Production of Bioaugmented Composts with *Trichoderma harzianum*

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ABSTRACT
Objective: To develop a bioaugmented compost in a pile system through the addition of submerged fermentation products derived from *Trichoderma harzianum*.
Methodology: Biodegradation kinetics of newspaper were monitored over a 30-day period in a cylindrical air-lift bioreactor inoculated with *Trichoderma harzianum* (IrV6S1C7), aiming to generate fungal biomass and cellulolytic enzymes. Subsequently, the fermentation products were incorporated into a 60 kg pile of organic waste comprising banana, red mango, leaf litter, and lettuce to assess waste degradation time in comparison to an uninoculated control.
Results: By day 30 of fermentation, biomass production of *T. harzianum* reached 67 g/L, while the activities of cellulolytic enzymes carboxymethyl cellulase (CMCase) and filter paperase (FPase) were 2095.51 U/L and 1471.75 U/L, respectively. The compost pile inoculated with *T. harzianum* suspension exhibited a more consistent stabilization of the carbon-to-nitrogen (C/N) ratio over time relative to the control.
Conclusions: The bioaugmented compost enriched with *T. harzianum* and its fermentation derivatives facilitated the production of a stable pre-compost with enhanced nitrogen availability, benefiting both plant growth and the proliferation of beneficial microbial communities.

Keywords: organic waste, bioreactor, submerged culture, cellulases.

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INTRODUCTION

In Mexico, it is estimated that approximately 120,128 tons of municipal solid waste are generated daily (SEMARNAT, 2022), posing significant environmental and economic challenges due to the lack of comprehensive waste management systems. This deficit contributes to the emission of greenhouse gases and the contamination of soils and water bodies caused by leachate discharge (De Anda *et al.*, 2021). Approximately 50% of this waste is composed of organic matter (OM), which can be utilized for compost production. Composting is a low-cost biotechnological process that enables the transformation of organic waste into stable organic matter (mature compost) through the activity of diverse microorganisms (Vargas-Pineda *et al.*, 2019). Despite its advantages, conventional composting techniques are time-consuming. Therefore, the inoculation of organic residues with selected microbial consortia has been proposed to accelerate the composting process and enhance the quality of the final product (Méndez-Matías *et al.*, 2018; Condori Nina, 2022).

Among the microorganisms that can be employed to bioaugment compost and improve its efficacy as a soil fertility enhancer, *Trichoderma* species stand out due to their adaptive capacity and production of a wide range of bioactive metabolites, including enzymes, plant growth-promoting compounds, and volatile organic substances (Hernández-Melchor *et al.*, 2019; Loayza-Dueñas & Gallegos-Jara, 2020). A relevant example was demonstrated by Bellini *et al.* (2023), who evaluated *Trichoderma*-enriched compost for lettuce production and biocontrol of *Fusarium oxysporum* f. sp. *lactucae*. Their findings revealed a 70% reduction in disease severity, increased crop yield, and no adverse impact on the native soil microbial community. Similarly, Méndez-Matías *et al.* (2018) assessed the effect of *Trichoderma harzianum* and *Aspergillus* sp. inoculation during the composting of mezcal agave bagasse and sugarcane bagasse in a pile system in Oaxaca, Mexico. Their results confirmed that the inoculation of lignocellulolytic fungi significantly reduced substrate degradation time and improved the physicochemical properties of the mature compost, compared to a non-inoculated control. Despite the demonstrated benefits of exogenous microbial inoculation in enhancing composting efficiency and product quality, research in Mexico remains at the experimental level. Outcomes are highly dependent on the substrate type and the microbial strains employed, indicating the need for a deeper understanding of the process to facilitate its standardization and large-scale implementation. Based on the above, the aim of the present study was to evaluate the production of pile compost bioaugmented with submerged fermentation products of a native *Trichoderma harzianum* strain, previously isolated from the maize rhizosphere in Irapuato, Guanajuato.

MATERIALS AND METHODS

Microorganisms used

The strain IrV6S1C7, isolated from the rhizospheric soil of maize cultivated in experimental plots in Irapuato, Guanajuato, Mexico (Herrera-Jiménez *et al.*, 2018), was employed in this study. This strain is preserved in glass tubes containing slanted PDA medium at 4 °C and was taxonomically identified as *Trichoderma harzianum* (GenBank accession number MN165442) (Hernández-Melchor *et al.*, 2023). The strain was propagated by point inoculation onto Petri dishes containing PDA medium and incubated at 28 °C for five days. Subsequently, the cultures were exposed to light and ambient temperature for two additional days to promote sporulation.

Newspaper degradation kinetics in bioreactor

A batch submerged culture degradation kinetics experiment was conducted over 30 days using the IrV6S1C7 strain in a cylindrical air-lift bioreactor with a total volume of 6 L and an effective working volume of 5.5 L. The culture medium used was a basal minimal medium (BMM) composed of (g/L): 6.0 Na_2HPO_4, 3.0 KH_2PO_4, 2.64 $(NH_4)_2SO_4$, 0.5 $MgSO_4 \bullet 7H_2O$, 0.015 $CaCl_2$, 3.0 $MnSO_4$, and 3.0 $ZnSO_4$, with the pH adjusted to 4.8 using a 0.05 M citrate buffer (García-Espejo *et al.*, 2016). A total of 27.5 g (0.5% w/v) of untreated newspaper cut into 1 cm×1 cm pieces was used as the sole carbon source. The inoculum consisted of a spore suspension at a concentration of 1×10^6 spores/mL. Newspaper was selected due to its proven efficacy in inducing cellulase production in

laboratory-scale submerged cultures using *Trichoderma harzianum* (Hernández-Melchor *et al.*, 2023). The bioreactor was operated under continuous aeration at a flow rate of 3 L/min via a perforated stainless-steel sparger located at the base. Air injection was regulated using a float-type rotameter with an aluminum float (Applikon Biotechnology, Netherlands) and a dual-outlet air pump (802 ELITE). The internal temperature was maintained at 24±1 °C, and pH was stabilized within the range of 4.2-5.0 without the addition of acid or base. Volume loss due to sampling and aeration was compensated weekly by adding sterile distilled water to maintain the working volume (~70 mL) of the bioreactor.

Analytical determinations

Aliquots (20 mL) were collected every third day to quantify cellulase activity, biomass production, residual cellulose, and reducing sugars. All assays were performed in triplicate.

Quantitative analysis of cellulases

Total cellulase activity was determined using the Filter Paper Assay (FPase), while endoglucanase activity (CMCase) was assessed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959; Ghose, 1987), employing D-glucose as the standard (calibration curve prepared from a 5 g/L glucose standard solution). The enzymatic activity protocols were conducted as described by Hernández-Melchor *et al.* (2022). Absorbance measurements were taken at 540 nm using a BioTek® Synergy 2 microplate reader. Enzymatic activities were expressed in IU/L (international units per liter).

Determination of biomass and residual cellulose

Fungal dry biomass and cellulose content in the bioreactor were determined according to the method described by Ahamed and Vermette (2009). Dry biomass was calculated by subtracting the residual cellulose weight from the total dry weight of solids, which includes both mycelium and cellulose.

Reducing sugar concentration

Reducing sugars were quantified using the DNS method (Miller, 1959). Optical density (OD) was measured at 540 nm using a BioTek® Synergy 2 spectrophotometer. A calibration curve was constructed from a 5 g/L D-glucose standard solution.

Pile compost production

Organic and garden waste were collected from the Colegio de Postgraduados, Montecillo campus, and the Central de Abastos in Mexico City (CDMX) to construct two composting piles (60 kg each). Each pile was composed of 18 kg of *Musa balbisiana* (plantain), 8 kg of *Mangifera indica* 'Tommy Atkins' (red mango), 23 kg of leaf litter, and 11 kg of *Lactuca sativa* (common lettuce). The piles measured 1×1×0.6 m and were covered with 70% black shade mesh, remaining exposed to outdoor environmental conditions. The waste materials were shredded and cut to an approximate particle size of 10 cm. Proportions of each component were homogenized to ensure uniform composition in the compost piles. One pile served as an uninoculated control, while the second was inoculated with the products derived from

the bioreactor-based newspaper degradation process (fungal biomass, enzymes, residual cellulose, and fermentation broth) at the end of the culture kinetics (day 30). One replicate was established for each treatment. The compost piles were aerated through weekly manual turning over a 35-day period to evaluate the effect of microbial inoculation and to obtain a pre-compost undergoing maturation. Moisture levels were maintained within a 70-80% range through the addition of tap water. pH and temperature were monitored in both piles using a multiparameter soil meter. Composite samples (20 g) were collected weekly for 35 days to determine total carbon and nitrogen content, as well as to quantify cellulase activity (CMCase and FPase).

Determination of total carbon and nitrogen

Total carbon content was determined using the Walkley-Black method, while total nitrogen was measured using the Kjeldahl method (Rodríguez & Rodríguez, 2015).

Quantitative analysis of cellulases

From the composite samples collected from the compost piles, 5 g were weighed and mixed with 20 mL of 0.05 M citrate buffer (pH 4.8). The mixtures were incubated at room temperature with agitation at 100 rpm for 1 hour. After incubation, the samples were centrifuged at 400 rpm for 10 minutes. Subsequently, 2 mL aliquots of the supernatant were taken to determine total cellulase activity (FPase and CMCase) as described by Hernández-Melchor et al. (2022).

Statistical analysis

The collected data were statistically analyzed using the SAS software package for Windows 8, version 6.2-9200. All analyses were conducted in triplicate, and the mean ± standard deviation was used to represent the statistical significance of each data set. A 95% confidence interval was applied, and statistical significance was considered valid at $p<0.05$. One-way ANOVA was employed for the analysis.

RESULTS AND DISCUSSION

Newspaper degradation kinetics

A degradation kinetics study was conducted using newspaper as the sole carbon source for the native *T. harzianum* strain (IrV6S1C7), cultivated in a batch-mode submerged system within a cylindrical air-lift bioreactor. Figure 1 presents the results for biomass concentration, residual cellulose, and reducing sugars obtained throughout the 30-day culture period. Biomass production by *T. harzianum* peaked on day 9, reaching a maximum concentration of 97 g/L. Subsequently, a decline in biomass concentration was observed as the culture entered the stationary phase, yielding a final biomass concentration of 67 g/L by day 30.

Figure 1 also illustrates the concentrations of residual cellulose and reducing sugars, both byproducts of newspaper degradation (Centeno Rumbos *et al.*, 2015). The residual cellulose content exhibited a trend similar to that of biomass, increasing proportionally and reaching a maximum of 95 g/L on day 9, followed by a reduction to 43 g/L by day 30.

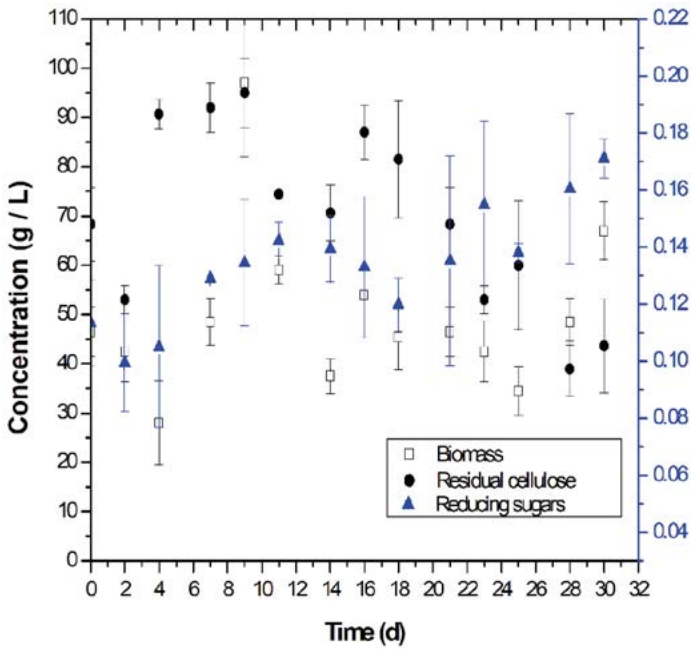

Figure 1. Biomass, residual cellulose, and reducing sugar concentrations over time during newspaper degradation in a cylindrical air-lift bioreactor. Mean values ± standard errors, n=3, ($p \leq 0.05$).

This trend indicates that the degradation of the carbon source (newspaper) was directly linked to biomass production over the course of the kinetics. Similarly, the concentration of reducing sugars steadily increased throughout the cultivation period, reaching a peak of 0.17 g/L on day 30 (Figure 1). This concentration is approximately 50 times higher than the value reported by Centeno Rumbos *et al.* (2015), who evaluated biomass production by *T. reesei* using paper sludge as a carbon source in submerged culture. Newspaper is primarily composed of cellulose, which can be enzymatically hydrolyzed into reducing sugars such as glucose and cellobiose (Hernández-Melchor *et al.*, 2019). Accordingly, the results of this study demonstrate a sequential degradation pattern of the substrate (newspaper), wherein *T. harzianum* biomass enzymatically breaks down the carbon source into its simplest form glucose. Figure 2 presents the enzymatic activity values for cellulases (FPase and CMCase) measured throughout the batch culture kinetics in the bioreactor during newspaper degradation by *T. harzianum*. As shown in Figure 2, CMCase activity increased over time, reaching a maximum of 2219 IU/L on day 7, after which it declined until the end of the cultivation period. In contrast, FPase activity continued to increase steadily over time, reaching its peak value of 1471 IU/L on day 30.

The presence of these enzymes during the biodegradation kinetics of newspaper indicates the breakdown of complex substrates into simpler forms that can be utilized by *T. harzianum* as a carbon source. The maximum cellulase activities observed in this study were 3.2 times lower for CMCase and 2.3 times higher for FPase compared to the values reported by Li *et al.* (2019), who assessed the cellulolytic enzyme production capability of *T. harzianum* LZ117 using cellulose as the carbon source. The differences between the present results and those reported in the literature can be attributed to the nature and

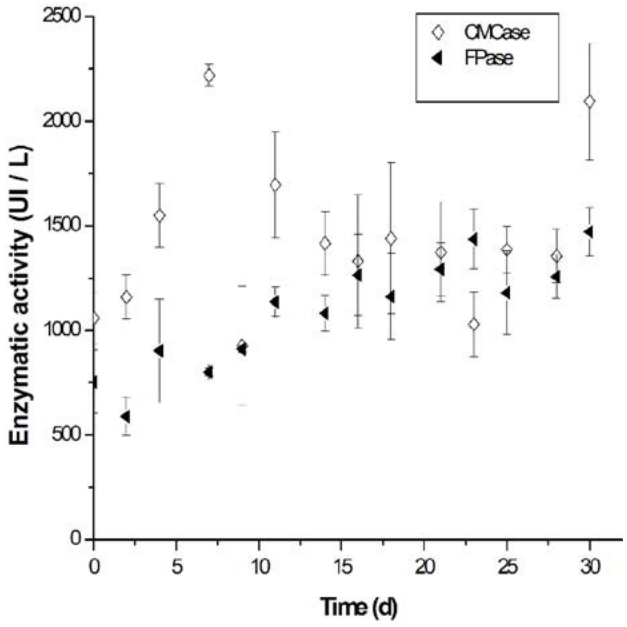

Figure 2. Cellulase activity (FPase and CMCase) over time during newspaper degradation in a cylindrical air-lift bioreactor. Mean values ± standard errors, n=3, ($p \leq 0.05$).

complexity of the substrate, as well as the operational parameters such as temperature, agitation, pH, and the bioreactor configuration all of which significantly influence the induction of enzymatic production in various fungal species (Adnan *et al.*, 2019; Bohacz-Kowalska, 2020). Given the enzymatic capacity of *Trichoderma* to degrade various carbon sources (Hernández-Melchor *et al.*, 2019), its incorporation as a bioaugmentation agent was evaluated for the degradation of organic matter (OM) during composting.

Pile compost production

The composting process was carried out over a period of 35 days to evaluate the effect of adding *T. harzianum* and the products derived from newspaper degradation in the bioreactor to the inoculated (bioaugmented) pile, in comparison to a non-inoculated control pile. Figure 3 presents the recorded values of temperature and pH throughout the composting process.

As shown in Figure 3A, the first five days correspond to the mesophilic phase in both piles. This was followed by the onset of the thermophilic phase, which lasted approximately 14 days and reached a maximum temperature of 44 °C. On day 23, a decrease in temperature was observed, marking the beginning of the cooling phase, which continued until day 35, when final temperatures of 22 °C and 21 °C were recorded in the inoculated and control piles, respectively. This behavior is consistent with that reported by Vargas-Pineda *et al.* (2019), who used waste from a local wholesale market to evaluate its degradation through composting. They described the microbial succession occurring at each stage of the process, highlighting the role of these communities in the degradation of organic matter (OM). In the present study, the early presence of *T. harzianum* in the inoculated pile had a significant effect on the timing of the different composting phases. The pH values in the inoculated

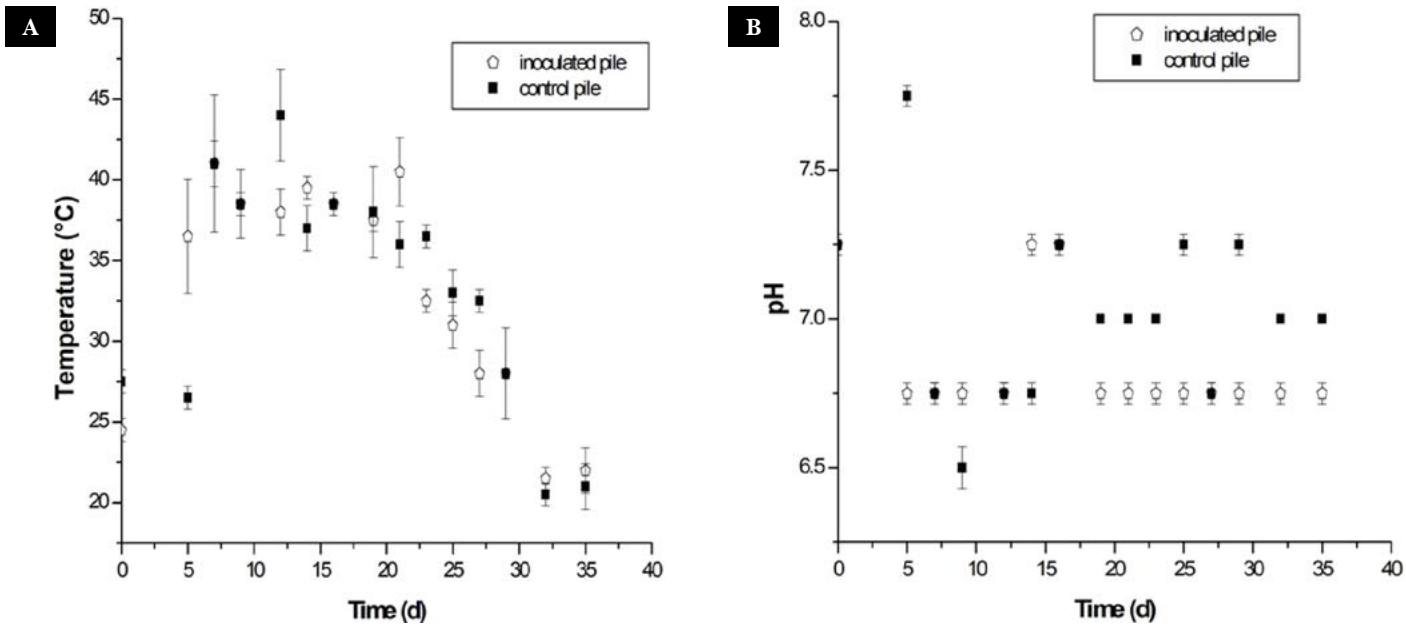

Figure 3. Temperature (A) and pH (B) profiles recorded during the composting process in the inoculated pile and the control pile. Mean values ± standard errors, n=3, ($p \leq 0.05$).

pile remained within a range of 6.7-7.2, while in the control pile they ranged from 6.5-7.7 (Figure 3B). pH plays a critical role in shaping microbial diversity during composting and affects nutrient availability. Generally, fungi tolerate pH levels between 5 and 8, whereas bacteria tend to thrive in slightly alkaline conditions, with optimal growth occurring in a pH range of 6-7.5 (Guano Andrade, 2017).

Carbon/Nitrogen (C/N) Ratio

Table 1 presents the C/N ratio values obtained from both the inoculated and control compost piles over the 35-day period. The C/N ratio in a composting process reflects the availability of carbon and nitrogen necessary for optimal microbial growth during the different composting stages (Bohórquez Santana, 2019). According to Hernández (2003), when the C/N ratio exceeds 33, the decomposition of plant residues tends to be slower; conversely, a ratio below 17 facilitates faster degradation. In the present study, the control pile initially (t_0) exhibited a C/N ratio of 34.3, while the inoculated pile started at 14.3. Over time, the ratios in both treatments fluctuated and eventually reached final values of 10.8 (control) and 11.6 (inoculated) by day 35 (Table 1). Iñiguez *et al.* (2011) indicate that a C/N ratio between 10 and 25 serves as a benchmark for compost maturity and stability. By day 35, both treatments produced a pre-compost undergoing maturation, as inferred from the final C/N ratios. However, additional analyses including heavy metal content, basal respiration, germination tests, and phytotoxicity assessment are required to fully evaluate the quality and maturity of the resulting product.

Based on these results, the pile inoculated with *T. harzianum* maintained a low C/N ratio from the beginning of the composting process through day 35, indicating greater nitrogen availability to support the growth of the fungus and other microorganisms.

Table 1. C/N ratio recorded during the composting process in a pile inoculated with *Trichoderma harzianum* (IrV6S1C7) and a control pile.

Time (d)	C/N ratio	
	Inoculated pile	Control pile
0	14.3	34.3
7	14.7	16.5
14	24.3	9.2
21	9.1	15.4
28	17.1	8.9
35	11.6	10.8

Méndez-Matías *et al.* (2018) conducted a similar study in which they evaluated the C/N ratio during the composting of mezcal agave bagasse and sugarcane bagasse inoculated with *T. harzianum*. At the start of the process, they reported an initial C/N ratio of 55.4, which decreased to a final value of 24.3 by day 133. Their findings demonstrated that the incorporation of lignocellulolytic microorganisms accelerated substrate degradation and led to the production of mature compost. According to the temperature, pH, and C/N ratio data presented, no significant differences could be established between treatments by day 35 as a result of the addition of submerged fermentation products of *T. harzianum*. Therefore, it is recommended to extend the composting period until maturity is achieved in order to properly assess the agronomic value of each treatment.

Quantitative Analysis of Cellulases

Due to the nature of the organic matter (OM) involved in the composting process primarily lignocellulosic materials a complex set of enzymatic mechanisms is required for its degradation (Gómez-García *et al.*, 2019). Among the key enzymes involved are cellulases, which are responsible for breaking down cellulose into simpler molecules. These enzymes are considered high-value bioproducts in various industrial sectors. Consequently, several research groups have explored cellulase production using low-cost lignocellulosic waste as raw material (Zhang *et al.*, 2017; Li *et al.*, 2019; Wang *et al.*, 2020). In light of this, the present study monitored cellulase enzymatic activity throughout the composting process. Furthermore, the presence of these enzymes may be associated with the specific microbial communities present at each stage and the nature of the decomposing materials (Guano Andrade, 2017). Figure 4 shows the enzymatic activity profiles of two cellulases (FPase and CMCase) quantified during composting in a pile inoculated with *T. harzianum* and in a non-inoculated control pile.

Figure 4 shows that the maximum cellulase activities (CMCase and FPase) occurred at the beginning of the composting process, decreasing progressively until day 35. Final values reached 28,123 and 14,098 IU/L for CMCase, and 12,402 and 1,829 IU/L for FPase in the control and *T. harzianum*-inoculated piles, respectively. The enzymatic activity trends over time are consistent with those reported by Guano Andrade (2017), who analyzed cellulase and amylase activity during the composting of sewage sludge and plant residues.

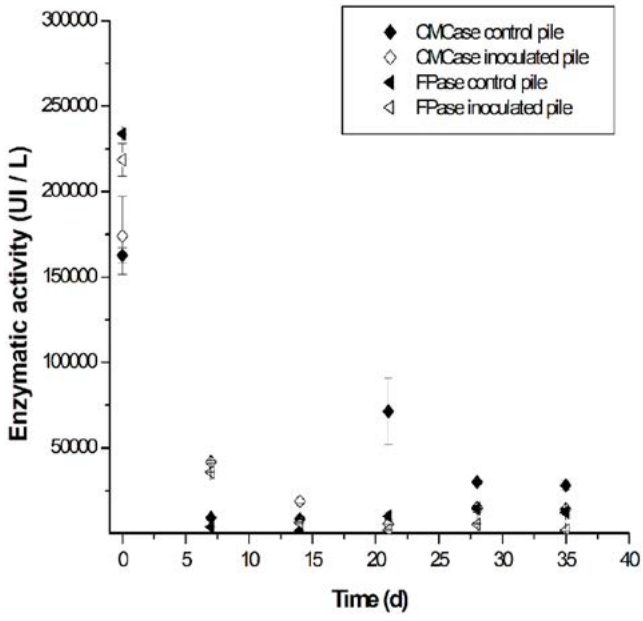

Figure 4. Cellulase activity (FPase and CMCase) over time during 35 days of composting organic matter in a pile inoculated with *T. harzianum* (IrV6S1C7) and a control pile. Mean values ± standard errors, n=3, ($p \leq 0.05$).

Similarly, Li *et al.* (2017) noted that the decrease in enzymatic activity during composting suggests a reduction in available substrates for microbial communities. This decline is also influenced by variables such as temperature and the type of raw material, which affect the dynamics of cellulolytic bacteria and fungi present in the composting material. These factors may help explain the lower enzymatic activity observed in the inoculated pile. Although both treatments exhibited comparable C/N ratios, the complexity of the carbon sources available may differ. Additionally, it is important to consider that the fermentation supernatant added to the inoculated pile contained active cellulases, which may have contributed significantly to the early degradation of substrates. This early activity could lead to the accumulation of simpler molecules by day 35, thereby reducing the need for continued high enzymatic activity. This hypothesis warrants further confirmation through molecular analysis of the compounds present in each pile. Despite the absence of statistically significant differences between treatments, it is worth highlighting *T. harzianum's* ability to degrade complex substrates and produce enzymes of industrial relevance. These findings support the potential for implementing a circular economy approach, whereby fungal biomass and enzymes can be generated from waste degradation and subsequently applied independently across different production scales.

CONCLUSIONS

The *T. harzianum* strain (IrV6S1C7) demonstrated the capacity to degrade newspaper under submerged culture conditions in a cylindrical air-lift bioreactor, effectively producing industrially relevant enzymes such as cellulases, along with fungal biomass. Although the initial addition of *T. harzianum* (IrV6S1C7) as a bioaugmentation agent during the

composting process did not yield statistically significant differences compared to the uninoculated control, a pre-compost exhibiting early signs of maturation was successfully obtained by day 35. Therefore, future studies should consider extending the composting period to assess compost maturity, field applicability, economic feasibility, and agronomic validation.

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