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Fungal biopesticide production: Process scale-up and sequential batch mode operation with *Trichoderma harzianum* using agro-industrial solid wastes of different biodegradability

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ABSTRACT

This work presents a sequential batch operational strategy (SBR) for fungal conidia production in solid-state fermentation (SSF) to improve the traditional batch operation, while also aiming to present a robust and scalable process. *Trichoderma harzianum* was fermented using two substrates with different biodegradability (rice husk and beer draff), scaling from 1.5 L to 22 L bioreactors. Before the SBR operation, the optimum time to get inoculum from each SBR batch was determined as 4 days. While single batch process scale-up was successful with both substrates, SBR strategy was only feasible using beer draff as substrate: conidia production was sustained during 3 consecutive batches in 1.5 L bioreactors and for 5 batches at 22 L. At both scales conidia production was around $2.0x10^9$ conidia g^{-1}dm, achieving maximum specific oxygen consumption rate (sOUR) values close to 4 g O_2 kg^{-1}dm h^{-1} in most reactors. Air filled porosity was found as a key parameter regarding process scale-up, with a minimum value of 80% as necessary to proper scaling up to 22 L. Process robustness was statistically demonstrated as no significant differences in conidia production, moisture and pH were found at different reactor heights using both substrates in most 22 L reactors tests. Consequently, SBR operation has been presented as a reproducible method to overcome traditional packed-bed drawbacks while also improving SSF performance in comparison to traditional industrial SSF processes.

1. Introduction

Massive use of chemical pesticides presents a threat to both human health and to the environment, urging a shift to greener options [1,2]. Fungal biopesticides represent one of the most attractive options among biocontrol agents, as they present no harm to humans, crops or ecosystems while being highly pathogenic to more than 1000 insect species [3,4]. A wide range of culture media can be used to produce fungal biopesticides, both by submerged fermentation (SmF) and solid-state fermentation (SSF). Although each system presents several advantages and drawbacks [5], SSF (defined as a process that occurs in the absence or near absence of free water) offers lower costs in comparison to SmF, mainly related to the use of agro-industrial wastes as substrates, serving both as nutrient and support for fungal growth and sporulation [6]. In addition to this, aerial conidia, which can only be produced by SSF, are the primary infective propagule of most fungal biopesticides [2], making SSF preferred over SmF as fungal biopesticide production method. One of the most interesting fungal biocontrol agents is *Trichoderma* spp., mainly due to its recognized antagonistic capabilities, presenting great success against soil-borne diseases [7,8]. Several agro-industrial residues have been used to produce fungal conidia by SSF using various *Trichoderma* strains, effectively generating an added value on obtained products due to waste valorization [9]. A comprehensive list of used substrates (including agro-industrial wastes) with the genera *Trichoderma* (among others) was reviewed by Sala et al. [4].

Different reactor configurations have been reported to produce fungal biocontrol agents by SSF. From static tray reactors to agitated rotatory drums and both static or agitated packed beds, each design has several advantages and drawbacks. Packed bed reactors (mainly presented as cylindrical columns) facilitate oxygen availability via continuous forced aeration as well as maintaining constant moisture when air supplied is previously saturated with water. However, the main disadvantage of using packed bed reactors lies in heat removal, causing important difficulties in process scale-up [10].

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A possible method to improve packed-bed reactors operation lies in sequential batch reactor operation (SBR) [11,12]. This strategy eliminates the requirements of preparing fresh inoculum for each batch (reducing the quantity of required inoculum to process the same amount of substrate) and has already been tested using SSF technology to produce cellulases using coffee husk [11] and to produce rose-like aroma compounds using sugarcane bagasse [12]. Regarding heat removal, the use of high porosity substrates to allow maximum heat transfer is a possible path to overcome, or at least reduce, heat transfer effects on fungal growth and sporulation. Achieving accurate porosity measurements is still difficult, which highlights the role of air-filled porosity (AFP_R) as a more reliable value of the space in the available space in the matrix for air content and movement [13]. Substrates presenting high AFP_R such as rice husk have been previously tested [14]; whereas the use of highly porous bulking agents of very low respiration activity just as wood chips also helps at maximizing heat removal throughout the packed bed [15], being necessary when working with residues presenting low AFP_R values such as beer draff [16]. Both rice husk and beer draff are mass produced worldwide. Rice husk is a by-product of rice, third agricultural commodity in terms of production by the year 2014, with production growing each year (estimated on 215 M tones on 2018 using the correlation of 0.28 kg rice husk obtained per kg of milled rice) [17–18]. Beer draff is easy to obtain as is a by-product of the brewery industry, one of the most productive beverage industries in the world, generating large quantities of brewer's spent grain or beer draff, a solid leftover obtained after the fermentation process which is primarily used as animal feed [19–20]. Additionally, inoculant age plays an important role in fungal growth since conidia germination tends to decrease as culture ages in most strains, leading to a quality loss [21]. As such, it should be investigated prior to the use of any SBR strategy when working with fungal cultures.

In a context of increasing relevance on environmentally-friendly production processes, biopesticide production using SSF emerges as a great tool to achieve this objective. However, most of the actual fungal SSF fermentations using packed bed bioreactors have been performed at lab scale [4], while achieving more results at higher scales has become a necessity [10]. Assuming its challenges, a viable process where inoculum production does not represent a hindrance neither for equipment nor for economy is needed. In addition, organic wastes with different biodegradability can be used as substrate in SSF. The proper scaling-up of the SBR strategy using packed-bed reactors would help at overcoming all of the previously mentioned SSF challenges. To the authors' knowledge, scale-up of fungal SSF in packed bed reactors under a SBR strategy has not been previously tested and it can be a starting point to reduce associated costs by overcoming inoculum production problems of solid-state operation at commercial scale, reducing the quantity of inoculum necessary to process the same amount of substrate by eliminating fresh inoculum preparation in each batch. The aims of this paper are: i) to develop a robust, reproducible and scalable process for *Trichoderma harzianum* (TH) conidia production in packed bed reactors using agro-industrial wastes as substrates, ii) to present and adapt SBR as a feasible operation strategy to substitute traditional batch in packed bed TH fungal growth and iii) to test SBR using substrates presenting different biodegradability and AFP_R in order to compare its effect towards the reactors' temperature.

2. Materials and methods

2.1. Fungal strain

All tests were carried out using TH strain CECT 2929 (isolated from soil in the United Kingdom before 23/01/1991) and provided by Spanish Type Culture Collection (CECT). The original strain was preserved at −80°C in sterile cryovials (Fisherbrand™, Fisher Scientific S. L.) containing 10% glycerol, as established by the strain provider. TH was cultured in malt extract agar at 25°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, Barcelona) 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. Different conservation conditions were used due to the different properties of the materials, with rice husk presenting a moisture lower than 10% while the value for beer draft was over 80%. In order to maintain values of 55–60%, 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: 70 beer draff/30 wood chips (w/w) when working with 1.5 L volume reactors and 40 beer draff/60 wood chips (w/w) when working at 22 L. Wood chips were added to beer draff in order to ensure a value of AFP_R in the range of 40–80%, stated as proper for composting processes [22], necessary to ensure a proper air circulation throughout the packed bed. Raw materials and initial fermentation mixtures characterization for all performed tests 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. Autoclaving of substrates was performed prior to their use at all reactor's scales studied.

2.3. Solid-state fermentation

Different SSF tests were performed at two packed-bed reactor scales (1.5 and 22 L) using two agro-industrial wastes with different biodegradability as main substrates (rice husk and beer draff). Previous studies in 0.5 L reactors were performed to determine the preferred

Table 1
Raw substrates and mixtures characterization for all used substrates.

Parameter/ Substrate	RH[1]	RH[2]	BDr[1]	WC[1]	70/30 w/w (BDr/ WC)[2]	40/60 w/w (BDr/ WC)[2]
MC (%)	10.2 ± 0.1	58.7 ± 0.4	76.4 ± 0.5	9.7 ± 0.3	63.8 ± 5.1	55.2 ± 4.8
OM (%)	82.6 ± 2.6	82.6 ± 2.6	93.6 ± 0.9	98.4 ± 0.6	95.0 ± 1.4	96.5 ± 1.2
pH	5.7 ± 0.3	6.3 ± 0.3	6.5 ± 0.3	5.1 ± 0.2	5.7 ± 0.2	5.1 ± 0.8
Carbon (%)	40.3 ± 0.8	40.3 ± 0.8	48.2 ± 0.7	46.9 ± 0.6	47.8 ± 0.6	47.4 ± 0.6
Hydrogen (%)	5.2 ± 0.2	5.2 ± 0.2	6.9 ± 0.3	6.2 ± 0.3	6.7 ± 0.3	6.5 ± 0.3
Nitrogen (%)	0.4 ± 0.1	0.4 ± 0.1	4.04 ± 1.2	0.4 ± 0.2	3.0 ± 0.6	1.9 ± 0.6
Sulphur (%)	<0.1	<0.1	0.1 ± 0.01	<0.1	<0.1	<0.1
C/N ratio	85.4 ± 15.2	85.4 ± 15.2	12.6 ± 2.5	117.4 ± 13.6	16.0 ± 6.2	25.0 ± 6.2
TSC (mg g^{-1}dm)	17.9 ± 0.2	17.9 ± 0.2	123.4 ± 9.3	13.1 ± 0.4	96.7 ± 10.3	67.9 ± 5.3
AFP_R (%)	90.3 ± 0.5	85.6 ± 0.6	64.0 ± 0.3	95.3 ± 0.5	70.3 ± 0.9	81.2 ± 0.5
DRI (g O_2 kg^{-1}OM h^{-1})	1.2 ± 0.1	(-)	6.5 ± 0.4	(-)	(-)	(-)
Max sOUR (g O_2 kg^{-1}dm h^{-1})	0.6 ± 0.2	0.8 ± 0.1	3.2 ± 0.4	0.4 ± 0.1	2.5 ± 0.7	1.7 ± 0.4
COC^{6d} (g O_2 kg^{-1}dm)	1.5 ± 0.3	1.5 ± 0.3	16.8 ± 2.2	1.0 ± 0.2	12.1 ± 1.0	7.3 ± 0.7

RH: rice husk; BDr: beer draff; OM: organic matter; WC: wood chips; w/w: weight/weight; MC: moisture content; C/N: carbon/nitrogen; TSC: total sugar content; AFP_R: air filled porosity; DRI: dynamic respirometric index; sOUR: specific oxygen uptake rate; COC^{6d}: cumulative oxygen consumption at day 6; [1]: raw material; [2]: initial batch material.

conditions for the fermentations presented in this paper, more information on them can be found in Sala et al [23]. Although finding these conditions using higher volume reactors (1.5 L) would have been more representative, it has not been considered due to the time and effort associated to each test. Moreover, Design of Experiments technique was applied with 0.5L reactors ensuring statistical reliability. First fermentations were carried out in 1.5 L reactors because of their easily operation. The 22 L reactor was used to validate and study in detail the main parameters of the proposed process. Previous inoculant age test was also performed. Most relevant parameters used to monitor SSF evolution were conidia counting, respiration analyses (by means of sOUR) and temperature, as explained later. pH and moisture were determined for initial and final SSF materials.

2.3.1. Inoculant age test

A test to determine the optimum day to extract conidia as inoculum for consequent SBR fermentations was performed. A 22 L rice husk batch was run for 9 days. Sampling of this reactor was performed in days 4, 6, 8 and 9. Sampling times were chosen according to results on optimal conidia production time obtained in previous experiments [23]. In all these samples (25 g each), conidia were extracted using the 125 mL of Tween 80 0.1% and used as inoculum for triplicate 0.5 L reactors using rice husk as substrate. To determine best inoculant age, conidia were counted 6 days from the start of the 0.5 L reactors' fungal growth using Neubauer chamber (Brand^{TM} 717805).

2.3.2. Experimental set-up for 1.5 L reactors

1.5L 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. A total of 300 g of each substrate were fermented per triplicate for 5–8 days, according to results on optimal conidia production time obtained in previous experiments [23]. 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 (0.18–0.33 mL min^{-1} g^{-1}dm for rice husk and 0.71–0.96 mL min^{-1} g^{-1}dm for beer draff) 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 2.3.3.

Reactors were loaded and mixed in a 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 for them being made of polyvinyl chloride.

2.3.3. Experimental set-up for 22 L reactor

In this case, the reactor consisted of a cylindrical stainless-steel vessel with a removable basket of 48 cm height × 24.5 cm diameter, presenting a total volume of 22 L. In all tests, 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. Constant specific aeration in the ranges of 0.27 – 0.42 mL min^{-1} g^{-1}dm for rice husk and 0.53 – 0.87 mL min^{-1} g^{-1}dm for beer draff 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 above. Room 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, as they could not be autoclaved due to the reactor's volume. Inoculation was performed in ambient conditions in the laboratory before loading the substrate directly into the basket, using previously cleaned trays and appropriate volumes to ensure homogeneous distribution of the inoculum throughout the packed bed.

2.3.4. SSF sequential batch operation

SBR operation was performed in the same way both at 1.5 L and 22 L scales. A total of 4–5 batches were charged in each SBR operation. A schematic representation of the SBR process is presented in Fig. 1.

The fungal inoculum of the first reactor in the series was cultured as aerial conidia as described in section 2.1. Conidia were harvested and resuspended using 10 mL Tween 80 0.1%. Conidia in the suspension were counted using Neubauer chamber (Brand^{TM} 717805) and diluted to 6.6x10^6 conidia per gram dry matter (g^{-1}dm) in all tests using Tween 80 0.1%. The inoculum volume in each reactor was 10% of its total volume.

Inoculum for the following batches of the sequence was obtained by liquid extraction of fungal conidia contained in the solid material of the previous batch in the day determined through to inoculant age test (section 3.1). Conidia were extracted from the calculated quantity of fermented solid material using a 1/5 ratio (solid/liquid, w/w, using Tween 0.1%) and diluted to 6.6x10^6 conidia g^{-1}dm.

To follow the evolution of the process and assuming 6 days as the maximum conidia productivity time as obtained in previous experiments [23], sampling was performed from two to three times in each reactor on days 4, 6 or 8 from the beginning of each batch. In 1.5 L reactors, samples were alternatively taken from a different reactor of the triplicate each day, leaving at least one reactor untouched for all the fermentation time and sampling all three reactors in the last sample of the fermentation. In 22 L reactors, samples during fermentation were taken from the surface area. A different approach was taken for samples corresponding to the end of each batch, being sampled as 10 equally weighted samples at different bed heights (assuming 0 cm height at the bottom of the bed and 40 cm as maximum height). These samples were divided into three groups: upper samples (28–40 cm height), medium samples (12–28 cm height) and bottom samples (0–12 cm height) and were used to analyse conidia production, temperature and moisture variability at different reactor heights using a one-way ANOVA ($p < 0.05$ confidence) with the Tukey test using the software Minitab 17 (Minitab Ltd.).

2.3.5. Oxygen uptake rate

On-line oxygen consumption has been considered as an indicator of the biological activity. Specific oxygen uptake rate (sOUR) was calculated according to Puyuelo et al. [24], expressed as 1 h average value (sOUR) (Equation (1)) and recorded on-line in order to provide an indicator of the biological activity:

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

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), also providing information on the biological activity in the SSF reactor and as a direct measure of the degraded carbon [25].

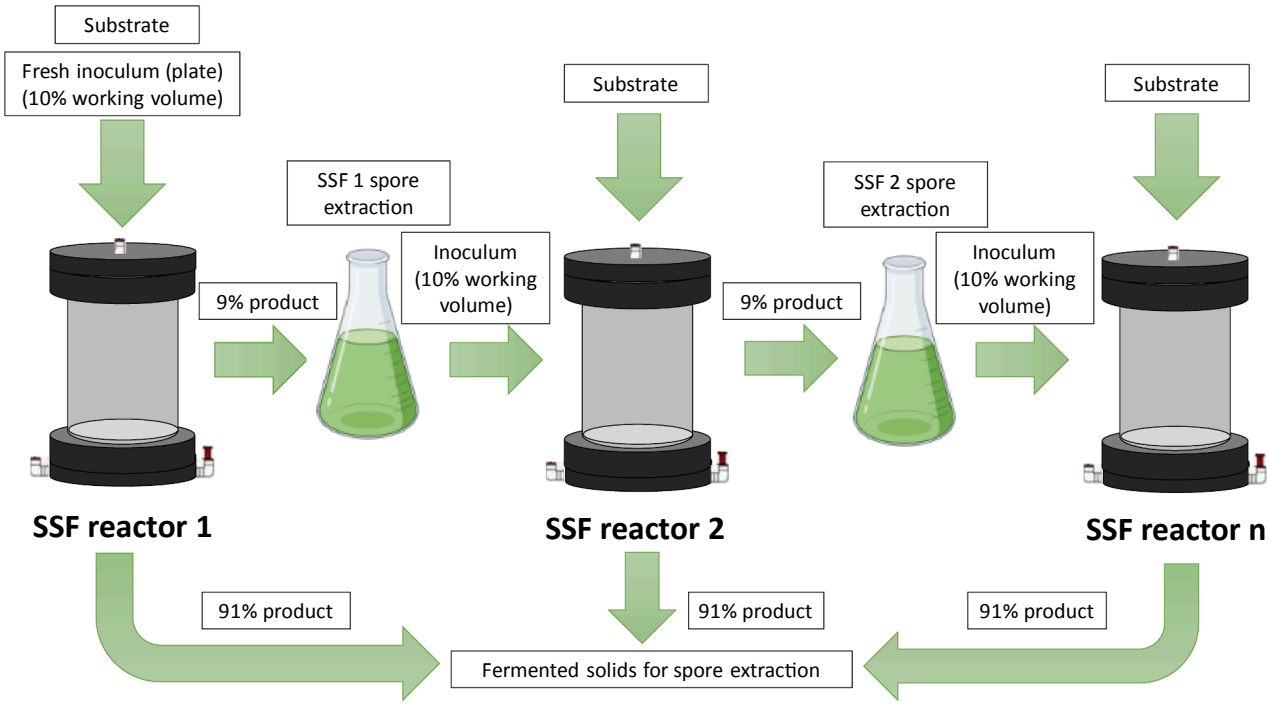

Fig. 1. Schematic representation of SBR operation on 1.5L or 22L reactors. Part of the **Figure** was provided by Biorender (https://biorender.com/).

2.4. Conidia counting

To determine fungal spore concentration, conidia counting method described in previous experiments [23] was used. Briefly, a Neubauer chamber (Brand^{TM} 717805) was used. 10 g of sample (conidiated substrate) were mixed with 50 mL of Tween 80 0.1%, shaken for 20 min at 150 rpm and appropriately 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. Total sugar content analysis

Total sugar content was empirically determined using the Anthrone method, using glucose for the calibration curve [26]. Total sugar content was expressed as gram of glucose equivalent per gram of dry matter according to equation: $Total sugar content = \frac{C}{P} \cdot V$ (3)

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

2.6. Analytical methods

Moisture (%), dry matter (%), organic matter (%) and pH have been determined for initial and final samples using standardized methods [27]. C/N analysis was performed by means of chemical elemental analysis in all initial samples. Results are shown in Table 1. Results are shown in Table 1. C/N analysis includes carbon, nitrogen, hydrogen and sulphur analyses for raw substrates and mixtures used in the presented fermentations.

AFP_R was calculated for all used substrates according to Equation (4) as presented by Richard et al. [13]:

$$AFP_R = 1 - BD_t\left(\left(\frac{1-DM}{D_W}\right) + \frac{DM^{*}OM}{PD_{OM}} + \left(\frac{DM(1-OM)}{PD_{ash}}\right)\right) \quad (4)$$

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}).

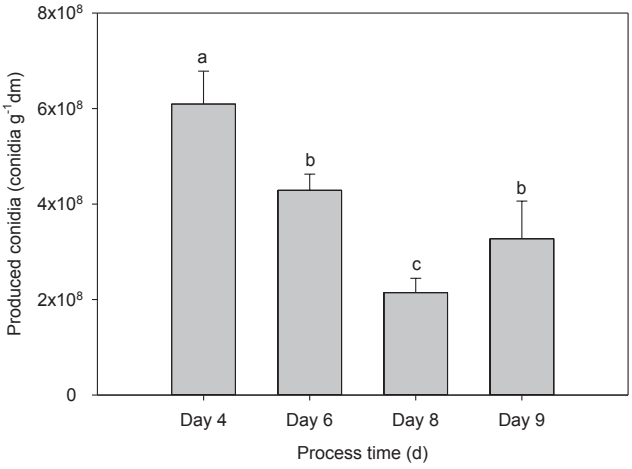

Fig. 2. Conidia production using TH inoculum obtained at different process times from a previous SSF assay. Different letters indicate significant differences between the evaluated groups ($p < 0.05$) based on the Tukey analysis.

3. Results and discussion

3.1. Determination of the optimal fermentation time for inoculum extraction to use in a sequential-batch operation

Fig. 2 shows conidia productions using inoculum extracted from the fermentation reactor at days 4, 6, 8 and 9. Conidia concentration from the inoculum extracted at day 4 was significantly higher to the rest of the tested inoculant ages'. Thus, 4 days was established as the optimum time for inoculum extraction.

Inoculant age relevance has been highlighted for various fungal strains, as conidia quality tends to diminish with age. This is the case of Smith and Edgington [28] using *Metarhizium* spp., Hallsworth and Magan [29] using *Beauveria bassiana* and Múñiz-Paredes et al. [30] using *Isaria fumosorosea*. However, and to our knowledge, this is the first time it has been tested using TH. Despite its relevance, inoculum age is not often studied in fungal optimization processes [21].

3.2. Rice husk sequential-batch reactor: Scaling from 1.5 L to 22 L

Fig. 3 shows obtained profiles in rice husk SBR operation in both scales; Fig. 3a shows conidia production, sOUR and temperature in 1.5 L reactors, while Fig. 3b shows same parameters for 22 L reactors.

In both scales, final conidia production on each reactor decreased halfway, starting around $1.4x10^9 \pm 5.0x10^8$ conidia g^{-1}dm in batch 1 and decreasing to values around $6.0x10^7 \pm 2.3x10^7$ conidia g^{-1}dm in batch 4 or 5. Maximum conidia production was achieved in day 6 of fermentation in all batches from 1 to 3, however, in batches 4 or 5 in both scales it was extended, achieving maximum conidia production in day 7. Conidia production reduction throughout the batches was apparently caused by the appearance of contaminant *Aspergillus niger* (AN) from batch 3 to batch 5. At both scales, AN conidia concentration (also presented in Fig. 3a and 3b as white dots) doubled in each batch, starting at values close to $9.0x10^6 \pm 2.0x10^6$ conidia g^{-1}dm and rising to $8.3x10^7 \pm 3.0x10^7$ conidia g^{-1}dm in batch 5 in 1.5 L SBR, surpassing TH

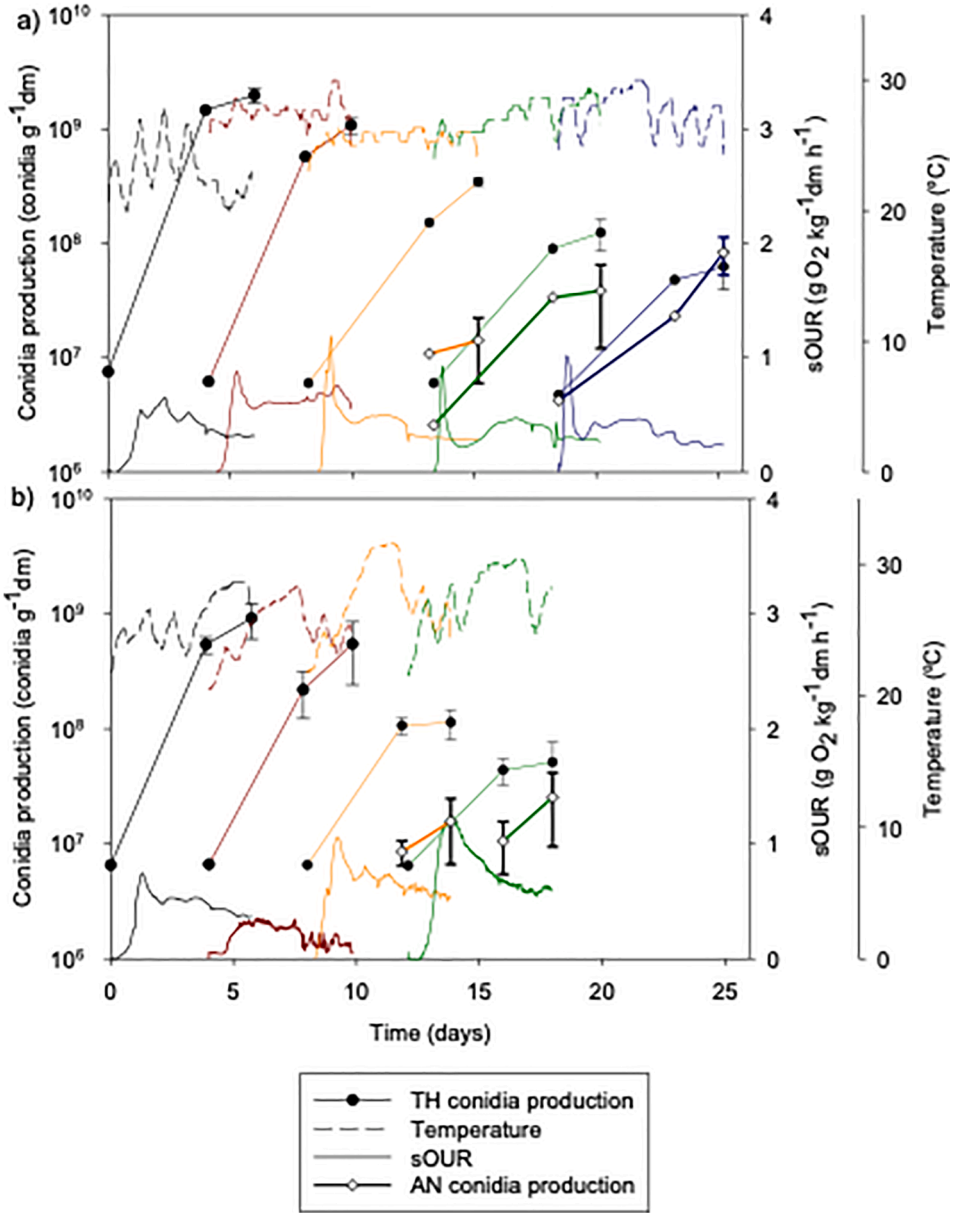

Fig. 3. Rice husk SBR profiles (conidia production, temperature, sOUR and AN conidia production) in 1.5L reactors (a) and 22L reactors (b). Batch 1: black. Batch 2: brown. Batch 3: orange. Batch 4: green. Batch 5: blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conidia production in the last batch. No fifth batch was performed at 22 L scale due to its behaviour being similar to 1.5 L. AN is a common contaminant in rice and its by-products [31–33]. Despite TH's anti-fungal properties [7], AN conidia were still able to grow in the substrate, being capable of withstanding autoclaving as reported in previous works [23]. Conidia production loss suggests AN growth started in the second batch, taking advantage of inoculum quality loss in comparison to pure inoculum extracted from plates used in the first batch, even though AN conidia could not be detected when counting due to their low numbers in comparison to TH conidia. With this result, production using rice husk at higher scales is still possible if working with single batch strategy using pure inoculum extracted from fresh plate.

In terms of biodegradability, respiration profiles were similar in all batches working at both scales, reaching maximum values close to 1 g O_2 kg^{-1}dm h^{-1} at similar times in most of the batches, being similar to the ones presented in previous works using the same substrate [23]. According to Barrena et al. [15], rice husk is a substrate that presents low biodegradability (DRI lower than 2 g O_2 kg^{-1} OMh^{-1}). In consonance, temperature profiles in both scales also show low potential biodegradability, as even at 22 L scale mean temperatures in the reactor never surpassed 32℃, starting approximately at 25℃ in all fermentations. Higher temperature variation is to be expected when using substrates that present high or even moderate biodegradability, according to Barrena et al. [15].

Obtained values for moisture, pH and total sugar content were similar between scales: moisture ranged from 50 to 60% in most batches and pH started at values close to 6 and raised to values close to 7 at the end of all batches. Similar values for both parameters were observed at both scales. AFP_R could easily be maintained at correct values in order to ensure proper oxygen transfer at both scales (around 85%, as shown in Table 1, being superior to the highest values of 80% indicated for the composting process by Ruggieri et al. [22]). Despite all parameters being adequate for TH growth and sporulation according to Zhang and Yang [34], co-culture growth was observed from batch 3 onwards. Regarding AN growth parameters and according to several authors [35,36], TH and AN co-culture growth was possible within the observed parameters' ranges, with both of them being present at least from batch 2 onwards. However, AN growth over TH suggests faster growth of contaminant in rice husk in comparison to TH, promoting its prevalence in the co-culture in batch 5 in the 1.5 L SBR and assuming similar results would have been obtained in case of performing a fifth 22 L batch.

Rice husk has been found as an easy to scale-up substrate due to its naturally high porosity and low biodegradability, greatly reducing possible drawbacks caused by heat accumulation. However, despite successful scaling-up of the process (achieving similar results between both tested scales), presence of AN in the substrate suggests not to follow

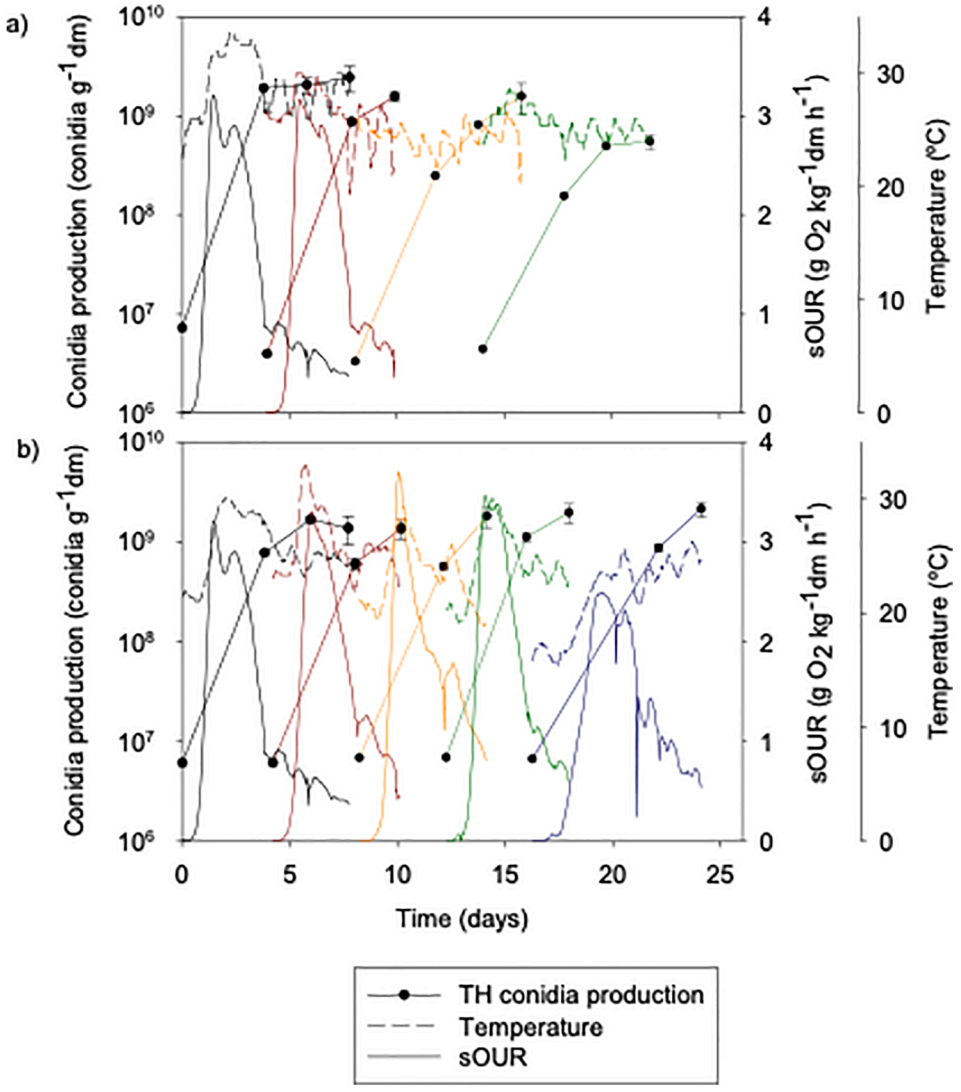

Fig. 4. Beer draff SBR profiles (conidia production, temperature and sOUR) in 1.5L reactors (a) and 22L reactors (b). Batch 1: black. Batch 2: brown. Batch 3: orange. Batch 4: green. Batch 5: blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a SBR strategy when using rice husk. In fact, AN gradually takes over the culture until it surpasses TH in terms of conidia concentration. Consequently, a batch strategy using fresh inoculum appears to be the most optimal to maintain conidia concentration at its maximum, as best performances were obtained in the first batch (fresh inoculum) at both scales and could not be replicated in subsequent batches. As such, this strategy could be performed not only using rice husk as substrate but also when working with substrates which pose similar difficulties in terms of sterilization, which are common when working in SSF with agro-industrial wastes due to substrate heterogeneity [37].

3.3. Beer draff sequential-batch reactor: Scaling from 1.5 L to 22 L

Fig. 4 shows obtained profiles in beer draff SBR scaling. Contrarily to rice husk SBR results, different performances were observed between 1.5 L and 22 L SBR reactors. In 1.5 L reactors (Fig. 4a) conidia concentration was sustained for 3 consecutive batches, showing no significant differences among them, obtaining values between $1.6x10^9$ conidia g^{-1}dm ± $3.2x10^8$ and $2.1x10^9$ conidia g^{-1}dm ± $3.7x10^8$. However, batch 4 yielded a significantly low conidia production of $5.6x10^8$ conidia g^{-1}dm ± $9.7x10^7$. Maximum conidia production was achieved in day 6 of fermentation in batches 1 and 2; however, in batches 3 and 4 fermentation time was extended and maximum conidia production was achieved in day 8. In contrast, conidia concentrations at 22 L (Fig. 4b) was sustained in all batches, obtaining values between $1.7x10^9$ conidia g^{-1}dm ± $1.1x10^8$ and $2.2x10^9$ conidia g^{-1}dm ± $3.7x10^8$, showing no significant differences between all 5 batches. Maximum conidia concentration was achieved within 6 days in 4 out of 5 batches, while in batch 5, 8 days were needed. Longer time might have been due to differences in temperature profiles between batch 5 and the rest of the SBR batches. AN contamination was not detected at any scale, confirming the substrate change as a valid decision.

Differences in performance between SBR at 1.5 L and SBR at 22 L were attributable to the combined effects of three parameters: AFP_R, pH and moisture. As presented in Table 1, initial AFP_R was different between scales: 72.6 ± 0.7 in 1.5 L vs 81.2 ± 0.5 in 22 L. These differences were caused by substrate proportions used at each scale, being 70/30 w/w (beer draff/wood chips) at 1.5 L and 40/60 w/w (beer draff/wood chips) at 22 L. A failed 22L batch using proportion 70/30 is shown in Figure S1 in the supplementary material. TH conidia production was not achieved in this batch. Mean temperatures superior to 40℃ were achieved, while sOUR reached values close to 8 g O_2 kg^{-1}dm h^{-1}. This behaviour completely differed in comparison to any of the batches shown in Figs. 3 and 4, achieving much higher values both for temperature and sOUR. 1.5 L substrate mixture was not adequate for 22 L reactor due to substrate compaction, which highly reduces AFP_R and oxygen transfer, subsequently difficulting fungal growth and sporulation and facilitating the appearance of contaminants [38]. AFP_R adjustment was key for the success of both scale-up and SBR strategy performance, as 22 L SBR had 5 consecutive batches which presented the same behaviour, with the possibility of lengthening the process even more, while 1.5 L SBR only had 3. In addition, pH was also significantly different between scales: while 1.5 L behaviour was similar to that observed for rice husk (with pH starting at values close to 5.5 and ending close to 7.5–8), in 22 L reactors pH started at more acidic values (4.5–5), ending in most cases at values lower than 6.0. These variations might also be attributed to the use of different proportions of substrate and bulking agent, being batch 1 in 22 L SBR the one with the lowest conidia concentration in this test, acidic pH might have been favourable both for TH fungal growth and conidia production, as presented by some authors [34,38]. Regarding moisture, while in 1.5 L SBR this parameter ranged from 60 to 67%, it was much lower in most batches of the 22 L SBR, ranging from 51 to 58%, both of them corresponding to the optimum range presented by most *Trichoderma* strains [39]. Optimum range for TH conidia production is of 55–60% according to previous works [23], suggesting better moisture adjustment in the 22 L SBR.

In terms of biodegradability, all obtained respiration profiles were similar, reaching maximum values close to 3.5 g O_2 kg^{-1}dm h^{-1} at comparable times in nearly all of the batches, with the only exception of batch 5 in 22 L SBR, which only reached 2.5 g O_2 kg^{-1}dm h^{-1}. These oxygen consumption rate values indicate higher biodegradability when comparing to rice husk. Much higher total sugars available (80–88 mg g^{-1}dm) combined with higher values of DRI, sOUR and COC^{6d} also indicated higher substrate biodegradability when comparing to rice husk. Mean temperatures in batch 5 were lower than those obtained in the rest of batches, being even lower than 20℃ at the beginning of the fermentation. Due to the relevance of temperature in fungal growth [23,29], lower values of this parameter might be the cause of fungal growth and conidia production lengthening time in batch 5. Even though mean temperature profiles at both scales did not highly differ from profiles when working with rice husk, using a substrate with higher potential biodegradability caused temperature gradients in the reactor. Packed bed bioreactors often present problems related to temperature gradients due to heat removal difficulties [40], as such, the effect of temperature in different areas of the reactor will be discussed in depth in section 3.4. It can be concluded that observed differences in batch 5 in 22 L SBR were caused by several non-optimal range values in the analysed parameters rather than lower inoculum quality as might have happened at 1.5 L scale. Optimal values for TH growth and sporulation for all process parameters will be discussed in section 3.4.

Successful scaling from 1.5 L to 22 L has been achieved using beer draff as substrate and wood chips as bulking agent in a SBR operation. However, SBR performance differed between scales: while at 1.5 L conidia concentration decreased from batch 4 onwards due to loss of inoculum quality, at 22 L scale 5 batches were performed achieving similar maximum conidia concentrations. Differences between the two scales have been mainly caused by the use of different substrate mixtures, being 70–30% in 1.5 L and 40–60% in 22 L (as presented in section 2.2). These results suggest that a minimum AFP_R value of around 80% is needed to ensure proper fungal growth when working with packed bed reactors, highlighting the need to find the optimal AFP_R value when scaling up SSF fungal conidia production processes using packed bed reactors operating with and SBR strategy. Particle size relevance in solid-state fermentation studies has been highlighted by Yazid et al. [37]. Small particle size provides larger surface area for fungal growth while being prone to agglomeration and difficulties in oxygen transfer. In contrast, large particle size provides better oxygen transfer and reduces heat accumulation at the cost of limiting surface growth area. Higher surface area helps at maximizing mycelial growth, which is necessary for correct fungal sporulation. Additionally, mycelial growth does not affect substrate porosity, meaning it should be maximized before sporulation [41]. Balance between different sizes in large reactors is mandatory to ensure proper fungal growth and sporulation. These findings are highly relevant to fungal SSF, as they establish a reproducible method to overcome SSF traditional drawbacks while defining biodegradability and AFP_R as the key parameters in SSF scale –up. The results presented open the possibility of performing the current process using packed-beds at higher scales.

3.4. 22 L reactor global analysis performance

In order to test the robustness and reproducibility for fungal conidia production in packed bed reactor operating of a SBR strategy, statistical analyses were performed using data collected from sampling of the final solid material of each 22 L batch performed with both substrates (4 batch using rice husk and 5 batch using beer draff). As explained in section 2.3.4, samples points were divided depending on their height in the packed bed.

Fig. 5 shows mean values and standard deviations obtained when analysing samples at the end of each batch depending on their height. Results are shown for conidia production, moisture and pH in all performed 22 L batches. When comparing process parameters within the

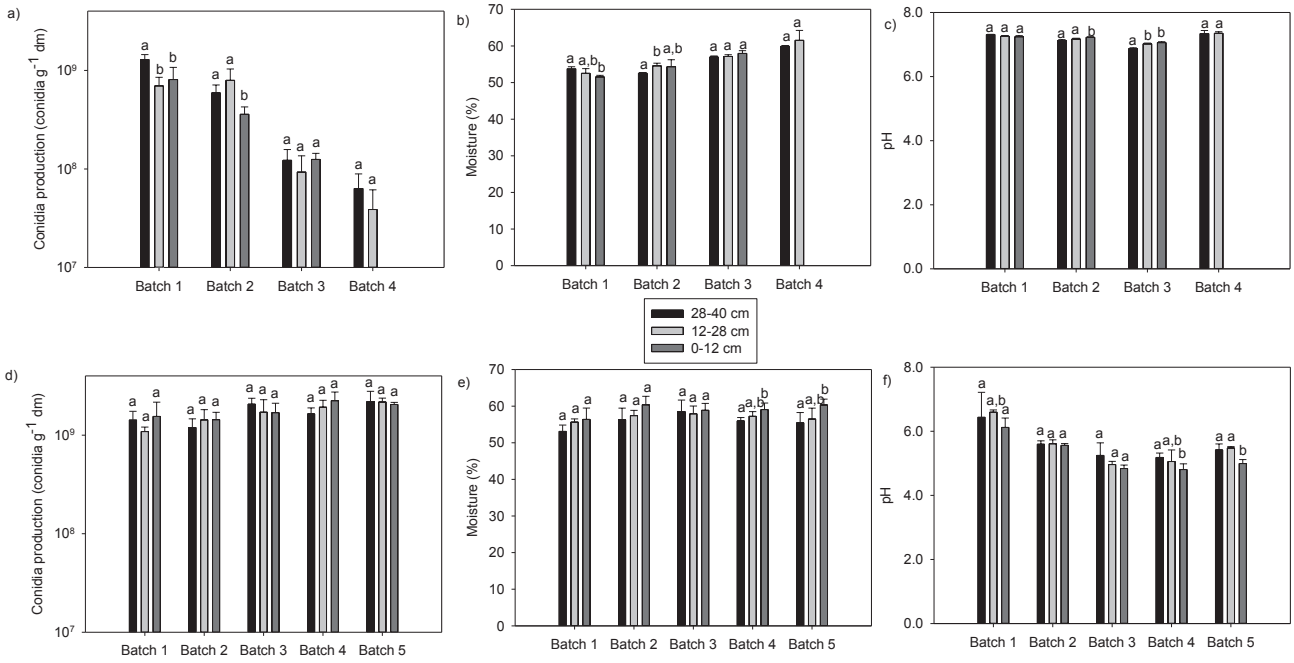

Fig. 5. Mean values and standard deviations obtained in samples collected at different reactor heights at the end of all 22L batches. a) Rice husk conidia production, b) Rice husk moisture, c) Rice husk pH, d) Beer draff conidia production, e) Beer draff moisture and f) Beer draff pH. Different letters indicate significant differences between the evaluated groups ($p < 0.05$) based on the Tukey analysis.

same batch, little significant differences are shown thorough the packed bed in both substrates, while no patterns are followed in terms of maximum conidia production height in the reactor. It can be assumed that conidia production does not depend on bed height in a 22 L rice husk or beer draff packed bed reactor, even though it could still be dependent on bed height at higher scales. This is consequent with little moisture and pH variations shown in all batches, with nearly all the compared samples not presenting significant differences with the rest of the samples in the same batch. When significant differences exist, it always corresponds to a slight variation in terms of absolute numbers. These data highlight the robustness of the process, as both tested substrates packed beds had similar physical properties at all heights. In addition, pH differences between substrates are clearly shown in Fig. 5c) and 5f). Lower pH values were achieved with beer draff in comparison to rice husk and corresponding to the batches which yielded highest conidia productions, meaning acidic pH might have been beneficial for TH growth and sporulation. These results differ from the majority of the references in bibliography, as TH growth and sporulation optimums are normally located near neutral pH [34,42], even though TH can grow and sporulate within an initial pH range of 3–9 according to Zhang and Yang [34]. These differences in behaviour when comparing with literature could have been caused by using a specific *Trichoderma* strain which could present better results when working at acidic pH. Some *Trichoderma* spp. strains have been demonstrated to work optimally under acidic pH conditions [43].

Temperature variation in different areas of the 22 L reactors (centre of the packed bed, close to the reactor's wall, mean values and external temperature) is shown in Figure S2 in the supplementary material. Despite differences between reactors and substrates, in most cases analysed temperatures were close to the optimal growth temperature range for most *Trichoderma* strains, being 25-30℃ according to Kubieck and Harman [44]. Reactors corresponding to rice husk showed little to no difference in temperature values between different reactor areas, with minimal temperature differences being of a maximum of 3℃, effectively achieving similar temperature on all the packed beds' volume. This behaviour is comparable to the one obtained by Barrera et al. [45], who reported advantageous temperature axial gradients using both rice husk and polyurethane foam as substrate and inert support for *Trichoderma asperellum* conidia production, although their analysis was based on airflow rate variation while the work presented in this paper is not focused in this parameter. However, in beer draff batches, radial temperature differences were observed, as temperatures at the reactors' wall and the centre of the bed were different during all the fermentation process, with differences ranging from minimal 2-3℃ to more than 10℃ depending on fermentation time, effectively creating different zones in the reactor in terms of radial temperature. Higher temperatures were achieved in the centre of the bed in beer draff reactors, which is consequent with the higher respiration indexes observed when using beer draff in comparison to rice husk, leading to an overall increase of the bed's temperature. Similar approach was presented by da Cunha et al. [46] working with *Metarhizium anisopliae* and a mixture of rice and sugarcane bagasse in a similar packed bed bioreactor to the one presented in this study. However, their analysis was focused on axial temperature, finding relevant temperature differences depending on the substrate axial position, behaviour that has not been studied in this paper. As no significant differences between axial conidia productions have been found, we can assume that observed axial temperature differences did not negatively affect conidia production, although in specific moments corresponding to the maximum biological activity, differences of 10-15℃ between reactor wall and centre of the packed bed were observed. Given the high influence of temperature on fungal conidia production [23,29], it can be assumed that observed differences did not significantly affect conidia production. Although it has not been analysed in this paper, Finkler et al. [47] described heat transfer in a SSF packed-bed reactor using data obtained at various reactor heights, demonstrating an axial uniformity of the packed-bed which might also have happened in this work using both presented substrates.

Fig. 6 shows contour and mesh graphs of obtained data corresponding to last samples of all 22 L beer draff performed batches, presenting conidia production dependence on moisture and pH. A defined area of maximum conidia production was found, corresponding to a moisture range of 56–60% and a pH range of 5–6. Both ranges were consequent with results obtained in other works performed with similar strains [23,34].

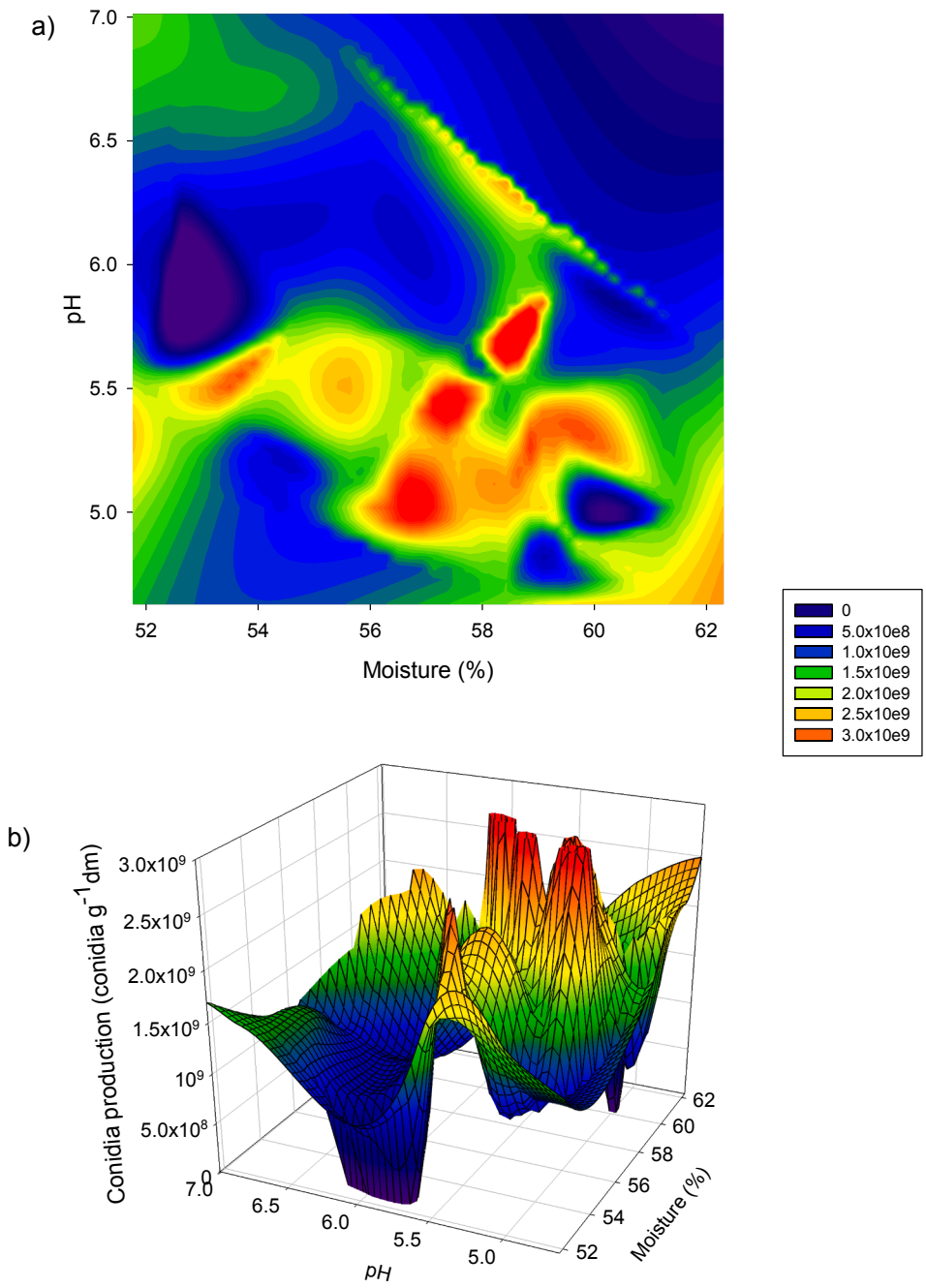

Fig. 6. 3D graphics (x axis moisture, y axis pH and z axis conidia production) corresponding to results obtained in last samples of all 22L beer draff batches. a) Contour graph, b) 3D mesh graph.

These analyses demonstrate that a reproducible and robust SBR process has been achieved when fermenting beer draff complemented with wood chips in 40/60% w/w proportion, as obtained results have not been significantly different for 5 consecutive batches. Aside from temperature, values of relevant parameters can be considered constant throughout the reactor bed, ensuring the robustness of the process. A minimum AFP_R value of 80% is needed to correctly perform fungal fermentation at 22 L scale, as results were better in terms of consecutive batches when comparing to 70% AFP_R tested at 1.5 L scale (Table 1), showing the high relevance of the parameter in the scaling process. These findings are highly relevant to fungal SSF, as most SSF processes performed using packed beds do not present uniformity due to heat transfer and bed packing issues [48], which is not only achieved in single batch in this work but also in a maximum of 5 consecutive batches by implementing a SBR strategy.

Future work should be focused on further scaling up the SBR strategy using beer draff complemented with the adequate quantity of wood chips. In order to ensure correct fungal growth and sporulation, AFP_R should be used as main scale-up parameter. Other relevant parameters such as temperature and moisture but also pH should be thoroughly monitored and controlled or adjusted when scaling the process to pilot plant scale.

4. Conclusions

A robust, reproducible, and scalable process to produce TH conidia in SSF packed bed bioreactors using substrates of different biodegradability has been achieved. Process scale-up has been successful using

both rice husk or beer draff and wood chips as substrates. SBR strategy has been successful using the mixture of beer draff and wood chips, sustaining conidia production for 5 consecutive batches at values close to $2.0x10^9$ conidia g^{-1}dm. Prioritizing the use of the safest and sterilisable substrate, rice husk was discarded in favour of beer draff under this operational strategy for beer draff not presenting AN contamination, despite sterilization. Differences in performance between scales when using beer draff allowed the definition of a minimum AFP_R value of 80%, determining AFP_R as a key parameter in SSF process scale-up. Process robustness was demonstrated with packed-bed uniformity in all 22 L reactors with both substrates despite their different biodegradability. No significant variations throughout the height of the reactor for conidia production, moisture and pH, were detected showing only minimum temperature rise when scaling beer draff. Implementing a SBR strategy with adequate AFP_R values helps at overcoming major scale-up drawbacks, at least up to a scale of 22 L, being a feasible alternative to traditional batch operation.

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2021.131620.

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