



Optimizing solid-state fermentation of *Trichoderma harzianum* in packed-bed bioreactors using beer spent grains and wheat straw for the production of metabolites related to phosphorus availability

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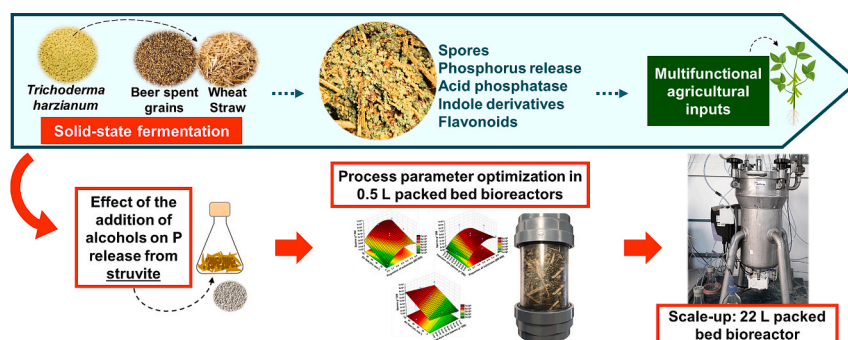
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HIGHLIGHTS

- High spore production (1×10^{10} spores g^{-1} DM) was achieved in SSF using 0.5 L PBBs.
- ACP, indole derivatives and flavonoids were produced by the strain during SSF.
- Airflow and substrate ratio were key factors in process performance.
- Scale-up in 22 L PBB maintained high spore and indole derivative levels.
- Findings support PBBs as scalable alternatives to plastic bag systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Phosphorus (P) availability is a key limiting factor for plant growth, and microbial-based strategies represent a promising approach for developing sustainable agricultural inputs. In this context, this study investigates the cultivation of *Trichoderma harzianum* using beer spent grains and wheat straw as substrates in a solid-state fermentation (SSF) process in packed bed bioreactors (PBBs) at lab and bench scales. The objective was to evaluate the strain's potential to enhance P availability by promoting medium acidification and the production of phosphatases, indole derivatives, and flavonoids. The optimized process in 0.5 L PBB resulted in fermented solids with high spore content (1.08×10^{10} spores g^{-1} of dry mass, DM), acid phosphatase (ACP) activity ($3.7 U g^{-1}$ DM), and phosphate mobilization. Additionally, the strain produced indole derivatives ($511.3 \mu g g^{-1}$ DM) and total flavonoids ($2.2 mg g^{-1}$ DM). Air flow and substrate ratio were the most significant factors, highlighting the role of adequate O_2 supply and efficient CO_2 removal in SSF processes. At bench scale (22 L PBB), cultivation also supported high biomass formation, at the order of 1×10^9 spores g^{-1} DM, and yielded appreciable amounts of

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indole derivatives (688 and 498 $\mu\text{g g}^{-1}$ DM in batches 1 and 2, respectively). Production of ACP and flavonoids was lower compared with lab scale, likely influenced by a 16.5°C temperature rise above the optimal range, underscoring the need to improve heat dissipation. These findings demonstrate the potential of PBBs for producing multifunctional *T. harzianum*-based products from agro-industrial residues under solid-state fermentation.

1. Introduction

Ensuring high levels of available phosphorus (P) in the soil is crucial for high agricultural productivity and to meet the growing global food demand (McDowell et al., 2024). However, P in soil is typically present in forms not readily assimilable by plants, necessitating extensive phosphatic fertilization (Urso and Gilbertson, 2018). Currently, commercial phosphate fertilizers are primarily derived from phosphate rocks, a finite resource, posing a threat to long-term global food security (IFA, 2023). Additionally, the high solubility of conventional mineral fertilizers results in substantial P losses through leaching, leading to eutrophication and negatively impacting local ecosystems and water quality (Weeks and Hettiarachchi, 2019). Therefore, improving the efficiency of P fertilizer use and exploring alternative P sources are crucial steps toward a more sustainable and circular agricultural system.

In this context, P-solubilizing microorganisms (PSMs) have gained attention for their ability to enhance P bioavailability in soil (Shi et al., 2024). These microorganisms facilitate P acquisition by plants through multiple mechanisms, including stimulation of root growth (via mycorrhizal associations or hormonal regulation), production of organic acids, secretion of phosphatases, and production of siderophores and exopolysaccharides (Kaur et al., 2024).

Solid-state fermentation (SSF) offers a promising approach for cultivating these PSMs while enabling the bioconversion of agro-industrial residues into biofertilizers. SSF can promote P mineralization from organic waste by generating hydrolytic enzymes (e.g., phosphatases, cellulases, and chitinases), which are beneficial for soil application (Wang et al., 2022). Filamentous fungi are particularly well-suited for SSF processes (Farinas, 2015). Among them, *Trichoderma* species have been extensively applied in agriculture, primarily as biocontrol agents, and have also shown potential to enhance plant P uptake both directly (Porrás et al., 2025).

Trichoderma harzianum stands out for its ability to synthesize metabolites that contribute to P mobilization, notably indole-3-acetic acid (IAA) and flavonoids. IAA regulates plant growth by stimulating root formation and root hair development (Prado et al., 2019a), thereby increasing root surface area and improving P acquisition. Flavonoids act as signaling molecules in the rhizosphere and as chelating agents that promote P release from metal-phosphate complexes (Shah and Smith, 2020).

Despite the industrial relevance of *T. harzianum*, its large-scale production is still predominantly performed using SSF on cereal-based substrates, often employing relatively simple cultivation systems, which can be labor-intensive and limit process scalability (Naeimi et al., 2020). However, SSF scale-up remains a challenge, mainly due to difficulties in controlling metabolic heat accumulation (Casciadori et al., 2024).

Different types of bioreactors have been developed for SSF, including rotating drum, tray, and packed-bed bioreactors (PBBs), each with specific advantages and limitations. Rotating drum systems improve mixing and heat distribution; however, low agitation velocities (<2 rpm) are often required to prevent damage to the mycelium, limiting their applicability (Grajales et al., 2025). In contrast, tray bioreactors avoid shear stress and allow better temperature control but require large areas and intensive labor at industrial scale, and their open configuration increases the risk of contamination (Arora et al., 2018). PBBs, in turn, offer a more compact and closed configuration, which reduces contamination risk, improves space utilization, potentially resulting in higher production per unit volume than tray systems, as reported by Sala

et al. (2022).

PBB operation is often limited by the development of temperature and moisture gradients within the bed, which tend to intensify during scale-up due to the greater difficulty of dissipating metabolic heat while maintaining adequate moisture conditions. Unlike submerged fermentation, scale-up in PBBs cannot rely solely on geometric similarity, since process performance depends strongly on heat and mass transfer within a heterogeneous solid matrix (Mitchell et al., 2023). Accordingly, strategies such as maintaining constant superficial air velocity, limiting bed height, and increasing bed porosity have been adopted to improve transport conditions (Pitol et al., 2016). However, even under these criteria, overheating and spatial heterogeneities may still occur, highlighting the intrinsic complexity of scaling SSF processes. Other approaches have also been explored, including changes in aeration and operation mode. Perez et al. (2019) evaluated different air injection configurations in a PBB and observed slight improvements in O_2 distribution, but limited effects on overheating. Similarly, Rodrigues et al. (2022) reported that continuous operation reduced CO_2 accumulation but did not significantly decrease maximum temperatures, likely due to the already high bed porosity. In contrast, Sala et al. (2022) used 60% wood chips as a bulking agent in a 22 L PBB and reported no overheating issues, highlighting porosity as a key factor.

Agro-industrial residues such as beer spent grain (BSG) and wheat straw (WS) are promising substrates due to their availability and complementary structural and nutritional properties. Both are lignocellulosic materials; however, BSG is rich in proteins, residual carbohydrates, and minerals that support fungal growth (Ikram et al., 2017), whereas WS presents a more fibrous structure that enhances bed porosity and improves heat and mass transfer in packed-bed systems (Casciadori et al., 2014), while also acting as a carbon source (Sala et al., 2021a). Around 20 kg of wet BSG are produced per 100 L of brewed beer, contributing to an estimated global production of 36.4 million tonnes per year (Nyhan et al., 2023). Regarding WS, approximately 1 kg of WS is produced for every 1.3 kg of wheat grain, and a significant fraction of this residue remains underutilized or is improperly managed, often being discarded or burned, which raises environmental concerns (Shanmugam et al., 2024).

Previous studies have demonstrated the potential of BSG as a substrate for *T. harzianum* cultivation in PBBs, particularly when combined with bulking agents such as wood chips (WC) (Sala et al., 2021b; Sala et al., 2022). WC are primarily used as structural bulking agents, with limited contribution as a carbon source due to their lower biodegradability under SSF conditions. In contrast, WS represents a promising alternative, as it improves bed porosity while also serving as a lignocellulosic substrate. Therefore, the combination of BSG and WS represents an effective strategy for SSF using *T. harzianum* to produce metabolites involved in P cycling.

In light of these considerations, this study aimed to optimize the production of *T. harzianum* spores through SSF in lab and bench scale PBBs using BSG and WS as substrates, and to evaluate its potential for P solubilization and mobilization. The study comprehensively evaluated the microorganism's capacity to solubilize inorganic and organic P through direct mechanisms (organic acid and phosphatase production) and indirect mechanisms (indole derivative and flavonoid synthesis). The findings can contribute to expanding knowledge on *T. harzianum* and its applications, supporting the development of multifunctional inputs for sustainable agriculture.

2. Material and methods

2.1. Microorganisms and solid substrates

Trichoderma harzianum CECT 2929 was provided by the Spanish Type Culture Collection (CECT). The original strain was preserved at -80°C in sterile cryovials containing 10% glycerol, as recommended by the strain provider. For use, the fungus was cultivated on plates with malt extract agar (MEA) at 25°C for 6–8 days. A spore suspension was then obtained by scraping the surface of the agar after adding 10 mL of 0.1% (v/v) Tween 80 (Sala et al., 2022). The spore concentration was determined using a Neubauer chamber.

The solid substrates used in the experiments were wheat straw (WS), obtained from the experimental farms of the Universitat Autònoma de Barcelona (UAB), and beer spent grains (BSG), a solid byproduct from beer processing that was kindly provided by Cervesa del Montseny S.L. (Sant Miquel de Balenyà). BSG from two different beer production batches were used in this study. For the experiments in Erlenmeyer flasks, BSG from the first batch was utilized, while for the bioreactor experiments, BSG from the second batch was employed. Additionally, the mixture with WS enhanced reactor's bed porosity and prevented compaction. For lab scale cultivation, WS was manually cut, whereas at bench scale it was coarsely ground using a knife mill. The particle size of the substrates is presented in Table 1, along with other physical and chemical characteristics.

Wood chips (WC) were also used in the cultivations in bench scale as inert bulking agent, following the approach described by Sala et al. (2022). BD was stored frozen (-20°C), while WS and WC were kept at room temperature (20 – 25°C). Before use, both substrates and the WC were autoclaved twice at 121°C for 30 min.

Struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), kindly provided by the Sostenipra Group (ICTA-UAB) from a wastewater treatment plant in Denmark, was also included in the solid matrix as a source of low-solubility P in tests of inorganic P solubilization. Prior to utilization, the struvite was ground to enhance its surface area. The total P content of the struvite was 13.9% (w/w). Table 1 presents the characterization of the materials used.

Table 1
Characterization of materials employed in the SSF processes.

Parameter	BSG ¹	BSG ²	WS	WC
MC (%)	73.70 $\pm$ 0.15	77.10 $\pm$ 0.20	8.66 $\pm$ 0.33	9.70 $\pm$ 0.30
OM (%)	96.95 $\pm$ 0.31	93.30 $\pm$ 0.30	96.81 $\pm$ 0.40	98.40 $\pm$ 0.60
pH	6.10 $\pm$ 0.01	6.60 $\pm$ 0.20	5.70 $\pm$ 0.02	5.10 $\pm$ 0.20
C (%)	45.62 $\pm$ 0.36	41.98 $\pm$ 0.16	42.10 $\pm$ 0.53	46.90 $\pm$ 0.60
H (%)	6.91 $\pm$ 0.28	6.51 $\pm$ 0.07	5.82 $\pm$ 0.44	9.70 $\pm$ 0.30
N (%)	3.20 $\pm$ 0.21	2.39 $\pm$ 0.07	0.57 $\pm$ 0.09	0.40 $\pm$ 0.20
S (%)	0.26 $\pm$ 0.06	0.23 $\pm$ 0.02	<0.10	<0.10
C/N ratio	14.27 $\pm$ 0.94	17.56 $\pm$ 0.54	73.74 $\pm$ 11.00	117.40 $\pm$ 13.60
Total P (%)	0.47 $\pm$ 0.01	0.54 $\pm$ 0.02	0.07 $\pm$ 0.00	–
BD (g L^{-1})	527.5 $\pm$ 31.2	533.0 $\pm$ 23.4	25.5 $\pm$ 3.7	50.0 $\pm$ 4.5
AFP (%)	52.7 $\pm$ 1.9	50.5 $\pm$ 2.7	98.4 $\pm$ 0.3	96.7 $\pm$ 0.4
PS-L/ls (cm)	0.5–1.0	–	2–4	–
PS-L/bs (cm)	–	0.5–1.0	5–10	0.7–7.1
PS-T (mm)	0.5–3.1	0.5–3.1	30–80	5–20

MC: moisture content, expressed on wet basis; OM: organic matter content, and elemental composition (C, H, N, S, and total P), expressed on dry basis; BD: bulk density; AFP: air filled porosity; PS-L/ls: length of particle size used at lab scale; PS-L/bs: length of particle size used at bench scale; PS-T: thickness of particle size; BSG¹: beer spent grains from the first batch of beer; BSG²: beer spent grains from the second batch of beer; WS: wheat straw; WC: wood chips.

2.2. Solid-state fermentation

The experimental work was organized in sequential steps. First, preliminary SSF assays were conducted in Erlenmeyer flasks to evaluate the overall performance of *T. harzianum* under different conditions, including the addition of low molecular weight alcohols to assess their effect on medium acidification and their potential impact on the solubilization of inorganic P, using struvite as a low-solubility P source. Based on the results of this stage, the suitability of including struvite and low-molecular-weight alcohols in later experiments was assessed.

In the following stage, experiments were conducted in PBBs at lab and bench scales. First, 0.5 L PBBs were used to optimize sporulation under controlled conditions, while also monitoring metabolites related to P availability. Then, the process was scaled up to a 22 L PBB using the optimal conditions identified at lab scale. Fig. 1 presents an overview of the experimental setup.

2.2.1. Evaluation of P release in flasks

To evaluate the microorganism's ability to solubilize P, a set of cultivations was carried out in 0.5 L Erlenmeyer flasks. In these experiments, the solubilization of both organic and inorganic P through SSF was assessed. Struvite was used as the inorganic P source, added at a concentration of 3.5% (w/w on a dry basis, d.b.). Similar concentrations of phosphate rock have been used in other studies, which reported positive solubilization results through this process with the fungus *A. niger* (Klaic et al., 2021; Rodrigues et al., 2024). The organic P source was provided by the substrates themselves, which were employed in a BSG:WS ratio of 7:3 (w/w on d.b.). This mixture resulted in a moisture content of 65%, ideal for growth of this strain (Sala et al., 2020). Total dry mass (DM) of substrates packed in each Erlenmeyer flask was 12.5 g.

Based on the work of Rodrigues et al. (2024), the addition of low molecular weight alcohols to the culture medium was tested to enhance inorganic P solubilization. Methanol was added at concentrations of 3%, 6%, and 9% (w/w on d.b.), while ethanol was added at concentrations of 4%, 8%, and 12% (w/w on d.b.). SSF processes were conducted in duplicate in incubators set at 25°C , the optimal temperature for sporulation of this strain (Sala et al., 2020). To maintain a humid environment during cultivation, trays containing water were placed inside the incubators. Inoculation was adjusted to 1×10^7 spores g^{-1} DM, and flasks were covered with filters to allow gas exchange. All procedures were performed under sterile conditions. The cultivation period was 4 days, which is sufficient to achieve high sporulation, as reported by Sala et al. (2021b). Subsequently, a time-course experiment was performed in duplicate (48–120 h), with samples taken every 24 h. The sacrificial sampling method was used, meaning that two flasks were prepared for each time point. The evaluated responses included sporulation, final pH, acid phosphatase (ACP) activity, alkaline phosphatase (ALP) activity and P release.

2.2.2. Optimization of spore production in 0.5 L PBB

For the optimization of spore production, a Box-Behnken design was applied. The process parameters evaluated were air flow (10 – 100 mL min^{-1}), BSG:WS ratio (2:8–8:2), and inoculum size (1×10^5 – 1×10^7 spores g^{-1} DM), according to the design matrix presented in Table 2. Inoculum size was included as a factor due to its known influence on bioprocess productivity, it is a key operational parameter with practical and economic relevance. The experiments were performed in 0.5 L PBBs, consisting of cylindrical Polyvinyl chloride (PVC) reactors 13 cm in height and 7 cm in diameter, corresponding to a working volume of 0.45 L and a total volume of 0.5 L, as described by Sala et al. (2021a). Before entering the bioreactor, the air was passed through a humidification system consisting of a water-filled flask to increase its relative humidity and prevent bed drying, as shown in Fig. 1b.

Considering that the different BD:WS ratios in each treatment result in beds with varying bulk densities due to the specific physical properties of each particle, the bed volume inside the bioreactor was stan-

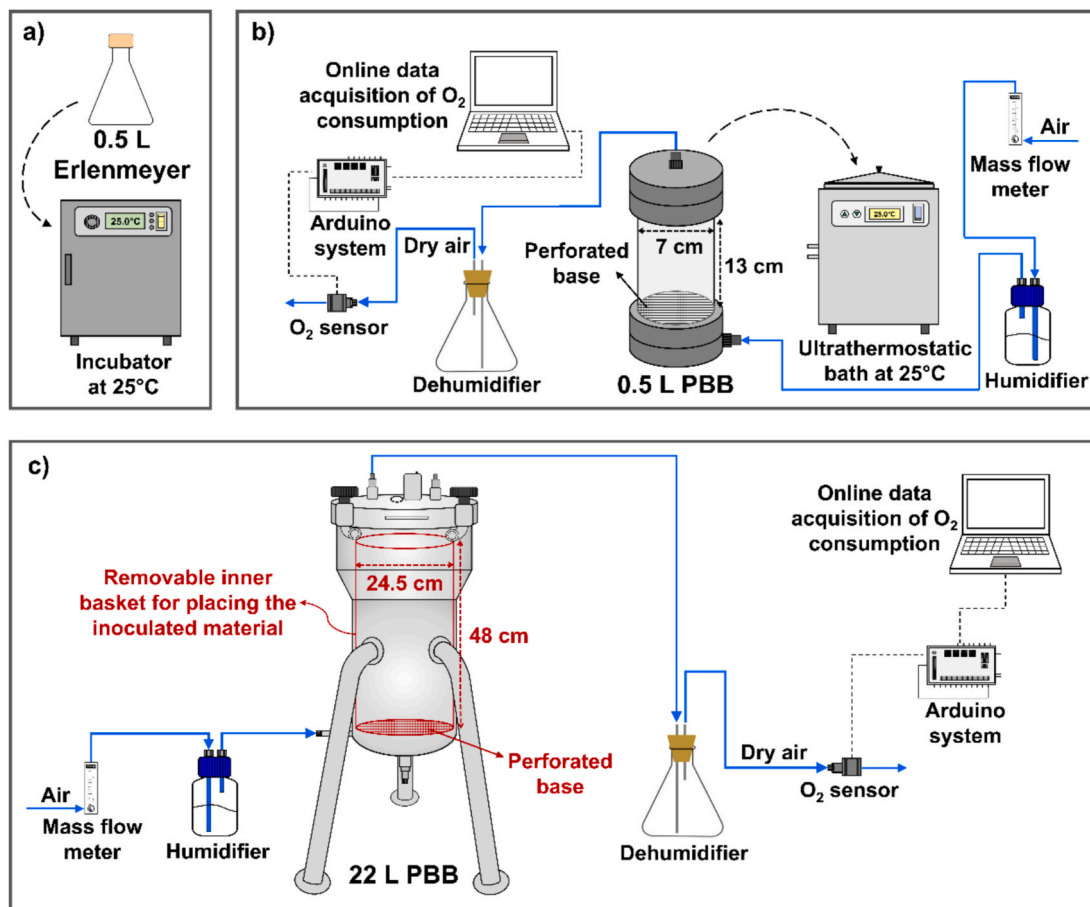


Fig. 1. Schematic representation of the experimental apparatus at lab and bench scales: (a) 0.5 L Erlenmeyer flask, (b) 0.5 L and (c) 22 L packed bed bioreactors (PBBs).

dardized instead of the mass (Casciadori et al., 2014). The bulk densities were calculated using Eq. (1) and the values are presented in Table 2. This approach was adopted because comparing beds with the same mass, but different heights would be inappropriate, given that the focus of this study is to analyze key variables for scale-up. Bed height, along with factors such as moisture content, affects bed porosity, as the weight of the upper layers can cause compaction in the lower layers (Casciadori et al., 2024).

$$\rho_{\text{bulk}} = \frac{m_{\text{bed}}}{V_{\text{rec}}} \quad (1)$$

where ρ_{bulk} , m_{bed} and V_{rec} represent the bulk density (g L^{-1}), bed mass (g) and the volume (L) occupied by the bed inside the bioreactor, respectively.

The moisture content of all treatments was adjusted to 70%, as this was the value obtained in the treatment with the highest proportion of BSG (BSG:WS ratio = 8:2), which is the more humid substrate. Prior to inoculation and packing, the bioreactors were sanitized with 70% ethanol and exposed to ultraviolet light for 15 min. The packing technique used was loose packing proposed by Casciadori et al. (2014), in which the substrates are smoothly accommodated without causing bed compression. All procedures were performed under sterile conditions. The bioreactors were then completely sealed, allowing only air flow in and out. Cultivation was carried out for 4 days, with temperature control maintained by immersing the bioreactors in a thermostatic water bath set to 25°C. Spore production was the response variable evaluated in this experiment.

2.2.3. Time course analysis under optimized conditions in 0.5 L PBB

Under the optimal conditions for sporulation, defined in the previous section (aeration, substrate ratio, and inoculum size), the production of metabolites of interest was evaluated over time. Cultivation was carried out in the 0.5 L PBBs for 6 days, with sampling every 24 h. The point corresponding to 4 days of cultivation was performed in triplicate, and the standard deviation obtained at this point was used as an error estimate for the other time points. The sacrificial sampling method was applied, meaning one bioreactor was sacrificed at each time point. The responses evaluated over time included O_2 consumption, spore production, ACP activity, P release, indole derivatives, and total flavonoids. The inoculation, packing, and temperature control procedures were performed as described in the previous section.

2.2.4. Process scale-up to a 22 L PBB

The experimental setup for the 22 L PBB is shown in Fig. 1b. The bioreactor consists of a cylindrical stainless-steel vessel with a removable basket of 48 cm height $\times$ 24.5 cm diameter, with a total volume of 22 L, as described by Sala et al. (2022). In all fermentations, the working volume was approximately 90% of the reactor capacity. As in the 0.5 L PBB, the incoming air passed through a humidification system before entering the bioreactor. While this approach is generally sufficient to maintain moisture under small-scale conditions, at larger scales metabolic heat accumulation may increase bed temperature, which can promote moisture loss and the development of gradients within the bed. A loose packing technique was used to prevent bed compaction.

To monitor temperature distribution and detect potential gradients, temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) were placed at six different positions inside the

Table 2

Matrix and results of the Box-Behnken design carried out to evaluate the influence of air flow, proportion of beer spent grains (BSG):wheat straw (WS) and inoculum size on the sporulation by *T. harzianum* in 0.5 L packed bed bioreactors.

Run	Bulk density (g L ⁻¹)	Independent variables			Dependent variables	
		Air flow (mL min ⁻¹)	BSG: WS (C/N ratio)	Inoculum size (spores g ⁻¹ DM)	Spores exp ^a (g ⁻¹ DM)	Spores pred ^b (g ⁻¹ DM)
1	122	55	2:8 (45)	1.00 × 10 ⁵	1.34 × 10 ⁹	2.42 × 10 ⁹
2	233	55	8:2 (21)	1.00 × 10 ⁵	5.91 × 10 ⁹	5.43 × 10 ⁹
3	122	55	2:8 (45)	1.00 × 10 ⁷	1.97 × 10 ⁹	8.76 × 10 ⁸
4	233	55	8:2 (21)	1.00 × 10 ⁷	1.82 × 10 ⁹	3.88 × 10 ⁹
5	122	10	2:8 (45)	5.05 × 10 ⁶	1.29 × 10 ⁹	1.01 × 10 ⁹
6	233	10	8:2 (21)	5.05 × 10 ⁶	3.79 × 10 ⁹	3.11 × 10 ⁹
7	122	100	2:8 (45)	5.05 × 10 ⁶	1.99 × 10 ⁹	3.19 × 10 ⁹
8	233	100	8:2 (21)	5.05 × 10 ⁶	7.09 × 10 ⁹	6.20 × 10 ⁹
9	133	10	5:5 (28)	1.00 × 10 ⁵	4.14 × 10 ⁹	4.42 × 10 ⁹
10	133	10	5:5 (28)	1.00 × 10 ⁷	1.56 × 10 ⁹	2.88 × 10 ⁹
11	133	100	5:5 (28)	1.00 × 10 ⁵	7.11 × 10 ⁹	7.51 × 10 ⁹
12	133	100	5:5 (28)	1.00 × 10 ⁷	6.97 × 10 ⁹	5.97 × 10 ⁹
13	133	55	5:5 (28)	5.05 × 10 ⁶	5.86 × 10 ⁹	5.19 × 10 ⁹
14	133	55	5:5 (28)	5.05 × 10 ⁶	4.25 × 10 ⁹	5.19 × 10 ⁹
15	133	55	5:5 (28)	5.05 × 10 ⁶	6.46 × 10 ⁹	5.19 × 10 ⁹
OC	156	100	6:4 (25)	1.00 × 10 ⁵	1.08 × 10 ¹⁰	7.78 × 10 ⁹

^a Spores exp corresponds to the value of spores obtained experimentally.

^b Spores pred corresponds to the value of spores obtained according to the fitted regression equation.

* OC means optimized condition.

** The value corresponds to cultivation performed under optimal conditions for a duration of 96 h.

bioreactor: two at the bottom (center and wall), two at mid-height (center and wall), and two at the top (center and wall). The room temperature was also recorded.

Two batches were conducted, each lasting 6 days. In batch 1, the optimal process conditions established in the 0.5 L PBBs were maintained, with a substrate ratio of BSG:WS 6:4 and an inoculum size of 1 × 10⁵ spores g⁻¹ DM. To scale the air flow while minimizing bed drying, the superficial air velocity was used as the scaling criterion, calculated using Eq. (2).

$$v_s = \frac{Q}{A_t} \quad (2)$$

where v_s is the air superficial velocity (cm min⁻¹), Q is the volumetric air flow rate (cm³ min⁻¹) and A_t is the cross-sectional area of the PBB (cm²).

In batch 2, the air flow rate was increased by 1.3 times, and 20% WC was added as an inert bulking agent. The values used, along with other cultivation characteristics, are presented in Section 3.4.

In summary, the engineering strategy adopted for scale-up consisted of maintaining similar gas–solid contact conditions across scales, while implementing additional modifications to mitigate heat and mass transfer limitations. In batch 2, this was achieved by increasing the air flow rate and incorporating a bulking agent to enhance bed porosity.

Simultaneously to the 22 L PBB batches, three 0.5 L PBBs were packed with the same inoculated material to assess potential scale-up issues. The responses evaluated included O₂ consumption, temperature profiles, spore production, ACP activity, indole derivatives, and total flavonoids.

2.2.5. Assessment of oxygen consumption

In the SSF processes conducted in the 0.5 L and 22 L PBBs, the flow rate was monitored using a mass flow meter (Mass-Stream D-6311, Bronkhorst, NL). The O₂ content in the outlet gases was assessed using an electrochemical O₂ sensor O₂-A₂ (Alphasense, UK). Data analysis was performed by a non-commercial tailor-made software Arduino® based that calculates the respiration rates.

The specific rate of O₂ consumption was calculated according to Eq. (3) using the data of the molar fraction of O₂ in the outlet gas stream collected online, as well as the process parameters, as described by Sala et al. (2022). The cumulative O₂ consumption (COC) was also determined by calculating the area under the curve of the specific rate of O₂ consumed.

$$sOUR = F \times (0.209 - y_{O_2}) \times \frac{P \times 32 \times 60 \times 10^3}{R \times T \times DW \times 10^3} \quad (3)$$

where $sOUR$ is the specific Oxygen Uptake Rate (g O₂ kg⁻¹ DM h⁻¹); F is the air flow (mL min⁻¹); y_{O_2} is the oxygen molar fraction in the exhaust gases (mol O₂ mol⁻¹); P is the pressure of the system assumed constant at 101325 Pa; 32 is the oxygen molecular weight (g O₂ mol⁻¹ O₂); 60 is due to the conversion factor from minute to hour; 10³ is due to the conversion factor from mL to L; R is the ideal gas constant (8310 Pa L K⁻¹ mol⁻¹); T is the temperature at which F is measured (K); DW is the initial dry weight of solids in the reactor (g); 10³ is due to the conversion factor from g to mg.

2.3. Analytical procedures

At the end of the SSF processes, the fermented solids were subjected to solid–liquid extraction for specific analytical procedures. This process involved mixing the sample with the respective liquid, followed by agitation for 30 min at 25°C in an orbital shaking incubator. For analyses requiring the removal of suspended solids, the samples were centrifuged at 9000 rpm for 15 min at 4°C. The details of each analysis are provided in the following sections.

2.3.1. Spore counting

Fermented solids were extracted with 0.1% Tween 80 (9 mL g⁻¹), diluted, and plated on potato dextrose agar (PDA) with 0.01 g L⁻¹ rose bengal. After 2 days at 30°C, colonies were counted, and spore number was calculated using Eq. (4), assuming one colony equals one spore, as confirmed by Neubauer chamber counts (data not shown).

$$\text{Spore count} = \frac{CN \times DF \times EV}{PV \times SDM} \quad (4)$$

where: CN is the colonies number; DF is the dilution factor of the plated sample; EV is the volume of 0.1% Tween 80 solution used for extraction; PV is the volume of the plated sample; SDM is the sample dry matter subjected to extraction.

2.3.2. Soluble and total P determination

To determine the soluble P, the fermented solids were extracted with distilled water (5 mL g⁻¹). After the extraction, the samples were centrifuged. Soluble P of the supernatant was measured using the equipment 115 VAC PHOSPHAX sc, Hach-Lange, which determines P in the form of PO₄ in a range of 0.05–15 mg L⁻¹. To calculate the released P, the soluble P initially present in each experiment was subtracted.

In experiments with struvite, the efficiency of P solubilization was calculated using Eq. (5), considering the total initial P present in the

medium. This included both the P introduced through struvite and the P naturally present in the substrates.

$$\text{P solubilization efficiency (\%)} = \frac{\text{Solubilized P}}{\text{Initial Total P}} \times 100 \quad (5)$$

Total P determination was performed by the chemical analysis service of UAB using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

2.3.3. Phosphatase activity assays

Fermented solids were extracted with sodium acetate buffer (pH 4.8) and sodium citrate dihydrate buffer (pH 9.0) (15 mL g⁻¹) to determine ACP and ALP activities, respectively. After centrifugation, supernatants were used for the assay. Phosphatase activity was measured as the p-nitrophenol released from 5 mM p-nitrophenyl phosphate (p-NPP) at 37°C. The reaction ran for 10 min and was stopped by adding 1 mL of 0.1 M NaOH. p-Nitrophenol was quantified at 410 nm using a calibration curve (10–60 μmol L⁻¹). One unit (U) of acid phosphatase activity was defined as 1 μmol p-nitrophenol released per hour under these conditions (Tabatabai and Bremner, 1969).

2.3.4. Quantification of indole derivatives

Fermented solids were extracted with distilled water (5 mL g⁻¹) to quantify indole derivatives. The reaction was carried out by mixing 1 mL of extract with 4 mL of Salkowski reagent (2% FeCl₃ in 35% perchloric acid) and incubating for 30 min. After incubation, the samples were centrifuged, and the supernatants were analyzed by spectrophotometry at 535 nm. The concentration of indole derivatives was determined using a standard curve prepared with indole-3-acetic acid (IAA) (Bric et al., 1991).

2.3.5. Quantification of total flavonoids

Fermented solids were extracted with acidified methanol (70% methanol, 10% acetic acid, v v⁻¹) in an ultrasonic bath to quantify the total flavonoid content. Then, 1 mL of the extract was mixed with 250 μL of 5% aluminum chloride and incubated in the dark for 30 min. After incubation, the samples were centrifuged, and the supernatants were analyzed by spectrophotometry at 425 nm. The concentration of total flavonoids was determined using a standard curve prepared with quercetin (Chang et al., 2002).

2.3.6. Air-filled porosity

Air-filled porosity (AFPR) was calculated for used substrates according to Eq. (6) as presented by Richard et al. (2004):

$$AFPR = 1 - BD_t \left[\left(\frac{1 - DM}{D_w} \right) + \frac{DM \times OM}{PD_{OM}} + \left(\frac{DM(1 - OM)}{PD_{ash}} \right) \right] \quad (6)$$

where: AFP (air-filled porosity, %); BD_t (total bulk density on a wet basis, kg m⁻³); DM (dry matter on a wet basis, %); OM (organic matter on a dry basis, %); D_w (water density, 1000 kg m⁻³); PD_{OM} (organic fraction particle density, 1600 kg m⁻³) and PD_{ash} (ash particle density, 2500 kg m⁻³).

2.4. Statistical analysis

Statistical analyses were performed using STATISTICA software, version 7 (StatSoft Inc., 2004). Analysis of variance (ANOVA) was conducted, followed by mean comparisons using Tukey's test, with a confidence level of 95%. The software was also used for the design of experiments (DoE) and the construction of surface plots.

3. Results and discussion

3.1. Effect of the addition of alcohols on P release

The presence of low molecular weight alcohols in small concentrations in the cultivation medium can enhance the permeability of microbial cell membranes, thereby increasing the excretion of metabolites into the extracellular medium. This strategy has proven effective to produce organic acids by *A. niger* (Dhillon et al., 2013). Rodrigues et al. (2024) reported a threefold increase in oxalic acid production after adding 7% methanol to the culture medium in SSF process. This approach allowed reaching an effective solubilization of P from phosphate rocks. However, the extent of this effect may vary depending on the microorganism and the culture conditions.

Several studies have demonstrated that certain strains of *T. harzianum* can produce organic acids and solubilize P, including citric, malic, oxalic, tartaric, succinic, and lactic acids (Zhang et al., 2014; Li et al., 2015; Bader et al., 2020). However, the potential of the strain studied here for these purposes remains unknown. Therefore, the effect of adding different concentrations of methanol and ethanol to the culture medium containing 3.5% struvite (a source of low-solubility inorganic P) was evaluated, as described in Section 2.2.1. Struvite is a byproduct obtained during the treatment of urban wastewater under alkaline conditions. Its recovery contributes to both sustainable agriculture and the prevention of P returning to water bodies (Ahmed et al., 2018).

Fig. 2a shows sporulation and final pH after 4 days at different alcohol types and concentrations. Spore production decreased with increasing alcohol, and 12% ethanol completely inhibited sporulation, yielding counts below the inoculum (1 × 10⁷ spores g⁻¹ DM). All alcohol treatments produced significantly fewer spores than the control (9.8 × 10⁹ spores g⁻¹ DM). This effect can also be observed in Fig. 2b, which shows the final appearance of the fermented solids without addition of alcohol and with the maximum addition of methanol and ethanol where sporulation occurred (9% methanol and 8% ethanol). In the control condition, where no alcohol was added, the microbial biomass exhibited a dark green color, characteristic of *T. harzianum* spore. On the other hand, with the addition of 9% methanol, a lighter greenish color was observed, while the addition of 8% ethanol resulted in a whitish appearance. During its growth stages, *T. harzianum* typically initiates colony development with white mycelium. As development proceeds, conidiophores are formed, generating and dispersing green conidia (Guzmán-Guzmán et al., 2025).

Rodrigues et al. (2024) also observed reduced sporulation with alcohol addition. Similarly, Anwar and Sardar (2009) reported that methanol can decrease sporulation while potentially enhancing metabolite yield. In the present study, alcohol did not stimulate acid production, as the final pH remained close to the initial value (Fig. 2a). Consequently, the P solubilization efficiency was less than 50% in all evaluated conditions, as shown in Fig. 2c, which shows the ACP activity, released P and the P solubilization efficiency for each condition. Li et al. (2015) reported that *T. harzianum* SQR-T037 solubilized phytate, Fe₂O₃, CuO, and Zn, but not Ca₃(PO₄)₂, and pH remained high despite organic acid production. This suggests that pH reduction is a key factor for inorganic P solubilization.

On the other hand, a higher acid phosphatase (ACP) activity was observed in the treatments with alcohol, as evidenced in Fig. 2c, with significant increases at 6% and 9% methanol and 8% ethanol, while other conditions did not differ from the control. The alkaline phosphatase activity (ALP) was also quantified, but the value was close to zero U g⁻¹ DM. Regarding released P, only the values corresponding to the addition of 9% methanol and 8% ethanol were significantly higher than those of the control. However, released P values followed the same trend as ACP activity, suggesting that the released P was primarily derived from organic sources through enzymatic action.

In this regard, addition of 6% and 9% methanol, as well as 8%

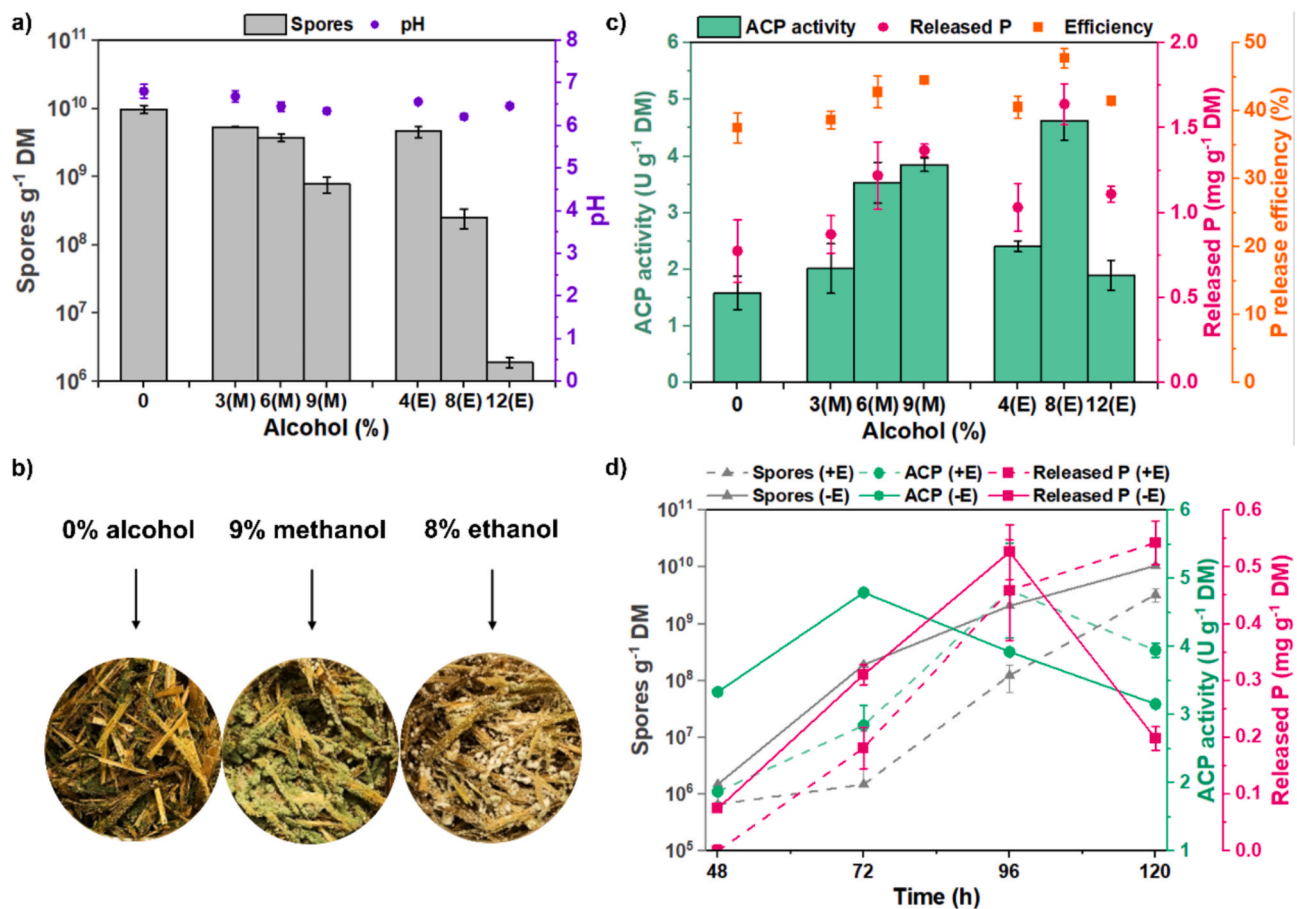


Fig. 2. Effect of different methanol and ethanol concentrations on *T. harzianum* SSF using beer spent grains:wheat straw 7:3 as substrates and 3.5% of struvite as inorganic P.

Source. The evaluated responses include a) spore production and final pH; b) phenotypic aspect of the fermented solids; c) Acid phosphatase (ACP) activity, released P, and P solubilization efficiency and d) time-course analysis (without struvite) comparing 8% ethanol addition versus no ethanol. SSF was conducted at 25°C for 4 days in Erlenmeyer flasks

ethanol, appears to favor higher ACP excretion, with a value of 4.6 U g^{-1} DM. There are few studies in the literature about the production of phosphatases by *T. harzianum*, especially through SSF. Some studies have investigated phosphatase production by other *Trichoderma* species and different conditions. Busato et al. (2021) investigated ACP production during a vermicomposting process enriched with phosphate rock, observing a maximum production of 3 U g^{-1} of vermicompost when simultaneously inoculating *Trichoderma asperellum* and *Trichoderma virens*. Mercl et al. (2020) studied soil P availability following inoculation with *Penicillium* sp. and *T. harzianum*, reporting an increase in ACP activity in soil by *T. harzianum*, with a maximum value of 2.4 U g^{-1} DM. Lima et al. (2022) evaluated ACP production by different *Trichoderma* species through submerged fermentation in soybean molasses, achieving a maximum activity of 2.5 U mL^{-1} with *T. harzianum* as inoculum. In this context, the ACP activity values obtained here demonstrate the significant potential of the studied strain to release organic P.

Given that the strain did not exhibit significant potential for solubilizing inorganic P, the next experiments were conducted without the addition of struvite. To better understand the influence of ethanol and evaluate the responses over time, two time-course experiments were conducted. Considering that the highest ACP activity was obtained with the addition of 8% ethanol, cultivation with 8% ethanol was compared with a control without ethanol, as shown in Fig. 2d.

The ACP activity profiles exhibit a distinct peak, followed by a decline in activity (Fig. 2d) which may result from enzymatic inactivation (e.g., product inhibition) or degradation by protease production

(Tang et al., 2022). Vassilev et al. (2007) and Lei et al. (2017), who evaluated phytase production by *Aspergillus niger* and ACP by *Trichoderma asperellum*, respectively, also reported a decrease in the activity of these enzymes at the end of fermentation. The peaks occurred at 96 h and 72 h of cultivation for treatments with and without ethanol, respectively, both reaching 4.8 U g^{-1} DM. In cultivation without ethanol, the released P profile decreased after 96 h, likely due to new P complexes or microbial P consumption.

Regarding spore production, maximum values were 3.3×10^9 and 1.1×10^{10} spores g^{-1} DM at 120 h for treatments with and without ethanol, respectively (Fig. 2d). These levels are among the highest reported for *Trichoderma* spp. in SSF (Hamrouni et al., 2023; Meng et al., 2024), highlighting the strain's strong sporulation capacity. Additionally, the spore production without ethanol increased faster than with 8% ethanol. Combined with the phenotypic characteristics of the treatments (Fig. 2b), these results suggest that alcohol delayed development without altering metabolic pathways or metabolite excretion.

Based on these results, the addition of ethanol did not provide significant advantages. Nevertheless, the strain exhibited efficiency in producing ACP and mobilizing organic P. Furthermore, the combination of BSG and WS supported high spore production, achieving levels on the order of 1×10^{10} spores g^{-1} DM. In view of this, subsequent experiments focused on optimizing spore production by investigating critical scale-up factors, as detailed in the next section.

3.2. Optimization of spore production in 0.5 L PBB

A Box-Behnken design was employed using 0.5 L PBBs to optimize spore production, evaluating BSG:WS ratio, inoculum size, and aeration over 4 days. The results from all experimental combinations are presented in Fig. 3, which shows surface and contour plots of spore production, and in Table 2, which provides the design matrix, experimental responses, and predicted values from the regression model (R^2 adj = 68.26%) described in Eq. (7). Among the linear parameters, only inoculum size showed no significant effect. The model showed insignificant lack of fit (p-value = 0.490) and was simplified by excluding non-significant quadratic terms and interactions.

$$\text{Spores} = -4090142778 + 277112934(P_{BD}) - 155,8(IS) + 34357712(AF) - 2269941(P_{BD})^2 \quad (7)$$

where P_{BD} , IS and AF represent the proportion of BSG (%), inoculum size (spores g^{-1} DM) and air flow (mL min^{-1}), respectively.

According to Fig. 3, the highest spore production values (1.08×10^{10} spores g^{-1} DM) were achieved at the maximum air flow tested (100 mL min^{-1}), a BSG:WS proportion of approximately 6:4, and the smallest

inoculum size evaluated (1×10^5 spores g^{-1} DM). By maximizing the response (spore production) using the model (Eq. (7)), these same conditions were determined as the optimal parameters. Under optimal conditions, a productivity of 2.7×10^9 spores g^{-1} DM d^{-1} was achieved. This value is significantly higher than that reported by Sala et al. (2022), who obtained of 3.6×10^8 spores g^{-1} DM d^{-1} in a 1.5 L PBB using the same strain and BSG as substrate.

Aeration is a key factor in SSF within PBBs, as it directly influences O_2 supply, CO_2 removal, and metabolic heat dissipation. Proper aeration supports aerobic metabolism and optimal microbial growth, whereas excessively high rates may dehydrate the substrate, reducing moisture for growth and sporulation (Mitchell et al., 2023). In this study, no adverse effect on moisture was observed at the maximum air flow (100 mL min^{-1}), with final values similar to the initial 70%. Under similar conditions, Sala et al. (2020) tested 20–60 mL min^{-1} for *T. harzianum* and found aeration non-significant, likely due to the restricted range.

Along with aeration, substrate proportion significantly impacts bio-process yield. As previously discussed, altering the ratio of BSG to WS results in beds with different bulk densities. This is supported by the structural properties of the substrates (Table 1), where WS exhibits low bulk density and high AFP, while BSG presents higher bulk density and

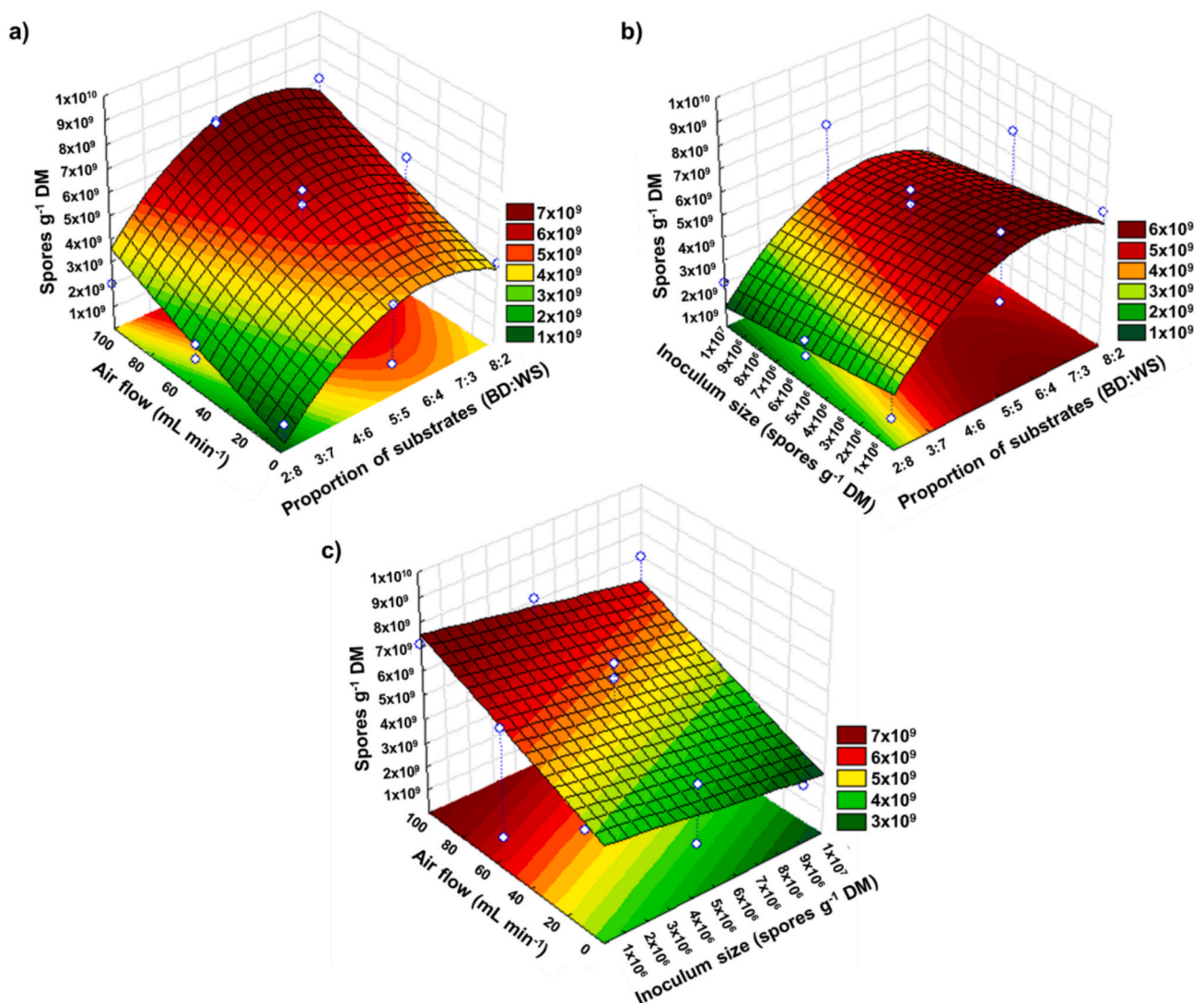


Fig. 3. Response surface plots, resulting from the Box-Behnken design experiments, showing the influence of air flow, proportion of beer spent grains (BSG) and wheat straw (WS) and inoculum size on sporulation, as predicted by the fitted regression model. The model showed an adequate fit to the experimental data, with an insignificant lack of fit (p-value = 0.490). a) Inoculum size fixed in 5.05×10^6 spores g^{-1} DM. b) Air flow fixed in 55 mL min^{-1} . c) Proportion of substrates fixed in 5:5 (BSG:WS). Solid-state fermentations were carried out at 25°C for 4 days in 0.5 L packed bed bioreactors.

lower AFP. These structural differences directly affect heat dissipation, air distribution, and ultimately bioprocess efficiency (Casciari et al., 2014; Perez et al., 2019). Beyond these physical factors, substrate composition also varies with the proportions used, leading to changes in the C/N ratio, as shown in Table 2.

The optimal C/N ratio for *Trichoderma* species varies widely, from 6 to 160 (Shahzad et al., 2018; Dorta-Vásquez et al., 2019). For *T. harzianum* in submerged culture, ratios between 14 and 24 are reported as optimal (Shahid et al., 2011; Kobori et al., 2015). However, carbon sources in liquid media are usually readily assimilable (e.g., glucose, sucrose), whereas complex solid substrates undergo gradual enzymatic degradation, releasing nutrients progressively. This suggests that the initial C/N ratio may not accurately reflect the ratio available over time, with degradation kinetics being an additional factor to consider. Therefore, the C/N ratio of 25, which yielded the highest sporulation here, may have balanced nutrient release, favoring *T. harzianum* sporulation (Shahzad et al., 2018).

As previously mentioned, the inoculum size was not significant in the model; however, the highest spore production was observed at the smallest inoculum size evaluated (1×10^5 spores g^{-1} DM). This is advantageous for scaling, as inoculum preparation often represents a major production cost. Ideally, the inoculum must ensure homogeneous and rapid colonization, but not necessarily at high levels ($>1 \times 10^7$ spores g^{-1} DM), since excessive amounts may reduce yield by depleting nutrients. The lack of significance likely reflects the fast, abundant growth of *Trichoderma*, consistent with other studies reporting no differences in sporulation across inoculum sizes (Dorta-Vásquez et al., 2019; Li et al., 2022).

3.3. Time-course analysis of sporulation and associated metabolic responses in 0.5 L PBBs

Time is another crucial factor in process scaling, as longer cultivation increases costs. After defining optimal aeration, substrate proportion, and inoculum size, O_2 consumption and sporulation were evaluated over 144 h, together with ACP activity, released P, indole derivatives, and total flavonoids (Fig. 4). Monitoring O_2 consumption provides crucial insights into the performance of aerobic microorganisms, particularly in SSF, where direct biomass quantification is not straightforward.

Fig. 4a shows the specific O_2 consumption rate (sOUR) and cumulative O_2 consumption (COC) over time. The final COC was $307.58 \text{ g O}_2 \text{ kg}^{-1} \text{ DM}$, with a maximum sOUR of $4.67 \text{ g O}_2 \text{ kg}^{-1} \text{ DM h}^{-1}$ at $\sim 43 \text{ h}$, followed by a smaller peak around 65 h, possibly indicating diauxic growth. This occurs when microorganisms sequentially utilize carbon sources, prioritizing the most readily assimilable. In SSF with BSG and WS, this behavior is expected, as the blend contains carbon sources of varying complexity. Although BSG and WS have similar total carbon (Table 1), BSG may provide a more accessible carbon fraction due to presence of residual soluble compounds and partially processed components from beer production (Ikram et al., 2017).

The sOUR values obtained in this study were higher than those reported in the literature for similar cultivations. Sala et al. (2021a) evaluated SSF of the same *T. harzianum* strain in a 0.5 L PBB using only BSG as a substrate and reported a maximum sOUR of approximately $2.2 \text{ g O}_2 \text{ kg}^{-1} \text{ DM h}^{-1}$. In a 1.5 L PBB using 70% of BSG and 30% of WC, Sala et al. (2021b) recorded a peak of $2.5 \text{ g O}_2 \text{ kg}^{-1} \text{ DM h}^{-1}$. Additionally, Ghoreishi et al. (2023), when evaluating grass clippings and pruning

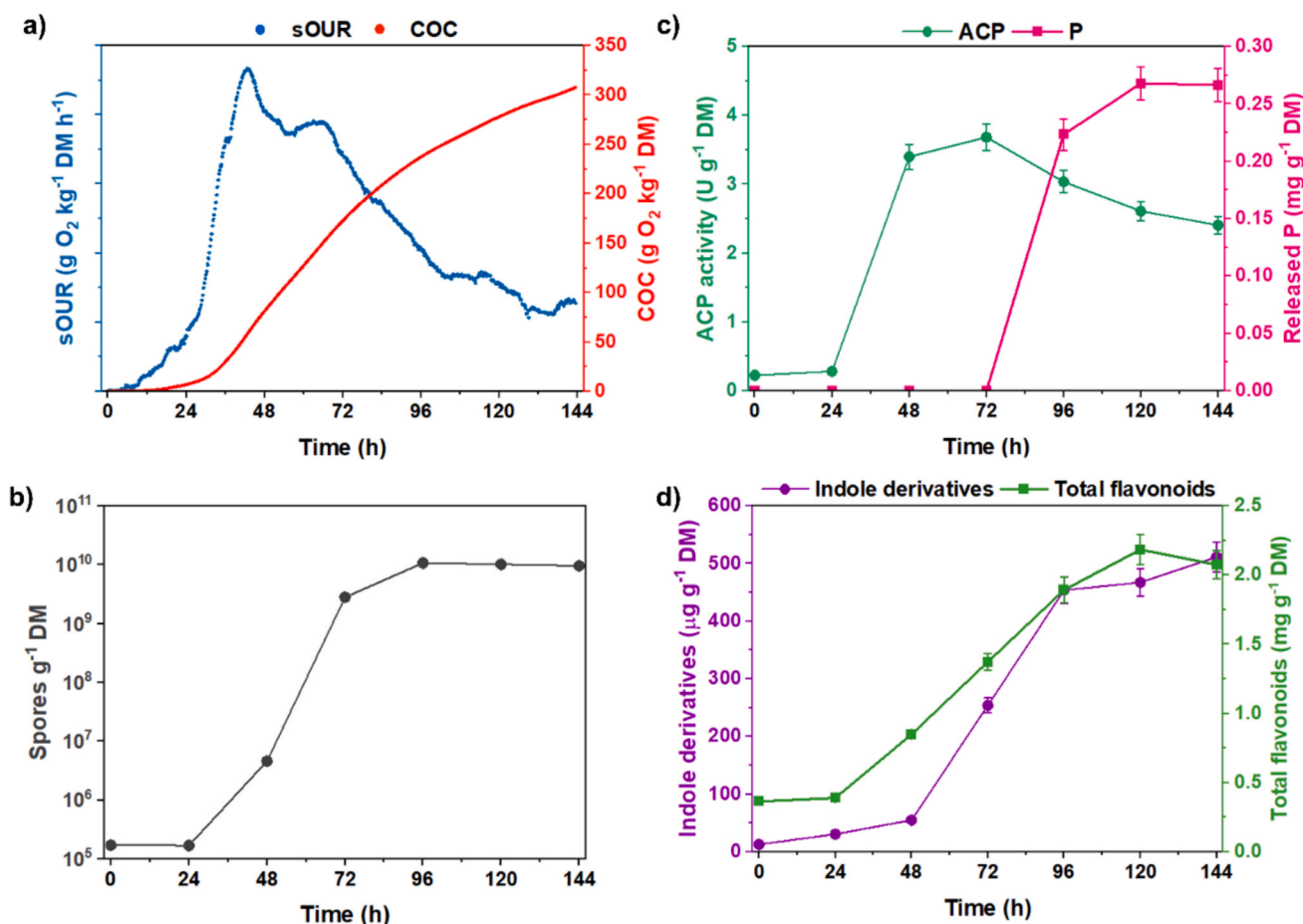


Fig. 4. Time course analysis under optimized conditions (air flow of 100 mL min^{-1} , BSG:WS proportion of 6:4, and inoculum size of 1×10^5 spores g^{-1} DM) in 0.5 L packed bed bioreactors. Profiles of (a) Specific oxygen uptake rate (sOUR) and Cumulative Oxygen Consumption (COC), (b) spore production, (c) Acid phosphatase (ACP) activity and released P, and d) indole derivatives and total flavonoids. Solid-state fermentations were conducted at 25°C .

waste as substrates, reported maximum sOUR values below $1.5 \text{ g O}_2 \text{ kg}^{-1} \text{ DM h}^{-1}$. The higher sOUR values observed in this study suggest intensified metabolic activity of *T. harzianum* under the tested conditions, potentially linked to adequate nutrient availability and favorable aeration. Although Sala et al. (2021a) used only BSG, which is a nutritionally rich medium, it results in lower porosity. These findings underscore the importance of bed porosity in SSF processes. Since O_2 availability and metabolic activity are closely linked to spore formation, it is essential to examine their influence on *T. harzianum* sporulation over time. The spore production profile under optimized conditions (Fig. 4b) shows a maximum at 96 h. Beyond this point, spore numbers remain constant, suggesting that extending the process past 96 h does not increase production, as previously observed (Sala et al., 2022).

The maximum spore production achieved in this study, 1.08×10^{10} spores $\text{g}^{-1} \text{ DM}$, is quite promising, particularly considering that no supplementation was used and the initial concentration was in the order of 1×10^5 spores $\text{g}^{-1} \text{ DM}$. This represents a 60,000-fold increase. Zhang and Yang (2015) optimized the cultivation of a *T. harzianum* strain in straw and wheat bran using Erlenmeyer flasks and obtained a sporulation of 1.49×10^{10} CFUs $\text{g}^{-1} \text{ DM}$, using an inoculum size of 1×10^7 CFU per bottle; however, their process required 8 days of cultivation and supplementation with ammonium persulfate. Under similar conditions to present study, Sala et al. (2021a) reported a maximum sporulation of 5×10^9 spores $\text{g}^{-1} \text{ DM}$, while Ghoreishi et al. (2023) observed a peak sporulation of 3.03×10^9 spores $\text{g}^{-1} \text{ DM}$, both using an inoculum size of 1×10^7 spores $\text{g}^{-1} \text{ DM}$.

The results for ACP activity and P release are presented in Fig. 4c. Maximum ACP activity was $3.7 \text{ U g}^{-1} \text{ DM}$ at 72 h, then declined to $2.4 \text{ U g}^{-1} \text{ DM}$ at 144 h. This result is lower than the $4.8 \text{ U g}^{-1} \text{ DM}$ observed in Erlenmeyer flasks without ethanol addition. However, the trend was similar: a peak after 72 h followed by a decline, which may be attributed to enzyme degradation or inhibition, as previously discussed.

The higher ACP activity observed in the Erlenmeyer flask cultivation was likely due to the medium composition, which had a higher proportion of BSG (BSG:WS 7:3), providing greater N and P (Table 1), which may have better supported growth and ACP production. Despite the lower structural substrate (WS), compaction was not an issue, as each flask contained only 12.5 g of material. Additionally, the higher P content may have stimulated ACP production and secretion, since this P is bound in organic molecules such as nucleic acids and phospholipids (Wang et al., 2022).

As a result of ACP activity, the released P profile increased after 72, reaching a maximum of $0.27 \text{ mg P g}^{-1} \text{ DM}$ (P_2O_5). This value is also lower than that previously obtained in Erlenmeyer flasks, likely due to the factors discussed.

The profiles of indole derivatives and total flavonoids are presented in Fig. 4d. It is well known that one of the main pathway microorganisms use to produce IAA and related molecules is derived from tryptophan; however, *T. harzianum* can also synthesize them from naturally occurring compounds in the substrate, particularly BSG, which is rich in proteins and phenolic compounds (Ikram et al., 2017). Studies indicate that process conditions and substrate composition affect IAA production, suggesting alternative biosynthetic pathways beyond direct tryptophan use (Nieto-Jacobo et al., 2017; Prado et al., 2019a). In this study, tryptophan was not added to reduce costs and simplify the process.

Both profiles increased over time and stabilized after 96 h. Indole derivatives reached a maximum of $511.3 \mu\text{g g}^{-1} \text{ DM}$ at 144 h, equivalent to $32.7 \mu\text{g mL}^{-1}$ considering the solid-liquid extraction ratio. Napitupulu et al. (2019), optimizing IAA production by *T. harzianum* in flasks with 1% tryptophan, also observed equilibrium after 96 h, with a maximum of $10.1 \mu\text{g mL}^{-1}$. In contrast, Ghoreishi et al. (2023), evaluating grass and pruning waste with 0.45% tryptophan in a 0.5 L PBB over 10 days, reported a peak of $101.46 \mu\text{g g}^{-1} \text{ DM}$ on day 3, followed by a decline likely due to IAA degradation as a nutrient source, given limited readily assimilable carbon and nitrogen.

Prado et al. (2019b) evaluated indole derivative production by

T. harzianum in SSF using agro-industrial by-products with 1% tryptophan, reporting a maximum of $97.3 \mu\text{g mL}^{-1}$ on wheat bran. Higher production was reported by Prado et al. (2019a) with *Aspergillus flavipes* on soybean bran, reaching $655 \mu\text{g mL}^{-1}$ with 1.5% tryptophan. These results highlight the variability in indole production depending on microorganism, medium, and process. Since no exogenous tryptophan was added here, the observed production is promising and may enhance P acquisition and plant growth.

Regarding the total flavonoid profile, a maximum of $2.2 \text{ mg g}^{-1} \text{ DM}$ was reached after 120 h, equivalent to 218.4 mg L^{-1} , representing a 6-fold increase compared to the initial flavonoid content in the substrates ($0.36 \text{ mg g}^{-1} \text{ DM}$). Microbial production of flavonoids under SSF conditions remains relatively underexplored; however, recent studies have demonstrated its feasibility. For instance, Oliveira et al. (2026) reported flavonoid production values up to $0.75 \text{ mg g}^{-1} \text{ DM}$ through a SSF process with *T. harzianum* in PBB using spent coffee and BSG as substrates, indicating that the results obtained in this study are noteworthy.

In addition, some studies indicate that *Trichoderma* application in plants can increase their flavonoid content. Imran et al. (2023) investigated the application of culture filtrates from three *Trichoderma* spp. in tomato leaves and found a significant increase in total flavonoid content. Similarly, Pascale et al. (2017) reported a significant increase in the polyphenol content in *Vitis vinifera* when applying spores of a *T. harzianum* strain as well as a secondary metabolite (6-pentylapyrone).

Other microorganisms have also been explored for their ability to enhance flavonoid content of some substrates. Zhao et al. (2023) investigated the transformation of total flavonoids in mulberry leaves via SSF and achieved a 3-fold increase (24.42 to $72.55 \text{ mg rutin equivalents/g dry weight}$) using *Aspergillus cristatus*. Likewise, Xiao et al. (2021) reported a 1.3-fold increase in total phenolic content in black soybeans after SSF with *Eurotium cristatum*. From a metabolic perspective, the increase in flavonoid content during SSF may be associated with both fungal biosynthesis and substrate biotransformation. In fungi, the shikimate pathway links central carbon metabolism to the biosynthesis of aromatic amino acids, which serve as precursors for a wide range of aromatic and phenolic metabolites (Shende et al., 2024). In addition, enzymatic activities enhanced under SSF conditions, particularly hydrolytic enzymes, can promote the release of bound phenolic compounds from lignocellulosic matrices (Selo et al., 2024). Thus, the 6-fold increase in total flavonoid content observed in this study is highly promising. It may contribute to several plant development benefits, including enhanced P and nutrient acquisition, increased resistance to pathogens and abiotic stress, and higher overall yield (Shah and Smith, 2020).

3.4. Scale-up: 22 L packed bed bioreactor

After optimizing key process parameters and analyzing the temporal profiles of biomolecules of interest in the 0.5 L PBBs, the scale-up to a 22 L PBB was evaluated. Based on the dimensions of the 0.5 L PBB, the superficial air velocity (Eq. (2)) corresponding to the optimal air flow rate (100 mL min^{-1}) was approximately 0.5 mm s^{-1} . To maintain this velocity in the 22 L PBB, an air flow rate of approximately 1500 mL min^{-1} was required. This same flow rate was previously used by Sala et al. (2022) using the same strain and bioreactor configuration, with no reports of harmful effect due to temperature rise, drying, or O_2 limitation. In contrast, in the present study, a pronounced temperature increase occurred during the first batch. To address this, two modifications were implemented for the second batch: the addition of 20% wood chips (WC) as bulking agent and an increase in air flow rate to 2000 mL min^{-1} .

Regarding the cultivation time, 4 days were initially selected, as Fig. 4 indicates that the profiles of spores, indole derivatives, and total flavonoids began to stabilize after 96 h. However, in the first 22 L PBB batch, O_2 consumption started later (Fig. 5a) than in 0.5 L PBBs (Fig. 4a), so cultivation was extended to 144 h (6 days). Operating conditions,

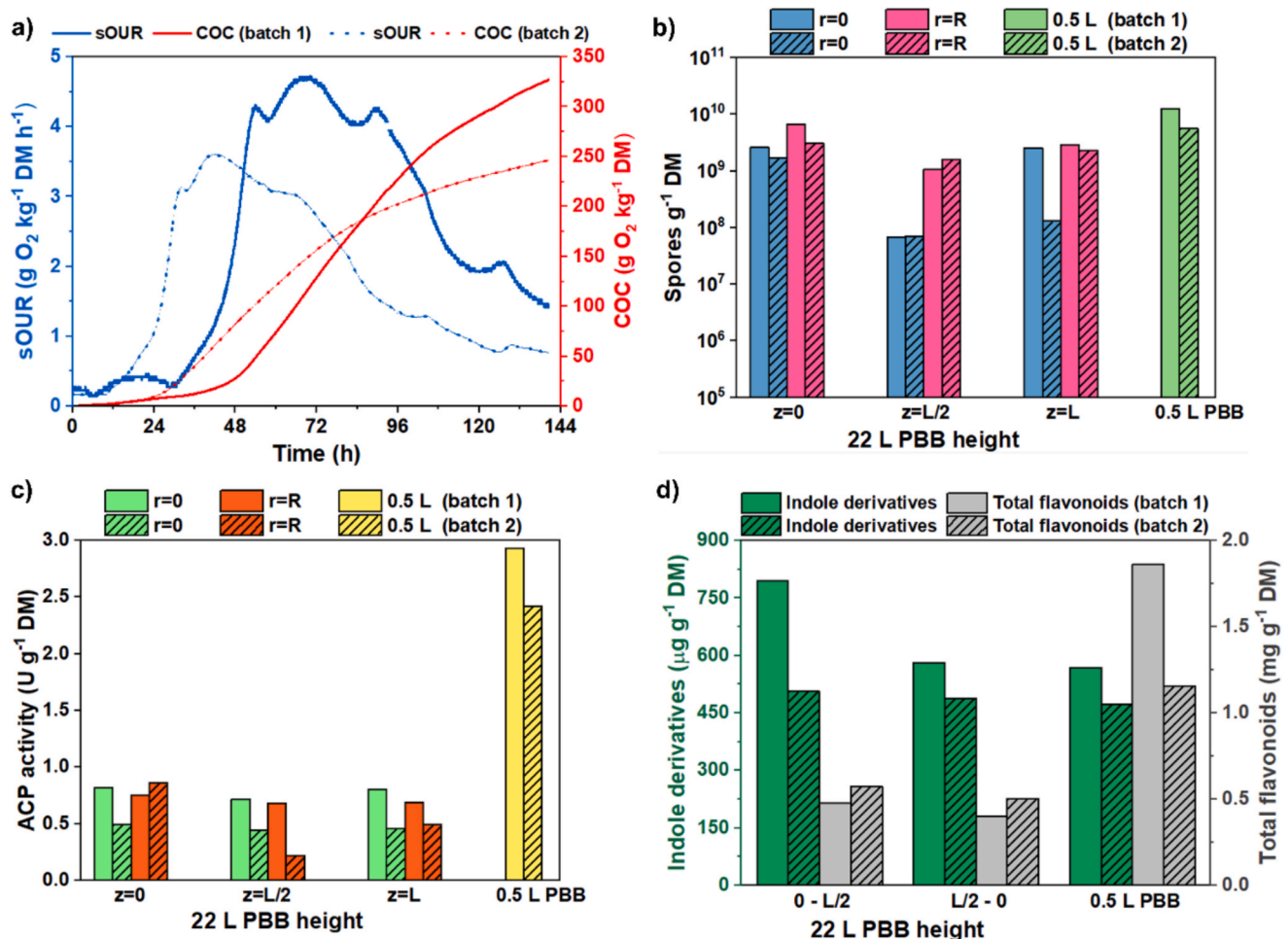


Fig. 5. Results from solid-state fermentation process in packed-bed bioreactors (PBB) of 22 L and 0.5 L. Profiles of (a) Specific oxygen uptake rate (sOUR) and Cumulative oxygen consumption (COC), (b) spore production, (c) ACP activity, and (d) production of indole derivatives and total flavonoids.

maximum and average temperature, peak O₂ consumption rates, and average yields of spores and metabolites for each batch are summarized in Table 3, while spatial variations are shown in Fig. 5. As mentioned in Section 2.2.4, the same inoculated material was distributed in three 0.5 L PBBs to compare results and identify scale-up issues; these results are also in Table 3.

Maximum sOUR and COC values in the 22 L bioreactor were similar to those in 0.5 L PBBs with the same material (Table 3), with peaks around 70 and 40 h for batches 1 and 2, respectively. The delayed peak in batch 1 likely resulted from lower initial temperatures (Fig. 6b). Lower sOUR and COC in batch 2 reflect the 20% WC addition, reducing the proportion of easily assimilated substrates and metabolic activity. Sala et al. (2022) and Ghoreishi et al. (2024) reported maximum sOUR of 3.3 and 1.1 g O₂ kg⁻¹ DM h⁻¹, respectively; here, rates were higher at 4.72 (batch 1) and 3.21 g O₂ kg⁻¹ DM h⁻¹ (batch 2), confirming vigorous microbial activity. Indeed, average spore production was 2.6×10^9 and 1.5×10^9 spores g⁻¹ DM in batches 1 and 2, respectively (Table 3). Sala et al. (2022) reported a maximum of 1.7×10^9 spores g⁻¹ DM after 6 days, highlighting the high sporulation obtained here.

When comparing results at bench and lab scale, sporulation was 4.8- and 3.8-fold lower in batches 1 and 2, respectively (Table 3). Considering that the scale-up represented a 44-fold increase in reactor volume, a reduction of less than 5-fold demonstrates that the scale-up was successful, especially considering that the inoculum size was on the order of 1×10^5 spores g⁻¹ DM, representing a growth of 4 orders of magnitude. The closer value to lab scale observed in batch 2 is likely related to the addition of 20% WC and the increase in air flow rate to 2000 mL min⁻¹,

modifications that enhanced both bed porosity and aeration.

The average production of indole derivatives was also maintained during scale-up and was even slightly higher in the 22 L PBB than in the 0.5 L PBBs, particularly in batch 1 (Table 3), where the higher BSG proportion provides more indole precursors (Ikram et al., 2017). This increase is noteworthy, as metabolite yields typically decline at larger scales due to mass and heat transfer limitations (Rodrigues et al., 2022; Casciatori et al., 2024), as observed for ACP activity and total flavonoids. The average ACP activity values were approximately 4.0- and 4.8-fold lower than those obtained in the 0.5 L PBBs for batches 1 and 2, respectively. Similarly, the average production of total flavonoids was approximately 4.2- and 2.1-fold lower than in the 0.5 L PBBs for batches 1 and 2, respectively (Table 3), likely due to an increase in temperature that occurred in both batches, reaching up to 41.5°C (Table 3).

Agro-industrial waste typically has low thermal conductivity, which, combined with the low air flow used to prevent bed drying, reduces both static and dynamic thermal conductivities, making heat removal difficult. This leads to axial and radial temperature gradients (Fig. 6) (Casciatori et al., 2016). Consequently, the increase in bed temperature enhances water evaporation, which may lead to progressive drying of the solid matrix and the development of moisture gradients within the bed. These effects contribute to heterogeneous patterns of sporulation and metabolite production (Fig. 5b, c and d).

Fig. 5b shows spore production at different positions inside the 22 L PBB for each batch. The maximum values of sporulation were 6.7×10^9 spores g⁻¹ DM and 3.1×10^9 spores g⁻¹ DM for batches 1 and 2, respectively, both occurred in the region near to the air inlet and the

Table 3

Operating conditions and main results for the two batches conducted in the 22 L packed bed bioreactor.

	Batch 1		–	Batch 2	
	22 L	0.5 L		22 L	0.5 L
Substrates proportion (BSG: WS)	6:4	6:4		6:4	6:4
WC (%)	–	–		20	20
Inoculum size (spores g ⁻¹ DM)	1.00 × 10 ⁵	1.00 × 10 ⁵		1.00 × 10 ⁵	1.00 × 10 ⁵
Air flow (mL min ⁻¹)	1500	100		2000	100
Cultivation time (hours)	144	144		144	144
Maximum sOUR (g O ₂ kg ⁻¹ DM h ⁻¹)	4.72	5.04 ± 0.44		3.65	3.21 ± 0.43
Maximum COC (g O ₂ kg ⁻¹ DM)	327.93	319.54 ± 30.72		246.40	219.25 ± 35.77
Maximum temperature (°C)	41.50	25.00		41.50	25.00
Average temperature (°C)	27.56	25.00		28.99	25.00
Average sporulation (spores g ⁻¹ DM)	2.64 × 10 ⁹	1.27 × 10 ¹⁰ ± 1.80 × 10 ⁹		1.47 × 10 ⁹	5.52 × 10 ⁹ ± 6.53 × 10 ⁸
Productivity (spores g ⁻¹ DM d ⁻¹)	4.40 × 10 ⁸	2.12 × 10 ⁹ ± 3.00 × 10 ⁸		2.45 × 10 ⁸	9.2 × 10 ⁸ ± 1.09 × 10 ⁸
Average ACP activity (U g ⁻¹ DM)	0.74	2.93 ± 0.27		0.50	2.42 ± 0.21
Average total flavonoids (mg g ⁻¹ DM)	0.44	1.86 ± 0.17		0.54	1.16 ± 0.09
Average indole derivatives (μg g ⁻¹ DM)	687.84	568.61 ± 62.55		497.50	471.95 ± 41.06
Average P released (mg g ⁻¹ DM)	0.21	0.24 ± 0.07		0.17	0.16 ± 0.03

BSG: beer spent grains; WS: wheat straw; WC: wood chips; DM: dry matter; sOUR: specific oxygen uptake rate; COC: cumulative oxygen uptake rate; ACP: acid phosphatase.

wall ($z = 0$, $r = R$), that is the coldest region inside the bioreactor, as shown in Fig. 6. This figure presents the temperature profiles along with the maximum and average values recorded at various radial and longitudinal positions within the 22 L PBB. Temperature peaks observed every 24 h in Fig. 6b and c were attributed to environmental fluctuations caused by direct sunlight received through laboratory windows. Conversely, the lowest values of sporulation were 6.9×10^7 spores g⁻¹ DM and 7.1×10^7 spores g⁻¹ DM – two orders of magnitude lower – for the batches 1 and 2, respectively, both occurring in the central region of the bioreactor ($z = L/2$, $r = 0$), which is the hottest area (Fig. 6). Sala et al. (2020), who evaluated the effect of temperature on the strain studied here, reported a substantial decrease in spore production above 32°C. Nevertheless, considering that the inoculum size was on the order of 1×10^5 spores g⁻¹ DM, even in the hottest region of the bioreactor the spore content still increased by two orders of magnitude.

As mentioned earlier, the metabolites most negatively affected by scale-up were ACP activity and total flavonoids, however, their values did not vary substantially across different positions within the bioreactor, as shown in Fig. 5c and d, respectively. In Fig. 5d, the initial flavonoid content was subtracted. This pattern suggests that even moderate temperature increase may have impaired the synthesis and excretion of ACP, and possibly its stability (Vasileva-Tonkova et al., 1996), as well as the production of total flavonoids. Indeed, even in the coldest regions of the bioreactor, the values were markedly lower than in the 0.5 L PBBs. Flavonoids are thermosensitive molecules, which explains the significantly reduced values observed in the 22 L PBB. In addition, their synthesis by fungi can be impaired at elevated temperatures (Jiang et al., 2018).

Another factor that may have contributed is bed compaction, which affects O₂ transfer, CO₂ removal, and metabolite production. Although

WS addition creates voids to facilitate heat removal, the bed was compacted by 6 cm at the end of batch 1. For batch 2, 20% WC was added, as it has greater mechanical resistance than WS and could prevent compaction. Notably, the percentage of WC added was relative to the total mass packed in the bioreactor. In addition to this, the air flow rate was increased to 2000 mL min⁻¹ (increase of approximately 33%). However, despite a lower compaction of 4 cm in batch 2, these strategies were not sufficient to prevent overheating. In contrast, Sala et al. (2022) reported a maximum temperature around 30°C and an average of 25.7°C after 6 days of cultivation using a bed composed of 40% BSG and 60% WC. Ghoreishi et al. (2024) also worked with a 22 L PBB, using 56% grass and 44% bulking agent (pruning waste or WC) and observed no overheating, with maximum temperatures of 31°C for both bulking agents. However, spore and IAA production were higher with WC, likely due to reduced compaction and improved aeration. The lower heat generation observed with grass as a substrate is consistent with its nutrient-poor composition, which supports slower microbial growth compared with BD and WS.

As mentioned previously, indole derivative production was not negatively affected by scale-up and was even higher than in the 0.5 L PBBs in batch 1, especially near the air inlet (Fig. 5d). This may be related to temperature increase during bench-scale cultivation. Environmental factors, particularly temperature, strongly influence IAA production, though effects vary by strain and conditions. Napitupulu et al. (2019) reported optimal IAA production at 27°C for a *T. harzianum* strain, differing from its 30°C growth optimum. In contrast, Ghoreishi et al. (2023) found that the highest IAA production by the strain studied here occurred at 30°C, whereas maximum sporulation was observed at 25°C. These findings highlight the need for further in-depth studies to clarify temperature influence on IAA production under different cultivation conditions.

Overall, the scale-up results were satisfactory, particularly for spore and indole derivative production. These findings demonstrate that the 22 L PBB can successfully support large-scale cultivation. Future work may focus on optimizing air flow rates and adjusting the proportion of WC to further enhance thermal management and maximize metabolite production.

4. Conclusions

This study demonstrated the potential of *Trichoderma harzianum* cultivated by solid-state fermentation (SSF) using beer spent grains (BSG) and wheat straw (WS) as substrates in packed-bed bioreactors (PBBs) to produce metabolites recognized as phosphorus (P) acquisition promoters in plants. BSG and WS proved to be effective substrates, offering a sustainable alternative to rice-based production. The strain produced acid phosphatase and mobilized organic P, confirming its role in P cycling. A Box–Behnken design showed that sporulation was mainly influenced by substrate composition and air flow, while inoculum size was not significant, indicating that low inoculum levels are sufficient. High sporulation was achieved at both lab and bench scales (1×10^{10} and 2×10^9 spores g⁻¹ dry matter (DM) in 0.5 and 22 L PBBs, respectively), along with the production of indole derivatives and flavonoids. Scale-up was successful, although improved heat dissipation may enhance performance.

CRedit authorship contribution statement

Natalia Alvarez Rodrigues: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jana Font:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Esther Molina Peña:** Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Arnau Sala:** Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Fernanda Perpétua Casciadori:** Writing – review & editing, Supervision. **Cristiane Sanchez Farinas:** Writing – review &

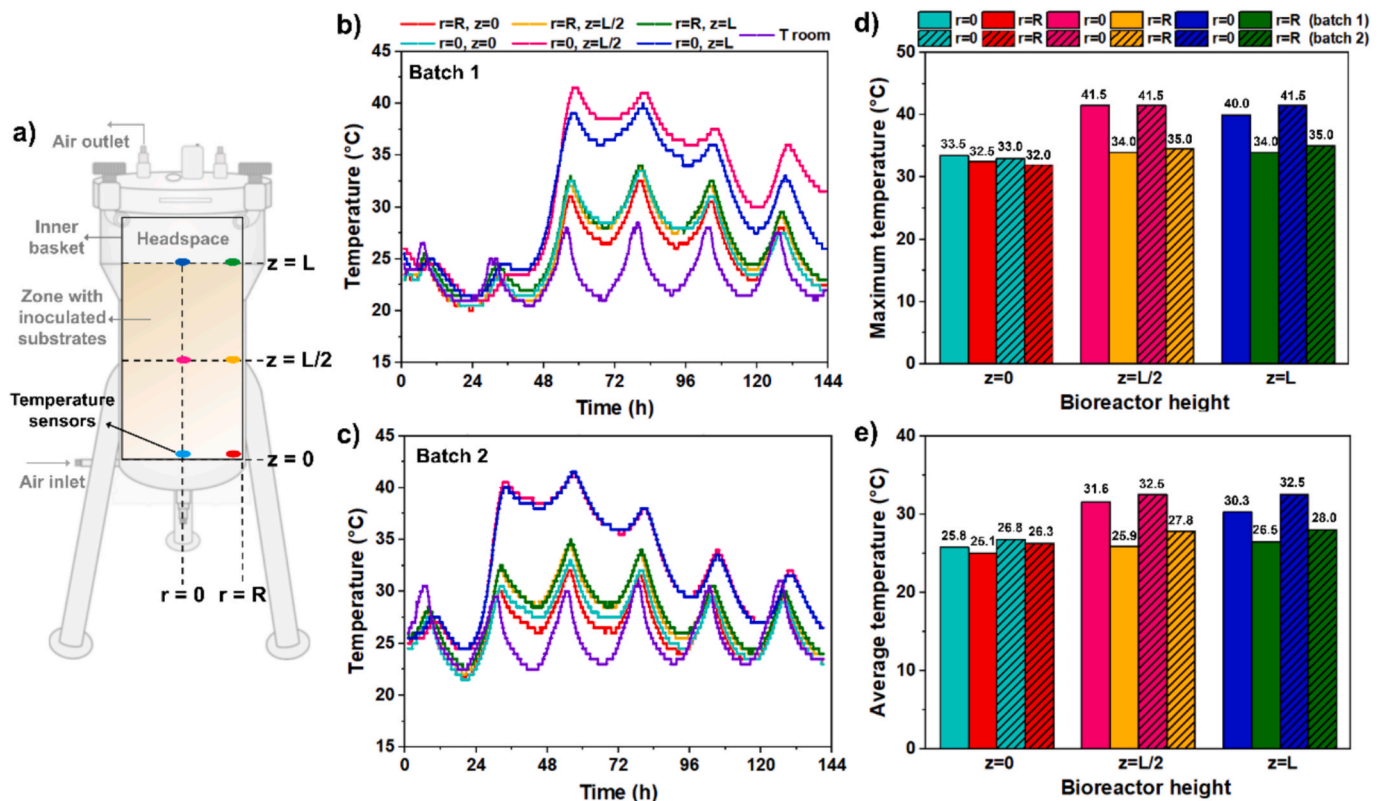


Fig. 6. Thermal behavior during solid-state fermentation in the 22 L packed bed bioreactor: (a) position of temperature sensors inside the bioreactor; (b) temperature profiles over time for the two batches; (c) maximum and (d) average temperatures recorded at each sensor position.

editing, Supervision. **Adriana Artola:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

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Data availability

Data will be made available on request.

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