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Biodegradation of Phenoxyacetic Acid Herbicides (MCPA AND 2,4-D) by *Escherichia Coli*: Insights from HPLC Detection

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***Abstract*:** The present study investigates the herbicide degradation potential of *Escherichia coli* strain LKDA3 under varying concentrations of two commonly used phenoxyacetic herbicides: 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA). Strain LKDA3, previously isolated using 500 mg/L herbicide, was evaluated for its degradation efficiency at 300, 500, and 700 mg/L concentrations over a 5-day incubation period in minimal salt medium supplemented with 0.2% glucose at 30 °C. Residual herbicide concentrations were quantified using high-performance liquid chromatography (HPLC), and degradation efficiency was determined by comparing chromatographic peak areas to those of untreated controls. The strain achieved complete (100%) degradation of 2,4-D at 300 mg/L, 99% at 500 mg/L, and 38.2% at 700 mg/L. A similar pattern was observed with MCPA, with ~99% degradation recorded across all concentrations. However, growth kinetics, measured via OD_{600}, showed concentration-dependent inhibition, particularly at 700 mg/L. Notably, MCPA induced a stronger inhibitory effect on bacterial growth compared to 2,4-D, despite high degradation efficiency. These results suggest that *E. coli* LKDA3 is capable of metabolizing both herbicides effectively, although elevated concentrations exert growth-limiting effects. The findings highlight the potential application of LKDA3 in bioremediation strategies targeting phenoxy herbicide-contaminated environments.

***Keywords*:** Biodegradation, *Escherichia coli,* Phenoxy herbicides, 2,4-D, MCPA, HPLC

Human activities have played a major role in accelerating climate change, primarily through the release of greenhouse gases into the atmosphere. This has contributed to a rise in extreme weather conditions, including prolonged droughts, reduced rainfall, and escalating global temperatures (Tejada et al., 2023). Since the 20th century, the use of agrochemicals-particularly pesticides has expanded considerably in agricultural practices. This overreliance has led to the contamination of both surface and groundwater resources (Barba et al., 2020, Zhang et al., 2021). In countries such as those in the European Union, the United States, and various developing nations, the herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) is commonly applied in agriculture to manage a broad spectrum of weeds. MCPA is a phenoxy-acid herbicide categorized under the auxins group, functioning by mimicking natural plant growth hormones. This imitation disrupts normal growth processes, resulting in tissue breakdown and ultimately plant death (Gorodylova et al., 2022). MCPA is effective in controlling weed populations, its removal from the soil is crucial before planting the next crop season. Residual MCPA in the soil can adversely affect subsequent crops, particularly sensitive ones such as cotton, soybeans, and corn (Zhang et al., 2021). Due to its high water solubility and low soil adsorption, MCPA easily leaches into surface and groundwater, posing a risk to water quality. This herbicide has been linked to adverse health effects in humans, particularly gastrointestinal and endocrine system disturbances (Wang et al., 2021). Similarly, 2,4-Dichlorophenoxyacetic acid (2,4-D), another member of the phenoxy herbicide group, is widely used to manage broadleaf weeds in agricultural lands and grasslands. As a hormonal herbicide, it disrupts plant growth by interfering with the hormonal balance within plants (Mustafa et al., 2015; Barba et al., 2020). Despite their effectiveness in weed control, both MCPA and 2,4-D are harmful to human health and the environment due to their persistence and toxicity. Therefore, there is an urgent need to develop efficient microbial solutions for their degradation. Studies have shown that 2,4-D residues can induce cytotoxic and genotoxic effects, trigger mutations in plants, and cause histological., physiological., and behavioral changes in animals (Aguiar et al., 2020). Researchers worldwide commonly rely on degradation technologies to eliminate environmental pollutants and their toxic byproducts. Among these methods, biodegradation stands out due to its cost-effectiveness and environmentally friendly nature (Singh et al., 2022). The effectiveness of a microbe in breaking down contaminants depends not only on its inherent biodegradation capacity but also on how accessible the pollutant is, which can be influenced by its interaction and binding with soil particles (Gorodylova et al., 2022). Microorganisms exhibit a wide range of capabilities in degrading various classes of pesticides. The natural breakdown of these substances can be enhanced through bioremediation techniques, which involve the addition of nutrients, carbon sources, or electron donors to support

microbial activity (Mustafa et al., 2015). Numerous studies have reported the occurrence of microorganisms capable of degrading pesticides and herbicides in environments such as soil, sewage sludge, and water. These microbes were often isolated from sites contaminated with such pollutants (Nguyen et al., 2021; Gorodylova et al., 2022).

An essential aspect of isolating herbicide-tolerant microbial strains involves carefully selecting suitable strains and creating favorable conditions that support their survival and activity. Typically, these microorganisms are cultured in environments containing pesticides and herbicides often at concentrations exceeding typical toxicity levels, to assess their resistance and degradation capabilities (Gorodylova et al., 2022). Although many studies have explored microbial degradation of herbicides, only a limited number of bacterial and fungal species have been successfully isolated when exposed to lower pesticide concentrations. The objective of the present study was to isolate microbial strains capable of tolerating the highest possible concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), both individually, within the shortest incubation period.

MATERIAL AND METHODS

Chemicals and solutions: HPLC grade 4-chloro-2-methylphenoxyacetic acid (MCPA) was procured from Merck, while 2,4-dichlorophenoxyacetic acid (2,4-D) of 99% purity was obtained from SRL. All other analytical-grade reagents were supplied by HiMedia. The mineral salt medium (MSM) used for microbial cultivation consisted of the following components (g/L): dipotassium phosphate (K_2HPO_4) - 1.5, potassium dihydrogen phosphate (KH_2PO_4) - 0.5, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) - 0.5, sodium chloride (NaCl) - 0.5, and ammonium nitrate (NH_4NO_3) - 1.5, as described by Li et al. (2008).

Enrichment and Identification of herbicide-degrading bacteria: Soil samples were obtained from sugarcane and wheat cultivation fields in the vicinity of Bakshi Ka Talab (26.9897° N, 80.9205° E), Lucknow, Uttar Pradesh, India, at a depth of approximately 10 cm. Prior to bacterial isolation, the samples were air-dried, passed through a sieve to remove debris, and stored at 4 °C for later use. Bacterial isolation was conducted using a minimal salt medium (MSM), in which herbicides served as the sole carbon source to promote the selective growth of herbicide-tolerant strains. For the enrichment process, 10 g of each soil sample was introduced into 250 mL Erlenmeyer flasks containing 100 mL of MSM supplemented with either 2,4-D or MCPA at a concentration of 500 mg/L. Enrichment was continued by transferring 1% (v/v) of the culture into fresh herbicide-containing MSM three times per week. After several enrichment cycles, cultures were serially diluted and spread onto MSM agar plates. Following a 4-day incubation period, morphologically distinct colonies were isolated and subjected to further screening for their ability to degrade the target herbicides.

Molecular identification of herbicide-degrading bacterial strains: The bacterial isolate designated as strain A3 was identified through amplification and sequencing of the 16S rRNA gene. Universal primers 16-GT-F (5'-AGAGTTTGATCATGGCTCA-3') and 16-GT-R (5'-TCGGATGGGAACTTTAAGT-3') were employed for PCR amplification and characterization of the bacterial strains. Each PCR reaction was prepared in a total volume of 25 µL, containing 1X Premix Taq, 0.4 µM of each primer, and 50 ng of genomic DNA extracted from the bacterial culture. Thermal cycling was performed on an Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany) with the following program: initial denaturation at 95 °C for 5 minutes; 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 90 seconds; followed by a final extension at 72 °C for 7 minutes. The PCR amplicons were confirmed by agarose gel electrophoresis and purified for subsequent Sanger sequencing. Sequence data were analyzed via the NCBI BLASTN tool to compare with sequences in the GenBank database, facilitating the identification or potential discovery of novel strains. In total, five bacterial strains exhibiting the ability to degrade the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) were isolated and classified based on the sequence similarity of their 16S rRNA genes to known taxa in GenBank.

Biodegradation kinetics of 2,4-D and MCPA by soil bacteria in MSM: Strain A3 was isolated using minimal salt medium (MSM) supplemented with 500 mg/L of the target herbicide. Post-identification, the strain was preserved by preparing a 70% glycerol stock, which was stored at –20°C for future use. Freshly cultured cells were employed for all experimental assays to ensure consistent and active microbial growth. To evaluate the effects of environmental parameters on strain A3 growth, experiments were performed by varying pH and temperature while maintaining a constant herbicide concentration in MSM. The growth kinetics of the isolated strain LKDA3 were investigated in Luria-Bertani (LB) broth under both herbicide-free and herbicide-supplemented conditions to evaluate the impact of herbicide exposure on bacterial proliferation. A control culture (without herbicide) was maintained alongside the treatment group to enable comparative analysis. Bacterial growth was assessed over a 26-hour period, with optical

density measurements at 600 nm (OD_{600}) recorded hourly using a UV/VIS spectrophotometer. Growth kinetics were monitored in MSM containing 500 mg/L herbicide and supplemented with 0.2% (w/v) glucose as an additional carbon source. Cultures were incubated at 30 °C with agitation at 150 rpm for five days. Daily optical density measurements at 600 nm (OD600) were recorded using a UV/VIS spectrophotometer to track microbial proliferation. For pH-dependent growth analysis, strain A3 was inoculated into MSM adjusted to pH values of 4.0, 5.0, 6.0, and 7.0, while maintaining the herbicide concentration at 500 mg/L and incubation temperature at 30 °C. Glucose supplementation remained constant at 0.2%. Cultures were incubated under shaking conditions for five days, with daily OD600 measurements to assess growth across different pH levels. To investigate the impact of temperature, strain A3 was cultivated in MSM (pH 7.0) at 20 °C, 30 °C, 40 °C, and 50 °C, with a fixed herbicide concentration and 0.2% glucose. Growth was monitored daily over a five-day incubation period by measuring OD600. All experiments, including those evaluating pH and temperature effects, were conducted in triplicate to ensure reproducibility and statistical reliability. Media blanks without bacterial inoculation served as controls in all spectrophotometric analyses. Additionally, the biodegradation potential of strain A3 was examined at varying herbicide concentrations (300 mg/L, 500 mg/L, and 700 mg/L) in MSM supplemented with 0.2% glucose. Cultures were initiated at an OD600 of 1.0 and incubated at 30 °C with shaking at 150 rpm for five days. Both microbial growth and herbicide degradation capacity were assessed at the end of the incubation period via OD600 measurements. This comprehensive experimental design facilitated an in-depth understanding of strain A3 growth dynamics and its capacity to degrade herbicides under diverse environmental conditions.

Analytical determination of 2,4-D and MCPA degradation products: During the biodegradation assays conducted in minimal salt medium (MSM) under optimized conditions, culture samples were periodically collected to monitor and analyze the degradation products. The culture broth was initially centrifuged at 12,000 × g for 15 minutes to separate bacterial cells from the supernatant. Both fractions were processed independently to extract metabolites and degradation compounds. The supernatant was concentrated by lyophilization and subsequently reconstituted in 3 mL of methanol. This methanolic solution was centrifuged at 6,000 × g for 15 minutes to remove particulate impurities, followed by filtration through 0.22 µm sterile syringe filters (Xia et al., 2017) to obtain a clear filtrate suitable for analysis. Concurrently, the bacterial cell pellet was resuspended in 3 mL of methanol and incubated overnight at room temperature to facilitate extraction of intracellular metabolites. Post-incubation, the suspension underwent sonication for 1 minute at ambient temperature to lyse the cells and enhance metabolite release. The mixture was then allowed to settle overnight to enable sedimentation of cell debris. Subsequently, the suspension was centrifuged at 6,000 × g for 15 minutes, and the supernatant was collected and filtered through sterile 0.22 µm PVDF syringe filters (AXIVA) to remove any residual particulates. Both extracellular and intracellular extracts were thus prepared for high-performance liquid chromatography (HPLC) analysis to characterize herbicide degradation products.

RESULTS AND DISCUSSION

Enrichment and identification of herbicide-degrading bacteria: A total of five bacterial isolates demonstrating herbicide-degrading potential were successfully obtained from agricultural soil samples using an enrichment culture technique, wherein herbicides served as the sole carbon and energy source to selectively enrich the degrading microbial population. Identification of all isolates was conducted through amplification and sequencing of the 16S rRNA gene. The resulting nucleotide sequences were analyzed using the NCBI BLASTN tool to determine taxonomic affiliations, and all validated sequences were submitted to the GenBank database. Among the five isolates, strain A3 was selected for detailed investigation due to its superior degradation capacity. Sequence alignment using BLASTN revealed that strain A3 shared 99% similarity with *Escherichia coli*, based on a 1438 bp region of the 16S rRNA gene. Consequently, the strain was identified as *Escherichia coli* and designated as *E. coli* LKDA3. The corresponding sequence was deposited in the NCBI GenBank under accession number OQ881067. To confirm the taxonomic position of strain A3, a phylogenetic analysis was performed by comparing its 16S rRNA gene sequence with those of closely related species retrieved from GenBank. The phylogenetic relationships are depicted in Figure 1.

Biodegradation kinetics of 2,4-D and MCPA by soil bacteria in MSM: The resulting growth curves (Fig. 2a) demonstrated that herbicide exposure did not significantly inhibit the growth of *E. coli* LKDA3 under the enriched conditions provided by LB medium. Both treated and control cultures exhibited similar growth trends, indicating a high level of tolerance to the tested herbicides. An initial lag phase of approximately 4 hours was observed, during which cellular activity was primarily focused on physiological adaptation rather than active division. This was followed by a pronounced exponential growth phase starting around the

6th hour, characterized by rapid cell proliferation and a steady increase in OD_{600}. The exponential phase persisted until approximately the 18th hour, with the OD_{600} reaching ~1.2 units. Maximum cell density was recorded at approximately OD_{600} = 2.5 after 20 hours of incubation. Subsequent measurements showed a plateau in optical density, marking the onset of the stationary phase, likely resulting from nutrient depletion and/or accumulation of inhibitory metabolic by products.

Following the growth kinetics assessment in Luria-Bertani (LB) medium, the growth behavior of *E. coli* strain LKDA3 was further evaluated in minimal salt medium (MSM) to investigate its ability to utilize herbicides under nutrient-limited conditions. Initially, the strain was cultured in MSM without any additional carbon source, both in the presence and absence of herbicides. No detectable growth was observed over a 5-day incubation period at 30 °C, suggesting that the strain was unable to metabolize either the herbicide compounds or the MSM constituents as sole sources of carbon and energy under these conditions. To enhance the growth potential, MSM was supplemented with 0.2% (w/v) glucose and 500 mg/L of either 2,4-dichlorophenoxyacetic acid (2,4-D) or 2-methyl-4-chlorophenoxyacetic acid (MCPA). In the control group (MSM + 0.2% glucose without herbicide), minimal growth was observed after 5 days, indicating that the limited glucose alone was insufficient for robust proliferation. When 2,4-D was present, a slight increase in OD_{600} was recorded on day 1 (~0.04), which subsequently declined by day 5, suggesting an initial adaptive response followed by inhibition due to herbicide toxicity. A comparable trend was observed in MCPA-supplemented cultures, where OD_{600} similarly peaked at ~0.04 on day 1 but exhibited a modest increase to ~0.08 by day 5 (Fig. 2b), indicating a delayed but partial adaptation to

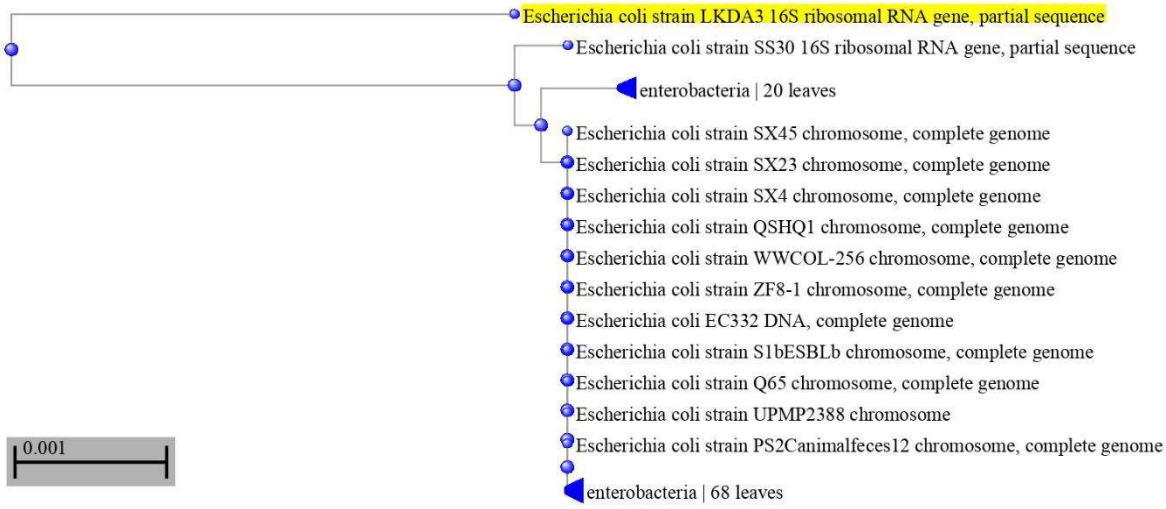

Fig. 1. Phylogenetic tree of strain LKDA3 with other microorganisms

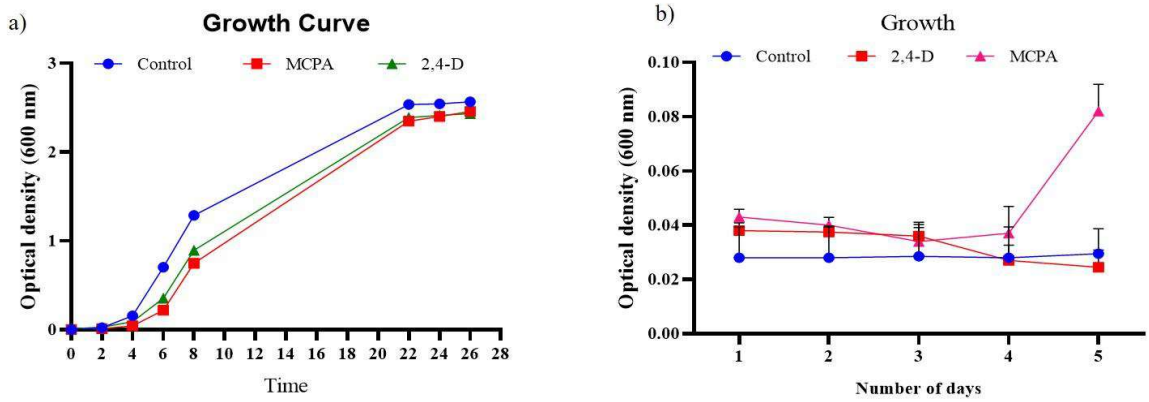

Fig. 2. Growth Curve in (a) enriched medium (LB broth) (b) Minimal salt medium with 0.2% glucose in the presence of 2,4-D and MCPA at 500 mg/L concentration

the herbicide-induced stress. These results suggest that both herbicides exert inhibitory effects on *E. coli* LKDA3 under minimal nutritional conditions. While the strain exhibited limited initial survival., it showed some capacity for physiological adaptation, particularly in the presence of MCPA. To further investigate environmental influences on growth, the effect of pH was assessed. Cultures were grown in MSM supplemented with 0.2% glucose and 500 mg/L of herbicide, across a pH gradient (5.0, 6.0, 7.0, and 8.0), at 30 °C under continuous shaking (150 rpm) for 3 days. In the presence of 2,4-D, the highest OD_{600} (~0.12) was observed at pH 6.0, indicating that mildly acidic conditions favor growth under herbicide stress. Reduced growth was observed at pH 7.0 and 8.0, while no growth occurred at pH 5.0 (Fig. 3a), indicating that strongly acidic conditions impair strain viability. In contrast, cultures exposed to MCPA demonstrated limited survival across all pH levels, with OD_{600} values remaining around ~0.05, and no detectable growth at pH 5.0 (Fig. 3b). These findings highlight the pronounced inhibitory effect of MCPA, particularly under acidic conditions, and suggest a narrow window of environmental tolerance for strain LKDA3 in herbicide-amended minimal media.

Following growth kinetics analysis in LB medium, the growth response of *Escherichia coli* strain LKDA3 was further examined in minimal salt medium (MSM) to assess its ability to utilize herbicides under nutrient-limited conditions. No visible growth was observed in MSM alone, with or without herbicides, after 5 days of incubation at 30 °C, indicating that the strain could not metabolize the herbicides or MSM constituents as sole carbon sources. When MSM was supplemented with 0.2% glucose and 500 mg/L of 2,4-D or MCPA, a slight increase in OD_{600} (~0.04) was recorded on day 1, followed by either stagnation or a marginal rise by day 5 (~0.08 in MCPA), indicating initial stress adaptation. Minimal growth was also noted in the glucose-only control, suggesting limited nutrient availability. To investigate environmental tolerance, growth was evaluated at different pH levels (5.0–8.0) in MSM with glucose and 500 mg/L herbicide. Optimal growth with 2,4-D was observed at pH 6.0 (OD_{600} ~0.12), while growth declined at neutral and alkaline pH and was absent at pH 5.0. In MCPA treatments, OD_{600} remained low (~0.05) across all pH levels, with no growth at pH 5.0. These findings suggest that strain LKDA3 shows limited growth under herbicide stress, with slightly better adaptation to 2,4-D than MCPA.

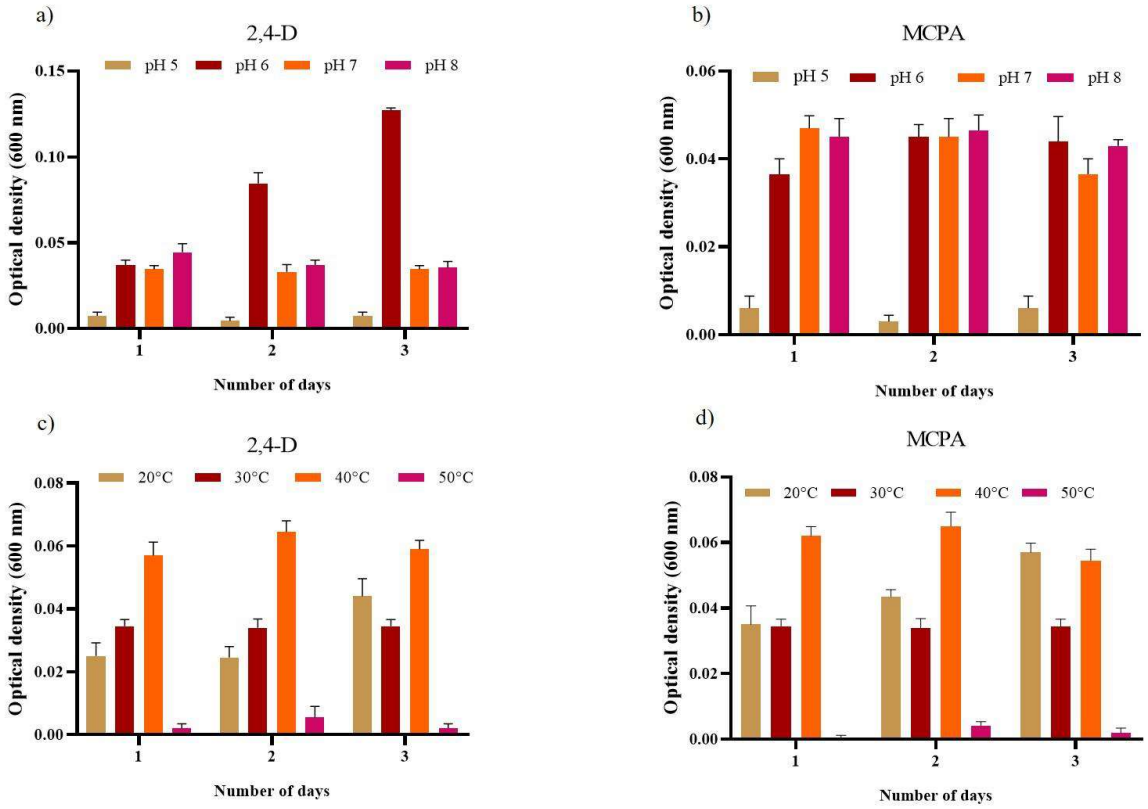

Fig. 3. Growth of strain LKDA3 in minimal salt medium + 0.2% glucose with different pH (a) 2,4-D (b) MCPA and temperatures (c) 2,4-D and (d) MCPA

Analytical determination of 2,4-D and MCPA degradation products: The herbicide degradation capability of *E. coli* strain LKDA3 was assessed at three initial concentrations (300, 500, and 700 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) following a 5-day incubation under optimized conditions (30 °C, 150 rpm). While agricultural usage typically involves herbicide concentrations between 200–300 mg/L, the strain was originally isolated using 500 mg/L, justifying the inclusion of higher concentrations to evaluate degradation efficiency under increased exposure. After incubation, culture supernatants were collected and analyzed via high-performance liquid chromatography (HPLC) to quantify residual herbicide levels. Degradation efficiency was calculated by comparing the sample peak areas to those of untreated standards (Fig. 5). Strain LKDA3 exhibited complete degradation of 2,4-D at 300 mg/L and 500 mg/L shows 99%, and 38.2% at 700 mg/L (Fig. 4b), indicating a concentration-dependent response. Concomitant growth measurements (OD_{600}) revealed more robust biomass accumulation at lower herbicide levels, with diminished growth (OD_{600} ≈ 0.12) observed at 700 mg/L (Fig. 4a), suggesting delayed adaptation or toxic effects at elevated concentrations. Comparable trends were noted for MCPA, where ~99% degradation occurred after 5 days at all concentrations. However, bacterial growth declined markedly with increasing MCPA levels. At 300 mg/L, OD_{600} reached ~0.5, while at 700 mg/L, it dropped to ~0.2, indicating stronger inhibitory effects of MCPA compared to 2,4-D.

Isolated *E. coli* LKDA3 shows 99% degradation rate at 300 mg/L and 500 mg/L concentration of 2,4-D herbicide, while at 700 mg/L it shows decline degradation rate of 38.2%, indicating potential toxicity at higher concentrations. Similarly, MCPA degradation was approximately 99% at 300 mg/L, with reduced bacterial growth observed at higher concentrations. These findings suggest that while strain LKDA3 can effectively degrade these herbicides at lower concentrations, higher levels may inhibit its activity.

Comparative studies have explored the degradation of 2,4-D by *E. coli* strains. Bhat et al. (2015) investigated the effects of sublethal levels of 2,4-D on *E. coli*, revealing that exposure induced oxidative stress, leading to filamentous cell morphology and alterations in metabolic pathways.

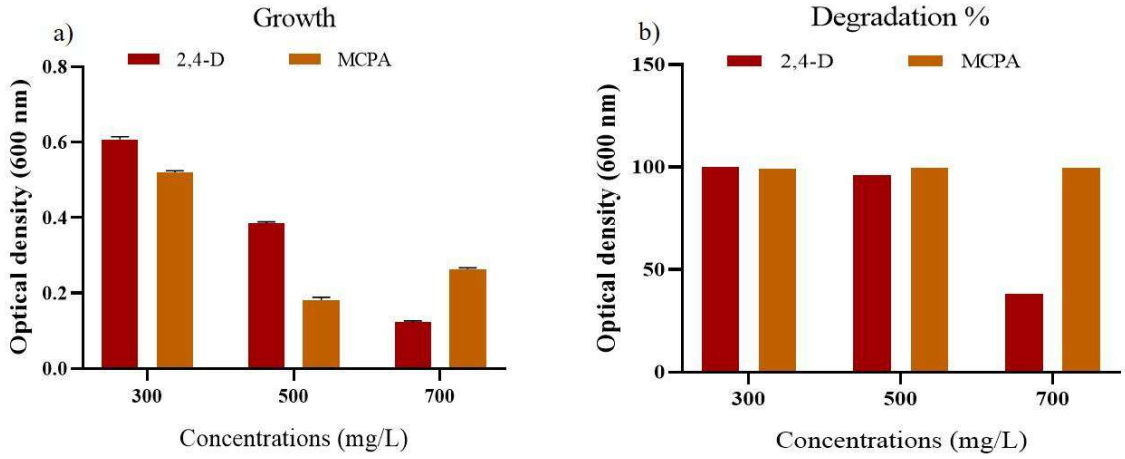

Fig. 4. Growth with different concentrations of herbicides (2,4-D and MCPA) after 5 days of incubation at 30˚C (a) degradation % of 2,4-D and MCPA by *E. coli* LKDA3 (b)

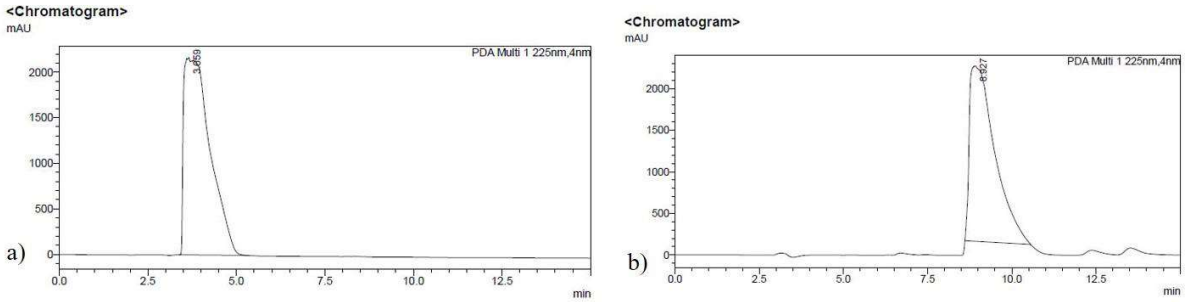

Fig. 5. Standard HPLC chromatograph of both herbicide (a) 2,4-D (b) MCPA

These stress responses could impact the bacterium's degradation capabilities. In contrast, a study by Wang et al. (2023) engineered *E. coli* strains with a complete 2,4-D degradation pathway, achieving rapid and complete degradation of 0.5 mM 2,4-D within 6 hours. This highlights the potential of genetic engineering to enhance degradation efficiency. Further, Bhat et al. (2018) demonstrated that sublethal concentrations of 2,4-D arrested cell division in *E. coli* by disrupting the divisome complex, leading to DNA damage and activation of the SOS response. These cellular disruptions could compromise the bacterium's viability and degradation capacity under herbicide stress. Additionally, a study by Singh et al. (2022) using Raman spectroscopy found that exposure to 2,4-D and glyphosate induced distinct biochemical changes in *E. coli*, potentially leading to phenotypic antibiotic resistance. Such stress-induced adaptations may influence the bacterium's metabolic functions, including herbicide degradation. Collectively, these studies underscore the complex interplay between herbicide concentration, bacterial stress responses, and degradation efficiency. While strain LKDA3 exhibits promising degradation capabilities at lower herbicide concentrations, its performance diminishes at higher levels, likely due to induced stress responses similar to those observed in other *E. coli* studies. Future research could focus on enhancing the resilience and degradation efficiency of such strains, potentially through genetic modifications or adaptive evolution strategies, to improve bioremediation outcomes in environments with varying herbicide concentrations.

CONCLUSION

The present study demonstrates the efficacy of selected strain *E. coli* LKDA3 in degrading phenoxyacetic herbicides 2,4-D and MCPA under varying environmental conditions. The results reveal that bacterial growth and degradation efficiency are significantly influenced by pH, temperature, and herbicide concentration. Optimal bacterial proliferation and maximum degradation were observed at pH 6 and temperatures between 30°C and 40°C, 40˚C being the best 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 to 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. Further studies involving molecular characterization of the degradation pathways and field-scale validation are recommended to enhance the application potential of strains. Overall, this research reinforces the viability of using microbial agents for sustainable and cost-effective remediation of phenoxy herbicide pollution. The ability of the bacteria to maintain high degradation performance under suboptimal conditions highlights their potential application in bioremediation of herbicide-contaminated soils and aquatic environments.

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