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Research article

Enhanced Biodegradation of phenoxyacetic herbicides 2,4-D and 4-chloro-2-methylphenoxyacetic acid by bacterial strains under variable environmental conditions

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ABSTRACT

Phenoxyacetic acid herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) are widely used in agriculture but pose significant environmental risks due to their persistence and potential toxicity. This study investigates the growth dynamics and degradation efficiency of bacterial strains exposed to varying concentrations of 2,4-D and MCPA under different pH and temperature conditions. Growth was monitored through optical density measurements (OD600), while degradation efficiency was calculated based on herbicide concentration decline over time. In control conditions, bacterial growth showed a typical sigmoidal curve, whereas the presence of herbicides slightly inhibited early growth, particularly for 2,4-D. Optimal bacterial growth and degradation occurred at neutral to slightly alkaline pH (pH 7–8), with significantly reduced growth at acidic pH (pH 5). Temperature profiles revealed that 30°C and 40°C supported the highest bacterial activity, while extreme temperatures (20°C and 50°C) negatively impacted growth in 2,4-D treatments. Interestingly, MCPA-exposed cultures exhibited more stable growth across varying conditions. Herbicide concentrations ranging from 300 to 700 mg/L showed minimal effect on degradation efficiency, which remained above 99% for both 2,4-D and MCPA. However, higher concentrations marginally suppressed bacterial growth. The findings suggest that the tested bacterial strains possess strong adaptive mechanisms and metabolic versatility for degrading phenoxyacetic herbicides, even under stressful environmental conditions. This study underscores the potential of microbial bioremediation as an effective approach for detoxifying herbicide-contaminated environments and highlights critical factors influencing biodegradation efficiency. These results contribute valuable insights into optimizing field-scale applications for sustainable agricultural practices.

1. Introduction

" Soil harbors a high abundance of microorganisms that play a crucial role in various biological processes essential for environmental sustainability. These include participation in nutrient cycling, biochemical pathways, organic matter formation and decomposition, promotion of plant growth, and maintenance of soil structure and fertility (Chaparro et al., 2012; Zhang et al., 2017). Over the past two decades, the global application of pesticides and herbicides has increased significantly. Among the most commonly used are phenoxyalkanoic acid herbicides, a class of xenobiotic compounds structurally related to plant hormones (Paszko et al., 2016; Singh et al., 2022). The widespread and intensive use of these agrochemicals has resulted in the accumulation of substantial residues in the environment, posing a serious threat to human health due to their potential entry into the food chain and contamination of drinking water sources (Mohamed 2009). Although pesticides and herbicides contribute significantly to global food production, certain compounds exhibit environmental persistence and exert deleterious effects on soil

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ecosystems (Mercurio et al., 2016). Phenoxyalkanoic acid herbicides, such as 4-chloro-2-methylphenoxyacetic acid (MCPA) and 2,4-dichlorophenoxyacetic acid (2,4-D), are widely used for controlling broadleaf weeds in both agricultural and non-agricultural settings (Nicolaisen et al., 2008). While these compounds have contributed to enhanced crop yields and agricultural productivity, their extensive and prolonged usage has raised environmental concerns. Notably, their persistence in surface and groundwater systems has been increasingly documented over the past several decades (Cox et al., 2001; Tejada 2009). To mitigate the risk of groundwater contamination, various bioremediation approaches have been developed, focusing particularly on enhancing the capacity of indigenous microbial communities to degrade these herbicidal residues. Due to its extensive global application, MCPA has been chosen as a representative compound in studies investigating the photodegradation of organic pollutants in aquatic environments (Topalov et al., 2001). Herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chloro-2-methylphenoxyacetic acid (MCPA), and mecoprop-P are commonly applied to protect a wide range of crops (Singh et al., 2022). Specifically, 2,4-D is used for corn, sugarcane, cranberries, and sorghum; both 2,4-D and MCPA are employed in rice and asparagus cultivation; MCPA and mecoprop-P are suitable for use on vines and forage crops; and applications also extend to clover and groundnuts (Paszko et al., 2016). For over six decades, 2,4-D has served as a systemic herbicide effective in managing dicotyledonous weeds in cereal and grass crops. Although it is known to exhibit a relatively short half-life in both soil and aquatic systems, its widespread usage continues to raise environmental and health concerns (Singh et al., 2022; Chinalia et al., 2007). Numerous bacterial strains capable of degrading 2,4-D have been isolated from diverse environmental sources. Several genes involved in the catabolism of 2,4-D have been characterized, and the metabolic pathways for its degradation are well-established. Notably, 2,4-D was the first herbicide for which the microbial agents responsible for in situ biodegradation were clearly identified, supported by extensive data on its environmental behavior, microbial physiology, and genetic basis (Cupples et al., 2007).

While numerous investigations have focused on the microbial degradation of herbicides, the successful isolation of bacterial and fungal strains from environments with low pesticide concentrations remains relatively limited. This highlights the need for further exploration of microbial communities capable of withstanding higher contaminant loads. The primary aim of the present study was to isolate and characterize microbial strains exhibiting tolerance to elevated concentrations of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), with an emphasis on achieving maximum herbicide resistance within a minimal incubation period.

2. Materials and methods

2.1 Bacterial strains and culture conditions

The bacterial strains utilized in this study were previously isolated from agricultural soils with known herbicide exposure histories. Soil samples were collected from sugarcane and wheat fields near villages of Bakshi ka Talab, Lucknow, Uttar Pradesh, India, at a surface depth of 10 cm after removing field waste. Samples were air-dried, sieved, and stored at 4˚C for isolation. The isolation process was performed in minimal salt medium conditions, utilizing herbicides (2,4-D and MCPA) as the sole carbon source to isolate microbial strains that are capable of tolerating herbicides. This approach aimed to ensure the survival of specific strains while eliminating undesired strains that can easily be available in various environments. The Minimal Salt Medium contains K_2HPO_4 - 1.5 g/L; KH_2PO_4 - 0.5 g/L; $MgSO_4.7H_2O$ - 0.2 g/L; NaCl - 0.5 g/L; NH_4NO_3 - 1.5 g/L (Li et al., 2008). The isolates were maintained on Luria Bertani agar plates at 4°C and subcultured regularly to preserve viability. For experimental assays, a single colony was inoculated into Luria Bertani broth and incubated at 30°C under agitation (120 rpm) for 24 hours to achieve exponential-phase cultures.

2.2. Herbicide preparation

High-performance liquid chromatography (HPLC) grade 99% purity 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) were procured from SRL and Merck, respectively. Stock solutions were prepared in HPLC grade methanol, filtered through 0.22 µm syringe filters, and stored at 4°C until use. All media component chemicals were purchased from HIMEDIA, and some chemicals from SRL, both of which are of analytical grade with a purity of 98.9%. Luria Bertani Broth Miller, Agar Powder, Di-potassium Phosphate (K_2HPO_4), Potassium di-hydrogen Phosphate (KH_2PO_4), Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$), Sodium Chloride (NaCl), Ammonium Nitrate (NH_4NO_3). Working concentrations (300, 500, and 700 mg/L) were prepared by diluting the stock solutions in minimal salt medium (MSM) before each experiment.

2.3 Experimental design

To evaluate the influence of environmental factors on bacterial growth and herbicide degradation, three independent set of experiments were conducted:

- **pH variation**: The pH of MSM was adjusted to 5, 6, 7, and 8 using sterile 1 N HCl or 1 N NaOH.
- **Temperature variation**: Cultures were incubated at 20°C, 30°C, 40°C, and 50°C in temperature-controlled orbital shakers.
- **Herbicide concentration**: The degradation potential was tested at 300, 500, and 700 mg/L concentrations of each herbicide.

Each experiment was carried out in 250 mL Erlenmeyer flasks containing 100 mL of MSM supplemented with the respective herbicide and inoculated with 1% (v/v) bacterial suspension. Controls without bacterial inoculation were included to account for abiotic losses. All treatments were performed in triplicate.

2.4 Measurement of bacterial growth

Bacterial growth was monitored by spectrophotometer by measuring optical density at 600 nm (OD600) using a UV/Visible Spectrophotometer system (LMSP-UV-1200). Measurements were recorded at regular intervals from 0 hr to 26 h, with each one-hour interval for the growth curve in enriched medium to determine growth kinetics under each treatment condition. While for pH and temperature the variation conditions exposed in MSM were recorded for up to 3

days. Three different herbicide concentrations were tested in MSM for 5 days at each day interval for three different herbicide concentrations (300 mg/L, 500 mg/L, and 700 mg/L).

2.5. Herbicide Degradation Analysis

Herbicide degradation efficiency was assessed by quantifying residual 2,4-D and MCPA concentrations from culture supernatants. The collected culture broth was first centrifuged at 12,000 g for 15 minutes to separate the bacterial cells from the supernatant. The supernatant and cell fractions were subsequently processed separately to extract potential metabolites and degradation products. The supernatant was subjected to lyophilization to concentrate the dissolved compounds. The lyophilized material was then reconstituted in 3 mL of methanol. This solution was further centrifuged at 6,000 g for 15 minutes to remove any particulate matter, and the resulting supernatant was carefully filtered through 0.22 µm sterile syringe filters (Xia et al., 2017) to obtain a clear filtrate containing the degradation products. Simultaneously, the separated bacterial cell pellet was re-suspended in 3 mL of methanol and incubated at room temperature overnight to allow complete extraction of intracellular components. Following incubation, the cells were subjected to sonication for 1 minute at room temperature to lyse the cells and enhance the release of intracellular metabolites into the methanol solution. After sonication, the samples were again left undisturbed overnight at room temperature to allow cell debris to settle naturally. The resulting suspension was then centrifuged at 6,000 g for 15 minutes to further clarify the extract. The supernatant, containing dissolved intracellular compounds, was carefully collected and filtered through 0.22 µm sterile PVDF syringe filters (AXIVA Syringe Filters, Sterile PVDF) to ensure removal of any residual particulate material. Both the supernatant and cell extract samples were thus prepared for subsequent chemical analysis via HPLC of the herbicide degradation products.

3. Results and discussion

3.1. Effect of Herbicides on Bacterial Growth

The presence of 2,4-D and MCPA impacted bacterial growth dynamics when compared to the control group. In the control (herbicide-free) condition, bacterial growth exhibited a standard sigmoid curve, with OD600 readings increasing from 0.02 at 0 hours to a peak of 1.2 at 8 hours, followed by a log phase to a maximum of 2.0 at 22 hours, later no increase was shown indicating entry into the stationary phase. In cultures treated with **2,4-D**, growth was initially suppressed, with an OD600 of 0.03 at 0 hours, increasing similar to control up to 0.90 at 8 h and reaching at stationary phase at 22 h with OD600 of 2.0 (Fig. 1a). This suggests a lag in adaptation due to the herbicide's toxic effect on early metabolic activity. Conversely, **MCPA-treated** cultures displayed relatively similar growth, increasing from 0.03 at 0 hours to 0.90 at 8 hours and a maximum of 2.0 at 22 hours, indicating that MCPA exerted the same effect on bacterial proliferation. Growth in MSM was monitored till 5 days, and no cell growth was observed in control conditions where no extra supplements were provided, not even any herbicide; it attained a maximum of OD600 0.08 on the 5th day. In the case of 2,4-D exposure, the bacterial culture initially exhibited limited survival, achieving an optical density (OD_{600}) of 0.18 on the first day; however, it was able to sustain viability, maintaining an OD_{600} of 0.15 by the fifth day (Fig. 1b). Similar pattern was observed in MCPA with higher number of cell density initially on 1st day (OD600 0.12) with maximum of OD600 0.16 at 5th day.

3.2. Influence of pH on growth and degradation

Bacterial activity varied significantly with pH. At **pH 5**, no growth was observed, and minimal growth was observed at pH 6 in both herbicide conditions (2,4-D and MCPA), likely due to acid-induced stress affecting enzyme systems. The highest growth was recorded at OD600 of 0.24 for 2,4-D and 0.31 for MCPA at 72 hours at pH 7 conditions (Fig. 2a & b). At pH 8 (alkaline condition) shows similar volume of cell growth in 2,4-D around 0.22 at 72 hours, while in MCPA pH 8 condition seem to be little hostile for the survival of strain only able to maintain a cell number of maximum around 0.18 at 72 hours indicating that slightly alkaline conditions somewhat favours both microbial proliferation and metabolic degradation.

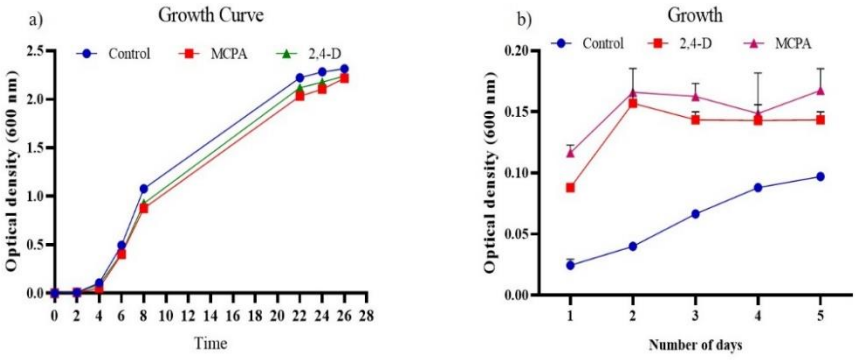

Fig. 1. Growth kinetics of isolated bacterial strain in Luria Bertani (LB) broth (a) and in Minimal Salt Medium (MSM) (b) at 30˚C

3.3. Effect of temperature on growth and degradation

Temperature played a pivotal role in modulating microbial activity. At **20°C**, bacterial growth was barely observed, suggesting limited metabolic activity under low temperatures. At 30˚C and 40˚C, bacterial growth was observed to some extent (OD600 of 0.26 for 2,4-D and 0.32 for MCPA),

maximum growth was observed at **40°C** for both herbicides, with OD600 values reaching 0.25 (2,4-D) and 0.32 (MCPA) (Fig. 2c &d). Interestingly, a transient growth spike was noted at **50°C** in 2,4-D cultures on day 1 (OD600 0.31), but this was not sustained, suggesting possible thermal stress.

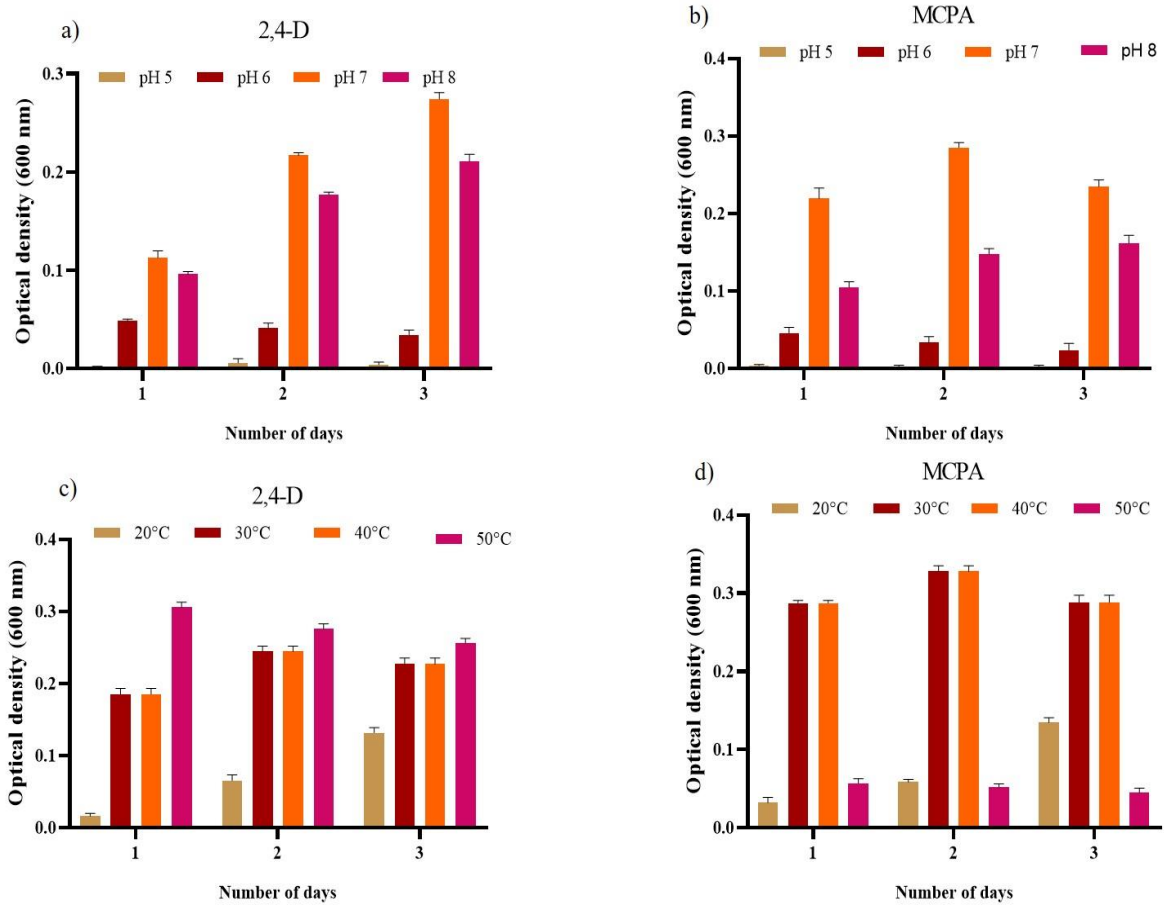

Fig. 2. Influence of pH (a) and temperature (b) on the growth of bacterial strain in minimal medium conditions at 30˚C for 3 days

3.4. Impact of herbicide concentration on growth and degradation

The bacterial growth response exhibited a concentration-dependent pattern when exposed to varying levels of the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA). At a concentration of 300 mg/L, the optical density at 600 nm (OD_{600}) reached 0.25 for 2,4-D and 0.17 for MCPA by the fifth day of incubation, indicating low cellular proliferation. However, as the herbicide concentrations increased, a modest reduction in bacterial biomass was noted for 2,4-D, with OD_{600} declining to 0.15 at 500 mg/L, followed by a slight recovery to 0.17 at 700 mg/L. In contrast, MCPA-exposed cultures demonstrated an atypical growth pattern: an increase in OD_{600} to 0.25 at 500 mg/L, followed by a decrease to 0.17 at 700 mg/L (Fig. 3). Despite these fluctuations in cell density, the biodegradation efficiency remained remarkably high, consistently surpassing 99% across all tested concentrations. Degradation was measured via reverse-phase HPLC. Fig. 4 showing the standard chromatograph of both herbicides 2,4-D and MCPA.Therefore, for degradation analysis, concentrations of 300, 500, and 700 mg/L were selected to assess the strain's degradation efficiency across a gradient of exposure levels. Post incubation, both the culture supernatant and the cells were separated, and the residual herbicide concentration in the supernatant was determined using HPLC. Degradation efficiency was calculated by comparing the peak area of the residual herbicide in the samples against that of the standard. This decoupling of biomass accumulation from degradation performance suggests that bacterial cells retained a highly active metabolic capacity under herbicide-induced stress. The persistence of degradation capability, even under potentially inhibitory conditions, implies the involvement of inducible catabolic enzymes or alternative metabolic pathways that confer resilience and functional robustness to the microbial consortium.

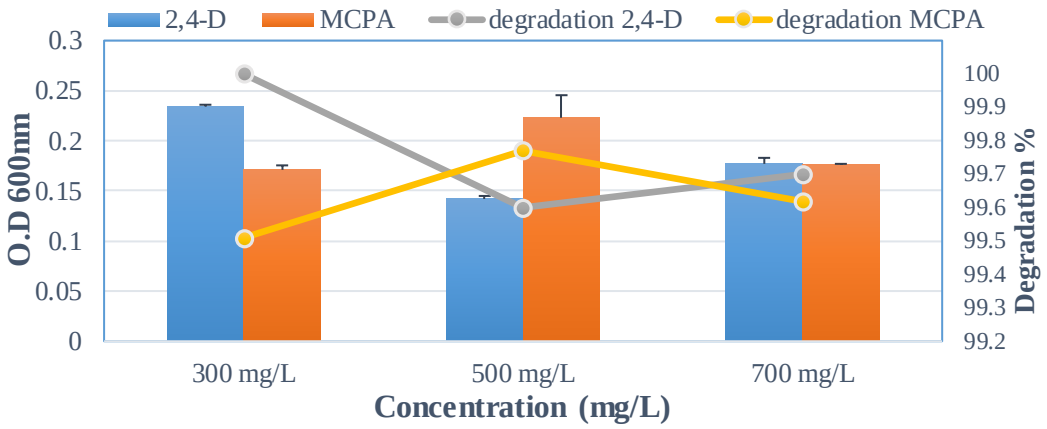

Fig. 3. Growth with different concentrations of herbicides (2,4-D and MCPA) after 5 days of incubation at 30°C Degradation % of 2,4-D and MCPA on right side of the graph and absorbance on left side

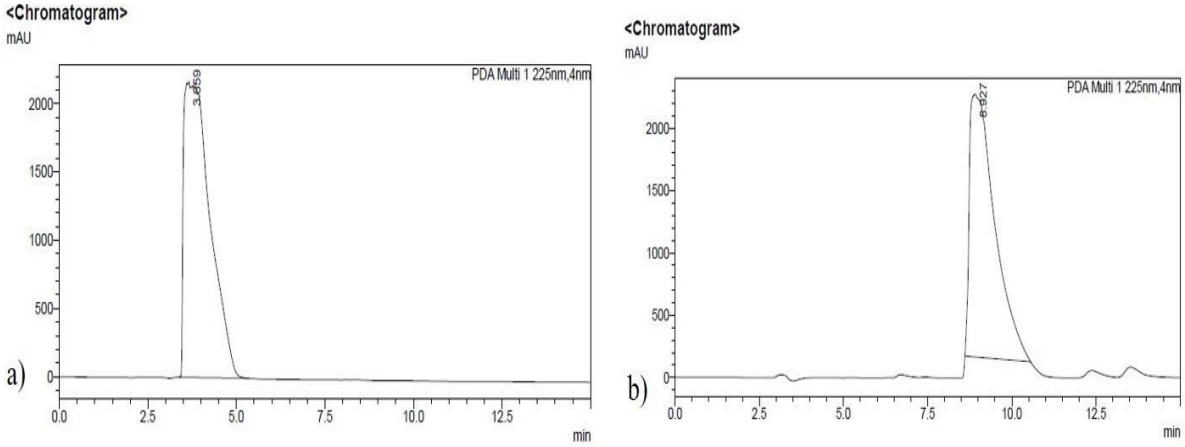

Fig. 4. HPLC Standard chromatogram of (a) 2,4-D and (b) MCPA

Numerous studies have documented the isolation and functional characterization of microbial strains capable of degrading the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) under diverse environmental conditions. For example, *Cupriavidus campinensis* strain BJ71 exhibited efficient degradation of 2,4-D, removing 99.57% of a 350 mg/L concentration within six days under optimal conditions (pH 7.0, 30 °C, 3% inoculum) (Han et al., 2015). However, its efficacy diminished in soil environments, achieving only 42.53% degradation in sterile soil and 87.13% in non-sterile soil after 14 days, indicating a potential influence of native microbial competition and soil matrix complexity on biodegradation performance. *Achromobacter sp.* LZ35 was also shown to metabolize both 2,4-D and MCPA as sole carbon sources, achieving over 75% degradation of 100 mg/L 2,4-D and 90% of 50 mg/L MCPA within a 12-day incubation period. Notably, approximately 50% of 2,4-D was removed within the first three days, suggesting rapid initial degradation kinetics (Yang et al., 2017). Further work by Gorodylova et al., (2021) investigated MCPA biodegradation using biocomposite-based systems, achieving degradation rates between 0.05 and 0.13 mg/h within 30 to 96 hours. However, the presence of planktonic microbial communities was associated with delayed herbicide breakdown. Using mass spectrometric analysis, two key MCPA metabolites—4-chloro-o-cresol (MCP) and 5-chloro-3-methylcatechol—were identified within 30 hours of incubation, confirming microbial enzymatic activity in the transformation pathway.

Brucha et al., (2021) extended this line of research by evaluating long-term 2,4-D degradation in microbial communities extracted from both surface and subsurface soil layers of the Amazon rainforest. Similarly, Yang et al., (2017) demonstrated that the addition of acetate to paddy soils enhanced 2,4-D degradation by approximately fivefold.

This enhancement was associated with a marked increase in the relative abundance of genera such as *Dechloromonas* (23.3%) and *Pseudomonas* (37.7%), implicating these taxa in acetate-mediated biostimulation. Recent work by Muhammad et al., (2023) reported that *Cupriavidus campinensis* was capable of degrading 94.69% of 0.72 g/L 2,4-D within six days at 40 °C

and pH 7, using a high inoculum density (200 g/L), further supporting its bioremediation potential. In another notable study, *Escherichia coli* was genetically engineered to completely degrade 0.5 mM 2,4-D within six hours by utilizing it as a sole carbon source, showcasing the promise of synthetic biology approaches for herbicide detoxification (Wu et al., 2017). Additionally, *Cupriavidus gilardii* demonstrated complete degradation of 2,4-D at lower concentrations (10, 50, and 100 mg/L) within 24 hours; however, its efficiency significantly declined at elevated herbicide levels, with only 53.1%, 36.3%, and 9% removal observed at 200, 250, and 350 mg/L, respectively (Xia et al., 2017). These findings underscore the concentration-dependent efficiency of microbial degradation and the importance of selecting appropriate strains for specific contamination levels.

4. Conclusion

The present study demonstrates the efficacy of selected bacterial strains in degrading phenoxyacetic herbicides under varying environmental conditions. The results reveal that bacterial growth is significantly influenced by pH, temperature, and herbicide concentration. Optimal bacterial proliferation was observed at pH 7–8 and temperatures between 30°C and 40°C, suggesting that these conditions enhance enzymatic activity and microbial adaptation. Although both herbicides exhibited some inhibitory effects on early bacterial growth, particularly at higher concentrations, the strains retained exceptional degradation capacities across all tested levels, consistently achieving degradation efficiencies above 99%. MCPA was found to be slightly less inhibitory than 2,4-D, as indicated by higher growth rates in its presence. This may reflect structural differences between the compounds affecting microbial uptake and enzymatic breakdown. The ability of the bacteria to maintain high degradation performance under suboptimal conditions underscores their potential application in bioremediating herbicide-contaminated soils and aquatic environments. These findings contribute to the growing body of evidence supporting microbial degradation as a reliable and environmentally friendly approach for mitigating the persistence of synthetic herbicides. Overall, this research reinforces the viability of using microbial agents for sustainable and cost-effective remediation of phenoxy herbicide pollution.

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Competing Interests:
We do not have any relevant financial or non-financial interests to disclose.

Author Contributions:
All authors contributed to the study conception and design. The preparation of materials, collection of data, and analysis of the data were carried out by Loveleen Kaur. Initially, Loveleen Kaur wrote the manuscript and all authors commented on previous versions. All authors read and approved the final manuscript.

Data Availability:
On reasonable request, the corresponding author can provide data sets generated and/or analyzed during this study.

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