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# Analysis of chlorpyrifos degradation by *Kocuria* sp. using GC and FTIR

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## ABSTRACT

An organophosphate (OP) pesticide is the most popular type of pesticide family, which effectively eliminates pests owing to its acute neurotoxicity. Organophosphorus hydrolase (*oph*) is a bacterial enzyme that is capable of degrading a wide range of neurotoxic OP pesticides. It was proved that *Kocuria* species was capable of degrading organophosphate (OP) pesticide, chlorpyrifos. The present study was aimed at analysing its ability in degrading chlorpyrifos using Gas Chromatography (GC) and Fourier Transform Infrared **(**FTIR) techniques.

**KEY WORDS** : Chloropyrifos, Fourier transform infrared, Gas chromatography, *Kocuria* sp.

## INTRODUCTION

Organophosphate pesticides (OP) are a group of highly toxic agricultural chemicals widely used in plant protection. Their usage has become an indispensable tool in agriculture for the control of weeds, insects and rodent pests. They are poisonous but play an important role in generating plenty of food to the world population (Kurzel and Certrulo, 1981; Akhtar and Ahmed, 2002). Compounds of this family are spontaneously hydrolyzed and cause neurotoxicity in mammals (Sogorb and Vilanova, 2002). Excessive pesticide usage resulted in accumulation of pesticide residues in crops, soils, and biosphere creating an ecological stress (Qiao *et al.,* 2003). Chlorpyrifos is a broad spectrum systemic phosphorothioate ester insecticide patented and introduced by Dow Chemical Company in United States of America in 1965 (Murray *et al.,* 2001). Chlorpyrifos is available in granules, wettable powder, dustable powder, emulsifiable concentrate (Swathi and Singh, 2002) and used for the control of a wide range of pests such as cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, aphids, lice, leptinotarsa and other insects. It is applied to different crops including cotton, nuts, corn, fruits, vegetables, ornamental plants and is highly persistent in foliar application. Chlorpyrifos causes hazardous effects to the environment and also toxic to human beings resulting in headache, nausea, muscle twitching, convulsions, birth defects and even death. It is toxic to a variety of beneficial arthropods including bees, beetles and parasitic wasps. It kills fishes and birds in minute concentrations. Plants are affected by delayed seedling emergence, fruit deformities and abnormal cell division (Thomas and Nicholson, 1989; Richards and Baker, 1993; Giesy *et al.*, 1999; Ragnarsdottir, 2000; Wood and Stark, 2002; Galloway and Handy 2003). It has antimicrobial property, hence prevents the proliferation of chlorpyrifos degrading microorganisms in soil (Shelton and Doherty, 1997).

In light of its importance in agriculture and a need to degrade it in the environment, the present study has been taken up to analyze the ability of *Kocuria* species to degrade OP *in vitro* using GC and FTIR techniques.

## MATERIALS AND METHODS

### Isolation of chlorpyrifos degrading *Kocuria* sp.

Soil samples were collected in January 2008 from agricultural fields where commercial crops like tobacco and cotton were extensively grown in the West Godavari District of Andhra Pradesh, India lies in the Latitude 20.0 North and Longitude 77.0 East and chlorpyrifos (United Chemicals, Gujarat, India) pesticide was used intensively, by contemplating such soil would contain pesticide contamination and natural micro-flora experiencing pesticide stress. The samples were pooled together and collected into a sterile polythene bag and enriched by adding 1ml of chlorpyrifos for enrichment of the soil and subjected for serial dilution. These samples were screened and identified as *Kocuria* sp. at the molecular level which closely resembled *Micrococcus* by phylogenetic analysis (Nagavardhanam and Vishnuvardhan, 2012a).

### Cloning of *opd* gene of *Kocuria* sp.

The organophosphate degrading gene (*opd*) gene of *Kocuria* sp was ligated into a vector pMAL-c2X placing *opd* gene at the downstream of the constructive *tac* promoter and a recombinant vector '*opd* pMAL-c2X' was constructed**.** This recombinant vector *opd* pMAL-c2X was then transformed into competent *E.coli* DH5a and was grown in the presence of X-gal (5-bromo-4-chloro-indolyl-ß-D-galactopyranoside). Successful ligations of *opd* gene into vector pMAL-c2X and transformed *E. coli* DH5a with *opd* gene were detected by blue-white screening molecular technique. White bacterial colonies which indicate the successful ligation and such positive recombinant colonies were selected and screened (Nagavardhanam and Vishnuvardhan, 2012b)

### Analysis of the culture filtrates for Chloropyrifos degradation by Gas Chromatography

#### *Sample preparation*

The Culture flasks containing *Kocuria* sp. and the cloned culture of *E.coli* containing OPH of *Kocuria* sp. containing $10^5$ cells per inoculum were incubated in the presence of 3.84 g/l Chloropyrifos for 48 hours, where the organophosphorus hydrolase enzyme activity was maximum were filtered through pre-weighed Whatmann No.1 filter paper to separate the biomass. The supernatant was collected and was subjected for degradation studies by Gas Liquid Chromatography. The flasks without inoculants served as control (Kothari and Rathore, 1990).

#### *Extraction of pesticide residues*

The culture filtrate is taken in a bottle and is added with 100ml of acetone and n-hexane in 1:1 ratio. It was mixed thoroughly and was filtered through a filter paper with the help of a suction pump. Then the filtrate was allowed to evaporate and then the culture extract was concentrated after which the concentrated extract was added with 50ml of 15% Sodium Chloride solution and 50ml of Di-Chloro Methane (DCM), shaken thoroughly and was allowed to settle for 15-20 minutes. Then the DCM layer was collected in to a bottle through a funnel arranged with cotton and Sodium sulphate ($Na_2SO_4$), then 50ml of DCM was added to it again and that layer was collected through cotton and $Na_2SO_4$ funnel. Then the filtrate was allowed to evaporate completely. Then 10ml of acetone or n-hexane was added to the bottle and the residue was collected in to a vial. The same process was repeated twice with 5ml of acetone or n-hexane each time. Then the amount of pesticide in the samples was estimated through GC analysis (McMillin et al., 1997).

The samples were quantified by comparing peak heights with standards of known concentration. Percentages of recoveries are given in the respective data tables with corrections for internal standards were appropriate.

***Instrumentation employed***

The amount of the pesticide residue in the samples was analyzed through GC technique. Varian CP 3800 model Gas Liquid Chromatography was employed with VF-1 MS column of length 15 meters, thickness of 0.25 µm and 0.25 mm internal diameter. The carrier gas used was Nitrogen wherein the injection temperature was 260 $^0$C. The detector system used was electron capture detector (ECD) tritium source electron capture detector and a Tracor model MT-220. Electron capture GLC determinations employed two columns (0.64 cm by 1.8 m): one contained 2% OV-101 and 100/120-mesh Gas-Chrom Q; the other contained equal parts of 0.75% OV-17 and 0.85% OV-210 on 100/120 Gas-Chrom Q. Column, detector, and inlet temperatures were 185, 250, and 250°C, respectively. The carrier gas was nitrogen, used at a flow rate of 25 ml/min. Respective columns, detectors (ignited), and injector temperatures were 180, 160, and 220°C, and gas flow rates for 02, air, H2, and N2 (carrier) were 20, 50, 200, and 60 ml/min, respectively.

***Calculation of percentage of degradation of the pesticide***

- Amount degraded = original amount added-amount recovered
- % degradation = amount degraded×100÷amount added
- % recovered = amount recovered in the control ÷original amount added ×100

Recovery data for GLC analyses are given in the respective tables. All calculations for GLC data are based on known standards.

***Fourier Transform Infrared (FTIR) Spectroscopic analysis of Chloropyrifos degradation***

FTIR Spectroscopy is used in this study to obtain qualitative bond and functional group information for Chloropyrifos samples (xi Chen et. al., 2007). The non-destructive nature of FTIR additionally allows sample analysis by alternative techniques that were not explored in this particular study. This method protocol, however, was developed with consideration of possible future adaptation to include both quantification and coupled analytical techniques.

## RESULTS AND DISCUSSION

### Analysis of the culture filtrates for Chloropyrifos degradation by GC

In the present investigation, bioremediation of the pesticide, chlorpyrifos was carried out using pure culture of *Kocuria* sp. and the cloned culture in a shake flask under controlled environmental conditions. The GLC data showed that chlorpyrifos was degraded upto 52% in the cloned culture containing 3.84 g/l chlorpyrifos while in native *Kocuria* sp. containing 3.84 g/l chlorpyrifos, it was degraded upto 75%. Thus, the microorganism *Kocuria* sp. and the cloned culture were found to be well adapted to chlorpyrifos. The percentage of degradation was much more than the previous observations (Huang *et al.*, 2000; Baskaran *et al.*, 2003; Fulekar and Geetha, 2008) in both the test organisms with reference to the concentration of Chloropyrifos used. The study indicated that *Kocuria* sp. was able to degrade Organophosphorus pesticides more rapidly (Fig.1).

### Analysis of Chloropyrifos degradation using FTIR

FTIR was applied to the study of the biodegradation process of

organophosphorous pesticides. After degradation, the residual pesticides were extracted by solvents, and the solution was then concentrated. The contracted solution was scanned and analyzed by FT-IR. During the biodegradation of Chlorpyrifos, the IR peaks of C-Cl with 600–800 $cm^{-1}$ and C-H with 2991 $cm^{-1}$ were obviously dropped. It could be concluded that the cleavages of C-Cl and C-H bonds occurred, and chlorine was further converted into chloride. The IR spectrum for pure Chloropyrifos showed five major peaks at 767.58, 829.78, 1658.33, 1588.96 and 1486.06 $cm^{-1}$, respectively. Correspondingly, the first and second peaks were ascribed to C-Cl and 1, 4-benzene substitution, and the last three peaks were contributed by the benzene special vibration. The intensity of the major peaks decreased and even disappeared after the UV irradiation, which indicated the aromatic ring of Chloropyrifos was destroyed in the photocatalytic degradation (Fig. 2).

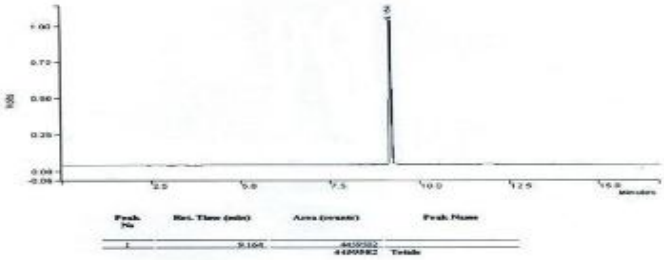

**Fig.1a: Control: Chloropyrifos without inoculum**

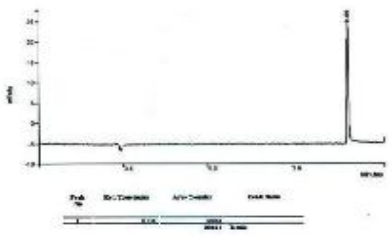

**Fig.1b: Kocuria sp. culture embedded with Chloropyrifos**

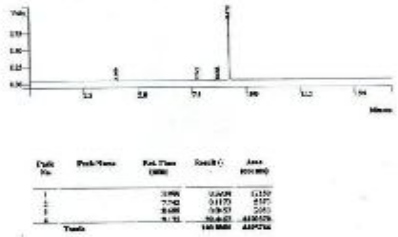

**Fig.1c: Cloned culture embedded with Chloropyrifos**

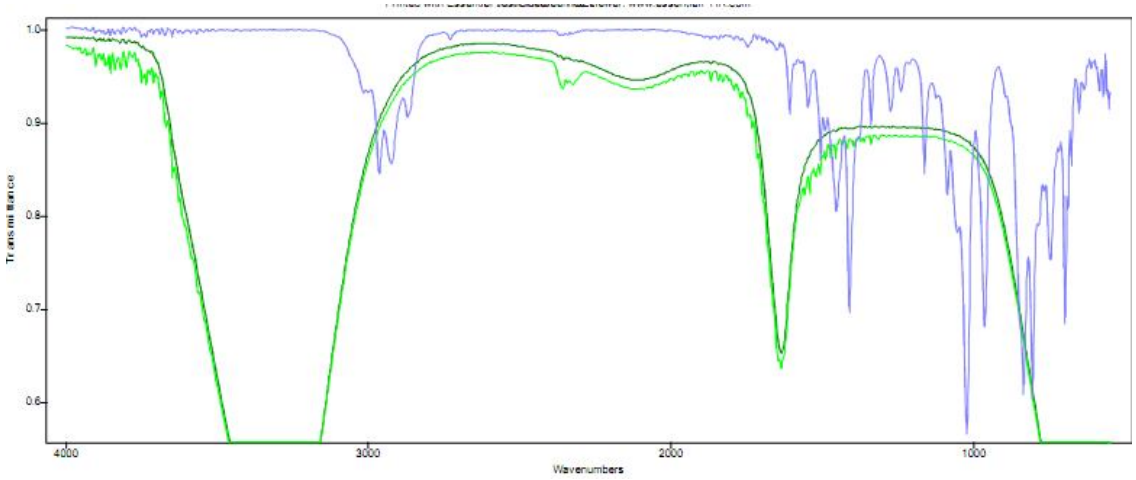


**Fig.2: FTIR spectra of Pesticide Degradation**

Blue : Pesticide – Chlorpyriphos; Light Green – Degraded Wild type; Green – Degraded Cloned

The results based on the peaks with GC and FTIR were in accordance with the previous studies on chloropyrofos degradation. A bacterium capable of degrading chlorpyrifos was isolated and it was *Alcaligenes faecalis* DSP3 (Yang *et al.,* 2005). The primary metabolite was found to be TCP. *Enterobacter* strain B-14 biotransformed chlorpyrifos up to TCP only (Singh *et al.,* 2004). A gene for organophosphorus hydrolase from *Flavobacterium* sp. and expressed it in *Synechococcus* PCC 7942 (Chungjatupornchai and Fa-Aroonsawat, 2008). Further, it was showed that this enzyme is located both on the surface as well as intracellular and also reported that phosphatases play an important role in the biodegradation of chlorpyrifos (Madhuri and Rangaswamy 2002; Thengodkar and Sivakami 2010).

The mechanism of chlorpyrifos degradation in bacteria and fungi is fairly understood and a number of degradation products such as diethylthiophosphoric acid, TCP, chlorodihydro-2-pyridone, dihydroxy pyridine, tetrahydro-2pyridone, and maleamide semialdehyde have been identified (Singh and Walker, 2006). The combined experiments of photolysis and microbial degradation, the degradation of TCP firstly into chlorodihydro2-pyridone by reductive dechlorination followed by its degradation into tetrahydro-2-pyridone and then to maleamide semialdehyde, which ultimately got mineralized into water, CO, and ammonium(Feng *et al.,* 1998). The occurrence of unidentified peaks in the chromatogram of present study may be byproducts of TCP, which needs further investigation.

## CONCLUSION

Organophosphorus hydrolase (*oph*) is a bacterial enzyme that is capable of degrading a wide range of neurotoxic OP pesticides. The present study proved that the percent degradation by *Kocuria* sp was higher when compared to the previous reports.

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