chlorpyrifos as a sole source of phosphorus. This ability indicates that the bacterium has phosphotriesterase that hydrolyzes the phosphotriester bond of chlorpyrifos. Cell free extract (CFE) obtained from *P. putida* CP-1 further confirmed the triesterase activity as it hydrolyzes chlorpyrifos and gives 3,5,6-trichloro-2-pyridinol (TCP) as a major metabolite.

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Authors' Contribution

TAN conceived and designed the study. MK, SR and AZ performed the experimental work. MB and RA helped in analysis of data. TAN wrote the article. JH and AP helped in preparation of manuscript.

Key words

Biodegradation, Organophosphorus insecticides, Chlorpyrifos, *Pseudomonas*, Phosphotriesterase.

INTRODUCTION

Organophosphorous pesticides (OPs) are groups of potent neurotoxins widely used across the world against a number of insect pests (Kenaga *et al.*, 1965; Rigterink and Kenaga, 1966). OP compounds constitute 38% of overall pesticides use (Ajaz *et al.*, 2013) and preferred over organochlorines pesticides due to their low persistence in the mammalian system (Kazemi *et al.*, 2012). These are basically anti-choline esterases and repress the neuro-muscular activities of both insects and humans (Nazarian and Amini, 2008).

Chlorpyrifos [O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioates] is extensively used pesticide and accounts for about 11 % of total pesticide used in the US. However, according to Malik *et al.* (2007) the use of chlorpyrifos has been strictly banned in the US and European countries, but, it is still one of the widely used OPs in developing countries. During 2003, it was one of the top ten insecticides sold in term of weight (Khooharo *et al.*, 2008). In general, OPs are most culprit agents among numerous pesticides and their extensive use cause various health problems by affecting the entire ecosystem. According to the report of the World Health Organization (WHO, 1990) 39 million people around the world have been affected by acute pesticide poisoning and about 200,000 deaths annually by pesticide poisoning as its exposure to humans results in many disorders including nervous breakdown (Jeyaratnam, 1990; Paudyal, 2008).

Microorganisms are key players that determine the fate of xenobiotics including pesticides. But unlike other organophosphorous compounds, chlorpyrifos has been found to be resistant to enhance degradation and suggested that 3,5,6-trichloro-2-pyridinol (TCP) has anti-microbial properties (Racke *et al.*, 1990). However in past, numerous attempts have been made to isolate chlorpyrifos degrading microbes (Mallick *et al.*, 1999; Mukherjee *et al.*, 2004; Singh and Walker, 2006). Several bacterial species belonging to different genera including *Pseudomonas, Agrobacterium, Flavobacterium, Arthrobacter, Enterobacter, Stenotrophomonas, Sphingomonas, Burkholderia, Serratia* and *Bacillus*, have been characterized possessing the capability to hydrolyze OP compounds (Horne *et al.*, 2002; Li *et al.*, 2007; Pakala *et al.*, 2007; Yang *et al.*, 2010; Ali *et al.*, 2012). Same species *i.e.*, *P. putida* has also been isolated from the cotton contaminated soil of Pakistan that has high efficiency to degrade chlorpyrifos (Ajaz *et al.*, 2009).

Enzyme based bioremediation is emerging technology to decontaminate pesticide residues from agricultural soils and wastewater and post harvest fruits and vegetables. Microbial remediation either biostimulation or bioaugmentation require certain pre-requisites which vary from environment to environment

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and could not be maintained in each case. Free enzyme bioremediation is most effective, fast and targeted way of detoxifying xenobiotics (Scott *et al.,* 2009, 2011). Enzymes can be obtained from a broad range of organisms including microbes, plants, insects etc. The characteristics of these enzymes can be improved by protein engineering or *in-vitro* evolution according to the requirements (Sutherland *et al.,* 2004; Russell *et al.,* 2011). Several types of enzymes have been reported that are involved in the detoxification of OPs, most important are esterases classified as phosphotriesterases (PTE) (Sogorb and Vilanova, 2002). Phosphotriesterases have also been isolated from various microorganisms including bacteria and fungi. The two most important and widely used PTEs are OPH (*Flavobacterium*, *Pseudomonas*) and OpdA (isolated from *Agrobacterium*) (Mulbry and Karns, 1989; Horne *et al.,* 2002). In this study, we have found the phosphotriesterase activity in cell free extract (CFE) obtained from *P. putida* strain CP-1 which can hydrolyze broad range of organophosphorus insecticides.

MATERIALS AND METHODS

Chemicals and reagents

All analytical grade chemicals and reagents are of >99 % purity and were purchased from Merck, Germany and Sigma-Aldrich, USA. Synthetic oligonucleotides were obtained from Integrated DNA Technologies, USA.

Isolation of chlorpyrifos degrading bacteria

Soil samples were collected from contaminated cotton fields of Faisalabad, Pakistan which have previous history of pesticide application including chlorpyrifos. The enrichment culture technique was used to isolate bacterial strains. Soil was treated with 50 µg/ml chlorpyrifos and incubated at 32°C in a shake flask incubator at 240 rpm. To enrich the soil with chlorpyrifos degrading native microflora, equal amount of chlorpyrifos was added into the soil after every seven days up till 60 days. Minimal salt medium (MSM) containing 0.1 % NH_4NO_3, 0.1 % NaCl, 0.15 % K_2HPO_4, 0.05 % KH_2PO_4, 0.01 % $MgSO_4.7H_2O$, 0.0025 % $FeSO_4$ (Hong *et al.,* 2007) supplemented with 20 µg/ml chlorpyrifos was used for isolation of bacterial strains. A suspension of 0.5 ml of chlorpyrifos treated soil was spread at fresh MSM agar and incubated at 32°C for two days to get prominent bacterial colonies. Pure colonies were obtained by further subculturing of isolated colonies on nutrient agar. Further confirmation was done by inoculating bacterial colonies in MSM without any carbon and phosphorous source where chlorpyrifos was provided as a sole source of carbon and phosphorus.

Taxonomic identification of bacterial strain

The 16S rDNA gene was amplified via colony PCR by slightly modifying the protocol reported by Pérez-Pérez and Hanson (2002), using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT -3'). PCR amplification was performed in a total volume of 25 µl by using 2720 Thermal Cycler of Applied Biosystems PCR mixture containing 10 mM dNTPs, 10 µM of each primer, 1xTaq buffer, 0.5 mM $MgCl_2$, 1 U Taq polymerase and bacterial colony as DNA template. The PCR conditions for amplification were: initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 90°C for 30 sec, primer annealing at 50°C for 30 sec, extension at 72°C for 2 min; and final polymerization at 72°C for 2 min. Amplified product was resolved electrophoretically using 1% (w/v) agarose gel prepared in TBE buffer and compared to a 2 kb DNA Ladder. Affymetrix PrepEase® Gel Extraction Kit from USB Corporation was used to purify DNA from agarose gel. The amplified products were sequenced and compared to other 16S rRNA gene sequences available in the National Center for Biotechnology Information (NCBI) public database by basic local alignment search tool (BLAST) searching.

Preparation of seed culture

Seed culture of isolated strain was grown in 15 ml LB broth by inoculating medium-sized colony and incubated overnight at 32°C on an orbital shaker. Cells were pellet down by centrifuging the broth culture at 3000 rpm for 10 min. The pellet was washed three times with sterilized distilled water and resuspended in 1 ml sterilized distilled water.

Extraction of enzyme

Bacterial strain was grown in LB broth to the late logarithmic phase; cells were harvested by centrifugation at 10,000 *g* at 4°C. The cell pellet was washed and re-suspended in 1 ml of 50 mM phosphate buffer (pH 7.0). The cells were disrupted by sonication for 10 min while bursting them for 30 sec allowing a 15 second cooling time between each burst. The disrupted cells were centrifuged at a speed of 30,000 *g* at 4°C for 30 min to remove all cell debris and the supernatant was used as enzyme source (Chaudhry *et al.,* 1988).

Enzyme expression

To determine the nature of the expression of enzyme, either it expresses constitutively or inducible an experiment was conducted in LB broth in the absence or presence of chlorpyrifos (0.75 mM) respectively. Cell free extract (CFE) was obtained by the method described

above and used as an enzyme source. Total cellular protein concentration was determined by Lowry method using bovine serum albumin as the standard (Lowry *et al.,* 1951). Each reaction was performed in replicates of three and contains 20 mg of CFE except the one which was used as blank. Hydrolysis of chlorpyrifos was monitored spectrophotometerically by measuring the formation of 3,5,6-trichloro-2-pyridyl (TCP) at a wavelength of 320 nm (Furlong *et al.,* 1989).

Enzyme activity against chlorpyrifos and methyl parathion

Hydrolysis of substrates were determined spectrophotometrically using T80 UV-Visible spectrophotometer, PG instruments, UK. Enzyme assay was performed in glass cuvettes while using a reaction volume of 2 ml. Reaction mixture contains 200 µl CFE, 200 µl of substrate (10 mM) and 1600 µl of phosphate buffer (pH 7.6), while blank was without CFE carrying buffer instead. The reaction was started by adding CFE and mixing the contents by inversion. Enzyme activity was determined by measuring the formation of 3,5,6-trichloro-2-pyridyl (TCP) the product of chlorpyrifos at a wavelength of around 320 nm and by measuring the formation of ρ-nitrophenol at a wavelength of 405 nm in the case of methyl parathion. An increase in TCP was monitored up till 2 h with an interval of 10 min between each reading.

Enzyme activity against malathion and profenofos

Hydrolysis of malathion and Profenofos was determined spectrophotometrically by monitoring the production of thiol groups at wavelength of 412 nm. After 2 h with an interval of 10 min between each reading assays were performed in Ellman's reagent. Reaction mixtures contain 200 µl of substrate (2 mM), 400 µl CFE and 1400 µl of Ellman's reagent making a total volume of 2 ml while blank sample was without CFE.

GC/MS analysis

The residual chlopyrifos and its possible metabolites were extracted from the reaction mixture. Each sample (3 ml) was taken in clean glass test tubes and centrifuged for 5 min at 6000 rpm. The supernatants were transferred to separate test tubes and an equal volume of hexane was added to them. After vigorous shaking , the organic layer was separated in clean test tubes. The same procedure was repeated twice and the organic layer was collected. The water was completely removed by passing the organic layer through anhydrous Na_2SO_4. The solvent was then allowed to evaporate completely at room temperature and reconstitute the organic layer in 500 µL ethyl acetate (Goda *et al.,* 2010). The samples were then stored at 4°C until their analysis.

The residues of chlorpyrifos and its metabolites were analyzed by Clarus 600 GC/MS manufactured by Perkin Elmer. Helium gas was used as the carrier gas at a flow rate of 1.0 mL/min. The chromatographic conditions for detecting malathion were as follows: no split ratio; injection volume, 3 µl; injector program, 100°C initially for 0.0 min and then increasing to 220°C at rate of 3°C min^{-1}. The temperature of injection was 180°C and the total analysis time was 40 min (Romeh and Hendawi, 2013).

RESULTS

Chlorpyrifos degrading bacteria

After successive enrichment process and subculturing on phosphate free media a single isolate has been obtained from the contaminated soil, named strain CP-1. The strain was gram negative showing capability to degrade chlorpyrifos. The strain CP-1 was identified on the basis of 16S rRNA technique and amplified sequence was about 1450 bp approximately. The sequence of strain CP-1 has 97% homology with 16S rRNA of *Pseudomonas putide* strains CSY-P1 and CSY-P2 (Gene Bank accession no. KF010920.1, and Gene Bank accession no. KF010922.1, respectively). Thus the strain CP-1 was designated as *P. putida* CP-1 which was involve in the biotranformation of OP insecticides.

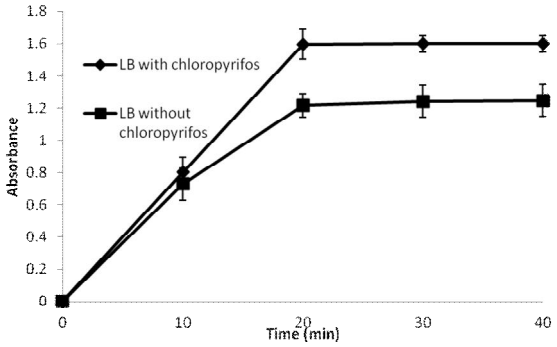

Fig. 1. Expression of enzyme in the presence and absence of chlorpyrifos.

Enzyme expression

The expression study was performed while using LB broth with and without chlorpyrifos to find the nature of enzyme either it is induced by the substrate or constitutive in nature. In each reaction mixture the quantity of CFE used was same *i.e.,* 20 mg/l. The results revealed that the enzyme can express constitutively. But the enzyme that expressed in medium containing chlorpyrifos has significantly high activity (Fig. 1).

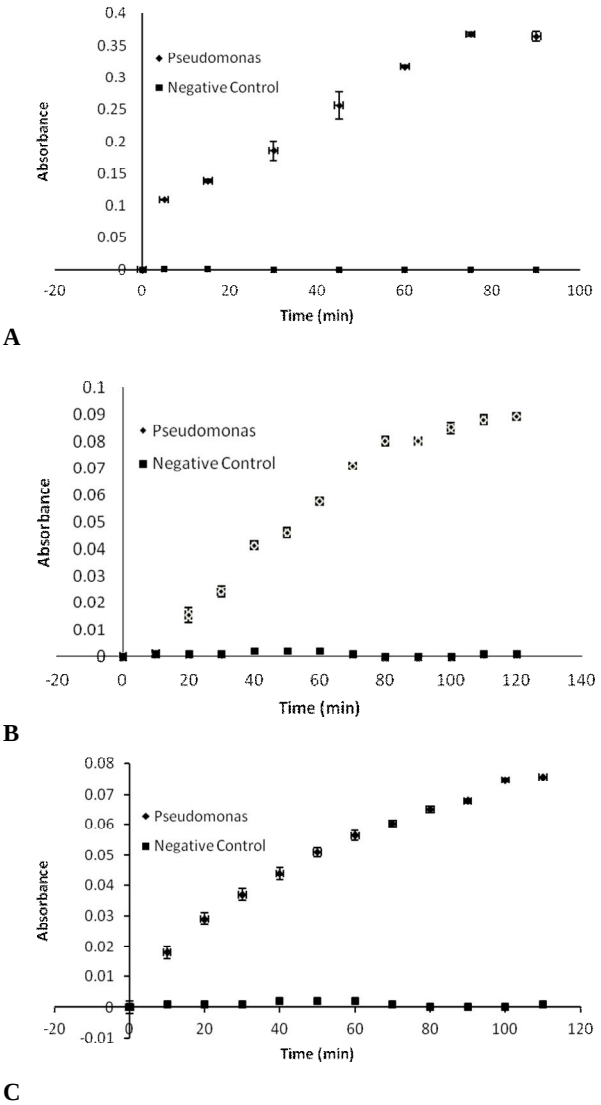

Fig. 2. Cell free extract obtained from *Pseudomonas aeruginosa* showing activity against chloropyrifos (A), malathion (B) and profenofos (C) while no activity observed in negative control. The bars show the standard deviation between each reading from the mean average.

Hydrolase activity against chlorpyrifos

CFE obtained from the strain-CP1 shown to be very active against the chlorpyrifos. Enzyme chlorpyrifos hydrolase obtained from the strain was belong to subclass phosphotriesterase that hydrolyzes the phosphotriester bond in chlorpyrifos, the mechanism of action is just like phosphotriesterases isolated from *Flavobacterium*, *Pseudomonas* and *Agrobacterium* (Mulbry *et al.,* 1986; Chaudhry *et al.,* 1988; Horne *et al.,* 2002). In the present study, we have found that the enzyme chlorpyrifos hydrolase efficiently converts chlorpyrifos into its product TCP shown in Figure 2 which was also confirmed by the GC/MS analysis. The samples treated with CFE has a major metabolite TCP which was confirmed while matching the data with MS library.

Analysis of substrate specificity

Substrate specificity of enzyme was determined by using CFE against different OP insecticides. The enzyme chlorpyrifos hydrolase shown to be active against malathion, profenofos and ethyl parathion but it has no activity against the methyl parathion. The assay was conducted against each substrate for about two hours until complete degradation of a substrate shown in Figure 2. In each experiment, three replicates were used along with blank contain no CFE. In each assay, it was found that the enzyme (CFE) hydrolyzes the substrate within two hours of incubation at room temperature.

DISCUSSION

Microbial bioremediation of insecticides is receiving considerable attention particularly due to their abilty to use them as nutrient source and metabolization into non toxic components (Naqvi *et al.,* 2009). The basis for similar investigation for microorganisms is to find some more efficient and indigenous microbes that can act more efficiently in native atmosphere. The study describes the enrichment of soil bacteria capable of degrading chlorpyrifos by using it as a sole source of phosphorus. Chlorpyrifos is one of the extensively used OP insecticide not only in Pakistan but also throughout the world. Ajaz *et al.* (2009b) has reported *Psedomonas putida* MAS-1 can able to degrade chlorpyrifos. The major pathway of degradation begins with cleavage of phosphorus ester bond and most bacteria utilize it as sole source of phosphorus (Mulbery and Karns, 1989; Horne., *et al.,* 2002). Mostly the enzyme that catalyze OP insecticides are PTE that hydrolyze the phosphotriester bond present in these compounds (Naqvi *et al.,* 2014). PTE have been isolated from numerous bacterial strains having varying catalytic activities against different OPs (Mulbry *et al.,* 1986; Chaudhry *et al.,* 1988; Horne *et al.,* 2002). PTE was first isolated from numerous *Flavobacterium* which can able to hydrolyze OPs by cleaving phosphotriester linkage (Mulbry *et al.,* 1986). The more efficient PTE was isolated by Horne *et al.* (2002) from *A. radiobacter* with comparatively broad substrate range. CFE obtained here from *P. putida* CP-1 has phosphotriesterase activity as it cleaves the triester bond in chlorpyrifos and produce TCP as a major metabolite.

The constitutive nature of enzyme is an extra benefit for enzymatic catalysis of xenobiotics. It makes enzyme more feasible for environmental application (Scott *et al.*, 2011). Here the enzyme phosphotriesterase expressed constitutively which also make it applicable for enzymatic bioremediation. `Substrate specificity of an enzyme is an important component in terms of environmental application (Scott *et al.*, 2010). Generally an enzyme used for enzymatic bioremediation is considered to be more applicable if it is broad substrate range (Scott *et al.*, 2009). The present work revealed that the PTE obtained from *P. putida* CP-1 has broad substrate range and can able to catalyze chlorpyrifos, malathion and profenofos as substrate. That indicates that the enzyme is quite promising in degrading various OP insecticides.

CONCLUSION

The isolated strain *P. putida* CP-1 shows promising attitude to utilize chlorpyrifos as sole source of phosphorus. Moreover the enzyme extracted shows promising activity against different types of OPs that indicates the enzyme is broad substrate range and can be used to detoxify various types of OPs in soil and water.

Statement of conflict of interest

Authors have declared no conflict of interest.

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