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Bioremediation of organophosphorus pesticide phorate in soil by microbial consortia

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ABSTRACT

Microbial consortia isolated from aged phorate contaminated soil were used to degrade phorate. The consortia of three microorganisms (*Brevibacterium frigoritolerans*, *Bacillus aerophilus* and *Pseudomonas fulva*) could degrade phorate, and the highest phorate removal (between 97.65 and 98.31%) was found in soils inoculated with mixed cultures of all the three bacterial species. However, the mixed activity of any of two of these bacteria was lower than mixed consortia of all the three bacterial species. The highest degradation by individual mixed consortia of (*B. frigoritolerans* + *B.aerophilus, B. aerophilus* + *P. fulva* and *B. frigoritolerans* + *P. fulva*) appeared in soil between (92.28–94.09%, 95.45–97.15% and 94.08–97.42%, respectively). Therefore, inoculation of highly potential microbial consortia isolated from in situ contaminated soil could result in most effective bioremediation consortia for significantly relieving soils from phorate residues. This much high phorate remediation from phorate contaminated soils have never been reported earlier by mixed culture of native soil bacterial isolates.

1. Introduction

From more than 40 years, the usage of Organophosphorus pesticides (OPPs) in agricultural practice is common. This insecticide inhibits acetylcholinesterase enzyme, crucial for the transmission of the normal nerve impulse. There are three phosphoester linkages in the structure of the most of the OPPs. As phophoester bonds hydrolysed, acetylcholinesterase inactivating properties are reduced and further reduces toxicity (Horne et al., 2002). One of them, Phorate is a board range pesticide; it has been proved as a most active insecticide against most insect pest species (Extension Toxicology Network, 1996; FAO, 2005; Maria, 2010). Phorate has been classified as a most hazardous insecticide according to the world health organization; so its constant usage is a rising alarm. In European communities, it has been banned and while used with limits in the US. Still many agricultural administrations are keenly working to suspend the prohibition on the usage of this enormously lethal insecticide (Misra, 2011). Phorate has water solubility (50 mg l^{-1}), hence percolate through the soil to groundwater. Till now, reported metabolites of phorate are phoratoxon, phoratoxon sulfone, phorate sulfoxide, and phorate sulfone that are more toxic in action (Henderson et al., 2004). With average LD_{50} of 2–4 mg kg^{-1}, Phorate is considered as one of the most toxic pesticides (Hazardous Substances Data Base, 1988). Therefore, complete removal of phorate in contaminated soil is the need of the hour.

In the environment, microbial degradation is considered to be one of an essential factor to determine fate of organophosphorus pesticides. Bacterial strains of the diverse taxonomic group have a great ability for the degradation of OPPs pesticides (Ghassempour et al., 2002; Sorensen et al., 2008; Maduri and Rangaswamy, 2009; Ratna et al., 2012; El-Helow et al., 2013). These studies are useful for the improvement of biodegradation strategies for the microorganisms mediated cleaning of OPPs pesticides. Bioremediation has received much consideration as an active biotechnological method to decontaminate polluted environments. Different methods have been used in bioremediation such as biostimulation, biosparging, and bioaugmentation (Vidali, 2001; Singh and Walker, 2006).

To remediate phorate contamination in soils, biodegradation remains the most natural and cost effective method. Till now, *Azotobacter*, *Pseudomonas, Flavobacterium* strains has been found to degrade phorate (Ortiz-Hernandez et al., 2003). In soil, phorate biodegradation results in the formation of phosphodithioate sulfoxide and phosphodithioate sulfone (Szeto et al., 1990). Degradation products, however, differ both with the nature of microflora as well as environmental conditions and may yield different products (Henderson et al., 2004).

Different bacterial species are known to be involved in biodegradation of organophosphorus insecticides. In our previously

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published work (Jariyal et al., 2014), we have isolated and evaluated of phorate degrading bacterial spp. in liquid culture and soil. Screening of these bacterial spp. for the degradation of phorate, resulted in the identification of bacterial species Imbl 2.1 as *Brevibacterium frigoritolerans* strain, Imbl 4.1 as *B. aerophilus* strain and Imbl 5.1 as *Pseudomonas fulva* strain. However, these bacterial species, causing complete phorate metabolization were regarded as potent phorate degraders and further studies were carried out on this bacterial spp. Taxonomic characterization resulted in the identification of Imbl 2.1 as *Brevibacterium frigoritolerans* (GenBank acc # JX8446361), strain Imbl 4.1 as *Bacillus aerophilus* (GenBank acc # JX844642) and strain Imbl 5.1 as *Pseudomonas fulva* (GenBank acc # JX844645) by National Center for Biotechnology Information (Jariyal et al., 2014).

Therefore, the purpose of this study to evaluate the bioremediation potential of native bacterial species in consortium in phorate contaminated soil.

2. Materials and methods

2.1. Soils

Sandy loam soil was collected from Entomological Research farm, PAU Ludhiana. In the present study, all physico chemical parameters of soil (sandy loam) were observed pH = 7.61; anion exchange capacity = 155^{-1}; sand = 81.0 per cent; organic carbon = 0.38 per cent; silt = 10.0 per cent; electrical conductivity = 0.12 dsm^{-1} and clay = 8.0 per cent.

2.2. Chemicals

Phorate, phoratoxon, phorate sulfoxide, and phorate sulfone (Sigma-Aldrich, India) were used for analytical analysis. Phorate 10 CG (Hind Crop Science, Mumbai, India) was dissolved in acetone and prepared 1000 µg/ml concentration. Through dilution, 50 µg/ml was prepared with the help of acetone. The purity of phorate formulation and its phorate contents were confirmed by the absence of any of phorate metabolites or any other interfering compounds as determined in acetone extracts of formulation using standard GLC analysis procedures as described later.

2.3. Microorganisms

In our previous studies (Jariyal et al., 2014), microorganisms (*Brevibacterium frigoritolerans*, *Bacillus aerophilus*, and *Pseudomonas fulva*), were isolated from sugarcane fields, which degraded 50 µg ml^{-1} phorate in liquid culture. These microrganisms were used in the present study for accessing their biodegradation potential in the soil.

2.4. Evaluation of Bacterial spp. for phorate degradation

2.4.1. In liquid cultures

For phorate degradation by isolated *bacterial* spp., 50 ml of Mineral salt medium (MSMP) at 50 µg ml^{-1} phorate in an Erlenmeyer flask (250 ml) was incubated with 1 ml (~ 10^8 cfu) of bacterial culture at 28 °C on an incubator (120 rpm) and 5 ml of aliquots were drawn for the analysis of phorate residual contents at different time periods.

2.4.2. Bacterial inoculum Preparation

The inoculum was prepared by growing bacterial *spp.*in 50 ml of MSMP overnight at 25 °C on a rotary shaker at 200 rpm. Cultures were pelleted by centrifugation at room temperature (6000 g for 10 min), cells were rinsed twice with sterilized phosphate buffer (pH 7.2) and quantified by plate count technique. All inoculations were made at 1% i.e. the equivalent of 1 ml inoculum to 100 ml water or 100 g soil.

2.4.3. Phorate degradation in phorate amended soil

The soil was sieved, sterilized in the container. And soil (100 g) was mixed with 100, 200, 300 mg phorate kg^{-1} in plastic cups. One ml of bacterial culture (~4.5×10^6 cells) is inoculated and moisture content is maintained at ~70% in each cup. Each treatment was replicated thrice. One cup (without bacterial inoculation) was kept as control. All the cups were incubated at 37 ± 1 °C for up to 42 days.

2.4.4. Estimation of phorate and metabolites: extracted from soil samples and quantified using gas liquid chromatography as given below

In an Erlenmeyer flask, the soil (5 g) sample was dissolved in acetone (50 ml) and left for overnight. After that, in a separatory funnel (1 l), soil extract was added with frequent acetone washing. To the 500 ml brine solution, the filtrate was added, partitioning was done by hexane, dichloromethane, and anhydrous sodium sulfate was used for the dehydration of extract. Five hundred mg activated charcoal powder is used for cleaning up of extract and the extract so obtained was filtered through Whatman filter paper No.1. Dehydrated phorate extracts were dissolved in about 20 ml acetone and concentrated using a rotary vacuum evaporator at < 35 °C. The final volume was reconstituted in acetone.

2.4.5. Analysis of extracts for phorate and metabolites estimation

Soil extracts were analyzed for the phorate and metabolites (phoratoxon, sulfone, and sulfoxide) by a prestandardized procedure in GLC (Shimadzu Model GC-2010) equipped with Flame Photometric Detector and a split-less mode of Rtx-5 capillary column (30 m × 0.53 mm i.d. × 0.25 µm film thickness of 5% diphenyl and 95% dimethyl polysiloxane). The operating settings of GLC were set: 290 °C temperature of the injector, 200 °C initial column temperature hold till 5 min then for 3 min at 270 °C and temperature of the detector were 320 °C. The flow of N_2 gas was kept at 30 ml min^{-1}. The compound was identified.

2.4.6. Confirmation

The metabolites and phorate were confirmed by (Jariyal et al. (2014) using a GCMS of Electron Multiplier with Quader pole) and column capillary (30 m × 0.25 mm i.d. × 0.25 µm) film thickness (GCMS-QP 2010 plus). The experiment was run in single ion monitoring mode and flow of Helium was kept at 0.94 ml min^{-1}. The data was processed by using software 'GCMS solution version 2.5'

2.4.7. Degradation kinetics study

The kinetics of the phorate degradation were calculated by plotting time against residual phorate concentration to derive correlation coefficients and the best fit curves equations were calculated by using correlation coefficients square as per DPR procedure (Johnson et al., 1992). Graphical representation of time along with logC was made to confirm dissipation kinetics of total phorate residues (Fig. 1).

3. Results and Discussion

3.1. Efficiency of phorate and metabolites recovery from soil samples

Sandy loam soils (100 g) were fortified with 100,200, 300 mg phorate kg^{-1}. The extracted samples from the soil were cleaned and examined using already described the method. The control samples were examined in a similar method to determine interferences, if some, because of any reagent, substrate. Recovery was found (> 85 per cent) in the case of phorate and sulfoxide, phoratoxon and sulfone metabolites from the spiked soil samples. Hence, there is no need to apply any correction factor in the presented data. Observed limit of detection (LOD) of phorate 0.003 mg kg^{-1} and 0.01 mg kg^{-1} was a limit of quantification (LOQ).