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Microbes-assisted Biodegradation of Chlorpyrifos-an Organophosphate Pesticide and Management of Bacterial Wilt in *Solanum melongena* L

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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Original Research Article

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Abstract

Background: The extensive use of pesticides, particularly organophosphorus compounds like chlorpyrifos, has become an integral component of modern agriculture but has resulted in persistent residues that pose serious risks to soil health and environmental safety. Microbial biodegradation has emerged as an eco-friendly and cost-effective strategy for detoxifying pesticide-contaminated soils while supporting sustainable crop production.
Aims: The study aims to identify efficient chlorpyrifos-degrading bacteria (CDB) and evaluate their compatibility with selected microbial biocontrol agents for simultaneous degradation of chlorpyrifos residues and management of bacterial wilt of brinjal (*Solanum melongena* L.) caused by *Ralstonia solanacearum* in contaminated soil.
Study Design: Laboratory isolation and screening followed by pot culture experiment under controlled conditions.

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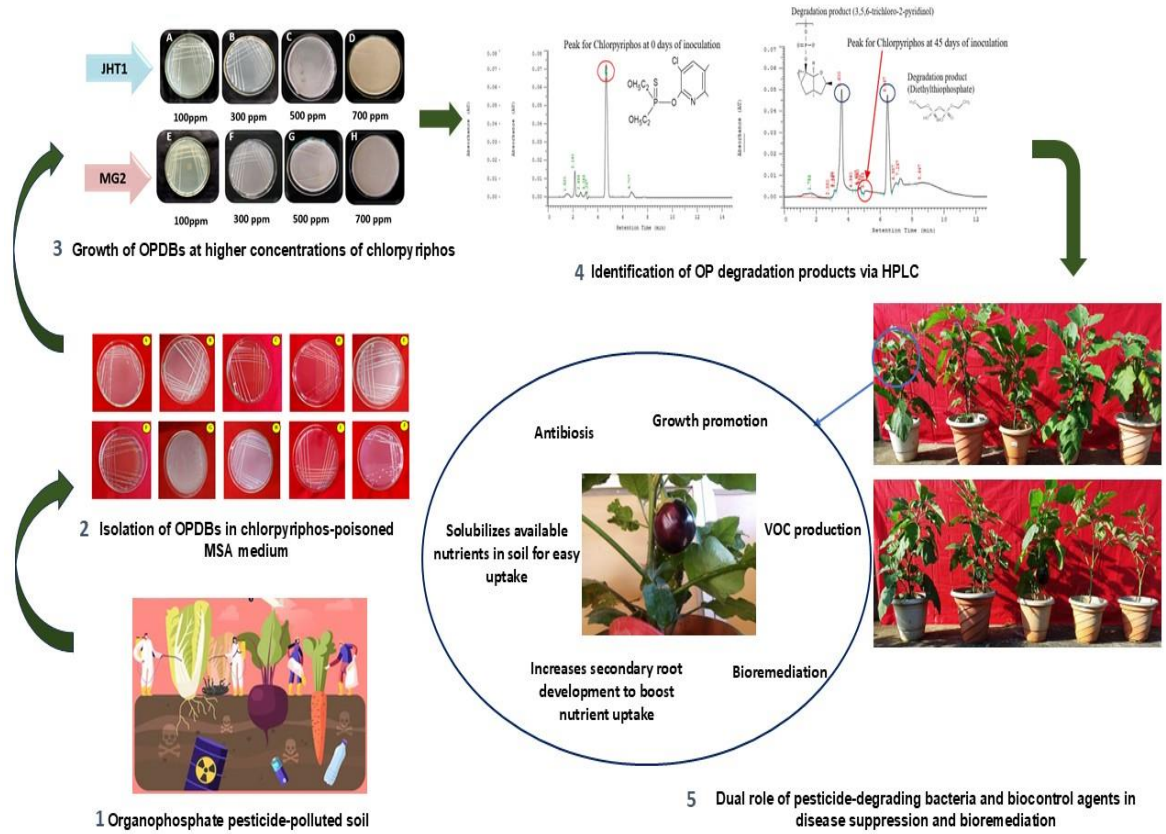
Place and Duration of Study: The study was conducted in the Department of Plant Pathology, Assam Agricultural University, during 2022-2024.

Methodology: Ten bacterial isolates capable of tolerating chlorpyrifos (25 ppm) were isolated using mineral salt medium amended with chlorpyrifos. Efficient isolates showing growth up to 700 ppm chlorpyrifos were screened for degradation efficiency. Two superior Chlorpyrifos-Degrading Bacteria (CDB), *Achromobacter marplatensis* JHT1 and *Pseudomonas azotoformans* MG2, were selected. Their *in vitro* compatibility was tested with each other and with two commonly used microbial biocontrol agents, *viz. Pseudomonas fluorescens* and *Trichoderma harzianum*. A compatible consortium containing the two CDBs and two MBCAs (*Pseudomonas fluorescens* and *Trichoderma harzianum*) was developed and evaluated in chlorpyrifos-contaminated soil challenged with *Ralstonia solanacearum*. Key parameters, including percent wilt incidence, fruit yield, and residual chlorpyrifos levels in the soil, were recorded to assess the effectiveness of the consortium.

Results: The selected isolates demonstrated growth up to 700 ppm chlorpyrifos and showed significant degradation potential under laboratory conditions. The microbial consortium comprising *A. marplatensis*, *P. azotoformans*, *P. fluorescens*, and *T. harzianum* was fully compatible in vitro. Application of the consortium in contaminated soil resulted in 80% reduction in percent wilt incidence compared to uninoculated control. Fruit yield increased 1.8-fold over the control. The consortium also significantly enhanced pesticide degradation, leading to a 71% reduction in Chlorpyrifos residues in the soil within 45 days of application, leaving only 20-28% residues. The integrated treatment performed significantly better than individual inoculations.

Conclusion: The compatible consortium of MBCA and CDB offers a promising eco-friendly strategy for residue reduction and effective management of bacterial wilt in brinjal grown in pesticide-contaminated soils, contributing to sustainable and residue-free vegetable production.

Graphical Abstract



Keywords: Bacterial wilt; biodegradation; biocontrol agents; chlorpyrifos; microbial consortium; soil bioremediation.

1. Introduction

The success of agriculture in providing food security is widely acknowledged in favor of high-yielding varieties coupled with extensive use of xenobiotics, mostly pesticides (Aktar et al., 2009) as a part of a full-proof crop protection strategy. The use of pesticides, hence, has become inevitable in modern agriculture to feed the consistently growing population from almost exhausted arable land and water resources to meet an additional food demand (Nicolopoulou-Stamati et al., 2016; Ahmed et al., 2023). Amongst different groups of chemical pesticides, organophosphorus pesticides (OP), as esters derived from phosphoric acids, are the most commercially favored class of pesticides, occupying 38% of the world market (Kumar et al., 2018). Chlorpyrifos, an extensively used OP, is considered to have moderately persistent residues in soil, even after months of application, despite its water-soluble nature and hence, served as a potential source of hazard to soil and water with varied genotoxic effects (Srivastava et al., 2022), comprising 385 million annual cases of unintentional pesticide poisoning worldwide (Boedekar et al., 2020; Kuma et al., 2025). These figures warrant an alarm about the accumulation of OP residues and their urgent detoxification. Microbes-mediated biodegradation of pesticide residues has received greater attention in the recent past, considering this process is economically more viable than other alternative processes, involving chemical or physical degradation wherein the participating microbes utilize pesticides as a carbon or phosphorus source (Kaushal et al., 2021).

The OP is widely used in field and horticultural crops against a diverse range of insect pests, apart from weeds (Kumar et al., 2018). Amongst the vegetables, the brinjal crop (*Solanum melongena* L.) receives a significant proportion of OP used against various insect pests, more particularly against brinjal fruit and shoot borers (*Leucinodes orbonalis*) (Das and Islam, 2014). The biocontrol agents such as *Trichoderma* spp., *Bacillus* spp., Pseudomonads, etc, have been considered as an integral part of crop production systems for plant and soil health management (Saikia et al., 2022). Besides disease and pest management, several rhizospheric microorganisms have been reported to have soil and plant health-promoting efficiency. Integrating such beneficial microbes in the detoxification of pesticides is a research priority for a sustainable environment and agricultural productivity. Although several soil microbes like *Bacillus*, *Flavobacterium*, *Trichoderma*, etc. have been reported to degrade pesticides, their field application study is minuscule. In our approach, we have attempted to isolate and screen a few effective CDBs from contaminated soils. Previous studies have shown that some strains of *Pseudomonas* and *Trichoderma* can reduce pesticide residues while simultaneously suppressing *R. solanacearum* through competitive exclusion and production of antimicrobial metabolites (Suresh et al., 2022). An effort was made to evaluate a cost-effective delivery mechanism through a combination of chlorpyrifos-degrading bacteria (CDB) and microbial biocontrol agents (MBCA) to detoxify pesticide-polluted soils and suppress bacterial wilt disease caused by *Ralstonia solanacearum* E.F. Smith in brinjal, which is a major cause of concern, imparting severe plant mortality coupled with heavy yield loss reported worldwide (Yendo et al., 2018). The present study was undertaken to (i) isolate and characterize efficient Chlorpyrifos-Degrading Bacteria (CDB) from pesticide-contaminated soils; (ii) evaluate their compatibility with established Microbial Biocontrol Agents (MBCA); (iii) To evaluate the combined efficacy of compatible microbial consortia in suppressing bacterial wilt of brinjal (*Ralstonia solanacearum*) and enhancing chlorpyrifos biodegradation in soil under pot culture conditions.

2. Materials and Methods

2.1 Isolation of Chlorpyrifos-Degrading Bacteria

The chlorpyrifos degrading bacteria were isolated from brinjal fields of Jorhat ($26.72/26.80^0$, 00/97'N; $94.12/94.22^0$00/88'E), Darrang ($26.52/26.53^0$, 00/58 'N;$92.13/92.16^0$00/22'E), and Sonitpur ($26.44/26.63^0$,12/45'N;$92.33/92.24^0$00/47'E) districts of Assam, where chlorpyrifos has been applied for over five years to control brinjal fruit and shoot borer (*Leucinodes orbonalis*). Farmers applied an average of 2,573 kg of pesticide active ingredients per season, with 68% exceeding recommended doses and a mean overuse of 0.88 kg per season. Prolonged application has led to residue accumulation in soils, creating selective pressure for indigenous bacteria capable of utilizing chlorpyrifos as a carbon source (Tripathy, 2022). Five topsoil samples (0-20 cm) were collected, packed in polythene bags with ice packs, and brought to the laboratory, Department of Plant Pathology, for further analysis. Soil samples were thoroughly homogenized and packed into soil columns. The native soil microflora was enriched by weekly supplementation with a synthetic chlorpyrifos solution (10 ppm) for six weeks, following the method of Latifi et al. (2012). After enrichment, a small portion of the treated soil was aseptically transferred into test tubes, serially diluted, and 1 mL aliquots ($x10^{-8}$) were spread onto ATCC Mineral Salt Medium (HiMedia, India) prepared as per Karpouzas and Walker (2000) and Singh et

al. (2004) that was supplemented with commercial-grade chlorpyrifos (trade name: TRICEL; 20 EC formulation) at 25 ppm as the sole carbon source. Plates were incubated at 27 ± 2 °C on a shaker for 24 h. Distinct colonies of chlorpyrifos-degrading bacteria were purified through repeated streaking and maintained on nutrient agar (NA) slants. The isolates were further evaluated for their growth on MSA supplemented with chlorpyrifos at varying concentrations (50, 100, 300, 500, 700, and 1000 ppm), following the procedure described by Verma (2016) and were subjected to preliminary morphological and biochemical characterization following standard protocols, including Gram staining, KOH solubility, and catalase/oxidase activities, alongside metabolic assays such as starch hydrolysis, citrate utilization, nitrate reduction, indole production, gelatin liquefaction, and arginine dihydrolase activity to establish their physiological profiles.

2.2 Growth Response of Efficient Chlorpyrifos-Degrading Bacterial Isolates

The growth response of Chlorpyrifos-degrading bacterial isolates capable of growing up to 700 ppm chlorpyrifos was evaluated in MS broth amended with four concentrations of chlorpyrifos (100, 300, 500, and 700 ppm). Each flask containing the respective medium was aseptically inoculated with the selected bacterial strains and incubated at 37 °C on an orbital shaker at 150 rpm. Growth was monitored spectrophotometrically (Double Beam Spectrophotometer 2203, Systronics) by recording the optical density (OD) at 600 nm at 24 h intervals for 9 days (0-9 days) (Yadav et al., 2015). MS broth without bacterial inoculation served as the control.

2.3 Identification of the CDB Isolates Capable of Growing at Higher Concentrations of Chlorpyrifos

The CDB isolates capable of growing at a higher chlorpyrifos concentration of 700 ppm were identified using 16S rRNA gene sequencing. The genomic DNA from oligotrophic pesticide-degrading bacterial isolates was extracted using the modified method of Maki et al. (2011). Briefly, a loopful of bacterial culture grown in nutrient broth was incubated at 37 °C until OD_{600} = 1.0, pelleted by centrifugation (12,000 rpm, 5 min), and lysed using lysis buffer (100 mM Tris-acetate, pH 7.8; 20 mM sodium acetate; 1 mM EDTA; 1% SDS) with NaCl (5 M). DNA was purified via phenol:chloroform: isoamyl alcohol and chloroform: isoamyl alcohol extraction, precipitated with chilled ethanol (–20 °C, overnight), washed with 70% ethanol, air-dried, and resuspended in TE buffer with RNase. DNA quality and concentration were assessed using a Nanodrop 1000 spectrophotometer ($A_{260}/A_{280} \geq 1.8$). PCR amplification of the 16S rRNA gene was performed in 25 µl reactions containing 2 µl of DNA template (50 ng/µl), 1.0 µl each of universal primers 27F (5′-AGAGTTTGATCCTGGCCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) at a concentration of 10 pM, 2.5 µl of dNTPs (2.5 mM each), 2.5 µl of 10X Taq buffer (containing 15 mM $MgCl_2$), and 0.5 µl of Taq DNA polymerase (5 U/µl). Thermal cycling was conducted with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min, concluded by a final extension at 72°C for 10 min. Amplification products were resolved on a 1.5% (w/v) agarose gel, visualized under UV light after ethidium bromide staining (0.5 µg/mL), and purified for sequencing. The purified PCR products were sequenced at the National Collection of Industrial Microorganisms (NCIM), Pune, India. For phylogenetic analysis, sequences showing the highest similarity were aligned using Clustal W with default parameters. A neighbor-joining phylogenetic tree was then constructed using MEGA version 7.0 software to determine the taxonomic position of the isolates (Handique et al., 2024; Saikia et al., 2022).

2.4 HPLC Analysis of Chlorpyrifos in CDB Inoculated Medium

The two most efficient bacterial isolates, capable of sustaining promising growth at chlorpyrifos concentrations up to 700 ppm, were selected for further high-performance liquid chromatography (HPLC) analysis. The chlorpyrifos-degradation assay was conducted following the method described by Mali et al. (2022), with minor modifications. Individual bacterial colonies were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of Mineral Salt (MS) medium amended with 25 ppm chlorpyrifos. The cultures were incubated at 37 ± 2 °C under constant shaking at 150 rpm in an orbital shaker to maintain aerobic conditions. Samples were periodically collected at 0, 3, 5, 7, and 14 days post-inoculation to monitor the degradation process and identify metabolic by-products. Residual chlorpyrifos and its degradation metabolites were quantified using HPLC. A control treatment without bacterial inoculation was maintained under identical conditions to account for non-biological degradation.

2.5 Compatibility Assay of CDBs with MBCAs

The efficient chlorpyrifos-degrading bacteria (CDBs) were evaluated for their compatibility with each other as well as with two microbial bioagents, *Trichoderma harzianum* (NCBI Accession No. ON364138) and *Pseudomonas fluorescens* (NCBI Accession No. KT258013). Both bioagents were obtained from the author's Biocontrol Laboratory, Department of Plant Pathology, Assam Agricultural University, Jorhat, India. These isolates had previously demonstrated antagonistic activity against *Ralstonia solanacearum* (GenBank Accession No.: OQ743450), the bacterial wilt pathogen, as reported by Rahman et al. (2023). Compatibility between these microbes was assessed through a dual culture assay; *P. fluorescens* was cultured on King's B (KB) medium at 28 ± 2 °C for 48 hours, while *T. harzianum* was maintained on Potato Dextrose Agar (PDA) at 25 ± 2 °C for 7 days. The interaction was evaluated by co-inoculating both agents on a shared Petri plate containing a 1:1 mixture of KB and PDA media, followed by incubation at 27 °C to accommodate the growth requirements of both species. CDBs were streaked at one side of the Petri plate, and the bioagent was inoculated on the opposite side, maintaining an equal distance from the plate's center. Plates were incubated at 25 ± 1 °C for five days, after which the development of inhibition zones, if any, was recorded. The experiment comprised four treatments, each replicated five times, and was arranged in a completely randomized design (CRD). The efficacy of treatments was determined based on colony growth measurements after 120 hours of incubation.

2.6 Preparation of MBCA and CDB Bioinoculants

Pure cultures of *P. fluorescens* and CDBs were maintained in NA, while *T. harzianum* was maintained on Potato Dextrose Agar (PDA) at 28±1°C. Sterile distilled water was added to the 24-hour growths of the bioagents and the pesticide-degrading isolates. From these stocks, bacterial suspensions amounting to 15 mL each were aseptically added to 1 liter of NA broth contained in different conical flasks. The flasks, after thorough stirring, were incubated at 28±1°C for 72 hr to obtain a concentration of 1×10^7 colony-forming units (CFU/mL) for each bacterium. The sterilized round-bottom flasks containing 780 mL of sterile distilled water were inoculated aseptically with 50 mL each of *P. fluorescens*, CDB1, and CDB2 cells (multiplied in Nutrient Broth) and *T. harzianum* cells (multiplied in Potato Dextrose Broth). To facilitate greater adherence property of the substrates, 10mL of sticker, carboxy-methyl cellulose (CMC @1%) was aseptically added. Similarly, 10mL of an osmoticant (mannitol @ 1%) was added to impart the substrates a higher moisture-retaining property. The flasks, after thorough mixing, were incubated at 28±1°C for 7 days to get the bioinoculants. Following the incubation period, the population count of the consortium was determined using the serial dilution pour plate technique; the final microbial load was found to be $2.8x10^9$ CFU/mL for the bacterial components (*P. fluorescens* and CDBs) and $1.5x10^7$ CFU/mL for *T. harzianum*, ensuring the high potency of the formulation.

2.7 Pot Experiment Set-up

The compatible combinations of bioagents and CDB thus developed were tested against bacterial wilt of brinjal and degradation of OP through pot (dimension: 45cm length x 25 cm diameter) grown brinjal (wilt susceptible variety, Navkiran) under net-house conditions (optimum temperature 27-30°C, photoperiod 7.2 hrs, and relative humidity 70-80%). The potting soil (Entisol, pH 6.5, 58.4% sand, 24.8% silt, 16.8% clay, and 1.8% organic carbon) was autoclaved at 121 °C and 15 psi pressure for two consecutive days. Five treatments comprising efficient pesticide-degrading isolates and biocontrol agents, each with five replications, were evaluated under a CRD. Seven-day-old seedlings were inoculated with a virulent culture of *Ralstonia solanacearum* ($1x10^8$ CFU/mL), which was collected from the Biocontrol Laboratory, Department of Plant Pathology, Assam Agricultural University, Jorhat. Inoculation was performed using the root clip method (Bora et al., 2016a, Bora et al., 2016b), after which seedlings were transplanted into sterilized pots (UV sterilized for 30 minutes for 3 consecutive days) containing sterilized soil treated with 600 mg of chlorpyrifos per kg of soil (equivalent to 600 ppm). Although 700 ppm chlorpyrifos was found to be the optimum concentration for degradation under in vitro conditions, a slightly lower concentration (600 ppm) was selected for the pot experiment to simulate a more realistic field-level contamination and to minimize possible phytotoxic effects on brinjal seedlings under controlled pot conditions, while still maintaining sufficient pesticide stress for evaluating the biodegradation potential of the microbial consortium. The pesticide was applied as a 60 mL aqueous solution of the commercial formulation (e.g., 20% EC) and thoroughly mixed with each 1 kg of soil to ensure a uniform distribution before transplanting. The soil was further amended with recommended fertilizers, including 20g N, 30g P_2O_5, 10g K_2O, and 5g $ZnSO_4$ per seedling per pot (Bora et al., 2024a), to simulate standard field conditions. The experiment was laid out in a Completely Randomized Design (CRD) consisting of five treatments, each replicated five

times with one plant per replication, totaling 25 plants for the study. Bioinoculants were applied via seed treatment (ST, 20 mL/100 seeds) for 1 hr followed by 2 hrs of shade drying, seedling root treatment (RT, 2% solution at 100 mL/100 seedlings) by root dip for 1 hr followed by 1 hr of shade drying, and soil application (SA, 20 mL of 2% consortia inoculant per plant) at 20 and 40 days after transplanting (DAT).

Soil bacterial and fungal populations were analyzed using the serial dilution and plate count technique on nutrient agar and potato dextrose agar, respectively. Microbial population was expressed as colony-forming units (CFU g^{-1} soil). For maintaining an inoculum density of 10^8 CFU mL^{-1} in the pot experiment, bacterial suspensions were standardized spectrophotometrically (OD_{600} = 0.8–1.0) and cross-verified by plate count method prior to application.

2.8 *In vivo* Assessment of MBCA and CDB Combination against Rs

The efficacy of the different bioformulations against *Ralstonia* wilt was evaluated by recording the number of wilted plants at weekly intervals until harvest. The per cent wilt incidence (PWI) was calculated by the following formula:

$$PWI\ (\%) = \frac{No.of\ plants\ wilted}{Total\ number\ of\ plants} x\ 100$$

Different yield attributing characters of pot-grown brinjal plant, *viz*., number of leaves, number of branches, shoot dry weight, root dry weight, shoot length, and root length were recorded in support of the yield record performance of the crop. The yield of each treatment (per plant) was recorded at harvest for onward statistical analysis.

2.9 *In vivo* Assessment of MBCA and CDB Combination in Chlorpyrifos Degradation

HPLC analysis of pot soils amended with chlorpyrifos was performed to check the persistence of pesticide residue. Soil samples were collected from each treatment at 0, 15, 30, and 45 days post-inoculation (DPI). In order to perform HPLC analysis of the soil samples, one gram of soil was taken every time from each treatment and mixed thoroughly with 20 mL of distilled water and stirred for 10 minutes at 250 rpm (Latifi et al., 2012) at room temperature. The filtrate was collected with Whatman no. 1 filter paper.

The residue was re-extracted with 20 mL of distilled water, followed by 10 min stirring at 200 rpm at room temperature, and the filtrate was collected. The residue was again extracted with 20 mL of distilled water, and the filtrate was collected. All the filtrates were mixed, and the volume was made up to 50 mL with distilled water. The combined filtrate was centrifuged at 5000 rpm for 15 min and passed through a 0.45 µm syringe filter into amber HPLC vials. For quantification, 10µL of each sample was injected into the HPLC system. The percent degradation was calculated using the formula: %Degradation = [(C*i* - C*t*) / C*i*] x100, where C*i* represents the initial concentration of chlorpyrifos at day 0 and C*t* represents the concentration at the specified sampling time *t* (0, 15, 30, 45, and 60 days post inoculation).

2.10 Statistical analysis

Data generated were subjected to statistical analysis for the computation of critical difference (F-test) using SAS software (v8.1, SAS Institute, North Carolina, USA). Duncan's Multiple Range Test was performed to separate the means at a 5% level of significance.

3. Results and Discussion

3.1 Isolated Chlorpyrifos Degrading Bacteria

A total of ten bacterial isolates (TZ1, TZ2, TZ3, JHT1, JHT2, JHT3, ICR1, ICR2, MG1, and MG2) were isolated from pesticide-polluted soils based on their ability to grow in Minimal Salt Medium (MSM) supplemented with chlorpyrifos, an organophosphate pesticide. Growth under selective conditions indicated their ability as chlorpyrifos-degrading bacteria (CDB). The isolates exhibited distinct morphological and biochemical profiles, reflecting inter-strain variability as described in Table 1.

Table 1. Morphological, cultural, and biochemical characteristics of the isolated chlorpyrifos-degrading bacterial isolates

Isolate	Gram reaction	Cell shape	Colony colour/pigmentation	KOH	Catalase	Starch hydrolysis	Citrate utilization	Nitrate reduction	Indole production	Gelatin liquefaction	Arginine dihydrolase
TZ1	–ve	Rod shaped	Creamy white, non-pigmented	+	+	–	+	–	+	+	+
TZ2	–ve	Rod shaped	Pale yellow, non-pigmented	+	+	+	+	+	–	+	+
TZ3	–ve	Short rods	Greyish white, non-pigmented	+	+	–	+	–	+	–	+
JHT1	–ve	Rod shaped	Greyish white, non-pigmented	+	+	–	+	–	–	+	+
JHT2	+ve	Rod shaped	Dull white, non-pigmented	+	+	+	+	+	–	+	+
JHT3	–ve	Rod shaped	Off-white, non-pigmented	+	+	–	+	–	+	–	+
ICR1	+ve	Rod shaped	Light cream, non-pigmented	+	+	+	+	+	–	+	+
ICR2	–ve	Short rods	Milky white, non-pigmented	+	+	–	+	–	+	+	+
MG1	–ve	Coccus shaped	Pale white, faint pigment	+	+	+	+	+	–	+	+
MG2	–ve	Rod shaped	Pale yellowish-green, green water-soluble pigment	+	+	–	+	+	+	+	+

+ indicates positive reaction; - indicates negative reaction

3.2 Identification of the CDB Isolates Growing at Higher Concentrations of Chlorpyrifos

The obtained sequences, compared with those available in the NCBI database using BLASTn, revealed that isolate JHT1 exhibited more than 99% sequence similarity with *Achromobacter marplatensis*, while isolate MG2 showed over 99% sequence similarity with *Pseudomonas azotoformans*. The validated sequences were deposited in the NCBI GenBank database, where they were assigned the accession numbers MW397524 (*A. marplatensis*) and MW397525 (*P. azotoformans*), respectively (Fig. 2a, b).

3.3 Growth Response of Chlorpyrifos-degrading Isolates

Growth response of the CDB isolates showing growth up to 700 ppm concentration of chlorpyrifos was studied spectrophotometrically and expressed in terms of optical density (OD) values at 600 nm. The maximum bacterial growth with 100 ppm was observed at day 7 in TZ2, JHT1, and MG2, whereas in JHT2 and MG1, maximum growth was observed by day 8. At 300 ppm, maximum growth was recorded at day 6 by JHT1, JHT2, and MG2, whereas TZ2 and MG1 showed maximum growth at day 7. At 500 ppm and 700 ppm, maximum growth was exhibited by JHT1 and MG2 at day 6, whereas TZ2, JHT2, and MG1 showed maximum growth at day 7. The growth curve reached a static after maximum growth, and thereafter declined with all CDB isolates. In contrast, the control sample showed no change at 600 nm for 9 days of incubation, demonstrating the potential of these isolates to utilize pesticides as a carbon source (Fig. 1).

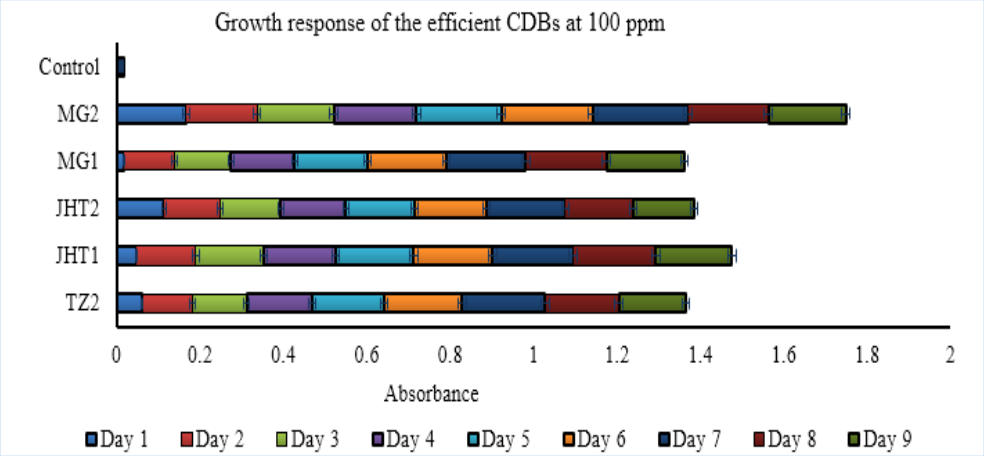


Fig. 1a. Growth response of the efficient Chlorpyrifos Pesticide-degrading bacteria at 100 ppm concentration

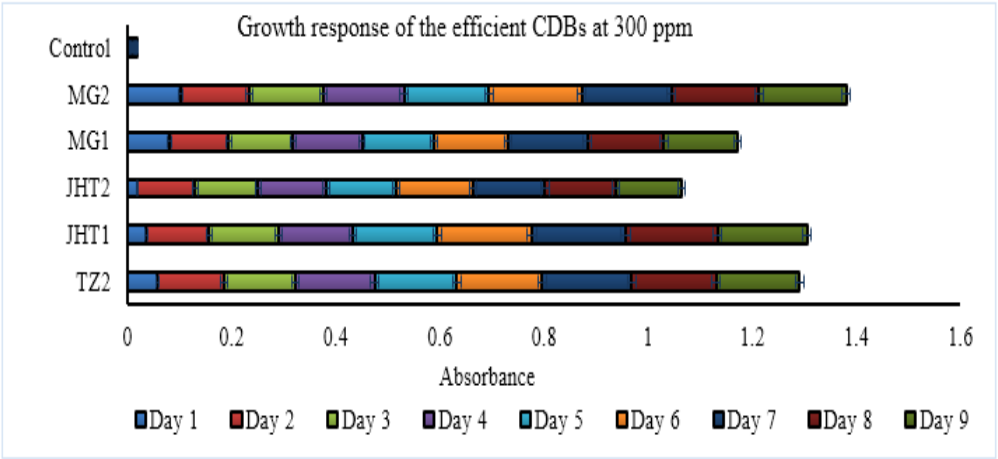


Fig. 1b. Growth response of the efficient Chlorpyrifos-degrading bacteria at 300 ppm concentration

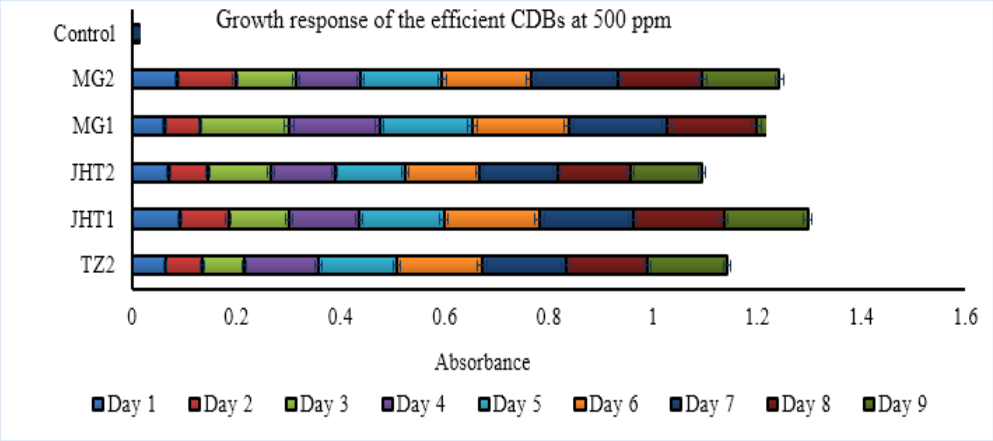


Fig. 1c. Growth response of the efficient Chlorpyrifos-degrading bacteria at 500 ppm concentration

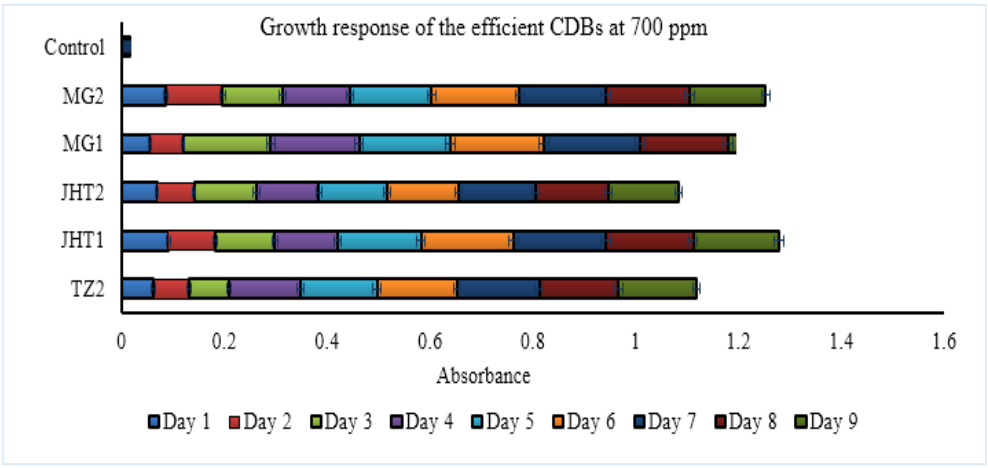


Fig. 1d. Growth response of the efficient Chlorpyrifos-degrading bacteria at 700 ppm concentration

Fig. 1 (a-d). Growth response of the efficient CDBs at four different concentrations (100, 300, 500, and 700 ppm) of chlorpyrifos

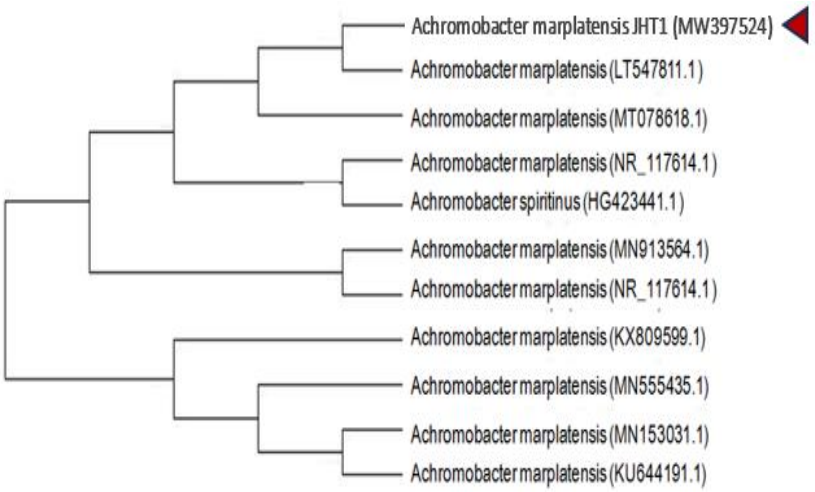


Fig. 2a. Phylogenetic tree illustrating the genetic relationship of the JHT1 isolate with closely related bacterial isolates constructed using the Maximum Likelihood method based on 500 bootstrap replicates. Bootstrap values are indicated at the nodes, representing the confidence level of each clade

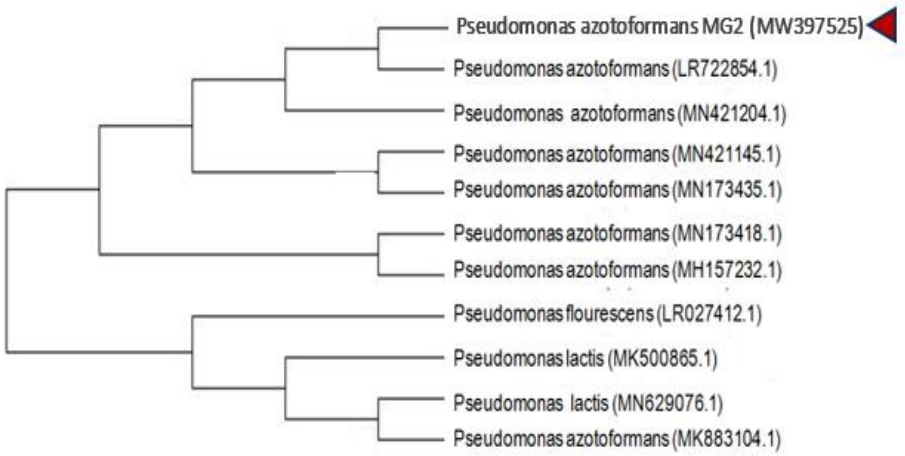


Fig. 2b. Phylogenetic tree illustrating the genetic relationship of the MG2 isolates with closely related bacterial isolates constructed using the Maximum Likelihood method based on 500 bootstrap replicates. Bootstrap values are indicated at the nodes, representing the confidence level of each clade

3.4 HPLC Analysis of the Degradation Efficacy

HPLC analysis confirmed efficient degradation of chlorpyrifos by *Achromobacter marplatensis* and *Pseudomonas azotoformans* by the 45th day after inoculation, with the formation of two major metabolites, 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DEP) (Fig. 3a-d). The appearance of these metabolites indicates hydrolytic cleavage of the parent compound, a characteristic pathway of microbial chlorpyrifos degradation. Comparable bioremediation efficiency has been reported by Melghani et al. in 2009, who demonstrated that *P. azotoformans* degraded 66.81% of profenofos within 48 h in contaminated soil, emphasizing its strong xenobiotic-degrading potential. In the present study, the detected metabolites TCP and DEP are known to be non-toxic or minimally toxic and were subsequently utilized by the isolates as carbon and phosphorus sources, thereby supporting their growth and metabolic activity (Kumar et al., 2023).

3.5 MBCA-CDB Compatibility

The pesticide-degrading bacteria *A. marplatensis* and *P. azotoformans* were found to be compatible, evident through the dual culture assay. Further, the microbial biocontrol agents *P. fluorescens* and *T. harzianum* also showed compatibility with the pesticide-degrading bacteria (Fig. 4a-d). The compatibility of *Pf* and *Th* is widely reported by several workers (Yendo et al., 2018), revealing their dual functional ability, growth promotion, and effectiveness against a variety of plant diseases. Such an observation provides further clues about the mutually positive relationship between OP-degrading microbes as major players participating in the microbial disintegration of pesticides. The compatibility between four CDB isolates, viz., *Pseudomonas putid*a (NII 1117), *Klebsiella* sp. (NII 1118)., *Pseudomonas stutzeri* (NII 1119) and *Pa* (NII 1120) with elevated organophosphorus hydrolase activity (0.171 units/mL/min) were previously reported for effective OP-degradation in the presence of metabolites like chlorpyrifos-oxon and diethylphosphorothioate (Ifediegwu et al. 2015).

3.6 *In-Planta* Response for Bacterial Wilt Management and Growth Promotion

Different combinations of microbial biocontrol agents and chlorpyrifos-degrading bacteria showed varying responses on per cent wilt incidence (PWI), being lowest with a combination of *Pf+Th+Am + Pa* (T5), followed by *Pf + Th + Pa* (T4). However, the magnitude of reduction in PWI was significantly higher with *Pf*+ Th (T2) compared to either *Pf+ Th + Am* (T3) *or Pf + Th + Pa* (T4), displaying the coordinated response of MBCA with CDB. The antagonists, *Th* and *Pf*, suppress *Rs* by extensively reporting via overcrowding of the pathogen, secreting lytic enzymes (β1,3-glucanases in lysis of the pathogen cell wall), antibiotics, and toxic metabolites like cyanide (Kohl et al., 2019). In our study, a combination of *Pf* and *Th* expanded the functional corridor of these two antagonists via ammonia production, phosphate solubilization, and indole acetic acid production, either in the absence or in the presence of chlorpyrifos, thereby facilitating the optimization of the performance of the host crop (Saikia et al., 2022). Some of the recent studies have strongly advocated the application of

microbes in a consortium mode proved far more efficacious (Singh et al., 2021). Plants primed with *Trichoderma* sp. and challenged with pathogens were observed having a higher amount of shikimic acid coupled with defense-related enzymes, *viz*., polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase (Sharma et al., 2020).

The reduction in PWI was associated with an enhanced yield of brinjal, and all the treated combinations of *Pf + Th + Am + Pa* were most effective in enhancing the yield of brinjal (Table 2), followed by a combination of *Pf + Th + Pa*. The other two combinations, *viz., Pf + Th* and *Pf + Th + Am*, were, however, observed to display a higher magnitude of response over the control treatment only. These observations followed a similar pattern of response concerning yield attributing parameters, such as root and shoot dry weight, suggesting the superior agronomic response along with a reduction in wilt disease of brinjal with a combination of bioagent and CDB over either of the two alone. Such a combination of MBCA and CDB is also likely to accelerate many of the fundamental plant physiological processes such as photosynthesis rate, stomatal conductance, transpiration, internal CO_2 concentration, water use efficiency, and nutrient uptake (Srivastava et al., 2022; Bora et al., 2025), besides solubilization of several plant nutrients, sequestration of iron through siderophore production, and growth hormones production (Woo and Pepe, 2018; Bora et al., 2024a) pivotal in agronomic crop response.ch observation hold a much greater promise for the commercial development of MBCA-CDB based formulations with a cost-effective delivery mechanism to ensure the development of residue residue-free vegetable crop production system.

3.7 *In vivo* Response of Microbes in Chlorpyrifos Degradation

In the pot experiment, different combinations of microbial biocontrol agents (MBCAs) and chlorpyrifos-degrading bacteria (CDB) evaluated for their ability to degrade chlorpyrifos in soils supporting brinjal plants, revealed that all microbial treatments significantly enhanced chlorpyrifos degradation compared to the uninoculated control. Among the tested combinations, the consortium comprising *A. marplatensis + P. azotoformans + P. fluorescens + T. harzianum* (T5) exhibited the highest degradation efficiency. This treatment reduced the residual chlorpyrifos concentration to 28.96% of the initial level after 45 days, corresponding to approximately 71% degradation (Fig. 5). Among the other treatment combinations, the consortium of *A. marplatensis* with *P. fluorescens* and *T. harzianum* (T3) showed greater degradation efficiency than the combination of *P. azotoformans* with *P. fluorescens and T. harzianum* (T4), leaving 33.45% and 41.54% residual chlorpyrifos, after 45 days of treatment corresponding to 66.55% and 58.46% degradation respectively. All treatment groups exhibited statistically significant improvements over the untreated control, affirming the potential of integrating MBCA and CDB for accelerated chlorpyrifos degradation in soil. The enhanced degradation observed in these treatments may be attributed to the functional diversity of the microbial strains used. While CDB strains directly degrade chlorpyrifos through enzymatic hydrolysis, MBCA strains contribute indirectly by altering the soil microenvironment, suppressing pathogens, and possibly co-metabolizing intermediate products. This supports the concept of bioremediation through microbial synergy, where multiple strains cooperate metabolically to achieve efficient pollutant breakdown. Our findings align with earlier reports, such as Yang et al. (2005), who demonstrated the capacity of *Alcaligenes faecalis* strain DSP3 to degrade chlorpyrifos and its metabolite TCP under varying 6 soil and culture conditions. Their study revealed a rapid decline in pesticide residues in treated soils compared to controls, highlighting the pivotal role of microbial intervention in pesticide dissipation. Furthermore, the chemical structure of OP-pesticides, characterized by diethyl phosphorothioate side chains and phosphotriester bonds (except in TCP), suggests a common degradation mechanism via hydrolysis of the phosphotriester bond (Kumar et al., 2018). Natural environmental factors such as sunlight and air exposure also facilitate the breakdown of these compounds. For instance, Singh (2004) reported up to 97% degradation of phosphamidon residues in mustard crops within 15 days post-application, indicating the role of natural attenuation. Comparable microbial consortia have also shown significant degradation potential. Olanrewaju et al. in 2017 observed 80% endosulfan degradation using bacterial strains from the Pseudomonas and Achromobacter genera. Similarly, Akbar and Sultan (2016) reported 93-100% chlorpyrifos degradation within 42 days at treatment concentrations ranging from 200–500 ppm. Collectively, the present study underscores the utility of integrating biocontrol and degradative microbial agents for the dual purpose of pest management and soil detoxification. This approach not only facilitates the breakdown of persistent OP-pesticide residues but also supports a healthier rhizospheric environment, potentially enhancing the biocontrol efficacy against soil-borne pathogens and improving plant growth and yield. Chlorpyrifos-degrading bacteria metabolize chlorpyrifos primarily through phosphotriesterase-mediated hydrolysis, generating TCP and DEP, which subsequently enter central metabolic pathways.

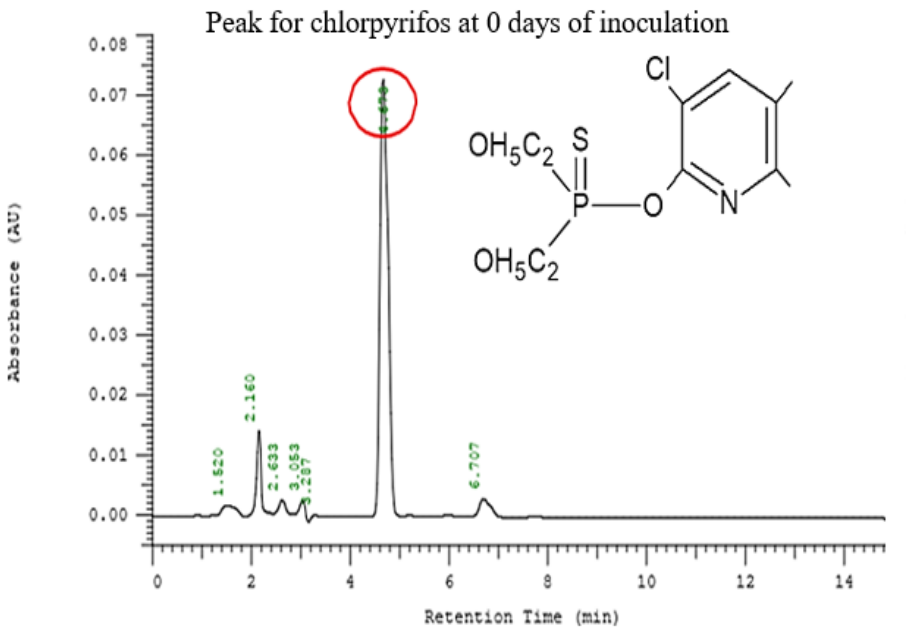


Fig. 3a. Chlorpyrifos degradation by Achromobacter marplatensis at 0 days of inoculation. Presence of one high peak represents the dominance of chlorpyrifos pesticide

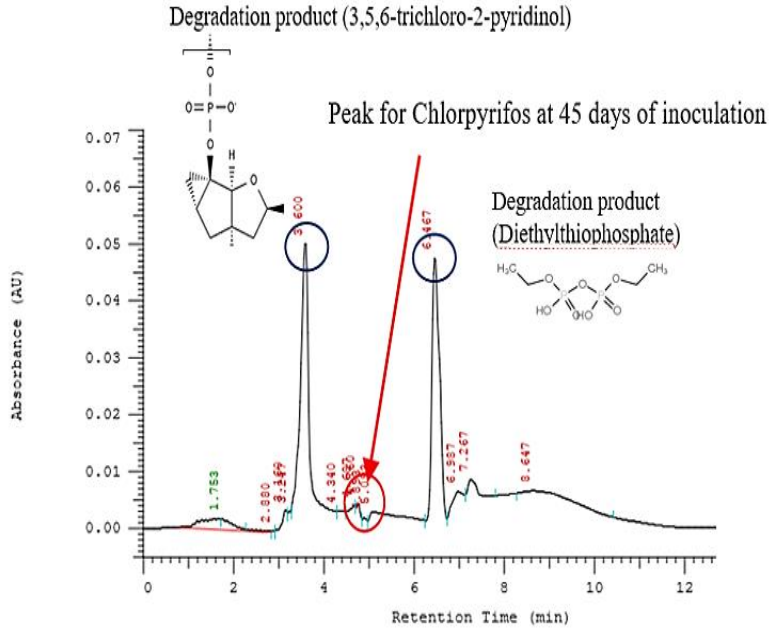


Fig. 3b. Chlorpyrifos degradation by Achromobacter marplatensis at 45 days of inoculation. The presence of two lower peaks marks the dominance of intermediate non-toxic metabolites 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DEP)

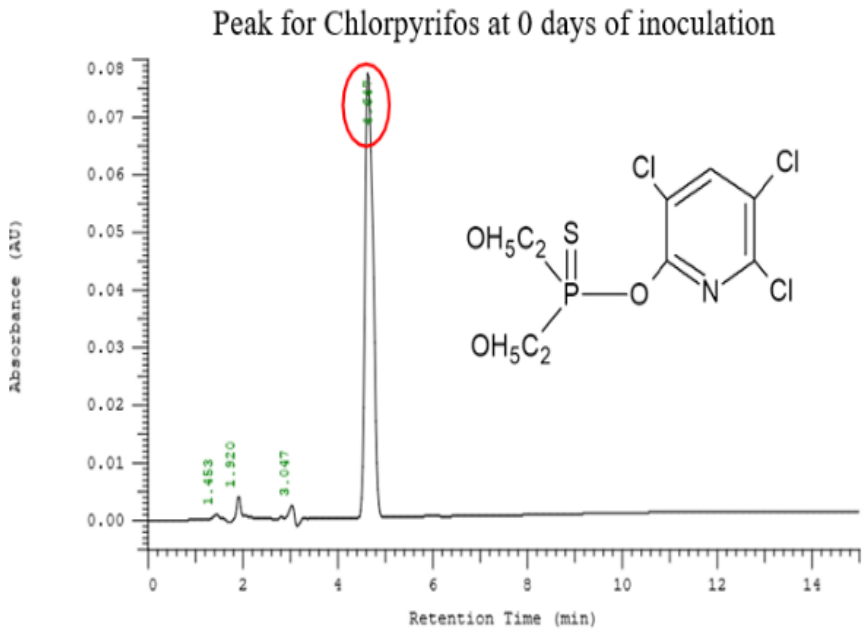


Fig. 3c. Chlorpyrifos degradation by Pseudomonas azotoformans at 0 days of inoculation. The presence of one high peak represents the dominance of the chlorpyrifos. Pesticide

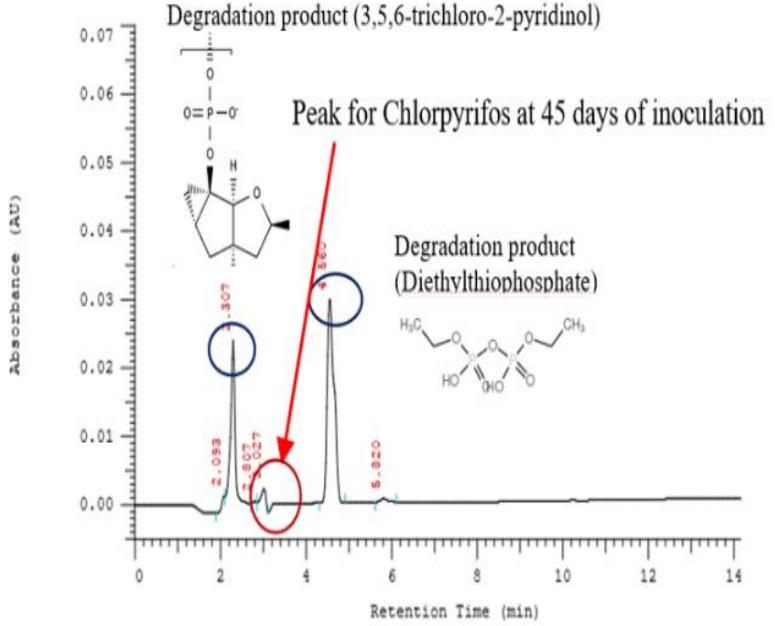


Fig. 3d. Chlorpyrifos degradation by Pseudomonas azotoformans at 45 days of inoculation. The presence of two lower peaks marks the dominance of intermediate non-toxic metabolites 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DEP)

Fig. 3(a–d). HPLC chromatograms showing *in vitro* chlorpyrifos degradation by *Achromobacter marplatensis* JHT1 and *Pseudomonas azotoformans* MG2. The reduced peaks corresponding to the intermediate, non-toxic metabolites 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DEP) in Fig. 3b and 3d indicate successful degradation of chlorpyrifos by both isolates

In contrast, microbial biocontrol agents operate mainly through biosynthesis of antibiotics, siderophores, lytic enzymes, and phytohormones that suppress pathogens and promote plant growth. The functional integration of CDB and MBCA thus represents a synergistic convergence of detoxification and disease-suppression pathways.

Table 2. Response of MBCA and CDB combinations on PWI, yield attributes, and yield of brinjal grown in chlorpyrifos-loaded potted soil

Treatments	PWI (%)	Yield attributing attributes		Yield (kg/plant)
		Shoot dry weight (g/plant)	Root dry weight (g/plant)	
T_1(Control)	85.0e	16.96e	3.52e	0.50de
T_2(*Pf*+*Th*)	15.0bc	28.75d	4.28c	1.10bc
T_3(*Am*+*Pf*+*Th*)	20.0bcd	27.95c	4.07cd	0.74d
T_4(*Pa*+*Pf*+*Th*)	10.0ab	31.46b	4.44b	1.23b
T_5(*Am*+*Pa*+*Pf*+*Th*)	5.0a	36.39a	5.35a	1.42a
S.Ed (+)	4.49	0.32	0.13	0.16
C.D. ($p \leq 0.05$)	9.86	0.67	0.27	0.33

Pf: *Pseudomonas flourescens*; *Th*: *Trichoderma harzianum*; *Am*: *Achromobacter marplatensis* and *Pa*: *Pseudomonas azotoformans.* Different superscript letters within a column indicate significant differences among treatments at $p \leq 0.05$

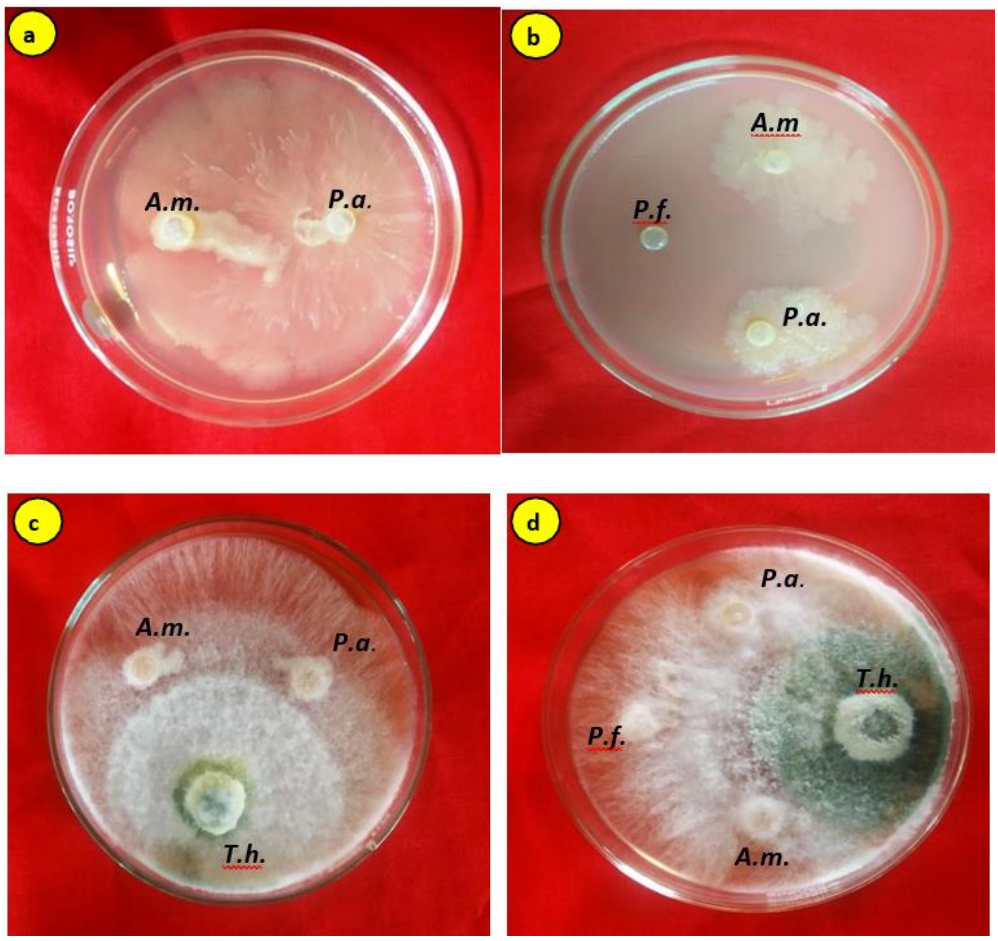


Fig. 4. *In vitro* compatibility between MBCAs (*Pf: Pseudomonas fluorescens* and *Th: Trichoderma harzianum)* and CDBs (*Am*: *Achromobacter marplatensis* and *Pa*: *Pseudomonas azotoformans*); 7a. *Am+Pa* 7b. *Am+Pa+Pf*; 7c. *Am+Pa+Th*, 7d. *Am+Pa+Pf+Th*. Plates demonstrate the absence of antagonistic zones, confirming physiological compatibility for consortium formulation

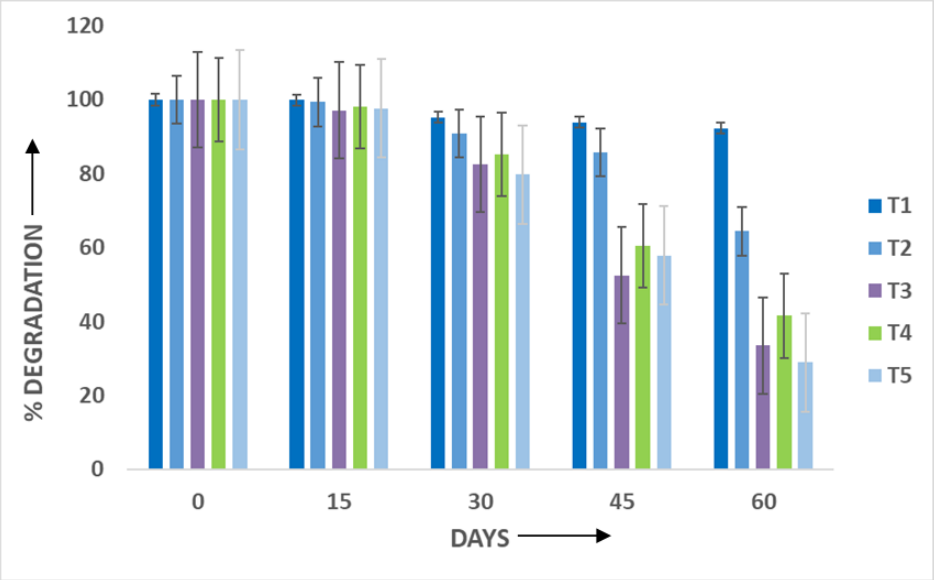


Fig. 5. Effect of different microbes in the biodegradation of Chlorpyrifos. Treatment T5 involving a combination of *Am* + *Pa* + *Pf* + *Th* significantly outplayed the rest of the other treatments, viz., T1, T2, T3, and T4, in terms of degradation. Treatments comprise of (T1- Control; T2- *Pf* + *Th*; T3- *Am*+ *Pf* + *Th;* T4- *Pa* + *Pf* + *Th;* T5- *Am* + *Pa* + *Pf* + *Th*). Data are presented as mean ± standard error of five replications. Error bars indicate significant differences among treatments at p ≤ 0.05 based on Duncan's Multiple Range Test

Am: *Achromobacter marplatensis, Pa*: *Pseudomonas azotoformans, Pf*: *Pseudomonas flourescens*, and *Th*: *Trichoderma harzianum*

4. Conclusion

Bioremediation of pesticide-contaminated soils while maintaining crop productivity represents an important challenge in sustainable agriculture. In this study, an enrichment method enabled the isolation of efficient chlorpyrifos-degrading bacteria (CDB) capable of utilizing pesticide residues and contributing to soil detoxification. The combined application of microbial biocontrol agents (MBCAs) and CDB demonstrated promising potential for the simultaneous degradation of chlorpyrifos residues and reduction of bacterial wilt incidence in brinjal. This integrated microbial approach offers a sustainable strategy to mitigate pesticide contamination while enhancing plant health. However, further validation through multi-location field trials and molecular characterization of pesticide-degrading genes is necessary to better understand the degradation mechanisms and confirm the large-scale applicability of the developed microbial consortium.

Disclaimer (Artificial Intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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Competing Interests

Authors have declared that no competing interests exist.

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