Biodegradation of chlorpyrifos by co-culture of *Cellulomonas fimi* and *Phanerochaete chrysosporium*

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Accepted 30 January, 2014

Chlorpyrifos is one of the major organophosphorous pesticides used in agriculture to control insect pests. Due to the widespread toxicity of chlorpyrifos in the environment, exploration of various chlorpyrifos-degrading microbes to clean-up the pollutant is of immense importance. A bacterial strain (*Cellulomonas fimi*), that could transform chlorpyrifos to 3,5,6-trichloro-2-pyridinol (TCP) and a TCP utilizing fungal strain (*Phanerochaete chrysosporium*) were obtained from microbial type culture collection (MTCC), microbial type of culture collection, Chandigarh. The fungus could also degrade 50 mg chlorpyrifos L⁻¹ within 6 days. Co-culture completely mineralized 50 mg chlorpyrifos L⁻¹ with 16 h at 33°C and at pH 8.4 using a total inoculum of 0.13 g biomass L⁻¹. These results showed that, the chlorpyrifos degrading bacterial and fungal strains had the potential to clean up the organophosphorous pesticide-contaminated environment.

Key words: Chlorpyrifos, organophosphorous, Mineralization, *Cellulomonas fimi*, *Phanerochaete chrysosporium*.

INTRODUCTION

Chlorpyrifos (O, O-diethyl O-(3, 5, 6-trichloro-2-pyridyl) phosphothioate) is one of the most widely used organophosphate insecticides. It is a broad spectrum and moderately toxic insecticide with half-life (persistence) of 10-20 days in the environment. It has a very low solubility in water (2 mg/L) but is readily soluble in most organic solvents (Racke et al., 1990). Excessive use of chlorpyrifos has contaminated several ecosystems (EPA, 1995). Additionally, the frequent application of chlorpyrifos may result in high levels of chlorpyrifos residues accumulated on agricultural crops, which poses a potential health hazard to consumers. Maximum residue limits of chlorpyrifos have been recommended by the joint FAO/WHO meeting on pesticide residues (IPCS, 2008). These problems highlight the need of biodegradation of chlorpyrifos residues from contaminated soil, water system or crop. Therefore, biodegradation using native microorganisms for its removal from the environment is quite attractive. Further, TCP, the main biodegradation product of chlorpyrifos, has antimicrobial properties, preventing the proliferation of chlorpyrifos-degrading microbes, so that enhanced degradation does not occur (Singh and Walker, 2006). TCP has been classified as persistent and mobile by the US EPA with a half-life ranging from 65 to 360 days in soil (Armbrust, 2001). TCP shows more mobility than the prent molecule due to its greater water solubility, thus causing widespread contamination of soil and aquatic environments (Feng, 1995). Therefore, study of biodegradation of TCP is also equally important.

A few chlorpyrifos-degrading bacteria, including *Enterobacter* strain B-14, *stenotrophomonas* sp Ye¹, *Serratia* and *Sphingomonas* sp. DSP-2 have been reported in earlier studies (Sing et al., 2006; Li et al., 2007, Xu et al., 2007). Several chlorpyrifos degrading fungi such as
**Phanerochaete chrysosporium, Aspergillus terreus and Verticillium sp.** DSP with varying degrees of success have been reported (Bumpus et al., 1993; Omar 1998; Yu et al., 2006). Unfortunately, bacterial degradation was only partial and TCP accumulated in the medium without further metabolism. Only one TCP mineralizing bacterium (*Pseudomonas* sp) has been described so far (Feng et al., 1997). Little information is available on the microbial metabolism of TCP. A chlorpyrifos and TCP-degrading *Alcaligenes fescalis* strain was reported (Yang et al., 2005), but was not efficient enough for chlorpyrifos removal.

The present study characterized the mineralization of chlorpyrifos by co-culture of *Cellulomonas fimi* that can transform chlorpyrifos to TCP and a fungal strain, *P. chrysosporium* that is capable of degrading TCP as well as chlorpyrifos, in liquid medium. The optimal conditions for efficient chlorpyrifos degradation by this co-culture were also investigated.

**MATERIALS AND METHODS**

**Chemicals**

Chlorpyrifos (98% purity), 3, 5, 6-trichloro-2-pyridinol (93% purity) were obtained from Sigma Aldrich chemical private limited, Bangalore, India. All other chemicals were analytical grade and commercially available.

**Culture**

*C. fimi* MTCC-24 and *P. chrysosporium* MTTC 787 were obtained from the microbial type culture collection (MTTC) Chandigarh, India.

**Microbial growth and biodegradation assays of two strains and their co-culture**

Strains were pre-cultivated at (30°C, 200 rpm, in the dark) in nutrient broth medium containing chlorpyrifos or TCP at 50 mg L⁻¹. Microbial growth was determined by the dry weight biomass (g L⁻¹). After full growth, the culture was harvested and inoculated into minimal medium with 50 mg chlorpyrifos L⁻¹ or TCP as sole carbon source. The biodegradation assays for each strain and their co-culture were conducted at 30°C pH 7 in the dark. The co-culture of strain *C. fimi* and *P. chrysosporium* was used to determine the optimal incubation conditions. A series of experiments was carried out with different degradation times (0-6 days), initial chlorpyrifos concentrations (25-400 mg L⁻¹), total inoculation biomass amount (0.05-0.25 g L⁻¹) each in half incubation temperature (15-35°C) pH (5-9) and additional carbon source (Glucose or Sucrose, 0-20 g L⁻¹). Cultures without inoculation were used as an abiotic control. All experiments were performed in triplicates.

**Extraction efficiency**

Samples were recovered from culture flasks at respective time intervals and centrifuged at 7,200 × g for 10 min to obtain cell free medium. The supernatant thus obtained was added to a separating funnel and chlorpyrifos was extracted with an equal volume of dichloromethane (Anwar et al., 2009). After partitioning, the organic layer of dichloromethane was evaporated in a solvent evaporator to obtain a powdery residue of the organic compound.

**Chromatographic analysis**

**GC-MS analysis**

A GC-MS-QP2010 system equipped with an electron capture detector (ECD) was used to analyze the residual chlorpyrifos in the sample after bacterial degradation. One micro-litre of each dichloromethane extract was manually injected onto a BPX5 column (30 m, 0.25 mm). The oven temperature was held initially at 180°C for 1.5 min, programmed from 180° to 260°C at 10°C/min, and held at 260°C for 12.5 min. The chromatographic analysis was complete in 22 min. Nitrogen was used as carrier gas at a column head pressure of 95.6 kpa giving a linear carrier flow of 36.4 cm/s and column flow rate of 0.90 mL/min. The injection mode was splitless, the injector temperature was 260°C and detector temperature was 280°C. The chromatograms were recorded using a computer and compared with the standard library of NIST-07 mass spectral data. External standards were used for quantification of the residual concentration of chlorpyrifos.

**High-performance liquid chromatography (HPLC) analysis**

TCP was analyzed by HPLC using a Zorbx SB-C18 column with detection at 230 nm (Feng et al., 1997) and with array detection from 200-600 nm to identify its possible metabolites. The CO₂ concentration in the gas sample drained from the flask heads space was analyzed by GC equipped with a thermal conductivity detector (Soares et al., 2005).

**RESULTS**

**Growth and degrading ability of Cellulomonas fimi and Phanerochaete chrysosporium**

*C. fimi* and *P. chrysosporium* were grown in liquid culture medium containing chlorpyrifos or TCP as the sole carbon source. The time course of chlorpyrifos metabolism by *C. fimi* is shown in Figure 1 where complete disappearance of 50 mg chlorpyrifos l⁻¹ was observed within 3 days. TCP (retention time 3.6 min by HPLC) was detected as the only major metabolites of chlorpyrifos degradation and it accumulated in the medium without further metabolism.

Strain *P. chrysosporium* grew rapidly when TCP was provided as the sole carbon source and 50 mg TCP l⁻¹ could be degraded within 4 days (Figure 2a) No intermediate metabolite was detected in the medium. The CO₂ levels increased with decrease of TCP showing the capability of degrading TCP. *P. chrysosporium* could also degrade chlorpyrifos but to a lesser extent; 50 mg L⁻¹ was degraded within 6 days Figure 2b. TCP accumulated to a peak value on day 2, but decreased thereafter and was undetectable by day 8. The growth of *C. fimi*, and *P. chrysosporium* stopped after the sole carbon source, chlorpyrifos or TCP, was consumed as shown in Figures 1 and 2.

**Mineralization of chlorpyrifos by co-culture of Cellulomonas fimi and Phanerochaete chrysosporium**

No growth inhibition was observed in the co culture of
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Figure 1. Bacterial growth and degradation of chlorpyrifos (50 mg L\(^{-1}\)) by *cellulomonas fimii* cultivated in mineral salts medium. Values are means ± standard deviations of three replicates.

Strain *C. fimii* and *P. chrysosporium*; complete degradation of 50 mg chlorpyrifos L\(^{-1}\) was achieved by the co-culture within 16 h. (Figure 3). This was much faster than the rate achieved by the pure fungal isolate, indicating that growth was stimulated by a synergistic interaction with the bacterial strain. The clearly lower concentration of accumulated TCP in the co-culture could be in part responsible for the significantly enhanced biodegradation of chlorpyrifos. The increase of CO\(_2\) level in the headspace indicated the mineralization of chlorpyrifos. (data not shown). Throughout all the studies degradation was accompanied by microbial growth as monitored by dry weight biomass. Under all experimental conditions chlorpyrifos and TCP loss was never greater than 10% in the abiotic controls. In most cases described to date the degrading bacteria tend to transform chlorpyrifos by hydrolysis to produce diethylthiophosphoric acid and TCP which in turn accumulate in the culture medium without further metabolism. Several soil fungi can apparently mineralize chlorpyrifos as well as TCP which are resistant to microbial attack (Singh and Walker, 2006).

Optimal conditions for biodegradation of chlorpyrifos by co-culture

Slower degradation rate was observed due to increased toxicity at higher chlorpyrifos concentrations; 100 mg chlorpyrifos L\(^{-1}\) required 24 h for complete mineralization. As shown in Figure 4a, b and c the optimal condition for chlorpyrifos degradation by the co-culture was 33\(^\circ\)C, pH 8.4 and total inoculum biomass 0.14 g dry weight L\(^{-1}\) for an assay time of 12 h in medium containing 50 mg chlorpyrifos L\(^{-1}\). Figure 4-d shows that biodegradation of chlorpyrifos was greatly enhanced by addition of sucrose.

GC-MS analysis for toxic intermediates

After degradation studies, the samples were analyzed by gas chromatographic - mass spectrometry (GC-MS) technique. The GC-MS analysis showed the presence of chlorpyrifos at R.T. of 9.51 min. The comparison with standard library of NIST-07 mass spectral database confirmed the matching of mass/charge ratio versus relative intensity at R.T 9.51 for the samples to standard spectra of chlorpyrifos. The mass spectra obtained showed that chlorpyrifos was degraded to small metabolites which could not be identified using the available library database. The presence of chlorpyrifos was observed at R.T. 9.51 min but no any intermediate was identified till R.T. 25 min. This indicated that chlorpyrifos is probably completely metabolized by the isolates into smaller intermediates.

DISCUSSION

In the present study, chlorpyrifos-degrading bacterial strain *C. fimii* and an effective TCP-mineralizing fungal strain *P. chrysosporium* were obtained from MTTC. Both the bacterial and fungal strains were co-cultivated in the medium containing 50 mg L\(^{-1}\) chlorpyrifos or TCP as sole source of carbon. Biodegradation occurring in an environment exposed to the chemical pollutant is a complex process in which many different metabolically active microbial communities take part. Several co-cultivated and mixed cultures have been reported for efficient mineralization of contaminants (Dejonghe et al., 2003; Bazot et al., 2007). The interaction between different microbial species on biodegradation of chlorpyrifos is an important consideration in developing bioremediation strategies for
chlorpyrifos removal. In this study, the optimal condition for chlorpyrifos degradation by the co-culture were observed to be 33°C pH 8.4 and total inoculum biomass 0.14 g dry wt L⁻¹; the complete mineralization of chlorpyrifos or TCP was observed within 16h and it was identified that this co-culture could rapidly mineralize chlorpyrifos. Degradation was possible under a broad range of temperatures and pH values, showing that microbial remediation should be possible under many environmental conditions. Xu et al. (2007) reported that 100 mg L⁻¹ chlorpyrifos required 24 h for complete mineralization by Serratia sp. and Trichosporon sp.

TCP is the major degradation product of chlorpyrifos (Meikle and Youngson, 1978; Tomlin, 2003). There have been few reports of enhanced degradation of chlorpyrifos by isolated microorganisms because of antimicrobial activity of TCP at high concentrations (Racke et al., 1990; Singh et al., 2004). Although mineralization of TCP has

Figure 2. Fungal growth and mineralization of (A) TCP (50 mg L⁻¹) and (B) chlorpyrifos by Phanerochaete chrysosporium cultivated with mineral salts medium. Values are means ± standard deviations of three replicates.
Figure 3. Microbial growth and mineralization of chlorpyrifos (50 mg L\(^{-1}\)) by the co-culture of *Cellulomonas fimi* and *Phanerochaete chrysosporium* cultivated with mineral salts medium. Values are means ± standard deviations of three replicates.

Figure 4. Effects of inocula biomass amount (a), temperature (b), pH (c) and additional carbon source (d) on biodegradation of chlorpyrifos (50 mg L\(^{-1}\)) over 12 h. Total inocula biomass amount (0.05 - 0.25 g L\(^{-1}\)), temperature (15-35°C), pH (5-9) and sucrose (0 - 20 g L\(^{-1}\)). Values are means ± standard deviations of three replicates.
been reported in soil (Racke and Robbins, 1991), very little information exists with respect to microbial degradation of TCP. Bacterial strains capable of mineralizing TCP in liquid medium and in soil, while utilizing TCP as a sole source of carbon and energy have been reported in A. faecalis (Yang et al., 2005) and Pseudomonas sp. (Feng et al., 1997). In this study, we report the degradation of chlorpyrifos by co-culture of C. fimi and P. chrysosporium which utilize 50 mg L\(^{-1}\) chlorpyrifos or TCP as the sole source of carbon. Less degradation at high concentrations of TCP might be attributed to cellular toxicity as stated above. Growth of A. faecalis and degradation of TCP is known to be unaffected by TCP at concentrations of more than 800 mg/L (Yang et al., 2005). Three other CP degrading bacteria, Enterobacter strain B-14, Stenotrophomonas sp.YC-1, and Sphingomonas sp. Dsp-2 failed to utilize TCP for growth and energy (Singh et al., 2004; Yang et al., 2006; Li et al., 2007). In the present investigation, the mineralization of chlorpyrifos by co-culture of C. fimi and P. chrysosporium under optimal conditions was studied. This result may prove to be promising for biotreatment of wastewaters and bioremediation of contaminated soils.

**Conclusion**

In the present study, chlorpyrifos-degrading bacterial strain C. fimi and an effective TCP-mineralizing fungal strain P. chrysosporium were employed for the mineralization of chlorpyrifos; complete degradation of chlorpyrifos was attained within 16 h. This may lead to the development of a possible bioremediation technology in the near future. However, extensive studies are still needed to suggest possible mechanism of chlorpyrifos biodegradation by the bacterial and fungal strains.

**REFERENCES**

Anwar S, Liaquat F, Khan OM, Khalid ZM, Iqbal S (2009). Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by Bacillus pumilus strain C2A1. J. Hazard Mater. 68:400-405.

Armbrust KL (2001). Chlorothalonil and chlorpyrifos degradation products in golf course leachate. Pest Manage. Sci. 57: 797-802.

Bazot S, Bois P, Joyeux C, Lebeau T (2007). Mineralization of diuron [3-(3,4-dichlorophenyl)-1, 1-dimethyleurea] by co-immobilized Arthrobacter sp. and Deltia acidovorans. Biotechnol. Lett. 29:749–754.

Bumpus JA, Kakar SN, Coleman RD (1993). Fungal degradation of organophosphorus insecticides. Appl. Biochem. Biotechnol. 39-40:715–726.

Dejonghe W, Berteloot E, Goris J, Boon N, Crul K, Maertens S, Hofte M, De Vos P, Verstraete W, Top EM (2003). Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading Variovorax strain. Appl. Environ. Microbiol. 69:1532-1541.

Environmental Protection Agency (1995). Review of chlorpyrifos poisoning data. US EPA. 1,46.

Feng Y (1995). Transformation of 3,5,6-trichloro-2-pyridinol, a metabolite of pyridine e-based pesticides. Ph.D. Thesis, The Pennsylvania State University.

Feng Y, Racke KD, Bollag J (1997). Isolation and characterization of a chlorinated-pyridinol-degrading bacterium. Appl. Environ. Microbiol. 63:4096-4098.

IPCS (2008). Data sheets on pesticides no. 33. Chlorpyrifos-methyl. In: The International Programme on Chemical Safety, Ottawa, ON. http://www.inchem.org/documents/pds/pds33_24e.htm.

Li X, He J, Li S (2007). Isolation of a chlorpyrifos-degrading bacterium, Sphingomonas sp. strain Dsp-2, and cloning of the mdp gene. Res Microbiol. 158:143-149.

Meikle RW, Youngson CR (1978). The hydrolysis rate of chlorpyrifos, O,O -diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, and its dimethyl analog, chlorpyrifos- methyl, in dilute aqueous solution. Arch. Environ. Contam. Toxicol. 7:13-22.

Omar SA (1998). Availability of phosphorus and sulfur of insecticide origin by fungi. Biodegradation. 9:327-336.

Racke KD, Laskowski DA, Schultz MR (1990). Resistance of chlorpyrifos to enhanced biodegradation in soil. J. Agric. Food Chem. 38:1430-6.

Racke KD, Laskowski DA, Schultz MR (1990). Resistance of chlorpyrifos to enhanced biodegradation in soil. J. Agric. Food Chem. 38:1430-1436.

Racke KD, Robbins ST (1991). Factors affecting the degradation of 3,5,6-trichloro-2-pyridinol in soil. In: Somasundaram L, Coats JR (eds) Pesticide transformation products: fate and significance in the environment. Am. Chem. Society, Washington, DC. pp. 92-107.

Singh BK, Walker A (2006). Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev. 30:428-471.

Singh BK, Walker A, Morgan JAW, Wright DJ (2004). Bio-degradation of chlorpyrifos by Enterobacter strain B-14 and its use in biodegradation of contaminated soils. Appl. Environ. Microbiol. 70:4855-4863.

Soares A, Jonasson K, Terrazas E, Guiyesse B, Mattiasson B (2005). The ability of white-rot fungi to degrade the endocrine-disrupting compound nonylphenol. Appl. Microbiol. Biotechnol. 66:719-725.

Tomlin CDS (2003). The pesticide manual, 13th edn. British Crop Protection Council, Alton.

Xu G, Li Y, Zheng W, Peng X, Li W, Yan Y (2007). Mineralization of chlorpyrifos by co-culture of Serratia and Trichosporon spp. Biotechnology Letters. 29:1469-1473.

Yang C, Liu N, Guo XM, Qiao CL (2006). Cloning of mdp gene from a chlorpyrifos-degrading bacterium and use of this strain in bioremediation of contaminated soil. FEMS Microbiology Letters. 265:118-125.

Yang L, Zhao YH, Zhang BX, Yang CH, Zhang X (2005). Isolation of a chlorpyrifos-degrading bacterium. FEMS Microbiol Lett. 251: 67–73.

Yu YL, Fang H, Wang X, Wu XM, Shan M, Yu JQ (2006). Characterization of a fungal strain capable of degrading chlorpyrifos and its use in detoxification of the insecticide on vegetables. Biodegradation. 17:487-494.