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Biodegradation Potential of Indigenous Soil Bacteria Against Chlorpyrifos in Suthamalli Reservoir, Tirunelveli, India.

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Abstract

The current study focuses on chlorpyrifos-degrading bacteria that were isolated from soil samples of Suthamalli Dam, Tirunelveli District, Tamil Nadu. The pesticide were extracted from the sample using the SLE-LTP method. UV-visible spectroscopy showed supreme absorbance at 280 nm, confirming the existence of chlorpyrifos in the soil. Eight bacterial species were isolated for bioremediation on nutrient-agar medium. Three isolates, RFS1, RFS4, and RFS6 (*Streptococcus*, *Rhodococcus* and *Bacillus* species respectively through morphology and biochemical tests) were selected based on their ability to grow in varying concentrations of chlorpyrifos (25, 50, 75, and 100 µl/ml). Chlorpyrifos biodegradation was studied using these bacterial cultures over 12 days. This study conclude that *Streptococcus* sp, *Rhodococcus* sp, and *Bacillus* sp could effectively degrade the chlorpyrifos in contaminated agricultural fields.

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Abbreviations

HPLC	High-Performance Liquid Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
TLC	Thin Layer Chromatography
SLE-LTP	Solid Liquid Extraction-Low Temperature Purification
FTIR	Fourier Transform Infrared Spectroscopy
UV	Ultraviolet
NIST	National Institute of Standards and Technology

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Introduction

Chlorpyrifos (CPF) is a widely used organophosphate pesticide that is commonly applied in rural areas to control pests in crops such as paddy, banana, and vegetables. However, it persists in soil and water, breaking down into harmful by-products such as 2-hydroxy-3,5,6-trichloropyridine, this leads to serious environmental and health issues. Over time, CPF can accumulate in rural environments, affecting soil and water quality as well as non-target organisms. Traditional methods for cleaning up CPF, such as chemical and physical treatments, are often expensive and ineffective. As a result, microbial degradation has emerged as a promising, eco-friendly, and cost-efficient alternative.[2]

Many studies have focused on isolating microbes that can break down CPF from contaminated sites. Bacterial genera such as *Bacillus*, *Pseudomonas* sp., *Klebsiella* sp., and *Acinetobacter* are particularly effective, as they utilize enzymes like organophosphate hydrolases and phosphodiesterase to degrade CPF, potentially leading to complete mineralization.[1,5] Additionally, bacterial consortia have been found to work together in a manner that accelerates CPF degradation, following first-order kinetics.[4] In the Indian context, indigenous bacterial isolates and optimized microbial consortia have shown high resistance and efficiency in degrading CPF, suggesting that using locally adapted strains may be beneficial for bioremediation efforts.[6]

These site-specific microbial resources are essential in flooded agricultural areas where pesticide use is high. The Suthamalli Store in Tirunelveli district, Tamil Nadu, serves as a key water source for flood-affected crops such as paddy, black gram, and banana.

The heavy use of pesticides in surrounding areas increases the likelihood of CPF accumulating in soil and sediment. Identifying and characterizing local CPF-degrading bacteria from this source soil could offer an effective natural solution for reducing pesticide pollution. Therefore, this study aims to (i) isolate CPF-degrading bacterial strains from the soil of the Suthamalli Store, (ii) characterize their taxonomic profile and degradation potential, and (iii) evaluate their suitability for sustainable bioremediation approaches.

Materials and Methods

Study Area

Suthamalli Reservoir is located at 11°03'53" N latitude and 79°13'35" E longitude. Suthamalli Reservoir was built primarily to provide water for domestic and agricultural uses. Banana, black gram, and paddy fields are the most cultivable vegetation around the reservoir. The agricultural land near the reservoir measures 2556.69 hectares. The Suthamalli reservoir is a subbasin of the Thamirabarani River, which flows from the Agasthiyar peak of the Pothigai hills in the Western Ghats and empties into the Gulf of Mannar.

Pesticide and Chemicals

Commercial-grade chlorpyrifos (Dursban, EC 20%), an organophosphate pesticide, was acquired for this study. It was purchased at a neighboring agrochemical store in the Tirunelveli area of Tamil Nadu. The analytical grade chemicals were purchased from HIMEDIA in Chennai.

Experimental Design

Sample Collection

In the Tamil Nadu district of Tirunelveli, soil samples were taken from the Suthamalli Reservoir's. The Chlorpyrifos pesticide is frequently used in the banana field to remove the pests and thus the pesticide residues were flow into the reservoir through the runoff. After removing the soil's surface of any residual debris from plants, grasses, and trash, the samples had been collected. Shovel was used to dig the soil and 10-15 cm deep soil was collected as a sample in clean and sterilized plastic bottles. Two distinct locations were used to collect the sample i.e. site 1 and site 2 from the banana field in sterilized bottles which were labeled with date, time and sample location as SR-1, SR -2. Samples were kept in a cold box containing ice and transported to the Environmental studies laboratory of Madurai Kamaraj University. Then the sample was air dried in the desk for 24 hours and stock in a icebox at 4˚C for microbial study.

Sample Preparation

The soil samples were taken from the fridge and big lumps were broken into fine powder. Soil sample was sieved through 200 micrometer sieve and the collected fine particles of soil as sample for isolation of bacteria tolerant to Chlorpyrifos pesticide.

Pesticide Residue Analysis Employed The Solid–Liquid Extraction Combined With Low-Temperature Purification Method (Sle-Ltp)

SLE-LTP involves separation of chemicals into organic phase- acetonitrile and other contaminants soluble in aqueous phase like dust, organic matter (eg. Plant residues) in aqueous phase. Aqueous phase consists of water which solidifies at -20°C and traps the water-soluble compounds and acetonitrile dissolves the chemical compounds like pesticides, which can be separated after 1 hr. The extracted acetonitrile is treated with anhydrous sodium sulfate to eliminate any remaining water. Samples can then be evaluated using a variety of qualitative and quantitative methods.[7]

Extraction of Pesticides from Soil

The 50 ml centrifuge tube was filled with four ml of soil sample and four ml of distilled water, followed by eight ml of acetonitrile. The material was homogenized using a vortex for 5 minutes before being stored at -20˚C for 1.5 hours. Two layers were observed, one was an aqueous layer at the bottom having soil at bottom and the other was an organic layer at the top. Organic layer consisting of acetonitrile was transferred to another 50 ml centrifugation tube and 0.375g of anhydrous sodium sulphate was added to it and the sample was subjected to a vortex for 30 seconds to remove the water from the sample. The sample was then centrifuged at 4000 rpm for ten minutes. The resulting solution was transferred to another 50 mL centrifuge tube and dried in beakers covered with holes in aluminum foil for 4-6 days. Dried samples of beakers are used for UV-Visible spectroscopy, TLC, FTIR and GC-MS analysis.[8]

Analytical Methods

UV- Visible Spectroscopy Analysis

Three ml of the extracted pesticide from soil sample by SLE- LTP method, was analyzed in the UV- Visible spectroscopy in 600 nm. Acetonitrile HPLC grade used as the control.

Gas Chromatography - Mass Spectrometry Analysis

A Shimadzu 2030 GC equipped with mass spectrometry was used to analyze the pesticides and its residues in the soil sample. The Sample was extracted in Acetonitrile HPLC grade solvent. The oven temperature was kept at one hundred degrees Celsius for five minutes before being increased to 300°C for 20 minutes. The detector temperature was 300 degrees Celsius. Nitrogen was used as a carrier gas. The chemicals were identified using mass spectra, and the results were compared using the National Institute of Standards and Technology (NIST) collection.[8]

Isolation of Bacteria

Soil bacteria were isolated via the standard and serial dilution method, with one gram of soil from each site suspended in 9 ml of sterile (double distillation water). The sequential dilution method was utilized up to a 10-7 dilution. A small aliquot (0.1ml) of each dilution (10-1, 10-2, 10-3, 10-4, 10-5, 10-6, and 10-7) was obtained and streaked over nutrient agar plates to obtain bacterial colonies. After 24 hours of incubation at 34˚C, isolated colonies were selected and pure cultures grew on nutrient agar plates.[9]

Identification of Bacteria

To characterize the physical characteristics of the bacterial isolates from the soil, microscopic and biochemical investigations were carried out. They were checked for pigmentation, colony morphology, and standard preliminary tests including Gram's staining and biochemical assays that were validated by Bergey's manual of determinative bacteriology.

Biodegradation Study

In a sterilized laminar air flow chamber, one milliliter of bacterial broth culture was inoculated into 100 milliliters of nutrient broth and 5 milliliters of 50 µL/ml of chlorpyrifos pesticide. The culture was kept in a growth chamber for ten days at 37°C. The degradation was determined by monitoring the absorbance of the 3 ml aliquot of sample at 320 nm at an interval of 24 hrs in UV - visible spectroscopy.[10,11]

Growth Efficacy Study

The growth performance of bacterial isolates in the presence of chlorpyrifos was assessed in liquid Minimal Salt Medium (MSM) supplemented with chlorpyrifos (50 µg/mL). Each 100 mL culture was infused with 1 mL of bacterial broth and cultured at 37 °C in a shaking incubator (150 rpm) for 10 days. Control flasks included: (C1) uninoculated MSM + chlorpyrifos (to monitor abiotic loss) and (C2) MSM + bacteria without pesticide (to monitor background growth). Growth was monitored at 24 h intervals by measuring optical density at 600 nm (OD600), and CFU counts were performed at selected intervals to validate bacterial growth.[12]

Results

Chlorpyrifos has been an efficient pesticide in agriculture, its detrimental impacts on the health of people and the environment have prompted more regulations and a move toward safer pest management techniques. Microorganisms found in the soil of chlorpyrifos-infected banana fields may break down this pesticide. Numerous harmful environmental consequences result from the excessive application of these organophosphate pesticides on banana fields. It is important to eliminate contaminants from the environment based on their level of toxicity. The most common way of removing pesticides is biologically, as soil microorganisms may metabolize these toxic substances into non- toxic substances.

Extract the pesticide from the soil and water sample by SLE-LTP method of suthamalli reservoir, in which at 288 nm the peak of chlorpyrifos is observed in the soil sample by UV-Visible spectrophotometer.

The UV-visible absorption spectrum of Chlorpyrifos pesticide demonstrates a characteristic strong absorbance peak at 225 nm, distinguishing it clearly from the soil sample spectrum. The soil sample shows moderate absorbance in the same region, which may indicate the presence of trace organic compounds or pesticide residues. The comparative profile confirms that Chlorpyrifos can be reliably detected and quantified in the UV range of 220–285 nm. Beyond 300 nm, spectral overlap diminishes due to negligible absorption. This peak indicates preliminary the presence of Chlorpyrifos residues in collected soil sample.

The extracted chlorpyrifos pesticide from the soil sample was identified by GCMS using the NIST library. Figure 1 showed that chromatogram image reveals a clear separation, three distinct analytes eluting between 5 and 17 minutes, and a solvent front at the start. The data can be further analyzed to identify substances based on mass spectral information.

Upon GCMS analysis shows in a (Table 1) a retention time of 12.434 min in the 3rd peak and 22.149 min in 5th peak as chlorpyrifos and Phosphoric acid, tris (2,4-di-tert-butylphenyl) ester respectively, which was used as a stabilizer and an antioxidant. The obtained soil sample encompassed chlorpyrifos, as verified by these findings.

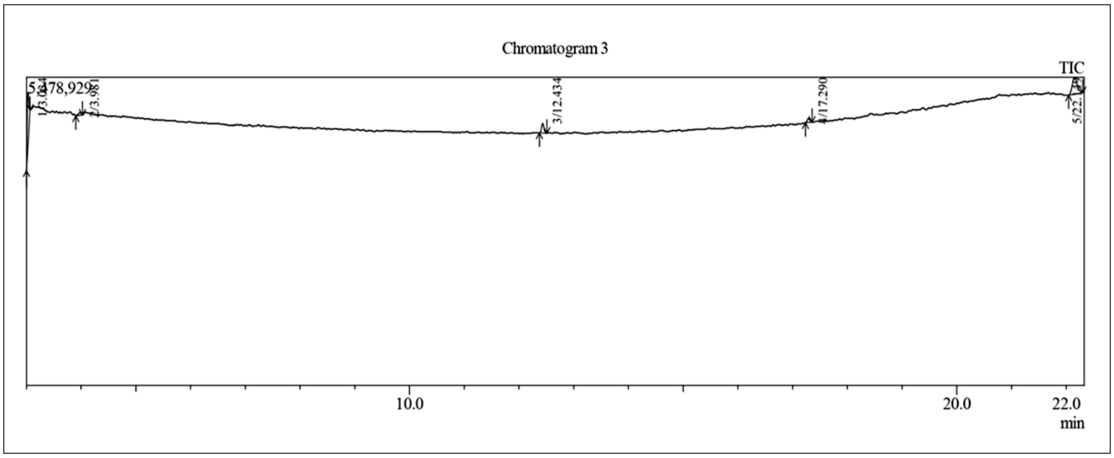

Fig. 1: GC scan showing the peaks for presence of chlorpyrifos in the soil sample

Chlorpyrifos (CAS No. 2921-88-2) is an organophosphate insecticide with the molecular formula $C_9H_{11}Cl_3NO_3PS$ and a molecular weight of 349 $g{\cdot}mol^{-1}$. It is also commercially known under trade names such as Dursban. The compound is systematically named as O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, according to IUPAC nomenclature.

Table 1: GC–MS Characterization of Environmental Contaminants and Additives

Peak ID	Retension Time (Min)	Initial Time (Min)	Final Time (Min)	Peak Area (counts.s^{-1})	Relative Peak Area (%)	Peak Height (Counts)	Base Ion (m/z)	Identified Compound
1	3.034	3.005	3.070	2219622	44.54	914872	28.05	Nickel tetracarbonyl
2	3.981	3.905	4.025	184733	3.71	53723	1.95	Hydrogen
3	12.434	12.375	12.510	513835	10.31	170278	96.95	Chlorpyrifos
4	17.290	17.235	17.355	239177	4.80	73127	149.00	Bis(2-ethylhexyl) phthalate
5	22.149	22.045	22.300	1826415 4983782	36.65 100.00	297797 1509797	647.45	Tris(2,4-di-tert-butylphenyl) phosphate

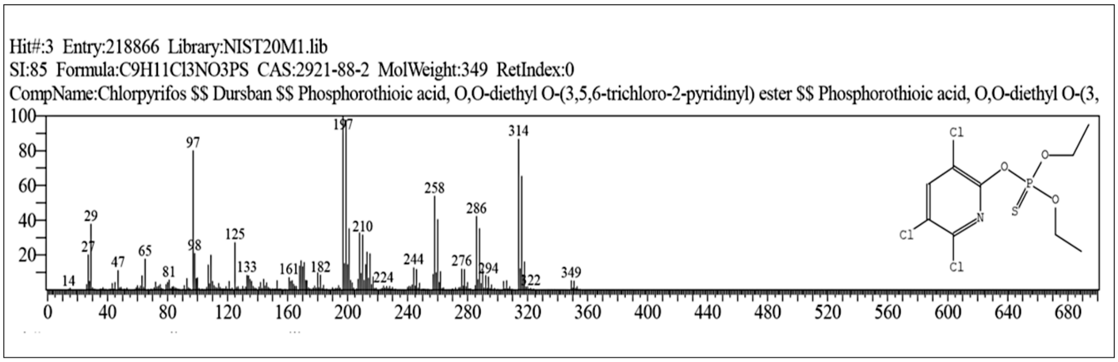

Fig. 2: Quantitative Report for the Chlorpyrifos

Tris (2,4-di-tert-butylphenyl) phosphate (CAS No. 95906-11-9) is an organophosphate compound with the molecular formula $C_{42}H_{63}O_4P$ and a molecular weight of 662 g·mol^{-1}. This compound is also referred to as Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphate or Phenol, 2,4-bis(1,1-dimethylethyl)-, 1,1',1''-phosphate, depending on the nomenclature system used. It is widely recognized as an antioxidant additive in polymers due to its thermal stability and resistance to oxidative degradation.

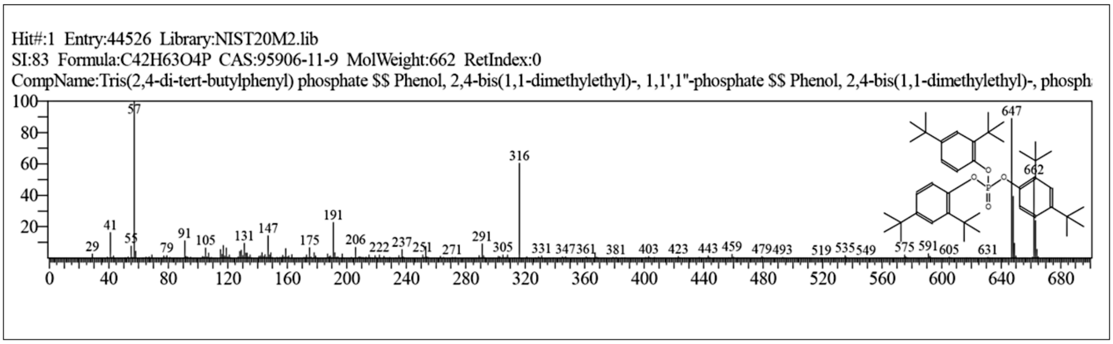

Fig. 3: Quantitative Report for the Tris (2,4-di-tert-butylphenyl) phosphate

Isolation of Chlorpyrifos Pesticide Degrading Bacterial Isolates

The soil sample from a banana field containing chlorpyrifos pesticide included all eight of the bacterial isolates that were able to use organophosphate pesticide as their only source of carbon for growth and development. They were presumptiviely identified according to their physical and biochemical characteristics as presented in the (Table 2.). The RFS1, RFS2, RFS3, RFS4, RFS5, RFS6, RFS7 and RFS8 isolates were identified as *Streptococcus* sp, *Arthrobacter* sp, *Enterobacter* sp, *Rhodococcus* sp, *Staphylococcus* sp, *Bacillus* sp, *Pseudomonas* sp and *Proteus* sp respectively.

Table 2: Phenotypic and Biochemical Characteristics of Bacterial Isolates

S.no	Shape	Gram Staining	Indole	Methyl red	Voges Proskauer	Citrate	Name of the species
RFS1	Spherical shape	Gram Positive	-ve	-ve	-ve	-ve	Streptococcus sp
RFS2	Rod shape	Gram Positive	-ve	-ve	-ve	-ve	Arthobacter sp
RFS3	Rod shape	Gram Negative	-ve	-ve	+ve	+ve	Enterobacter sp
RFS4	Cocci shape	Gram Positive	-ve	-ve	-ve	-ve	Rodococcus sp
RFS5	Spherical & grape-like clusters	Gram Positive	-ve	-ve	-ve	+ve	Staphylococcus sp
RFS6	Rod shape	Gram Positive	-ve	-ve	+ve	+ve	Bacillus sp
RFS7	Rod shape	Gram Positive	- ve	-ve	-ve	+ve	Pseudomonas sp
RFS8	Rod shape	Gram Negative	-ve	+ve	-ve	+ve	Proteus sp.

Bacterial isolates RFS1, RFS4 and RFS6 were observed that they are be tolerant in pesticide tolerance test so further they were used for bio-degradation study for monitoring their ability to degrade chlorpyrifos. significant changes in the growth curve (observed at 600 nm) of the bacteria were observed in addition to chlorpyrifos to the bacterial culture. The addition of chlorpyrifos insecticide to the culture medium increases bacterial growth because it gives an additional carbon source.

Each isolate's optical density at 600 nm was measured at various time intervals. i.e., 24, 48, 72, 96, 120, 144, 168, 192, 216 and 264 on nutrient broth. Out of 8 isolated bacterial strains only 3 isolates- RFS1, RFS4 and RFS6 were selected on their ability to grow in different concentration of chlorpyrifos (25,50,75 and100µL/ml) for the bio-degradation study shown in (Table 3). The outcome of the isolates' growth reaction while chlorpyrifos existed showed in the table 3 RFS1, RFS4 and RFS6 named as *Streptococcus*, *Rodococcus* and *Bacillus* were utilized the pesticide as the only carbon and energy source. *Streptococcus* showed maximum growth (OD=1.936) (Fig 4.) and *Bacillus* (OD= 1.842) (Fig 5.) in 10th days. The same results was found that *Bacillus* tropicus degrade 60% chlorpyrifos after 48 hrs. of incubation found by Rayu *et al*.[5] while *Rodococcus* recorded highest growth in 9th days incubation (Fig 6). that Chlorpyrifos was subjected to biodegradation study with RFS1, RFS4 and RFS6 bacterial isolates for a period of 12 days. In the light of this fact, biodegradation especially microbial degradation has proven to be a suitable method for pesticide elimination. Most of the previous study indicated that Rhodobacter could effectively degrade the pesticide like 2,4- D and chlorpyrifos.[3] Chlorpyrifos is changed into chlorpyrifos oxon by the oxidation reaction, which is one of the steps involved in the mechanism of chlorpyrifos degradation.

Table 4 shows that the calculation value of growth efficacy as the relative increase in OD of the *chlorp-yrifos-treated culture* compared to its respective control at each time point.

Growth Efficacy (%)= $OD_{treatment}$ − OD control X 100/ OD control

Table 3: Growth curve and Biodegradation study for chlorpyrifos using RFS1, RFS4 and RFS6 bacterial culture

Time	RFS1 and chlorp-yrifos	RFS1 control	Chlorp-yrifos with RFS1	RFS4 and chlorp-yrifos	RFS4 control	Chlorp-yrifos with RFS4	RFS6 and chlorp-yrifos	RFS6 control	Chlorp-yrifos with RFS6
0	0	0	1.611	0	0	1.441	0	0	1.441
24	0.648	0.108	1.507	0.522	0.213	1.325	0.584	0.192	1.405
48	0.748	0.153	1.732	0.636	0.245	1.391	0.668	0.201	1.628
72	0.721	0.241	1.596	0.609	0.267	1.486	0.697	0.245	1.523
96	0.776	0.366	1.724	0.623	0.301	1.491	0.701	0.312	1.673
120	0.799	0.388	1.776	0.627	0.367	1.507	0.733	0.345	1.687
144	0.829	0.393	1.81	0.63	0.345	1.539	0.714	0.367	1.685
168	0.838	0.418	1.873	0.619	0.387	1.652	0.696	0.421	1.711
192	0.831	0.457	1.936	0.622	0.402	1.503	0.705	0.456	1.842
216	0.599	0.462	1.445	0.628	0.412	1.565	0.689	0.49	1.605
264	0.605	0.472	1.471	0.69	0.423	1.595	0.696	0.523	1.634

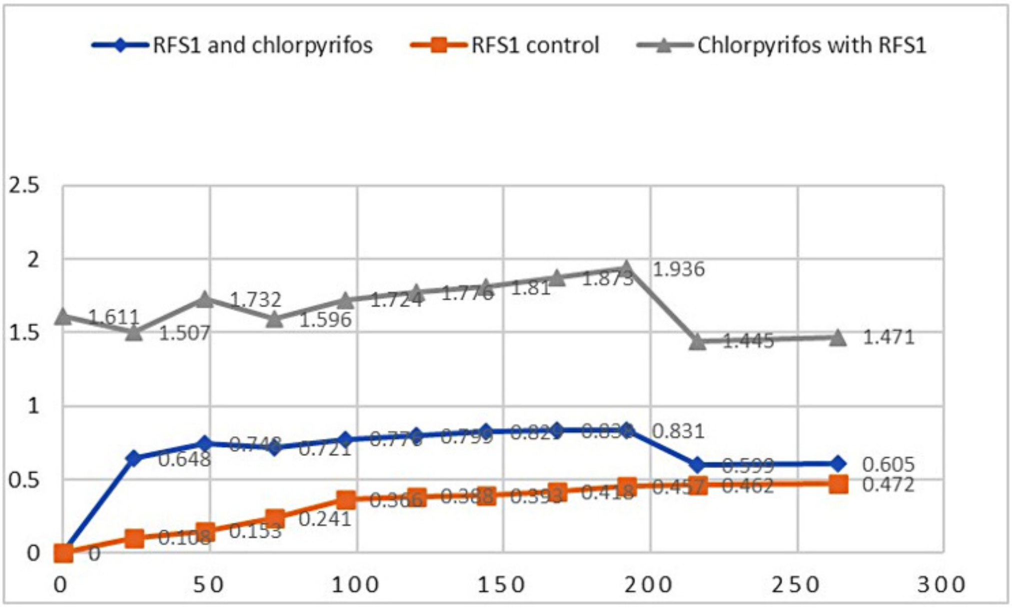

Fig. 4: Growth curve and Bio-degradation study for chlorpyrifos using RFS1 bacterial culture

RFS1 (*Streptococcus*) showed the highest initial growth efficacy (500%) at 24 h, but then gradually declined after 72 h. RFS6 (*Bacillus*) maintained strong growth efficacy (200–230%) up to 48 h, and still showed above 90% up to 144 h, confirming its robustness. RFS4 (*Rhodococcus*) had moderate efficacy (70–160%), with more stable performance across the incubation period compared to RFS1. This indicates that RFS1 is the fastest responder, but RFS6 sustains growth better over time, making it a strong candidate for long-term chlorpyrifos biodegradation.

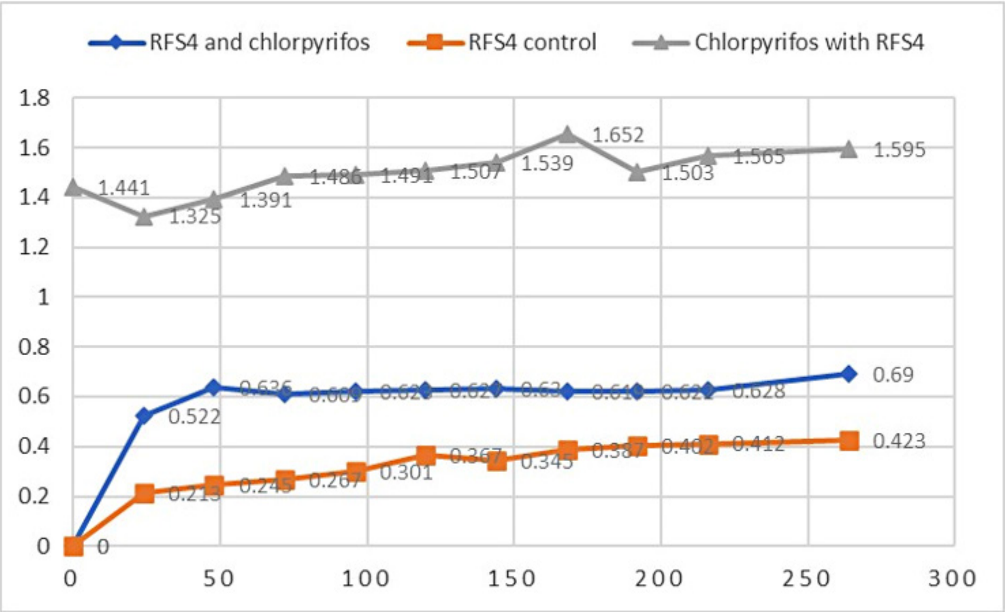

Fig. 5: Growth curve and Bio-degradation study for chlorpyrifos using RFS4 bacterial culture

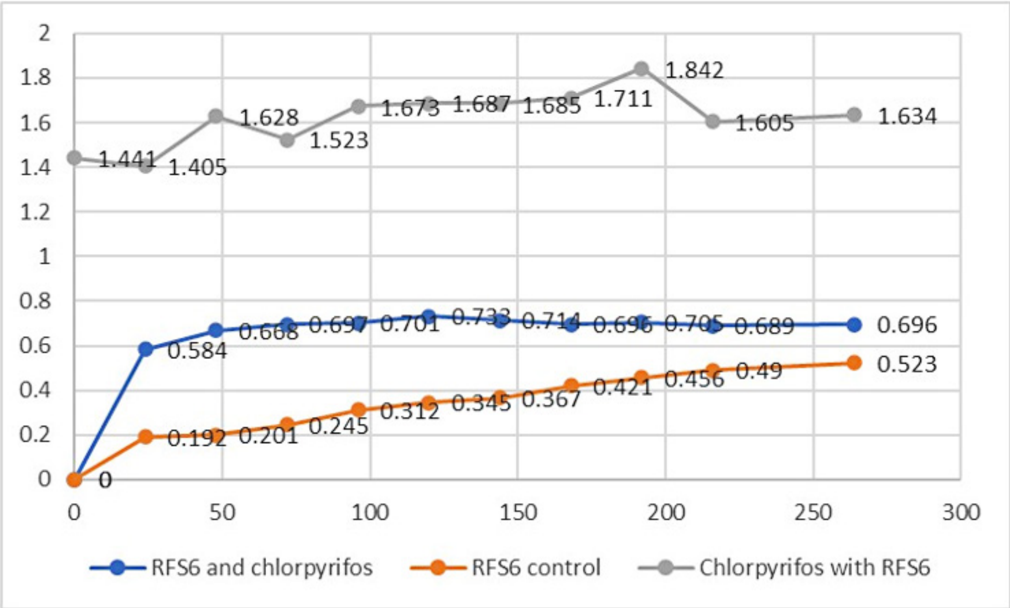

Fig. 6: Growth curve and Bio-degradation study for chlorpyrifos using RFS6 bacterial culture

Table 4: Growth efficacy of bacterial cultures expressed as the relative increase in optical density (OD) of chlorpyrifos-treated samples compared to untreated controls at different time intervals.

Time (h)	RFS1 (%)	RFS4 (%)	RFS6 (%)
0	0.00	0.00	0.00
24	500.00	145.07	204.17
48	388.89	159.59	232.34
72	199.17	128.09	184.49
96	112.02	106.98	124.68
120	105.93	70.84	112.46
144	110.94	82.61	94.55
168	100.48	59.95	65.32
192	81.84	54.73	54.61
216	29.65	52.43	40.61
264	28.18	63.12	33.08

Discussion

Chlorpyrifos, an organophosphate pesticide, has long been valued for its broad-spectrum efficacy in agriculture. However, its persistence and toxicity have raised significant concerns regarding environmental and human health, resulting in stricter regulations and growing interest in sustainable pest management strategies.[18] In the current investigation, chlorpyrifos residues were found in soil samples from the Suthamalli reservoir region using UV-Visible spectroscopy and GC-MS. A distinct absorbance peak at 225 nm confirmed the pesticide's presence in the UV spectrum, consistent with previous findings that chlorpyrifos exhibits strong UV absorption in the 220–285 nm range.[16] GC–MS analysis further validated the presence of chlorpyrifos at a retention time of 12.434 min, alongside additional contaminants such as tris (2,4-Di-Tert-Butylphenyl) phosphate, indicating that pesticide residues often co-exist with industrial additives in agricultural soils.[15] The isolation of eight bacterial strains capable of utilizing chlorpyrifos as the exclusive carbon source underscores the role of indigenous soil microbiome in natural detoxification processes. Among these, *Streptococcus* (RFS1), *Rhodococcus* (RFS4), and *Bacillus* (RFS6) exhibited significant growth responses when exposed to chlorpyrifos. Growth efficacy analyses revealed that RFS1 displayed rapid initial adaptation, reaching 500% growth efficacy at 24 h, whereas RFS6 demonstrated more sustained degradation potential, maintaining over 90% efficacy beyond 144 h. This finding is consistent with prior studies where *Bacillus*[13] and *Rhodococcus* species[17] were identified as efficient degraders of organophosphate due to their diverse enzymatic pathways, including hydrolysis and oxidation.[14]

The differential growth responses of the isolates suggest complementary degradation strategies: fast responders like *Streptococcus* initiate rapid breakdown, while robust degraders such as *Bacillus* ensure prolonged activity. These results highlight the potential of harnessing native bacterial consortia for bio-remediation of chlorpyrifos-contaminated soils. Similar microbial-mediated degradation pathways have been reported, where chlorpyrifos is oxidized to chlorpyrifos-oxon and subsequently metabolized into less toxic products.[19] Thus, microbial bio-degradation represents a practical, eco-friendly strategy for pesticide removal in agricultural systems. Future work should focus on the genetic characterization of degradation enzymes and the development of bioaugmentation models for large-scale field applications.

Conclusion

Our findings' showed the isolated bacterial strains that could use the chlorpyrifos pesticide as a soil carbon source. One important mechanism by which xenobiotic chemicals are eliminated from the environment and avoid contamination of the environment is the use of these compounds by soil bacteria. Result of this study suggest that the isolates *Streptococcus*, *Rodococcus* and *Bacillus* capacity to use and proliferate in the presence of chlorpyrifos. It shows that they might be applied to the bioremediation of soil and water damaged by pesticides.

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Conflict of Interest

The author(s) do not have any conflict of interest.

Data Availability Statement

The Manuscript incorporates all datasets produced.

Ethics Statements

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Permission to Reproduce Material from other Sources

Not Applicable

Author Contributions

- **M. Mathar Fathima:** Designed the experiment and methodology, writing, executed experiment, organised the data, and prepared the manuscript.
- **S. Kannan:** Reviewed the data along with the whole manuscript.
- **P. Dheivanai:** Data analysis

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