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Antagonistic activity and hydrolytic enzyme production by endophytic *Trichoderma asperelloides* for the biological control of soilborne phytopathogens

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Abstract

Trichoderma asperelloides, a foliar endophytic fungus, was evaluated for its antagonistic potential against soil-borne phytopathogens and its ability to produce hydrolytic enzymes, aiming for biotechnological applications in sustainable plant disease management. Dual-culture assays demonstrated significant inhibition of mycelial growth in *Fusarium oxysporum* (66%), *Rhizoctonia solani* (89%), *Macrophomina phaseolina* (55%), and *Sclerotinia sclerotiorum* (90%), with interactions primarily characterized as direct mycelial contact inhibition. Notably, *T. asperelloides* outperformed the tested commercial fungicide, showing 19%, 39%, 33%, and 37% inhibition rates, respectively. Enzymatic assays revealed the production of amylases (6.36×10^{-4} U/mL), cellulases (4.34×10^{-3} U/mL), proteases (5.90×10^{-1} mg/mL), and when cultured with milhocina, chitinases (6.72×10^{-3} U/mL). Bench-scale fermentation induced higher chitinase production compared to the standard induction medium, highlighting milhocina as an efficient alternative substrate. The results indicate that *T. asperelloides* controls phytopathogens primarily through space competition and secondarily *via* hydrolytic enzyme production. Furthermore, the use of milhocina as a fermentation substrate holds promise for both industrial optimization and sustainable practices.

Key-words: Endophyte; Dual culture; Bench-scale fermentation; Enzymatic analyses.

Atividade antagônica e produção de enzimas hidrolíticas por *Trichoderma asperelloides* endofítico para o controle biológico de fitopatógenos de solo

Resumo

Trichoderma asperelloides, um fungo endófito foliar, foi avaliado quanto ao seu potencial antagônico contra fitopatógenos de solo e à capacidade de produção de enzimas hidrolíticas, visando aplicações biotecnológicas no manejo sustentável de doenças vegetais. Ensaios de cultivo pareado demonstraram inibição significativa do crescimento micelial de *Fusarium oxysporum* (66%), *Rhizoctonia solani* (89%), *Macrophomina phaseolina* (55%) e *Sclerotinia sclerotiorum* (90%), com interações predominantemente caracterizadas como inibição por contato micelial direto. Notavelmente, a eficácia de *T. asperelloides* superou a do fungicida comercial testado, com índices de inibição 19%, 39%, 33% e 37%, respectivamente. As análises enzimáticas revelaram produção de amilases ($6{,}36 \times 10^{-4}$ U/mL), celulases ($4{,}34 \times 10^{-3}$ U/mL), proteases ($5{,}90 \times 10^{-1}$ mg/mL) e, em cultivo com milhocina, quitinases ($6{,}72 \times 10^{-3}$ U/mL). A fermentação em escala de bancada evidenciou maior produção de quitinases em comparação ao meio indutor padrão, destacando a milhocina como um substrato alternativo eficiente. Os resultados indicam que *T. asperelloides* atua no controle de fitopatógenos principalmente por competição por espaço e, secundariamente, pela produção de enzimas hidrolíticas. Adicionalmente, o uso da milhocina como substrato fermentativo apresenta potencial tanto para a otimização industrial quanto para práticas sustentáveis.

Palavras-chave: Endófito; Cultura pareada; Fermentação em escala de bancada; Análises enzimáticas.

Introduction

Microorganisms represent one of the most diverse and ecologically versatile groups on Earth, thriving in a wide range of environments and playing essential roles in ecosystem functioning (KUSARI et al., 2012; RANA et al., 2020). Despite their ubiquitous presence and ecological significance, a vast portion of microbial diversity remains untapped. Only a small fraction of existing microbial species has been isolated, taxonomically characterized, and systematically evaluated for their biotechnological potential. This unexplored microbial reservoir holds immense promise as a source of bioactive compounds, enzymes, and metabolic pathways with applications across agriculture, medicine, industry, and environmental sustainability (RHODEN et al., 2012; CORRÊA et al., 2014; ORLANDELLI et al., 2015; FELBER et al., 2016; TOGHUEO et al., 2017; RIBEIRO et al., 2018; OLIVEIRA et al., 2020; OLIVEIRA et al., 2021).

Among these microorganisms, fungi, particularly species within the genus *Trichoderma*, have garnered attention for their robust antagonistic potential in biological control (ABBAS et al., 2017). These fungi employ a variety of mechanisms to inhibit plant pathogens, including mycoparasitism, nutrient competition, and the production of antimicrobial metabolites and hydrolytic enzymes. Such mechanisms often act synergistically, enhancing their effectiveness in suppressing a broad spectrum of phytopathogens (PASCALE et al., 2017; SCHOFFEN et al., 2020; ELSHAHAWY; MARREZ, 2024).

The growing demand for sustainable agricultural practices has intensified interest in the use of microbial antagonists as biological control agents (OLIVERIA et al., 2020). Unlike chemical pesticides, these biological alternatives offer environmentally safe and ecologically balanced solutions for plant disease management, particularly against economically impactful pathogens such as *Fusarium, Rhizoctonia, Macrophomina,* and *Sclerotinia* species (DEAN et al., 2012).

To translate the promising traits of antagonistic fungi into practical applications, it is essential not only to screen and validate their biocontrol potential under controlled conditions but also to evaluate their scalability for industrial production (MARKS et al., 2025). This process begins with bench-scale fermentation, which enables the optimization of growth conditions and metabolite yield. Furthermore, the use of alternative substrates such as milhocina, a nutrient-rich by-product of corn processing, can serve as a cost-effective and environmentally sustainable strategy, adding value to agro-industrial residues and contributing to a more circular bioeconomy.

In this context, the present study aimed to investigate the biotechnological potential of the endophytic fungus *Trichoderma asperelloides* as a biological control agent. Specifically, we evaluated its antagonistic activity against the phytopathogens *F. oxysporum, R. solani, M. phaseolina, and S, sclerotiorum*, in addition to its capacity for enzymatic production under bench-scale fermentation conditions using milhocina as a substrate, an essential step toward sustainable scale-up and potential commercial application.

Materials and methods

Microorganisms

The *T. asperelloides* isolate SF001 (GenBank accession number: PV688052) used in this study was originally isolated as a foliar endophyte from soybean and is part of the Collection of Endophytic and Environmental Microorganisms (CMEA) of the Laboratory of Bioprospecting, Biotechnology, and Biochemistry of Microorganisms (LBBBM), affiliated with the Department of Research, Innovation, and Development at Síntese Agro Science (Maringá, Paraná, Brazil). The research involving this strain was registered with the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under registration number AF5C89E.

The phytopathogenic fungal strains *F. oxysporum, R. solani, M. phaseolina,* and *S. sclerotiorum* were also obtained from the CMEA collection. All strains were preserved using the Castellani method (Castellani, 1967) and reactivated on Potato Dextrose Agar (PDA, pH 6.6) supplemented with Tetracycline (50 μg/mL), followed by incubation at 28 °C for 7 days.

In vitro assessment of antagonistic activity of *Trichoderma asperelloides*

The antagonism assay was conducted based on the method described by Campanile et al. (2007), with modifications. The endophytic fungus and the phytopathogens were previously cultured on Potato Dextrose Agar (PDA) for 7 days at 28 °C. For dual culture assays, 5 mm² mycelial plugs of *T. asperelloides* and each of the four tested phytopathogens were inoculated on opposite sides of PDA plates, maintaining a 4 cm distance between inocula. Plates were incubated at 28 °C for 7 days. Experiments were performed in triplicate, including negative controls (phytopathogen alone) and positive controls (phytopathogen + commercial fungicide at 50 μg/mL).

Mycelial growth inhibition index (Im%) was determined using ImageJ software (v.1.46r), by measuring the area occupied by the pathogen in treatment *versus* control conditions. The inhibition index was calculated using the formula: Im%=100x(1-MT/MC) where Im% is the mycelial growth inhibition index, MT is the mean mycelial area (cm²) in the treatment group, and MC is the mean mycelial area in the control group.

Fungal interaction types between the endophyte and the pathogens were categorized according to the classification system proposed by Badalyan et al. (2002), with modifications by Ribeiro et al. (2018), as follows:

- A: contact inhibition;
- B: inhibition at a distance;
- C: overgrowth of the pathogen by the endophyte without initial inhibition;
- CA1/CA2: partial/complete overgrowth after initial contact inhibition;
- CB1/CB2: partial/complete overgrowth after initial distance inhibition;
- D: overgrowth of the endophyte by the pathogen without initial inhibition;
- DA1/DA2: partial/complete overgrowth by the pathogen after contact inhibition;
- DB1/DB2: partial/complete overgrowth by the pathogen after distance inhibition.

Mycelial growth data (cm²) were subjected to analysis of variance (ANOVA), and treatment means were compared using the Scott-Knott clustering test at a 5% significance level.

Enzymatic activity of *Trichoderma asperelloides via* cup plate assay

The endophytic fungus *T. asperelloides* was cultivated in liquid media formulated to induce the production of amylases, cellulases, and proteases. The basal medium contained yeast extract (5 g/L), magnesium sulfate (0.5 g/L), calcium chloride (0.2 g/L), iron sulfate (0.02 g/L), and zinc sulfate (0.01 g/L). For enzyme induction, the medium was supplemented with 1% soluble starch (for amylases), 1% carboxymethylcellulose (for cellulases), or 1% skim milk (for proteases).

Each 100 mL flask was inoculated with two 5mm² mycelial discs from actively growing *T. asperelloides* colonies and incubated at 28 °C under agitation (150 rpm) for 7 days. After incubation, cultures were filtered through gauze to remove mycelial debris and centrifuged at 4,000 rpm for 15 minutes. The supernatants were assayed for enzyme activity using the cup plate method.

Aliquots of 100 μL of the supernatant were placed in wells on Petri dishes containing solid medium (1% starch, 1% carboxymethylcellulose, or 1% skim milk in 1000 mL distilled water, pH 7.0) and incubated at 28 °C for 24 hours. Enzymatic activity was revealed by specific staining:

- Amylase activity: immersion in iodine solution for 30 seconds followed by rinsing with distilled water;
- Cellulase activity: staining with Congo red (1 g/L) for 30 minutes and destaining with 5 M NaCl for 15 minutes.

Enzyme activity was quantified by measuring the diameter of the clear hydrolysis zones and expressed in centimeters.

Quantification of enzymatic activity by UV-Vis spectrophotometry

Enzymatic activity in the culture supernatant was also quantified using colorimetric assays and UV-Vis spectrophotometry. The release of reducing sugars, indicative of amylase and cellulase activity, was measured by the 3,5-dinitrosalicylic acid (DNS) method as described by Miller (1959), with modifications. 1 mL of enzymatic supernatant was mixed with 1 mL of DNS reagent and heated at 95 °C for 5 minutes. After cooling (± 4 ºC), absorbance was read at 540 nm. Reducing sugar concentrations were determined using a standard curve of glucose (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). The results of the enzymatic activity were expressed in U/mL.

Protease activity was assessed by gelatin hydrolysis. Aliquots of 100 µL of the supernatant were incubated with 900 µL of 1% gelatin solution prepared in 50 mM phosphate buffer (pH 7.0) at 28 °C for 24 hours. Soluble proteins released were quantified using the Bradford assay (Bradford, 1976) with Coomassie Brilliant Blue G-250 dye. Absorbance was measured at 595 nm after 5 minutes of reaction, and protein concentration was determined from a Bovine Serum Albumin (BSA) standard curve (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). The results of the enzymatic activity were expressed in mg/mL.

Bench-Scale Fermentation Using an Alternative Substrate for Enzyme Production

Following the evaluation of the hydrolytic enzyme production potential, a bench-scale fermentation was conducted using a 5 L bioreactor with milhocina (a corn-based by-product) as an alternative substrate for fungal biomass and enzyme production. The fermentation medium was composed of yeast extract (5 g/L), magnesium sulfate (0.5 g/L), calcium chloride (0.2 g/L), iron sulfate (0.02 g/L), zinc sulfate (0.01 g/L), and milhocina (10 g/L), and was prepared in a BIOENG-B (ENGCO) fermenter.

To maintain pH stability throughout the fermentation, sterile solutions of 2.5 M NaOH and 0.065 M phosphoric acid were prepared and autoclaved separately. The culture medium was sterilized *in situ* at 121 °C for 30 minutes. After sterilization, the medium was cooled to 60 °C, then maintained at 30 °C using a circulating water bath. The pH was adjusted to 6.0 using peristaltic pumps that delivered acid or base at a rate of 50 mL/min. Once temperature and pH were stabilized, a 1% (v/v) inoculum of *T. asperelloides* (OD_{600} = 0.2), previously cultured under agitation (150 rpm) at 28 °C, was added to the bioreactor using feed flasks and peristaltic pumps (20 mL/min).

Throughout the fermentation process, a pH cascade control system was employed, and dissolved oxygen levels were monitored continuously. Optical density at 600 nm was measured every 24 hours. At the end of the fermentation, the culture broth was centrifuged at 4,000 rpm for 10 minutes to separate the fungal biomass from the enzymatic extract. The resulting supernatant was evaluated for enzyme activity using both the cup plate and UV-Vis spectrophotometric methods.

To characterize the specific enzymatic activities, 100 µL aliquots of the enzymatic broth were incubated in 50 mM phosphate buffer (pH 7.0) containing 1% of the appropriate substrates: soluble starch, carboxymethylcellulose, gelatin, and colloidal chitin, corresponding to amylase, cellulase, protease, and chitinase activities, respectively. The reactions were incubated for 24 hours at 28 °C. The activities of cellulases, amylases, and chitinases were quantified using the DNS assay, as previously described, while proteolytic activity was determined using the Bradford assay.

Results

Antagonistic Activity

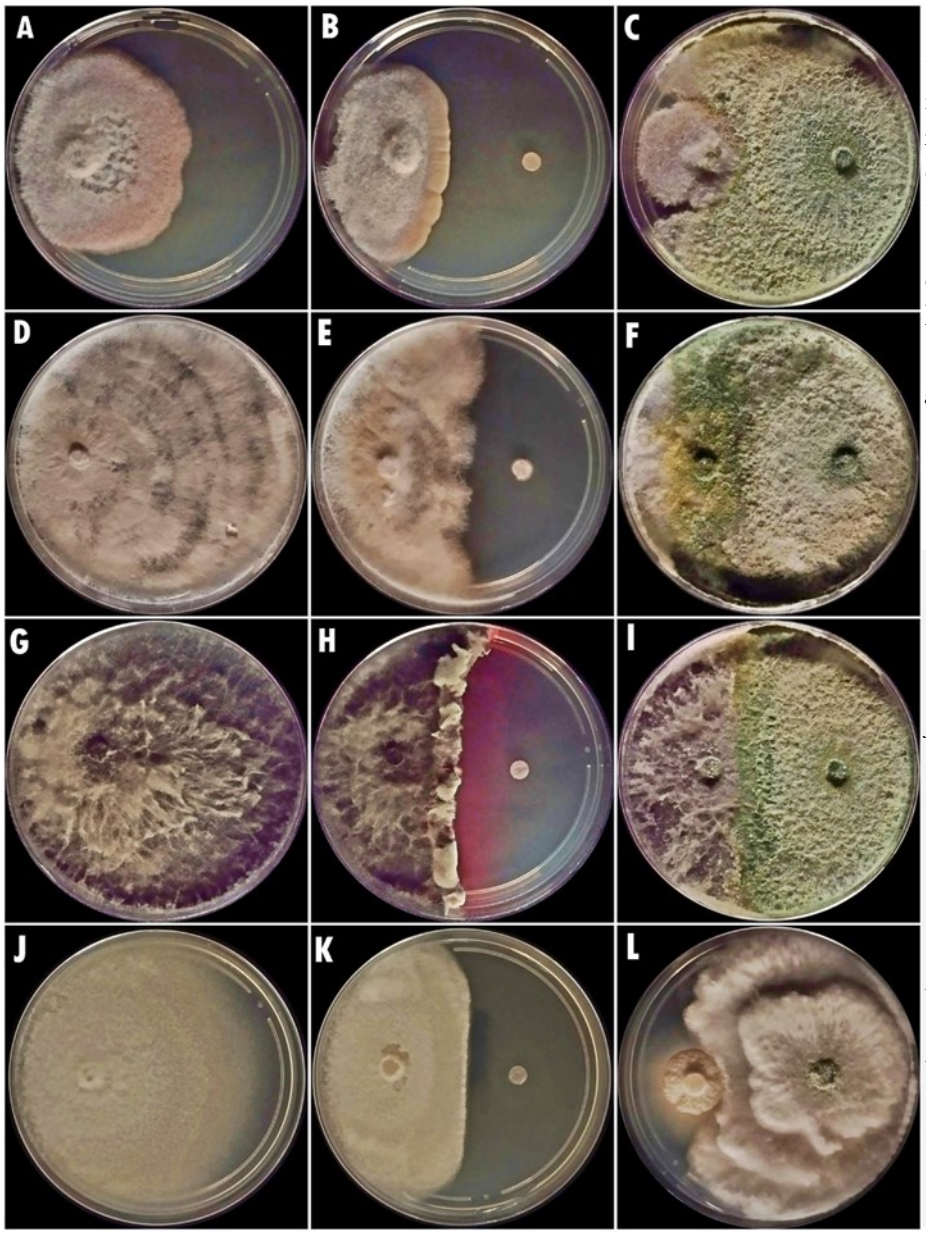
The mycelial growth inhibition rates promoted by the endophytic fungus *T. asperelloides* against the phytopathogens *F. oxysporum, R. solani, M. phaseolina*, and *S. sclerotiorum* are presented in Table 1 and Figure 1. Statistically significant differences were observed among all tested pathogens, highlighting the consistent yet variable antagonistic capacity of *T. asperelloides*.

In the case of *F. oxysporum, T. asperelloides* inhibited mycelial growth by 66%, an effect primarily associated with direct mycelial contact inhibition, classified as type A. This result was notably more effective than that of the commercial fungicide used as a positive control, which reached only 19% inhibition.

For *S. sclerotiorum*, inhibition reached 90%, also mediated by direct mycelial contact, whereas the fungicide control showed considerably lower efficacy, with only 37% inhibition. A similar pattern was observed against *M. phaseolina*, with an inhibition rate of 55%. In the interaction with *R. solani*, a distinct pattern was observed. After initial growth suppression by mycelial contact, *T. asperelloides* was able to partially overgrow the pathogen, a response classified as interaction type CA1. This interaction resulted in an inhibition rate of 89%.

Table 1. *In vitro* antagonistic activity of *Trichoderma asperelloides* against soilborne phytopathogens based on mycelial growth inhibition in paired culture. Values represent means ± standard deviation (n = 3). Means followed by different letters in the same group differ significantly according to the Scott-Knott test ($p < 0.05$). Negative Control: Pathogen alone; Positive Control: Pathogen with commercial fungicide. Interaction types classified according to Badalyan et al. (2002): A – mycelial contact inhibition; CA1 – initial contact inhibition followed by partial overgrowth.

Phytopathogen	Assay	Mycelial Area (cm²)	Inhibition	Interaction Type
	Negative Control	27.7 ± 2.25	—	—
	Positive Control	22.4 ± 1.69[b]	19%	—
F. oxysporum	*T. asperelloides*	9.50 ± 0.94[a]	66%	A
	Negative Control	61.9 ± 3.86	—	—
	Positive Control	37.3 ± 3.10[b]	39%	—
R. solani	*T. asperelloides*	6.52 ± 0.38[a]	89%	CA1
	Negative Control	60.5 ± 0.70	—	—
	Positive Control	40.0 ± 6.41[b]	33%	—
M. phaseolina	*T. asperelloides*	27.2 ± 4.32[a]	55%	A
	Negative Control	53.0 ± 0.69	—	—
	Positive Control	33.9 ± 1.00[b]	37%	—
S. sclerotiorum	*T. asperelloides*	3.17 ± 0.39[a]	90%	A



Overall, the results suggest that the predominant antagonistic mechanism employed by *T. asperelloides* is inhibition through direct mycelial contact, indicating a strong competitive ability for space and nutrient resources. This reinforces the potential of *T. asperelloides* as an effective biological control agent against a broad spectrum of soilborne phytopathogens.

Figure 1. Paired culture between the endophytic *Trichoderma* and phytopathogenic fungi. **A.** Negative control *Fusarium oxysporum*. **B.** *F. oxysporum* exposed to commercial fungicide. **C.** *F. oxysporum* challenged with *T. asperelloides*. **D**. Negative control *Rhizoctonia solani*. **E.** *R. solani* exposed to commercial fungicide. **F.** *R. solani* challenged with *T. asperelloides*. **G**. Negative control *Macrophomina phaseolina*. **H.** *M. phaseolina* exposed to commercial fungicide. **I.** *M. phaseolina* challenged with *T. asperelloides*. **J.** Negative control *Sclerotinia sclerotiorum*. **K.** *S. sclerotiorum* exposed to commercial fungicide. **L.** *S. sclerotiorum* challenged with *T. asperelloides*.

Enzymatic Production

The fungus *T. asperelloides* demonstrated a promising capacity for the production of hydrolytic enzymes, with notable activity observed for amylases, cellulases, and proteases. In the semi-quantitative assay conducted using the agar diffusion method (cup plate), the isolate exhibited particularly strong amylolytic activity, as evidenced by the formation of more prominent hydrolysis halos compared to those produced by cellulases and proteases (Table 2, Figure 2).

Quantitative assessment of enzymatic activity, based on the release of reducing sugars, corroborated the results obtained in the semi-quantitative test. The highest level of enzymatic activity was observed for amylases (6.36 x 10^{-04} U/mL), followed by cellulases (4.34 x 10^{-04} U/mL), and proteases (5.90 x 10^{-01} U/mL), as shown in Table 2.

Table 2. Enzymatic activity of *Trichoderma asperelloides*. T1: Erlenmeyer production. T2: Bench-scale production. Hydrolytic halo diameters (cm) were measured using the semi-quantitative cup plate assay. Amylase, cellulase, and chitinase expressed in U/mL. Protease expressed in mg/mL. Values are expressed as means ± standard deviation (n = 3).

	Method	Amylase	Cellulase	Protease	Chitinase
T1	Cup plate assay	1.35±0.120	0.6±0.08	0.8±0.15	---
	Spectrophotometry	$6.36\pm0.00\times10^{-4}$	$4.34\pm0.014\times10^{-3}$	$5.90\pm0.00\times10^{-1}$	---
T2	Cup plate assay	0.31±0.04	0.18±0.04	0.45 ±0.08	0.25±0.05
	Spectrophotometry	$1.90 \pm 0.07 \times10^{-2}$	$4.66\pm0.00 \times10^{-3}$	$1.00\pm0.19\times10^{-1}$	$6.72\pm0.06\times10^{-3}$

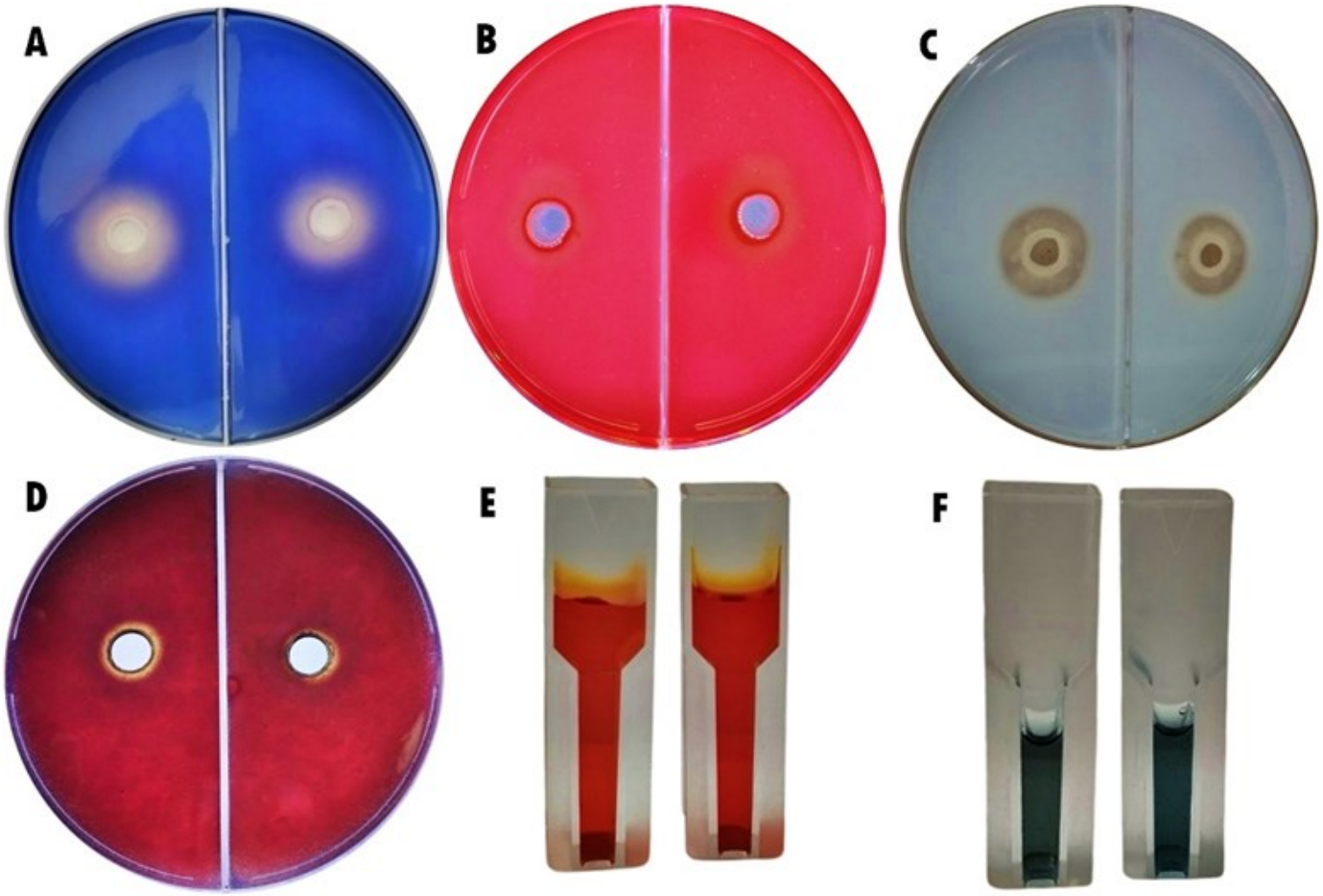


Figure 2. Production of hydrolytic enzymes by *Trichoderma asperelloides*. **A.** Amylase production: clear halos after staining with Lugol's solution indicate positive enzymatic activity. **B.** Cellulase production: yellow halos formed after Congo red staining demonstrate enzymatic activity. **C.** Protease production: the presence of clear halos indicates proteolytic activity. **D.** Chitinase production: yellowish halos after staining with Lugol's solution indicate positive enzymatic activity. **E.** DNS assay for quantification of reducing sugars. **F.** Bradford assay for quantification of proteolytic activity.

A well-defined fungal growth profile was observed during the 40-hour bench-scale fermentation process. At the end of fermentation, a total fungal biomass of 143.47 g was obtained. As shown in Figure 3, a growth peak occurred at 24 hours, under pH 7.4, with an optical density (OD_{600}) of 0.800. Enzymatic broth analysis at 40 hours revealed significant activities of amylases, cellulases, and proteases, corroborating the results previously obtained in enzyme -specific selective media (Table 2). Additionally, the incorporation of milhocina into the fermentation medium promoted the induction of chitinolytic activity, as evidenced by the detection of chitinases, a finding not observed in preliminary assays (Table 2, Figure 2).

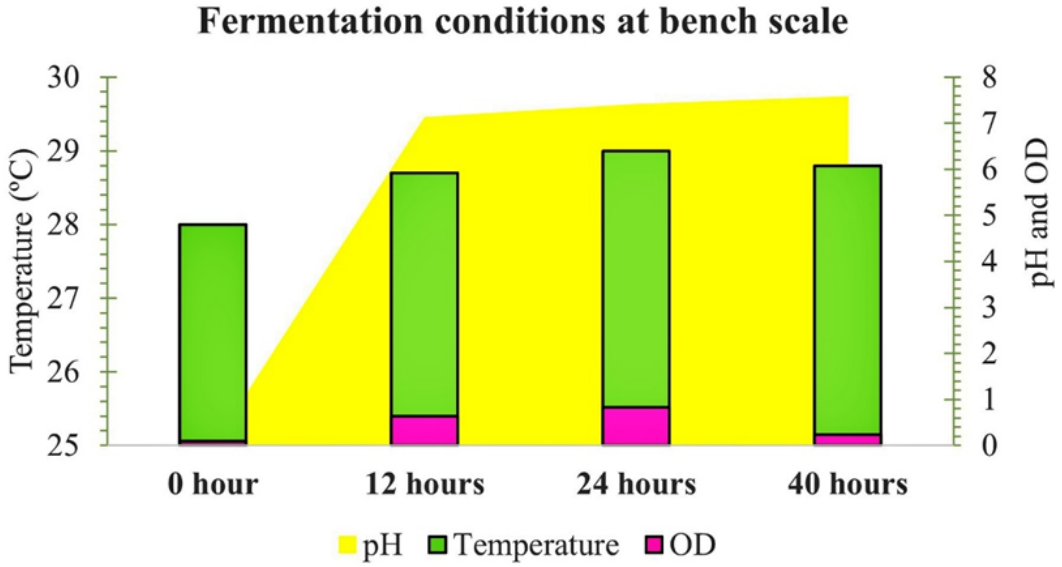


Figure 3. Changes in bench-scale fermentation conditions over time. Bars represent temperature (°C, green), optical density (OD, pink), and pH (yellow) measured at 0, 12, 24, and 40 hours during the fermentation process.

Discussion

The antagonistic activity demonstrated by the *Trichoderma* strain in this study confirms and expands upon previous reports of the genus' efficacy as a biological control agent against diverse phytopathogenic fungi. Our results align with established findings showing *Trichoderma*'s inhibitory effects against *F. oxysporum* (CHEN et al., 2021), *Sclerotinia* spp. (CARVALHO et al., 2019), *M. phaseolina* (GUPTA, 1998; ALY et al., 2007), and *R. solani* (ANEES et al., 2010; HU et al., 2022), reinforcing its broad-spectrum biocontrol potential. These consistent observations across multiple pathogen systems highlight *Trichoderma*'s reliability as a foundation for integrated disease management strategies.

The observed antagonism appears to operate through a sequential, multifactorial mechanism. Primary inhibition occurs through competitive exclusion, where *Trichoderma*'s characteristically rapid mycelial growth (Table 1 and Figure 1) enables superior substrate colonization and nutrient acquisition. This competitive advantage creates an ecological barrier that physically and nutritionally restricts pathogen establishment. The biotechnological significance of this trait lies in its preventive potential, allowing *Trichoderma* to establish protective dominance in the host environment prior to pathogen exposure.

Following establishment, secondary mechanisms involving bioactive or enzyme production enhance biocontrol efficacy. As corroborated by Elshahawy and Marrez (2024), enzymatic hydrolysis plays a crucial role in this phase. Our strain demonstrated robust production of cellulases (cleaving β-1,4-glycosidic bonds), amylases (depleting carbohydrate reserves), and proteases (degrading structural proteins) (Table 2 and Figure 2) - enzymatic activities that collectively compromise pathogen cell wall integrity (Samuels et al., 2006). This enzymatic profile aligns with reports from Lorito et al. (1993), Dienes et al. (2007), Ghasemi et al. (2020), and Naher et al. (2021).

A particularly significant finding involves the enhanced enzymatic activity observed when using milhocina as a fermentation substrate. This corn processing byproduct, rich in Cu, Mn, Fe, and Zn (MATEI et al., 2021), appears to serve dual functions: as a nutritional substrate and as a metabolic inducer. The increase in enzyme production compared to conventional media suggests these trace minerals may act as cofactors or genetic expression

modulators in biosynthetic pathways. This observation supports the growing body of evidence (NAEIMI et al., 2020) that substrate composition can significantly influence biocontrol agent efficacy through metabolic regulation.

The implications of these findings extend beyond basic biocontrol mechanisms. The successful use of milhocina demonstrates how agricultural byproducts can be strategically employed to both reduce production costs and enhance functional performance in microbial biocontrol agents. This approach aligns with circular economy principles while potentially improving field efficacy. Future research should explore the molecular mechanisms underlying substrate-induced metabolic changes and their consistency across different *Trichoderma* strains and cultivation systems.

Conclusions

This study highlights the significant biotechnological potential of *T. asperelloides* as a multifunctional biocontrol agent with dual antagonistic mechanisms. The isolate demonstrated remarkable efficacy against key soilborne pathogens, achieving inhibition rates of 90% against *S. sclerotiorum* and 89% against *R. solani*. The observed dominance of direct mycelial contact inhibition suggests superior competitive abilities for both spatial dominance and nutrient acquisition.

Beyond physical antagonism, the strain exhibited exceptional metabolic versatility, producing a comprehensive suite of hydrolytic enzymes including amylases, cellulases, proteases, and chitinases - all critical for degrading structural components of pathogen cell walls. Particularly noteworthy was the enhanced enzymatic profile obtained through bench-scale fermentation using milhocina, a corn-processing byproduct.

These findings position *T. asperelloides* as a prime candidate for commercial biofungicide development, offering: (i) high efficacy through combined physical and enzymatic antagonism, (ii) production scalability using low-cost agro-industrial substrates, and (iii) environmental advantages by reducing chemical pesticide dependence. Future research should focus on field validation of these laboratory results and optimization of formulation processes to translate this potential into practical agricultural applications.

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