

Dynamic temperature regimes induce sporulation of *Trichoderma harzianum* AMUTH-1 on diverse substrates for bioformulation development

Ziaul Haque*, Qasid Ali, Ruman Ahmad, Vaibhav Pathak, Mohd Shahid Anwer Ansari and Naved Azum

ABSTRACT

This study evaluated the influence of temperature (5–40 °C) on the sporulation of *Trichoderma harzianum* AMUTH-1 across five substrates (mustard, sorghum, finger millet, rice, and wheat) to optimise their application in solid-state fermentation-based bioformulation development. AMUTH-1 successfully colonised all substrates, though the extent of growth varied from minimal to complete coverage. Sporulation levels differed significantly among substrates; rice supported the highest sporulation, with a mean log₁₀ value of 7.55 CFU/g substrate at 20–30 °C, followed by sorghum, wheat, mustard, and finger millet. Temperature was identified as a critical determinant of fungal spore performance, with optimal sporulation observed at 25 °C. In contrast, extremely low (≤ 15 °C) and high (≥ 35 °C) temperatures markedly suppressed conidial production. Notably, a temperature downshift (30 $\rightarrow$ 20 °C) significantly enhanced sporulation relative to static incubation, indicating that dynamic temperature regimes can stimulate conidial yield more effectively than constant conditions. Collectively, these findings underscore the importance of substrate selection and temperature management in the development of robust *Trichoderma*-based biocontrol formulations.

Keywords: Biocontrol, conidiation, mustard, optimisation, plant disease, rice, wheat

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INTRODUCTION

The use of fungi as biocontrol agents has gained considerable attention in recent years as an effective strategy for plant disease management (Mukherjee *et al.*, 2019; Kumar *et al.*, 2023; Haque *et al.*, 2024). Among these, *Trichoderma* species are well-documented for their ability to protect agricultural and horticultural crops against diverse plant pathogens (Ghazanfar *et al.*, 2018; Khan *et al.*, 2020; Haque *et al.*, 2024). Their antagonistic activity is mediated through multiple mechanisms, including antibiosis, competition, cross-protection, hyperparasitism, induced resistance, and direct predation (Khan and Mohiddin, 2018). Several *Trichoderma* species, notably *T. asperellum*, *T. hamatum*, *T. harzianum*, and *T. viride*, have demonstrated

strong efficacy against pathogens such as *Alternaria solani* and *Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *ciceris*, *Macrophomina phaseolina*, *Meloidogyne* spp., *Pythium aphanidermatum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Pradhan *et al.*, 2022; Haque *et al.*, 2023; Swathy *et al.*, 2024). Despite extensive literature on *Trichoderma* and its bioformulations (Mbarga *et al.*, 2014; Intana *et al.*, 2023; Haque *et al.*, 2025b), two major bottlenecks hinder its large-scale application under field conditions: (i) challenges in mass production, particularly spore (conidial) yield with minimal contamination, and (ii) the development of ideal bioformulations that are cost-effective, easy to apply, contain high spore

loads, maintain viability, and exhibit long-term storage stability (Martinez *et al.*, 2022; Haque *et al.*, 2025c). Optimisation of sporulation under solid-state fermentation remains underexplored, particularly with respect to substrate type and temperature (Steyaert *et al.*, 2010; Maurya *et al.*, 2024). Previous studies have highlighted the importance of temperature in spore density of *Trichoderma* (Zhang and Yang 2015; Sachdev *et al.*, 2018; Maurya *et al.*, 2024). Previously, Mulatu *et al.* (2021) reported that the optimum temperature range for rapid growth of *Trichoderma* spp. was 25–30 °C, reflecting the genus mesophilic nature. However, Mohiddin *et al.* (2017) observed a contrasting trend, in which 10 °C supported the maximum growth of *T. harzianum* compared with 20 and 30 °C. These findings underscore the need for systematic evaluation of temperature conditions to enhance spore yield during mass production.

In this context, the present study investigated the sporulation performance of *Trichoderma harzianum* AMUTH-1 under varying temperature conditions (5–40 °C) across five substrates, viz., finger millet, mustard, sorghum, rice and wheat, using a multifactorial approach under solid-state fermentation. Pure conidial production via solid-state fermentation offers several advantages, including reduced risk of inoculum contamination during storage and application, enhanced metabolic activity of the formulation, and minimised off-site drift during field use. This work aims to establish optimised conditions for robust bioformulation development of *Trichoderma*-based biocontrol agents.

MATERIALS AND METHODS

Isolate

Trichoderma harzianum isolate AMUTH-1 (NCBI GenBank Accession no. KM435269; NAIMCC-India depository Accession no. NAIMCC-F-04335) was selected for this study because of its multifaceted nature and biocontrol capability against various plant pathogens (Haque *et al.*, 2024). For the mass production of conidia (spores) of *T. harzianum* AMUTH-1, solid-state fermentation technology was used as described by Haque *et al.* (2025a). The *T. harzianum* sporulation on different substrates was assessed by mixing 1 g of colonised

substrate with 9 mL of distilled water, agitating at 150 rpm in a rotary shaker at room temperature for 1 hr. The suspension was filtered through two layers of muslin cloth, and the spore count was determined using a Neubauer Haemocytometer as spores/g of substrate and presented as a log₁₀ value.

Sporulation of *T. harzianum* AMUTH-1

A multifactorial experiment was carried out to optimise the sporulation of *T. harzianum* AMUTH-1 using the solid-state fermentation method at different temperatures (5, 10, 15, 20, 25, 30, 35, 40, 20→30, and 30→20 °C) on finger millet, mustard, sorghum, rice, and wheat. The flasks containing 100 g of substrates with 13–15% moisture content were humidified by adding 45–47 mL of distilled water, bringing the moisture content to 60%. The substrates in the flasks were autoclaved at 15 psi (121.6°C) for 20 minutes and inoculated with 5 mm discs from 7-day-old cultures of *T. harzianum* AMUTH-1. Then the flasks were placed in a biological oxygen demand (BOD) incubator at the designated temperature. The dynamic temperature shift occurred at 10 days of incubation. The sporulation of *T. harzianum* AMUTH-1 was assessed after 25 days of incubation. All the above treatments were replicated three times.

Spore viability test

At the end of each treatment, spore viability of the harvested conidia was determined by the procedure described by Cavalcante *et al.* (2025). Viable and active spores were defined as those that germinated and developed a germ tube. Non-viable spores were those that did not develop germ tubes and showed no changes in size or morphology.

Data analysis

The study was repeated to assess reproducibility, and the observations from six replicates were used to calculate means in Microsoft Excel 2016. Data were analysed by analysis of variance (ANOVA) using the R Program at $P \leq 0.05$, 0.01 and 0.001 levels. ANOVA data are not presented, as graphs were prepared. The data on the effect of temperature on individual substrates were presented as a scatter plot in Origin Pro. To show how sporulation of *T. harzianum* AMUTH-

1 varies with combinations of temperature and substrates, a 3D surface plot was created using Origin Pro software.

RESULTS

Temperature conditions on individual substrates for the sporulation of *T. harzianum* AMUTH-1

The results showed that temperature and substrate strongly influenced the sporulation of *T. harzianum* during solid-state fermentation. Among the five substrates tested, *T. harzianum* AMUTH-1 colonised all substrates, with growth ranging from scant to complete coverage.

Initially, the substrates were white due to mycelial development. The colour gradually changed, and later various shades of green were observed among the substrates (Fig. 1). However, the level of sporulation was significantly different among the substrates and temperature conditions (Fig. 1). The highest sporulation of *T. harzianum* AMUTH-1 occurred

in rice with mean $\log_{10}$ value of 7.55 CFU/g substrate at 20-30 °C (Fig. 2C). Sorghum exhibited the next highest colonization and sporulation (Fig. 2B), followed by wheat with $\log_{10}$ value of 5.92 CFU/g (Fig. 2C). The pattern of sporulation declined on mustard (Fig. 2E) and trailed by finger millet ($\log_{10}$ = 3.57 CFU/g, Fig. 2D), which exhibited the least fungal population with low colonization and sporulation (Fig. 1). Higher temperatures (>35 °C) suppressed sporulation, while extremely low temperatures (<15 °C) inhibited growth altogether across substrates. Notably, a temperature downshift (30 → 20 °C) or upshift (20 → 30 °C) significantly enhanced sporulation compared to static temperature incubation, indicating that dynamic temperature regimes can stimulate conidial yield more effectively than constant conditions (Fig. 1A-E).

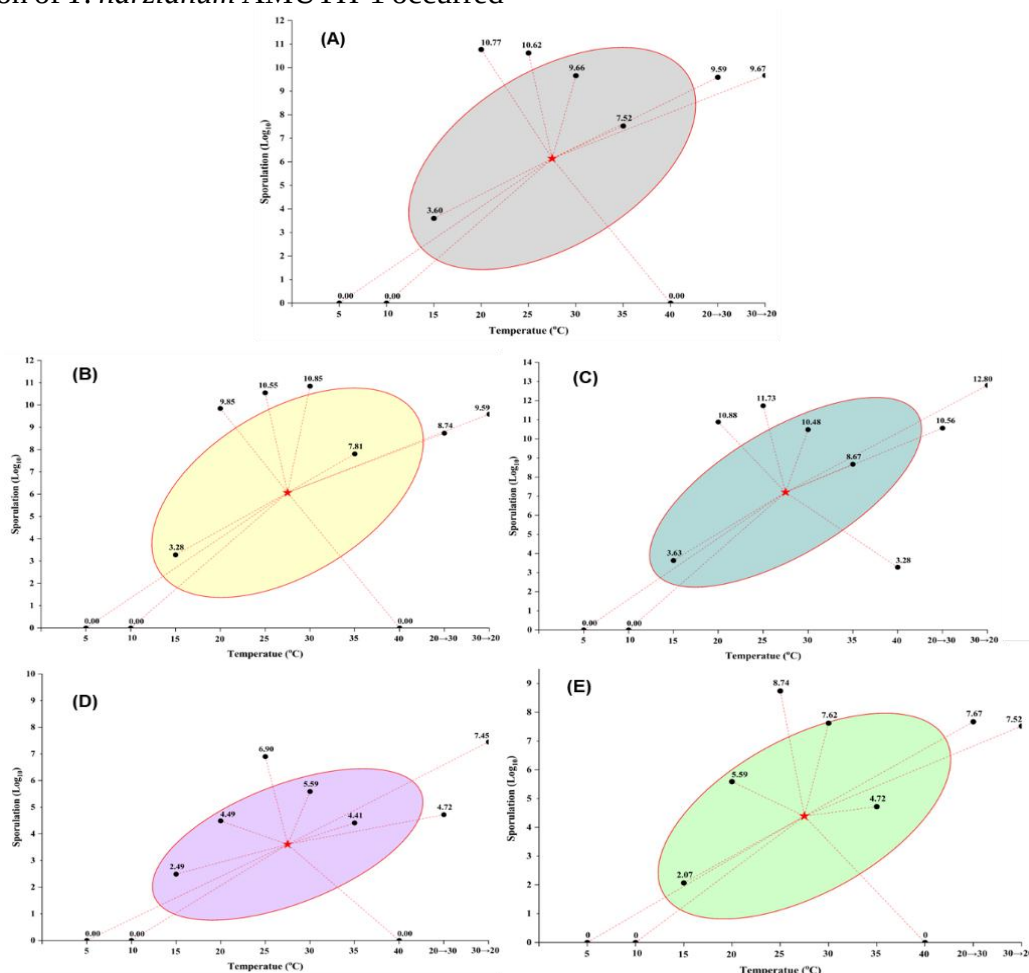


Figure 1. Scatter dot plot showing the effect of temperature levels on sporulation of *Trichoderma harzianum* AMUTH-1 on sorghum (A), wheat (B), rice (C), finger millet (D) and mustard (E) under solid-state fermentation after 25 days of incubation.

Combined impact of temperature and substrates

The 3D surface plot showed that sporulation increased gradually with temperature from 10°C and reached an optimum around 25–30°C across most substrates (Fig. 2). At low temperature (10–15 °C), sporulation was minimal, whereas a

sharp decline was observed at 40°C (Fig. 3). Among substrates, rice supported the highest sporulation, followed by wheat, sorghum and finger millet (ragi), while mustard showed comparatively lower sporulation. The temperature changes (20→30°C and 30→20°C) also led to enhanced sporulation (Fig. 2).

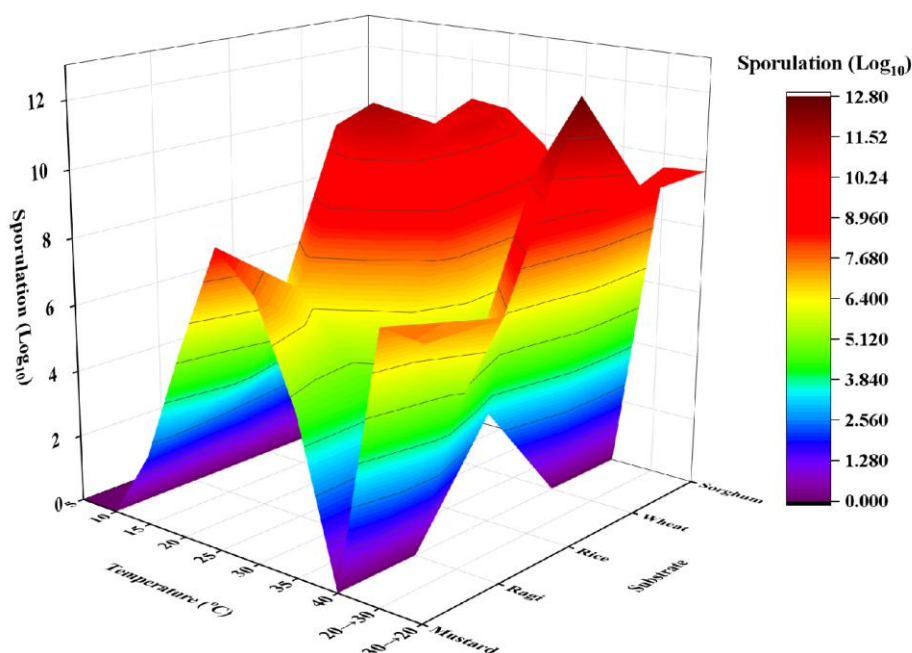


Figure 2. 3D surface plot showing the combined effect of temperature and substrates (finger millet/ragi, mustard, sorghum, rice and wheat) on sporulation of *Trichoderma harzianum* AMUTH-1 under solid-state fermentation after 25 days of incubation. The colour gradient and surface height indicate the level of sporulation, where warmer colours (yellow to red) and higher peaks represent greater spore production, as measured in log₁₀ values.

Overall, the plot demonstrates that sporulation is strongly influenced by both substrate type and temperature, with maximum production occurring on rice/wheat at around 25–30°C, representing ideal conditions for fungal multiplication (Fig. 2). Statistical analysis using two-way ANOVA showed significant differences among treatments. The calculated p-value was less than 0.001, indicating that temperature and substrate significantly influenced sporulation at $P \leq 0.001$, and the F-value was also significant at $P \leq 0.001$.

Spore viability

The spore viability tests revealed that temperature regimes and substrate types did not significantly affect conidial viability, and conidia remained viable at the time of harvesting across all test regimes except the very low (5–10 °C) and high-moisture (40 °C) regimes (Table 1).

The moderate temperature (20–30 °C) not only supports sporulation but also maintains spore viability (Supplementary Fig. S1). Extreme temperature led to desiccation and no spore formation, while very low temperature caused dormant spore formation (Table 1).

DISCUSSION

Trichoderma harzianum has proven to be an effective natural biocontrol agent against several fungal pathogens in various crops (Pani *et al.*, 2021; Xiao *et al.*, 2023). The present study demonstrates that both substrate type and temperature regimes are critical determinants of *T. harzianum* AMUTH-1 colonisation and sporulation. The results highlight clear substrate-specific preferences, with rice consistently supporting prolific sporulation across a wide temperature range. Wheat, sorghum and mustard also supported sporulation across the

temperature range, but their performance declined at higher or lower temperatures. This pattern indicates sensitivity to extreme temperatures, likely due to effects on active metabolic pathways (e.g., those involved in conidiophore development). At low temperatures, enzyme activity slows, reducing energy flow and biosynthesis. At high temperatures, enzymes may denature, halting sporulation altogether (Sachdev *et al.*, 2018). Furthermore, fungal cell membranes require a certain degree of fluidity to transport nutrients and signals. Cold stiffens membranes, while heat makes them too fluid, disrupting normal cellular processes (Cavalcante *et al.* 2025).

Table 1. Effect of pH, moisture, temperature and incubation period on spore viability of *Trichoderma harzianum* under solid-state fermentation.

Temperature (°C)	Spore Viability				
	Rice	Sorghum	Wheat	Mustard	Finger millet
5	–	–	–	–	–
10	+	+	–	–	–
15	+	+	+	–	+
20	+	+	+	+	+
25	+	+	+	+	+
30	+	+	+	+	+
35	+	+	+	+	+
40	–	–	–	–	–
20→30	+	+	+	+	+
30→20	+	+	+	+	+

+ indicates no change in spore viability, – indicates no sporulation or more than 10% decrease in spore viability.

Rini and Sulochana (2007) also reported that *T. harzianum* and *T. viride* achieved maximum spore density at $28 \pm 2^\circ\text{C}$ after 30 days of incubation, but on different substrates such as coir pith combined with neem cake, and mixtures of cow dung, neem cake, and wheat flour. The recovery at saturation may reflect adaptive sporulation under stress, but the overall inconsistency suggests that these substrates are best suited for controlled temperature regimes rather than field conditions with fluctuating weather (Pang *et al.*, 2006). This finding underscores the importance of substrate selection in biocontrol formulations, as unsuitable carriers can compromise inoculum quality and field

efficacy (Cavalcante *et al.* 2025).

Sporulation of *T. harzianum* AMUTH-1 was maximized at 25°C , consistent with the mesophilic nature of *Trichoderma* spp. (Harman *et al.*, 2004; Srivastava *et al.*, 2021). Higher temperatures ($>35^\circ\text{C}$) suppressed sporulation, while extremely low temperatures ($<15^\circ\text{C}$) inhibited growth altogether. This result is consistent with Mulatu *et al.* (2021), who reported that the optimal temperature range for rapid growth of *Trichoderma* spp. was $25\text{--}30^\circ\text{C}$, reflecting the genus's mesophilic nature. However, Mohiddin *et al.* (2017) observed a contrasting trend, in which 10°C supported the maximum growth of *T. harzianum* compared with 20 and 30°C . Such discrepancies may be attributed to moisture levels and nutrient availability. Notably, in our study, the temperature downshift ($30 \rightarrow 20^\circ\text{C}$) produced the highest sporulation overall, suggesting that dynamic temperature changes may act as stress signals, triggering enhanced sporulation. Similar stress-induced sporulation has been reported in other fungi, in which environmental fluctuations stimulate the formation of reproductive structures as a survival mechanism (Su *et al.*, 2012; Sachdev *et al.*, 2018). This novel observation offers a potential strategy to improve conidial yield in industrial solid-state fermentation.

From a practical standpoint, these results have direct implications for biopesticide production and deployment. Rice emerges as the most reliable substrate for large-scale multiplication, particularly under optimum temperature conditions ($20\text{--}30^\circ\text{C}$), which is common in tropical and subtropical climates. Wheat and sorghum may be used under controlled fermentation systems where temperature can be optimized, while ragi appears unsuitable for commercial application. Furthermore, the novel finding of enhanced sporulation under a temperature downshift ($30 \rightarrow 20^\circ\text{C}$) at 10 days of incubation, further boosting yield, adds new insight into stress-induced conidiation and offers practical strategies for industrial-scale production. These results provide a framework for designing fermentation protocols that maximize conidial yield, ensuring cost-effective

and scalable production of *T. harzianum*-based biopesticides for sustainable agriculture.

AUTHORS CONTRIBUTIONS

Z. Haque conceived the idea, developed the research facility, supervised the execution and data recording and prepared the original draft. Q. Ali, V. Pathak and R. Ahmad collected the data and executed the experiments. M.S.A. Ansari helped in the execution of the experiments, performed the word processing and data analysis. N. Azum performed data analysis and reviewed the manuscript. All authors read and approved the final manuscript.

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