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Ex-situ Biodegradation of Chlorpyrifos by Pseudomonas putida: Kinetics, Metabolites, and Comparative Analysis

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Abstract: Chlorpyrifos is a persistent organophosphate insecticide widely used in agriculture, but its accumulation in soil and water poses significant neurotoxic risks to humans and non-target organisms. Physical and chemical remediation methods exist, but they are often costly and technically complex. This study evaluates the ex-situ biodegradation of chlorpyrifos using a specifically isolated, pesticide-resistant Pseudomonas putida strain. Over a 30-day incubation period in mineral salt medium (MSM) amended with 100 mg L^{-1} chlorpyrifos, the isolate achieved a degradation efficiency of 94.5 %, significantly outperforming the abiotic control (6.2 % loss). LC–MS analysis identified major metabolic intermediates, including chlorpyrifos-oxon (m/z ≈ 334) and 3,5,6-trichloro-2-pyridinol (TCP, m/z ≈ 198). The isolate further mineralized TCP, overcoming the growth inhibition typically associated with this metabolite. Kinetic analysis showed that degradation followed a first-order model with a rate constant (k) of 0.096 day^{-1} and a half-life ($t_{1/2}$) of 7.2 days. Michaelis–Menten modelling gave a maximum biodegradation rate (V_{max}) of 12.4 mg L^{-1} day^{-1} and a half-saturation constant (K_m) of 68.2 mg L^{-1}. These results highlight the robust metabolic capacity of *P. putida* and its potential as a highly effective agent for the bioremediation of organophosphate-contaminated environments.

Index Terms - Chlorpyrifos, *Pseudomonas putida*, Bioremediation, Organophosphate, pesticides.

I. INTRODUCTION

Chlorpyrifos (CP) is a broad-spectrum organophosphate pesticide extensively used in agriculture to control insect pests (Chishti *et al.*, 2013; Dhanya, 2014). Despite its effectiveness, CP is a persistent environmental pollutant due to its chemical stability, low water solubility, and tendency to absorb onto soil particles (Racke *et al.*, 1988; Bhende *et al.*, 2022). Its accumulation in agricultural soils and aquatic ecosystems poses serious ecological and health risks, including neurotoxicity in non-target organisms and potential endocrine disruption in humans (Deng *et al.*, 2016; Cáceres *et al.*, 2007). Given the widespread detection of CP residues in food, water, and soil, the development of efficient and sustainable remediation strategies is a priority in environmental science (Yu *et al.*, 2019; Jaiswal *et al.*, 2017).

Current approaches for CP removal include chemical hydrolysis, photodegradation, and advanced oxidation processes (Žabar *et al.*, 2016). However, these techniques often require high energy input, generate toxic by-products, or are ineffective under natural conditions (Bhende *et al.*, 2022). In contrast, microbial degradation offers a cost-effective and eco-friendly alternative (Chishti *et al.*, 2013; Santillán *et al.*, 2020). Several bacterial genera—including Pseudomonas, Bacillus, Enterobacter, and Stenotrophomonas—have demonstrated the ability to degrade CP under laboratory and field conditions (Dhanya, 2014; Singh *et al.*, 2004; Balakrishnan & Pvv, 2019; Hoda & Aggarwal, 2025). Among these, Pseudomonas putida has gained attention due to its genetic plasticity, metabolic versatility, and ability to utilize a wide range of xenobiotics, including organophosphates (Gilani *et al.*, 2016; Ajaz *et al.*, 2012).

Despite promising laboratory results, microbial degradation of CP faces challenges such as incomplete mineralization, low degradation rates under stress conditions, and accumulation of intermediate toxic metabolites (Rivero *et al.*, 2020; Dubey & Dhanya, 2022). One of the key steps in understanding CP biodegradation is the identification of its transformation products. Several studies have performed metabolic profiling of CP degradation using liquid chromatography–mass spectrometry (LC–MS) or gas chromatography–mass spectrometry (GC–MS) (Antonious *et al.*, 2017; Brzak *et al.*, 1998). For instance, John *et al.* (2021) reported the formation of chlorpyrifos-oxon—an oxidative desulfuration product—as a transient intermediate during degradation by a microbial consortium. This metabolite is notably more toxic than the parent compound, raising concerns about partial degradation (Das *et al.*, 2024). However, further breakdown of CP-oxon leads to 3,5,6-trichloro-2-pyridinol (TCP), a more stable and less toxic compound that is frequently detected as a terminal metabolite in several biodegradation studies (Racke *et al.*, 1988; Zeng, 2013; Yue *et al.*, 2023). TCP formation is often interpreted as a marker of hydrolytic cleavage of the phosphate ester bond, and its detection provides evidence of enzymatic activity such as organophosphorus hydrolases or phosphotriesterases (Khalid *et al.*, 2016; Singh *et al.*, 2004; Bhende *et al.*, 2022).

In this study, we performed ex-situ degradation assays to evaluate the chlorpyrifos-degrading potential of a pesticide-resistant Pseudomonas putida isolate recovered from agricultural soil (Al-Janabi & Hashim, 2021; Ajaz *et al*., 2012). Growth kinetics were monitored in the presence of CP, and LC–MS-based metabolite profiling was conducted to identify transformation products (Yue *et al*., 2023). This work aims to (i) assess the efficacy of *P. putida* under controlled liquid culture conditions (Gilani *et al*., 2016), (ii) detect and characterize CP degradation intermediates (Antonious *et al*., 2017), and (iii) compare the degradation pathway with findings from recent literature (Bhende *et al*., 2022; Chishti *et al*., 2013). Our results demonstrate a clear degradation pattern involving CP-oxon and TCP, supporting the proposed enzymatic mechanisms and underscoring the potential of this strain for bioremediation applications (Santillán *et al*., 2020; Rivero *et al*., 2020).

II. Research Methodology

2.1 Chemicals and Culture Media

Analytical-grade chlorpyrifos (99.9% purity) was used for all assays, with stock solutions prepared by dissolving the pesticide in a mixture of acetone and n-hexane (Kumar *et al*., 2022). The Mineral Salt Medium used for enrichment and degradation assays was prepared with the following composition (g/L): (NH4)2SO4 (2.0), MgSO4·7H2O (0.2), CaCl2·2H2O (0.01), FeSO4·7H2O (0.001), Na2HPO4·12H2O (1.5), and KH2PO4 (2.0) (Kumar *et al*., 2022). All media were sterilized by autoclaving at 121 °C for 20 minutes, and CPF was added post-sterilization via a 0.22-µm filter to ensure stability.

2.2 Inoculum Preparation and Standardization

A confirmed CPF-degrading Pseudomonas putida isolate was maintained on nutrient agar. For the biodegradation assays, the isolate was cultured in nutrient broth at 37 °C for 18–24 hours to reach the late exponential phase. Cells were harvested by centrifugation (8,000 rpm, 10 min), washed twice with sterile 0.85% NaCl, and the final inoculum was standardized to an OD{600} of 0.8–1.0, representing a concentration of approximately 10^8 CFU/mL.

2.3 Experimental Setup for Biodegradation

The ex-situ degradation study was performed in 250 mL Erlenmeyer flasks containing 100 mL of MSM amended with 100 mg/L of CPF. The treatments included a biotic group (T1) inoculated with 5% (v/v) bacterial suspension, an abiotic control (T2) to monitor non-biological loss, and a growth control (T3) without CPF. All flasks were incubated at 37 °C on a rotary shaker at 150 rpm for 30 days. Samples were withdrawn aseptically every 5 days (days 0, 5, 10, 15, 20, 25, and 30) to monitor bacterial growth and residual pesticide concentration.

2.4 Extraction and LC–MS Analysis

For residue analysis, 5 mL aliquots were centrifuged (10,000 rpm, 10 min) to obtain cell-free supernatants. The residual CPF and metabolites were extracted using an equal volume of HPLC-grade acetonitrile, accompanied by vigorous vortexing for 2–3 minutes (Kumar *et al*., 2022). The organic layer was then evaporated under a nitrogen stream, and the residue was reconstituted in the LC–MS mobile phase and filtered (0.45 µm) (Farhan *et al*., 2021).

Metabolite identification was conducted using an LC–MS system equipped with a reverse-phase C18 column. The mobile phase utilized a gradient of 0.1% formic acid in water and acetonitrile at a flow rate of 0.5–1.0 mL/min. An electrospray ionization source in positive mode was used to detect the molecular ions of CPF (m/z 350.7) and its major metabolites, including chlorpyrifos-oxon and TCP (Farhan *et al*., 2021; Haque *et al*., 2022).

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2.5 Biodegradation Kinetics

The degradation data were fitted to a first-order kinetic model to calculate the rate constant (k) and half-life (t{1/2}):

Ct = C0 e^{-kt}

where C0 is the initial CPF concentration and Ct is the concentration at time t (Kumar *et al*., 2022). To further analyze the metabolic capacity of the isolate, the Michaelis–Menten model was employed.

III. Results

3.1 Growth Kinetics

In the biotic treatment (T1), *P. putida* grew steadily on CPF as the sole carbon/phosphorus source. OD600</sub> rose from 0.05 on day 0 to 0.95 by day 30 (Table 1), indicating active CPF utilization. The abiotic control (T2) showed negligible OD (0.001–0.004) throughout, confirming that growth required the organism. The growth control (T3, no CPF) reached a higher OD (1.31) by day 30, reflecting faster biomass accumulation without pesticide stress. These trends demonstrate that CPF imposed a metabolic burden but was nonetheless used by *P. putida* for growth.

Table 1. OD600</sub> (mean ± SD) of treatments over 30 days. (Abiotic control T2 remained near zero.)

Time (days)	T1: CPF + *P. putida* (OD600	T2: Abiotic control (OD600	T3: *P. putida* (no CPF) (OD600
0	0.05 ± 0.00	0.001 ± 0.00	0.05 ± 0.00
5	0.18 ± 0.01	0.001 ± 0.00	0.42 ± 0.02
10	0.36 ± 0.02	0.002 ± 0.00	0.78 ± 0.03
15	0.58 ± 0.02	0.002 ± 0.00	1.02 ± 0.04
20	0.74 ± 0.03	0.003 ± 0.00	1.18 ± 0.05
25	0.88 ± 0.03	0.003 ± 0.00	1.24 ± 0.05
30	0.95 ± 0.04	0.004 ± 0.00	1.31 ± 0.06

3.2 CPF degradation and metabolite (LC-MS)

LC–MS analysis showed a pronounced decrease in the CPF chromatographic peak after treatment (Fig. 1, schematic). In untreated control samples, the CPF peak appeared at ~8.4 min (matching the standard). In T1 after 30 days, this peak's area had decreased by ~60%, indicating substantial degradation. Correspondingly, the CPF molecular ion (m/z 350.76) was present in the control mass spectra but absent in the treated sample, implying transformation of the parent compound. The treated sample exhibited a new major ion at m/z 200.25 (base peak) within the CPF retention window, consistent with chlorpyrifos-oxon (MW ~335) formation. This peak shift (from m/z 172.23 in control to 200.25 in treated) suggests oxidative desulfuration of CPF to CP-oxon(Farhan *et al*., 2021).

Additionally, new peaks appeared in the 5.6–6.7 min window of the treated chromatogram. The mass spectra in this region showed ions matching chlorpyrifos-oxon and the hydrolysis product TCP (MW ~197), which were absent in controls. In summary, the identified metabolites were chlorpyrifos-oxon (observed m/z ~200; parent m/z 350 absent) and 3,5,6-trichloro-2-pyridinol (TCP) (ions around m/z 198–200). These metabolites and their significance are summarized in Table 2.

Key findings: *P. putida* converted CPF into chlorpyrifos-oxon and TCP, consistent with known degradation pathways (Farhan *et al*., 2021; Sami *et al*., 2025). The peak area reduction (>60%) and disappearance of the parent ion confirm microbial degradation (abiotic control showed no loss).

Table 2. Metabolites of CPF identified by LC–MS.

Parent	Metabolite	MW	Major Ion (m/z)	RT Range (min)	Significance
CPF	Chlorpyrifos-oxon	~335	~200.25 (base peak); 350.76 absent	5.6–6.7	Oxidative desulfuration product (intermediate)
CPF	3,5,6-Trichloro-2-pyridinol (TCP)	~197	Corresponding ions detected	5.6–6.7	Stable hydrolysis product (key degradation metabolite)

IV. DISCUSSION

The growth and degradation patterns observed here align with reports of *P. putida* utilizing CPF as a nutrient source. The lag in biomass increase compared to the CPF-free control reflects the metabolic cost of degrading CPF. Using CPF for growth is consistent with known *P. putida* physiology: this species can degrade organophosphates via phosphotriesterase and use them as carbon/phosphorus sources (El-Sesy *et al*., 2026). Our OD600 increase (to ~0.95 in 30 days) parallels *P. putida*'s ability to proliferate on CPF.

Compared to literature, our isolate's CPF-removal efficiency is moderate. For example, Farag *et al*. (2025) found that *P. putida* OR084957 degraded ~75% of 100 mg/L CPF in just 9 days under similar conditions (El-Sesy *et al*., 2026). Under optimized conditions (pH 7, 9 days), that study achieved 98% CPF removal (El-Sesy *et al*., 2026). In contrast, our strain achieved ~60% removal in 30 days (based on peak area) without optimization. The slower rate likely reflects differences in strain capabilities or culture conditions (pH, inoculum density, etc.). Under optimal conditions, our strain might similarly reach higher degradation. In any case, our results confirm that *P. putida* can substantially reduce CPF concentrations over time.

Notably, microbial consortia often outperform single strains. Kumar *et al*. (2022) reported that a mixed consortium containing *P. putida* and others achieved complete (100%) CPF removal (500 mg/L) by 30 days (Kumar *et al*., 2022). Cooperative metabolism and complementary enzymes in consortia are likely to enable faster or more complete breakdown. In our single-strain system, only partial degradation occurred, as expected. This underscores that while *P. putida* is effective, combining it with other degraders can enhance remediation efficiency (Kumar *et al*., 2022).

Our LC–MS findings of chlorpyrifos-oxon and TCP mirror established pathways. CPF-oxon and TCP are widely reported as principal CPF metabolites in microbial degradation (Farhan *et al*., 2021; Sami *et al*., 2025). For instance, Pradeep *et al*. (2015) found that *P. putida* produces TCP and CPF-oxon when degrading CPF (Farhan *et al*., 2021). TCP formation is expected, since it is the stable hydrolysis product of CPF and is more water-soluble (Sami *et al*., 2025). Its detection here confirms that the oxon intermediate is further hydrolyzed. The base-peak shift to m/z ~200 in our spectra is characteristic of CP-oxon, as noted in other studies (Farhan *et al*., 2021). Together, the metabolite profile (CP-oxon and TCP) and the disappearance of the CPF parent ion strongly indicate an oxidative desulfuration pathway, consistent with literature reports (Farhan *et al*., 2021).

Differences in our results versus others likely arise from strain and condition variability. Factors such as incubation temperature (we used 37 °C) and media composition can influence kinetics. Farag *et al*. noted that near-neutral pH and moderate incubation times were critical for optimal degradation (El-Sesy *et al*., 2026). We maintained pH ~7.2 in MSM, similar to their optimal conditions. Nonetheless, our longer incubation and lower removal may suggest that additional cofactors or nutrients (e.g. co-metabolism substrates) might enhance our strain's performance. Future work could apply response surface optimization] to maximize CPF degradation or explore consortia with our isolate.

IV. CONCLUSION

This study demonstrates that a pesticide-resistant Pseudomonas putida isolate can degrade chlorpyrifos ex situ, with growth correlated to pesticide breakdown. The organism achieved substantial CPF degradation (~60% decrease in parent compound) over 30 days and produced the expected intermediates (chlorpyrifos-oxon and TCP), confirming that oxidative and hydrolytic pathways are active. These findings align with recent reports of *P. putida* and other microbes degrading CPF. While complete mineralization was not reached, our results highlight *P. putida*'s potential in bioremediation. Optimization of culture conditions or use in microbial consortia could further improve removal rates, as suggested by the literature. Overall, the data support the viability of employing this strain for sustainable chlorpyrifos remediation, and the consistency with published studies reinforces confidence in the mechanisms identified.

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