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Optimal biodegradation of the herbicide haloxyfop-*R*-methyl by the fungus *Mucor circinelloides* YMM22

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ABSTRACT

The widespread use of the herbicide haloxyfop-*R*-methyl poses environmental risks due to its persistence and toxicity, necessitating the development of effective bioremediation strategies. This study investigated the biodegradation of this herbicide by the fungus *Mucor circinelloides* YMM22. The fungus was isolated from Egyptian soil which can grow in the presence of haloxyfop-*R*-methyl up to 500 mg/L. The fungus was identified as *M. circinelloides* YMM22 by phenotypic and molecular procedures. The fungus was cultivated in an optimized Czapek Dox agar medium containing haloxyfop-*R*-methyl (25-500 mg/L) as the sole carbon source for 6 days at 28°C. The highest growth of 5.25 cm was recorded at 100 mg/L haloxyfop-*R*-methyl. The effects of glucose, shaking speed, and pH on the biodegradation (%) were tested using a Box-Behnken design, and the pesticide residues were determined by HPLC. The optimal biodegradation of 92.13, 85.10, and 86.11% were obtained for 100, 200, and 300 mg/L haloxyfop-*R*-methyl, respectively at 5.4 g/L glucose, pH 8, and 150 rpm after 5 days of incubation. These findings confirm that *M. circinelloides* YMM22 is a highly effective and robust biological agent for the detoxification of haloxyfop-*R*-methyl, showing great potential for application in the bioremediation of contaminated environments.

Keywords: Polluted water; Pesticide residues; Removal; HPLC analysis.

1. Introduction

Herbicide is a chemical that is used to manipulate or control undesirable vegetation, usually known as weeds. An herbicide is typically applied before or during planting to minimize competition with other vegetation in row-crop farming such as cash and tree crop cultivation, herbicides are essential for weed management (Green 2014; Obayagbona et al. 2023; Pervaiz 2024). This practice aims to maximize crop yield and economic returns, supporting the growing global population. Herbicides have become a significant environmental concern due to their low effectiveness against targeted flora. This has increased the risk of negative health impacts on non-target organisms exposed to such chemicals (Das et al. 2024; Thiour-Mauprivez et al. 2019). Herbicides, while crucial for agricultural practices, can also pose environmental risks. These chemicals, when used improperly or under certain conditions, can contaminate soils, groundwater, and surface water bodies, leading to negative ecological consequences (AbuQamar et al. 2024; Li et al. 2024).

Haloxyfop-R-methyl (commonly referred to as haloxyfop) is indeed an aryloxyphenoxypropionate herbicide, which are selective post-emergence herbicides, used to control annual and perennial weeds in fields where crops like sugar beets, fodder beets, potatoes, leafy vegetables, onions, sunflowers, soybeans, vines, strawberries, and other broadleaf plants are grown (Sinha et al. 2025; Zhou et al. 2018). It inhibits lipid biosynthesis and induces oxidative stress. It thus has selective targets within plants, with very little or no side effects on non-target plants. However, the misuse of pesticides pollutes the environment and negatively affects plants, humans,

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and animals. Excessive use of haloxyfop-*R*-methyl pollutes aquatic environments and negatively affects ecosystems (Wei et al. 2023; Zhou et al. 2025). Haloxyfop-*R*-methyl causes growth failure in the eyes and bodies of zebrafish embryos (Lee et al. 2024). In addition, non-target crops such as maize and wheat may be negatively affected by this herbicide. Furthermore, haloxyfop-*R*-methyl has been detected in drinking water.

Mineralization, in the context of pesticide degradation, is the process where microorganisms (microbiota) break down a pesticide into simpler, inorganic compounds like carbon dioxide (CO_2), water (H_2O), and minerals (Doukani et al. 2022; Li et al. 2024). These microbes utilize the pesticides chemical structures as a source of carbon and energy for their growth and reproduction. This process effectively eliminates the pesticide from the environment (Obayagbona et al. 2023; Rohilla et al. 2025). Microbial biodegradation of organic contaminants is used as an environmentally safe means of removing pesticides from the aquatic environment. Fungi are a potentially strong competitor compared to other microbes in the biodegradation of pesticides (Barros do Nascimento et al. 2025). However, to our knowledge, no article has investigated the biodegradation of haloxyfop-*R*-methyl using fungi.

The key objectives of this study were to isolate and identify a novel microbial agent capable of degrading the environmentally persistent herbicide haloxyfop-*R*-methyl, and to systematically evaluate its bioremediation potential. This involved the isolation of a fungal strain from contaminated soil, its phenotypic and molecular identification, and an assessment of its tolerance to high concentrations of the herbicide. Furthermore, we sought to investigate the fungus's capacity to utilize haloxyfop-*R*-methyl as a sole carbon source and to determine the optimal cultural conditions, specifically the concentration of a co-substrate (glucose), pH, and agitation speed, for maximizing the biodegradation efficiency using a statistical response surface methodology (Box-Behnken design). The ultimate goal was to confirm the efficacy of this fungal isolate as a robust and effective biological agent for the detoxification of haloxyfop-*R*-methyl, thereby proposing a viable strategy for the bioremediation of contaminated environments.

2. Materials and Methods

2.1. Chemicals and microbiological media

Analytical grade haloxyfop-*R*-methyl with a purity of 99.3 % (Figure 1) was obtained from Shoura Chemicals Co. (Giza, Cairo- Alexandria Desert Road, Egypt). Potato Dextrose Agar (PDA) was purchased from Oxoid (Basingstoke, Hampshire, RG24 8PW, UK). Czapek Dox agar were purchased from Hi-Media Leading Biosciences Company (India). Other chemicals and solvents were purchased from El-Nasr Pharmaceutical Chemicals Co. (Abu Zaabal Area 491, Qalyub, Egypt) and used without further purification. All media were prepared immediately and autoclaved at 121°C for 20 min.

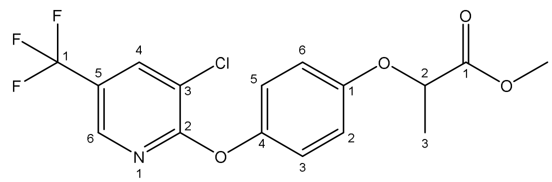

Figure 1. Chemical structure of the herbicide haloxyfop-*R*-methyl.

2.2. Isolation of haloxyfop-*R*-methyl degrading fungi

A soil sample was collected from the wheat rhizosphere in El-Beheira Governorate, Egypt (31.147158°N, 30.239593°E). To isolate herbicide-degrading fungi, 1 mL of a 10^{-2} soil dilution was spread onto Czapek Dox agar (CDA) plates, wherein haloxyfop-*R*-methyl (50 mg/L) served as the sole carbon source. Plates were incubated at 28°C for 7 days. Emerging fungal colonies were aseptically transferred to potato dextrose agar (PDA) for purification. Pure cultures were subsequently established on PDA slants and stored at 4°C for long-term preservation.

2.3. Identification of fungal isolate

The fungal strain was identified based on morphological (Domsch et al. 1980; Moubasher 1993) and molecular characteristics. Genomic DNA of YMM22 (≈100 mg of fresh fungal biomass) was extracted according to the Miniprep Fungal/Bacterial Quick-DNA Kit (Zymo Research), following the kit manufacturer's procedures. PCR reactions were performed according to the manufacturer's instructions using COSMO PCR RED Master Mix (W1020300X). The PCR primer pair was NL1 and NL4 which are used to amplify the 5′ end of 28S rDNA. Finally, PCR reaction products were detected on 1.5% agarose gel, electrophoretically. Amplified products were sequenced in both directions using Sanger technology using an ABI 3730xl DNA sequencer and the same primers pair. The sequence of YMM22 was identified by comparing it to nucleotide databases, using BLASTN software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition, the nucleotide sequence of YMM22 has been deposited in GenBank under accession number OQ067101. Neighbor-joining tree (1000 replicates bootstrap) was also performed for the YMM22 sequence using MEGA 6 software.

2.4. Biodegradation of haloxyfop-*R*-methyl on agar plates

Agar plates of CDA containing 25 to 500 mg/L haloxyfop-*R*-methyl (as sole carbon source) were inoculated with 0.9 cm plugs of *M. circinelloides* YMM22 in the center of the plates and then incubated at 28 ± 2°C for 4, 7, and 15 days. Controls were CDA plates without pesticide. Finally, the average linear growth of the fungus and the clear degradation area around the colonies were recorded.

2.5. Biodegradation of haloxyfop-*R*-methyl in carbon-limited broth medium

Erlenmeyer conical flasks (250 mL) containing 50 mL Czapek Dox broth (CDB) medium were inoculated with 0.5 mL of spore suspension (10^6 spore/mL), and incubated at 28 ± 2°C for 5 days. The CDB culture medium consisted of D-glucose (20 g/L), $NaNO_3$ (3 g/L), KH_2PO_4 (1 g/L), $MgSO_4.7H_2O$ (0.5 g/L), KCl (0.5 g/L), and $FeSO_4.7H_2O$ (0.01 g/L), then, haloxyfop-*R*-methyl was added separately to the sterile medium. The pH of the media was adjusted with 0.1 N NaOH or 0.1 N HCl solutions. At the end of the experiment, the dry biomass of the YMM22 culture was obtained by filtration on Whatman filter paper, dried for 12 h at 60°C, and re-weighed (El-Sayed 2015). To determine biodegradation (%), a water sample (1 ml) was taken separately at the end of the incubation and centrifuged at 6000 rpm for 10 min. The pesticide concentration was determined by the HPLC. The biodegradation (%) of haloxyfop-R-methyl was determined using the following equation.

2.6. HPLC analysis of haloxyfop-*R*-methyl

Haloxyfop-*R*-methyl residues were quantified using high-performance liquid chromatography (HPLC) on an Agilent 1260 Infinity system equipped with a variable wavelength ultraviolet (UV) detector. Separation was achieved on a reversed-phase ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 µm) maintained at 40°C. The isocratic mobile phase, consisting of acetonitrile, methanol, and water (60:20:20, v/v/v), was delivered at a flow rate of 1.0 mL/min. Detection was performed at 280 nm, and haloxyfop-*R*-methyl eluted at a retention time of 5.298 min. Quantification was based on a five-point external standard calibration curve (0.125-1.0 µg on-column) constructed by plotting peak area against concentration. The calibration curve for haloxyfop-*R*-methyl was linear up to 1.0 µg and the correlation coefficient (R^2) was 1.0 (Figure 2).

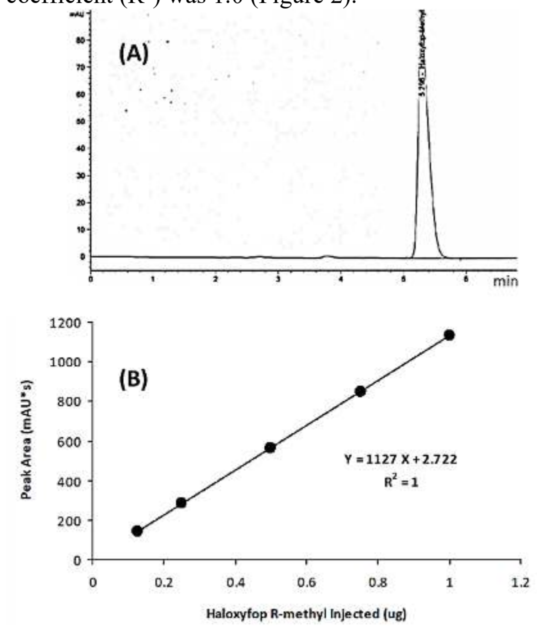

Figure 2. Chromatogram (A), and calibration curve (B) of haloxyfop-*R*-methyl using HPLC connected with a variable wavelength ultraviolet detector (VWD).

2.7. Improving the degradation of haloxyfop-*R*-methyl using a Box-Behnken design

A Box-Behnken experiment (Box and Behnken 1960) was created using Minitab 17.1.0 (Minitab Inc., USA) to optimize the biodegradation of 100 mg/L haloxyfop-*R*-methyl (Table 1). Three independent variables (glucose, pH, and shaking) were examined and their response (biodegradation, %) adjusted to a polynomial quadratic equation. This equation was used to find out the relationship between the independent and dependent variables.

2.8. Biodegradation of haloxyfop-*R*-methyl in optimized medium

The time course for biodegradation of haloxyfop R-methyl (100, 200, and 300 mg/L) was achieved with *M. circinelloides* YMM22 under optimized conditions (glucose, 5.45 g/L; pH 8; and shaking, 150 rpm). Biodegradation (%) was routinely monitored daily for 5 days by determination of haloxyfop R-methyl residues by HPLC analysis. Finally, the remaining haloxyfop R-methyl was plotted with time.

Table 1. Biodegradation (%) optimization of haloxyfop-*R*-methyl using Box-Behnken design

Run order	Point type	Glucose (g/L)	pH	Shaking speed (rpm)	Degradation (%) ± SE	Dry biomass (g/L) ± SE
1	2	(-) 0.0	(-) 4	(0) 75.0	87.38±40.82	20.4±0.33
2	2	(+) 20	(-) 4	(0) 75.0	88.93±41.49	20.8±0.16
3	2	(-) 0.0	(+) 8	(0) 75.0	89.26±40.91	20.6±0.16
4	2	(+) 20	(+) 8	(0) 75.0	89.53±41.77	22.4±1.14
5	2	(-) 0.0	(0) 6	(-) 0.00	88.10±41.10	20.6±0.16
6	2	(+) 20	(0) 6	(-) 0.00	90.00±42.14	21.8±0.33
7	2	(-) 0.0	(0) 6	(+) 150	89.78±42.06	20.8±0.33
8	2	(+) 20	(0) 6	(+) 150	90.00±41.86	22.0±0.49
9	2	(0) 10	(-) 4	(-) 0.00	89.04±41.54	20.9±0.41
10	2	(0) 10	(+) 8	(-) 0.00	89.99±42.13	21.5±0.57
11	2	(0) 10	(-) 4	(+) 150	89.95±41.84	21.0±0.33
12	2	(0) 10	(+) 8	(+) 150	91.00±42.47	21.4±0.49
13	0	(0) 10	(0) 6	(0) 75.0	88.39±41.08	20.9±0.24
14	0	(0) 10	(0) 6	(0) 75.0	88.99±41.49	20.9±0.24
15	0	(0) 10	(0) 6	(0) 75.0	88.99±41.48	20.9±0.24

2.9. Statistical analysis

Statistical analysis was performed using Minitab 17.1.0 (Minitab Inc., USA). During the entire study, all experiments were performed in triplicate. All data were analyzed by analysis of variance (ANOVA). Data means were compared by the Fisher's least significance difference ($p \leq 0.05$).

3. Results and Discussion

3.1. Identification of the fungal isolate YMM22

The evolutionary position of the fungal isolate YMM22 was determined through phylogenetic analysis of its gene sequence. The relationship among related taxa was inferred using the Neighbor-Joining method in MEGA6 software. The optimal tree is shown in Figure 3, with the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) displayed next to the branches. The resulting phylogenetic tree clearly demonstrates that the isolate YMM22 clusters with high statistical support within a clade containing reference strains of *Mucor circinelloides*. Specifically, the isolate formed a robust monophyletic group with known *M. circinelloides* sequences, supported by a high bootstrap value of 100%. This strong nodal support provides confidence in the taxonomic assignment, confirming that the isolated YMM22 is a strain of *M. circinelloides*. The tree also delineates the position of *M. circinelloides* relative to other closely related species within the Mucor genus, such as *M. racemosus* and *M. ellipsoideus*, which formed distinct, separate clades with significant bootstrap support, underscoring the genetic distinction between these species.

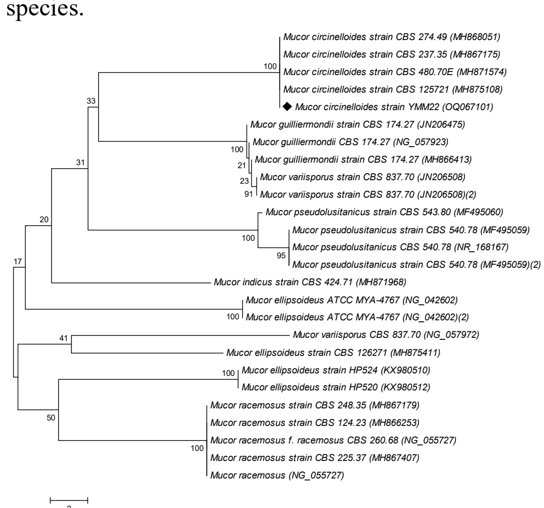

Figure 3. Phylogenetic tree according to the Neighbor-Joining method showing the evolutionary relationships of taxa (MEGA6). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

The high bootstrap support for the clade containing YMM22 and authenticated *M. circinelloides* strains provides conclusive molecular evidence for its identification. Bootstrap values above 70% are generally considered to indicate strong support for a node, and the value of 99% leaves little doubt about the taxonomic assignment (Felsenstein 1985). The close relationship with other strains suggests a potential common lineage or ecological adaptation, possibly related to their shared origin from soil (Hoffmann et al. 2009). This identification is significant as M. circinelloides is a widespread fungus of agricultural, clinical, industrial, and scientific importance (Fazili et al. 2022; Mohamed et al. 2025; Rodrigues Reis et al. 2019). Its accurate identification is crucial for further studies, whether they concern its role as an opportunistic pathogen, its use in biotechnological applications such as lipid production, or its function in biotransformation processes (Mohamed et al. 2025). The reliable phylogenetic placement of YMM22 established in this study provides a solid foundation for subsequent functional and comparative genomic investigations. Furthermore, the clear separation from other Mucor species in the tree validates the use of the internal transcribed spacer (ITS) sequence as a reliable barcode for the accurate differentiation of species within this genus.

3.2. Growth of *M. circinelloides* on haloxyfop-*R*-methyl on agar plates

Biodegradation and tolerance of M. circinelloides YMM22 were examined against different amounts of haloxyfop-*R*-methyl (25, 50, 100, 250, and 500 mg/L) on agar plates (Figures 4-6). Figure 4 shows the growth response of M. circinelloides strain YMM22 to different concentrations of haloxyfop-*R*-methyl (0-500 mg/L) on Czapek Dox agar with different time intervals. Fungal growth was observed across the entire range of haloxyfop-R-methyl concentrations tested, reaching a maximum radial extension of 5.25 cm at 100 mg/L. However, M. circinelloides YMM22 growth (0.7 cm) was very weak at 500 mg/L. On the other hand, zones of haloxyfop-*R*-methyl biodegradation were observed around fungal mycelium at all incubation times and all pesticide concentrations. In addition, the diameter of the biodegradation zone decreased with increasing pesticide concentration. Moreover, the diameter of the biodegradation zone increases with the increase in incubation time (Figure 5).

Figure 6 shows the growth response of *M. circinelloides* YMM22 to different concentrations (0-500 mg/L) of haloxyfop-R-methyl after 7 days on CDA medium at 28±2°C. The growth was significantly influenced by the concentration of herbicide. The fungus exhibited a clear dose-dependent response to herbicide. At the lowest concentration tested (25 mg/L), fungal growth was not significantly different from the control (0 mg/L). However, as the herbicide concentration increased to 50-500 mg/L, a significant reduction in growth was observed. The highest concentrations of 250 mg/L and 500 mg/L resulted in the most substantial inhibition of growth, with these two treatments being statistically similar to each other but significantly different from all lower concentrations. The LSD test confirmed that all treatments with different letters are significantly distinct ($P \leq 0.05$), indicating a clear progression of inhibitory effects.

Figure 4. Growth response of *M. circinelloides* strain YMM22 to different concentrations of haloxyfop-*R*-methyl (0-500 mg/L) on Czapek Dox agar with different time intervals. (a) 4 days, (b) 7 days and (c) 15 days of growth at 28±2°C.

Figure 5. Haloxyfop-*R*-methyl (500 mg/L) degradation zone around *M. circinelloides* YMM22 colonies on Czapek Dox agar after 4, 7 and 15 days of growth at 28±2 °C.

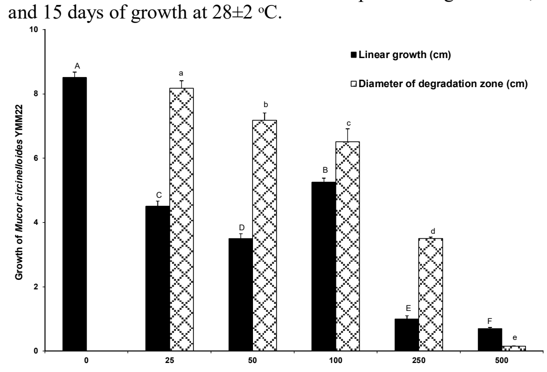

Figure 6. Growth response of *M. circinelloides* YMM22 in the presence of haloxyfop-*R*-methyl (0-500 mg/L) after 7 days on Czapek Dox agar at 28±2°C. Bars that do not share the same letter are significantly different according to the LSD test ($P \leq 0.05$).

The results demonstrate that *M. circinelloides* YMM22 possesses a notable tolerance to haloxyfop-*R*-methyl at low concentrations. However, its growth is significantly impaired at concentrations of 100 mg/L and above. This pattern of dose-dependent inhibition is a common response of microorganisms to toxicants, where a threshold concentration is surpassed, leading to metabolic disruption and reduced biomass accumulation (Silva et al. 2019). The ability of the fungus to grow up to 100 mg/L suggests potential mechanisms of tolerance. Fungi can employ various strategies to survive in the presence of herbicides, including enzymatic degradation, efflux pumps, and metabolic avoidance (Gao et al. 2024; Satish et al. 2017). Zygomycetes, the phylum to which Mucor belongs, have been reported for their metabolic versatility and ability to biotransform or degrade complex organic pollutants, including pesticides (Banu et al. 2024; Satish et al. 2017). The significant growth reduction at higher concentrations (250-500 mg/L) indicates that these potential detoxification mechanisms are likely overwhelmed, leading to the herbicide's primary toxic action. Haloxyfop-*R*-methyl is an acetyl-CoA carboxylase (ACCase) inhibitor, which disrupts lipid biosynthesis in plants. While the specific target in fungi may differ, the disruption of crucial metabolic pathways likely leads to the observed growth inhibition (Depetris et al. 2024; Takano et al. 2020). The finding that *M. circinelloides* can withstand and grow at substantial concentrations of a widely used herbicide has important ecological and biotechnological implications. Ecologically, it highlights the role of soil fungi in the resilience of the microbial community and the natural bioremediation of herbicide-contaminated soils (Hao et al. 2025; Huang et al. 2018). From a biotechnological perspective, this strain warrants further investigation for its potential in the bioremediation of environments polluted with aryloxyphenoxypropionate herbicides. Future research should focus on elucidating the precise degradation pathway, identifying the responsible enzymes, and optimizing conditions for large-scale application.

3.3. Enhanced biodegradation of haloxyfop-*R*-methyl by *M. circinelloides* YMM22

The effective bioremediation of pesticide residues like haloxyfop-*R*-methyl is crucial for mitigating environmental pollution. This study successfully employed a Response Surface Methodology (RSM) framework, specifically a Box-Behnken Design (BBD), to model, optimize, and understand the interactive effects of key physiological parameters on the biodegradation of haloxyfop-R-methyl. A Box-Behnken experimental design was employed to optimize the biodegradation of haloxyfop-*R*-methyl by investigating the effects of glucose concentration, pH, and shaking speed. Other media components were maintained as fixed factors (Table 1). The observed response was modeled by a quadratic regression equation that describes the relationship between the independent variables and the biodegradation rate: Biodegradation (%) = 88.790 + 0.493 Glucose + 0.560 pH + 0.450 Shaking - 0.270 Glucose×Glucose + 0.255 pH×pH + 0.950 Shaking×Shaking - 0.320 Glucose×pH - 0.420 Glucose×Shaking + 0.025 pH×Shaking.

The derived quadratic regression model demonstrated an excellent fit to the experimental data, as indicated by a high coefficient of determination (R^2 = 97.48%). This implies that the model accounts for 97.48% of the variability in the biodegradation response, leaving only a minor 2.52% attributable to random error or variables not included in the study. An R^2 value exceeding 0.9 is widely considered indicative of a highly reliable and predictive model in bioprocess optimization (Bezerra et al. 2008). The model's structure, containing linear, interaction, and quadratic terms, allows for a nuanced understanding of the system's behavior beyond simple linear relationships. Analysis of the model equation provides direct insight into the individual and interactive roles of the variables. The positive linear coefficients for glucose concentration (+0.493), pH (+0.560), and shaking speed (+0.450) indicate that, within the experimental range studied, an increase in any single factor initially promotes a higher biodegradation rate. This suggests that adequate carbon supplementation (glucose), an alkaline pH, and sufficient aeration (shaking) are all favorable conditions for the microbial consortium or enzyme responsible for the degradation. The positive quadratic terms for pH×pH (+0.255) and, most notably, Shaking×Shaking (+0.950) reveal a significant convex (upward-curving) curvature. This is a critical finding, as it demonstrates that the positive effect of these factors, especially shaking speed, accelerates as their values increase, without reaching a plateau or decline within the tested domain (Jensen 2017). Conversely, the negative quadratic term for Glucose×Glucose (-0.270) suggests a concave relationship, where beyond a certain concentration, additional glucose may provide diminishing returns or even begin to slightly inhibit the process. This is a common phenomenon in microbial degradation, where high concentrations of a readily available carbon source can lead to catabolite repression, suppressing the expression of enzymes needed to metabolize more complex compounds like pesticides (Nair and Sarma 2021). Furthermore, the negative interaction coefficients for Glucose×pH (-0.320) and Glucose×Shaking (-0.420) highlight significant two-factor interactions. These negative terms imply that the optimal level of one factor is dependent on the level of the other. For instance, the negative Glucose×Shaking interaction suggests that at high glucose concentrations, the benefit of increased shaking is less pronounced, or vice versa, possibly due to shifts in microbial metabolism or oxygen demand under different nutrient conditions.

Table 2. Analysis of variance for biodegradation (%) optimization of haloxyfop-R-methyl using Box-Behnken design

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	11.1155	11.1155	1.23506	21.50	0.002
Linear	3	6.0693	6.0693	2.02308	35.21	0.001
Glucose	1	1.9405	1.9405	1.94045	33.78	0.002
pH	1	2.5088	2.5088	2.50880	43.67	0.001
Shaking	1	1.6200	1.6200	1.62000	28.20	0.003
Square	3	3.9286	3.9286	1.30952	22.79	0.002
Glucose×Glucose	1	0.4733	0.2692	0.26917	4.69	0.083
pH×pH	1	0.1229	0.2401	0.24009	4.18	0.096
Shaking×Shaking	1	3.3323	3.3323	3.33231	58.00	0.001
Interaction	3	1.1177	1.1177	0.37257	6.49	0.036
Glucose×pH	1	0.4096	0.4096	0.40960	7.13	0.044
Glucose×Shaking	1	0.7056	0.7056	0.70560	12.28	0.017
pH×Shaking	1	0.0025	0.0025	0.00250	0.04	0.843
Residual Error	5	0.2872	0.2872	0.05745		
Lack-of-Fit	3	0.0473	0.0473	0.01575	0.13	0.933
Pure Error	2	0.2400	0.2400	0.12000		
Total	14	11.4028				

Source: The source of variation (e.g., Regression, specific factors, Error). DF: Degrees of freedom. Seq SS/Adj SS: Sequential and Adjusted Sums of Squares (measure of variation). Adj MS: Adjusted Mean Square (SS/DF), used to calculate the F-statistic. F: F-statistic (Adj MS term / Adj MS Residual Error), tests if the term is signifcant. P: P-value; a value below 0.05 (typically) indicates a statistically significant effect.

The analysis of variance (ANOVA) was conducted to evaluate the significance and adequacy of the developed quadratic model, and to understand the individual and interactive effects of the independent variables: Glucose concentration, pH, and shaking speed. The ANOVA for the quadratic model (Table 2) reveals a highly significant regression model, as evidenced by a high F-value of 21.50 and a very low probability value ($P = 0.002$). This indicates that the model is statistically significant at a 95% confidence level ($\alpha = 0.05$) and that the terms in the model have a substantial effect on the biodegradation efficiency. The model explains a high proportion of the variance in the data, with a high coefficient of determination (R^2) calculated as 0.9748 (from Seq SS Regression / Seq SS Total = 11.1155 / 11.4028). This implies that the model accounts for 97.48% of the total variation in the biodegradation response, demonstrating an excellent fit to the experimental data. Furthermore, the lack-of-fit test, which compares residual error to pure error, is non-significant ($P = 0.933$). A non-significant lack-of-fit is desirable as it suggests the model is well-fitted to the data and that any unexplained variation is likely due to random error rather than an inadequate model (Box et al. 2005; Montgomery 2017). Furthermore, the surface and contour plots (Figure 7) visually corroborate these complex interactions, allowing for the intuitive identification of regions where the biodegradation percentage is maximized. By analyzing the model's response surface, the optimal conditions for haloxyfop-*R*-methyl biodegradation were precisely determined to be 5.4 g/L glucose, pH 8, and 150 rpm shaking speed. The selection of pH 8 aligns with the model's positive linear and quadratic coefficients for pH, indicating a strong preference for alkaline conditions, which may favor the activity of specific hydrolytic enzymes or the growth of alkaline-tolerant degraders. The high optimal shaking speed of 150 rpm underscores the importance of oxygen transfer, which is essential for aerobic metabolism and the enzymatic breakdown of complex molecules (Liu et al. 2019). The moderate optimal glucose concentration of 5.4 g/L strikes a balance, providing necessary energy and co-metabolic support without inducing significant catabolite repression, as hinted at by the negative quadratic term. Validation under these optimized parameters resulted in 92.13% biodegradation within five days, a significant efficiency that confirms the robustness and predictive power of the developed model. This high degradation rate under optimized conditions demonstrates the potential of this microbial system for practical bioremediation applications.

3.4. Biodegradation of high doses of haloxyfop-*R*-methyl under optimal conditions

The time course of haloxyfop-*R*-methyl biodegradation by *M. circinelloides* YMM22 at three different initial concentrations (100, 300, and 500 mg/L) is presented in Figure 8. The fungus demonstrated a remarkable capacity to degrade the herbicide across all concentrations tested, though the degradation kinetics and final removal efficiency were significantly influenced by the initial pesticide load. At the lowest concentration of 100 mg/L, haloxyfop-*R*-methyl was rapidly and almost completely removed. The degradation occurred at the fastest rate, with the concentration dropping to near-undetectable levels within the first 7 to 10 days of the experiment. The degradation profile for the 300 mg/L concentration also showed a substantial decrease, following a similar rapid initial phase, though the time required to reach a very low residual concentration was slightly longer compared to the 100 mg/L treatment.

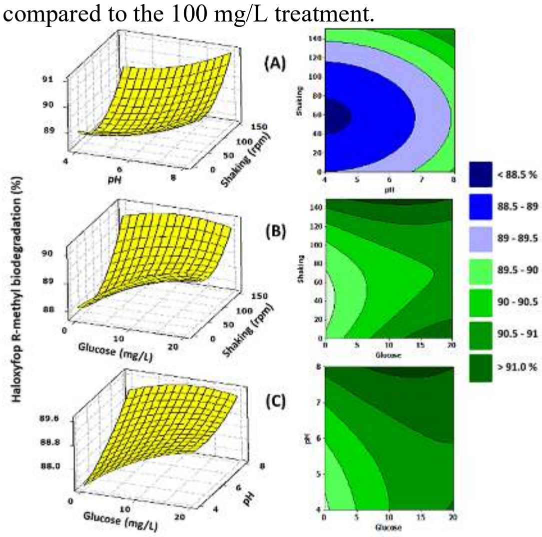

Figure 7. Surface and contour plots of independent variables affecting biodegradation (%) of haloxyfop-*R*-methyl by *Mucor circinelloides* YMM22 based on the Box-Behnken design. A: shaking and pH. B: shaking and glucose. C: pH and glucose.

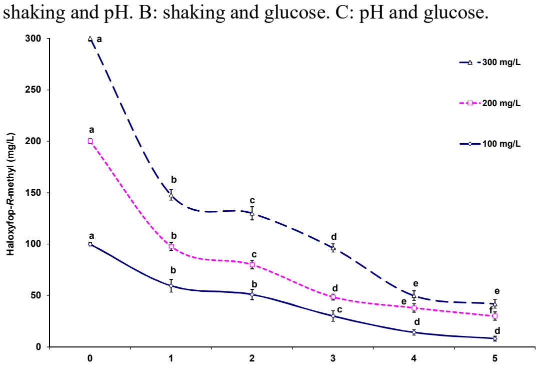

Figure 8. Time course of biodegradation of haloxyfop-*R*-methyl (100, 300, and 500 mg/L) by *M. circinelloides* YMM22 in an optimized medium at 28°C. Line values that do not share the same letter are significantly different according to the LSD test ($P \leq 0.05$).

In contrast, the degradation at the highest concentration of 500 mg/L exhibited a different pattern. While a significant amount of haloxyfop-*R*-methyl was ultimately removed, the initial rate of degradation was slower, and a small but statistically significant residual concentration persisted until the end of the incubation period. The LSD test ($P \leq 0.05$) confirmed that the degradation curves for the three concentrations were significantly different from one another, particularly during the mid to late phases of the experiment, as indicated by the differing letters on the plot lines.

The results clearly indicate that *M. circinelloides* YMM22 is a highly effective agent for the bioremediation of the herbicide haloxyfop-*R*-methyl. The efficient degradation observed, especially at lower concentrations, suggests that the fungus possesses a robust enzymatic machinery capable of metabolizing this compound. The rapid initial degradation phase across all concentrations is typical of microbial degradation processes, where the compound serves as a readily available carbon and energy source before the metabolic pathways become saturated or secondary metabolites accumulate (Alexander 1999). The inverse relationship between the initial haloxyfop-*R*-methyl concentration and the degradation rate/extension is a common phenomenon in pesticide biodegradation studies. This can be attributed to several factors. Firstly, high concentrations of pesticides can exhibit cytotoxic effects, potentially inhibiting microbial growth and enzymatic activity (Singh 2008). At 500 mg/L, HFM may have initially exerted a suppressive effect on the fungal biomass or the synthesis of specific catabolic enzymes, leading to the observed lag and slower degradation kinetics. Secondly, the slower degradation at higher concentrations could be due to the saturation of the microbial degradative capacity. The finite number of microbial cells or specific enzyme systems can only process a limited amount of substrate per unit of time (Bhandari et al. 2021; Singh 2008).

The near-complete removal of haloxyfop-*R*-methyl at 100-300 mg/L is a promising finding for bioremediation applications, as it falls within or above the range of concentrations that might be encountered in contaminated environments. The persistence of a high residual concentration at 500 mg/L is also noteworthy. This could be due to the formation of less degradable metabolites, the depletion of essential nutrients in the medium, or the induction of a dormancy phase in the fungus as the toxicant is reduced to a sub-inhibitory level (Fragoeiro and Magan 2008). The findings of this study align with previous research on fungal biodegradation of pesticides. For instance, various species of Trichoderma and Penicillium have shown similar concentration-dependent degradation profiles for other herbicides (Correa et al. 2021; Matúš et al. 2023; Parween et al. 2016). The efficacy of *M. circinelloides* YMM22 positions it as a strong candidate for the development of bioremediation strategies for sites contaminated with aryloxyphenoxypropionate herbicides.

4. Conclusion

This study successfully demonstrates the high efficacy of *M. circinelloides* YMM22 in degrading the herbicide haloxyfop-R-methyl. Under optimized cultural conditions, the fungus exhibited a remarkable capacity to remove the herbicide, achieving near-complete degradation at concentrations of 100 and 300 mg/L. The biodegradation process was found to be highly concentration-dependent, with lower initial concentrations resulting in faster and more extensive removal. Although a slightly slower degradation rate and a small residual concentration were observed at the highest tested level of 500 mg/L, the overall removal was still substantial, confirming the resilience and potency of the YMM22 strain. The ability of *M. circinelloides* YMM22 to tolerate and efficiently metabolize high concentrations of haloxyfop-R-methyl underscores its significant potential for application in the bioremediation of contaminated agricultural soils and wastewater. The concentration-dependent kinetics highlight the importance of inoculum density and environmental optimization for effective in-situ deployment. Future work should focus on elucidating the complete metabolic pathway of haloxyfop-R-methyl degradation by this fungus, identifying the key enzymes involved, and validating its efficacy in pilot-scale soil microcosm or bioreactor studies. Therefore, *M. circinelloides* YMM22 emerges as a promising and robust biological agent for the eco-friendly cleanup of haloxyfop-*R*-methyl pollution.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate

Not applicable.

Competing interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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