RESEARCH ARTICLE

# Characterization of Chlorpyrifos Degrading Bacteria Isolated from Rhizosphere of *Oryza sativa* L.

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## Abstract

Chlorpyrifos is an organophosphate insecticide widely used for pest management for many decades. Due to its persisting nature and ill effects on living organisms, the clean-up of chlorpyrifos from polluted areas has become a major priority. Bioremediation is the preferred approach for this purpose. The study was undertaken to extract bacteria capable of digesting chlorpyrifos employing an enrichment technique. The bacteria were isolated from the soil around the rice-growing areas in Raichur and Koppal districts of Karnataka, India. The isolates were examined and classified based on their physical and chemical characteristics. Efficient isolates were obtained through screening at progressively higher doses of chlorpyrifos. The study also confirmed the plant growth-promoting potential of these isolates, as they produced indole-3-acetic acid (IAA), siderophores, and solubilized phosphate. The isolates were analyzed to determine the extent of chlorpyrifos degradation, which showed a maximum degradation rate of 61.3%. The findings of this research indicate that the strains capable of degrading chlorpyrifos have the capability to be a viable option for enhancing agricultural yields in soils polluted with pesticides.

**Highlights**

1. Eight chlorpyrifos-degrading bacteria were isolated from rice rhizosphere soils.
2. The isolates grew efficiently even at 600 ppm chlorpyrifos concentration.
3. CDB-19 degraded up to 61.3% of chlorpyrifos in 10 days.
4. The isolates produced IAA, siderophores, and solubilized phosphate.

**Keywords:** Chlorpyrifos; Rhizosphere; Enrichment; Screening; Characterization.

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## Introduction

The increased incidence of pests and disease attacks on crops has led to greater reliance on insecticides and pesticides (Vijaykumar *et al*., 2015). These chemical agents, used globally, are employed to control diseases, pests, or weeds, maintaining high-quality products. There are positive outcomes of pesticide application, such as improved food productivity and a significant reduction in vector-borne diseases (Moorman, 2018).

The use of pesticides has become essential despite their drawbacks. Environmental contamination resulting from pesticides and their degradation products is a significant ecological concern (Galloway and Handy, 2003). Pesticide residues persist in various environmental matrices, including air, soil, groundwater, and surface water, for varying durations (Chishti *et al*., 2013). Due to their prolonged persistence, bioaccumulative properties, and potential toxicity to non-target organisms, organochlorine pesticides have been largely replaced by less persistent organophosphorus (OP) compounds, which are still effective. The OP insecticides represent the largest group of pesticides used globally, accounting for approximately 38% of total pesticide usage worldwide (Singh *et al.,* 2006).

Despite being classified as moderately toxic, chlorpyrifos (CPF) is recognized for its neurotoxic and immunotoxic effects, posing risks to both humans and animals (Liu *et al.* 2009). The widespread application of pesticides poses a significant risk of severe soil contamination. CPF negatively affects soil microbial communities and disrupts nitrogen mineralization. The presence of CPF in surface water and associated sediments has raised substantial public concern, emphasizing the need for immediate attention and remediation efforts to address this issue. (Hamzah *et al.,* 2017).

Bioremediation harnesses the potential of microbial degradation to provide a cost-effective and reliable method for pesticide abatement (Pankaj *et al*., 2016). Numerous soil and aquatic ecosystems have been successfully restored from pesticide contamination through the use of microbes that break down these pollutants. It is an environmentally sustainable approach and an effective way to minimize the risk of secondary contamination. The process of pesticide bioremediation represents a natural and widespread phenomenon observed across various environments, including soils, surface and groundwater, and sewage sludge (Rayu *et al*., 2017).

Soil microorganisms, particularly those inhabiting the rhizosphere, play a crucial role in pesticide degradation. The

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rhizosphere of rice harbours a diverse microbial community capable of metabolizing xenobiotic compounds, including organophosphates, through enzymatic transformations (Lakshmi *et al*., 2008). Several bacterial genera, such as *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Burkholderia*, have been reported to possess chlorpyrifos-degrading capabilities, primarily mediated by enzymes such as organophosphorus hydrolase and phosphotriesterases. Understanding the diversity and metabolic potential of these bacteria is crucial for developing effective bioremediation strategies. Given the widespread use of chlorpyrifos and the associated risks to human health and the environment, we experimented to investigate chlorpyrifos degradation through biological methods to develop a potential cost-effective bioremediation technology.

## Materials and Methods

### Collection of samples

A survey was conducted for the selection of various sites of the rice-growing regions in the TBP command area of Hyderabad Karnataka. Raichur and Koppal districts are the two major rice-growing districts selected for the present study. Fourteen geographical locations were marked with a minimum of 10 km distance between each. Soil samples were collected and preserved for the isolation of bacteria.

### Physico-chemical analysis of soil samples

The measurement of soil pH and electrical conductivity is essential as it enables the assessment of soil fertility, availability of nutrients, the activity of microorganisms, and concentration of soluble salts in the soil. The samples were analyzed for their chemical properties like pH, EC, and organic carbon by following standard procedures as given by Piper (1966), Jackson (1973), and the wet oxidation method of Walkley and Black (1934), respectively.

### Isolation of bacteria

Minimal salts medium (MSM) amended with chlorpyrifos as the sole carbon source was used for isolating chlorpyrifos-degrading bacteria. The media has the following composition ($L^{-1}$): $KH_2PO_4$, 4.8 g; $K_2HPO_4$, 1.2 g; $NH_4NO_3$, 1.0 g; $MgSO_4.7H_2O$. 0.2 g; Ca $(NO_3)_2.4H_2O$, 0.04 g; and Fe $(SO_4)_3$, 0.001 g with pH 7.0 (Gireesh *et al.,* 2016).

The bacteria were isolated from the collected soil samples using the enrichment culture technique followed by serial dilution. The soil samples were serially diluted to $10^{-3}$ and plated on minimal salt agar plates amended with 200 ppm of chlorpyrifos. The plates were incubated at room temperature (30 ± 2°C) for 7 days. The colonies exhibiting prolific growth on the plates were selected and purified.

### Characterization of isolates

The selected isolates were examined for colony morphology, such as color, size, and form. Surface, cell shape, motility, and Gram reactions were carried out as per the methods outlined by Bartholomew and Mittewer (1950). The biochemical characterization of isolates was done as per the standard procedures outlined by Cappuccino and Welsh (2018). The isolates were also studied for plant growth-promoting traits, *viz*., production of IAA and siderophore and solubilization of mineral phosphate.

### *In-vitro* screening

The bacterial isolates were inoculated into flasks containing 100 mL MSM broth supplemented with 300 ppm of chlorpyrifos and incubated on a shaker incubator for seven days at 30°C. Later, one ml of an aliquot was drawn and the turbidity was measured. Simultaneously, 10 µL of the broth was diluted and plated on nutrient agar to verify the bacterial growth. The isolates exhibiting growth were further tested at higher concentrations of chlorpyrifos (400 and 600 ppm) to obtain efficient isolates.

For the quantitative estimation, the isolates were grown in MSM broth containing 200 ppm of chlorpyrifos. After 2 days of incubation, the cultures were centrifuged, washed, and then diluted with distilled $H_2O$. A cell concentration corresponding to $1 \times 10^7$ CFU $mL^{-1}$ was used to maintain uniformity. The flasks containing 100 ml of MSM supplemented with 200 ppm of chlorpyrifos were inoculated with bacterial cell suspension. The flasks were incubated at 30°C in a shaker incubator. Uninoculated flasks served as control. An aliquot of the culture was withdrawn at 2-day intervals and centrifuged for quantitative estimation of degradation as per the method outlined by Venugopal *et al*., (2012).

## Results

### Characterization of soil samples

The rhizosphere soil samples collected from 14 different locations in the TBP command area were analyzed for chemical properties, *viz.,* pH, electrical conductivity (EC), and organic carbon content, using standard protocols. The physicochemical analysis of the soil samples showed a pH range of 6.98 to 8.52, EC values from 0.37 to 0.89 $dSm^{-1}$, and organic carbon percentages ranging from 3.18 to 6.75% (Table 1).

**Table 1:** Chemical properties of rhizospheric soil samples collected from the Tungabhadra command area

| *S. No.* | *Location* | *pH* | *EC (dS $m^{-1}$)* | *OC (g $kg^{-1}$)* |
|---|---|---|---|---|
| 1 | Maliyabad | 7.85 | 0.57 | 5.73 |
| 2 | Yeragera | 7.60 | 0.38 | 5.70 |
| 3 | Mantralaya | 6.98 | 0.37 | 4.97 |
| 4 | Manavi | 7.82 | 0.55 | 3.18 |
| 5 | Siravar | 7.34 | 0.51 | 3.97 |
| 6 | Neer manavi | 7.73 | 0.55 | 3.65 |
| 7 | Kallur | 7.57 | 0.60 | 3.77 |
| 8 | Kapagal | 7.91 | 0.41 | 3.56 |
| 9 | Kavital | 8.10 | 0.68 | 5.16 |
| 10 | Sindhanur | 8.02 | 0.74 | 4.41 |
| 11 | Gangavati | 8.52 | 0.89 | 3.54 |
| 12 | Siddapur | 8.23 | 0.75 | 4.85 |

**Table 2:** Morphological characteristics of the chlorpyrifos degrading bacteria

| *Sl. No.* | *Isolate* | *Colony character* | *Gram reaction* | *Motility* |
|---|---|---|---|---|
| 1 | CDB-1 | Creamy white, circular, medium, smooth, flat | -ve, rod | Motile |
| 2 | CDB-2 | Yellow, irregular, large, smooth, flat | -ve, rod | Motile |
| 3 | CDB-3 | Creamy, circular, medium, smooth, convex | +ve, rod | Motile |
| 4 | CDB-12 | White, irregular, large, smooth, convex | +ve, rod | Motile |
| 5 | CDB-14 | Light yellow, circular, small, rough, flat | -ve, rod | Motile |
| 6 | CDB-16 | White, circular, small, smooth, raised | -ve, rod | Motile |
| 7 | CDB-17 | Creamy white, circular, large, smooth, flat | +ve, rod | Non-motile |
| 8 | CDB-19 | White, irregular, small, rough, spreading, raised | -ve, rod | Motile |

**Table 3:** Biochemical characteristics of the chlorpyrifos degrading bacteria isolated from rhizospheric soil of rice

| *Sl. No.* | *Isolate* | *1* | *2* | *3* | *4* | *5* | *6* | ***7*** | ***8*** | ***9*** | ***10*** | ***11*** | ***12*** | ***Tentative genus*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CDB-1 | + | + | - | - | - | + | + | - | - | + | + | + | *Pseudomonas sp.* |
| 2 | CDB-2 | + | + | - | - | - | + | + | - | - | + | + | + | *Pseudomonas sp.* |
| 3 | CDB-3 | + | + | - | - | + | + | + | - | + | + | + | + | *Bacillus sp.* |
| 4 | CDB-12 | + | + | - | - | + | + | + | - | + | + | + | + | *Bacillus sp.* |
| 5 | CDB-14 | + | + | - | - | - | + | + | - | - | - | + | + | *Pseudomonas sp.* |
| 6 | CDB-16 | + | + | - | - | - | + | + | - | - | + | + | + | *Pseudomonas sp.* |
| 7 | CDB-17 | + | + | - | - | + | + | + | - | + | + | + | + | *Bacillus sp.* |
| 8 | CDB-19 | + | + | - | - | - | + | + | - | - | + | + | - | Pseudomonas sp. |

1 - Starch hydrolysis, 2 - Catalase test, 3 - Urease activity, 4 - Methyl red test, 5 – Voges Proskauer test, 6 - Citrate utilization test, 7 - Denitrification test, 8 - Indole test, 9 - $H_2S$ production, 10 – Casein hydrolysis test, 11 - Gas production, 12 - Gelatin liquefaction

## Isolation and purification

Eight bacteria capable of degrading chlorpyrifos were isolated from collected rhizosphere soils. After 7 days of incubation at $30^0$C, the bacterial isolates showed prolific growth on MSM agar plates. The colonies showing distinct morphology were selected and purified by four-way streaking.

## Characterization of the isolates

The cell morphology, colony morphology, and gram reactions were studied for eight chlorpyrifos-degrading isolates. Among them, three isolates were found to be gram-positive, and the remaining five isolates were gram-negative. Microscopic observations showed that all eight isolates were rod-shaped, seven isolates were motile and one was non-motile (Table 2). Each isolate was identified according to specific characteristics described in Bergey's Manual of Systematic Bacteriology (1994).

The results of the different biochemical tests conducted for the bacterial isolates revealed that all 08 isolates were positive for starch hydrolysis, catalase activity, citrate utilization test, denitrification, gas production, and negative results for methyl red test and indole test. Three isolates tested positive and the remaining five scored negative for Voges-Proskauer and $H_2S$ production tests. One isolate showed negative and the remaining seven isolates showed positive results for the casein hydrolysis and gelatin liquefaction tests. The isolates were provisionally identified as *Pseudomonas* and *Bacillus spp* owing to their biochemical characteristics (Table 3).

## Elucidation of growth-promoting traits

Bacterial isolates degrading chlorpyrifos in culture medium with tryptophan precursor produced IAA, detected by Salkowaski's reagent using a spectrophotometer at $OD_{530}$. IAA production from the isolates ranged from 10.59 to 17.04 µg $mL^{-1}$, with CDB-19 producing the highest concentration, followed by CDB-1. The values of siderophore units ranged between 51.33 and 60.33%, as shown in Table 4. The isolate CDB-14 produced the highest siderophore units, followed by the CDB-1 and CDB-12. Similarly, all the isolates solubilized phosphate (as indicated by

**Table 4:** Plant growth promoting traits of chlorpyrifos degrading bacteria

| *Sl. No.* | *Isolate* | *IAA (µg $mL^{-1}$)* | *Siderophores* | *Phosphate solubilization* | |
|---|---|---|---|---|---|
| | | | | *Efficiency* | *Index* |
| 1 | CDB-1 | 15.59 | 59.33 | 124.97 | 1.25 |
| 2 | CDB-2 | 13.30 | 54.33 | 124.00 | 1.24 |
| 3 | CDB-3 | 12.47 | 51.33 | 113.64 | 1.14 |
| 4 | CDB-12 | 10.59 | 59.33 | 115.96 | 1.26 |
| 5 | CDB-14 | 15.48 | 60.33 | 113.64 | 1.35 |
| 6 | CDB-16 | 11.40 | 56.66 | 112.87 | 1.16 |
| 7 | CDB-17 | 14.51 | 54.66 | 103.96 | 1.14 |
| 8 | CDB-19 | 17.04 | 58.33 | 121.43 | 1.13 |

**Table 5:** Percent degradation of chlorpyrifos by efficient bacterial isolates

| Time of interval | CDB-1 | | CDB-14 | | CDB-16 | | CDB-19 | |
|---|---|---|---|---|---|---|---|---|
| | Conc. (ppm) | (%) | Conc. (ppm) | (%) | Conc. (ppm) | (%) | Conc. (ppm) | (%) |
| Initial | 200 | Nil | 200 | Nil | 200 | Nil | 200 | Nil |
| 2nd day | 185.8 | 7.1 | 183.4 | 8.3 | 193.4 | 3.3 | 185.2 | 7.4 |
| 4th day | 171.6 | 14.2 | 148 | 26.0 | 167.4 | 16.3 | 150 | 25.0 |
| 6th day | 136.4 | 31.8 | 124.4 | 37.8 | 142 | 29.0 | 119.6 | 40.2 |
| 8th day | 118 | 41.0 | 98.8 | 50.6 | 114 | 43.0 | 94.8 | 52.6 |
| 10th day | 99 | 50.5 | 82.6 | 58.7 | 88.8 | 55.6 | 77.4 | 61.3 |

the zone of solubilization) from insoluble tri-calcium phosphate when inoculated onto Pikovskaya's media plates. The highest solubilization efficiency was shown by CDB-1 (124.97%), followed by CDB-2, but the highest solubilization index was recorded in CDB-14.

### *In-vitro* screening

The data indicates that eight isolates were able to grow on the media supplemented with 300 ppm of CPF. Among the eight isolates exposed to 400 ppm, six isolates showed growth, which was confirmed by plating on nutrient agar. There was no retardation of growth observed at high concentrations, indicating that the isolates tolerated up to 600 ppm of chlorpyrifos. Four isolates, namely, CDB-1, CDB-14, CDB-16, and CDB-19, could grow at 600 ppm and were studied for quantitative assessment of chlorpyrifos degradation.

The total degradation of chlorpyrifos at the end of the 10th day was between 50.5 and 61.3%. The highest degradation was showed by CDB-19, followed by CDB-14 and CDB-16 (58.7and 55.6%, respectively). The lowest (2%) values were observed in control. The inoculation of CDB-19 resulted in a decrease of the CPF concentration from 200 to 185.2 ppm within 2 days. By the end of the 10th day, the concentration was reduced to 77.4 ppm (Table 5).

## Discussion

Chlorpyrifos degrading microbes can be isolated from various sources like soil, corals, municipal waste, agrochemical industry waste, wastewater, etc. (Li *et al.,* 2007). The perusal of the literature has revealed that chlorpyrifos degrading microorganisms have been extensively isolated from various sources like contaminated agricultural soils (Singh *et al*., 2004; Yang *et al*., 2005), from sludge and wastewater from pesticide manufacturing units (Lu *et al*., 2013). Previously, Ahmad and Khan (2011) isolated 53 rhizobacteria belonging to the genera viz., *Pseudomonas, Enterobacter,* and *Klebsiella* capable of tolerating pesticide concentration in the range of 400 to 3200 µg $mL^{-1}$.

Morphological and biochemical tests identified the isolated colonies. During the starch hydrolysis test, the plates flooded with iodine solution formed a blue-black color as an indication of starch-iodine complex formation. The clear zone around the colony indicates starch degradation by amylase. In the gelatin liquefication test, gelatin becomes solid below 22°C while the degraded form of gelatin remains liquid. In the casein hydrolysis test, casein imparts white color to the media, which, upon degradation by the caseinase enzyme, causes the media to loses color and become halo. During $H_2S$ production, a black ring in the medium is formed due to the conversion of ferrous sulfate to ferrous sulfide. Based on these biochemical tests, the isolates were identified as *Pseudomonas* and *Bacillus* species. The obtained results agree with the observations of several other workers. Bhagobaty and Mallick (2008) reported that four bacterial isolates belonging to the *Pseudomonas* genus tested positive for oxidase and negative for indole. Dilfuza (2005) also identified the isolated bacteria as *Pseudomonas* species based on the biochemical tests. Previously, Mehta *et al.* (2021) isolated and screened several microbes to obtain the efficient strains among which, *Kocuria assamensis* showed chlorpyrifos degradation of 71.3%.

The CPF-degrading bacterial isolates exhibited significant plant growth-promoting traits, reinforcing their potential role in sustainable agriculture. IAA production is well-documented for enhancing root development and nutrient uptake, while siderophore production aids in iron acquisition, benefiting plant health under nutrient-limited conditions (Glick, 2012). Phosphate-solubilizing bacteria, like those observed in this study, improve soil phosphorus bioavailability, thereby supporting plant growth (Rodríguez & Fraga, 1999). The tolerance to high CPF concentrations suggests metabolic adaptation and enzymatic degradation capabilities, which are previously linked to microbial bioremediation of pesticides (Cycon *et al*., 2009). The efficient CPF degradation observed aligns with reports of microbial enzymatic pathways that break down organophosphates, reducing environmental toxicity (Singh & Walker, 2006).

The substrate availability is a key factor determining the rate of degradation (O'Conner, 1994). Microbes need to adapt to the new habitat and require time to produce the necessary degradative enzymes (Mulbry *et al*., 1996). This might cause a prolonged lag phase at high concentrations of chlorpyrifos. Biodegradation of different pesticides at high concentrations has been reported previously (Affam and Chaudhuri, 2013; Karpouzas *et al*., 2000). High concentrations of pesticides can stress bacteria, potentially slowing their growth. The paddy soil in this study had been exposed to continuous chlorpyrifos application for several years, which may have led to bacterial tolerance. Biodegradation of pesticides by the addition of bacteria has been reported for several compounds, including DDT (Nadeau *et al*., 1994), Monochrotophos (Acharya *et al*., 2015), and Carbofuran.

## Conclusion

Bioremediation techniques are more cost-effective than traditional methods. They allow for in situ treatment of contaminated sites, making the process safe and straightforward for handling harmful xenobiotics. This study identifies efficient bacteria viz., CDB-14, CDB-16, and CDB-19 capable of degrading chlorpyrifos and thriving at high concentrations (600 ppm). These isolates can degrade nearly 60% of the chlorpyrifos within 10 days, which makes them suitable candidates for biodegradation studies in vivo. In addition to their primary functions, the isolates display plant growth-promoting characteristics, supporting

their potential as beneficial inoculants. Their robust metabolic potential and adaptability to contaminated environments highlight their promise in large-scale field applications.

## Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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