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# Isolation of Pesticide-Degrading Microorganisms Using Winogradsky Columns: A Sustainable Approach to Bioremediation

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## Abstract

In India, pesticides play a significant role in modern agriculture by enhancing crop yields and protecting plants from pests. However, their toxic and persistent nature poses a serious threat to the environment and human health. Among them, chlorpyrifos, an organophosphate pesticide, is of particular concern due to its wide use and long-term residual presence in soil and water. This calls for urgent and eco-friendly solutions such as biodegradation through microbial action. In the present study, Winogradsky columns were employed as a natural, cost-effective enrichment system to isolate native bacterial strains capable of degrading chlorpyrifos. Soil samples were collected from a farm, nursery, and garden in pesticide-contaminated regions and were used to construct Winogradsky columns. These columns allowed for the development of nutrient and oxygen gradients, facilitating the selective enrichment of pesticide-degrading microbial communities over 8 weeks.

Following enrichment, five bacterial isolates—designated as G1, F2, F3, F4, and F5—were obtained. These isolates underwent both qualitative (clear zone formation) and quantitative (UV spectrophotometric) assays to evaluate their pesticide degradation potential. Further studies assessed the effect of environmental conditions such as temperature, pH, and NaCl concentration on bacterial growth to determine the robustness of the isolates.

Morphological and biochemical characterization, along with reference to Bergey's Manual of Systematic Bacteriology, identified the isolates as *Staphylococcus aureus* (G1), *Pseudomonas aeruginosa* (F2 and F5), and *Micrococcus luteus* (F3), while isolate F4 remained unidentified. Antibiotic susceptibility testing using the Kirby-Bauer disc diffusion method revealed sensitivity to most common antibiotics, ensuring their biosafety for environmental use.

To investigate the genetic basis of pesticide degradation, plasmid DNA isolation and plasmid curing were performed. Four isolates (F2, F3, F4, F5) were found to possess plasmids, but after curing with ethidium bromide, all strains retained their ability to degrade chlorpyrifos, suggesting that the responsible genes are located on the bacterial chromosome, ensuring genetic stability. This study demonstrates the effectiveness of using Winogradsky columns to enrich and isolate indigenous chlorpyrifos-degrading bacteria, highlighting their potential application in sustainable bioremediation of pesticide-contaminated soils.

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**KEYWORDS:** Winogradsky Column, Chlorpyrifos, Biodegradation, Bacteria, Plasmid Curing, Bioremediation

## 1. INTRODUCTION

Agriculture forms the backbone of the Indian economy, providing livelihood and food security to over a billion people. However, with declining cultivable land and increasing demand for food production, modern agricultural practices have become heavily reliant on chemical inputs, particularly pesticides. These chemicals, though essential for controlling pests and improving yields, also pose serious risks to human health and environmental sustainability.

Pesticides such as organophosphates are widely used to protect crops from insects, weeds, and microbial pathogens. Among them, Chlorpyrifos (CPF) is one of the most extensively applied broad-spectrum insecticides in agriculture, horticulture, and even in domestic settings. It acts by inhibiting cholinesterase, an enzyme responsible for breaking down the neurotransmitter acetylcholine, thus affecting the nervous system of pests and, unintentionally, other non-target organisms.

However, studies show that nearly 90% of pesticides applied to crops never reach their intended targets. Instead, they accumulate in soil, water, and air, contaminating ecosystems and entering the food chain. CPF residues have been linked to serious health problems, including infertility, miscarriages, neurological damage, immune dysfunction, and cancer. Its toxic effects extend beyond humans to birds, aquatic life, and beneficial microorganisms, making its persistence in the environment a major concern.

To address this growing threat, bioremediation has emerged as an eco-friendly and cost-effective solution. Traditional remediation techniques like soil excavation or chemical oxidation are costly, invasive, and often produce secondary pollution. This has driven the demand for sustainable, eco-friendly alternatives. However, natural attenuation is often too slow in highly contaminated or stressed environments. Hence, this study emphasizes bioaugmentation the introduction of indigenous new method degrading bacteria isolated from contaminated soil.

One particularly effective method to study and enrich such microorganisms is through the Winogradsky column.

Originally developed by Sergei Winogradsky, this column is a self-contained soil ecosystem that creates nutrient and oxygen gradients over time, fostering the growth of diverse microbial communities. The Winogradsky column serves as a natural enrichment system, enabling the selective growth of pesticide-tolerant and degrading microbes from native soils without the need for artificial selective agents.

In this study, the Winogradsky column is used as a model system to isolate chlorpyrifos-degrading microorganisms from soil samples collected from agricultural and nursery environments. Over a period of 8 weeks, the columns provide the conditions for microbial succession and selection based on the pesticide stressor added. This low-cost and sustainable method not only helps in identifying efficient degraders but also mimics the natural habitat, ensuring the adaptability of isolated strains to real-world environmental conditions.

By coupling the Winogradsky column approach with biochemical, molecular, and environmental analysis, this research aims to isolate potent pesticide-degrading bacteria and evaluate their potential for future bioremediation applications in contaminated soils.

## 2. LITERATURE REVIEW

### A. National Status

In India, extensive research has been undertaken to address the environmental challenges posed by pesticide pollution, especially chlorpyrifos, through microbial bioremediation techniques. Indian agricultural soils, due to heavy pesticide usage, have become reservoirs of potential pesticide-degrading microbes.

Naphade *et al*. (2012) [1] conducted a study in sugarcane fields of Maharashtra, where *Pseudomonas aeruginosa* was isolated and shown to degrade up to 90% of chlorpyrifos. This study highlighted the adaptability of indigenous microbes under pesticide pressure and laid a foundation for using native bacteria in bioremediation.

Sharma *et al*. (2013) reported successful isolation of *Bacillus* and *Micrococcus* spp. from contaminated soils in Pune using enrichment techniques in mineral salt medium (MSM) containing chlorpyrifos. These isolates demonstrated pesticide degradation ranging from 46% to 71.6%, supporting the potential of Indian soil microbes in bioremediation.

Bartakke *et al*. (2019) applied plasmid curing techniques to isolates obtained from contaminated sites in Mumbai. Their findings suggested that some degradation genes are chromosomally encoded, ensuring greater genetic stability of degrading traits.

Ramesh *et al*. (2017) emphasized the utility of Winogradsky columns in Indian research and education. They highlighted how the column serves not only as a teaching tool but also as an effective method for isolating pollutant-tolerant microbial populations from Indian soils.

These national studies underline the potential of Indian agricultural environments as sources of microbial strains with significant biodegradation capabilities and support the integration of Winogradsky column techniques for indigenous isolate enrichment.

### B. International Status

Globally, pesticide bioremediation has been widely explored with significant advancements in the understanding of microbial degradation mechanisms, genetic determinants, and environmental applications.

Singh *et al*. (2004) [2] in the USA demonstrated effective chlorpyrifos degradation by *Pseudomonas putida* strains isolated from contaminated agricultural fields. Their work was among the first to systematically examine the use of soil bacteria for organophosphate bioremediation.

Li *et al*. (2015) from China identified the *mpd* gene on plasmids in bacteria capable of degrading organophosphates. Their research revealed the role of plasmid-borne genes in rapid adaptation and enhanced degradation capabilities in mixed microbial communities.

Silva *et al*. (2018) in Brazil developed a microbial consortium from river sediments which showed synergistic degradation of chlorpyrifos, reporting over 90% degradation in just seven days. This highlighted the advantage of using consortia over pure cultures for efficient bioremediation.

Schmidt *et al*. (2010) in Germany used Winogradsky columns to explore microbial diversity and spatial metabolic differentiation. Their study validated the column as an excellent tool for enriching bacteria capable of various functions, including pollutant degradation, under gradient conditions.

Yang *et al*. (2006) cloned the *mpd* gene from a chlorpyrifos-degrading bacterium and expressed it in recombinant strains, illustrating the global interest in genetic and synthetic biology approaches to bioremediation.

## 3. METHODOLOGY

**Collection of Soil Samples**

Soil samples were collected from three different locations in Thane district, Maharashtra, India, each representing a potentially pesticide-contaminated environment:

Sample F: pesticide-exposed rice farm in Sopara Village, Nallasopara Sample N: Green Garden Nursery in Vasai, Sample G: Garden in Nallasopara.

**Fig 1:** Sample F: Collection from Pesticides exposed rice farm in Nallasopara

**Fig 2:** Sample N: Collection from Green Garden Nursery in Vasai, Palghar.

**Fig 3:** Sample G: Collection of soil sample from a Garden in Nallasopara

Approximately 100–150 grams of soil were collected from the top 15 cm using a sterile spatula and transferred into sterile plastic bags. Samples were stored at 4°C until further use to preserve microbial viability.

**Preparation of Winogradsky Columns for Enrichment**

To selectively enrich pesticide-degrading microbial communities, Winogradsky columns were constructed for each soil sample:

Transparent glass columns were filled two-thirds with respective soil samples.

A small amount of shredded newspaper (as a carbon source), calcium carbonate, and calcium sulfate was mixed into the soil to promote microbial growth.

Each column was supplemented with 100 µL of chlorpyrifos pesticide to act as a selective agent.

Sterile pond water was added until the soil was fully saturated, and the columns were sealed with cotton plugs.

The columns were incubated at room temperature in indirect sunlight for 8 weeks to allow nutrient and oxygen gradients to form, supporting microbial diversity and pesticide tolerance.

**Fig 4:** Winogradsky Column Assembly

**Enrichment of Pesticide-Degrading Bacteria Using Mineral Salt Medium (MSM)**

After incubation, soil and water samples were aseptically withdrawn from different zones of the Winogradsky columns.

1 g of soil from each zone was inoculated into 30 mL of sterile Mineral Salt Medium (MSM) in a conical flask.

MSM was supplemented with 100 µL of chlorpyrifos as the sole carbon source.

Composition of MSM (g/L):

$KH_2PO_4$ – 4.8

$K_2HPO_4$ – 1.2

$NH_4NO_3$ – 1.0

$MgSO_4 \cdot 7H_2O$ – 0.2

$Ca(NO_3)_2 \cdot 4H_2O$ – 0.04

$FeSO_4 \cdot 3H_2O$ – 0.001

pH – 7.0

The flasks were incubated at 28°C on a rotary shaker (100 rpm) for 7 days (First enrichment).

5 mL of enriched broth was transferred aseptically to fresh MSM with chlorpyrifos and incubated under the same conditions for another 7 days (Second enrichment).

**Isolation of Pesticide-Degrading Bacteria**

Loopfuls of the second enrichment broth were streaked on sterile Nutrient Agar (NA) plates using the 5-sector streaking method.

Plates were incubated at 28°C for 48 hours to obtain isolated colonies.

**Qualitative Screening of Isolates**

All isolated colonies were spot inoculated on sterile NA plates supplemented with chlorpyrifos.

Plates were incubated at 28°C for 3–4 days.

Clear zones around colonies indicated chlorpyrifos degradation capability.

**Quantitative Pesticide Degradation Assay**

Isolates with positive qualitative results were inoculated into MSM containing chlorpyrifos.

Incubation was carried out at 28 °C, and the pesticide degradation was monitored over 10 days.

At regular intervals, cell-free supernatant was extracted and optical density measured at 300 nm using a UV-Visible spectrophotometer (V-630) to determine the degradation efficiency.

**Identification of Isolates**

**Isolates were characterized through:**

**Morphological studies:** Gram staining, motility

**Biochemical tests:** Indole, Methyl Red, Voges-Proskauer, Citrate, Catalase, Oxidase, Nitrate reductase.

Identification was confirmed by referring to Bergey's Manual of Systematic Bacteriology.

## 4. RESULTS

**Isolation of Pesticide-Degrading Bacteria**

After 8 weeks of incubation in Winogradsky columns, a total of 13 bacterial colonies were isolated following enrichment in mineral salt medium (MSM) supplemented with chlorpyrifos. The isolates were designated as G1, F2, F3, F4, and F5, based on their source locations:

**Table 1:** Numbers of isolated pesticide degrading bacteria.

| Site of soil | No. of isolates obtained |
|---|---|
| F | 06 |
| G | 04 |
| N | 03 |

**Qualitative Analysis**

**(Clear Zone Formation)**

The isolates were spot-inoculated on nutrient agar plates containing chlorpyrifos. Formation of clear zones around colonies indicated their pesticide-degrading ability.

**Table 2:** For qualitative analysis.

| Bacterial isolates | Clear zone (mm) |
|---|---|
| G1 | 12 |
| F2 | 36 |
| F3 | 30 |
| F4 | 20 |
| F5 | 32 |

**Table 3:** For quantitative analysis.

| Isolates designation | G1 | F2 | F3 | F4 | F5 |
|---|---|---|---|---|---|
| Size | 1 mm | 2 mm | 1 mm | 1 mm | 1 mm |
| Shape | Circular | Circular | Circular | Circular | Circular |
| Color | White | Green | Yellow | White | Green |
| Margin | Entire | Entire | Entire | Entire | Entire |
| Elevation | Flat | Flat | Convex | Flat | Flat |
| Opacity | Opaque | Opaque | Opaque | Opaque | Opaque |
| Consistency | Mucoid | Butyrous | Butyrous | Butyrous | Butyrous |
| Gram's nature | Gram positive | Gram negative | Gram positive | Gram negative | Gram negative |

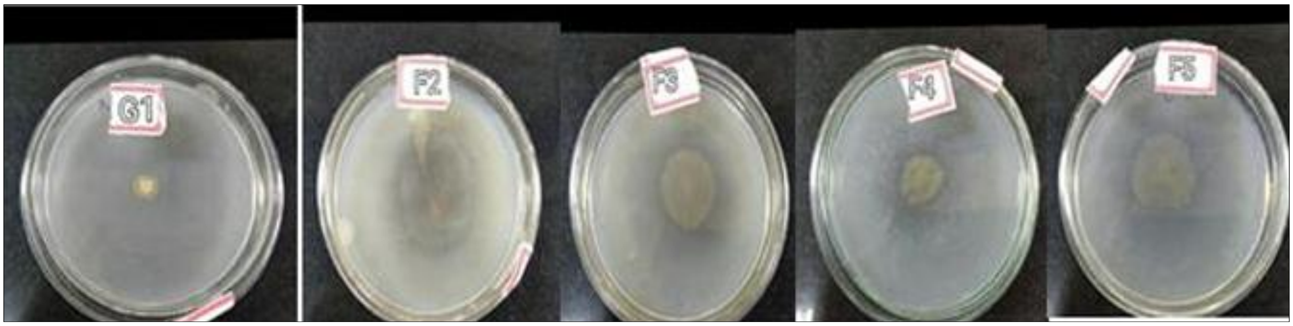


**Fig 5:** Clear zone of isolated pesticide-degrading bacteria.

**Quantitative Analysis**

All five isolates were evaluated for quantitative degradation using UV spectrophotometry at 300 nm. The percentage of degradation was calculated based on the reduction in optical density from Day 0 to Day 10.

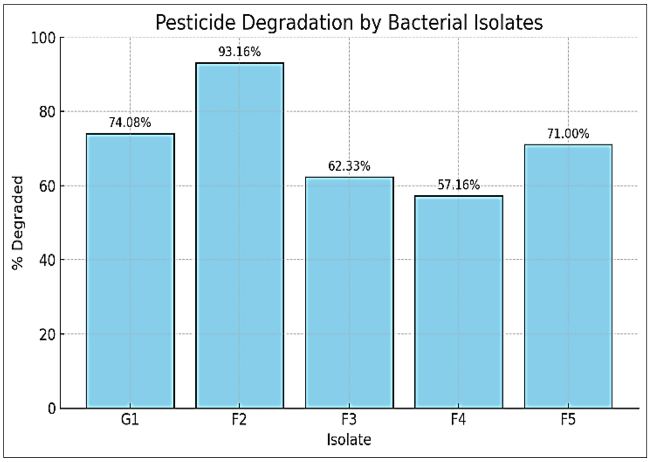


**Fig 6:** Percentage of Degradation

**Identification of isolated pesticide degrading bacteria**

The colony characteristics and biochemical tests of potential pesticides were studied. The results are summarized in the tabular format below (Table 4) (Table 5).

**Table 4:** Colony characters of isolated pesticide degrading bacteria.

| Isolate | OD Day 0 | OD Day 10 | % Degraded |
|---|---|---|---|
| G1 | 1.000 | 0.259 | 74.08% |
| F2 | 1.000 | 0.068 | 93.16% |
| F3 | 1.000 | 0.377 | 62.33% |
| F4 | 1.000 | 0.428 | 57.16% |
| F5 | 1.000 | 0.290 | 71.00% |

**Table 5:** Biochemical tests of isolated pesticide degrading bacteria

| Biochemical tests | G1 | F2 | F3 | F4 | F5 |
|---|---|---|---|---|---|
| Indole test | - | - | - | - | - |
| Methyl red test | - | - | - | - | - |
| Voges-Proskour test | - | - | - | - | - |
| Citrate utilization test | + | + | + | + | + |
| Catalase test | + | + | + | + | + |
| Oxidase test | + | - | + | - | - |
| H2S production | + | - | - | - | - |

## 5. CONCLUSION AND RECOMMENDATION

This study demonstrated the effective use of Winogradsky column-based enrichment for isolating chlorpyrifos-degrading bacteria from diverse environmental sources such as farm soil, garden soil, and nursery soil. The enrichment in Minimal Salt Medium (MSM) supplemented with chlorpyrifos allowed for the selective growth of pesticide-tolerant microorganisms. Among the five isolates obtained—Staphylococcus aureus (G1), Pseudomonas aeruginosa (F2 and F5), Micrococcus luteus (F3), and an unidentified isolate (F4)—Pseudomonas aeruginosa (F2) exhibited the highest pesticide degradation potential, with 93.16% degradation in quantitative analysis and a 36 mm clear zone in qualitative screening.

The results affirm the effectiveness of Winogradsky columns as a sustainable and selective technique for enriching native microbial populations with specific metabolic capabilities, such as pesticide degradation. The adaptability of these isolates to varying pH, temperature, and salt concentrations further supports their resilience and potential for field-scale bioremediation.

By isolating efficient degraders like *P. aeruginosa*, this study highlights the role of indigenous microorganisms in natural attenuation and site-specific bioremediation strategies. Furthermore, the eco-friendly and cost-effective nature of this method provides a strong alternative to conventional physico-chemical treatments for pesticide-contaminated environments. This approach also contributes to restoring soil fertility, promoting safer agricultural practices, and reducing the environmental impact of persistent agrochemicals like chlorpyrifos.

## 6. RECOMMENDATIONS

Molecular Identification of Unidentified Isolate (F4) - Isolate F4, which could not be identified through biochemical tests, should undergo molecular characterization (e.g., 16S rRNA sequencing) to determine its taxonomic identity and potential novelty as a pesticide-degrading microorganism.

Optimization of Enrichment Conditions in Winogradsky Columns Further optimization of the Winogradsky column composition—including substrate type, carbon sources, and redox gradients—can enhance the selective growth of highly efficient pesticide-degrading microbes.

**Development of Bacterial Consortia**

Based on degradation efficiency, consortia comprising F2 (Pseudomonas aeruginosa), F5 (Pseudomonas aeruginosa) and other compatible isolates could be formulated to increase degradation rates and target broader pesticide classes.

**Field Application Studies**

Isolates with high degradation potential, especially F2, should be tested under field conditions to evaluate their survival, adaptability, and effectiveness in real agricultural soils contaminated with chlorpyrifos.

**Bioaugmentation Strategy Design**

The high-performing isolates enriched via Winogradsky columns should be used in bioaugmentation strategies where these microbes are introduced into contaminated soils to accelerate the degradation process.

Soil Health and Fertility Assessment Post-Bioremediation

Long-term monitoring of treated soils should be done to assess restoration of microbial diversity, fertility, and crop productivity, ensuring the sustainability of bioremediation practices.

**Awareness and Training for Farmers**

Educational programs and demonstrations should be conducted to promote on-site bioremediation using locally enriched bacterial isolates from Winogradsky columns as a cost-effective and eco-friendly alternative to chemical cleanup.

Further Exploration of Winogradsky Columns for Other Pollutants

The success of chlorpyrifos degradation encourages extending the use of modified Winogradsky columns for isolating microorganisms capable of degrading other persistent pollutants like plastics, heavy metals, or other organophosphates.

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