Characterization of chlorpyrifos-degrading bacteria from paddy field soil for bioremediation

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Abstract. Chlorpyrifos a widely used organophosphate pesticide poses significant environmental hazards due to its persistence, toxicity and efficiency in contaminating soil and water resources. The urgent need for effective remediation strategies has led to increased interest in microbial bioremediation which utilizes the natural ability of microorganisms to degrade environmental pollutants. This study aimed to isolate and characterize chlorpyrifos degrading bacteria from the paddy field soil. Soil sample was collected from chlorpyrifos-treated fields and subjected to enrichment culture technique. The resulting bacterial isolates were screened for their degradation capabilities to assess their ability to metabolize chlorpyrifos. Based on the pesticide degradation potential, one isolate, PDB-1 was selected for further molecular characterization using 16S rRNA gene sequencing. This analysis revealed that among the chlorpyrifos-degrading bacterial community, *Acinetobacter baumannii* demonstrated significant pesticide degradation activity. The findings of this research enhance our understanding of microbial pathways involved in chlorpyrifos degradation and contribute to the development of sustainable bioremediation strategies for contaminated agricultural environments.

Keywords: agricultural soil, bioremediation, chlorpyrifos, environmental pollution, organophosphate, 16S rRNA gene sequencing

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1. Introduction

Agricultural practices worldwide increasingly depend upon chemical pesticides to safeguard crops and maximize yields. Organophosphates (OPs) like chlorpyrifos is among the most widely used pesticide due to their immediate effective against a broad spectrum of pests [1]. From 1960, chlorpyrifos is one of the effective pesticide due to its efficiency in targeting wide insect populations in various agricultural crops. However, the continuous usage leads to significant environmental pollution and public health concerns. Chlorpyrifos residues can persist in soils and water bodies leading to environmental contamination with prolonged periods [2]. Chemical persistence is further influenced by factors *viz.,* soil pH, temperature and microbial activity which affect the degradation processes of the chemical compound [3]. Such environmental contamination leads to serious risks not only to ecosystems but also to human health. Exposure to chlorpyrifos has been linked to neurological disruptions through the inhibition of acetylcholinesterase (AChE), an enzyme vital for neurotransmission. Inhibition of AChE activity results in overstimulation of nerve pathways causing symptoms like dizziness, nausea and respiratory complications [4].

Long-time persistence of chlorpyrifos in agricultural soils threatens soil quality and biodiversity. Studies have demonstrated that chlorpyrifos residues can adversely affect soil microbial communities which are important for nutrient cycling and plant growth. Also, pesticides act on non-target organisms *viz*., insects, birds and mammals which significantly alter ecosystem dynamics [5]. In humans, chronic exposure to chlorpyrifos has been associated with neurodevelopmental disorders in children and other chronic health conditions [6]. As a result, regulatory bodies in several countries, including the United States and the European Union have restricted chlorpyrifos due to its potential health impacts [7].

Traditional methods for removing pesticides from contaminated environments such as chemical degradation, thermal treatment and incineration are not considered due its high cost and secondary pollution [8]. Recently, bioremediation has emerged as a promising alternative that exploits the metabolic capabilities of microorganisms to naturally destroy chemical contaminants. This approach offers a sustainable and eco-friendly solution, as bacteria can enzymatically break down pesticides which results in non-toxic byproducts [9]. Bioremediation is particularly well-suited for addressing pesticide pollution allowing for in-situ degradation of chlorpyrifos in soil and water without extensive chemical intervention.

By considering the urgent need for sustainable remediation strategies, this study focuses on isolating and characterizing chlorpyrifos-degrading bacteria from paddy field soil. The research aims to identify bacterial strains with chlorpyrifos degradation capabilities and evaluate their potential for bioremediation applications. Agricultural soils particularly those with a history of pesticide application, harbor diverse microbial communities capable of metabolizing xenobiotic compounds like chlorpyrifos. Isolating pesticide degrading bacteria provides an opportunity to enhance bioremediation efforts and develop ecologically sound practices for managing pesticide residues.

2. Material and Methods

2.1 Soil sample collection, media and pesticide used

The soil sample was collected from topsoil layer (0-15cm) from the paddy field of Guruvoyal, Thiruvallur Dt, Tamil Nadu, India, an area with a nearly 10-year history of chlorpyrifos application. Soil sample was collected in sterile containers and transported to the laboratory for analysis. Commercial-grade chlorpyrifos (50% EC) was obtained from Sun Agro Chemical Industries, Madurai, Tamil Nadu, and used throughout the study. The choice of commercial-grade chlorpyrifos reflects the conditions to which soil microorganisms are naturally exposed [10].

The Mineral Salts Medium (MSM) enriched with chlorpyrifos served as the

primary medium for isolating chlorpyrifos-degrading bacteria. MSM was prepared with the following components per liter: 1 g NH_4NO_3, 1.73 g K_2HPO_4, 0.68 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 4 g NaCl, 0.03 g $FeSO_4 \cdot 7H_2O$, and 0.02 g $CaCl_2 \cdot 2H_2O$. Nutrient agar was also used to support bacterial growth during the isolation process [11].

2.2 Isolation and screening of chlorpyrifos-degrading bacteria

Air-dried soil samples (10 g) were added to 50 mL of MSM with chlorpyrifos at concentrations of 1, 5, 10, and 20 ppm. The flasks were incubated at 30°C on a rotary shaker at 250 rpm for 7 days. Bacterial cultures were periodically streaked onto mineral agar plates and incubated at 37°C for 24 hours to obtain pure isolates. Screening for chlorpyrifos degradation was conducted by spotting isolates on MSM agar plates supplemented with chlorpyrifos. Bacterial growth after 24 hours indicated chlorpyrifos-degrading potential. Identified bacterial isolates were sequenced for further phylogenetic analysis using BLAST and CLUSTALW [12].

3. Molecular characterization of chlorpyrifos-degrading bacteria

3.1 Genomic DNA extraction

Bacterial cultures were grown overnight in 100 mL of LB medium. Two milliliters of the culture were centrifuged at 7000 rpm for 10 minutes at 37°C and the supernatant was discarded. The pellet was resuspended in 10 mL of lysis buffer (0.15 M NaCl, 0.05 M sodium citrate buffer) and treated with 200 µL of lysozyme (10 mg/mL) at 37°C for 60 minutes. Lysis was completed by adding 500 µL of 20% SDS followed by gentle shaking for 5 minutes. 15 mL of extraction buffer containing sodium perchlorate (2.5 mL of 5 M) and a chloroform:isoamyl alcohol mixture (12.5 mL, 24:1) was added. The preparations were incubated at –20°C for 2 hours, thawed at room temperature with gentle shaking for 30 minutes, and centrifuged at 5000 rpm for 10 minutes. The supernatant was precipitated with isopropanol, washed with 70% ethanol, air-dried and dissolved in 1 mL of TE buffer. The DNA was quantified spectrophotometrically and analyzed on a 0.8% agarose gel following the method described [13].

3.2 PCR amplification of 16s rRNA gene

16S rRNA gene amplification was achieved with forward primer 5'-GAGTTTGATCCTGGCTCAG-3' and reverse primer - 5'-AGAAAGGAGGTGATCCAGCC-3'. Each reaction contained 100 ng genomic DNA, 1 µL of each primer (40 pmol/µL), 200 µM dNTPs, 1 U Taq DNA polymerase, and 1× Taq buffer. The PCR cycle included an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 5 min, annealing at 55°C for 15 s, and extension at 72°C for 3 min, and a final extension at 72°C for 5 min. Amplified PCR product was confirmed by 1.2% agarose/EtBr gel electrophoresis [14].

3.3 16S rRNA sequencing and phylogenetic analysis

The 16S rRNA gene sequences were edited and aligned using BioEdit version 7.0. The BLASTn tool (available at http://www.ncbi.nlm.nih.gov/blast/) was utilized to compare the sequences obtained in this study with previously published sequences, identifying similarities and evolutionary relationships [15]. Phylogenetic relationships were established using MEGA X software to construct a phylogenetic tree based on aligned sequences [16].

4. Results

4.1 Isolation of chlorpyrifos-degrading bacteria with different concentrations of chlorpyrifos

Soil samples (10 g) were suspended in 50 mL of MSM supplemented with varying concentrations of chlorpyrifos (1 ppm, 5 ppm, 10 ppm, and 20 ppm). A control was maintained with MSM devoid of chlorpyrifos. No bacterial colonies were observed on the MSM plates containing 10 ppm and 20 ppm chlorpyrifos. Four morphologically distinct bacterial colonies were identified on MSM supplemented with 5 ppm chlorpyrifos. Among these, one isolate showed the fastest and most robust growth along with the highest capability for chlorpyrifos hydrolysis (Figure. 1). This isolate (PDB-1) was selected for further characterization through partial sequencing of the 16S rRNA gene.

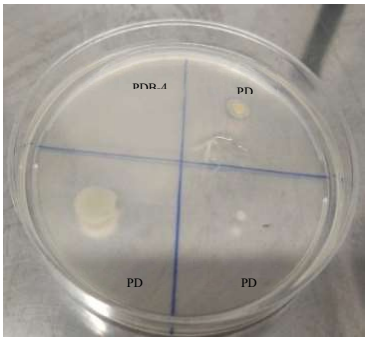

Fig. 1. Screening of potential chlorpyrifos-degrading bacteria on mineral salts agar

4.2 Molecular characterization of chlorpyrifos-degrading bacteria

Genomic DNA was successfully isolated from the selected bacterial isolate and the amplified 16S rRNA gene (Fig. 2) was further purified and sequenced. The sequences of the 16S rRNA gene from the bacterial strain was aligned and analyzed. The isolates was identified as *Acinetobacter baumannii*. Identification was confirmed by comparing the sequences against high-scoring 16S rRNA sequences in BLASTn searches. A phylogenetic tree was generated to analyze and illustrate the evolutionary relationships of PDB-1, *Acinetobacter baumannii*. The analysis indicated that this strain shares a close genetic relationship with other *Acinetobacter* species, highlighting its potential ecological significance in chlorpyrifos degradation (Fig. 3).

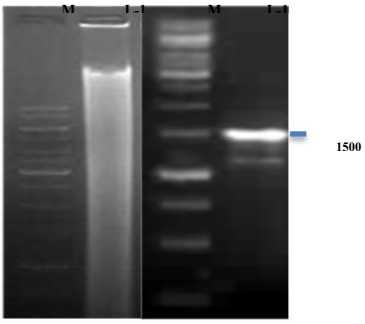

Fig. 2. Genomic DNA Isolation and 16S rRNA gene amplification from the bacteria PDB-1. a) M-1 kb DNA Marker; L-1- Separation of genomic DNA from PDB-1 b) M-1 kb DNA Marker; L-1- PCR amplification of 16S rRNA gene-1500 bp.

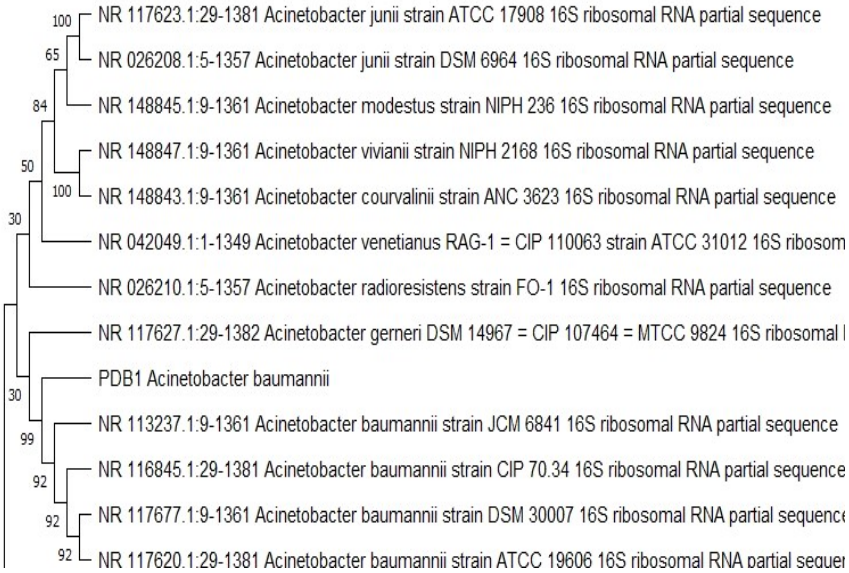

Fig. 3. Phylogenetic tree showing evolutionary relationships of *Acinetobacter baumannii*.

5. Discussion

Chemical pesticides including chlorpyrifos plays an important role in modern agriculture but their improper and continuous use raises considerable environmental concerns due to their persistence in soil and toxicity to non-target organisms. The isolation of chlorpyrifos-degrading bacteria is important for developing bioremediation strategies to mitigate pesticide contamination. In this study, MSM was used to enrich soil samples with varying concentrations of chlorpyrifos as a sole carbon source. The presence of four morphologically distinct bacterial colonies on MSM agar indicates the resistance or adaptability of microbial communities to organophosphate pollutants. The rapid growth and chlorpyrifos-hydrolyzing capability of the isolated bacteria underscore their potential degradation potential as biocontrol agents. This finding aligns with previous research that highlights the role of soil bacteria in detoxifying pesticides and organic pollutants [17], [18].

Molecular characterization using 16S rRNA gene sequencing uncovered that

the predominant chlorpyrifos degrading bacterial isolate was identified as *Acinetobacter baumannii*. Acinetobacter is known for their versatile metabolic capabilities and resistance to various environmental stresses, including toxic compounds. *Acinetobacter baumannii* has been reported to possess chlorpyrifos-degrading enzymes, contributing to its survival in contaminated environments [19]. The construction of a phylogenetic tree for *Acinetobacter baumannii* provides insights into its evolutionary relationships with other closely related species. Furthermore, the phylogenetic placement of the isolate indicates its adaptation to similar ecological niches, supporting the need for further studies to explore its metabolic pathways and enzymatic mechanisms.

Similar to our study, a soil bacterium capable of using chlorpyrifos as its sole carbon source was isolated through selective enrichment on a mineral medium containing chlorpyrifos. This bacterium identified as *Providencia stuartii* based on 16S rRNA sequencing, demonstrated efficient growth at lower concentrations of chlorpyrifos (50–200 mg/L) with significant degradation potential [20]. The ecological significance of chlorpyrifos-degrading bacteria extends beyond bioremediation. These microorganisms can enhance soil health by promoting microbial diversity and improving nutrient cycling in agroecosystems. By degrading harmful pesticides, they moderate the adverse effects on beneficial soil fauna and flora ultimately contributing to sustainable agricultural practices [21].

6. Conclusion

In conclusion, the identification and characterization of *Acinetobacter baumannii* as a chlorpyrifos-degrading bacterium focus its potential for bioremediation of contaminated soil. Future research should focus on elucidating the metabolic pathways involved in chlorpyrifos degradation and evaluating the efficacy of these bacterial strains in field applications. Also, exploring the potential of enzymes such as laccase and peroxidase to enhance pesticide degradation efficiency is needed. These enzymes can significantly improve the bioremediation process by targeting persistent pollutants. Additionally, overcoming challenges related to microbial consortia including their adaptation to various environmental conditions and scalability for practical applications is very important. Addressing these aspects will help to develop more sustainable bioremediation

strategies paving the way for effective management of pesticide-contaminated environments.

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8 Author contributions

The author contributed significantly to the conceptualization, design, data collection, analysis and interpretation of the study. They actively drafted and revised the manuscript and take full responsibility for all aspects of the work.

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