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Insights on fungal solid-state fermentation for waste valorization: Conidia and chitinase production in different reactor configurations

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ABSTRACT

Different reactor configurations are paired with a wide variety of agro-industrial wastes of different biodegradability to produce fungal conidia by solid-state fermentation.

This work presents a preliminary comparative study between packed-bed and tray reactor configurations to produce *Beauveria bassiana* and *Trichoderma harzianum* conidia using two different substrates in terms of biodegradability: rice husk or beer draff complemented with wood chips. Conidia production, mean temperature and respiration indexes have been analysed in most of the presented reactor configurations. Both strains showed higher conidia production when using beer draff complemented with wood chips as substrate due to the use of a mixture as substrate. When working with beer draff, chitinase analyses obtained similar profiles in both strains but higher overall values using TH. Conidia and chitinase production maximums were not achieved at the same time, having 2–3 days of difference depending on the strain. No significant differences in mean temperature were shown between most of the performed fermentations. As a result of the present work, further scaling of both packed bed and tray configurations using beer draff and wood chips to produce BB or TH conidia would be advisable. More experiments should be performed to optimize both conidia and chitinase productions to enhance the quality of the final product.

1. Introduction

The traditional use of chemical pesticides for pest management has led to numerous problems as they are harmful both for human health (due to their toxicity and mutagenic capabilities) and the environment (due to their toxicological effect). Biopesticides are considered an environmentally friendly alternative due to their harmless nature both to humans and to the environment. Among them, biocontrol agents represent a promising replacement due to their effectiveness on more than 1000 species while presenting no harm to humans or to the ecosystem (Thakore, 2016; Mascarin and Jaronski 2016; Araújo and Huges 2016).

Among biocontrol agents, fungal biopesticides represent one of the most interesting options. Fungal biopesticides can be produced

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both by submerged fermentation (SmF) or solid-state fermentation (SSF). Although each system presents several advantages and drawbacks (Pandey, 2003), SSF (defined as a process that occurs in the absence or near absence of free water) remains as the most used method as it is the only way to produce aerial conidia, the most infective fungal propagule (Mascarin and Jaronski 2016). In addition, lower costs mainly related to the use of agro-industrial wastes as substrates (serving both as nutrient and support for fungi) also make SSF preferred over SmF, as their products present added value due to residue valorization (De la Cruz Quiroz et al., 2015; Ballardo et al., 2017). A comprehensive list of used substrates was reviewed by Sala et al. (2020), specially using genera *Beauveria* spp. and *Trichoderma* spp. *Beauveria* spp. have become one of the most-studied and used fungal entomopathogens worldwide due to its pathogenicity on more than 700 host species (Mascarin and Jaronski 2016). *Trichoderma* spp. have become a widely recognised biocontrol agent due to their antagonistic properties, being especially effective against soil-borne diseases (Verma et al., 2007; Sharma et al., 2014).

Different reactor configurations have been used to produce fungal biopesticides by SSF. Among them, packed bed and tray reactors are some of the most studied designs, presenting different advantages and drawbacks. Packed bed reactors (mainly presented as cylindrical columns) facilitate oxygen availability via continuous forced aeration, as well as maintaining constant moisture which is achieved when supplied air is previously saturated with water. However, the main disadvantage lies in heat removal, causing important difficulties in process scale-up (Krishania et al., 2018). Tray reactors have been traditionally used for SSF spore productions due to its simplicity. They can also allow aeration when bottom of the trays is perforated and moisture control when placed in closed chambers. Bed height is often between 5 and 15 cm. However, they also present mass transfer and heat limitations, leading to internal temperature gradients and large gas concentrations depending on bed height (Jou and Lo 2011; Krishania et al., 2018). Typically, substrates with high potential biodegradability are the ones which cause more difficulties in terms of heat removal, which in most cases tend to have low porosity (Barrena et al., 2011). On the other hand, use of high porosity substrates to increase heat transfer is a possible path to reduce negative effects on fungal growth and sporulation, with both alternatives already been tested in SSF (Mishra et al., 2016). In addition, starchy substrates (which are preferred for fungal growth) combine adequate porosity levels with moderate biodegradability (Ramachandran et al., 2008).

Mycoparasitism, which is closely related to fungal biopesticides insecticidal activity, depends on the presence of various enzymes. Conidia formation occurs without overall protein synthesis, but with the formation of wall. Consequently, chitin content at the end of growth is considered as a good indicator of conidia formation in fungi (Desfarges et al., 1987). Chitinases partially degrade various insects, nematodes or fungi cell wall (González et al., 2010), presenting high relevance in pests' control due to its high abundance in insects, arthropods and fungi (Berini et al., 2018). Recent attention has been given to the capacity presented by fungal biocontrol agents of producing chitinase and other hydrolytic enzymes. In the case of *Trichoderma*, chitinase has been stated as the enzyme responsible of its biocontrol capabilities (Anand and Reddy. 2009). Despite fungal growth and enzyme production close relation with fermentation conditions, there is still a lack of studies on both of them (Aita et al., 2019), specially related to SSF systems.

In previous works, main process parameters and their values for SSF using BB or TH were determined using rice husk as substrate (Sala et al., 2020). A scan of different substrates for the same process was also performed resulting in rice husk and beer draff as the most adequate for both fungal strains among other relevant outcomes (Sala et al., 2021a). Additionally, process scale-up and operational strategies have been studied for TH fermentations on rice husk and beer draff (Sala et al., 2021b). All mentioned experiments were performed on packed bed reactors. The present work is an attempt to study another reactor configuration with two different substrates using the two fungal strains also introducing the determination of chitinases as a biopesticide potential indicator. The aims of this work are: i) to compare the suitability of two substrates with different biodegradability (rice husk and beer draff) for fungal conidia production by SSF using both *Beauveria bassiana* (BB) or *Trichoderma harzianum* (TH) as inoculum. ii) to present an initial comparison between packed-bed and tray reactor configurations for fungal conidia production. iii) to provide a first approach on chitinase role in conidia production with both tested strains. With these objectives in mind, both BB and TH were cultivated using rice husk or beer draff as substrates in three bioreactors: two packed beds (1.5 L and 22 L) and one tray (8.5–13.5 L working volume, 43.5 L total volume).

2. Materials and methods

2.1. Fungal strains

Tests were carried out using two different strains, *Beauveria bassiana* (BB) (CECT, 20374) and *Trichoderma harzianum* (TH) (CECT 2929). The original strain was preserved at −80 °C in sterile cryovials containing 10% glycerol. Fungal strains were cultured in potato dextrose agar (PDA) (BB) or in malt extract agar (MEA) (TH) at 30 °C for 6–8 days before use.

2.2. Raw materials

Rice husk (Husk Ventures S.L., Barcelona) and beer draff (Cervesa del Montseny S.L., Sant Miquel de Balenyà) were used as substrates for fungal conidia production. Rice husk was stored at room temperature (20–25 °C) and beer draff was stored frozen before its use. In order to maintain values of 55–60% (Sala et al., 2020), initial moisture was adjusted before inoculation by adding the necessary volume of water when using rice husk and the necessary quantity of wood chips (Acalora, Ivars d'Urgell) when using beer draff. Wood chips added to beer draff also ensured a proper air filled porosity (AFP_R) in beer draff fermentations according to the results presented in Sala et al. (2021a) (70 beer draff/30 wood chips (w/w) when working with 1.5 L packed bed or with tray bioreactor and 40 beer draff/60 wood chips (w/w) when working with 22 L packed bed). Raw materials characterization of all substrates used in all presented fermentations (expressed as mean values for all supplies of the same substrate) is presented in Table 1. All substrates were autoclaved (121 °C for 30 min) prior to inoculation, considering the 10% inoculum volume in the initial moisture

calculations.

2.3. Solid-state fermentation

Experimental set-ups for all reactor configurations are presented in Fig. 1.

2.3.1. Experimental set-up for 1.5 L packed-bed bioreactors

1.5 L experimental set-up is shown in Fig. 1a). 1.5 L reactors consisted of polyvinyl chloride cylindrical reactors of 0.21 m height and 0.105 m internal diameter, corresponding to a working volume of 1.35 L. 300 g of each substrate were fermented per triplicate for a maximum of 8–9 days depending on the used strain. Minimum fermentation time was always chosen according to results on optimal conidia production time obtained in previous works (Sala et al., 2020). Temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) were used to obtain accurate temperature profiles at different reactor heights (0, 5, 10, 15 and 20 cm in each reactor) and ambient temperature. Constant aeration of 60 mL/min for rice husk (0.18–0.33 mL min^{-1} g^{-1}dm) and 100 mL/min for beer draff (0.71–0.96 mL min^{-1} g^{-1}dm) was continuously provided by means of a mass flowmeter (Mass-Stream D-6311, Bronkhorst, NL). The oxygen percentage in the output gases was measured by an electrochemical O_2-A_2 oxygen sensor (Alphasense, UK). Data analysis was performed by a non-commercial tailor-made software Arduino® based that calculates the respiration rates as explained in section 2.3.4.

Reactors were loaded and mixed in laminar flow chamber with the appropriate volume of inoculum, ensuring a homogeneous distribution and sterile conditions. Prior to the start of each test, all reactors were cleaned with water and bleach to prevent possible contamination, as they could not be autoclaved.

2.3.2. Experimental set-up for 22 L packed-bed bioreactors

22 L experimental set-up is shown in Fig. 1b). In this case, the reactor consisted of a cylindrical stainless-steel vessel with a removable basket of 48 cm height x 24.5 cm diameter, presenting a total volume of 22 L. In all fermentations, the working volume was approximately 90% of the reactor capacity. When working with rice husk, 3000 g of non-inoculated substrate were loaded, while when working with beer draff, 4000 g of mixture with wood chips were loaded into the basket. Air supply and acquisition data system were the same as in section 2.3.1. Fermentation time was adjusted following the same conditions as in 1.5 L reactors. Constant aeration of 500 mL/min for rice husk (0.28–0.42 mL min^{-1} g^{-1}dm) and of 1500 mL/min for beer draff (0.53–0.87 mL min^{-1} g^{-1}dm) was provided. Temperature of the solid media was monitored on-line in the lower half of the bed by means of a temperature probe (Pt-100 sensors, Sensotrans), while also obtaining accurate temperature profiles at different heights of the bed (0, 12, 24 and 36 cm) both at the centre of the packed bed and at the basket wall using the temperature sensors described in section 2.3.1. Ambient temperature was also monitored.

To work in conditions as sterile as possible, the reactor and the basket were cleaned with water, bleach and alcohol before and after every batch. When working with BB, inoculation and basket loading were performed in laminar flow cabinet using autoclaved material. When working with TH, inoculation was performed in ambient conditions in the laboratory before loading the substrate directly into the basket, using previously cleaned trays.

2.3.3. Experimental set-up for tray bioreactors

Tray bioreactor experimental set-ups are shown in Fig. 1c) and d). Tray bioreactor consisted of an incubator (Memmert ® GmbH + Co.KG P.O. Box 1720 91107 Schwabach Bundesrepublik Deutschland/Germany) adapted as tray bioreactor with 2 trays (set-up a, rice husk test) or 3 (set-up b, beer draff tests). Each tray dimensions were 39.5 cm length x 27.5 cm width, with 4 cm substrate bed height in all fermentations. Loaded substrate quantity was of 900 g in rice husk fermentation (450 g per tray), of 1.5 kg in BB beer draff fermentation (500 g per tray) and of 2.25 kg in TH beer draff fermentation (750 g per tray). When numbering trays, tray 1 was always the one closer to air sprinklers, and tray 2 or 3 were located further, maintaining the same distance between consecutive trays as shown in Fig. 1c) and d). To ensure proper air distribution, airflow was provided on the bottom part of the reactor by means of four sprinklers. Two set-ups were used depending on the sprinklers' distribution: while in set-up c) sprinklers were facing the trays, in set-up d) sprinklers were positioned facing the bottom of the reactor, to improve air circulation throughout the reactor. Constant aeration of 1000 mL/min (1.75–2.68 mL min^{-1} g^{-1}dm for rice husk and 0.76–0.91 mL min^{-1} g^{-1}dm for beer draff) was provided for both substrates.

Table 1
Raw material characterization of rice husk, beer draff and wood chips used in SSF fermentations. Presented values correspond to mean values of 3 substrate batches used for each substrate, one for each tested scale/reactor conformation.

Parameter	Rice husk	Beer draff	Wood chips
Moisture (%)	10.2 ± 0.1	77.1 ± 0.2	10.4 ± 0.2
Organic matter (%)	83.5 ± 1.4	93.3 ± 0.3	99.0 ± 0.8
pH	5.7 ± 0.3	6.6 ± 0.2	4.1 ± 0.4
Carbon (%)	41.0 ± 0.6	47.1 ± 1.1	48.3 ± 0.8
Nitrogen (%)	0.4 ± 0.1	3.0 ± 0.4	0.4 ± 0.1
C/N ratio	111.2 ± 17.6	15.9 ± 1.8	120.8 ± 14.2
Bulk density (kg m^{-3})	161 ± 2	355 ± 5	109 ± 4
Total sugar content (mg g^{-1}dm)	17.7 ± 0.3	118.7 ± 6.1	90.4 ± 7.2
AFP_R (%)	90.3 ± 0.5	63.5 ± 1.6	95.1 ± 0.2

Values are the average of independent samples and its standard deviation.

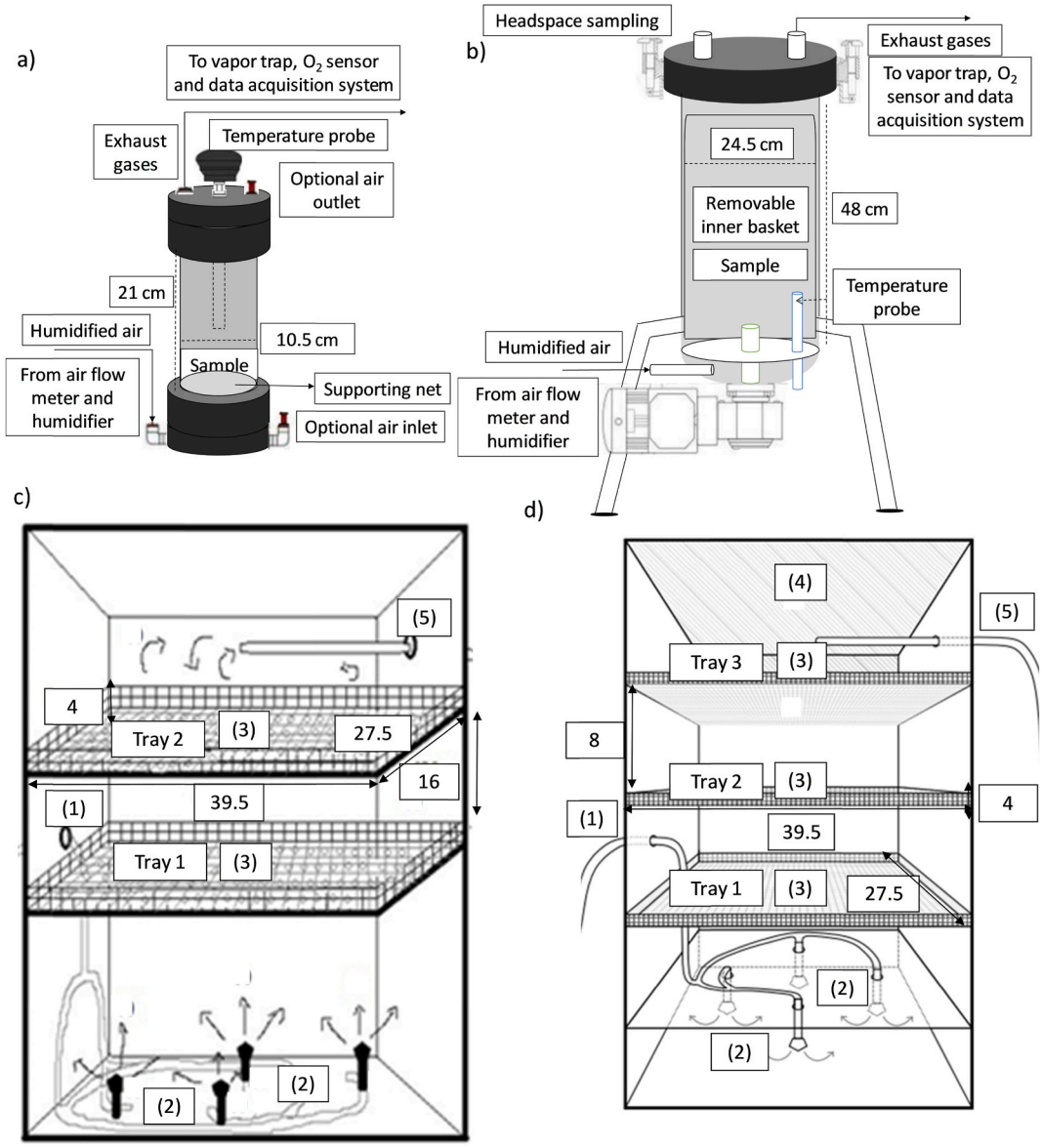

Fig. 1. Reactor set-ups for all presented fermentations. a) 1.5 L bioreactor set-up; b) 22 L bioreactor set-up; c) Tray bioreactor set-up for rice husk fermentation and d) Tray bioreactor set-up for beer draff fermentations. In c) and d) set-ups: air inlet (1); air sprinklers (2); trays with 1 cm diameter holes in the bottom (3); adsorbent (4) (only design d) and air outlet (5).

When obtained, respiration profiles correspond to the total oxygen consumption presented by all the trays in the system, as it was not possible to adapt the system to obtain respiration data corresponding to each individual tray. Adsorbent material (Vileda Professional, Freudenberg Home and Cleaning Solutions Ibérica, S.L.U.) was added to the top of the reactor (set-up d) to prevent water from exhausted air to drop onto the closest tray. Temperature profiles were obtained in all trays in different positions, placing 2 sensors in each tray and comparing to ambient temperature. Prior to all tests, both incubator and trays were cleaned and inoculated using the same method presented in section 2.3.3 for 22 L bioreactor.

2.3.4. Oxygen uptake rate

Specific oxygen uptake rate (sOUR) was calculated according to Puyuelo et al. (2010), expressed as 1h average value (sOUR) (Equation (1)) and recorded on-line in order to provide an indicator of the biological activity:

$$sOUR = F \cdot (0.209 - yO_2) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW} \quad (1)$$

where: sOUR is the specific Oxygen Uptake Rate (g O_2 kg^{-1} dm h^{-1}); F, airflow (mL min^{-1}); yO_2, is the oxygen molar fraction in the

exhaust gases (mol O_2 mol^{-1}); P, pressure of the system assumed constant at 101325 Pa; 32, oxygen molecular weight (g O_2 mol^{-1} O_2); 60, conversion factor from minute to hour; 10^3, conversion factor mL to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, temperature at which F is measured (K); DW, initial dry weight of solids in the reactor (g); 10^3, conversion factor g to mg.

The area below the O_2 consumption curve was also determined, which represents the cumulative oxygen consumption (COC).

2.4. Conidia counting

To determine fungal conidia, a Neubauer chamber (Brand™ 717805) was used. Conidiated substrate samples were mixed with Tween 80 solution (0.1% for BB or 0.01% for TH) in a 1:5 (v:v) proportion, shaken at room temperature in a shaker/incubator for 20 min (ZWYR-200D, Labwit Scientific) and diluted before counting. All cell counts were performed per triplicate and related to the dry matter present in the reactor at the counting time, following the equation:

$$\text{Concentration} = \frac{\text{N° of conidia}}{\text{CV} \cdot \text{DF}} \cdot \frac{EV}{SWW} \cdot \frac{SWW}{SDM} \quad (2)$$

where: Concentration is the conidia concentration in the initial tube (conidia g^{-1}dm); n° of conidia, the counted conidia in the Neubauer chamber at a known dilution; CV, Neubauer chamber counting volume (mL); DF, dilution factor of the counting tube; EV, extraction volume (mL); SWW, sample wet weight (g ww); SDM, sample dry matter(g dm).

2.5. Chitinase activity assay

2.5.1. Reagent preparation

Colloidal chitin was used as substrate for the reaction. For its preparation, 100 mg of colloidal chitin were weighted, mixed with 1.2 mL concentrated HCl and left overnight in the fridge with magnetic stirring. The following day, 40 mL of cold EtOH were added and the obtained solution was left overnight at room temperature with magnetic stirring. The following day, the solution was centrifuged for 25 min at 6000 g and 4 °C and the supernatant was discarded. Last step was repeated by adding 40 mL of distilled water, until achieving a pH 6.0 in the obtained solution (Berna et al., 2012).

DNS was used as reagent to determine the absorbance activity of its reduction to 3-amino-5-nitrosalicilic acid at 540–570 nm. In a covered beaker to prevent light exposure, 60 mL of distilled water were magnetically stirred while adding 1.0 g of DNS. When dissolved, 1.6 g NaOH were gradually added. In the following 20–30 min, 30 g of Rochelle salts were slowly added. Obtained solution was diluted to a final volume of 100 mL by adding distilled water (Miller 1959).

Phosphate buffer was prepared by adding 3.0 g $NaPO_2$ to 400 mL distilled water in agitation. When dissolved, pH was measured and adjusted to 6.0 by adding NaOH.

Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL sulphuric acid 95% at close to 0 °C temperature.

2.5.2. Activity determination

To perform chitinase activity assay, 10 g of sample were incubated in 30 mL phosphate buffer pH 6.0 at ambient temperature for 2.5 h without agitation. 50 mL of the liquid extracted were mixed with 450 ml phosphate buffer 50 mM and 500 mg colloidal chitin 1% w/v. Sample was incubated for 30 min at 37 °C, 750 mL DNS were added, incubated for 10 min at 100 °C and centrifuged.

Supernatant absorbance was measured at 540 nm. Using equation (3), enzymatic concentration is estimated using a previously prepared calibration curve.

$$\textit{Chitinase activity}\left(\frac{U}{gdm}\right) = \frac{(m\Delta ABS + h) \cdot DF \cdot B}{gdm} \quad (3)$$

where: m: calibration curve slope; Δabs: sample Abs – controls Abs; DF: extract dilution factor; B: total extract volume; gdm: grams of dry matter of the initial sample.

Controls abs have to be diminished from sample abs. In extract control, colloidal chitin is replaced with phosphate buffer. In chitin control, liquid extract is replaced with phosphate buffer. In the blank, all the process is performed using phosphate buffer.

2.6. Total sugar content analysis

Total sugar content was estimated using the Anthrone method (Scott and Melvin, 1953). Total glucose content was expressed as gram of glucose equivalent per gram of dry matter according to Equation (4):

$$\textit{Total sugar content} = \frac{C}{P} \cdot V \quad (4)$$

where: Total sugar content (g g^{-1}dm); C, concentration of glucose equivalents (g L^{-1}); P, weight of the dry sample (g); V, total volume of the supernatant (L).

2.7. Analytical methods

Moisture (%), dry matter (%), organic matter (%) and pH have been determined for initial and final samples using standardized methods (U.S. Composting Council, 2001). C/N analysis was performed by means of chemical elemental analysis.

AFP_R was calculated for all used substrates according to Equation (5) as presented by Richard et al. (2004):

$$AFP_R = 1 - BD_t \left(\left(\frac{1 - DM}{D_W} \right) + \frac{DM^{*}OM}{PD_{OM}} + \left(\frac{DM(1 - OM)}{PD_{ash}} \right) \right) \quad (5)$$

where: AFP_R, 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}).

2.8. Statistical analysis

Statistical difference between samples was analysed by means of a one-way ANOVA ($p < 0.05$ confidence) with the Tukey test using Minitab 17 (Minitab Ltd) software. Results were classified in letter groups. Those with different letter groups were significantly different.

3. Results and discussion

3.1. Fungal conidia production in packed bed bioreactor conformation

Fig. 2 (BB) and 3 (TH) show obtained profiles in packed bed reactor fermentations. In both Figures, a) and c) show rice husk fermentations and b) and d) beer draff fermentations, while a) and b) correspond to 1.5 L and c) and d) to 22 L. Conidia production, sOUR and temperature profiles are presented in all graphs. Successful conidia production and batch scaling were achieved in all packed bed fermentations.

Production differences were shown when working with rice husk. In BB fermentations, conidia productions were of almost $4.0 \times 10^8 \pm 4.7 \times 10^7$ conidia g^{-1}dm in 1.5 L and of $6.0 \times 10^8 \pm 5.4 \times 10^7$ conidia g^{-1}dm in 22 L, both reached at 7.8 d. When using TH, conidia productions were of almost $1.1 \times 10^9 \pm 1.7 \times 10^8$ conidia g^{-1}dm in 1.5 L and of $9.1 \times 10^8 \pm 3.1 \times 10^8$ conidia g^{-1}dm in 22 L, both reached at 5.8 d.

In terms of biodegradability, respiration profiles differed between scales. When working with BB, reaching both maximums at similar fermentation times (around 1.5 d) but with different values (0.2 vs 0.72 g O_2 kg^{-1}dm corresponding to 1.5 and 22 L). TH profiles reached similar maximum values (0.65 vs 0.74 g O_2 kg^{-1}dm corresponding to 1.5 and 22 L) but at different process times (2.3 vs 1.3 d for 1.5 and 22 L). All maximum values were always lower than 2 mg O_2 g $om^{-1}h^{-1}$, corresponding to substrates with low potential biodegradability according to Barrena et al. (2011). Temperature was kept within optimal values (around 25 °C for both strains) for conidia growth and sporulation in all fermentations, as determined in previous tests using rice husk and the same strains (Sala et al., 2020).

In comparison, both strains conidia production was far superior when using beer draff as substrate. When using BB, conidia production reached $1.5 \times 10^9 \pm 3.7 \times 10^8$ conidia g^{-1}dm at 6.8 days in 1.5 L fermentations and $2.5 \times 10^9 \pm 6.5 \times 10^8$ conidia g^{-1}dm at 7.8 days in 22 L fermentation. When using TH, productions of the same order of magnitude were obtained, being of $2.1 \times 10^9 \pm 5.0 \times$

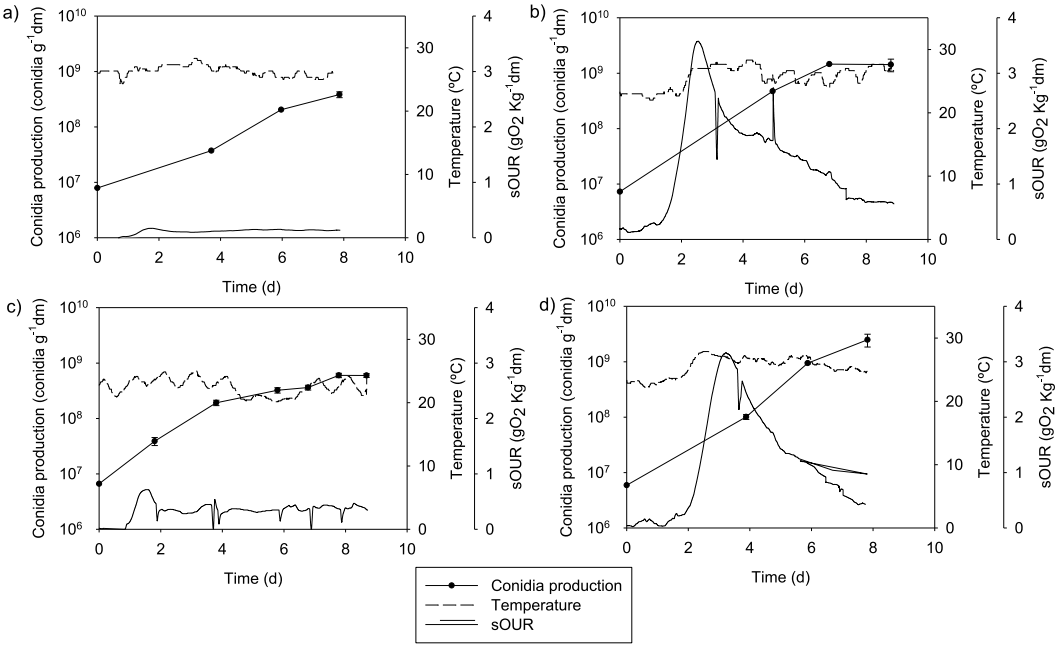

Fig. 2. BB profiles (conidia production, sOUR and mean temperature) obtained in packed bed bioreactors. a) 1.5 L rice husk; b) 1.5 L beer draff; c) 22 L rice husk and d) 22 L beer draff.

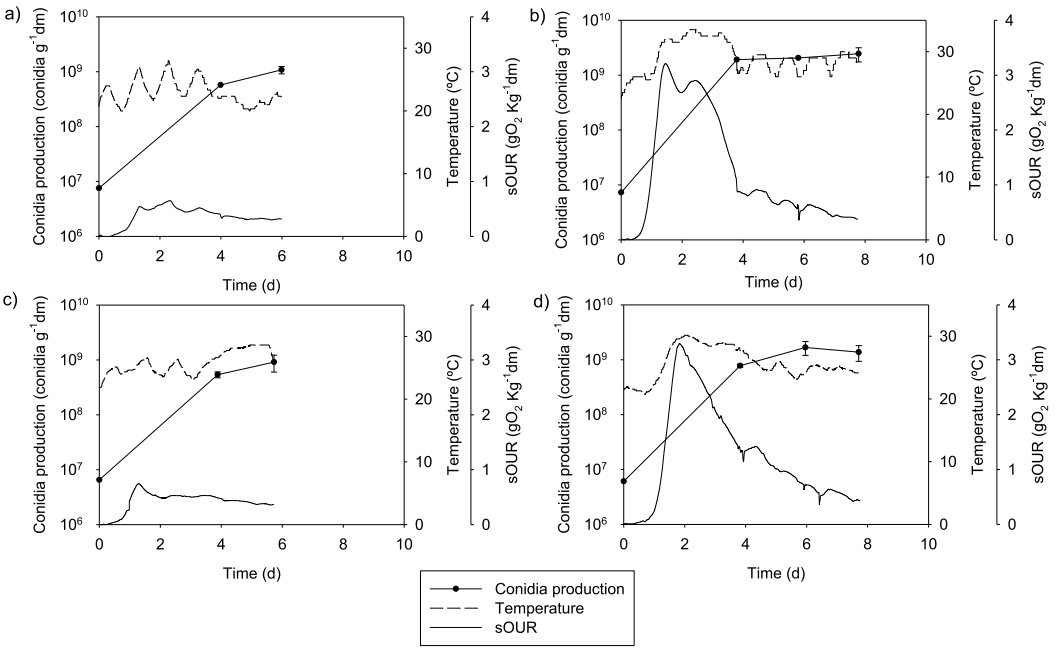

Fig. 3. TH profiles (conidia production, sOUR and mean temperature) obtained in packed bed bioreactors. a) 1.5 L rice husk; b) 1.5 L beer draff; c) 22 L rice husk and d) 22 L beer draff.

10^8 conidia g^{-1}dm in 1.5 L fermentations and $1.7 \times 10^9 \pm 4.7 \times 10^8$ conidia g^{-1}dm at in 22 L fermentation, both at nearly 6 days of fermentation time. All conidia productions were superior to their rice husk equivalents, even one order of magnitude higher for BB. Similar TH results were obtained in first batches presented in Sala et al. (2021b) for both strains. For BB, this is the first time to report conidia production using packed-bed bioreactor of 22 L with both substrates.

Temperature maintenance was better when using BB, as mean temperatures roughly surpassed the optimal value of 25 °C, whereas when using TH, they surpassed 30 °C in both scales during the first days of the fermentation. When operating with packed beds, temperature differences between different areas of the reactor are normally achieved, which might be as high as 20 °C according to Krishania et al. (2018). As both 1.5 L and 22 L packed-bed reactors were not insulated, improved results could be expected in case of heat exchange reduction.

In terms of biodegradability, respiration profiles were similar for all strains and reactors but achieved their maximums at different fermentation times. These differences were noteworthy when working with BB: maximum sOUR was achieved at 2.5 and 3.2 d in 1.5 and 22 L respectively (ranging from 3.2 to 3.6 g O_2 kg^{-1}dm), while when working with TH similar values were achieved at similar times in both scales (1.5–1.8 d and 3.2–3.3 g O_2 kg^{-1}dm). Differences in maximum sOUR time in BB had no effect on maximum conidia production time when scaling. Despite differences in biodegradability, mean temperatures profiles were similar between scales using both strains when comparing between substrates, being very close to optimal values when working with BB. This result is remarkable, as problems associated with heat transfer are the main cause of difficulties in SSF scale-up, even with works presented at laboratory scale (Pandey et al., 2008).

3.2. Fungal conidia production in tray bioreactor conformation

Fig. 4 shows profiles obtained in tray bioreactor fermentations. Fig. 4a) shows conidia production and temperature profiles obtained in rice husk fermentation inoculated with TH. Fig. 4b) and c) show conidia, temperature, sOUR and chitinase profiles obtained for beer draff fermentations using BB (4b) or TH (4c) as inoculum. Conidia production and chitinase activity are shown independently for all trays, being 2 trays in rice husk fermentation and 3 for beer draff fermentations.

When fermenting rice husk inoculated with TH, maximum conidia production was not significantly different between trays, obtaining $5.5 \times 10^8 \pm 1.4 \times 10^8$ and $8.3 \times 10^8 \pm 2.3 \times 10^8$ conidia g^{-1}dm respectively in trays 1 and 2, both at 5–6 days after the start of the fermentation. Similar conidia profiles were obtained between trays. However, conidia concentrations were lower in all cases than those obtained in PBBs, which could be due to the use of non-optimized air distribution, causing possible O_2 deficiency in some parts of the reactor. However, this hypothesis could not be confirmed, as no respiration profile was obtained in both fermentations. This behaviour might be related to the direction of the air sprinklers, which were faced up in this fermentation according to set-up shown in Fig. 1c). Consequently, the tray bioreactor design was changed from Figure 1c) to Figure 1d) in the rest of the presented tray bioreactor tests.

Mean temperature profile showed an increase during the first 3 days, stabilising in values close to the optimal of 25 °C (Sala et al., 2020) for the rest of both fermentations. The low biodegradability of rice husk (resulting in very low respiration values) difficulted

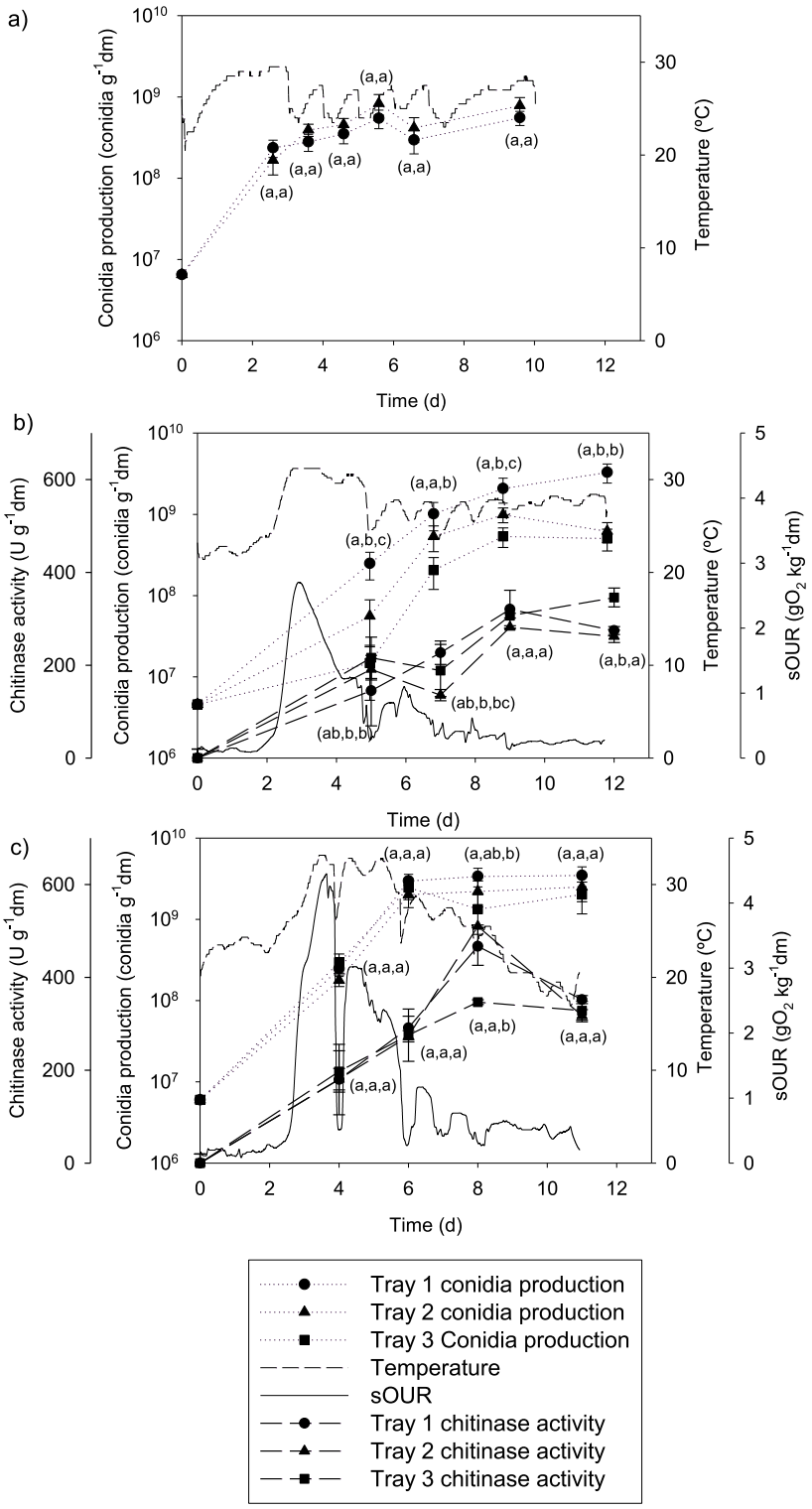

Fig. 4. Process parameters evolution (produced conidia, chitinase activity, mean temperature and sOUR) obtained in all tray bioreactors. Produced conidia (rice husk and beer draff) and chitinase activity (beer draff) are shown for each tray. Statistical difference between trays is shown in format (tray 1, tray 2, tray3).

monitoring respiration profiles. Much of the incubator's volume was not filled with substrate, leaving a huge dead volume. However, specific airflow in this test was the highest in this work (1.68–2.55 mL min^{-1} g^{-1}dm), much higher than PBB values using the same substrate (maximum of 0.6 mL min^{-1} g^{-1}dm). Thus, air leaks, coupled with rice husk's low respiration values, might be the reasons behind not obtaining respiration profile.

When fermenting beer draff, different behaviour was observed depending on the fermented strain. When working with BB, significant conidia production differences in at least two out of three trays were observed in all samplings. Although maximum conidia production was reached at 11.8 d in tray 1, trays 2 and 3 reached its maximum at day 8.8. As such, assuming 8.8 days as maximum conidia production time (at least for two out of three trays), conidia production for tray 1 was of 2.1×10^9 conidia g^{-1}dm, of 9.9×10^8 conidia g^{-1}dm for tray 2 and of 5.4×10^8 conidia g^{-1}dm for tray 3. Mean value of the three trays was $1.2 \times 10^9 \pm 7.9 \times 10^8$ conidia g^{-1}dm. This behaviour cannot be explained by lack of O_2 in trays 2 and 3, as O_2 percentages for the whole reactor only dropped to values close to 15%, demonstrating sufficient O_2 availability for all trays in the whole fermentation. However, differences between trays could be explained by air distribution through the reactor, as preferred paths might have been originated, difficulting air distribution for trays located further from the sprinklers. It is also possible that changes to air dispersion caused by the use of a different tray bioreactor set-up (facing the sprinklers down) might have influenced conidia production. However, as same substrate and strain were not tested using both set-ups, this statement remains as a hypothesis. Achieved conidia production was similar to the one obtained by Xie et al. (2013) using rice as substrate, confirming the potential of beer draff.

Similar chitinase activity profiles were achieved in all trays, with maximum chitinase production time being of around 9 days. Chitinase activities were not significantly different between trays in most of the analysed samples. Highest values in all trays were near 300 U g^{-1}dm. Maximum chitinase activity was achieved at the same time of maximum conidia productivity. In most of the presented BB

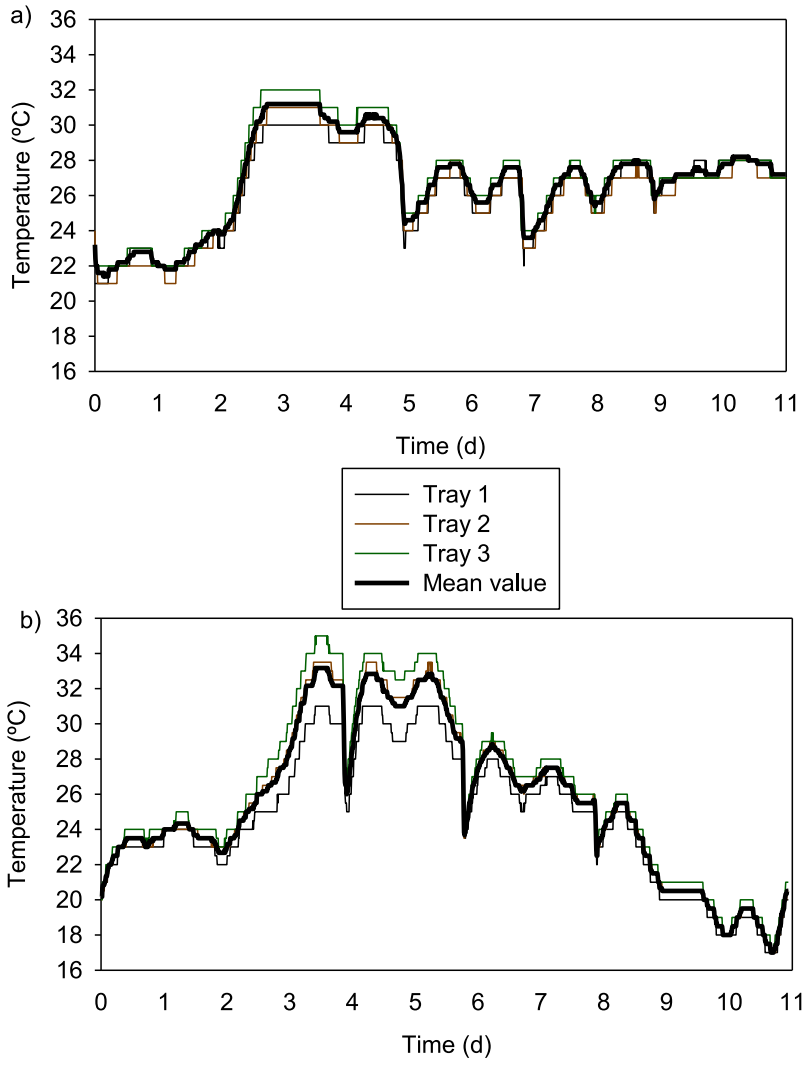

Fig. 5. Temperature profiles obtained for each tray in beer draff BB (a) and TH (b) tray bioreactors. Mean values are shown in bold.

fermentations in this thesis, this optimum has been of 7.5–8 days. With these results, this optimal could still be possible in this test. However, this statement cannot be assumed, as there is no analysis between 6.8 and 8.8 days, meaning maximum chitinase production could be achieved after maximum conidiation, as suggested by Desfarges et al. (1987). Contrary to observed behaviour in conidia production, airflow role in chitinase production seemed independent due to not presenting significant differences between trays. Before this work, no studies on chitinase role in BB conidia production have been found.

Opposing to BB beer draff results, no significant differences were observed between most of conidia productions in the different TH tray bioreactor samples. Maximum conidia production was obtained in day 6 and stabilized afterwards, being the same optimum conidia productivity time found in TH PBBs tests. Maximum conidia production was of 3.0×10^9 conidia g^{-1}dm for tray 1, 2.0×10^9 conidia g^{-1}dm for tray 2 and 2.5×10^9 conidia g^{-1}dm for tray 3 (mean value, $2.5 \times 10^9 \pm 4.9 \times 10^8$ conidia g^{-1}dm). Although maximum conidia production was still achieved in tray 1, it was not significantly different in comparison to the rest, suggesting no relevant effect of distance from air inlet when using TH, same behaviour previously found when using rice husk as substrate. Used TH strain demonstrated higher versatility in comparison to the used BB strain.

Similar chitinase activity profiles were achieved in all trays, with maximum chitinase production time being around 8 days. Chitinase activities were not significantly different between trays in most of the analysed samples, with the only exception of maximum chitinase production time. Highest values were achieved in trays 1 and 2 (465–510 U g^{-1}dm), being significantly different from tray 3 chitinase activity of 350 U g^{-1}dm. Similar to BB test, maximum chitinase activity was achieved after maximum conidia production (2 days). TH chitinase profiles were similar to the ones obtained by Sandhya et al. (2004) using SmF and mycelia as inoculum, opposed to spores used in this work. At maximum production time, chitinase production was different between trays, as the lowest activity was achieved in the tray located further from the air sprinklers, being significant in comparison to the rest. However, this difference was not observed in any other sampling. Although positive airflow influence when producing chitinases had been previously observed by other authors using various *Trichoderma* strains (De la Cruz Quiroz et al., 2017), it cannot be assumed that distance from airflow has negatively affected chitinase production when observing this difference only at one sampling time. More experiments should be performed before reaching a final conclusion on its importance. These studies should aim at maximizing both chitinase and conidia concentrations.

Beer draff fermentations temperature profiles are presented in Fig. 5, with Fig. 5a) corresponding to BB and Fig. 5b) to TH. Profiles were similar between strains, both achieving their maximums at the moment of highest respiration activity (between days 2 and 4) and decreasing after that period to stablish at values closer to the optimum. Although temperature variation between trays in BB reached only 2 °C, temperature differences might have reduced conidia production in tray 3 due to temperature relevance in fungal conidia production. BB strain is susceptible to temperature changes, even if they are only of 3 °C. Consequently, this difference might have negatively affected conidia production in trays located further from the sprinklers due to having lower heat dissipation. Temperature differences were higher when using TH, being of 5 °C between trays 1 and 3 (30–35 °C). When temperatures were not at its peak, differences between trays were of a maximum of 2 °C. Despite temperature relevance in fungal conidia production, these differences did not negatively affect conidia production in tray 3, opening the possibility of fermenting more quantity of material per tray. This behaviour is noteworthy to mention, as temperature differences between trays (or even in the same tray) would be expected in case of increasing bed height (Xie et al., 2013; Krishania et al., 2018). Differences between strains were also observed in the respiration profiles: maximums were of 2.7 and 4.2 g O_2 kg^{-1}dm for BB and TH respectively, reached at days 2.9 and 4.2 (BB and TH). Lag phase of almost 2 days was observed with both strains.

3.3. Comparison between conformations

A comparative approach between all presented fermentations in this work is shown in Tables 3–5. All conidia production values in this section correspond to mean values at maximum conidia production time.

Table 3 results allow a comparison based on parameters other than conidia production (conidia g^{-1}dm). While in terms of productivity (conidia g^{-1}dm d^{-1}) results follow a similar pattern when comparing with conidia production, different patterns are observed with total produced conidia and total produced conidia L^{-1}. When total conidia production is not corrected with total volume, 1.5 L reactors show lowest obtained productions, as expected from the lowest volume. When comparing tray results with same substrate in 22 L PB configuration, total conidia production obtained are, most of the times, in the same order of magnitude, considering differences in fermented substrate quantity. These results suggest similar conidia productions can be reached with both configurations. When correcting total conidia production using total volume, both 1.5 and 22 L PBB show higher values in comparison to tray in the majority of the reactors. This behaviour is explained by differences in working volume between reactors: while almost all the PB volume in both configurations was used as working volume, only 8 (rice husk) or 13.5 (beer draff) out of 43.5 L were used in tray configurations. As such, better spatial usage of tray configuration might improve conidia production, with possibility of reaching

Table 2
Initial parameter characterization for all substrates in all presented fermentations.

Fermentation/parameter	Moisture (%)	pH	Total sugar content (mg g^{-1}dm)	Air filled porosity (%)	Mixture (substrate/wood chips) (%/%)
1.5 L rice husk	64.2 ± 1.0	6.1 ± 0.1	12.4 ± 0.8	83.8 ± 0.4	100/0
22 L rice husk	58.7 ± 0.3	6.6 ± 0.1	13.6 ± 0.8	86.2 ± 0.4	100/0
Tray rice husk	58.5 ± 0.1	6.8 ± 0.1	13.9 ± 0.3	87.4 ± 0.6	100/0
1.5 L beer draff	63.8 ± 5.1	5.7 ± 0.2	91.1 ± 7.3	69.6 ± 0.7	70/30
22 L beer draff	55.2 ± 4.8	5.1 ± 0.8	67.9 ± 5.3	80.1 ± 0.9	40/60
Tray beer draff	63.4 ± 3.6	4.9 ± 0.3	103.5 ± 13.5	72.2 ± 2.5	70/30

Table 3
Conidia production and productivities comparison of all fermentations.

Test	Produced conidia (conidia g^{-1}dm)	Time (d)	Grams dry matter (g dm)	Total volume (L)	sAF (mL min^{-1} g^{-1}dm)	Productivity (conidia g^{-1}dm d^{-1})	Volumetric production (conidia g^{-1}dm L^{-1})	Total produced conidia	Total produced conidia L^{-1}
Tray bioreactor RH TH	7.0×10^8	5.8	387	43.5	1.75–2.68	1.2×10^8	1.6×10^7	2.7×10^{11}	6.2×10^9
Tray bioreactor BDr BB	1.2×10^9	8.8	540	43.5	0.76–0.91	1.4×10^8	5.5×10^7	6.5×10^{11}	1.5×10^{10}
Tray biorreactor BDr TH	2.5×10^9	5.8	765	43.5	1.09–1.31	4.3×10^8	1.1×10^8	1.9×10^{12}	4.4×10^{10}
Packed bed 1.5 L RH BB	3.8×10^8	7.8	105	1.5	0.18–0.33	4.5×10^7	2.5×10^8	4.0×10^{10}	2.7×10^{10}
Packed bed 1.5 L RH TH	2.0×10^9	5.8	105	1.5	0.18–0.33	3.4×10^8	1.3×10^9	2.1×10^{11}	1.4×10^{11}
Packed bed 1.5 L BDr BB	1.5×10^9	7.8	105	1.5	0.71–0.96	1.9×10^8	1.0×10^9	1.5×10^{11}	1.0×10^{11}
Packed bed 1.5 L BDr TH	2.1×10^9	5.8	105	1.5	0.71–0.96	3.6×10^8	1.4×10^9	2.2×10^{11}	1.4×10^{11}
Packed bed 22 L RH BB	6.0×10^8	7.8	1050	22	0.28–0.42	7.7×10^7	2.7×10^7	6.3×10^{11}	2.9×10^{10}
Packed bed 22 L RH TH	9.1×10^8	5.8	1050	22	0.28–0.42	1.6×10^8	4.1×10^7	9.6×10^{11}	4.4×10^{10}
Packed bed 22 L BDr BB	2.5×10^9	7.8	1400	22	0.53–0.87	3.2×10^8	1.1×10^8	3.5×10^{12}	1.6×10^{11}
Packed bed 22 L BDr TH	1.7×10^9	5.8	1400	22	0.53–0.87	2.9×10^8	7.7×10^7	2.4×10^{12}	1.1×10^{11}

sAF: specific airflow; RH: rice husk; BDr: beer draff; BB: *Beauveria bassiana*; TH: *Trichoderma harzianum*.

Table 4
Conidia production, mean temperature and maximum sOUR obtained in all BB fermentations. Statistical analyses are shown for conidia production and mean temperature.

Test/parameter	Conidia production (conidia g^{-1}dm)	Mean temperature (°C)	Max sOUR (g O_2 kg^{-1}dm)
Packed bed 1.5 L RH BB	$3.8 \times 10^8 \pm 1.7 \times 10^{7(a)}$	$26.4 \pm 0.9^{(a)}$	0.2
Packed bed 22 L RH BB	$6.0 \times 10^8 \pm 5.4 \times 10^{7(b)}$	$22.5 \pm 1.3^{(b)}$	0.7
Tray bioreactor BDr BB	$1.2 \times 10^9 \pm 8.0 \times 10^{8(c)}$	$26.9 \pm 2.6^{(a)}$	2.7
Packed bed 1.5 L BDr BB	$1.5 \times 10^9 \pm 3.8 \times 10^{8(c)}$	$26.0 \pm 1.7^{(a)}$	3.6
Packed bed 22 L BDr BB	$2.5 \times 10^9 \pm 6.5 \times 10^{8(c,d)}$	$25.5 \pm 1.6^{(a,b)}$	3.5

Table 5
Conidia production, mean temperature and maximum sOUR obtained in all TH fermentations. Statistical analyses are shown for conidia production and mean temperature.

Test/parameter	Conidia production (conidia g^{-1}dm)	Mean temperature (°C)	Max sOUR (g O_2 kg^{-1}dm)
Tray bioreactor RH TH	$7.0 \times 10^8 \pm 2.1 \times 10^{8(a)}$	$25.8 \pm 1.9^{(a,b,c)}$	(–)
Packed bed 1.5 L RH TH	$2.0 \times 10^9 \pm 3.0 \times 10^{8(b)}$	$23.0 \pm 1.9^{(a,b)}$	0.7
Packed bed 22 L RH TH	$9.1 \times 10^8 \pm 3.1 \times 10^{8(a)}$	$25.8 \pm 1.8^{(a,b,c)}$	0.7
Tray bioreactor BDr TH	$2.5 \times 10^9 \pm 4.8 \times 10^{8(b)}$	$25.6 \pm 4.4^{(a,b,c)}$	4.5
Packed bed 1.5 L BDr TH	$2.1 \times 10^9 \pm 3.7 \times 10^{8(b)}$	$29.1 \pm 2.6^{(a,c)}$	3.2
Packed bed 22 L BDr TH	$2.1 \times 10^9 \pm 3.6 \times 10^{8(b)}$	$25.7 \pm 2.6^{(a,b,c)}$	3.3

higher values. It must also be considered that, when working with beer draff, the substrate/bulking agent mixture used in tray and 22 L was different (70/30 in tray vs 40/60 in 22 L packed bed w/w beer draff/wood chips). Results could vary if using same proportions, which might be needed in case of increasing bed height, which was kept constant at 4 cm in all tray bioreactor tests. According to some authors, this value could be higher without compromising airflow through the bed (Jou and Lo, 2011; Krishania et al., 2018). Increasing bed height up to the same working volume used in the 22 L packed-bed (corresponding to 8 cm bed height per tray) configuration should lead to a better comparison.

Comparing specific airflow rates, values were proportional between PBB scales. In tray fermentations, much higher values were provided for rice husk fermentations, despite its higher AFP_R in comparison to beer draff. However, conidia production was much higher in TH beer draff tray fermentation rather than in rice husk. These results confirm the relevance of biodegradability as a key parameter in TH fermentations, as presented in previous works (Sala et al., 2021a).

Tables 4 and 5 present a comparison of conidia production and biodegradability, including mean values for conidia production and temperature (with the correspondent statistical analyses) and maximum sOUR for the fermentations.

Comparing between substrates, conidia productions on rice husk were always lower compared to those on beer draff using the same configuration and strain. This behaviour was coupled with much lower biodegradability presented by rice husk in comparison to beer draff. Rice husk respiration values never surpassed 0.75 g O_2 kg^{-1}dm at both tested scales, while beer draff varied between 2.70 to a maximum of 4.45 g O_2 kg^{-1}dm in tray bioreactor. Comparing initial values from sections 6.3 and 7.3, initial parameters at the start of the fermentation were similar between substrates (with the only exception of AFP_R in beer draff fermentations, determined as key parameter in beer draff process scale up in previous works (Sala et al., 2021b)). These results suggest conidia production is highly dependent on substrate biodegradability.

Comparing between strains, BB and TH present different behaviours. Although significant differences between reactor configurations were observed, they were not always between the same reactors. Maximum mean conidia production achieved using BB and beer draff was obtained in 22 L fermentation, while both tray and 1.5 L fermentations obtained more similar values. This result is remarkable, as most of BB aerial conidia production is not performed using packed-bed bioreactors as fermenters but by superficial production, which ranges from polypropylene bags and tray bioreactors to environmentally prepared chambers for fungal growth and sporulation (Jaronski and Mascarin, 2016). The use of different substrate/bulking agent ratios between 22 L beer draff bioreactors and the rest of the tested conformations (which results in different AFP_R values as presented in Table 2) could possibly have affected conidia production. Use of AFP_R values around 80% improved 22 L packed-bed performance in comparison to 1.5 L or tray performances (Sala et al., 2021b). This gives higher relevance to a correct AFP_R adjustment when working with BB, in agreement with BB PCA analysis results presented in previous works (Sala et al., 2021a). In contrast, TH conidia production was more equal between different reactor configurations when looking at the same substrate. TH conidia production has overall been superior to BB's, suggesting better use of the substrate by this fungal strain. This analysis is consequent with obtained results in TH PCA analysis obtained in previous works (Sala et al., 2021a), where parameters related to the biodegradability of the substrate were the most relevant for TH conidia production. This behaviour might also be attributed to the superior enzymatic production capabilities of TH (Verma et al., 2007) in comparison to BB. Aside from chitinases, the genera *Trichoderma* has been previously used to produce several enzymes, most of them being lignocellulosic enzymes such as cellulases, xylanases and endoglucanases (Lopez-Ramirez et al., 2019; Kar et al., 2013; Ortiz et al., 2015; Ahmed et al., 2016), whereas there are no reports on the use of BB to produce similar enzymes.

Achieved mean temperatures were similar among most of the reactors, presenting higher deviations in tray bioreactor than in packed bed and in beer draff than in rice husk. Although overall temperatures were a little bit lower when using rice husk, they were not always significantly different from beer draff. Most of the observed mean temperatures in all reactors and strains were around

25 °C. Interestingly, lowest mean temperature with both strains was observed in PBBs. Considering the thickness of the 22 L packed bed reactor in comparison to one tray bed's height (40 vs 4 cm), obtaining similar mean temperatures in both designs with both strains opens scaling-up possibilities for packed-beds. At the same time, higher bed thickness should also be tested for tray configurations, as demonstrated by several authors in different SSF tray fermenters (Krishna, 2005; Xie et al., 2013; Zhang et al., 2014). It is noteworthy to mention that tray bioreactor insulation capabilities were probably superior to PBB, as tray bioreactor was adapted from an incubator, meaning potential better heat insulation. However, PBB were almost full of substrate, whereas tray bioreactor had a huge dead volume in all fermentations, meaning generated heat per volume unit was higher in PBB than in tray.

4. Conclusions

Successful conidia production has been achieved using both BB or TH in both packed bed and tray reactor conformations. Higher productions with both strains were obtained when fermenting beer draff complemented with wood chips, being superior to rice husk due to the use of a substrate mixture, while also obtaining much higher respiration profiles. While significant differences in terms of conidia production were shown between 22 L packed bed reactor inoculated with BB and the rest, they were not observed when working with TH, suggesting TH as a most versatile strain than BB. Differences in conidia production between trays were shown when working with BB, although they were not present when working with TH. Chitinase analysis in tray bioreactors revealed different optimal production times for conidia and for chitinase production. Chitinase activity values were similar between strains. However, maximum chitinase production was 1.5–1.75 times higher in TH fermentation in trays 1 and 2. BB strain production was affected by little temperature and moisture variation between trays, while TH overall performance was more similar between trays despite experimenting higher variations between trays for both parameters. Total conidia production was similar between tray and 22 L packed beds when using same substrate. Although most reactors did not present significant differences in terms of mean temperature, better comparison could be made if adapting tray bed's thickness while also providing better heat insulation in 22 L PBB.

Future work should focus on scaling-up and improving both packed-bed and tray configurations with both substrates, but specially with beer draff. Packed bed should be scaled up to bench scale and tray should improve spatial usage by increasing the number of trays and its bed height, as packed-bed reactors have demonstrated the feasibility of using larger bed heights to produce fungal conidia with both tested strains. Additionally, more analyses on chitinase production should be performed in order to find the most optimal production time in terms of both conidia and chitinase activity, especially in packed-bed conformation.

Author statement

A. Sala: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing (original draft) **T. Echegaray:** Investigation **G. Palomas:** Investigation **M.J. Boggione:** Investigation, Validation, Writing (Review and Editing) **G. Tubio:** Writing (Review and Editing) **R. Barrena:** Conceptualization, Supervision, Formal analysis, Writing (Review and Editing), Methodology **A. Artola:** Conceptualization, Supervision, Writing (Review and Editing), Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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