Int.J.Curr.Microbiol.App.Sci (2025) *14*(11): 86-95

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 14 Number 11 (2025)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2025.1411.010

Enzymatic and Antagonistic Characterization of a Soil-Derived *Aspergillus aflatoxiformans* from Madhubani, Bihar with Non-Phytotoxic Effect on Marigold

Pretty Kumari* and M. Singh

Department of Botany, Patna University, Patna 800005, Bihar, India

**Corresponding author*

Keywords

Aspergillus aflatoxiformis, Mucor irregularis, biocontrol, enzymatic activity, marigold, Madhubani soil.

Article Info

Received:
15 September 2025
Accepted:
28 October 2025
Available Online:
10 November 2025

A B S T R A C T

Aspergillus aflatoxiformans was isolated from the rhizosphere soil of Madhubani, Bihar, India, and identified through ITS sequencing and phylogenetic analysis (GenBank accession number: PV789347). Antagonistic activity against *Mucor irregularis* (GenBank accession no. PV789341) was evaluated on potato dextrose agar (PDA) using the dual culture technique, which showed a mean percentage inhibition of radial growth (PIRG) of 80. 9 ± 4. 08%. Chitinase activity was quantified by measuring the hydrolysis zone diameter in six replicate plates, with a mean value of 2. 99 ± 0. 21 cm and a coefficient of variation of 7. 12%. Protease activity showed a mean proteolytic zone diameter of 3. 34 ± 0. 37 cm (n = 9). For plant interaction assessment, a 5-day-old broth culture containing approximately 1 × 10^6 spores mL^{-1} was prepared and 10 ml volume applied as a foliar and root spray to *Tagetes erecta* (marigold) every 15 days during evening time. No significant differences were observed in treated plants compared to controls, indicating the absence of phytotoxic effects. These findings highlight A. aflatoxiformis from Madhubani as a potentially safe and enzymatically active antagonist against plant pathogens, supporting its further exploration as a biocontrol agent in sustainable agriculture.

Introduction

A. aflatoxiformans, part of the A. flavus complex, is known for aflatoxin production and biocontrol potential (Klich & Pitt, 2012). Aflatoxins are highly carcinogenic secondary metabolites that pose serious threats to food safety, particularly in tropical and subtropical regions where crops such as maize, groundnut, and sorghum are vulnerable to contamination (Alejandro Ortega-Beltran *et al.,* 2023). However, recent advances in microbial ecology and biotechnology have highlighted the utility of Non-aflatoxigenic strains suppress toxigenic fungi via niche displacement (Jung *et al.,* 2024).

In India, native soil fungi like A. *aflatoxiformans* remain underexplored. Enzymes such as chitinase and protease contribute to pathogen suppression (Wang *et al.,* 2024; Ornela & Guimarães, 2024). Plant interaction studies ensure ecological safety (Ortega-Beltran *et al.,* 2023; Ortega-Beltran *et al.,* 2024).

In this study, we report the isolation and molecular characterization of a native A. *aflatoxiformis* strain from agricultural soils of Madhubani. Molecular identification was confirmed via ITS sequencing and phylogenetic analysis, establishing its placement within the A. *flavus* clade.

To assess its biocontrol potential, we performed dual culture assays against phytopathogenic fungi, alongside enzymatic profiling (Suyan Wang *et al.,* 2024) for chitinase and protease activity. —key indicators of mycoparasitic and antagonistic capabilities (Ortega-Beltran *et al.,* 2024). Chitinases and proteases are hydrolytic enzymes that degrade fungal cell walls and proteins, contributing to the suppression of pathogenic fungi and improve soil health. Furthermore, to assess its ecological safety and plant compatibility, we investigated the effects of this isolate on the growth and development of *Tagetes erecta* (marigold), a representative horticultural crop. Such plant interaction studies are crucial to ensure that *Aspergillus aflatoxiformans* biocontrol agents do not exert phytotoxic effects or disrupt beneficial plant-microbe relationships (Ortega-Beltran *et al.,* 2023, Ortega-Beltran *et al.,* 2024). This integrative approach connect molecular taxonomy, functional assays, and plant interaction studies to explore the ecological and agricultural relevance of A. aflatoxiformis. The outcomes of this research contribute to the growing body of knowledge on native fungal resources with potential applications in aflatoxin alleviation, crop protection, and soil microbiome improvement. By characterizing indigenous strains, we aim to assist the development of safe, effective, and locally adapted biocontrol agents for sustainable agriculture.

Materials and Methods

Isolation and Identification of the Fungal Isolate

Rhizospheric soil samples were collected from agricultural fields of Madhubani, Bihar, India. The samples were serially diluted and plated on Potato Dextrose Agar (PDA) and incubated at 28 ± 2 °C for 5–7 days. Fungal colonies showing distinct morphology were sub-cultured and purified. Genomic DNA was extracted, the Internal Transcribed Spacer (ITS) region was amplified and sequenced for molecular identification. The obtained sequence was compared using BLAST (NCBI), and a phylogenetic tree was constructed in MEGA X software using the Neighbor-Joining method with 1000 bootstrap replications to confirm species identity as *Aspergillus aflatoxiformis* (Kumar *et al.,* 2018).

Dual Culture Antagonism Assay

Antagonistic potential of A. aflatoxiformis was evaluated against *Mucor irregularis* using the dual culture technique on PDA (Skidmore & Dickinson, 1976).

Detection of Chitinase Activity

Chitinase was detected using colloidal chitin agar (Monreal & Reese, 1969; Nawani & Kapadnis, 2001). Wells (5 mm) were made in solidified agar, inoculated with fungal culture, and incubated at 28–30 °C for 48–72 h. The diameter of the clear zone surrounding each well, indicating chitin hydrolysis, was measured. The mean hydrolysis zone diameter from six replicate plates (n = 6) was recorded.

Detection of Protease Activity

Protease activity was assessed using casein agar (Vishwanatha *et al.,* 2009). Fungal inocula were placed into wells and incubated at 28–30 °C for 48–72 h. Clear zones around the wells indicated casein hydrolysis, confirming protease production. The mean diameter of the proteolytic zones from nine replicates (n = 9) was recorded.

Phytotoxicity Assay on *Tagetes erecta*

Marigold plants (Tagetes erecta) were treated with spore suspensions following protocols for biocontrol safety (Amaike & Keller, 2011; Karthikeyan *et al.,* 2020). The isolate was grown on PDA for 7 days at 28 ± 2 °C. Conidial suspensions were prepared in sterile distilled water containing 0. 01% Tween 80, and spore density was adjusted to 1×10^6 spores mL^{-1} using a Neubauer haemocytometer. Ten milliliters of this suspension were applied to the rhizosphere of each plant. Treatments were administered twice at 15-day intervals during evening hours to minimize evaporation and ensure proper absorption. Control plants received sterile distilled water with Tween 80. Growth parameters, including shoot length, root length, leaf number, and overall vigor, were recorded periodically and compared with untreated controls to determine any phytotoxic symptoms.

Statistical Analysis

All experiments were performed in replicates, and data were expressed as mean ± standard deviation (SD). Mean, and SD were calculated for each parameter using Microsoft Excel. Data were graphically represented and compared to assess variability and reliability.

Results and Discussion

Colony Morphology and microscopic observation

Colony and conidial development were consistent with Aspergillus spp. (Klich & Pitt, 2012) (Figure 1).

Dual culture Assay

Antagonistic activity of *Aspergillus aflatoxiformans* (GenBank accession no. PV789347) against *Mucor irregularis* (GenBank accession no. PV789341) was assessed using the dual culture technique on potato dextrose agar (PDA). In control plates, *M. irregularis* exhibited uniform radial growth. However, in dual culture plates, a marked inhibition of *M. irregularis* was observed in the presence of *A. aflatoxiformans*. The mean percentage inhibition of radial growth (PIRG) was calculated to be 80. 9 ± 4. 08%, indicating strong antagonistic potential of *A. aflatoxiformans* under in vitro conditions.

Upper row: Control plates with Mucor irregularis (GenBank accession no. PV789341) exhibiting normal growth.

Lower row: Plates showing inhibited growth of *Mucor irregularis* in presence of *Aspergillus aflatoxiformans* (GenBank accession no. PV789347).

Chitinase and Protease Assays

Hydrolytic enzyme activity supports mycoparasitic potential (Meena *et al.,* 2017; Verma *et al.,* 2021).

In the same plate, the well inoculated with *Aspergillus aflatoxiformans* culture exhibited a distinct zone of clearance around the well, indicating active chitinase production by the fungal mycelium.

In the remaining two plates, all wells received culture filtrate of A. aflatoxiformans. Clear hydrolysis zones were observed around each well, confirming the presence of extracellular chitinase activity in the filtrate.

Molecular confirmation and phyloglenetic analysis

ITS sequencing and phylogenetic placement confirmed identity (Kumar *et al.,* 2018; Klich & Pitt, 2012).

Phylogenetic Placement of *Aspergillus aflatoxiformans*

Phytotoxicity Assessment

To evaluate the phytotoxic effects of *Aspergillus aflatoxiformans* on marigold (Tagetes spp.), plants were treated with spore suspensions (2×10^6 cells/mL; 10 mL per plant) applied to the root zone every 15 days. No adverse effects on marigold suggest compatibility (Pal & Gardener, 2006; Ortega-Beltran *et al.,* 2023).

The isolate's antagonism and enzyme profile align with known biocontrol fungi (Pal & Gardener, 2006; Verma *et al.,* 2021). Chitinase and protease disrupt fungal cell walls (Meena *et al.,* 2017; Wang *et al.,* 2024). Marigold tolerance may be due to antioxidant compounds (Karthikeyan *et al.,* 2020).

This study presents the successful isolation and characterization of *Aspergillus aflatoxiformis* from rhizospheric soil in Madhubani, Bihar, and explores its potential as a biocontrol agent through antagonistic and enzymatic activities. Despite its close taxonomic relationship to aflatoxin-producing members of the *Aspergillus flavus* complex (Klich & Pitt, 2012), A. aflatoxiformis remains largely understudied in terms of its ecological role and beneficial applications.

One of the most striking findings is its strong antagonism against *Mucor irregularis*, demonstrated by a mean PIRG of 80. 9% in dual culture assays. This level of inhibition suggests that A. aflatoxiformis can effectively suppress soil borne fungal pathogens. Similar antagonistic behavior has been documented in other Aspergillus species like A. niger, A. terreus, and A. flavus, which are known to produce extracellular hydrolytic enzymes and antifungal metabolites (Pal & Gardener, 2006; Verma, Kharwar, & Singh, 2021).

Figure. 1 On the third day of incubation, the colony exhibited a white, velvety texture with moderate aerial mycelium. By the fourth day, the colony began to develop green pigmentation centrally, indicating the onset of sporulation. The transition from white to green suggests active conidial formation typical of sporulating fungal species.

Figure. 2 Microscopic analysis of *Aspergillus aflatoxiformans* cultures stained with lactophenol cotton blue revealed on Day 3 culture (40× magnification): The fungal colony displayed actively proliferating hyphae with early signs of conidiophore formation. Hyphal filaments were septate, branched, and moderately dense. Conidial heads were in nascent stages, with limited spore formation.

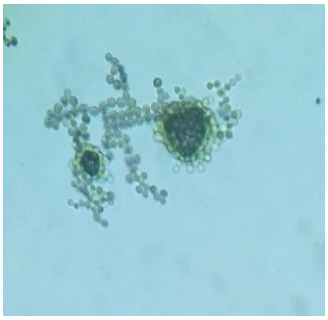


Figure. 3 Day 5 culture (40× magnification): A marked increase in conidiation was observed. Conidiophores were well-developed, terminating in globose to radiate conidial heads. Chains of conidia were distinctly visible, arranged in basipetal succession. Hyphae appeared more compact and mature, with enhanced septation and branching.

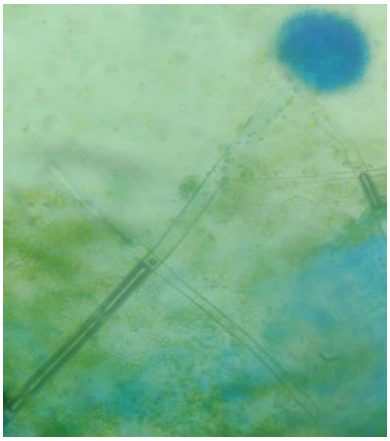


Figure. 4 Dual culture assay showing inhibition of *Mucor irregularis* by *Aspergillus aflatoxiformans*.

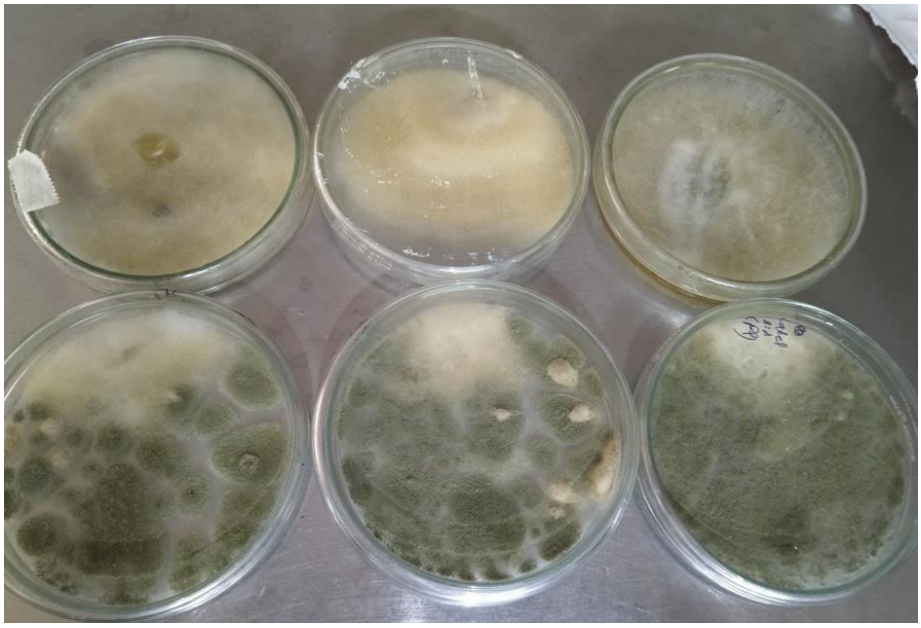


Figure. 5 Chitinase activity was assessed using agar plates supplemented with colloidal chitin and stained with Congo red show:Control well (first plate, left position well) showed no zone of clearance, confirming the absence of chitinase activity in the uninoculated medium.

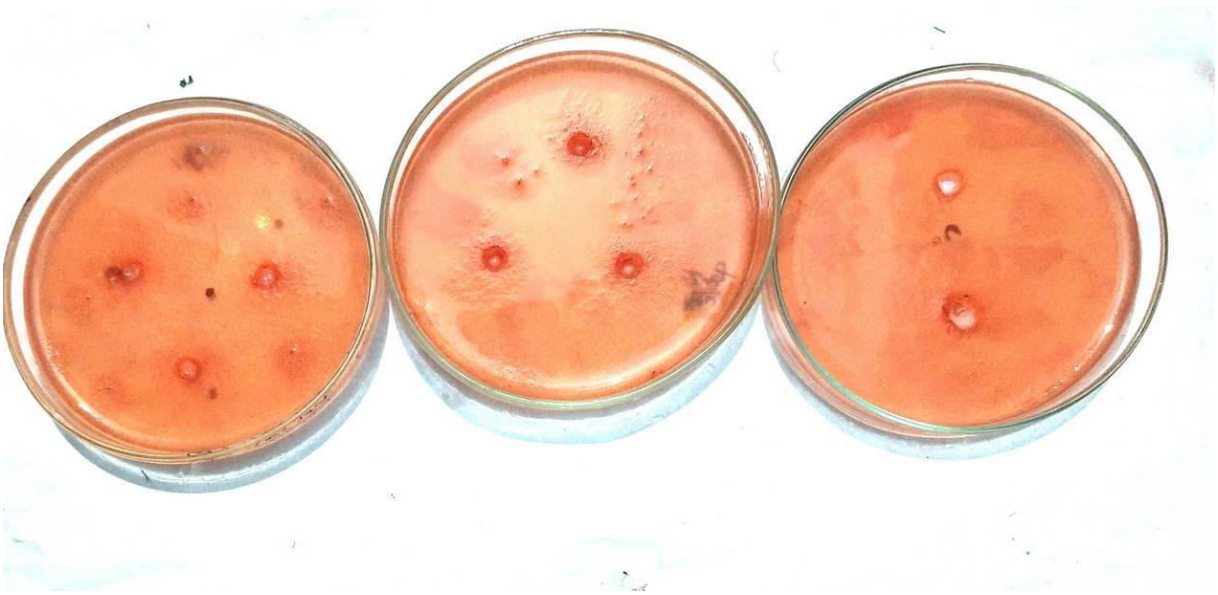


Figure. 6 Chitinase activity zones of *Aspergillus aflatoxiformis* measured from six replicate Petri dishes. Zone diameters were calculated as (length + width) /2 and plotted with standard deviation error bars. The mean diameter was 2. 99 ± 0. 21 cm, indicating moderate variability in enzymatic diffusion. X-axis: Zone numbers (Zone 1 to Zone 6) Y-axis: Zone diameters in centimeters. Bars: Represent individual zone diameters Error bars: ±0. 21 cm (standard deviation).

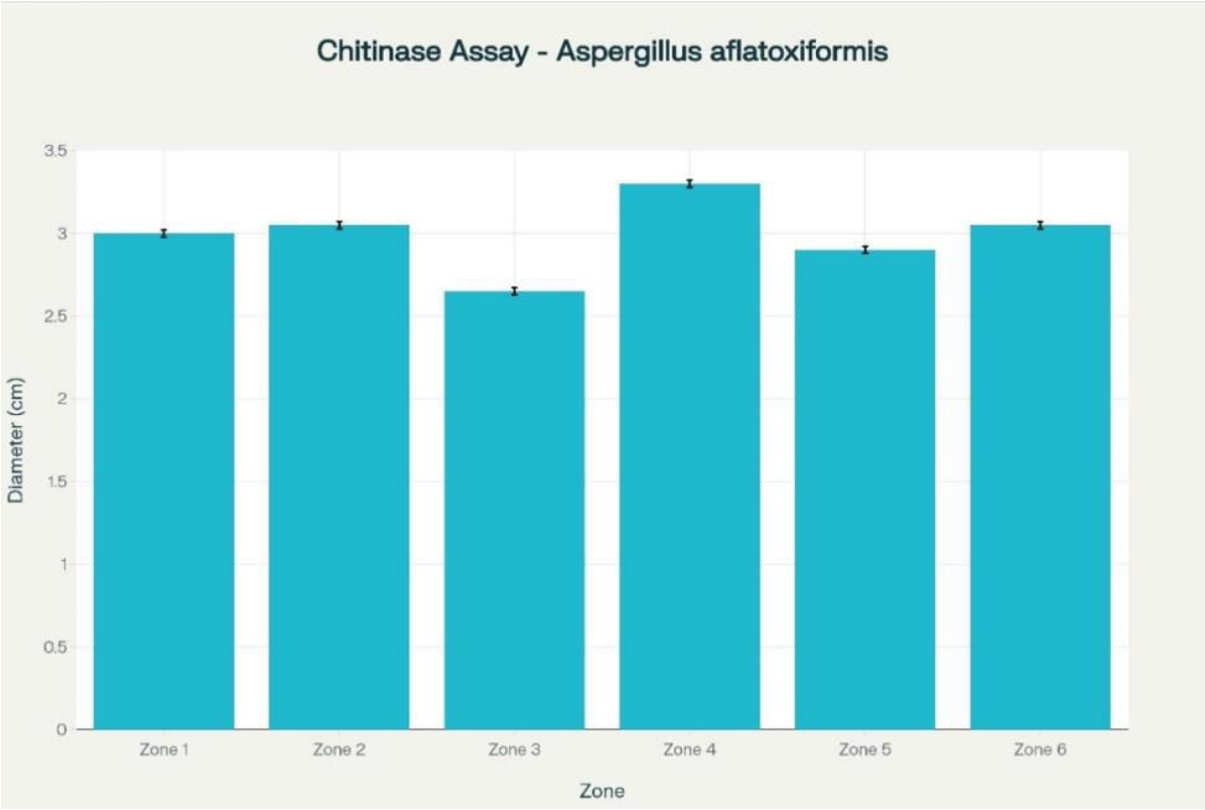


Figure. 7 Protease activity of *Aspergillus aflatoxiformans* on skim milk agar. Petri dishes 1–3 (top row and bottom left) show three clear hydrolytic zones each, indicating active protease secretion. The bottom-right dish represents the control, with no visible clearance zones. Mean zone diameter was 3. 34 ± 0. 37 cm across nine replicates (n = 9).

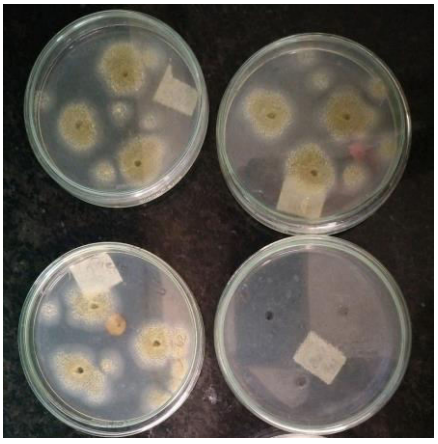


Figure. 8 Bar graph depicting mean proteolytic zone diameters (cm) formed by *Aspergillus aflatoxiformans* on skim milk agar. Each bar represents the average of three wells per Petri dish, with error bars indicating standard deviation (±0. 37 cm; n = 9).

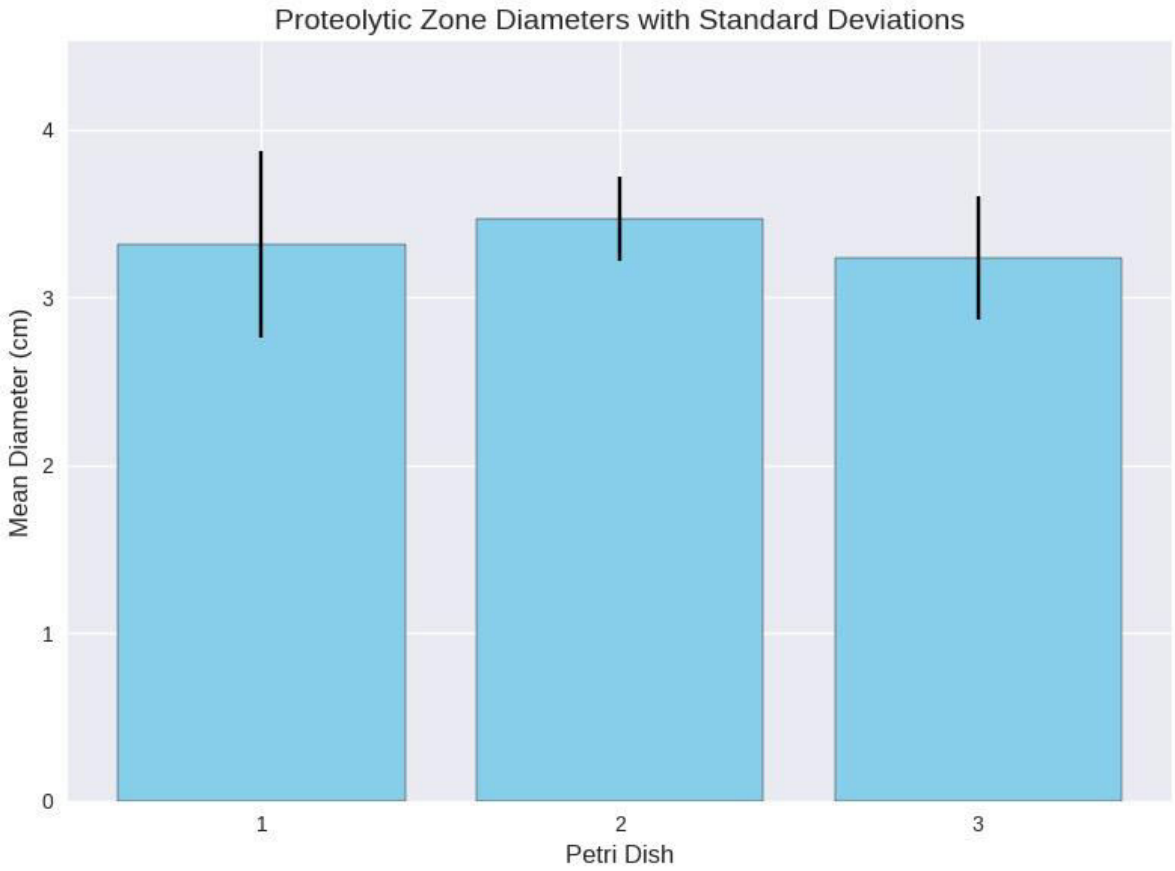
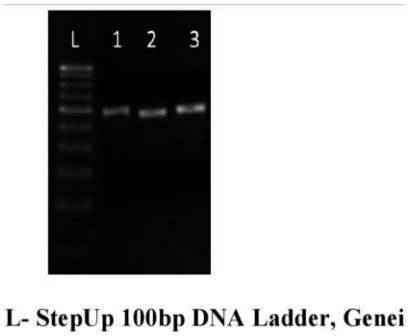


Figure. 9 Genomic DNA of the isolate corresponding to lane A1 was successfully extracted and visualized using agarose gel electrophoresis.

L- StepUp 1Kb DNA Ladder, Genei

Figure. 10 PCR amplification of the 16S/ITS region yielded distinct amplicons, Lane A1 confirming the presence of PCR product of isolate (*Aspergillus aflatoxiformans*).



L- StepUp 100bp DNA Ladder, Genei

Figure. 11 Phylogenetic tree based on ITS region sequences showing the placement of isolate A1 (ITS4RC ITS1 Seq85) within the *Aspergillus flavus* clade. The tree includes reference sequences from GenBank with accession numbers. *Penicillium citrinum* was used as an outgroup.

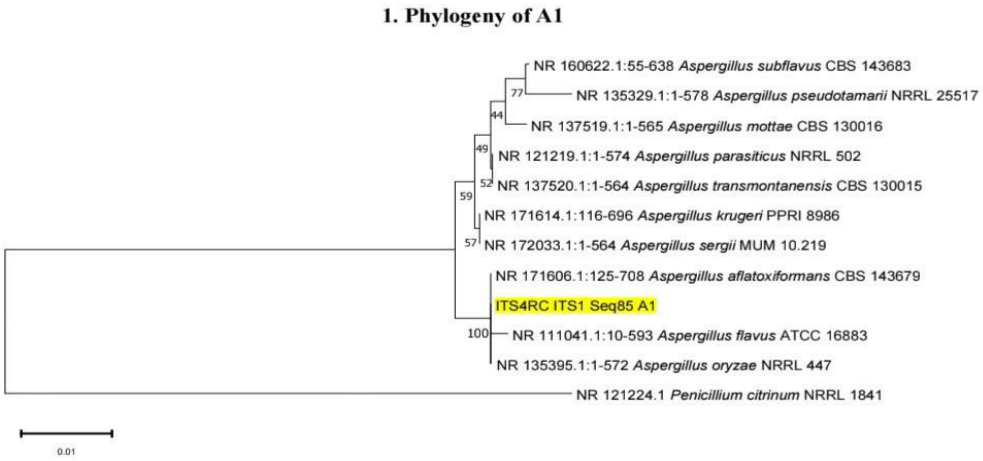


Figure. 12 The left panel displays untreated control marigold plants, while the right panel shows marigolds subjected to A. aflatoxiformans treatment.

The enzymatic profile of the isolate further supports its biocontrol potential. Notably, the production of chitinase (zone diameter: 2. 99 ± 0. 21 cm) and protease (zone diameters 3. 34 ± 0. 37 cm (n = 9) aligns with mechanisms commonly observed in mycoparasitic fungi. Chitinase targets the chitin-rich fungal cell wall, while protease breaks down proteinaceous structures, both contributing to pathogen lysis (Meena *et al.,* 2017). These activity are well-established for biocontrol agents like Trichoderma spp. and non-aflatoxigenic Aspergillus strains (Verma *et al.,* 2021).

Equally encouraging is the absence of phytotoxic effects on *Tagetes erecta* (marigold) following repeated spore applications. The treated plants showed no signs of stress or growth inhibition, suggesting that the isolate is either *non-aflatoxigenic* or produces negligible toxin levels. Marigolds, known for their antioxidant-rich thiophenes and flavonoids, may also play a role in mitigating any potential fungal stress. This compatibility underscores the strain's safety for use in ornamental horticulture.

Taken together, these findings position A. aflatoxiformis as a promising candidate for biocontrol applications—combining potent antifungal activity with plant safety. However, before advancing to large-scale deployment, it is critical to confirm its non-aflatoxigenic status through molecular screening of aflatoxin biosynthesis genes (e. g., aflR, aflD, omtA) and quantitative toxin assays (Amaike & Keller, 2011; Karthikeyan, Sankaralingam, & Kalaiselvi, 2020).

In conclusion, this study demonstrates that the isolated strain of *Aspergillus aflatoxiformis* from Madhubani, Bihar, exhibits strong antagonistic and enzymatic activity while remaining non-phytotoxic to marigold (*Tagetes erecta*). Such characteristics suggest its potential application in the management of diseases affecting ornamental plants, particularly marigold and other related species. Further studies on its genetic traits could enhance its utility through genetic engineering approaches and field trials enabling its broader use in sustainable disease control of diverse plant pathogens without compromising plant health.

Author Contributions

Conceptualization, P. K.; methodology, P. K.; investigation, P. K.; writing—original draft, P. K.; reviews and editing, P. K. and M. S.; supervision, M. S.

Funding

Supported by university Grants commission (UGC), India, through Junior research fellowship [Ref ID: 1052/CSIR-UGC NET June 2019].

Data Availability

ITS sequence deposited in NCBI GenBank (PV789344). Additional data available upon request.

Acknowledgements

Thanks to Patna University's Department of Botany, Dr. Meenakshi Singh, Dr. Birendra Prasad, and lab members. Gratitude to UGC for JRF support.

Conflicts of Interest

None declared.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

References

Amaike, S. and Keller, N.P., 2011. Aspergillus flavus. Annual Review of Phytopathology, 49, pp.107–133. https://doi.org/10.1146/annurev-phyto-072910-095221

Jung, K.S., Kim, H.M., Lee, J., Ganbat, D. and Lee, S.E., 2024. Biocontrol of aflatoxin-producing Aspergillus flavus ATCC 22546 by a non-aflatoxigenic Aspergillus flavus ATCC 9643. Applied Sciences, 14(14), p.6142. https://doi.org/10.3390/app1414612

Karthikeyan, V., Sankaralingam, S. and Kalaiselvi, N., 2020. Evaluation of non-aflatoxigenic Aspergillus flavus as biocontrol agents against toxigenic strains in groundnut. Biological Control, 147, p.104289. https://doi.org/10.1016/j.biocontrol.2020.104289

Klich, M.A. and Pitt, J.I., 2012. Differentiation of Aspergillus flavus from A. parasiticus and other closely related species. International Journal of Food Microbiology, 156(3), pp.236–241. https://doi.org/10.1016/j.ijfoodmicro.2012.04.015

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35(6), pp.1547–1549. https://doi.org/10.1093/molbev/msy096

Meena, M., Gupta, S.K., Swapnil, P., Zehra, A., Dubey, M.K. and Upadhyay, R.S., 2017. Alternaria toxins: Potential virulence factors and genes related to pathogenesis. Frontiers in Microbiology, 8, p.1451. https://doi.org/10.3389/fmicb.2017.01451

Monreal, J. and Reese, E.T., 1969. The chitinase of Serratia marcescens. Canadian Journal of Microbiology, 15(6), pp.689–696. https://doi.org/10.1139/m69-123

Nawani, N.N. and Kapadnis, B.P., 2001. Chitinase production by Streptomyces sp. NK1057. Brazilian Journal of Microbiology, 32(4), pp.295–297. https://doi.org/10.1590/S1517-83822001000400003

Ornela, P.H. and Guimarães, L.H.S., 2024. Purification, characterization and antifungal activity of the Aspergillus niveus chitinase produced using shrimp shells. Applied Biosciences, 3(2), pp.220–232.

Ortega-Beltran, A. and Bandyopadhyay, R., 2023. Aflatoxin biocontrol in practice requires a multidisciplinary, long-term approach. Frontiers in Sustainable Food Systems, 7, p.1110964. https://doi.org/10.3389/fsufs.2023.1110964

Ortega-Beltran, A., Aikore, M.S., Kaptoge, L., Agbetiameh, D., Moral, J. and Bandyopadhyay, R., 2024. Impact of storage conditions on the shelf life of aflatoxin biocontrol products containing atoxigenic isolates of Aspergillus flavus as active ingredient applied in various countries in Africa. CABI Agriculture and Bioscience, 5(1), p.78. https://doi.org/10.1186/s43170-024-00178-7

Pal, K.K. and Gardener, B.M., 2006. Biological control of plant pathogens. The Plant Health Instructor. https://doi.org/10.1094/PHI-A-2006-1117-02

Skidmore, A.M. and Dickinson, C.H., 1976. Colony interactions and hyphal interference between Septoria nodorum and phylloplane fungi. Transactions of the British Mycological Society, 66(1), pp.57–64. https://doi.org/10.1016/S0007-1536(76)80008-5

Verma, R.K., Kharwar, R.N. and Singh, R.P., 2021. Enzymatic and antifungal potential of Aspergillus niger against soil-borne pathogens. Archives of Phytopathology and Plant Protection, 54(1–2), pp.1–12. https://doi.org/10.1080/03235408.2020.1865791

Vishwanatha, K.S., Rao, A.G.A. and Singh, S.A., 2009. Acid protease production by solid-state fermentation using Aspergillus oryzae MTCC 5341: Optimization of process parameters. Journal of Industrial Microbiology & Biotechnology, 36, pp.129–139. https://doi.org/10.1007/s10295-008-0485-5

Wang, S., Wang, Y., Shi, X., Herrera-Balandrano, D.D., Chen, X., Liu, F. and Laborda, P., 2024. Application and antagonistic mechanisms of atoxigenic Aspergillus strains for the management of fungal plant diseases. Applied and Environmental Microbiology, 90(10), p.e01085-24. https://doi.org/10.1128/aem.01085-24

How to cite this article:

Pretty Kumari and Singh M. 2025. Enzymatic and Antagonistic Characterization of a Soil-Derived *Aspergillus aflatoxiformans* from Madhubani, Bihar with Non-Phytotoxic Effect on Marigold. *Int.J.Curr.Microbiol.App.Sci.* 14(11): 86-95. **doi:** https://doi.org/10.20546/ijcmas.2025.1411.010