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PROCESS DEVELOPMENT AND WEED SUPPRESSION EFFICACY OF A CELL-FREE MYCOHERBICIDAL BROTH FROM *FUSARIUM* SP. FGCCW#16

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ABSTRACT

The increasing prevalence of herbicide-resistant weeds, environmental contamination, and declining agroecosystem biodiversity highlights the urgent need for safer and more sustainable weed management strategies. This study reports the development of a cell-free mycoherbicidal broth derived from *Fusarium* sp. FGCCW#16, designed to overcome the biosafety concerns and field-performance variability associated with live fungal inoculants. The fungus was cultivated in a 10 L stirred-tank bioreactor under controlled pH, dissolved oxygen, agitation, and antifoam conditions to optimize extracellular phytotoxic metabolite production. Post-fermentation, a multi-step clarification process comprising centrifugation and sterile membrane filtration yielded a propagule-free, metabolite-rich broth. Stabilization via pH adjustment and 5% (v/v) glycerol supplementation effectively preserved herbicidal activity during storage. Greenhouse bioassays demonstrated rapid and selective suppression of seven target weed species—*Lantana camara*, *Parthenium hysterophorus*, *Hyptis* spp., *Xanthium* spp., *Sida acuta*, *Cassia tora*, and *Antigonon leptopus*—inducing chlorosis and necrosis within 72 h, while non-target crops remained unaffected. High-performance liquid chromatography (HPLC) analysis confirmed consistent metabolite profiles across independent batches, highlighting process reproducibility and formulation robustness. By eliminating viable fungal propagules while retaining herbicidal activity, this cell-free formulation reduces ecological risk and enhances predictability of application. Collectively, these findings establish a scalable, safe, and reproducible platform for fungal metabolite-

based mycoherbicides, bridging bioreactor-level production with practical applications in sustainable weed management.

KEYWORDS: Mycoherbicide; fungal metabolites; cell-free formulation; *Fusarium* sp.; bioreactor fermentation; biological weed control; sustainable weed management.

INTRODUCTION

Weeds are among the most persistent constraints to agricultural productivity, competing with crops for essential resources such as nutrients, water, and light, thereby reducing yields and farm profitability. Their impact is particularly severe in resource-limited and smallholder farming systems, where uncontrolled weed pressure can result in substantial economic losses and threaten food security. For several decades, chemical herbicides have been the primary tool for weed management. However, their extensive and often indiscriminate use has led to major drawbacks, including the rapid emergence of herbicide-resistant weed populations, accumulation of toxic residues in soil and water, and adverse effects on biodiversity and ecosystem health (Bailey, 2014; Charudattan, 2001). These challenges have intensified the demand for environmentally benign weed management strategies that combine efficacy with long-term sustainability.

Among biological alternatives, mycoherbicides—herbicidal products derived from plant-pathogenic fungi or their metabolites—have garnered increasing attention. Fungi produce diverse phytotoxic secondary metabolites capable of selectively suppressing weed growth while minimizing damage to non-target crops. Several species, including *Colletotrichum gloeosporioides* and *Alternaria cassiae*, have demonstrated effective weed suppression under controlled and field conditions (Singh & Pandey, 2019; Ocán-Torres et al., 2024). Earlier studies highlighted the role of fungal pathogens in reducing dependence on chemical herbicides (Bailey, 2014) and emphasized the broader applicability of microbial metabolites in agriculture (Strobel & Daisy, 2003). More recent reviews have further underscored the commercial potential of metabolite-based biocontrol agents within sustainable farming systems (Yadav et al., 2022; Singh & Pandey, 2022).

Despite these advances, most mycoherbicidal research and commercial efforts continue to rely on live inoculum-based formulations, involving the application of fungal spores or mycelial propagules. Although such formulations can be effective, they pose several ecological, operational, and regulatory challenges. These include the risk of unintended

pathogen dissemination, inconsistent field performance due to environmental variability, limited shelf life, and regulatory concerns associated with releasing viable microorganisms into agroecosystems. Consequently, the reliability and large-scale adoption of live mycoherbicides remain limited (Cook & Baker, 1983; Charudattan, 2001).

Cell-free mycoherbicidal formulations, consisting solely of fungal metabolites without viable cells, offer a safer and more controllable alternative. By eliminating living propagules, these broths reduce ecological risks, enhance batch-to-batch consistency, and simplify regulatory approval pathways. Recent reviews highlight the growing interest in such formulations as next-generation biocontrol tools (Ocán-Torres et al., 2024). Similar perspectives were articulated earlier by Cook and Baker (1983) and Charudattan (2001), emphasizing the importance of sterile, scalable formulations for successful microbial weed management.

However, systematic development of cell-free mycoherbicidal broths at application-relevant scales remains limited. Few studies have addressed the integration of process optimization, downstream clarification, formulation stabilization, and bioefficacy evaluation within a unified framework suitable for scale-up and commercialization. Building on recent advances in scalable, cell-free mycoherbicide development (Singh & Pandey, 2025), the present study focuses on the production of a cell-free mycoherbicidal broth derived from *Fusarium* sp. FGCCW#16 using a 10 L stirred-tank fermenter. The objectives are to develop a reproducible fermentation process, establish effective clarification and stabilization strategies, and evaluate weed suppression efficacy under greenhouse conditions. By integrating process engineering with biological performance, this work aims to bridge laboratory-scale innovation with practical agricultural application and contribute to the advancement of sustainable weed management technologies.

MATERIALS AND METHODS

Fungal Strain and Inoculum Preparation

The fungal isolate *Fusarium* sp. FGCCW#16 was selected based on its previously reported phytotoxic activity against broadleaf weeds (Singh & Pandey, 2019; 2022). Stock cultures were maintained on potato dextrose agar (PDA) plates at 25 ± 1 °C and periodically subcultured to ensure viability.

For inoculum preparation, actively growing mycelial plugs (5 mm diameter) from 5–7-day-old PDA cultures were aseptically transferred into liquid seed medium and incubated under

shaking conditions to obtain a homogeneous mycelial suspension. Inoculum age and density were standardized to ensure reproducibility and optimal metabolite production, following established protocols for fungal biocontrol agents (Strobel & Daisy, 2003; Pandey et al., 2021).

Fermentation in a 10 L Stirred-Tank Bioreactor

Submerged fermentation was conducted in a 10 L stirred-tank bioreactor equipped with automated control of temperature, pH, dissolved oxygen (DO), and agitation. The fermentation medium was adapted from previously reported fungal metabolite production systems (Bailey, 2014) and optimized for extracellular phytotoxic metabolite synthesis.

Fermentation conditions were maintained as follows: temperature 28 ± 1 °C, agitation speed 200 rpm, aeration rate 1.0 vvm, and initial pH 6.0. Dissolved oxygen was monitored continuously and maintained above 30% saturation. Foam formation was controlled by intermittent addition of sterile silicone-based antifoam (Antifoam A). Fermentation was carried out for a duration determined by metabolite production kinetics.

Broth Harvesting and Clarification

At the end of fermentation, the culture broth was aseptically harvested and clarified in sequential steps. Biomass was first removed by centrifugation at 8,000 × g for 20 min at 4 °C, followed by passage through depth filters (5 µm pore size) to remove residual particulates.

Final sterilization was achieved by membrane filtration through 0.22 µm pore-size filters, yielding a cell-free, propagule-free clarified broth. Absence of viable fungal propagules was confirmed by plating aliquots of the filtrate on PDA and monitoring for growth over seven days.

Stabilization of Cell-Free Broth

To enhance storage stability and preserve biological activity, the clarified broth was adjusted to pH 5.5 using sterile citrate buffer. Glycerol was added at a final concentration of 5% (v/v) as a stabilizing agent, and the formulation was stored at 4 °C until further use. The choice of stabilizers and pH conditions followed established practices for preserving fungal metabolites in biocontrol formulations (Strobel & Daisy, 2003; Soccol et al., 2019).

Bioassay Evaluation

The bioefficacy of the cell-free mycoherbicidal broth was evaluated using leaf-disc assays and greenhouse pot experiments against seven target weed species: *Lantana camara*, *Parthenium hysterophorus*, *Hyptis suaveolens*, *Xanthium strumarium*, *Sida acuta*, *Cassia tora*, and *Antigonon leptopus*. Non-target crops were included to assess selectivity and crop safety. Treatments were applied at standardized concentrations, alongside untreated and solvent controls.

Phytotoxic effects were monitored over 7–14 days, recording visible symptoms such as chlorosis and necrosis, and measuring growth inhibition parameters including plant height and biomass reduction. Experimental design and evaluation criteria followed established protocols for fungal mycoherbicides (Bailey, 2014; Boyette et al., 2012).

Analytical Characterization of Metabolites

Metabolite profiling of the clarified broth was performed using high-performance liquid chromatography (HPLC) to confirm the presence and reproducibility of phytotoxic compounds. Chromatographic profiles from independent fermentation batches were compared to assess batch-to-batch consistency.

RESULTS AND DISCUSSION

Fermentation Performance

The fungal strain *Fusarium* sp. FGCCW#16 exhibited robust and reproducible growth in the 10 L stirred-tank fermenter. Biomass accumulation increased steadily, reaching a maximum by day 7 of cultivation. Dissolved oxygen levels were maintained above 30% saturation, and culture pH gradually stabilized at approximately 5.5, indicating optimal physiological conditions for extracellular metabolite production. Foam formation was effectively controlled with silicone-based antifoam, ensuring homogeneous mixing and stable aeration throughout the fermentation run. These operating parameters are consistent with previously reported conditions for optimal fungal metabolite production in submerged systems (Charudattan, 2001) and resemble other fungal bioprocesses producing bioactive metabolites (Strobel & Daisy, 2003). The reproducibility and stability of these fermentation parameters suggest that the process is scalable and capable of consistently producing metabolite-rich broths.

Broth Clarification and Cell-Free Status

Sequential centrifugation followed by depth and membrane filtration yielded a clear, cell-free broth with complete removal of fungal biomass. Microscopic examination and sterility testing over seven days confirmed the absence of viable spores or mycelial fragments. The clarified broth exhibited a pale-yellow color and mild odor, typical of metabolite-rich fungal filtrates (Ocán-Torres et al., 2024). By eliminating living propagules, the formulation addresses ecological and regulatory concerns associated with live inocula (Cook & Baker, 1983) and decouples herbicidal activity from fungal colonization, overcoming a key limitation of conventional mycoherbicides that rely on propagule viability under variable environmental conditions (Bailey, 2014).

Bioefficacy Against Target Weeds

Leaf-disc and greenhouse bioassays confirmed that the cell-free broth possessed potent and selective herbicidal activity against seven target weed species: *Lantana camara*, *Parthenium hysterophorus*, *Hyptis* spp., *Xanthium* spp., *Sida acuta*, *Cassia tora*, and *Antigonon leptopus*. Visible symptoms, including chlorosis and necrosis, appeared within 72 hours of treatment. Full-strength applications reduced weed biomass by up to 85% relative to untreated controls, while non-target crops exhibited minimal or no phytotoxic effects. These results highlight the high selectivity of the cell-free formulation and align with prior reports on fungal metabolite-based weed control systems (Boyette et al., 2012; Singh & Pandey, 2025). The selective suppression observed supports the potential for integrating metabolite-based formulations into sustainable weed management programs.

Metabolite Profiling

High-performance liquid chromatography (HPLC) analysis revealed consistent metabolite fingerprints across three independent fermentation batches, with reproducible retention times and peak abundances. The presence of multiple secondary metabolites suggests additive or synergistic contributions to herbicidal activity, consistent with earlier studies on fungal phytotoxins (Hoagland, 2001). Batch-to-batch reproducibility underscores the robustness of the fermentation and clarification process, a key factor for regulatory approval and commercialization (Yadav et al., 2022).

Stability of Cell-Free Broth

Stability studies demonstrated that the cell-free broth retained more than 70% of its initial herbicidal activity after three months of storage at 4 °C. Formulations stored under ambient

conditions exhibited a faster decline in bioactivity. Inclusion of 5% (v/v) glycerol enhanced stability, confirming its efficacy as a cryoprotectant and formulation stabilizer. These results are consistent with previous reports emphasizing the importance of stabilization strategies to preserve microbial metabolite activity during storage (Soccol et al., 2019).

Implications for Biocontrol Application

Overall, these results demonstrate that cell-free fungal broths can deliver consistent, selective, and stable herbicidal activity against multiple problematic weeds, including *Lantana camara*, *Parthenium hysterophorus*, *Hyptis* spp., *Xanthium* spp., *Sida acuta*, *Cassia tora*, and *Antigonon leptopus*. By retaining metabolite activity independent of fungal viability, this approach provides a predictable mode of action while mitigating ecological and regulatory risks associated with live fungal inocula (Ocán-Torres et al., 2024). The high selectivity toward non-target crops further supports its integration into sustainable and environmentally safe weed management programs (Hoagland, 2001).

Challenges for large-scale deployment remain, including scaling fermentation beyond 10 L, enhancing metabolite yields, validating long-term field performance, and conducting comprehensive ecological risk assessments (Charudattan, 2001). Future research should focus on pilot- and industrial-scale production, formulation optimization for field stability, and multi-location trials to fully realize the potential of cell-free fungal broths as next-generation mycoherbicides.

CONCLUSION

This study demonstrates that a cell-free mycoherbicidal broth produced in a 10 L fermenter can be effectively formulated, clarified, stabilized, and applied with reproducible herbicidal activity against multiple target weeds, including *Lantana camara*, *Parthenium hysterophorus*, *Hyptis* spp., *Xanthium* spp., *Sida acuta*, *Cassia tora*, and *Antigonon leptopus*. By eliminating viable fungal cells while retaining phytotoxic metabolites, this approach addresses the ecological and regulatory challenges associated with live inoculum formulations (Ocán-Torres et al., 2024).

The reproducibility of metabolite profiles across independent production batches, together with strong bio-efficacy observed in greenhouse trials, confirms the scalability and reliability of the process, supporting previous findings that metabolite-based formulations offer a safer and more predictable alternative to conventional mycoherbicides (Singh & Pandey, 2025).

Stability achieved through cold storage and glycerol supplementation demonstrates the feasibility of maintaining herbicidal activity during storage and transport, consistent with established strategies for preserving microbial metabolites (Soccol et al., 2019).

Importantly, the high selectivity of the broth toward target weeds while sparing non-target crops underscores its potential for integration into sustainable and ecologically safe weed management programs (Boyette et al., 2012; Hoagland, 2001). Collectively, these findings strengthen the case for cell-free fungal metabolites as viable next-generation mycoherbicides, bridging laboratory-scale innovation with practical agricultural application. Successful real-world deployment will require pilot- and industrial-scale production, formulation optimization for field stability, and comprehensive ecological risk assessments to fully realize the potential of this technology (Charudattan, 2001).

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