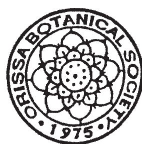
Plant Science Research 34 (1&2) : 17-22, 2012



Plant Science Research

ISSN 0972-8546

# Isolation and characterization of a chlorpyrifos degrading bacterium from rice soil

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ARTICLE INFO

*Article history:*
Received : 01 November 2012
Received in revised form : 02 December 2012
Accepted : 03 December 2012

*Keywords*:
chlorpyrifos
biodegradation
rice soils
bacteria
*Bacillus* sp.

ABSTRACT

The organophosphorus insecticide, chlorpyrifos has been widely used in agriculture, in veterinary against house hold pests, and in field agriculture for subterranean termite control. Due to its slow rate of degradation in soil it can persist for extended periods in soil with significant concern to the environment. A soil bacterium capable of utilizing chlorpyrifos as the sole source of carbon and energy was isolated from rice soil enriched with repeated application of chlorpyrifos (10 mg/kg). The strain named CRRI NF3, was preliminarily identified as *Bacillus* sp. based on its morphological, physiological and biochemical tests as well as 16s rRNA gene sequence analysis. Bioremediation of chlorpyrifos was examined using CRRI NF3 inoculated to the soil treated with 10 mg/kg chlorpyrifos and resulted in faster degradation than control soils without any inoculation. The optimum pH, temperature could have promoted degradation.

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## 1. Introduction

Organophosphorus (OP) compounds are used worldwide for pest control since 1937 (Dragun *et al.,* 1984) as substitute for highly persistent and toxic organochlorine pesticides. Organophosphorus insecticide chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinol) phosphate; trade name Dursban or Lorsban], is used worldwide as an agricultural insecticide (Chao *et al.,* 2002) in veterinary against house hold pests, and in field agriculture for subterranean termite control. Extensive use of chlorpyrifos has led to widespread environmental contamination resulting in damage to non-target species. In the environment, chlorpyrifos undergoes hydrolysis to tri-chloro pyridinol (TCP) and diethyl thiophosphoric acid (Racke and Coates, 1987). TCP is considered to inhibit further hydrolysis of chlorpyrifos resulting into its accumulation in the environment (Somasundaram and Coates, 1990). Studies indicated that wide range of water and terrestrial ecosystem might be contaminated with chlorpyrifos (EPA, 1997; Sapozhnikova *et al.,* 2004; Yang *et al.,* 2005) and demanded the public choice to establish an efficient, safe, and cost effective method to remove or detoxify chlorpyrifos residues from contaminated environment. In a singular report, Chlorpyrifos was degraded co-metabolically in liquid medium by *Flavobacterium* sp. ATCC 27551 (Mallick *et al.,* 1999) that was isolated form diazinon enriched soil planted to rice paddy (Sethunathan and Yoshida, 1973).

Recently, chlorpyrifos has been reported to be more amenable to biodegradation. Singh *et al.* (2004) isolated one *Enterobacter* sp. Strain B-14 which was isolated from contaminated soil of Australia and having the capability to hydrolyze chlorpyrifos. The bacteria hydrolysed chlorpyrifos and metabolized di-ethylthio-phosphate (DETP) as carbon, phosphorus and energy source for growth and proliferation. Yang *et al.* (2005) isolated a *Alcaligenes faecalis* strain DSP3 from chlorpyrifos contaminated soil around a chemical factory that was able to degrade both chlorpyrifos and its hydrolysis product TCP. Li *et al.* (2008) reported that seven

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bacterial isolates including *Sphingomonas* sp., *Stenotrophomonas* sp., *Bacillus* sp. and *Brevandimonas* sp. were capable of degrading chlorpyrifos using it as carbon and energy source. An aerobic bacterial consortium consisting of *Pseudomonas fluroscence*, *Bacillus melitensis*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*, developed from pesticide contaminated soil was able to degrade chlorpyrifos in liquid medium (Vidya Lakshmi *et al.,* 2008).

In the present study attempts were made to isolate, characterize and identify novel chlorpyrifos degrading bacteria capable of utilizing chlorpyrifos as the sole carbon and energy source, form rice soil enriched with chlorpyrifos and to assess the optimization of growth and degradation potential in liquid medium under the influence of different environmental factors like pH, temperature, etc.

## 2. Materials and methods

### 2.1 Soil

Soil from rice-growing areas of CRRI experimental field, was used in the study. The soil was air-dried in shade and ground to pass through a < 2-mm sieve before use. Soil sample was classified as alluvial and soil properties were as follows: sand, 52.5%; silt, 21.6%; clay, 25.9%; organic carbon content, 0.86%; cationic exchange capacity, 15.0 meq/100g soil; total nitrogen, 0.09%, and a pH of 6.21

### 2.2 Insecticide and chemicals

For studies under laboratory conditions and for greenhouse studies, a commercial formulation of 94% chlorpyrifos 20 EC (Force, Nagarjuna Chemicals, Hyderabad, India) was used. For degradation studies in culture medium, certified standard of chlorpyrifos (Dursban, AccuStandard Inc, New Haven, CT, USA) of 99.99% purity was used.

### 2.3 Enrichment of chlorpyrifos degrading bacteria

A mineral salt medium (MSM) (Mallick *et al.,* 1999) was used in preparing the enrichment of chlorpyrifos degrading bacteria from soils previously retreated with chlorpyrifos. Portions of 10 ml MSM containing chlorpyrifos (10 µg/ml) were inoculated with 100 µl suspension of soil from chlorpyrifos retreated flooded soil and incubated at room temperature ($30^0 \pm 2^0$C) on an orbital shaker at 110 rpm. After complete disappearance of chlorpyrifos from the inoculated medium in about 10 days, 1 ml of this medium was added to a fresh batch of 10 ml of MSM supplemented with chlorpyrifos as the sole source of carbon and energy and incubated for a further period of 5 days. This was repeated 4 times at 5 day intervals for selective enrichment of chlorpyrifos degrading bacteria. After 4th transfer, 1 ml sample was withdrawn aseptically from flask after 5 days incubation and analyzed for chlorpyrifos residue by GLC after the extraction with hexane. Un-inoculated medium served as control. Inoculated flask was incubated at room temperature at $30^0 \pm 2^0$C. After 5 days when chlorpyrifos disappeared completely, 100 µl sample from the culture was spread plated on MSM agar containing 10 µg/ml chlorpyrifos as the sole source of carbon and energy.

### 2.4 Characterization and identification of chlorpyrifos-degrading bacteria

The most efficient bacterial isolate growing on chlorpyrifos containing agar was subjected to morphological, cultural and biochemical characterization. The bacterial strain was further identified by using standard methods (Kreg and Holt, 1984), combined with 16S rDNA sequence analysis. Genomic DNA was isolated from bacterial cells grown overnight in MSM medium supplemented with glucose and peptone using standard CTAB method. The 16S rRNA gene was amplified by PCR using universal primers 5'-AGAGTRTGATCMTYGCTWAC-3' and 5'-CGYTAMCTTWTTACGRCT-3'. The purified PCR product was outsourced for sequencing of both the strands using ABI 3130 Genetic Analyzer and a Big Dye Terminator version 3.1"Cycle sequencing kit (ABI, USA). The determined sequence was compared with those available in the GenBank/EMBL database using the BLAST program (Altschul *et al.,* 1990). The nucleotide sequence coding for 16S rDNA sequence of the bacterium was deposited in the GenBank database with accession number.

### 2.5 Growth of chlorpyrifos-degrading bacteria vis-a-vis degradation of chlorpyrifos

One hundred µl of the bacterial suspension ($10^6$ cells/ml) was inoculated into MSM supplemented with 10 µg/ml chlorpyrifos and incubated for 3 days at room temperature at $30^0 \pm 2^0$C on a shaker at 110 rpm. At periodic intervals individual flask was sacrificed and the contents were used to determine the chlorpyrifos residues. All experiments were conducted in triplicate. Microbial growth was monitored by plate counting. Un-inoculated medium containing chlorpyrifos served as control.

### 2.6 Influence of various factors on chlorpyrifos degradation in liquid culture

To determine the effect of pH and temperature on biodegradation of chlorpyrifos by CRRI NF3, 10 ml of MSM supplemented with chlorpyrifos (10 µg/ml) was maintained at pH 6.0, 6.5, 7.0 and 7.5 at $30^0$C. To determine the influence of temperature on chlorpyrifos biodegradation experiments were conducted in 100 ml flasks containing 10 ml of the MSM supplemented with chlorpyrifos (10 mg/ml) at pH 6.2

and maintained at 25, 30 and 35 $^0$C respectively. All flasks were inoculated with $10^6$ cell/ml and incubated at room temperature of $30^0 \pm 2^0$C in a BOD incubator. At periodic interval triplicate of the flasks were sacrificed and analyzed for microbial growth and chlorpyrifos degradation.

*2.7 Degradation of chlorpyrifos in soil*

Soil from rice-growing areas of CRRI experimental field, was used in the study. Soil sample (40 g) was taken in a 250 ml conical flask and sterilized thrice on consecutive days and treated with chlorpyrifos (10 mg/kg). One set of soil was inoculated with CRRI NF3 ($10^8$ cells/ml) while another set without inoculation was kept as control. The inoculum was thoroughly mixed into the soil under sterile condition. The soil moisture was adjusted by the addition of sterile distilled water to 60% of its water holding capacity. The soil were incubated at room temperature in a laboratory condition. Chlorpyrifos was extracted from whole conical flask and determined by gas chromatography.

*2.8 Extraction and analysis of chlorpyrifos residues*

In the experiment on the degradation of chlorpyrifos in mineral salt medium, the whole flasks were sacrificed and extracted with equal volume (10 ml) of hexane dried over sodium sulfate (pinch of amount) and analyzed by GLC. Extraction of residues and sample preparation were done in the laboratory in diffuse light.

Chlorpyrifos residues extracted in hexane were analyzed by Perkin-elmer AutoSystem XL (USA) with electron-capture detector (ECD) for separation and quantitative analysis. The GLC fitted with metal packed column (2 m length, 3 mm OD) packed with 3% OV-17 on Chrome WHP 80/100 mesh. The operating conditions were as follows: carrier gas was nitrogen XL grade (99.99% purity), carrier flow was 20 ml/min, injector temperature was 240$^0$C, column temperature fixed at 220$^0$C, and detector temperature was 350$^0$C.Under these condition, the retention time of chlorpyrifos was 4.1 min. All the results presented are the mean of duplicate observations.

*2.9 Statistical analysis*

Mean and standard deviation of the data were calculated using Excel (Microsoft,USA). The kinetics data of all treatment were obtained using a first order model. Individual data sets on residues of chlorpyrifos was statistically analyzed using statistical package CropStat Ver. 7.2 (International Rice Research Institute, Philippines).

## 3. Results and discussion

*3.1 Isolation, selection and characterization of strains*

The degradation of chlorpyrifos was rapid in the CRRI soil after repeated application of chlorpyrifos (data not shown). A chlorpyrifos degrading bacterium was isolated by enrichment culture from such soil retreated with chlorpyrifos. The bacterial strain (coded CRRI NF3) was gram positive, aerobic and positive to oxidase and catalase activity. It was motile rod and its colony was white in colour. The biochemical characterization of the bacterium is given in table 1. Sequence analysis its 16S rRNA gene showed that strain CRRI NF3 was most closely related to *Bacillus* sp (GenBank Accession No JN592473). Bacterila speices belonging to genus *Bacillus* have previously been reported to degrade chlorpyrifos (Li *et al.,* 2008; Vidya Lakshmi *et al.,* 2008, Rani *et al.,* 2007, Anwar *et al.,* 2009), parathion (Yasuno *et al.,* 1965), methyl parathion and fenitrothion (Miyamoto *et al.,* 1966; Sharmila *et al.,* 1989, Ou and Sharma, 1989).

*3.2 Growth and degradation ability* Bacillus *sp. CRRI NF3*

Degradation pattern of bacterial strain was studied in the liquid culture medium with chlorpyrifos as the sole source of carbon. The time course of chlorpyrifos degradation by *Bacillus* sp. CRRI NF3 is shown in Fig.1, complete disappearance of 10 µg/ml was observed within 12 days with regular increment of bacterial population which could grow taking this insecticide as their energy sources.

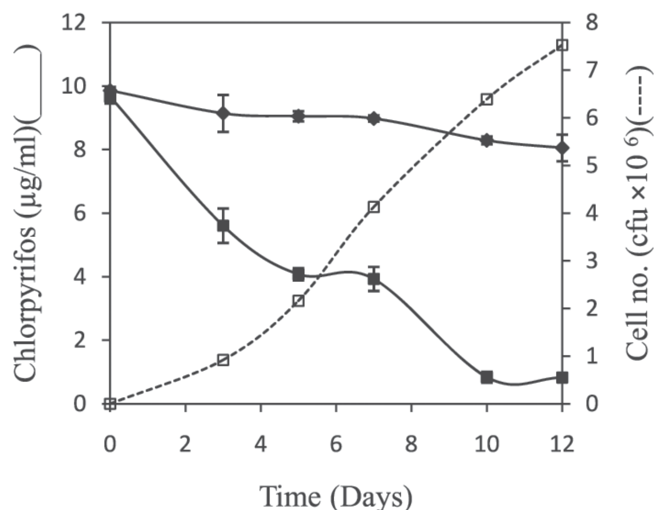


Fig. 1 Degradation of chlorpyrifos by strain CRRI NF3 in mineral salts medium. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: control (◆), CRRI NF3 (■), cell no. (□)

Table 1
Biochemical characterization of chlorpyrifos degrading strain CRRI NF3

| Characters | Isolate |
|---|---|
| Morphological | |
| Gram reaction | + |
| Cell shape | rod |
| Cell length | 2.0±0.1 |
| Colony color | White |
| Motility | + |
| Biochemical | |
| MR | + |
| MRVP | + |
| Nitrate reduction | + |
| Oxidase | + |
| Catalase | + |
| Tributyrin hydrolysis | + |
| Trehalose | + |
| Melibiose | + |
| ONPG | + |
| Esculin hydrolysis | + |
| D-arabinose | + |
| Malonate utilization | + |
| Dextrin | + |
| Gelatine | + |
| D-gluconic acid | + |
| Chemical sensitivity assay | |
| PH 6 | + |
| PH 5 | + |
| 1% NaCl | + |
| 4% NaCl | + |
| 8% NaCl | + |
| 1% Sodium lactate | + |
| Lithium chloride | + |
| Potassium tellurite | + |
| Aztreonum | + |
| Sodium butyrate | + |
| Sodium bromate | + |

*3.3 Effect of pH on biodegradation of chlorpyrifos*

The degradation patterns of chlorpyrifos in mineral salt medium at different pH are presented in Fig. 2. After 12 days of incubation at 30$^0$C the degradation rates of chlorpyrifos were found to varied with pH and there was corresponding variation of half-lives (Table 2). The hydrolysis percentages of chlorpyrifos were less than 5% in all controls. It is well known that the rates of base-catalysed hydrolysis for many organophosphorus insecticides are often greatly accelerated in water at pH values above 7.5 (Greenhalgh *et al.,* 1980). Wang *et al.* (2006) had also reported that the degradation rate of chlorpyrifos by *B. latersprorus* DSP in pure cultures was affected by higher as well as low pH.

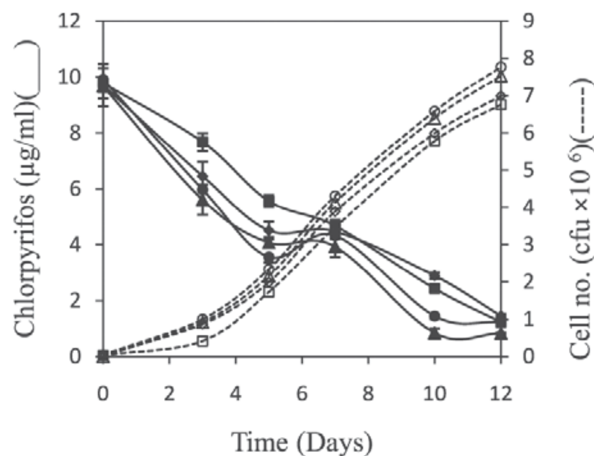


Fig.2 Effect of pH on degradation of chlorpyrifos in the mineral salt medium. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: CRRI NF3 at (■) 6.0, (▲) 6.5, (●) 7.0 and (◆) 7.5; Cell no. at (□) 6.0, (△) 6.5, (○) 7.0 and (◇) 7.5

Table 2
Kinetic data of chlorpyrifos degradation by the bacterial strain CRRI NF3

| Impact Factor | | | | |
|---|---|---|---|---|
| Chlorpyrifos concentration (µg.ml-1) | pH | Temperature ($^0$C) | $t_{1/2}$ (days) | $r^2$ |
| 10 | 6.0 | 30 | 4.12 | 0.9397 |
| 10 | 6.5 | 30 | 3.10 | 0.8707 |
| 10 | 7.0 | 30 | 3.93 | 0.9372 |
| 10 | 7.5 | 30 | 4.83 | 0.9412 |
| 10 | 6.5 | 25 | 5.80 | 0.9552 |
| 10 | 6.5 | 30 | 5.15 | 0.9291 |
| 10 | 6.5 | 35 | 3.91 | 0.9400 |

*3.4 Effect of temperature on biodegradation of chlorpyrifos*

The effect of different temperatures (25, 30 and 35 $^0$C) on chlorpyrifos biodegradation in mineral salts medium of

pH 6.5 is presented in Fig. 3. After 12 days of incubation, the degradation rate of chlorpyrifos at 25, 30 and 35$^{0}$C were examined to be 0.120, 0.135, and 0.177 and the corresponding half-lives were 5.80, 5.15 and 3.91, respectively (Table 2). Degradation rate at 35$^{0}$C was faster than lower temperature. The ANOVA analysis confirmed that half-lives of chlorpyrifos at 35$^{0}$C were significantly shorter than other two temperatures ($P < 0.05$). The results showed that the increase in temperature enhances the degradation rate of chlorpyrifos. Liu *et al.* (2003) reported that degradation of chlorpyrifos by *Aspergillus* sp. and *B. latersprorus* DSP (Wang *et al.,* 2006) was enhanced with the increase in temperature.

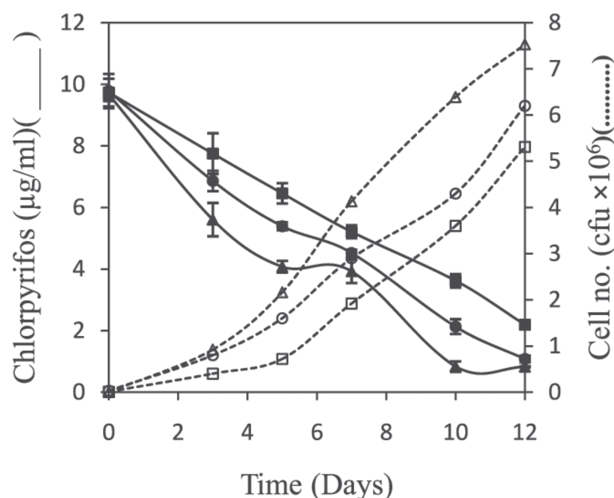


Fig. 3 Effect of temperature on degradation of chlorpyrifos in the mineral salt medium. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: CRRI NF3 at (■) 25$^{0}$C, (▲) 30$^{0}$C and (●) 35$^{0}$C; Cell no. at (☐) 25$^{0}$C, (△) 30$^{0}$C and (○) 35$^{0}$C.

### *3.5 Degradation of chlorpyrifos in laboratory soil*

Inoculation of CRRI NF3 to sterile field soil and incubated under laboratory conditions exhibited a more rapid degradation of chlorpyrifos than that by un-inoculated sterile soil (Fig. 4). The concentration of chlorpyrifos in inoculated flasks reduced to 2 mg/kg upon incubation for 25 days while in uninoculated soil, concentration of chlorpyrifos came down to only 8 mg/kg indicated enhanced degradation of chlorpyrifos upon inoculation with the chlorpyrifos-degrading *Bacillus* sp.

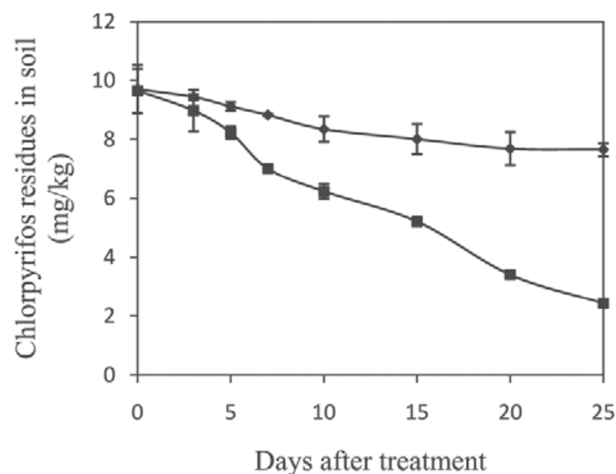


Fig.4 Degradation of chlorpyrifos by the bacterial strain CRRI NF3 on CRRI soil in the laboratory condition. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Legends: (◆) sterile soil suspension, (■) inoculated with CRRI NF3.

## 4. Conclusion

A *Bacillus* sp. degrading chlorpyrifos was obtained from a rice soil following enrichment by repeated addition of chlorpyrifos. Rice soil represents a unique ecosystem and acts as reservoir of unique microbial population. Rice plants with its actively growing roots released organic compounds into the rhizosphere that supported growth of the microbial community in the rhizosphere. The role of microorganisms specially *Bacillus* sp., was studied in the degradation of chlorpyrifos in liquid culture medium and in the soil. It is known that microorganisms are a major component of the ecosystem and play a considerable role in the degradation of several xenobiotics including chemical insecticide molecules. However, more research is needed to better understand the interactions among the rice plants, the rhizosphere bacterial communities and the population dynamics of the chlorpyrifos degrading bacterium.

**Acknowledgements**

This work was supported in part by the ICAR Networking Project, "Application of Microorganisms in Agriculture and Allied Sciences (AMAAS) - theme Agrowaste Management, Bioremediation and Microbes in Post-Harvest Processing" by the Indian Council of Agricultural Research, New Delhi.

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