

RESEARCH ARTICLE

Characterization of Chlorpyrifos-degrading bacteria from agricultural farmland

Sanjo Olaosun^{1,*}, Toyin Olayemi Agbabiaka¹, Najeem Adekunle Abdulraheem¹ & Gbadebo Monday Elebiyo²

¹ Department of Microbiology, Faculty of Life Science, University of Ilorin, Nigeria

² Department of Crop and Soil Science, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

* Corresponding Author, Email: 22-68fc004pg@students.unilorin.edu.ng

Received 13 February 2026 | Accepted 24 August 2026 | Published online 23 October 2026

Abstract

Chlorpyrifos is one of the frequently used pesticides with an immediate or long term adverse effect on human health and the ecosystem functioning. This study investigated the chlorpyrifos degradation capability of bacteria isolated from agricultural soil. Screening and identification of chlorpyrifos-degrading bacteria showed five isolates (*Pseudomonas monteilii*, *Moraxella* sp., *Enterobacter* sp., *Pseudomonas aeruginosa*, and *Staphylococcus* sp.) using enrichment culture technique, cultural morphology, biochemical tests, and molecular analysis. Biodegradation experiments were conducted in mineral salt medium supplemented with chlorpyrifos as the sole carbon source over a 24-day incubation period. Bacterial growth was monitored spectrophotometrically, while degradation was evaluated using High-Performance Liquid Chromatography analysis. Residual chlorpyrifos and degradation metabolites were characterized by gas chromatography–mass spectrometry. Highest optimal growth response was observed on Day 12 for all isolates with the highest seen in *Pseudomonas monteilii* (0.93 ± 0.01) while *Moraxella* sp. had the least (0.41 ± 0.35). *Pseudomonas monteilii* had the highest degradation rate (57 %) with $t_{1/2}$ of 19.71 days while *Moraxella* sp. had the lowest (18 %) with $t_{1/2}$ of 83.83. *Pseudomonas aeruginosa*, *Enterobacter* sp., and *Staphylococcus* sp. also showed considerable degradation rate of 33 %, and 29 % and 26 % respectively. Mass fragments detected in the control and bacterial-treated samples following chlorpyrifos degradation showed principal ions corresponding to chlorpyrifos and its major metabolites, 3,5,6-trichloro-2-pyridinol and diethyl phosphorothioate in all samples further established biotransformation process. The findings demonstrate that bacterial isolates, particularly *Pseudomonas monteilii*, could serve as promising candidates for the bioremediation of chlorpyrifos-contaminated environment.

Keywords Organophosphate | Chlorpyrifos | Microorganisms | Degradation | Human health | Environment

1 Introduction

The overuse of pesticides and herbicides is one of the most prevalent agricultural practices in Nigeria. These insecticides frequently have detrimental impacts on the ecosystem functions (Adebayo et al., 2023). Chlorpyrifos

(CP) is a member of the class of pesticides known as organophosphates. Farmers employ CP which is a white, crystalline, odoriferous insecticide to eradicate pests including worms and insects. Massacre (20 % Chlorpyrifos), Kolar (20 % Chlorpyrifos Emulsifiable Concentrate (EC)), Terminator (50 % Chlorpyrifos/5 %



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Cypermethrin), Predator (50% Chlorpyrifos EC), Perfect Killer (20% Chlorpyrifos EC), and ACT FORCE 55 (35% Chlorpyrifos + 10% Cypermethrin EC) are popular brands of chlorpyrifos used in Nigeria (Fabunmi & Adogame, 2022). Environmental related long term exposure to chlorpyrifos can pose significant health risks on food safety, consumers and agricultural workers (SRADeV Nigeria, 2020). CP specifically targets the neurological system by blocking the enzyme cholinesterase, which is essential for nerve coordination, researches have shown that it is somewhat harmful to people (Jaiswal et al., 2017). CP has been shown to further disrupt gene transcription, DNA synthesis, neurotrophin signaling cascade, and synaptic function (Sherif et al., 2025). On August 18, 2021, the U.S. Environmental Protection Agency (EPA) announced a ban on the use of chlorpyrifos on food crops in the United States due to its reported toxicity nature (Trager, 2021). Prolonged use of chlorpyrifos can affect soil productivity profile by altering nutrient cycling, microbial populations, plant growth and the ecosystem (Wolejko et al., 2022).

The degradation of chlorpyrifos is often through photo-degradation, bio-degradation by microorganisms, and chemical methods (Dafale et al., 2022). Chlorpyrifos is broken down via aforementioned routes to persistent toxic metabolite such as 3,5,6-trichloro-2-pyridinol (TCP) and DETP (di-ethyl-thio-phosphoric acid) (Kumar et al., 2018; Farhan et al., 2021). Most of the reported studies on chlorpyrifos degradation usually resulted to microbial transformation of chlorpyrifos into toxic metabolites that can have similar toxicity as the parent compound (Aswathi et al., 2019). While numerous studies have reported the degradation of chlorpyrifos through photodegradation, biodegradation, and chemical processes, a major challenge remains in identifying bacterial isolates capable of completely mineralizing the pesticide without producing toxic or persistent intermediate metabolites. Existing research has focused on bacterial strains isolated from temperate or non-African environments, creating a significant gap in knowledge regarding the degradative potential and functional diversity of indigenous bacterial communities in Nigerian agricultural soils, which are influenced by unique climatic conditions, soil physicochemical characteristics, and pesticide usage practices. Furthermore, while the adverse effects of chlorpyrifos on human health and non-target organisms are well documented, relatively few studies have integrated assessments of both bacterial degradation efficiency and the toxicological properties of the metabolites generated during degradation. Such combined evaluation is essential to determine whether a bacterial isolate is a truly effective and

environmentally safe bioremediation agent rather than one that merely transforms the pesticide into more harmful compounds. Therefore, there is a pressing need to isolate, characterize, and evaluate chlorpyrifos-degrading bacteria from Nigerian agricultural soils, with emphasis on both degradation performance and the toxicity profile of the resulting metabolites.

2 Materials and methods

2.1 Sample collection

Soil samples were collected from three agricultural farms (Location A: 8.51178°N, 4.599215°E ; B: 8.510855°N, 4.599485°E ; C: 8.510712°N, 4.599842°E) with history usage of chlorpyrifos (>6 years) within Lower Niger River Basin Authority, Ilorin, Kwara state, Nigeria. Composite samples were obtained by combining subsamples taken from the four corners and centre of each farm plot to reduce spatial heterogeneity and measurement bias (Arias-Navarro et al., 2013). Soil samples were collected on the 24th November, 2024 (start of dry season) at a depth of 0-15cm and moved immediately to the Laboratory of the Department of Microbiology, University of Ilorin, Kwara state, Nigeria using a sterile labeled polyethylene bag for analysis.

2.2 Pesticide used

Commercial grade of Chlorpyrifos containing 20 g/L of the active ingredient was used since it is often used at the same location where samples were gotten. The pesticide with brand name Perfect Killer was purchased from Oja-Oba market Ilorin, Kwara state, Nigeria.

2.3 Media used

The mineral salts medium (MSM) used in this study was composed of (g/L) 1.5g of K_2PO_4 , 0.5g of NaCl, 0.6g of Na_2HPO_4 , 2g NH_4SO_4 , 0.2g $MgSO_4 \cdot 7H_2O$, 0.01 $CaCl_2$ and 0.001g $FeSO_4 \cdot 7H_2O$ for the isolation of bacteria as described by Emeribe et al. (2023). To prepare a mineral salt medium (MSM) for microbial cultures, mineral salts were dissolved in distilled water. The pH was then adjusted to 6.5 before sterilizing the solution. Other media also used in this study were Nutrient Agar (NA) and Nutrient Broth (NB).

2.4 Isolation of chlorpyrifos-degrading bacteria

Little changes were made to the Emeribe et al. (2023) approach for isolating bacteria that degrade down chlorpyrifos. Chlorpyrifos was used as the only carbon source to fortify the mineral salt medium in order to carry out the enrichment culture process. In a 250 mL Erlenmeyer's flask, 100 mL of the mineral salt medium and 1 mL (20 mg/L) of the insecticide based chlorpyrifos made up one cycle enrichment preparation followed by adding 5g of the soil sample, the mixture was autoclaved for 15 minutes at 121°C to sterilize it. This set up was done in triplicate. The flasks were incubated at 30°C and 120 revolutions per minute (rpm) in a rotary shaker for seven days. After incubation, 0.1 mL of the culture was then inoculated on chlorpyrifos mineral salt agar and incubated at 30°C for 48-72 hours as described by Ifediegwu et al. (2015) but with little modification of the total chlorpyrifos concentration to 100 mg/L serving as a secondary phase of screening chlorpyrifos-degrading bacteria at higher pesticide concentration than 10 mg/L. Distinct bacterial colonies observed on the agar plates after incubation were selected and sub-cultured repeatedly until pure isolates were obtained. Pure isolates were maintained on nutrient agar slant for further studies.

2.5 Identification of the isolates

Pure isolates were identified using colonial morphology, microscopy, biochemical and molecular methods. The tests carried out include: Cultural morphology, gram staining, spore staining, catalase test, citrate utilization, oxidase test, indole production, mannitol test, methyl-red test, starch hydrolysis, Voges-Proskauer test and hydrogen sulphide production as described by Fawole and Oso (2004).

2.6 Growth response of the isolates in chlorpyrifos mineral salts medium

Identified isolates were sub-cultured into a fresh nutrient broth and incubated for 24 hours. One milliliter (mL) of the 24 hour old culture was used to inoculate 100 mL mineral salt medium fortified with chlorpyrifos to a concentration of 100 mg/L to further screen isolates for their high degrading capacity. Each setup was done in triplicates and uninoculated mineral salt medium fortified with chlorpyrifos served as controls. Incubation was done on a rotary shaker at 120 rpm at 30°C for 24 days. Bacterial growth rate was

monitored for 24 days at an interval of 4 days using Spectrophotometer (OD 600 nm) to establish the optical density as described by Ezeonu et al. (2011).

2.7 Chlorpyrifos degradation rate of isolates

A standard stock solution of 1000 ppm was obtained by weighing one 100 mg of chlorpyrifos standard, transferring it to a 100 mL volumetric flask, and adjusting the volume with acetonitrile to the appropriate level. From the stock solution, different concentrations of working standard were prepared and diluted to 10 mL to have (5, 10, 20, 30 and 40 ppm). The working solutions were used for the calibration curve. About 3.0 mL of the sample matrix was taken and transferred to a 10 mL clean dry glass test tube. The same volume of absolute ethyl acetate was added, capped and shaken vigorously to extract the pesticide. After intervals of shaking, the mixture was allowed to stand for 30 min where two layers separated. After being moved to a different test tube, the top layer containing the chlorpyrifos was filtered using double Whatman filter paper. For analysis, the filtrate was moved to a High-Performance Liquid Chromatography (HPLC) vial. The chromatographic analysis of chlorpyrifos was performed on a Biobase Machine (China) with reversed phase HPLC column that consists of a microprocessor-controlled 200 μ L eluent delivery pump. The system consists of a fixed wavelength Biobase model UV-Vis detection system. The mobile phase consisted of water, acetonitrile (70:30 v/v). The mobile phase solution was filtered with a 0.45 μ m vacuum pump, degassed, and pumped at a flow rate of 1 mL/min. The injection volume was 40 μ L and the separation was monitored using UV detection at 230 nm under isocratic condition. Separation was achieved on a 4.6 mm $\times$ 250 mm C18 reversed phase column with 5 μ m ultra-pure silica gel packing material (vertex). All the system was maintained at room temperature. Data collection and handling were carried out by Biobase software (N2000 chromatographic Data system). Identification of each component was determined by the peak value and retention time under similar condition with standards. The quantification of the compound was done using the calibration curves plotted (Atego et al., 2021). The rate of degradation of chlorpyrifos was measured only at the initial (Day 0) and final (Day 24) for all isolates and control. The degradation rate was calculated using the below equation (Oyega et al., 2024). Biodegradation studies showing the first-order degradation kinetics, half-life calculation and the degradation constant (k) was also reported.

$$\% \text{ Degradation} = \left(\frac{X - Y}{X} \right) 100$$

X= Initial concentration of chlorpyrifos

Y= Final concentration of chlorpyrifos

2.8 First-Order Kinetic Model

Pesticide biodegradation in aqueous mineral salt media is commonly modeled using first-order kinetics, where the rate of compound disappearance is directly proportional to its instantaneous concentration (Ramírez et al., 2023). The governing differential equation is:

$$dC/dt = -kC$$

where C is the chlorpyrifos concentration at time t (mg/L) and k is the first-order rate constant (day⁻¹). Integration of this equation with the boundary condition C = C₀ at t = 0 yields the exponential decay function:

$$C(t) = C_0 \cdot e^{(-kt)}$$

where C₀ is the initial concentration (mg/L) and C(t) is the residual concentration at time t

2.9 Degradation Rate Constant (k)

Rearranging the integrated rate law provides the working equation for calculating k from experimental endpoint data:

$$k = -\ln(C / C_0) / t$$

where C is the final residual chlorpyrifos concentration (mg/L) at day 24, C₀ is the initial concentration (100 mg/L), and t = 24 days. Larger k values indicate faster degradation kinetics (Cycoń et al., 2013).

2.10 Half-Life (t_{1/2})

The biological half-life is the time required for the pesticide concentration to decrease by 50% from its initial value and it is derived directly from the rate constant:

$$t_{1/2} = \ln(2) / k = 0.6931 / k$$

The half-life provides an intuitive and standardized basis for comparing degradation efficiency across isolates (Hafner et al., 2023).

2.11 Determination of metabolites

The metabolites were determined using an Agilent USA gas chromatography that was hyperphased to a mass spectrophotometer (5975C) with a triple axis detector and an auto injector (10 µL syringe). The carrier gas was helium gas. All chromatographic separations were carried out on capillary columns that were treated with phenyl methyl silox and had the following dimensions: length: 30 m, internal diameter: 0.25mm, thickness: 0.25 µm. Ion source temperature (EI) (250°C), interface temperature (300°C), pressure (16.2 psi), out time 1.8 mL/min, 1 µL injector in Split mode with split ratio 1:50, injection temperature of 280°C, and column temperature that began at 50°C for two minutes before changing to 100°C at a rate of 20°C/min are additional gas chromatography–mass spectrometry (GC-MS) conditions at a rate of 20°C per minute, the temperature was increased to 250°C and maintained for five minutes. Elution took 19 minutes in total. The system was controlled and data was collected using Ms. Solution software, which was supplied by the supplier. By comparing the collected mass spectra with the standard mass spectra from the NIST library, the chemicals were identified (NIST, 2023).

2.12 Molecular identification

Proper confirmation of chlorpyrifos-degrading isolates was done through molecular identification which involved steps such as extraction of DNA from the bacterial isolates, amplification of extracted DNA using polymerase chain reaction (PCR), and gene sequencing. DNA was extracted from bacterial samples using QIAamp® Mini Kit in accordance with the manufacturer's procedure (QIAGEN, 2023). The primer pair 27F and 511R was used to amplify the 16S rRNA gene, with an expected amplicon of ~ 512bp. Sanger sequencing procedure was used to carry out gene sequencing. Resulted nucleotide sequence was uploaded and compared to similar gene sequence on the database using the National Center for Biotechnology Information (NCBI) BLAST program (Li et al., 2022).

2.13 Statistical analysis

The experiments were done in triplicate (n=3:Soil samples) and in a completely randomized design. Results were recorded as mean ± SD. The data were analyzed using ANOVA and Tukey's HSD test (*p* < 0.05) and graphs were plotted using Microsoft Excel package.

3 Results

A total of five chlorpyrifos-degrading bacteria were isolated from the soil samples and tagged (A1, A2, N, X, and L1). Table 1 shows the cultural morphology while Table 2 shows the biochemical test result of the isolates. Isolate A1, A2, N, X, and L1 were identified through cultural morphology and biochemical test as *Pseudomonas* sp, *Moraxella* sp, *Enterobacter* sp, *Pseudomonas* sp, and *Staphylococcus* sp. respectively.

3.1 Growth response of isolates to chlorpyrifos medium (100 mg/L)

Optical Density (OD) at 600 nm shows different growth response of isolates in chlorpyrifos medium at 100 mg/L as seen in Table 3. Optical density values further establish the bacterial growth phases of each isolate in chlorpyrifos medium at 100 mg/L as shown in Fig. 1. The highest optimal growth rate was seen in *Pseudomonas* sp. at day 12 (0.93 ± 0.01) while the least optimal growth rate was seen in *Moraxella* sp. (0.41 ± 0.35) at same day 12.

Table 1. Colonial morphology of isolates from soil samples.

Isolate	Size	Form	Elevation	Margin	Colour
A1	Small	Circular	Raised	Undulate	Light Yellow
A2	Large	Circular	Flat	Entire	Creamy
X	Small	Circular	Raised	Undulate	Greenish
N	Small	Irregular	Flat	Lobate	Creamy
L1	Large	Circular	Raised	Curled	Whitish

Table 2. Cultural morphology and biochemical result of isolates from soil samples.

Test	Isolates				
	A1	A2	N	X	L1
Gram Staining	- Rod	- Cocci	- Rod	- Rod	+ Cocci
Catalase	+	+	+	+	+
Methyl-Red	-	+	-	-	+
Citrate	+	+	+	+	+
H ₂ S	-	+	-	-	+
Mannitol	+	+	+	+	+
Starch Hydrolysis	-	-	-	-	+
Indole	-	-	-	-	-
Oxidase	+	+	+	+	+
Voges-Proskauer	-	+	+	-	-
Spore	-	-	-	-	+
Probable Organism	<i>Pseudomonas</i> sp.	<i>Moraxella</i> sp.	<i>Enterobacter</i> sp.	<i>Pseudomonas</i> sp.	<i>Staphylococcus</i> sp.

Keys: - denote negative reaction, + denote positive reaction.

Table 3. Growth response of isolates to chlorpyrifos medium (100 mg/L)

Isolate	Day						
	0	4	8	12	16	20	24
<i>Pseudomonas</i> sp.	0.12 ± 0.01	0.20 ± 0.01	0.82 ± 0.03	0.93 ± 0.01	0.89 ± 0.01	0.67 ± 0.03	0.62 ± 0.04
<i>Moraxella</i> sp.	0.09 ± 0.01	0.08 ± 0.01	0.26 ± 0.51	0.41 ± 0.35	0.35 ± 0.25	0.22 ± 0.01	0.20 ± 0.01
<i>Enterobacter</i> sp.	0.11 ± 0.01	0.18 ± 0.01	0.66 ± 0.05	0.79 ± 0.01	0.78 ± 0.02	0.64 ± 0.01	0.39 ± 0.01
<i>Pseudomonas</i> sp.	0.11 ± 0.02	0.16 ± 0.00	0.76 ± 0.05	0.80 ± 0.01	0.79 ± 0.01	0.66 ± 0.03	0.48 ± 0.02
<i>Staphylococcus</i> sp.	0.08 ± 0.01	0.07 ± 0.01	0.40 ± 0.01	0.56 ± 0.02	0.46 ± 0.01	0.31 ± 0.02	0.29 ± 0.02
Control	0.01 ± 0.06	0.01 ± 0.03	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.07 ± 0.01

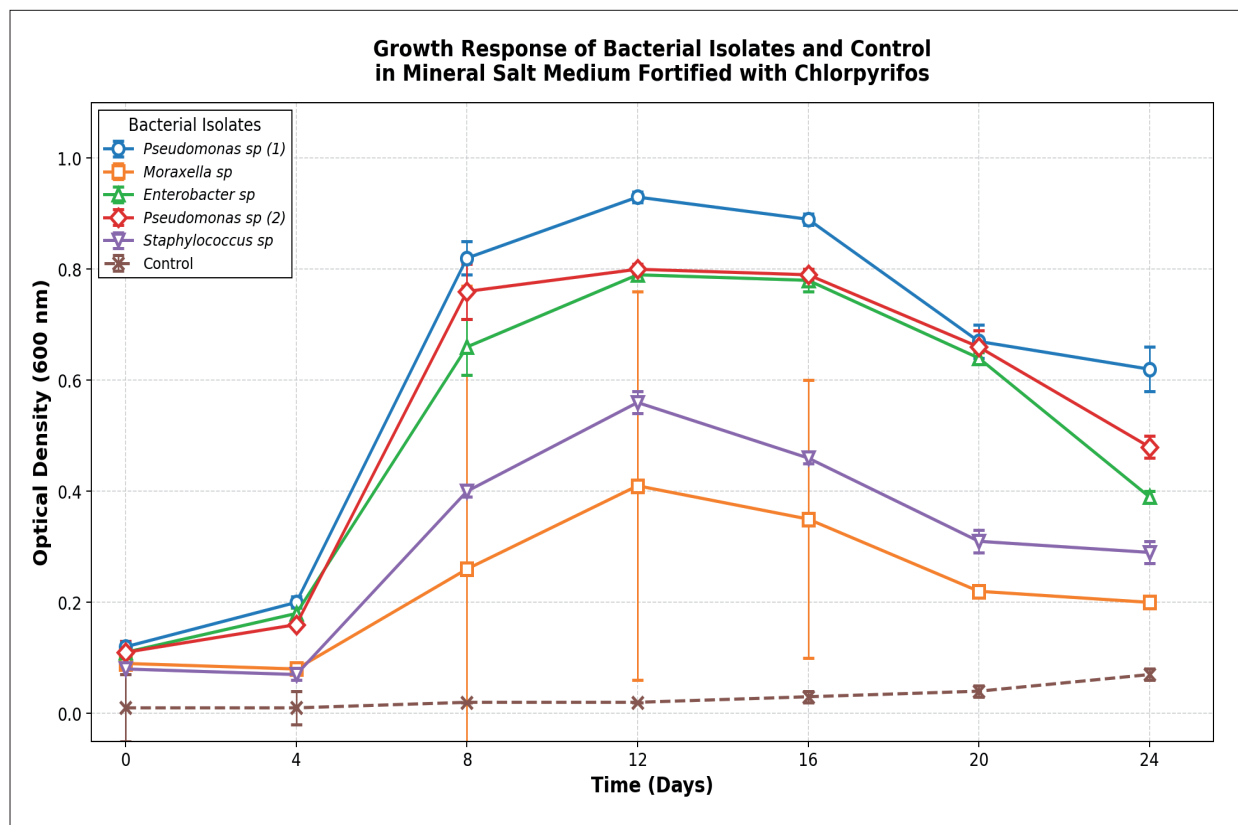


Figure 1. Growth response of isolates in mineral salt medium fortified with chlorpyrifos

Table 4. Rate of chlorpyrifos degradation by isolates at 100 mg/L.

Isolate	Initial concentration of chlorpyrifos (X) mg/L	Final concentration of chlorpyrifos (Y) mg/L	% Degradation = $\left(\frac{x-y}{x} \right) 100$
<i>Pseudomonas sp.</i>	100	43	57
<i>Moraxella sp.</i>	100	82	18
<i>Enterobacter sp.</i>	100	71	29
<i>Pseudomonas sp.</i>	100	67	33
<i>Staphylococcus sp.</i>	100	74	26
Control	100	98	2

Table 5. First-Order Biodegradation Kinetics of Chlorpyrifos at 100 mg/L for 24 Day Incubation Period

Isolate	C ₀ (mg/L)	C (mg/L)	% Degradation	k (day ⁻¹)	t _{1/2} (days)
<i>Pseudomonas sp.</i>	100	43	57	0.0352	19.71
<i>Moraxella sp.</i>	100	82	18	0.0083	83.83
<i>Enterobacter sp.</i>	100	71	29	0.0143	48.57
<i>Pseudomonas sp.</i>	100	67	33	0.0167	41.54
<i>Staphylococcus sp.</i>	100	74	26	0.0125	55.25
Control	100	98	2	0.0008	823.43

Note: k = first-order degradation rate constant; t_{1/2} = biological half-life; C₀ = initial concentration; C = residual concentration at day 24. Calculations: $k = -\ln(C/C_0)/24$; $t_{1/2} = 0.6931/k$.

Table 6. Comparison of GC–MS peaks between the control and *Pseudomonas* sp. (A1) treatment.

Peak (/A1/ Control)	RT (min)	Compound Identified	Area % (Control)	Area % (A1 Treated)	Quality (Control)	Quality (A1 Treated)	Observation
Pk 2/10	6.858/6.864	1,3-Di-tert- butylbenzene	4.57	0.79	91	94	Antioxidant; Present in both
Pk 7/18	9.919	2,4-Di-tert- butylphenol	8.06	1.41	96	96	Antioxidant; Present in both
Pk 14/6	8.632	Tetradecane	1.18	0.28	97	94	Alkane; Present in both
Pk 32/12	13.782	Methyl hexadecanoate (Methyl palmitate)	6.43	1.33	98	98	Fatty acid methyl ester; Present in both
Pk 34/14	14.182	n-Hexadecanoic acid (Palmitic acid)	11.06	0.97	99	99	Major fatty acid; Present in both
Pk 35/15	14.268/14.319	Chlorpyrifos	4.47	89.16	99	99	Organophosphate pesticide; dominant peak in A1 treated (>89%)
Pk 36/16	15.424	Methyl stearate	5.00	1.06	98	98	Fatty acid ester; Present in both
Pk 37/17	15.807/15.801	Octadecanoic acid (Stearic acid)	7.14	0.77	95	91	Fatty acid; Present in both
Pk 40	20.442	1-Hydroxy- cyclododecane- carbonitrile	8.55	—	60	—	Only in control; absent in A1 treated
Pk 31	13.473	Hexadecane	1.08	—	83	—	Alkane; only in control
Pk 13	14.108	Butyl 2-ethylhexyl phthalate	—	0.81	—	86	Only in A1 treated; possible plasticizer contamination
Pk 18	15.996	Ethyl stearate	—	0.30	—	95	Only in A1 treated, Bacterial cell membranes can release fatty acids during incubation.

Table 7. Comparison of GC–MS peaks between the control and *Pseudomonas* sp. (N) treatment.

Peak (Control/N2)	RT (min)	Compound Identified	Area % (Control)	Area % (N2 Treated)	Quality (Control)	Quality (N)	Observation
Pk10/Pk6	6.858/6.863	Di-tert-butylbenzene (1,3- or 1,4-)	4.57	1.16	91	91	Aromatic hydrocarbon; Present in both
Pk18/Pk14	9.919	2,4-Di-tert-butylphenol	8.06	2.00	96	96	Antioxidant phenol; Present in both
Pk14/Pk10	8.632	Tetradecane	1.18	0.39	97	94	Saturated alkane; Present in both
Pk32/Pk27	13.781–13.782	Methyl hexadecanoate (Methyl palmitate)	6.43	2.89	98	98	Fatty acid methyl ester; Present in both
Pk34/Pk29	14.182	n-Hexadecanoic acid (Palmitic acid)	11.06	1.70	99	99	Dominant fatty acid in control; Present in both
Pk35/Pk30	14.268/14.296	Chlorpyrifos	4.47	77.68	99	99	Organophosphate pesticide; overwhelmingly dominant in N (>77%)
Pk36/Pk32	15.424	Methyl stearate	5.00	2.33	98	99	Fatty acid ester; Present in both
Pk37/Pk33	15.807/15.801	Octadecanoic acid (Stearic acid)	7.14	1.21	95	93	Saturated fatty acid; Present in both
Pk40	20.442	1-Hydroxy-cyclododecane-carbonitrile	8.55	—	60	—	Present only in control; absent in N treated
Pk31	13.473	Hexadecane	1.08	—	83	—	Alkane only in control; absent in N treated
Pk23	11.991	3-Hexene, 2,2,5,5-tetramethyl-(Z)	1.51	—	49	—	Present in control as cluster of peaks; not detected in N
Pk13	9.581	Heneicosane / Heptacosane	—	0.33	—	86	Long-chain alkanes detected only in N treated
Pk28	14.096	Dibutyl phthalate	—	0.90	—	94	Phthalate plasticizer; only in N treated
Pk34	15.990	Ethyl stearate	—	0.27	—	89	Fatty acid ester detected only in N treated
Pk19	11.716	Octacosane	—	0.33	—	86	Long-chain alkane unique to N treated sample

Table 8. Comparison of GC–MS peaks between the Control and *Enterobacter* sp. (X) treatment.

Peak (Control/X2)	RT (min)	Compound Identified	Area % (Control)	Area % (X2 Treated)	Quality (Control)	Quality (X)	Observation
Pk10/Pk2	6.858	Di-tert-butylbenzene (1,3- or 1,4-)	4.57	2.04	91	91	Aromatic hydrocarbon; Present in both
Pk18/Pk9	9.919	2,4-Di-tert-butylphenol	8.06	3.78	96	97	Antioxidant phenol; Present in both
Pk14/Pk6	8.632	Tetradecane	1.18	0.90	97	96	Saturated alkane; Present in both
Pk32/Pk17	13.782	Methyl hexadecanoate (Methyl palmitate)	6.43	3.95	98	98	Fatty acid methyl ester; Present in both
Pk34/Pk19	14.182	n-Hexadecanoic acid (Palmitic acid)	11.06	4.00	99	99	Major fatty acid; Present in both
Pk35/Pk20	14.268–14.274	Chlorpyrifos	4.47	60.86	99	99	Organophosphate pesticide; dominant in X (>60%)
Pk36/Pk22	15.424	Methyl stearate	5.00	3.38	98	98	Fatty acid ester; Present in both
Pk37/Pk24	15.802–15.807	Octadecanoic acid (Stearic acid)	7.14	2.80	95	46	Fatty acid Present in both
Pk40	20.442	1-Hydroxy-cyclododecane-carbonitrile	8.55	—	60	—	Unique to control; absent in X treated
Pk31	13.473	Hexadecane	1.08	—	83	—	Alkane; present only in control
Pk38	16.259	Tetratriacontyl trifluoroacetate	0.95	—	52	—	Only in control;
Pk8	9.582	Heptadecane / Heneicosane	—	1.01	—	83	Long-chain alkanes unique to X treated sample
Pk18	14.091	Phthalic acid hexyl tetradecyl ester	—	1.07	—	90	Phthalate ester; only in X treated sample
Pk21	15.350	2-Methylhexacosane / Hentriacontane	—	0.68	—	91	Branched/long-chain alkane only in X treated
Pk26	20.454	Hexadecanoic acid 1-(hydroxymethyl)-1,2-ethanediyl ester	—	3.22	—	64	Glycerolipid-type ester unique to X treated

Table 9. Comparison of Mass Fragment between Control, *Pseudomonas* sp. (A1), *Pseudomonas* sp. (N), and *Enterobacter* sp. (X) treatment.

Compound	Control (mz) with percentage (%)	Sample A1(mz)	Sample N (mz)	Sample X (mz)	Observation
Chlorpyrifos/ 3,5,6-Trichloro-2- pyridinol (TCP)	Present (196.90) and (198.90) at 89 and 88 % respectively	Present (197.00) and (199.00) at 100 % and 97 % respectively	Present (197.00) and (199.00) at 100 % and 98 % respectively	Present (197.00) and (199.00) at 100 % and 97 % respectively	Occurs as parent compound with Pyridinic N, thiophosphate fragments and also as major degradation product.
diethyl phosphorothioate	Present (97.00) at 100 %	Present (97.00) at 96 %	Present (97.00) at 95 %	Present (97.00) at 95 %	N-methyl group removed from parent compound.

3.2 Chlorpyrifos degradation rate of isolates

HPLC analysis of the residual concentration of chlorpyrifos showed that each isolate degraded 100 mg/L concentration of chlorpyrifos at different rate as seen in Table 4. The two *Pseudomonas* species isolated from agricultural soil showed considerable degradation rate (57% and 33%) while *Moraxella* sp. showed low chlorpyrifos degradation profile (18%).

Applying the first-order kinetic equations to the 24-day experimental data, the degradation rate constants and half-lives were calculated for each isolate (Table 5).

3.3 Determination of metabolites

After the last day of incubation, GC-MS analysis of mineral salt medium fortified with chlorpyrifos of the best three chlorpyrifos-degrading bacteria (two *Pseudomonas* species and *Enterobacter* sp.) (Tabs 6–8) revealed the presence of chlorpyrifos metabolites such as 3,5,6-trichloro-2-pyridinol (TCP) and di-ethyl-thio-phosphoric acid (DETP) (Figs 2–5; Tab. 9).

3.4 Molecular identification

Molecular analysis of the best two chlorpyrifos-degrading bacteria isolate A1 and X (the two *Pseudomonas* species) revealed the 16S rDNA Sequence of the two microorganisms and Basic Local Alignment Search Tool (BLAST) identified isolate A1 and X to be *Pseudomonas monteilii* and *Pseudomonas aeruginosa* respectively through their percentage of identity, E-value which represents a statistical significant value, alignment score, and highest query coverage. The gene sequences of the bacteria were submitted to gene bank and were assigned the accession number PV946157 and PV946140 respectively (Fig. 6).

4 Discussion

In this study, primary screening for Chlorpyrifos-degrading bacteria at 10 mg/L revealed 21 isolates which were further screened for their high degrading capacity at 100 mg/ml. This helped to screen down these isolates to 5 (A1, A2, N, X, and L1) based on their rapid growth on the chlorpyrifos agar medium (100 mg/L). Isolate A1, A2, N, X, and L1 were identified through cultural morphology and biochemical test as *Pseudomonas* sp, *Moraxella* sp, *Enterobacter* sp, *Pseudomonas* sp, and *Staphylococcus* sp. respectively. This result established that bacteria can utilize chlorpyrifos as the sole carbon source for their growth and proliferation which is similar to that obtained by Kumar et al.(2017) and Wepukhulu et al. (2024). Bosu et al. also reported that many bacteria can metabolize chlorpyrifos under minimal nutrient conditions, confirming that the pesticide can serve as a sole carbon and energy source during biodegradation.

The bacterial growth response observed with spectrophotometer on the basis of the optical density from day 0 to day 24 showed the bacterial growth phases. The growth patterns observed indicates that the isolates were able to adapt to and utilize chlorpyrifos in the medium. The substantial increase in optical density relative to the control suggests occurrence of metabolic activity associated with chlorpyrifos utilization. Studies have established that species of *Pseudomonas*, *Enterobacter*, and *Staphylococcus* possess enzymes capable of degrading chlorpyrifos into less toxic intermediates, thereby supporting microbial growth (Cycoń et al., 2017;Fulekar & Geetha, 2008;Rathour et al., 2024). At the start of incubation period (day 0), relatively low optical densities ranging from 0.08 ± 0.01 to 0.12 ± 0.01 were observed in all isolates but slight increases (0.07 ± 0.01 - 0.20 ± 0.01) were observed in *Pseudomonas* species and *Enterobacter* sp., while *Moraxella* sp. and *Staphylococcus* sp. showed little or

no increase at day 4. This period likely represents the lag phase, during which the microorganisms adapted to chlorpyrifos stress and synthesized the enzymes required for pesticide metabolism. Similar adaptation periods have been reported for chlorpyrifos-degrading

bacteria grown in mineral salt media (Ewadi et al., 2023). A marked increase in growth occurred between Days 4 and 8 for all isolates. *Pseudomonas* sp. (A1) increased from 0.20 ± 0.01 to 0.82 ± 0.03 , while *Enterobacter* sp. increased from 0.18 ± 0.01 to 0.66 ± 0.05 . *Pseudomonas*

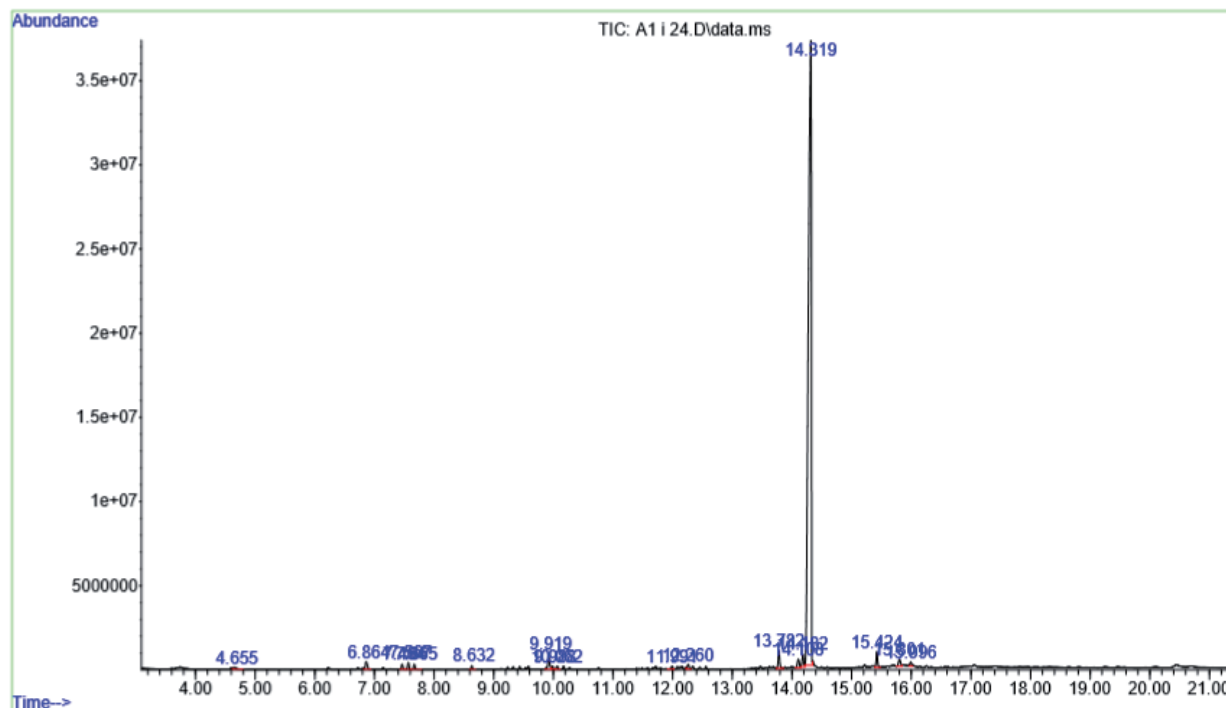


Figure 2. 18 Peaks Showing Tentatively Identified Compounds (TIC) by GC-MS in Chlorpyrifos Medium Containing *Pseudomonas* sp.

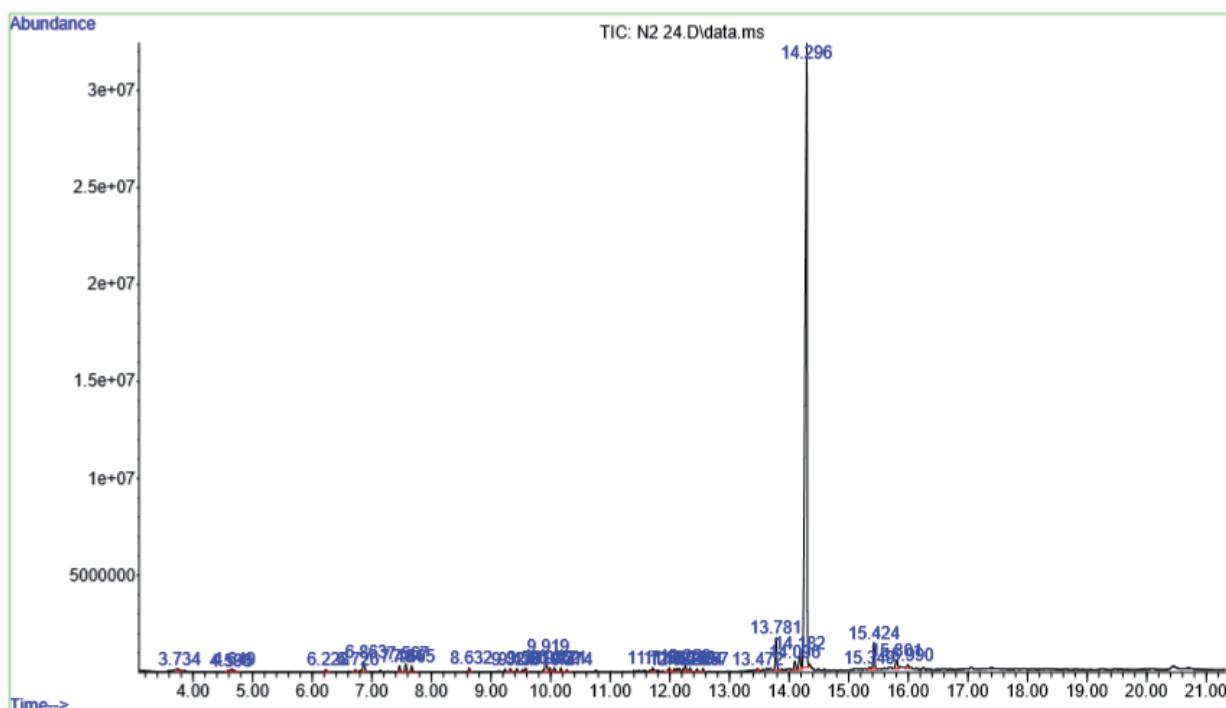


Figure 3. 34 Peaks Showing Tentatively Identified Compounds (TIC) by GC-MS in Chlorpyrifos Medium Containing *Enterobacter* sp.

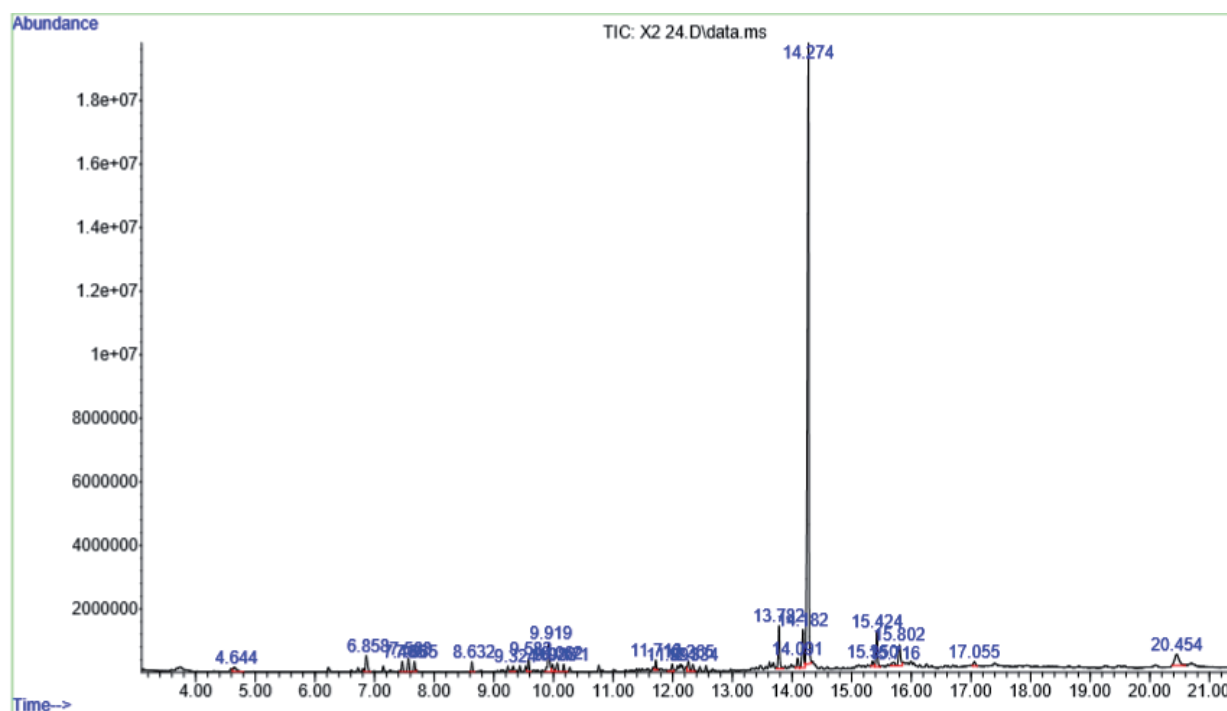


Figure 4. 26 Peaks Showing Tentatively Identified Compounds (TIC) by GC-MS in Chlorpyrifos Medium Containing *Pseudomonas* sp.

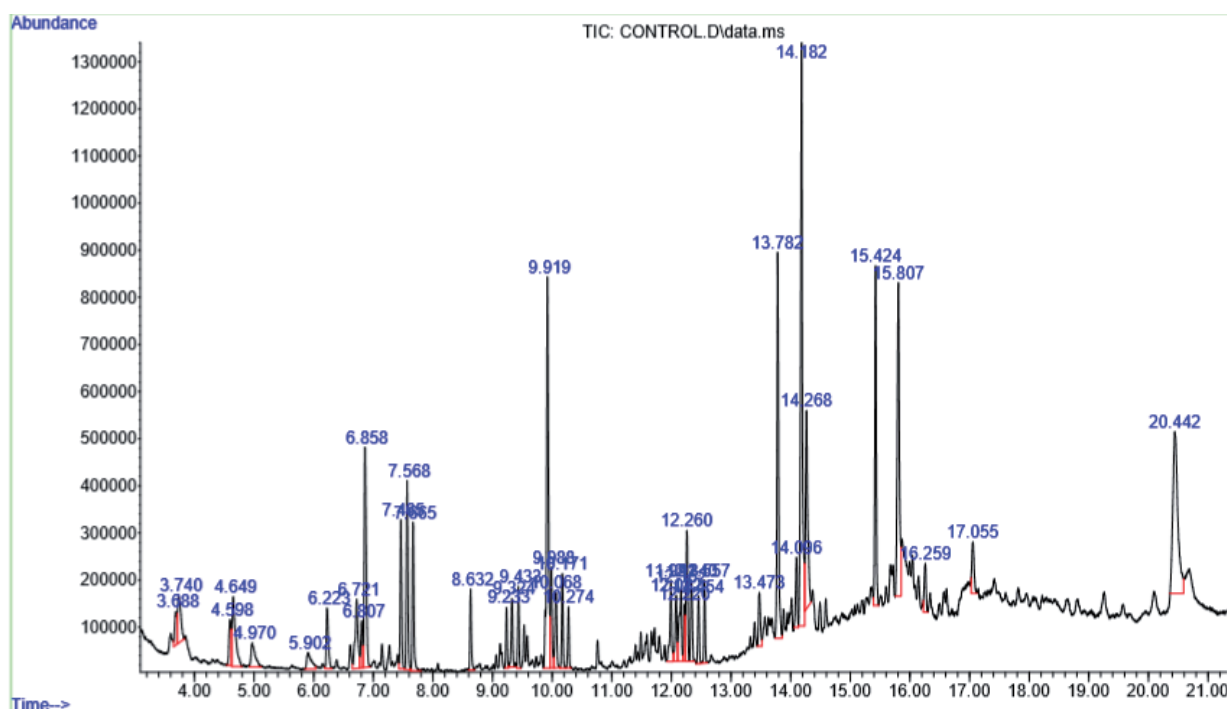


Figure 5. 40 Peaks Showing Tentatively Identified Compounds (TIC) by GC-MS in uninoculated Chlorpyrifos Medium (Control).

sp. (N) also showed substantial growth, increasing from 0.16 ± 0.00 to 0.76 ± 0.05 . This rapid increase suggests active utilization of chlorpyrifos and its metabolites, resulting in biomass accumulation. The higher growth of the *Pseudomonas* isolates suggests better adaptation

and metabolic capacity for chlorpyrifos degradation compared to the other isolates. This enhanced growth may be attributed to the possession of highly efficient degradative enzymes, including organophosphorus hydrolases and phosphotriesterases, which enable

Pseudomonas spp. to hydrolyze chlorpyrifos into less toxic metabolites that can subsequently be assimilated into central metabolic pathways (Bosu et al., 2024). Optimal growth was observed on Day 12 for most isolates. *Pseudomonas* sp. (A1) recorded the highest optical density (0.93 ± 0.01), followed by *Pseudomonas* sp. (N) (0.80 ± 0.01) and *Enterobacter* sp. (0.79 ± 0.01). The peak growth rate suggests maximum chlorpyrifos utilization and microbial activity. The better performance of *Pseudomonas* species is consistent with numerous reports identifying members of this genus as efficient degraders of organophosphate pesticides due to their diverse catabolic pathways (Cycoń et al., 2017). Between Days 12 and 16, growth values remained relatively stable, particularly for the two *Pseudomonas* isolates and *Enterobacter* sp. This indicates the start of the stationary phase, where microbial growth and cell death occur at approximately equal rates. After Day 16, all isolates exhibited declining optical densities. *Pseudomonas* sp. (A1) and *Pseudomonas* sp. (N) decreased from 0.89 ± 0.01 to 0.62 ± 0.04 and 0.79 ± 0.01 to 0.48 ± 0.02 respectively, while *Enterobacter* sp. declined from 0.78 ± 0.02 to 0.39 ± 0.01 by Day 24. The decline likely resulted from depletion of available nutrients other than chlorpyrifos, possible accumulation of toxic metabolites such as 3,5,6-trichloro-2-pyridinol (TCP), or increased competition among microbial cells. Recent studies have reported that prolonged incubation of chlorpyrifos-degrading bacteria often results in reduced biomass despite ongoing degradation, primarily because nutrient limitation and metabolite accumulation create less favorable growth conditions (Bosu et al., 2024; Ewida et al., 2023; Kumar et al., 2023).

Pseudomonas species showed considerable rate of chlorpyrifos degradation of 57% and 33% while *Moraxella* sp. had the low rate of degradation (18%). This

result is in agreement with what was reported by Ifediegwu et al. (2015) indicating the involvement of different Gram negative bacteria in chlorpyrifos-degrading-bacteria as they are efficient degraders of organophosphate pesticide through adoption of broad metabolic versatility, high environmental tolerance and production of different enzymes such as Organophosphorus hydrolase (OPH), Phosphotriesterase (PTE), Esterases, Monooxygenases Dioxygenases, and Hydrolases (Bosu et al., 2024; Chia et al., 2014). Vidya et al. (2008) reported *Pseudomonas aeruginosa* degrading 92% of 50mg/L of chlorpyrifos in soil sample. Ranu, et al. (2024) also reported that *Pseudomonas monteilii* showed significant potential by degrading 98% of chlorpyrifos (50mg/L). *Enterobacter* sp, and *Staphylococcus* sp. also showed considerable degradation rate of 29% and 26% respectively. Different species of bacteria from the genera are capable of degrading chlorpyrifos (Nandhini et al., 2022). *Enterobacter* sp. strains produce organophosphorus acid anhydrolase, facilitating the degradation of chlorpyrifos (Zia et al., 2012). Biodegradation of Chlorpyrifos by *Staphylococcus* sp. Isolated from Agricultural Soil was earlier reported by Sonal et al. (2020). Raman et al.(2015) also reported *Moraxella* sp. as part of bacteria isolated from rice field contaminated by pesticide. The uninoculated Control sample also showed slight rate of degradation (2%), this can be as a result of chlorpyrifos been prone to photo-degradation condition directly or indirectly (Ifediegwu et al., 2015).

First-order kinetic analysis of chlorpyrifos biodegradation over a 24-day incubation period revealed clear differentiation in the degradation capacity of the five tested bacterial isolates. *Pseudomonas monteilii* emerged as the most efficient degrader ($k = 0.0352 \text{ day}^{-1}$; $t_{1/2} = 19.71 \text{ days}$), achieving 57% chlorpyrifos removal and meeting the 'readily

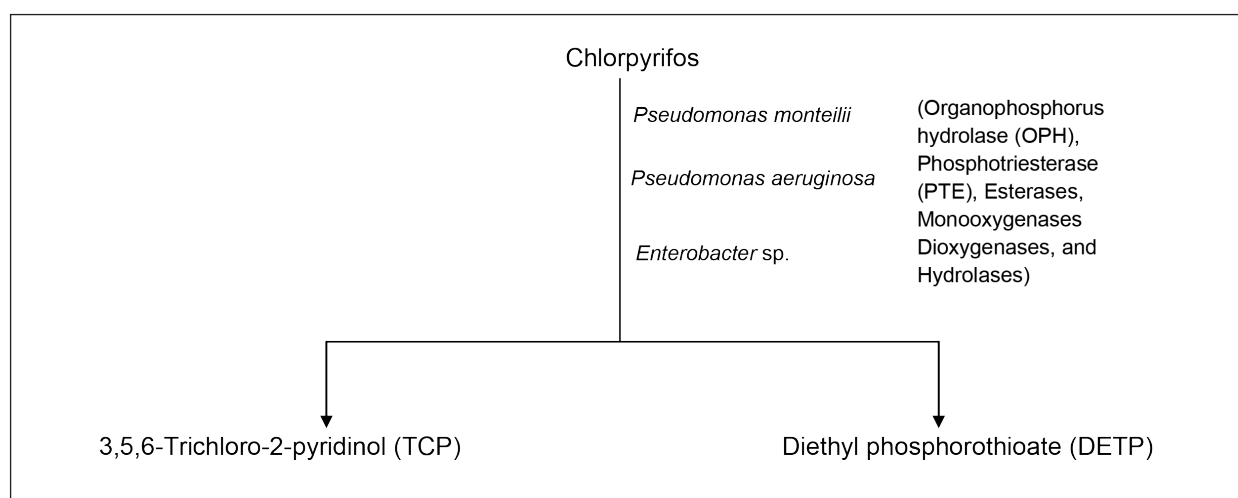


Figure 6. Proposed degradation pathway of Isolates.

degradable' classification threshold (USEPA, 2011). Anwar et al. (2009) similarly reported efficient chlorpyrifos biodegradation by *Pseudomonas putida* within comparable timeframes at equivalent initial concentrations, underscoring the consistent high-performance phenotype of this genus.

GC-MS analysis of the chlorpyrifos containing medium of control sample and best three of the chlorpyrifos-degrading isolates (*Pseudomonas monteilii*, *Enterobacter* sp, and *Pseudomonas aeruginosa*) showed several peaks indicating the presence of different compounds. Control sample had 40 groups of compounds while *Pseudomonas monteilii* had the least groups of compounds (18), *Enterobacter* sp. and *Pseudomonas aeruginosa* had 34 and 26 groups respectively. This decline in chromatographic complexity is consistent with active microbial catabolism, whereby the parent compound and its initial breakdown products are progressively channeled into simpler, and in some cases undetectable, metabolic end-products rather than accumulating as a diverse pool of intermediates (Abo-Amer et al., 2026).

The comparative analysis of the characteristic mass fragments detected in the control and bacterial-treated samples following chlorpyrifos degradation showed principal ions corresponding to chlorpyrifos and its major metabolite, 3,5,6-trichloro-2-pyridinol (TCP) in all samples. The persistence of the ions at m/z 197 and 199 in the treated samples indicates that chlorpyrifos underwent hydrolysis to form TCP, which is recognized as the primary intermediate of chlorpyrifos biodegradation. The detection of these ions in all bacterial cultures demonstrates that *Pseudomonas monteilii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp. possess the enzymatic capability to initiate cleavage of the phosphorothioate ester bond of chlorpyrifos. This reaction is typically catalyzed by enzymes such as organophosphorus hydrolase (OPH) or phosphotriesterase (PTE), yielding TCP and diethyl phosphorothioate (Adams et al., 2024; Ali et al., 2023). The presence of the fragment ion at m/z 97 in both the control and treated samples corresponds to diethyl phosphorothioate (DETP), a well-established degradation product generated following hydrolysis of chlorpyrifos. In the control, the ion exhibited 100% relative abundance, whereas slightly lower abundances (95–96%) were recorded in the bacterial treatments. The slight reduction in abundance may indicate that once formed, diethyl phosphorothioate underwent further microbial transformation, suggesting continued metabolism by the bacterial isolates rather than simple accumulation.

5 Conclusion

Five Isolates (*Pseudomonas monteilii*, *Moraxella* sp, *Enterobacter* sp, *Pseudomonas aeruginosa*, and *Staphylococcus* sp.) were found to be indigenous bacteria capable of using chlorpyrifos as their sole carbon source. The best two chlorpyrifos-degrading isolate were *Pseudomonas monteilii* and *Pseudomonas aeruginosa*, degrading 57% and 33% of 100mg/L of chlorpyrifos respectively. *Enterobacter* sp. and *Staphylococcus* sp. also showed considerable degradation rate of 33%, 29% and 26% respectively with accumulation of less toxic chlorpyrifos metabolites such as 3,5,6-trichloro-2-pyridinol (TCP) and di-ethyl-thio-phosphoric acid (DETP) thereby implying their usefulness in bioremediation of chlorpyrifos.

Funding

This work is a self-sponsored project.

Conflict of interest

The authors declare no conflict of interest. All the contributors were captured in either in the authorship or acknowledgment base on their contribution.

Acknowledgement

The authors wish to thank the Managing Director of Lower Niger River Basin Authority, Ilorin, Kwara state, Nigeria for granting access to farmlands for soil samples collection for the research.

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