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Utilization of Chlorpyrifos as a Sole Source of Carbon by Bacteria Isolated from Wastewater Irrigated Agricultural Soils in an Industrial Area of Western Uttar Pradesh, India

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Abstract: In the present study wastewater irrigated agricultural soil with a previous history of chlorpyrifos use was examined for its capacity to harbor bacteria capable of utilizing it as a sole source of carbon. Four bacterial isolates designated as RA-3, RA-5, RA-10, RA-20, isolated from the soil, using enrichment culture technique showed promising capability to utilize chlorpyrifos as a carbon source for their growth. Morphological and biochemical tests performed on the bacteria indicated that they might belong to the genus *Pseudomonas*. Thin layer chromatography and tetrazolium reduction assay showed that the strains were capable of degrading chlorpyrifos. All the chlorpyrifos degrading bacterial isolates were also tested for their antibiotic sensitivity against 10 antibiotics/drugs. All the isolates were sensitive to gentamycin and methicillin. RA-10 and RA-3 were sensitive to ampicillin whereas RA-5 was resistant and RA-20 showed intermediate range of sensitivity. RA-5 and RA-3 were sensitive to chloramphenicol whereas, RA-10 and RA-20 showed intermediate sensitivity. RA-5 and RA-20 showed resistance against co-trimoxazole and nalidixic acid. RA-5 and RA-10 showed intermediate sensitivity to tetracycline whereas RA-20 was resistant and RA-3 sensitive to it. All the bacterial isolates were also found to harbor a single plasmid. This leads us to believe that the soils with previous exposure to chlorpyrifos contain a diverse range of bacteria having novel organophosphorus hydrolase enzyme systems for causing the enhanced biodegradation of this toxic pesticide in the environment. Further elucidation of the enzymatic and molecular mechanisms involved in the process will help in creating possible bioremediation technologies using the soil bacteria.

Key words: Chlorpyrifos, organophosphorus, bacteria, carbon source, biodegradation

INTRODUCTION

Pesticide is a general term for substances, which are used to poison pests (weeds, insects, molds, rodents etc.).The indiscriminate uses of pesticides, has led to widespread contamination of water, soil and air with pesticide residues. Leading pesticides used in India include monocrotophos (10700 Million Tonnes (MT)-highest consumed), acephate (6400 MT), endosulfan (5600 MT) and chlorpyrifos (5000 MT-fourth highest consumed) (Source: Pesticide Information, Vol. XXVIII, No. 3, Oct-Dec 2002).

Organophosphates belong to a class of highly toxic neurotoxins that are commonly used as insecticides and chemical warfare agents such as Sarin and Soman. The effectiveness of the

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organophosphorus pesticides can be attributed to their ability to act as potent acetylcholinesterase inhibitors leading to an efficient inhibition of neuro transmission in living organisms (Cho *et al*., 2002).

Chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate) is a broad-spectrum organophosphorus insecticide. Extensive use of chlorpyrifos contaminates air, ground water, rivers, lakes, rainwater and fog water. Symptoms of acute poisoning include headache, nausea, muscle twitching and convulsions and in extreme case death also. Human birth defects have also been associated with exposure to chlorpyrifos products. It also affects the male reproductive system. Chlorpyrifos is toxic to a variety of beneficial arthropods including bees, ladybird beetles and parasitic wasps. It kills fishes at concentrations as low as a few parts per trillion. Birds are also susceptible with effects ranging from reduced weight of nestlings, deformities and death. In plants there have been reports of delayed seedling emergence, fruit deformities and abnormal cell division upon prolonged exposure to chlorpyrifos (Cox, 1995).

Chlorpyrifos is characterized by a P-O-C linkage as in other organophosphate pesticides, such as diazinon, parathion and methyl parathion. Unlike other organophosphates, there have been very few reports of enhanced biodegradation of chlorpyrifos since its first use in 1965 (Singh *et al*, 2003). Chlorpyrifos has been shown to be degraded co-metabolically in liquid medium by bacteria (Mallick *et al*., 1999; Horne *et al*., 2002) and various *opd* genes have been isolated from different geographical regions, some of which have been shown to hydrolyze chlorpyrifos (Serdar *et al*., 1982; Mulbry *et al*., 1986; Richnis *et al*., 1997; Horne *et al*., 2002). However, the first report of enhanced biodegradation by a chlorpyrifos degrading bacterium isolated from Australian soils was made by Singh *et al*. (2003). In 2004 the same author has reported enhanced biodegradation of chlorpyrifos by an *Enterobacter* strain B-14. Yang *et al*. (2005) isolated *Alcaligenes faecalis* DSP3, which is capable of degrading chlorpyrifos and 3, 5, 6-trichloro-2-pyridinol (TCP). More, recently Yang *et al*. (2006) were successful in cloning the *mpd* gene from a chlorpyrifos -degrading bacterium and using it for bioremediation of contaminated soil.

The human health and ecological risks associated with chlorpyrifos and its widespread use in Indian agriculture, prompted us to investigate the utilization of chlorpyrifos by soil bacteria as a sole source of carbon in a waste water irrigated agricultural soil, so that possible cost effective bioremediation technologies for the environmental clean-up of this toxic pesticide may be developed in the near future.

MATERIALS AND METHODS

Collection of Soil Samples

The soil sampling was done from the wastewater irrigated agricultural fields of village Dunda Haira (19 km from Delhi) in Delhi-Hapur Bypass Road, National Highway-24 of Ghaziabad district of Uttar Pradesh, India.

Composite soil samples were collected from various wastewater irrigated agricultural fields with a previous history of chlorpyrifos application for pest control. Samples were collected in sterilized polythene bags with the help of sterilized spatula as described by Singh *et al*. (2003).

Determination of Soil pH

The pH of the supernatant was measured by the method as described by Alef and Nannipieri (1985).

Enumeration of Microorganisms

Enumeration of microorganisms from soil was carried out as described by Reddy *et al*. (1986) and Malik and Ahmad (2002).

Isolation of Chlorpyrifos Degrading Bacteria from Soil

Ten gram of composite soil samples were taken in a petri plate to which 5 mL of distilled water and chlorpyrifos at the concentration of 100 μg g^{-1} of soil was added and mixed thoroughly. The petri plates were incubated at 28°C for 5 days in the dark. Technical grade chlorpyrifos was obtained from Bharat Rasayan Limited, New Delhi. One gram of soil from each replicate was used to inoculate 250 mL conical flasks containing 100 mL of minimal salt medium (Potassium dihydrogen orthophosphate 1.0 g L^{-1}, Di potassium hydrogen orthophosphate 1.0 g L^{-1}, Ammonium nitrate 1.0 g L^{-1}, Magnesium sulphate 0.2 g L^{-1}, Calcium chloride 0.02 g L^{-1}, Ferrous sulphate 0.01 g L^{-1}, pH 6.5) with 100 μg mL^{-1} of chlorpyrifos. The samples were incubated in the dark on a shaker incubator at 28°C and 150 rpm. After 5 days of incubation, 0.1 mL from each replicate was transferred to fresh minimal agar (1.5%) medium supplemented with 100 μg mL^{-1} of chlorpyrifos. After 3 days of incubation at 28°C, a few colonies appeared on the plates. The colonies were purified by several transfers on the same minimal salt agar medium supplemented with the same concentration of chlorpyrifos and were characterized on the basis of their morphological, cultural and biochemical characteristics (Collins and Lyne, 1987).

Individual bacterial colonies on minimal salt medium supplemented with chlorpyrifos were picked up randomly and purified by repeated sub culturing on the same minimal medium. The growth of all the isolated bacteria was further checked on minimal salt agar medium with chlorpyrifos in the concentrations of 25, 50, 100, 200, 400, 800, 1600 and 3200 μg mL^{-1}, as described by Shafiani and Malik (2003).

Time Course for the Growth of Bacterial Isolates

Bacteria, which were able to tolerate high concentrations of chlorpyrifos in minimal agar plates, were selected. The stock solution of chlorpyrifos was prepared in DMSO (HPLC grade). Exponentially grown cultures of the test organisms were inoculated into treated (25, 50, 100, 200 μg mL^{-1}) and an untreated liquid minimal medium and incubated at 30°C in a rotary shaker (150 rpm) for different time intervals. Solvent control was also run simultaneously. Growth was determined turbidometrically at different time intervals by measuring optical density (OD) in a Spectronic 20 spectrophotometer at 540 nm.

Carbohydrate Utilization Test

Utilization of carbohydrates as a sole source of carbon was determined in the minimal salt medium having the tested carbohydrates as the single carbon source. The following carbohydrates: glucose, sucrose, fructose, maltose, lactose, arabinose, mannose, raffinose, adonitol, dulcitol, inositol and mannitol (all from Hi media, India) were incorporated at the concentration of 1 g L^{-1}.

Amino Acid Utilization Test

Utilization of the amino acids as a sole source of nitrogen was determined in the minimal salt medium lacking ammonium salts. The following amino acids, arginine, asparagine, cystine, glycine, histidine, methionine, phenylalanine, threonine and tyrosine (all from Hi Media, India) were incorporated at the concentration of 1 g L^{-1}.

Detection of Organophosphorus Hydrolase Activity in the Bacterial Isolates

The detection of organophosphorus hydrolase activity in the selected bacterial isolates was performed as described by Cho *et al*. (2002) with some modifications. The bacterial isolates were grown in minimal agar plates supplemented with 100 μg mL^{-1} methyl parathion as the single carbon source. Upon incubation at 30°C for 24-48 h, appearance of a yellow coloration (due to *p*-nitrophenol formation) around the bacterial colony was reported as a positive test for the presence of organophosphorus hydrolase activity in the bacterial isolates.

Test for Determining the Biodegradability of Chlorpyrifos

Reduction of tetrazolium chloride by the bacterial isolates for determining the biodegradability of chlorpyrifos was performed as described by Yu and Yu (2000) with some modifications. The test organisms were inoculated into liquid minimal salt medium (in boiling tubes) supplemented with 100 μg mL^{-1} chlorpyrifos, serving as the sole carbon source and incubated at 30°C in a rotary shaker for 24 h. One milliliter of freshly prepared (0.02%) tetrazolium chloride was added to each tube containing the test organisms. The boiling tubes were then, incubated at 30°C in a BOD incubator for 4 h. After 4 h of incubation, the tubes were visually observed for color change (i.e., from white color of the minimal medium to red color of formazon). Appearance of red coloration was indicative of a positive test.

Extraction of Pesticides from Soil Samples

Extraction of pesticides in soil was done according to the method described by Gan *et al.* (1999).

Isolation and Characterization of Chlorpyrifos and its Biodegraded Products

The bacterial isolates were grown in minimal salt medium supplemented with 100 μg mL^{-1} concentration of chlorpyrifos. Cultures were incubated at 30°C for 2 days in a shaker incubator at 150 rpm. After 2 days of incubation, the cultures were extracted for chlorpyrifos and its biodegraded products, by acetonitrile (HPLC- grade) (Singh *et al*., 2003), with the help of a separatory funnel. The extract was evaporated to dryness under pressure with the help of vacuum pump and finally redissolved in the same solvent.

Thin Layer Chromatography

Thin layer chromatography was performed using the crude extracts (in acetonitrile) on Merck Silica gel 60(F_{254}) plastic TLC sheets. TLC plates were developed in solvent system having Heptane-Acetone-Ethyl Acetate in 80:10:10 ratio and compounds were visualized under UV light and by spraying with Kovac's colorizing reagent. Chlorpyrifos was visualized as a blue to purple-red spot on the plates. A standard TLC plate with different concentrations of chlorpyrifos i.e., 5, 20 and 50 μg dissolved in acetonitrile (HPLC grade) was also run.

Antibiotic Sensitivity Test

All the bacterial isolates were also tested for their sensitivity to different antimicrobial agents by means of disc diffusion method as described by Khan and Malik (2001). The following antibiotics (all from Hi media, India) were used: ampicillin (A), chloramphenicol (C), co-trimoxazole (Co), cloxacillin (Cx), gentamycin (G), kanamycin (K), methicillin (M), nalidixic acid (Na), streptomycin (S) and tetracycline (T). *E. coli* B was used as a sensitive strain.

Isolation of Plasmid DNA and Agarose Gel Electrophoresis

Plasmids were isolated from all the chlorpyrifos degrading bacterial isolates. The miniprep method as described by Kado and Liu (1981) was used. The isolated plasmids were characterized by agarose gel electrophoresis according to the standard procedure (Sambrook *et al*., 2001). The DNA bands were stained with ethidium bromide and fluorescent profile was photographed by gel documentation system (Vilber Lourmat, France).

RESULTS AND DISCUSSION

The population of various microbial communities in wastewater irrigated agricultural soil is shown in Table 1. Present study indicated that the total aerobic heterotrophs were 2.14×10^7 and

Table 1: Enumeration of microorganisms from agricultural soil

	Soil samples	
Parameters	SS1	SS2
pH	7.72	7.8
Total aerobic heterotrophs	$2.14\times10^{7}\pm 0.02\times10^{7}$	$1.95\times10^{7}\pm0.01\times10^{7}$
Asymbiotic nitrogen fixers	$3.65\times10^{4}\pm0.08\times10^{4}$	$1.98\times10^{4}\pm0.05\times10^{4}$
Actinomycetes	$5.25\times10^{4}\pm0.18\times10^{4}$	$4.38\times10^{4}\pm0.20\times10^{4}$
Fungi	$3.10\times10^{2}\pm0.21\times10^{2}$	$1.57\times10^{2}\pm0.85\times10^{2}$

*: Values are mean± SD in cfu g^{-1} of soil (n = 3×2)

Table 2: Tolerance of selected bacterial isolates to different concentrations of chlorpyrifos in minimal salt agar medium

	Conc. of chlorpyrifos (mg mL^{-1})							
Isolates	25	50	100	200	400	800	1600	3200
RA-3	+	+	+	+	+	+	+	±
RA-5	+	+	+	+	+	+	±	-
RA-10	+	+	+	+	+	+	+	±
RA-20	+	+	+	+	+	+	+	+

+ = Growth; ± = Mottled growth; - = Absence of growth

1.95×10^{7} cfu g^{-1} of soil for SS1 and SS2 samples, respectively. The results of total aerobic heterotrophs are in agreement with those of Malik *et al.* (2002). They reported total aerobic heterotrophic count as 5.39×10^{7} cfu g^{-1} in agricultural soil, whereas industrial soil contained 3.14×10^{7} cfu g^{-1} of total aerobic heterotrophs. Total actinomycetes count were 5.25×10^{4} for SS1 and 4.38×10^{4} for SS2, whereas total asymbiotic nitrogen fixers were 3.65×10^{4} for SS1 and 1.98×10^{4} for SS2. Similar trends in actinomycetes and asymbiotic N_2 fixers were also observed by Juwarkar *et al.* (1988) but Malik *et al.* (2002), reported lower counts of these organisms in agricultural soils of Aligarh (India).

The pH of the wastewater irrigated agricultural soil was determined as 7.72 for SS1 and 7.80 for SS2 (Table 1). The pH of the soil was comparable to those reported by Juwarkar *et al.* (1988) who reported the pH in the range of 6.9-8. Khasim *et al.* (1989) reported the pH of agricultural soil in the range of 7.5-8.6.

We have also screened a large number of bacterial isolates from wastewater irrigated agricultural soil for chlorpyrifos degrading ability. Effective chlorpyrifos biodegraders were selected by growing the organisms in a minimal salts medium supplemented with different concentrations of chlorpyrifos (as a sole source of carbon and energy) (Table 2). Four bacterial isolates showing appreciable chlorpyrifos degrading ability were selected and designated as RA-3, RA-5, RA-10 and RA-20. Time courses of growth of the bacterial isolates with different concentrations of chlorpyrifos are presented in (Fig. 1-4).

Growth of the isolates at the concentration used is similar or comparable to that of control. Kale *et al.* (1989) reported that the growth of *Azotobacter chroococcum* in nitrogen containing culture medium was not affected at 0.5 and 5 ppm concentrations but the growth of the same isolate was inhibited by pesticides at higher concentrations. Tu (1994) studied the effect of pesticides on soil bacteria at concentrations of 10 μg g^{-1} of soil for herbicides and 100 μg g^{-1} for fumigants and observed that most of the pesticide treatments affected nitrification of ammonium during two weeks of incubation. Nawab *et al.* (2003) also tested the growth of *Pseudomonas* isolates in the presence of γ-HCH at the concentrations ranging from 15-120 μg mL^{-1} and also found that growth declined at higher concentrations. Shafiani and Malik (2003) studied the growth of two *Pseudomonas* isolates (PSS 3 and PSS 20) in endosulfan containing medium at different time intervals. They also found that growth of the isolates at the lowest concentration (25 μg mL^{-1}) was comparable to that of control. They further reported that the growth declined at 100 μg mL^{-1} and dropped more sharply at 200 μg mL^{-1}.

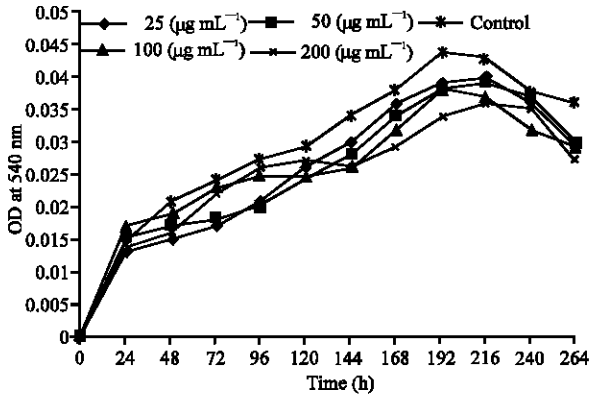

Fig. 1: Growth pattern of bacterial isolate RA-3 at different concentrations of chlorpyrifos

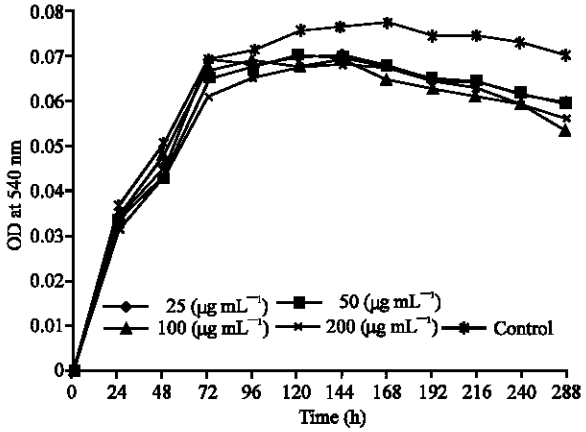

Fig. 2: Growth pattern of bacterial isolate RA-5 in different concentrations of chlorpyrifos

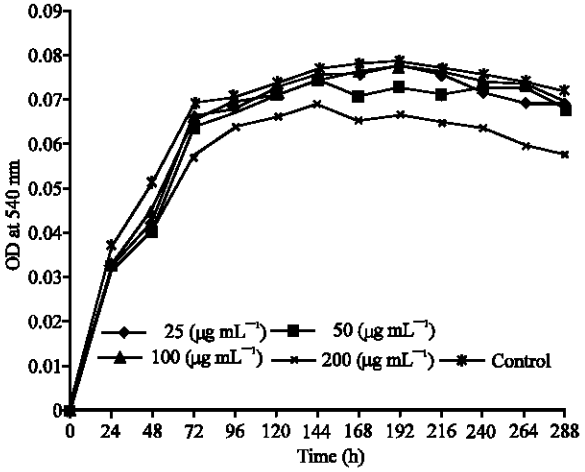

Fig. 3: Growth pattern of bacterial isolate RA-10 at different concentrations of chlorpyrifos

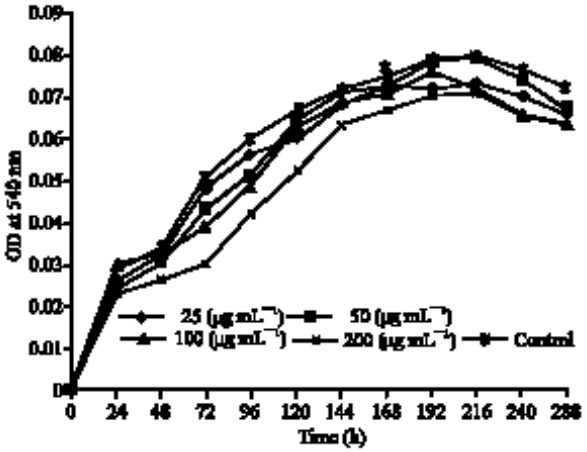

Fig. 4: Growth pattern of bacterial isolate RA-20 at different concentrations of chlorpyrifos

Fig. 5: Bacterial isolate RA-20 showing yellow coloration after 24 h of growth in minimal medium supplemented with 100 µg mL^{-1} of methyl parathion. Methyl parathion is degraded to p-nitrophenol by organophosphorus hydrolase

Many researchers have reported both innocuous and inhibitory effects of certain pesticides on soil bacteria, depending on the concentrations used (Rajagopal *et al*., 1984).

We have also tested all the selected bacterial isolates for the presence of organophosphorus hydrolase (OPH) activity. All the four isolates produced a visible yellow coloration upon growth on the minimal salt agar plates supplemented with methyl parathion (as the sole source of carbon and energy) (Fig. 5). Organophosphorus hydrolase (OPH), an enzyme isolated from *Pseudomonas diminuta* MG and *Flavobacterium* sp. strain ATCC 27551, has the ability to hydrolyze different organophosphorus insecticides (Dumas *et al*., 1989). Hydrolysis of OP compounds by OPH dramatically reduces their toxicity (DiSoudi *et al*., 1999), providing a promising enzymatic

decontamination technology. Though chlorpyrifos is characterized by a P-O-C linkage as in methyl parathion the hydrolyzed products of chlorpyrifos upon hydrolysis by OPH do not have a visible color. Methyl parathion on the other hand, is hydrolyzed to *p*-nitrophenol, which is yellow in color. Keprasertsup *et al*. (2001) and Cho *et al*. (2002) have also reported similar results, as ours while screening bacteria with OPH activity against organophosphorus pesticides.

The tetrazolium chloride reduction test was performed to evaluate the biochemical oxidation of chlorpyrifos by the test organisms. All the four bacterial isolates were able to reduce tetrazolium chloride into the highly colored product, formazon, indicating that they were able to drive the biochemical oxidation of chlorpyrifos in the liquid minimal salt medium. Yu and Yu (2000) using the same principle, to evaluate the biodegradability of *p*-nitrophenol by *Pseudomonas aeruginosa* in Biolog microplates, have reported that the formation of color is directly related to the amount of substrate mineralized and maximum color intensity is observed when all the biodegradable carbon sources are completely utilized in the culture medium.

Tetrazolium dyes can be used as colorimetric indicators of the biochemical oxidation of an organic substrate (Bochner and Savageau, 1977). Bacterial oxidation of a substrate generates reduced NAD; if the electrons are donated to an electron transport system, a tetrazolium dye may function as an artificial electron acceptor. Virtually any chemical substrate that is oxidized by a cell will result in the formation of NADH, which in turn will reduce redox dyes such as tetrazolium to a highly colored formazon (Red colored), which can be observed visually and quantified spectrophotometrically (at 590 nm) (Yu and Yu, 2000). A number of specific assays can serve adequately to evaluate biodegradability. Oxygen uptake is widely used as a surrogate parameter for biodegradability. Although the structures of electron transport chains of various microorganisms differ widely, the tetrazolium redox chemistry can be applied for virtually every species of bacteria as reported by Bochner (1989).

The thin layer chromatographic profile of chlorpyrifos and its biodegraded products by the bacterial isolates after 2 days of incubation, along with soil sample (SS2) are shown in Fig. 6 and 7.

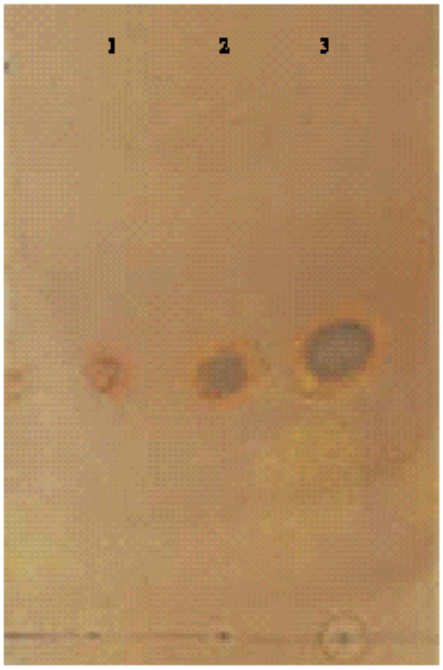

Fig. 6: Standard TLC profile for chlorpyrifos. Lane 1: 5 µg; Lane 2: 20 µg; Lane 3: 50 µg

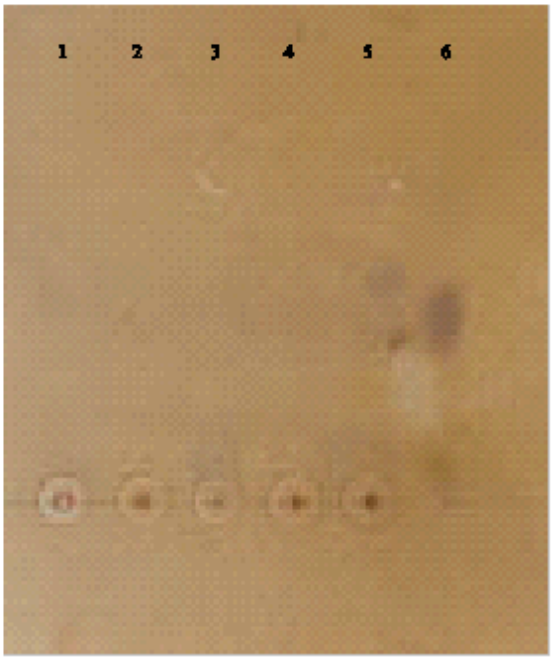

Fig. 7: TLC profile for the test samples. Lan 1: RA-3; Lan 2: RA-5; Lan 3: RA-10; Lan 4: RA-20; Lan 5: SS2; Lane 6: standard chlorpyrifos

The thin layer chromatography on Merck silica gel 60 (F_{254}) TLC sheets showed no presence of chlorpyrifos, as visualized by spots on the TLC plates, when compared with the standard TLC profile (Fig. 6) for chlorpyrifos. However, the environmental soil sample (SS2) showed a spot with similar migration behavior as that of chlorpyrifos, indicating the presence of a related chemical species within a detectable range in the sample.

Many other organophosphorus pesticides are susceptible to enhanced degradation in soil such as isophenfos (Racke and Coats, 1987), diazinon (Forrest *et al*., 1981), fensulfothion (Read, 1983), parathion and methyl parathion (Singh *et al*., 1999), but there have been very few reports of enhanced degradation of chlorpyrifos *per se*. There have, however, been reports of different rates of degradation of chlorpyrifos in soils, with a half-life ranging from 10-120 days (Racke *et al*., 1988). Singh *et al*. (2003) identified a robust bacterial population that utilized chlorpyrifos as a source of carbon in an Australian soil and the enhanced ability to degrade chlorpyrifos was successfully transferred to the United Kingdom soils. Only soils with a pH ≥ 6.7 were able to maintain this degrading ability 90 days after inoculation. Transfer and proliferation of degrading microorganisms from the Australian soil to the UK soils was monitored by molecular fingerprinting of bacterial 16sRNA genes by PCR-Denaturing Gradient Gel Electrophoresis (DGGE).

Recently Singh *et al*. (2004) reported the enhanced degradation of chlorpyrifos by an *Enterobacter* strain B-14 and found that the strain responsible for enhanced biodegradation of chlorpyrifos showed greatest similarity to *Enterobacter asburiae* based on 16s rRNA studies of the bacterium. This strain was shown to utilize chlorpyrifos as a sole source of carbon and phosphorus and hydrolyzed chlorpyrifos to diethylthiophosphoric acid (DETP) and 3, 5, 6-trichloro-2-pyrinidol. Further studies by them with B-14 revealed that the strain possessed a novel phosphotriesterase enzyme system, as the gene coding for this enzyme had a different sequence from the widely studied organophosphate degradative gene (*opd*). The authors also reported that addition of the strain B-14 to chlorpyrifos contaminated soils resulted in higher degradation rate than that observed in non-inoculated soils.

Yang *et al*. (2005) isolated *Alcaligenes faecalis* DSP3, which is capable of degrading both chlorpyrifos and TCP. More, recently Yang *et al*. (2006) were successful in cloning the *mpd* gene from a chlorpyrifos -degrading bacterium and using it for bioremediation of contaminated soil. Six chlorpyrifos-degrading bacteria were isolated using chlorpyrifos as the sole source of carbon by enrichment procedure. Their strain YC-1 showed the highest degrading capability and was putatively identified as the genus *Stenotrophomonas*. The strain YC-1 degraded 100 mg L^{-1} of chlorpyrifos within 24 h to DETP and TCP. DETP was utilized as a source of carbon and phosphorus, but it did not degrade TCP. Addition of the strain YC-1 to fumigated and non-fumigated soils resulted in a more rapid rate of chlorpyrifos degradation than that of uninoculated controls. 100 mg/kg of chlorpyrifos was degraded completely within 15 days. Degradation of chlorpyrifos in control non-fumigated soils (without inoculation) was lower with less than 24% of the applied concentration degraded in 15 days incubation studies. The rate of degradation in inoculated soils increased with increasing soil pH from 4.3 to 7.0 but there was no significant difference in degradation rate in soil with pH 7.0-8.4. The degradation rate of chlorpyrifos in acidic soils was found to be slower than in neutral and alkaline soils.

Mallick *et al*. (1999) studied the degradation of chlorpyrifos by *Flavobacterium* and *Arthrobacter* sp. and reported that the strains were able to use chlorpyrifos as a source of carbon. However, due to the absence of the standard metabolites for chlorpyrifos in the samples tested they were not able to characterize and quantify the biodegraded products of chlorpyrifos. Various authors have also reported that chlorpyrifos is degraded cometabolically in liquid medium by a *Flavobacterium* sp. and also by an *E. coli* clone with an *opd* gene (Richnis *et al*., 1997; Mallick *et al*., 1999; Wang *et al*., 2002).

Swati and Singh (2002) isolated fungal strains from the groundnut fields in Rajasthan (India) and found that *Aspergillus niger* and *A. flavus* utilized chlorpyrifos as a sole source of carbon and phosphorus. They reported that when *A. flavus* was provided with 200 mg kg^{-1} of chlorpyrifos, it utilized about 96.2% of chlorpyrifos as nitrogen and phosphorus source within 24 h of incubation. However, another species of the same fungus (*A. niger*) utilized 52.2% of the total chlorpyrifos thereby reducing its concentration to 95.6 mg kg^{-1} within 24 h of inoculation. Yu *et al*. (2006) isolated and characterized a fungal strain capable of degrading chlorpyrifos.18S rDNA gene analysis revealed that the fungal strain had a high level of homology (99%) to those from other *Verticillium* species. They further used the cell free extracts of the strain to detoxify chlorpyrifos in vegetables and reported that the cell free extracts of the fungus can used for enhanced degradation in vegetables.

We have also tested all the four bacterial isolates (RA-3, RA-5, RA-10 and RA-20) for their morphological, cultural and biochemical characteristics. All the isolates were gram negative, short rods and showed growth at 4°C as well as 41°C. All the isolates exhibited oxidase positive reaction. Indole test was negative for all the isolates. RA-3 and RA-20 showed positive test for methyl red and only RA-5 was found to be positive for Voges-Proskeur test. RA-5 and RA-20 were also found to utilize citrate as the source of carbon. All the bacterial isolates were positive for nitrate reduction except RA-20.The bacterial isolates were negative for urease, H_2S and gelatin hydrolysis tests (Table 3). Based on comparison of these characteristics with the standard description in Bergey's Manual of Determinative Bacteriology (1994), the selected isolates were tentatively identified as species of *Pseudomonas*.

Further, we have also tested all bacterial isolates for the utilization of amino acids as a sole source of nitrogen in a minimal salt medium. RA-20 was found to utilize all the amino acids tested. RA-5 also utilized all the amino acids except histidine, methionine and tyrosine. RA-10 was found to utilize arginine, cystine, glycine, methionine, phenylalanine and tyrosine. Amino acids cystine, histidine, phenylalanine, threonine and tyrosine were not utilized by RA-3 (Table 4).

Table 3: Morphological, cultural and biochemical characteristics of bacterial isolates from soil

	Isolates			
Tests	RA-3	RA-5	RA-10	RA-20
Gram's staining	-	-	-	-
Morphology	Short rods	Short rods	Short rods	Short rods
Growth at 4°C	+	+	+	+
Growth at 41°C	+	+	+	+
Indole test	-	-	-	-
Methyl red test	+	-	-	+
Voges Proskeur test	-	+	-	-
Citrate utilization	-	+	-	+
Starch hydrolysis	-	-	+	+
Catalase test	+	+	+	+
Nitrate reduction test	+	+	+	-
Gelatin hydrolysis	-	-	-	-
Oxidase test	+	+	+	+
Urease test	-	-	-	-
TSI test	-	-	-	-

+ = Positive; - = Negative

Table 4: Utilization of amino acids as a source of nitrogen by the bacterial isolates

	Isolates			
Amino acids	RA-3	RA-5	RA-10	RA-20
Arginine	+	+	+	+
Asparagine	+	+	-	+
Cystine	-	+	+	+
Glysine	+	+	+	+
Histidine	-	-	-	+
Methionine	+	-	+	+
Phenylalanine	-	+	+	+
Threonine	-	+	-	+
Tyrosine	-	-	+	+

+ = Positive; - = Negative

We have also tested the bacterial isolates for the utilization of carbohydrates. All the isolates showed similar properties in utilizing glucose and sucrose. RA-5 did not utilize arabinose, raffinose, adonitol and dulcitol whereas, RA-10 failed to utilize fructose, lactose, arabinose, raffinose, dulcitol and inositol (Table 5).

All the chlorpyrifos degrading bacterial isolates were also tested for their antibiotic sensitivity against 10 antibiotics/drugs. The results were recorded on the basis of the inhibition zone from the zone size interpretative chart supplied by the manufacturer. Table 6 shows the antibiotic sensitivity of chlorpyrifos degrading bacterial isolates. All the isolates were sensitive to gentamycin and methicillin. RA-10 and RA-3 were sensitive to ampicillin whereas RA-5 was resistant and RA-20 showed intermediate range of sensitivity. RA-5 and RA-3 were sensitive to chloramphenicol whereas, RA-10 and RA-20 showed intermediate sensitivity. RA-5 and RA-20 showed resistance against Co-trimoxazole and nalidixic acid. RA-10 and RA-20 showed intermediate sensitivity to nalidixic acid. RA-5 and RA-10 showed intermediate sensitivity to tetracycline whereas RA-20 was resistant and RA-3 sensitive to it.

Guha *et al.* (1997) tested the antibiotic susceptibility of *Micrococcus* sp., capable of degrading malathion and chlorpyrifos against 12 commonly used antibiotics and reported nalidixic acid resistance in *Micrococcus* strain M-36.

Shafiani and Malik (2003) also tested pesticide tolerant bacteria for their antibiotic susceptibility against different antibiotics namely nalidixic acid, cloxacillin, chloramphenicol, tetracycline, amoxicillin, methicillin and doxycycline. They reported that 100% of the pesticide tolerant *Pseudomonas* isolates

Table 5: Utilization of carbohydrates as carbon source by the bacterial isolates

	Isolates			
Carbohydrates	RA-3	RA-5	RA-10	RA-20
Glucose	+	+	+	+
Sucrose	+	+	+	+
Fructose	+	+	-	+
Maltose	+	+	+	+
Lactose	+	+	-	+
Arabinose	+	-	-	+
Mannose	+	+	+	+
Raffinose	+	-	-	+
Adonitol	+	-	+	+
Dulcitol	+	-	-	+
Inositol	+	+	-	+
Mannitol	+	+	+	+

+ = Positive; - = Negative

Table 6: Antibiotic sensitivity profile of chlorpyrifos degrading bacterial isolates

	Diameter of Inhibition zone (in mm) and Sensitivity									
Isolates	A	C	Co	Cx	G	K	M	Na	S	T
RA-3	30 (S)	22 (S)	26 (S)	18 (IM)	26 (S)	22 (S)	30 (S)	14 (IM)	16 (S)	24 (S)
RA-5	10 (R)	18 (S)	10 (R)	0 (R)	16 (S)	20 (S)	26 (S)	10 (R)	16 (S)	16 (IM)
RA-10	18 (S)	16 (IM)	14 (IM)	12 (IM)	19 (S)	16 (IM)	20 (S)	14 (IM)	16 (S)	18 (IM)
RA-20	16 (IM)	14 (IM)	0 (R)	7 (R)	15 (S)	18 (S)	22 (S)	11 (R)	14 (IM)	14 (R)

Sensitivity. (S) = Sensitive, (IM) = Intermediate, (R) = Resistant; Diameter of disc: 6 mm

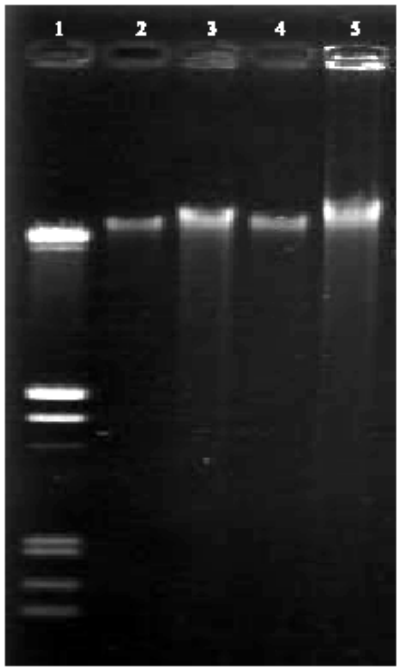

Fig. 8: Agarose gel electrophoretic profile of plasmid DNA isolated from the four chlorpyrifos-degrading bacterial isolates. Lane 1: Lambda DNA digested with *Eco*RI and *Hind*III Lane 2-5: Plasmids isolated from RA-3, RA-5, RA-10 and RA-20, respectively

were resistant to methicillin. 7.5% of their isolates exhibited multiple resistance to 5 different antibiotics in 3 different combinations. Nawab *et al.* (2003) isolated *Pseudomonas* strains from agricultural soils of Aligarh (India), which were found to possess γ-HCH degrading ability when the isolates were grown in a mineral salt medium containing γ-HCH as the sole source of carbon. A number of metabolites were produced and detected by gas chromatography. They also reported that these isolates were also resistant to different antibiotics.

We have also screened the bacterial isolates for the presence of plasmid DNA. Figure 8 shows the agarose gel electrophoretic profile of plasmid DNA isolated from the chlorpyrifos degrading bacteria. All the bacterial isolates were found to contain a single plasmid.

Guha *et al.* (1997) also reported the involvement of plasmids in degradation of malathion and chlorpyrifos by *Micrococcus* sp., They confirmed the involvement of plasmid in the degradation of chlorpyrifos by performing the curing experiment with well known curing agents (acridine orange and SDS). They reported that the elimination of plasmid from the bacterial strains had led to the loss of the capacity of the strains to degrade and utilize malathion and chlorpyrifos as a sole source of carbon for growth. Malik *et al.* (2002) studied the plasmid incidence in bacteria from agricultural and industrial soils of India and reported higher incidence of plasmid harboring strains in industrial soil (91.4%) whereas, only 40% of the bacterial isolates from agricultural soil contained plasmids.

Our study reveals that the bacteria isolated from wastewater irrigated soils were capable of utilizing chlorpyrifos for their metabolism and growth. TLC analysis of crude extracts of the metabolites of the isolated bacteria indicates their chlorpyrifos degrading capability. The presence of plasmids in all the chlorpyrifos degrading bacterial strains is significant since it points to the fact that soil bacteria can exchange or acquire the chlorpyrifos metabolizing property from bacteria in its immediate environment or from the microbial flora associated with agricultural inputs like irrigation water i.e., wastewater in the case of the present study. These lead us to expect that the soil bacteria possess numerous enzyme systems capable of metabolizing and degrading the toxic organophosphorus compounds.

To our knowledge this is the first report of isolation of chlorpyrifos metabolizing bacteria from wastewater irrigated crop fields in India. These isolates or their enzymes may be useful in the detoxification of chlorpyrifos contaminated soil/effluents and may lead to the development of a possible bioremediation technology in the near future for reclamation of damaged habitats. However, extensive studies are still needed to delineate the exact molecular mechanism of chlorpyrifos degradation by these bacteria.

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