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Harnessing Packed-Bed Bioreactors' Potential in Solid-State Fermentation: The Case of *Beauveria bassiana* Conidia Production

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Abstract: The packed-bed bioreactor is among the most promising reactor configurations for solid-state fermentation. However, the bed thickness poses several limitations involving mass and energy transfer, heat generation, and the homogeneity of the material, hampering its development at the industrial scale. Fungi are among the most promising microorganisms used in this configuration; however, only polypropylene bags and trays are used at the industrial scale. In this work, *Beauveria bassiana* is used to demonstrate the potential of solid-state fermentation for conidia production. A scale-up from 0.5 L to 22 L is presented, starting with substrate selection, optimization via design of experiments and 22 L batches. The optimized parameters were 70% moisture, 6.5×10^6 conidia mL^{-1} inoculum concentration, 20 mL min^{-1} airflow, 25 °C; temperature, and 40 C/N ratio. After optimization, beer draff was chosen as the preferred substrate for scale-up. Air-filled porosity was found to be the key parameter in fungal solid-state fermentation scale-up, establishing values of around 80% as necessary for fungal conidia production when working in a 22 L packed-bed bioreactor. When compared with the tray bioreactor, the packed bed obtained higher conidia production due to its better use of the total reactor volume. Our study harnesses the potential of the packed-bed bioreactor and serves as a base for further scale-up to industrial scale.

Keywords: solid-state fermentation; packed-bed bioreactor; *Beauveria bassiana*; conidia production; air-filled porosity

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1. Introduction

In recent years, the circular bioeconomy (CBE) has gained attention as an emergent strategy to cope with the important environmental challenges the world is facing [1]. In particular, CBE is related to the fulfillment of several sustainable development goals (SDGs) proposed by the United Nations [2]. For instance, SDG2 (sustainable agriculture), SDG6 (sustainable water management), SDG7 (sustainable energy), SDG8 (sustainable growth), SDG12 (sustainable production and consumption), and, above all, SDG13 (climate action) are closely related to CBE. In this framework, the development of CBE using organic wastes as raw materials achieves two main objectives. On the one hand, it changes the paradigm "from waste to resource", closing the organic loop [3]. On the other hand, it is an effective approach to the worldwide problem of the increasing generation of waste [4].

The most suitable strategy to take advantage of organic waste in a CBE-based waste valorization scheme is probably in the form of biorefineries. Biorefineries are complex facilities where organic waste is used as the raw material to be transformed into a wide variety of bioproducts and renewable energy [5]. The research and implementation related to these installations are experiencing an exponentially increasing trend, from pilot plants to the first full-scale facilities [6]. Biorefineries include both biotechnological and physicochemical processes. Among the biological technologies, composting and anaerobic digestion are the most relevant because of their early development at full scale some decades ago [7].

In the context of modern biorefineries, however, novel technologies have emerged to obtain highly valuable products beyond compost or biogas. This is the case for solid-state fermentation (SSF), in which organic waste acts as a substrate for obtaining a specific bioproduct with the inoculation of a specific strain, which can be bacteria, yeast, or fungi. Producing enzymes and biosurfactants are clear examples of SSF-oriented strategies [8]. More recently, fungi have revealed their potential to produce biostimulants from lignocellulosic organic waste, and several studies are available in the recent literature using a wide variety of agricultural waste or by-products [9–12]. Food waste (including household, agricultural, and industrial waste) constitutes a severe issue worldwide (between one-third and one-half of the total produced food is not consumed, depending on the consulted study) [13,14]. In Europe alone, around 88 million tons of food are generated annually, with the food industry producing a very high ratio of organic-specific waste [15,16]. SSF emerges as a technology to treat organic waste for the generation of highly valuable bioproducts. In this case, SSF has clear advantages. For example, the waste is used as obtained (involving a reduction in cost when compared with the use of complex media) and needs less water, especially when compared with traditional submerged fermentation. Other advantages of SSF include the similarity with natural conditions found in microorganisms, the reduction of substrate inhibition possibility, cost reduction due to the use of wastes as substrates, and easier downstream possibility when the product can be applied directly to soil [17]. However, SSF faces challenges when included in full-scale biorefinery schemes. The main challenge is scale-up, as SSF is a complex process where the biological process coexists with typical engineering limitations such as mass (especially oxygen) and heat transfer [18].

When focusing on fungi, the most obtained bioproducts are aerial conidia, which constitute the most infective propagule against insects. Being produced by SSF and using organic wastes as substrates, aerial conidia are cheaper to produce and require less water in comparison with submerged propagules. In terms of control, aeration in SSF is also easier due to the use of high porous material to ensure high oxygen transfer. In terms of application, aerial conidia are the propagules presenting higher resistance to abiotic factors (temperature, UV), higher virulence, and overall longer viability [19–22]. Among all fungal genera, *Beauveria bassiana* stands as the most produced biocontrol agent due to its high infective capacity (it is a pathogen of more than 700 species) and mode of action (penetrating the cuticle and killing the insect from the hemolymph) [19]. All *Beauveria* species are accepted as GRAS (generally recognized as safe), with several by-products being commercially available in several countries, including in Europe, not only for *Beauveria* but also for other genera such as *Trichoderma* or *Metahizium* [10]. Although conidia produced by *Beauveria bassiana* and other genera have been scaled-up to industrial volumes, achieved reactor configurations include trays or bags. These productions involve extensive chambers with controlled temperature and humidity, involving huge volumes that are not used for conidia production [19]. Industrial application of the packed-bed configuration could solve this issue while yielding higher volumetric production. In this study, an effective approach to developing a reliable SSF to fill the scale-up gap is presented. Two typical SSF bioreactor configurations (packed-bed and tray), coupled with a wide variety of organic waste and rejects from agriculture and food processing, are used. *Beauveria bassiana* conidia production is optimized and scaled up to 22 L. As a result, a physical property of the mixture, porosity, appears to be critical for the proper scale-up of the process, which should be carefully considered in further biorefinery schemes in parallel with biological issues.

2. Materials and Methods

2.1. Fungal Strain and Inoculum Preparation

Beauveria bassiana (strain CECT 20374) (BB) was used in all experiments. The original strain was preserved in cryovials containing 10% glycerol at −80 °C. The strain was selected due to its potential application as a biopesticide against several insect pests [10], as it was originally isolated from *Dociostaurus maroccanus* (Moroccan locust). Before use, the strain was cultured in potato dextrose agar (PDA) at 25–30 °C for 8–10 days. Aerial conidia were

harvested using 10 mL of Tween 80 0.1%. Conidia were counted using a Neubauer chamber and diluted to the appropriate concentration for each experiment using Tween 0.1%. The inoculum volume used in all reactors was 10% of the total working volume [23].

2.2. Raw Materials

Rice husk (waste from rice harvesting, Husk Ventures S.L., Barcelona, Spain), apple pomace (waste of apple juice production, Mooma, Fontanilles, Spain), spent soy fiber from the vegetable beverages industry (Liquats Vegetals S.L., Viladrau, Spain), beer draff (waste of beer production, Cervesa del Montseny S.L., Sant Miquel de Balenyà, Spain), orange peel (waste from orange juice machine, Escola d'Enginyeria at UAB), and potato peel (waste from potato peeling, Patatas Torres, Montmeló, Spain) were provided as substrates for fungal conidia production. Except for rice husk (stored at room temperature), all substrates were stored frozen at −20 °C until use. When necessary, all substrates (except for rice husk) were mixed with either rice husk (substrate selection) or wood chips (scale-up and tray experiments) to enhance their porosity. All substrates were autoclaved thrice before inoculation (121 °C, 30 min) and stored at 4 °C until use.

2.3. Solid-State Fermentation Experimental Setups

Experimental setups corresponding to all reactor configurations used are presented in Figure 1. In all bioreactors, inoculation was performed ensuring maximum homogeneity inside the laminar flux chamber, guaranteeing sterility.

2.3.1. Packed-Bed Reactors: 0.5 L

The experimental setup for 0.5 L is shown in Figure 1a. SSF was performed in cylindrical PVC reactors. The reactors measured 13 cm in height and 7 cm in diameter, corresponding to a working volume of 0.45 L. A total of 100 g of each substrate was loaded into each reactor. A constant temperature was ensured by means of heated water and was maintained at 25 °C when the parameter was not subjected to optimization. As they could not be autoclaved, all reactors were cleaned with water and bleach to prevent contamination before starting all experiments.

The 0.5 L reactors were used for substrate selection and optimization experiments (design of experiments (DoE) and time course). Substrate selection experiments were performed in triplicate. The experiments from the DoE were presented in a previous work by the same authors [13]. Briefly, we performed optimization experiments using a selected substrate. We used the Box–Behnken design (DesignExpert 11, StatEase Inc., Minneapolis, MN, USA) to perform a total of 2 DoEs [24]. We optimized the following parameters: initial moisture, inoculum concentration, and airflow in DoE1; and temperature, C/N ratio, and moisture in DoE2. The parameters and ranges to optimize were selected according to previous research [10]. Optimized results were used in a subsequent time course test that lasted 16 days.

2.3.2. Packed-Bed Reactors: 22 L Bioreactor

The 22 L experimental setup is shown in Figure 1b. The reactor consisted of a cylindrical stainless-steel vessel with a removable cylindrical basket of 48 cm height and 24 cm internal diameter with a working volume of 19.8 L, corresponding to 4000 g of substrate. The temperature in the solid media was monitored using a temperature probe (Pt-100 sensors, Sensotrans, Barcelona, Spain). Accurate temperature profiles at different heights of the bed (0, 12, 24, and 36 cm) were obtained using temperature sensors (standard Thermochron iButton device, Maxim Integrated, San Jose, CA, USA). The room temperature was also monitored. The fermentation time and aeration rate were adjusted depending on the results obtained in 0.5 L (see Section 3.3).

To ensure maximum sterile conditions, the cleaning of both the basket and reactor was performed using bleach and alcohol, both before and after all fermentations. The basket was autoclaved thrice before loading and inoculating inside the laminar flow chamber.

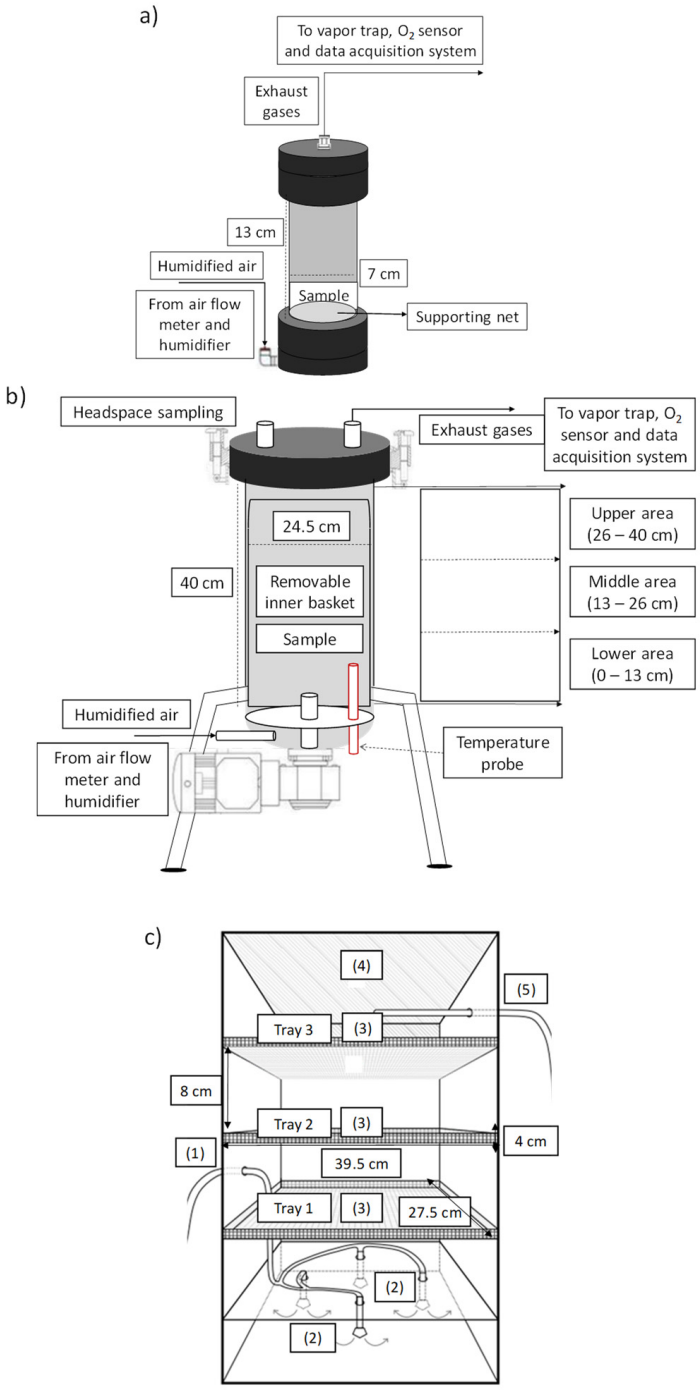

Figure 1. Reactor setup of (**a**) 0.5 L, (**b**) 22 L packed-bed, and (**c**) tray bioreactors. In (**b**), the reactor division used when sampling the complete reactor is shown. In (**c**), air inlet (1), air sprinklers (2), trays with 1 cm diameter holes in the bottom (3), adsorbent (4), and air outlet (5) are shown.

2.3.3. Tray Bioreactor

The tray bioreactor experimental setup is shown in Figure 1c. An incubator (Memmert ® GmbH + Co.KG, P.O. Box 1720, 91107 Schwabach, Germany) was adapted to work as a tray bioreactor, presenting a total volume of 43.5 L. The experiment was performed using 3 trays with the following dimensions: 39.5 cm length, 27.5 cm width, and 4 cm substrate bed thickness, corresponding to a total working volume of 4.35 L. The loaded substrate quantity was 1.5 kg (500 g per tray). When the trays were numbered, tray 1 was always the one closest to the air inlet (sprinklers), with the same distance maintained between

trays. Proper air distribution was ensured using four sprinklers located at the bottom part of the reactor facing downward. The top of the reactor was loaded with adsorbent material (Vileda Professional, Freudenberg Home and Cleaning Solutions Ibérica, S.L.U., Weinheim, Germany) to prevent condensing water from the exhausted air from dripping onto the closest tray. Each tray was provided with 2 temperature sensors to obtain accurate temperature profiles and to compare with room temperature. Before the experiment, all trays and the incubator were cleaned using the method presented in Section 2.3.3 for the 22 L bioreactor.

2.3.4. Oxygen Uptake Rate

The air supply and acquisition data systems were the same for all bioreactor configurations. To provide a respiration indicator of the biological activity, the specific oxygen uptake rate (sOUR) was calculated online according to Puyuelo et al. [25] and expressed as average values for 1 h following Equation (1):

$$\text{sOUR} = \text{F}\cdot(0.209 - \text{yO}_2)\cdot \frac{\text{P}\cdot 32\cdot 60\cdot 10^3}{\text{R}\cdot\text{T}\cdot\text{DM}\cdot 10^3} \quad (1)$$

where sOUR is specific oxygen uptake rate (g O_2 kg^{-1} dm h^{-1}); F is airflow (mL min^{-1}); yO_2 is exhaust gases oxygen molar fraction (mol O_2 mol^{-1}); P is system pressure assumed constant at 101,325 Pa; 32 is oxygen molecular weight (g O_2 mol^{-1} O_2); 60 is minute to hour conversion factor; 10^3 is mL to L conversion factor; R is ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T is temperature at which F is measured (K); DM is initial dry matter of solids in the reactor (g); 10^3 is g to mg conversion factor. In all bioreactor configurations, the obtained respiration profile corresponded to the total oxygen consumption presented by the full reactor.

2.4. Conidia Counting

A Neubauer chamber (Brand™ 717805) was used to determine fungal spore concentration. A total of 10 g of the sample (conidiated substrate) was mixed with 50 mL of Tween 80 (0.1% for BB), shaken for 20–25 min at 180 rpm at room temperature, and appropriately diluted before counting (adapted from [26,27]). Results were expressed in function of the dry matter present in the reactor at the counting time, following Equation (2):

$$\text{Concentration} = \frac{\text{N}^\circ \text{ spores}}{\text{CV}\cdot\text{DF}}\cdot\frac{\text{EV}}{\text{SWM}}\cdot\frac{\text{SWM}}{\text{SDM}} \quad (2)$$

where concentration is the initial tube spore concentration (spores g^{-1} dm); N° of spores is the counted spores using a Neubauer chamber at a specific dilution; CV is the Neubauer chamber counting volume (mL); DF is the counting tube dilution factor; EV is the volume of extraction (mL); SWM is the sample wet matter (g ww); SDM is the sample dry matter (g dm).

2.5. Total Sugar Content Analysis

The total sugar content of all substrates used in the substrate screening experiment was estimated using the anthrone method [28]. First, sugar extraction was performed using 3 g of sample and mixing with distilled water in a 1:10 (w/v) ratio to extract from dry solid samples. To incubate the mixture (15 min, 50 °C), a shaker/incubator (ZWYR-200D, Labwit Scientific, Melbourne, Australia) was used, followed by recovery of the supernatant via centrifugation (10 min, 2600× g). The whole extraction process was performed twice. The recovered supernatant volume was filtered using a 0.45 µm membrane filter. Before use, a fresh anthrone reagent was prepared by dissolving 200 mg of reagent in 100 mL of ice-cold 95% sulfuric acid. In 25 mL glass tubes, 4 mL of reagent was added to 1 mL of sample supernatant. After 8 min of heating in boiling water, the mixture was cooled rapidly by leaving samples on ice for 5–10 min. The absorbance was measured at 630 nm using a

spectrophotometer (Varian Cary50 Bio, Agilent Technologies, Inc., Tokyo, Japan). The blank was performed using 1 mL of distilled water. Six different glucose concentrations were used to prepare the calibration curve, ranging from 0 to 0.1 mg/mL. The total glucose content was expressed as grams of glucose equivalent per gram of dry matter (Equation (3)):

$$\text{Total sugar content} = \frac{C}{P} \cdot V \quad (3)$$

where total sugar content is expressed in g g^{-1} dm; C is the glucose equivalent concentration (g L^{-1}); P is the weight of the dry sample (g); V is the supernatant total volume (L).

2.6. Analytical Methods

The moisture (%), dry matter (%), organic matter (%), and pH were determined using standardized methods [29]. Chemical elemental analysis was used to perform C/N analysis. Air-filled porosity (AFP) was calculated according to Equation (4) [30]:

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

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

2.7. Statistical Analyses

Statistical differences between samples were analyzed using Minitab 17 (Minitab Ltd., Pennsylvania, PA, USA) software, with one-way ANOVA ($p < 0.05$ confidence) followed by the Tukey test for separation of means. Mean values, sample sizes, and standard deviations were provided for the analysis. The results were classified in letter groups. Those with different letter groups were significantly different.

3. Results

3.1. Substrate Selection

Six different substrates (rice husk, apple pomace, soy fiber, beer draff, orange peel, and potato peel) were selected to perform a substrate screening experiment. The initial inoculum concentration was fixed at 1×10^7 conidia g^{-1} dm for all substrates, in a range of values reported as optimal for Beauveria bassiana by several authors [23,26,31]. The characterization of wastes used as substrates in the substrate screening experiment is presented in Table 1. Rice husk was added to some substrates to enhance gas transfer via an increase in porosity.

Table 1. Initial parameter values for substrates/mixtures used in screening tests.

Parameter/ Substrate	MC (%)	OM (%)	C/N Ratio	pH	AFP (%)	TSC (mg g^{-1} dm)	Mixture (*w*:*w*) (%:%) (RH: Other)
RH	62.2 ± 0.4	83.5 ± 0.5	95.3 ± 13	5.6 ± 0.2	85.7 ± 2.2	17.9 ± 0.2	100:0
AP	73.7 ± 0.5	89.3 ± 0.7	86.6 ± 4.1	5.1 ± 0.1	60.2 ± 1.8	163 ± 7.1	20:80
SF	74.2 ± 2.5	92.2 ± 1.5	12.2 ± 0.4	6.6 ± 0.2	45.4 ± 1.2	22.1 ± 0.4	5:95
BDr	79.9 ± 3.4	95.0 ± 0.5	10.6 ± 2.5	5.5 ± 0.3	65.2 ± 1.3	120 ± 4.4	5:95
OP	84.2 ± 0.8	94.6 ± 1.9	41.1 ± 2.7	4.6 ± 0.4	59.8 ± 1.2	242 ± 9.5	0:100
PP	91.1 ± 0.7	81.6 ± 1.2	23.8 ± 0.9	5.4 ± 0.3	59.4 ± 0.7	40.8 ± 2.4	0:100

MC—moisture content; OM—organic matter; AFP—air-filled porosity; TSC—total sugar content; RH—rice husk; AP—apple pomace; SF—soy fiber; BDr—beer draff; OP—orange peel; PP—potato peel.

The produced conidia and respiration profiles obtained in the substrate screening experiment are presented in Figure 2. As shown in Figure 2, all substrates but soy fiber achieved conidia production between one and two orders of magnitude above the inoculum concentration. Potato peel achieved the highest conidia production, reaching 1.3×10^9 conidia g^{-1} dm, with only rice husk achieving a similar production of 9.0×10^8. The rice husk result was remarkable, with the second highest conidia production, lowest respiration profile, and second lowest lag phase, reaching its maximum respiration on day 1.6 at a value of 0.37 gO_2 kg^{-1} dm h^{-1} with a lag phase of 0.65 d. In comparison, potato peel doubled the respiration values (0.81 gO_2 kg^{-1} dm h^{-1}) at 2.8 days after a lag phase of 1.67 d. Most of the other substrates reached lower conidia production and higher respiration. As respiration is an indirect measure of substrate biodegradability, it serves as an indicator of the potential for heat generation when scaling up SSF processes, as reported in similar process as composting [32].

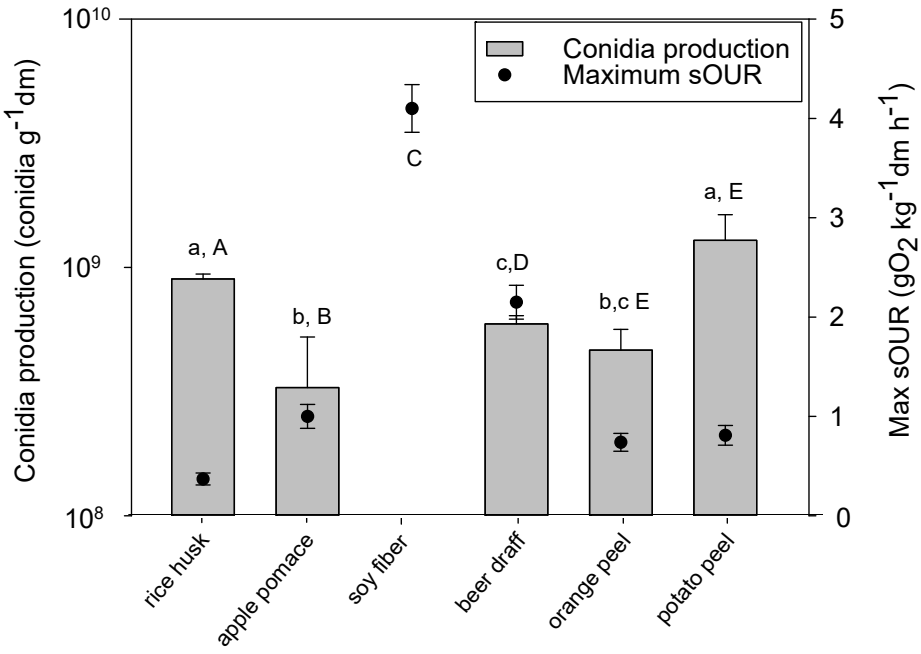

Figure 2. Results from substrate screening tests. Bars show conidia production and dots show maximum sOUR obtained for each substrate. Significant differences between samples are shown in lowercase (conidia production) and uppercase (maximum sOUR).

Soy fiber was the only substrate that did not exhibit *Beauveria bassiana* growth and sporulation. The respiration profile indicated the presence of bacterial contamination, reaching values of around 4 gO_2 kg^{-1} dm h^{-1} around day 2.6. The lag phase experienced by this substrate was 0.45 d, the shortest of all the substrates. Some successful substrates also presented a short lag phase (around 0.7 d for rice husk and beer draff) in comparison with the rest (1.3 d apple pomace, 1.67 d potato peel, and 2.4 d orange peel).

Moisture was maintained with all substrates, as final moisture values differed by less than 5% compared with the initial values.

As rice husk obtained high spore production coupled with a short lag phase and low respiration index, it was selected for the subsequent optimization experiments considering its potential for scale-up experiments.

3.2. Optimization in 0.5 L Reactors

Parameters and ranges tested in all batches of both DoEs are presented in Table 2. According to our previous work [24], the best results were obtained in reactor 6 in DoE1 and in reactor 7 in DoE2.

Table 2. DoE 1 and 2 performed batches. DoE 1 and DoE 2 parameters are separated by a vertical bar.

	DoE1			DoE2		
Run/ Parameter	**MC (%)**	**IC (Conidia g^{-1} dm)**	**AF (mL min^{-1})**	**T (°C)**	**C/N**	**MC (%)**
1	45	1×10^6	40	25	25	60
2	65	1×10^6	40	39	25	60
3	45	1×10^7	40	25	55	60
4	65	1×10^7	40	39	55	60
5	45	5.5×10^6	20	25	40	50
6	65	5.5×10^6	20	39	40	50
7	45	5.5×10^6	60	25	40	70
8	65	5.5×10^6	60	39	40	70
9	55	1×10^6	20	32	25	50
10	55	1×10^7	20	32	55	50
11	55	1×10^6	60	32	25	70
12	55	1×10^7	60	32	55	70
13	55	5.5×10^6	40	32	40	60
14	55	5.5×10^6	40	32	40	60
15	55	5.5×10^6	40	32	40	60

MC—moisture content; IC—inoculum concentration; AF—airflow; T—temperature; C/N—carbon/nitrogen ratio.

Optimal conditions found in previous DoEs were used to perform a time course test; profiles are shown in Figure 3. Despite using optimal production conditions (70% moisture, 6.5×10^6 conidia ml^{-1} inoculum concentration, 20 mL min^{-1} airflow, 25 °C temperature, and 40 C/N ratio) [23], the maximum conidia production was 9.0×10^8 conidia g^{-1} dm (peak at day 7.5–8), the same value obtained in the substrate screening experiment and half of the conidia production produced during DoEs. Between days 12 and 18, conidia production stabilized around 6.0×10^8 conidia g^{-1} dm. The pH range was between 6 and 8, and the moisture profile was maintained around 60%.

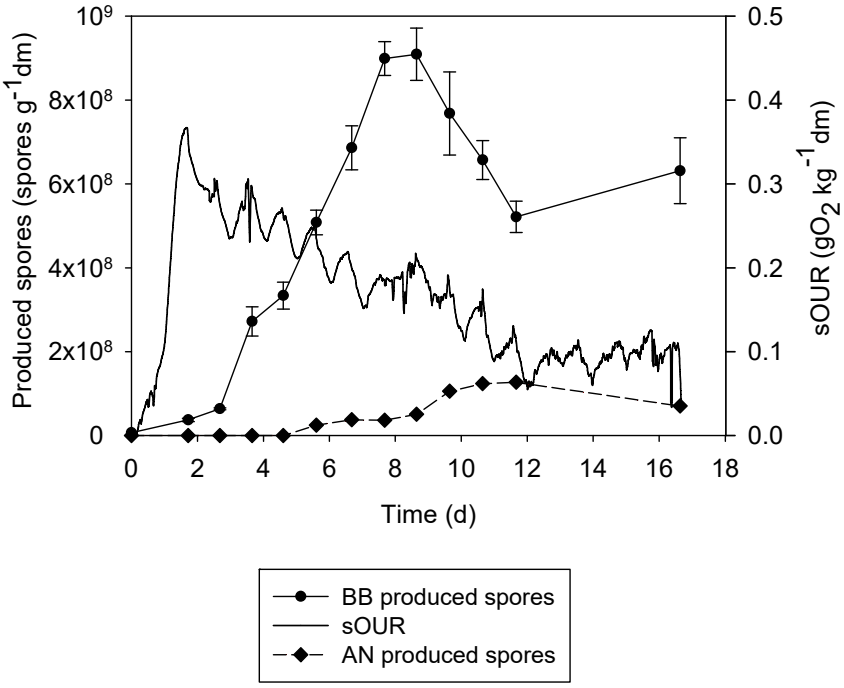

Figure 3. Time course profiles: BB (*Beauveria bassiana*) and AN (*Aspergillus niger*) produced conidia and sOUR.

Fungal contamination by non-inoculated *Aspergillus niger* was detected from day 5 onwards, reaching values close to 1×10^8 conidia g^{-1} dm, corresponding to approximately 10% of the total *Beauveria bassiana* production obtained. This contaminant was not found in previous fermentations using the same substrate.

Due to the presence of the contaminant in the time course experiment, another substrate was selected for scale-up. As potato peel was difficult to obtain, beer draff was selected alongside rice husk for further experiments.

3.3. The 22 L Experiments

Scale-up to 22 L was performed using two different substrates: rice husk and beer draff. Except for AFP, the parameters were maintained as found in the previous optimization experiments [24] for both substrates, as their enhancement was mandatory to ensure proper fungal growth. As demonstrated in the substrate screening and optimization experiments, rice husk could be used as the sole substrate due to its inherent high AFP. In contrast, beer draff was mixed with wood chips in a 40/60 *w*/*w* beer draff/wood chips ratio to adjust the AFP to values high enough to prevent compaction and allow a fungal SSF monoculture, reaching values higher than 80% with both substrates. In accordance with the AFP, the specific airflow (sAF) values also differed between substrates, in the range of 0.31–0.45 mL min^{-1} g^{-1} dm for rice husk and between 0.54 and 0.79 mL min^{-1} g^{-1} dm for beer draff.

The conidia production and respiration profiles obtained with both substrates are presented in Figure 4. Figure 4a corresponds to rice husk and Figure 4b to beer draff. Conidia production was higher when working with beer draff (Figure 4b), reaching values close to 2.5×10^9 conidia g^{-1} dm on day 8. This value was clearly superior to the one obtained at 0.5 L, which did not reach 1.0×10^9 conidia g^{-1} dm. It was also higher than the production obtained with rice husk at the same scale, rising to values of around 6.0×10^8 conidia g^{-1} dm. Although conidia production had already stabilized on day 8 when working with rice husk, it was still rising in the beer draff reactor. Fungal growth and sporulation were at the same timings as observed in the time course test.

As expected, differences in sOUR were observed between substrates. When working with rice husk, sOUR values never surpassed 0.9 gO_2 kg^{-1} dm h^{-1}, while maximum respiration with beer draff was around 3.5 gO_2 kg^{-1} dm h^{-1}, higher than values observed at 0.5 L. Differences were also observed in other parameters. Temperature profiles differed between substrates. While in rice husk, the temperature in all the reactors was kept similar to the mean temperature (20.3–24.8 °C), it was different in beer draff, where mean temperatures (22.5–27.5 °C) were a little bit higher. In addition, with beer draff, the center of the packed bed reached temperatures close to 33 °C at the time of maximum biological activity. Moisture also differed, between 57.4% and 60.9% with rice husk and 50.5% and 54.7% with beer draff. Only the pH was similar between substrates, remaining in the range of 6.0–6.8. While the mean temperature was closer to the optimum when working with beer draff, the moisture was closer when working with rice husk.

Final samplings from the 22 L reactors were performed, as shown in Section 2.3. All samples were analyzed for conidia production, moisture, and pH and statistically compared in relation to the height of the reactor. There were no statistical differences for the tested parameters when analyzing samples obtained at different reactor heights, with the independence of the substrate used. This result highlights the homogeneity and robustness of both SSF processes.

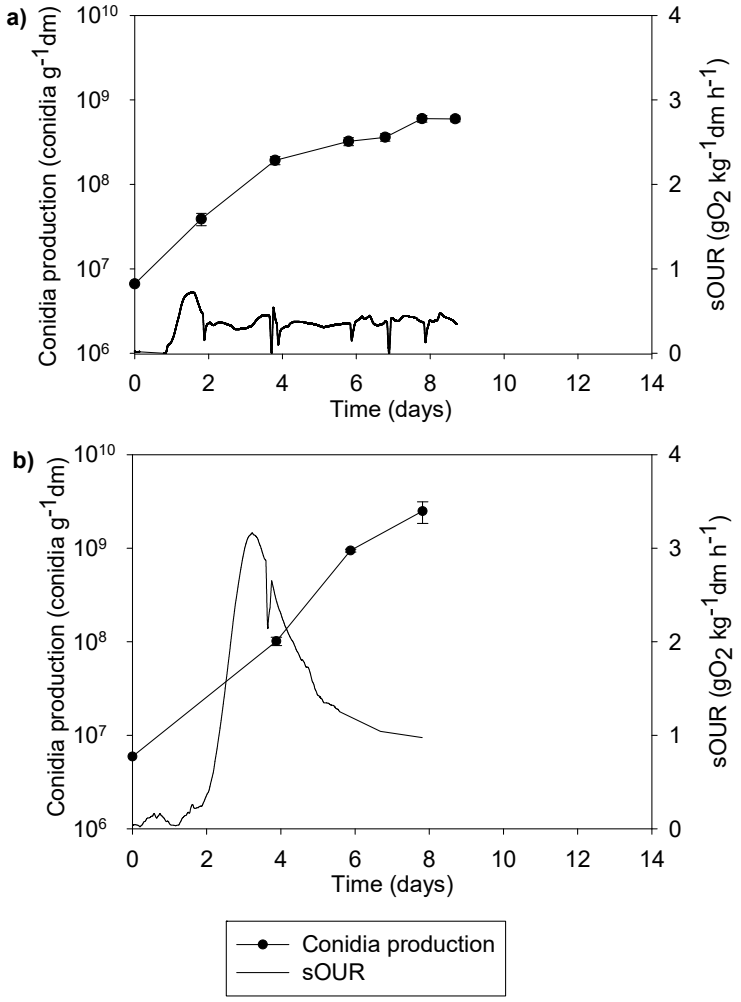

Figure 4. Process parameters evolution (conidia production and sOUR) in 22 L experiments. (**a**) Rice husk and (**b**) 40/60 *w*/*w* beer draff/wood chips.

3.4. Tray Bioreactor Comparison

Tray bioreactor fermentation was performed to compare with the results obtained in the packed bed scale-up. Figure 5 shows the reactor appearance of all beer draff bioreactor experiments, including 22 L and all trays in the tray bioreactor. When viewing the trays, conidia production differences can be observed; higher sporulation was visually confirmed in the tray closest to the sprinklers. Tray 1 (closest to the air supply) reached its maximum production on day 12. However, production in trays 2 and 3 reached its highest values on day 9. At that time, tray 1′s conidia production was 2.1×10^9 conidia g^{-1} dm, tray 2′s was 9.9×10^8 conidia g^{-1} dm, and tray 3′s was 5.4×10^8 conidia g^{-1} dm. The fermentation time was slightly higher when compared with packed-bed bioreactors. For respiration, the values obtained corresponded to the respiration of all trays, with a maximum sOUR of around 2.7 gO_2 kg^{-1} dm h^{-1} around day 3–3.5, in contrast with the time observed in both packed-bed bioreactor scales. Oxygen values never dropped below 15%, indicating oxygen availability throughout the full fermentation period. The temperature was maintained in the range of 25–32 °C, with small differences between trays of about 3 °C during the maximum biological activity time.

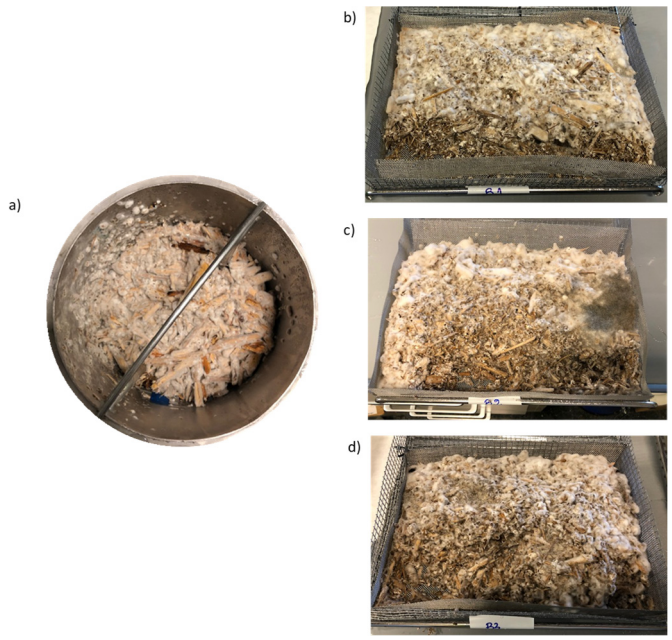

Figure 5. Bioreactor appearance at final process time in beer draff packed-bed scale-up and tray bioreactor experiments. (**a**) 22 L; (**b**) tray 1; (**c**) tray 2, and (**d**) tray 3.

A comparison of conidia production and productivity obtained in all beer draff reactors is shown in Table 3. Overall, the 22 L packed-bed bioreactor outperformed the tray in most analyzed responses. Maximum mean conidia production and productivity were both obtained when working with 22 L, showing higher values in the same order of magnitude. However, when analyzing total produced conidia and total produced conidia L^{-1}, the patterns were similar, but the 22 L results were one magnitude above. These results highlight the better spatial distribution of the packed-bed bioreactor and the relevance of AFP adjustment in *Beauveria bassiana* conidia production via SSF.

Table 3. Conidia production and productivities of all beer draff fermentations.

Parameter/Bioreactor	Tray Bioreactor	22 L Bioreactor
Mean produced conidia (conidia g^{-1} dm)	1.2×10^9	2.5×10^9
Time (d)	8.8	7.8
Grams dry matter (g dm)	540	1400
Total volume (L)	43.5	22
Productivity (conidia g^{-1} dm d^{-1})	1.4×10^8	3.2×10^8
Total produced conidia	6.5×10^{11}	3.5×10^{12}
Total produced conidia L^{-1}	1.5×10^{10}	1.6×10^{11}

4. Discussion

In this study, we present an optimized fermentation process for the production of fungal conidia for *Beauveria bassiana* using the packed-bed bioreactor configuration. We began with substrate selection and optimization, reaching a total volume of 22 L, and compared it with the tray production performance. Overall, our findings demonstrate the potential of the packed-bed bioreactor for *Beauveria bassiana* fungal conidia fermentation and highlight the role of several key parameters in the production process, particularly AFP and substrate biodegradability.

This work demonstrates the potential of the packed-bed configuration for fungal SSF conidia production. When compared with the tray bioreactor production, the 22 L packed-bed bioreactor showed the potential to surpass the tray if optimal conditions were

achieved. The conidia produced per liter of volume indicated better space utilization when working with a packed bed in comparison with a tray. This result is highly relevant, as most of the actual *Beauveria bassiana* production was superficial production using tray bioreactors or polypropylene bags put into environmentally controlled chambers [19]. Due to the AFP effect, the use of different mixtures between reactor scales might have affected the final conidia production results. A change to AFP values of around 80% improved the results in 22 L, to the point that fermentation was not possible when working at values of around 70%. This result demonstrates the relevance of AFP in *Beauveria bassiana* SSF when working with substrates similar to the ones used in this work, emphasizing its relevance for future works at higher volumes.

We have not found studies that used a similar strain (*Beauveria bassiana*), reactor configuration, and substrates for comparison. Other works used Erlenmeyer flasks, working with volumes between 250 and 500 mL and similar substrates (rice husk or coffee husk) [26,27], reaching spore productions close to the values obtained in this study when working at 22 L using rice husk. Using the same reactor configuration (22 L packed-bed bioreactor), beer draff complemented by wood chips as the substrate and *Trichoderma harzianum* as the fungal strain, Sala et al. [18] obtained conidia production values similar to the ones shown in this study. This comparison highlights the effect of proper AFP adjustment, allowing two different fungal strains to reach similar conidia production values.

The relevance of AFP as a key parameter has also been observed in 0.5 L experiments. In substrate selection tests, rice husk (presenting the highest AFP values, around 86%) was among the two best substrates in terms of conidia production. The obtained values were similar to the ones reported by Mishra et al. [27] using the same substrate. Substrates like rice husk are referred to as bulking agents, presenting low biodegradability according to Barrena et al. [33]. These substrates are promising for SSF scale-up, as they generate low amounts of heat due to their low biodegradability and high AFP. However, they also have low quantities of easily biodegradable carbon and nitrogen present, conditioning fungal growth and colonization. Therefore, they are mostly used as support and AFP enhancers rather than as the main substrates [34–36]. As demonstrated by our findings, once AFP is correctly adjusted, substrates such as beer draff (medium biodegradability, according to Barrena et al. [33]) can outperform them, as they possess higher sugar and nitrogen availability even when they are mixed with bulking agents. Better results at 0.5 L should also be expected in case of AFP adjustment using the same substrate/bulking agent ratio used in 22 L. Higher AFP coupled with sufficient specific aeration possibly contributes to maximizing fungal growth and sporulation, with the possibility of outperforming the results in substrate selection experiments [37]. To our knowledge, AFP relevance as a scale-up parameter has not been previously demonstrated by other authors, although the relevance of other parameters, such as specific aeration and superficial velocity, has been highlighted by several authors, a summary of them can be found in Finkler et al.'s review [38].

AFP adjustment not only helps maximize conidia production but also contributes highly to overcoming several SSF hindrances, including heat generation, the presence of contaminants, and homogeneity.

One of the main factors hampering SSF scale-up is heat generation [38]. Similar mean temperature profiles were obtained in both reactor configurations, despite 10 times the difference in bed thickness (40 vs. 4 cm). The generated heat per unit volume was much higher in a packed-bed bioreactor, as its working volume was much closer to the total volume in comparison with the tray bioreactor. This fact opens scale-up possibilities for packed beds. In addition, greater bed thickness should be tested for the tray configuration, as other authors [39] have already demonstrated its relevance for fungal growth and sporulation.

When working with rice husk during the time course test, the contaminant *Aspergillus niger* was detected. In the substrate screening experiment, the presence of contaminants was not detected in any other substrate producing *Beauveria bassiana* conidia, except for soy fiber. Growth in this substrate was faster (0.54 d lag phase) and yielded higher oxygen

values (around 4 gO_2 Kg^{-1} dm h^{-1}) in comparison with the rest of the tested substrates. The presence of contaminants is one of the issues most hampering SSF [40,41]. *Aspergillus niger* is a common food contaminant and is usually present in rice husk, a by-product of rice [42–44]. When working with soy fiber, low values of AFP (around 50%) favored the presence of bacterial contamination, which was identified as *Burkholderia gladioli*, a genus usually pathogenic to humans, plants, and animals that can be found in food by-products, which presents growth parameters similar to *Beauveria bassiana* but with faster growth [45–47]. Substrate selection must avoid the presence of contaminants, as dealing with them becomes more difficult as the volume increases, especially when referring to fungal spores capable of withstanding autoclave conditions. In addition, correct AFP adjustment also diminishes contamination probability, as demonstrated with the successful 22 L beer draff batch obtained after AFP optimization.

Achieving scale-up overcomes another of the most common problems of SSF, which is homogeneity [48]. The obtained *Beauveria bassiana* product was homogeneous throughout the whole height of the reactor, in terms of conidia production, moisture, and pH. In the axial gradient, temperature differences were also not observed, similar to the behavior observed by other authors [49,50]. Most works using similar quantities of substrate show differences in temperature throughout the bed, whereas the ones showing fewer differences show adjusted superficial velocity [48]. This parameter was not adjusted in this work; hence, temperature differences were not controlled. Although temperature differences were present in the radial axis (around 10 °C between the center of the packed bed and the wall of the reactor), the homogeneity obtained in the axial axis indicates that its effect on conidia production was not significant. Moreover, the results in this work correspond to one successful batch. As such, more batches should be performed using a 22 L packed-bed configuration to assess not only the homogeneity but also the reproducibility of the SSF bioprocess. In addition, better adjustment of superficial velocity should be tested to ensure better heat transfer.

Given the promising results, future research should focus on demonstrating the reproducibility of the proposed fermentation and on the further scale-up of the packed-bed bioreactor configuration using *Beauveria bassiana* or similar fungal strains, maintaining the chosen substrate. This work focused on scaling up the fermentation; thus, the biopesticide effect of the obtained spores was not tested. Despite homogeneous production throughout the reactors' height, performance differences between different sections of the reactor should be analyzed and compared with the effect of the same strain cultivated in a plate. The effect of the produced biopesticide against pests should also be tested. In the case of scaling the production, the biopesticide effect should also be tested. To ensure proper scale-up and to promote the use of packed-bed bioreactors at the industrial level, product performance should be comparable to that of current commercial products.

5. Conclusions

Successful conidia production was achieved using *Beauveria bassiana* and the packed-bed bioreactor configuration, scaling the production up to a 22 L volume. AFP was highlighted as the most relevant parameter for fungal conidia production scale-up in SSF, stablishing a value of 80% as sufficient. Proper AFP adjustment helped overcome several SSF scale-up drawbacks, including heat generation, contamination, and product homogeneity. Moreover, a model to predict conidia production was also obtained, highlighting the relevance of other parameters (temperature, moisture, inoculum concentration, and C/N ratio) on *Beauveria bassiana* conidia production, obtaining optimized parameters of 70% moisture, 6.5×10^6 conidia mL^{-1} inoculum concentration, 20 mL min^{-1} airflow, 25 °C temperature, and 40 C/N ratio. When compared with the tray bioreactor, the 22 L packed bed showed better results, especially in terms of total conidia produced, due to a better use of the total volume. Conidia production when working at 22 L was of 2.5×10^9 conidia g^{-1} dm, superior to obtained values when working with tray bioreactor. Overall, our results harnessed the potential of packed-bed bioreactors for fungal conidia production and will

serve as a base to further scale-up this process using packed-bed reactor configurations. Future prospects should focus both on scaling the fermentation up to a minimum of 100 L volume and on testing the biopesticide effect of the obtained bioproduct.

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