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Research article

Conidia production of the entomopathogenic fungus *Beauveria bassiana* using packed-bed bioreactor: Effect of substrate biodegradability on conidia virulence

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ABSTRACT

This work presents the scale-up of the conidia production of the entomopathogenic fungus *Beauveria bassiana* using two different wastes, coupled with concentration and virulence tests of the produced conidia against the pest *Tenebrio molitor*. *Beauveria bassiana* CECT 20374 was used in solid state fermentation (SSF) operating under batch strategy. Two substrates with different biodegradability (rice husk and beer draff) were tested, successfully scaling from 1.5 L to 22 L bioreactors. Higher conidia production was reached using beer draff as substrate (2.5×10^9 and 6.0×10^8 conidia g^{-1} dry matter in 1.5 and 22 L reactors respectively) highlighting air free porosity relevance as scale-up parameter. Concentration and dose-response tests against larvae and adult *Tenebrio molitor* were performed to compare strain CECT 20374 with control strain KVL 13–39 (a *B. bassiana* strain previously tested against *T. molitor*). Virulence effect of the 22 L fermentation product of strain CECT using rice husk or beer draff was tested against *T. molitor* adult stage. However, quality loses between conidia produced in agar plates and fermented products were observed (from 75 to 80% mortality in plates to 40% in rice husk and 50–60% in beer draff fermented products respectively). The differences between plate and fermented samples also indicated fermentation process, extraction and conservation steps as possible causes for quality losses, highlighting the need to optimize them to maximize virulence maintenance.

1. Introduction

The traditional use of chemical pesticides for pest management has led to numerous problems as they are harmful both for human health and the environment. Biopesticides have been recognized as an alternative to chemical pesticides for biological pest control. Entomopathogenic fungi represent an attractive alternative among biological control agents due to their ability to infect more than 1000 insect species while presenting no harm to humans or to the ecosystem (Mascarin and Jaronski, 2016; Araújo and Huges, 2016; Zheng et al., 2022).

Fungal biopesticides can be produced by solid-state fermentation (SSF). Despite having its drawbacks, SSF is the only method capable of producing aerial conidia (the most infective propagule) in a relatively inexpensive way, while also being associated with lower costs related to the use of agro-industrial wastes as substrates, serving as nutrient and support for fungal growth (De la Cruz Quiroz et al., 2015). An extensive list of wastes used for production of conidia of fungal biocontrol agents was reviewed by Sala et al. (2019), especially for genera *Beauveria* spp. and *Trichoderma* spp. Although conidia production of *Beauveria* spp. has been scaled-up to industrial scale, it has mainly been achieved using bags or trays as reactor configuration (Mascarin and Jaronski, 2016). Packed bed reactors (mainly presented as cylindrical columns) represent a promising alternative to traditional reactors. Packed-beds facilitate oxygen availability via continuous forced aeration, as well as maintaining constant moisture when supplied air is previously saturated with water. However, the main disadvantage of using packed bed reactors lies in the temperature control of the solid mass. Important difficulties in process scale-up can be caused by limitations due to heat removal from the reactor (Pandey et al., 2008).

In terms of insect pathology, studies of pathogenicity and virulence are of major relevance to properly evaluate mortality against insect pests. Pathogenicity is defined as the quality of being pathogenic, while

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virulence is the degree of pathogenicity within a group or species (Shapiro-Ilan et al., 2005; Kaya and Vega, 2012). Previous works involving *Beauveria* spp. have normally focused on identification of the most virulent strain against a specific pest, testing its effectiveness at different levels from laboratory to *in planta* assays (e.g. Keyser et al., 2016; Barta, 2018; Seid et al., 2019). In most works, comparisons were made on conidia directly obtained from plate cultures rather than fermentation production. Despite its relationship, high biocontrol potential strain identification and strain conidia production are most frequently reported separately (Toledo and Brentassi, 2022). Although several substrates have been optimized for *B. bassiana* conidia production, the effect of substrate characteristics that are key at production stage (such as biodegradability) have not been studied at product application stage, despite their potential effect on final product virulence.

The aims of this study were: i) to upscale *B. bassiana* conidia production on packed-bed bioreactors (up to 22 L) using two substrates of different biodegradability; ii) to compare virulence presented against the model pest insect *Tenebrio molitor* of the up-scaled *B. bassiana* strain with a reference *B. bassiana* strain of demonstrated high virulence and iii) to assess relative virulence differences between conidia produced using the two substrates of different biodegradability.

2. Materials and methods

2.1. Fungal strains

Two different *B. bassiana* strains were used in this work: CECT 20374 (CECT) in SSF (acquired from Spanish Type Culture Collection CECT, produced by SSF) and KVL 13–39 (KVL) used as reference in mortality and virulence tests (isolate obtained from the commercial biocontrol product BotaniGard containing the *B. bassiana* strain GHA). Both strains were stored at −80 °C in their respective culture collections. CECT was cultured in potato dextrose agar (PDA) and KVL in Saboraud dextrose agar supplemented with Yeast Extract media (SDAY) diluted 4 times (SDAY/4) at 25 °C for 7–9 days in darkness before use. SDAY/4 was used for both strains when cultured for virulence tests (see section 2.7).

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 SSF. Wood chips (Acalora, Ivars d'Urgell, Lleida) were used for air filled porosity (AFP) and moisture adjustment in the case of beer draff. Conservation conditions and substrate pretreatment (moisture adjustment, autoclaving and substrate mixture with wood chips) were described previously in Sala et al. (2021a). As presented in the same work, beer draff specific oxygen uptake rate (sOUR) was 3–4 times superior to rice husk (4.0 vs 1.0 $gO_2\ kg^{-1}$ dry matter (dm) h^{-1}), testing 2 substrates of different biodegradability. Raw substrate and mixtures characterization is presented in Table S1.

2.3. Solid-state fermentation

Fermentation tests were carried out using CECT strain as inoculum. Different SSF tests were performed at two packed-bed reactor volumes (1.5 and 22 L) using rice husk or beer draff as main substrates. The preferred conditions for the fermentations were 65–70% moisture, 25 °C temperature, 5.5×10^6 conidia g^{-1}dm inoculum concentration and 20 mL min^{-1} airflow rate, as described in Sala et al. (2020). pH was maintained in range of 6.2–8.0 for rice husk and 6.1–7.2 for beer draff. Total sugar content ranged from 16.6 to 4.2 for rice husk and from 83.2 to 12.2 for beer draff.

Experimental set-ups for both 1.5 and 22 L batch reactors were similar to previous works (Sala et al., 2021a). Same procedure was also followed both for temperature and final sample analyses. At both reactor volumes, sterile conditions were ensured by loading and inoculating in laminar flow cabinet. All fermentations were run for at least 8 days.

2.3.1. Reactor experimental set-up

Reactors of 1.5 L were made of polyvinyl chloride, cylindrical, 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. When working with beer draff, 70/30 w/w beer draff/wood chips was used as mixture. Constant specific aeration (0.40–0.58 mL min^{-1} g^{-1}dm for rice husk and 0.80–0.96 mL min^{-1} g^{-1}dm for beer draff) was continuously provided by means of a mass flowmeter (Mass-Stream D-6311, Bronkhorst, NL).

Oxygen percentage of 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® that calculates the respiration rates as explained in section 2.3.2.

Reactor of 22 L consisted of a cylindrical stainless-steel vessel with a removable cylindrical basket of 48 cm height x 24.5 cm diameter, presenting a total volume of 22 L (90% working volume), corresponding to 3000 g of rice husk and 4000 g of beer draff mixture with wood chips. Different substrate/bulking agent mixtures (70/30 and 40/60 w/w) were tested. Air supply and acquisition data system were the same as above. Constant specific aeration in the ranges of 0.31–0.45 mL min^{-1} g^{-1}dm for rice husk and 0.54–0.79 mL min^{-1} g^{-1}dm for beer draff was provided. When working with beer draff, specific aeration values were different between reactor volumes due to using different substrate/bulking agent mixtures.

2.3.2. Specific Oxygen uptake rate monitoring

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

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

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.

2.4. Conidia production assessment

Fungal conidia concentration was determined by the conidia counting method described in previous works (Sala et al., 2020). Briefly, 10 g fermented sample were mixed with 50 mL of Tween 80 0.1%, shaken for 20 min at 150 rpm and diluted to 10^5–2.5×10^6 conidia/mL before counting using a Neubauer chamber (Brand™ 717805). All conidia counts were performed in triplicates and related to the dry matter present in the reactor at counting time, following Equation (2):

$$\text{Concentration} = \frac{\text{Nº of conidia}}{\text{CV} \cdot \text{DF}} \cdot \frac{EV}{SWW} \cdot \frac{SWW}{SDM} \quad (2)$$

Where: Concentration is the conidia concentration in the initial tube (conidia g^{-1}dm); nº 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

Total sugar content of the fermented substrate was empirically determined using the Anthrone method, using glucose for the calibration curve (Scott and Melvin, 1953), expressed as gram of glucose equivalent per gram of dry matter following Equation (3):

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

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

2.6. Standard 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 in all initial fermentation samples. Chemical elemental analysis includes carbon, nitrogen, hydrogen and sulphur determination for raw substrates and mixtures used in fermentations. AFP was calculated according to Richard et al. (2004).

2.7. Assays against insects

Virulence assays were performed to compare the performance of the conidia of the produced strain (CECT) and the strain GHA from the commercialized product BotaniGard (referred to accession KVL). The strain GHA was used as positive control in all virulence tests, as it previously has shown high virulence against insects (Wraight et al., 2010, 2016; Swiergiel et al., 2016).

2.7.1. Conidia suspensions

Conidia suspensions were obtained from agar plates and from SSF experiments. When harvested from agar plates, conidia were transferred into 50 mL centrifuge tubes and lyophilised, while 10–20 g samples of the different SSF batches were lyophilised and vacuum packed. Lyophilised conidia were stored at room temperature (maximum 20 °C) for a maximum time of 2–3 months.

Conidia rehydration and dilution were performed using diluent Triton X 0.05%. When using plate samples, lyophilised conidia powder was rehydrated using 100 mL of Triton X 0.05%. When using solid lyophilised samples from SSF processes, rehydration was performed by mixing a known amount of dried sample with 5 times the volume of Triton X 0.05%. Samples were agitated at 150 rpm for 20–25 min. Later, suspensions were filtrated twice: first through a conventional sieve filter (around 1 mm porous) to separate large solid particles and second through a 100 μm laboratory sieve (Endecotts Ltd, London, England). After filtration, samples were centrifuged at 3000 rpm for 10 min using 15 mL centrifuge tubes (1:5 vol) (Sigma 2–16 KL, Buch Holm, Denmark). The supernatant was discarded and the pellet was resuspended in 10 mL Triton X 0.05%. The conidia were diluted to the appropriate concentration for counting and stored at 4 °C until use within a maximum of 7 days. All conidia suspension treatments were performed in sterile conditions and adapted from Inglis et al. (2012).

All CECT strain samples (plate or fermented samples) were lyophilised. The KVL strain was used as fresh conidia extracted from plates without being lyophilised.

2.7.2. Conidia germination tests

Conidia germination tests were performed prior to the use of all suspensions. Briefly, after conidia counting (section 2.4), 100 μL of 10^6 diluted conidia suspension were transferred to a PDA plate and incubated at 25 °C for 24 h. Following, three glass cover slips were placed on the agar surface of each plate and 100 conidia were counted under each cover slip, distinguishing between germinated (Gc) and non-germinated (NGc) conidia by identification of presence of germ tube in Gc. Conidia germination (%) under each cover slip was calculated following Equation (4):

$$CG(\%) = 100 * \frac{G_c}{(G_c + NG_c)} \quad (4)$$

Where: CG: conidia germination (%); G_C: germinated conidia; NG_C: non-germinated conidia.

2.7.3. Insect culture

Insect culture consisting of larvae of the yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) was obtained from Avifauna (Denmark) and kept in darkness at constant temperature of 25 °C (Series BD Classic Line Model 400, Binder) in ventilated plastic containers (30 × 21 × 20 cm^3). Each container was filled with 160–175 g of larvae, 500 g of organic oatmeal and 150–200 g slices of organic potatoes. Potato slices were replenished every 2–3 days. Pupae were collected regularly and kept separated in a medium-sized ventilated plastic box (22 × 17 × 6 cm^3). Upon adult emergence, beetles were separated from the pupae and maintained in a plastic container (30 × 20 × 12 cm^3) with oatmeal and potato slices.

For experiments using larvae, individuals were selected at 2–3 weeks after reception, measuring between 1 and 1.5 cm, colouring homogeneously yellow-tan. When using adults, individuals were selected between 1 and 3 weeks after adult eclosion, ensuring complete black colour of the cuticle.

2.7.4. Insect assays set-up

Medicine cups of 30 mL were used as set-up for assays with individual larvae and adults. When working with larvae, each *T. molitor* individual (previously introduced in a medicine cup) was exposed by pipetting 2 μL of the conidia suspension onto the anterior part of the body and left inside the medicine cup with a moist filter paper at 25 °C in darkness for 24 h, to maintain sufficient humidity for germination of conidia. Each cup was sealed with a lid perforated with three holes. After 24 h, the filter paper was removed and replaced with a 1% water agar cube (0.5–1 cm edge). Each larva was fed with 0.75–1 g wheat bran (Coop Danmark A/S, Denmark) and left in the medicine cup at 25 °C for up to 14 days. Mortality was checked daily. Dead individuals were collected and surface sterilized by immersion in 5% sodium hypochlorite for 30 s, followed by two rinses of 30 s in deionized water. The rinsed cadavers were then left individually inside parafilm-closed Petri dishes with moist filter paper at 25 °C for 2–7 days in order to stimulate fungal emergence from the dead insects (mycosis).

Adults of *T. molitor* were exposed by pipetting 5 μL of the conidia suspension onto the intersegmental membrane between the head and the pronotum. Each beetle was then left at 25 °C in the medicine cup with a potato slice for 24 h in darkness, to maintain enough moisture for fungal germination. Potato slices were removed and replaced with moist filter paper inside the medicine cups and the beetles were fed with 1.5–2 g oatmeal and left at 25 °C for up to 14 days. Mortality was checked daily. Moist filter papers were replaced if dry. Dead individuals were collected and surface sterilized as for the larvae.

2.7.5. Dose-response tests

For testing the effects of different concentrations of *B. bassiana* conidial suspensions from plate-grown samples listed in Table 1, a dose-response assay was performed using both larvae and adults of *T. molitor*. The assays were performed in triplicate with 10 individuals for each concentration. Using *B. bassiana* conidia harvested from agar media plates, four concentrations of the CECT strain (10^5, 10^6, 10^7, 10^8 conidia mL^{-1}) were tested against *T. molitor* larvae and adults (10^6 was not tested against adults). Triton X 0.05% suspension was used as control and diluent. For KVL strain, only 10^5 and 10^7 conidia mL^{-1} were tested,

Table 1
Germination test results for all *Beauveria bassiana* samples (n = 3).

Sample	Germination (%)
KVL 13–39 (P)	96 ± 1^{a}
CECT 20374 (P)	85 ± 1^{b}
RH 22 L (CECT-SSF)	65 ± 2^{c}
BDr 22 L (CECT-SSF)	75.5 ± 1.5^{d}
BDr 22 L (WV 10 L) (CECT-SSF)	74 ± 2^{d}

P: plate; RH: rice husk; BDr: beer draff; WV: working volume, SSF: solid-state fermentation. Statistical significance between tests is shown in superscript.

both for larvae and adults.

2.7.6. Comparative virulence test

Based on the dose-response assays, an experiment to compare virulence of conidia obtained from fermented rice husk or beer draff against *T. molitor* was performed, using setup as previously described (Keyser et al., 2016; Seid et al., 2019). The virulence assays were performed using two concentrations, low (X1) and high (X2), respectively, previously found to be in the lower and higher range of mortality in the dose-response assays in 2.7.5. The virulence assay was performed with 12 adult individuals for each concentration in triplicates. Procedures and conditions as described in 2.7.5.

2.8. Statistical analysis

Abbott's formula was used to correct insect mortality in all insect assays where the control mortality serves as inbuilt decrease of the background population. Corrected mortality was calculated according to Equation (6) as presented in Capinera (2004):

$$Corrected\ mortality = \frac{\%\ test\ mortality - \%\ control\ mortality}{100 - \%\ control\ mortality} \quad (6)$$

Time to reach 50% mortality of the total population (LT_{50}) was calculated by means of probit analysis (Finney, 1971). Briefly, the

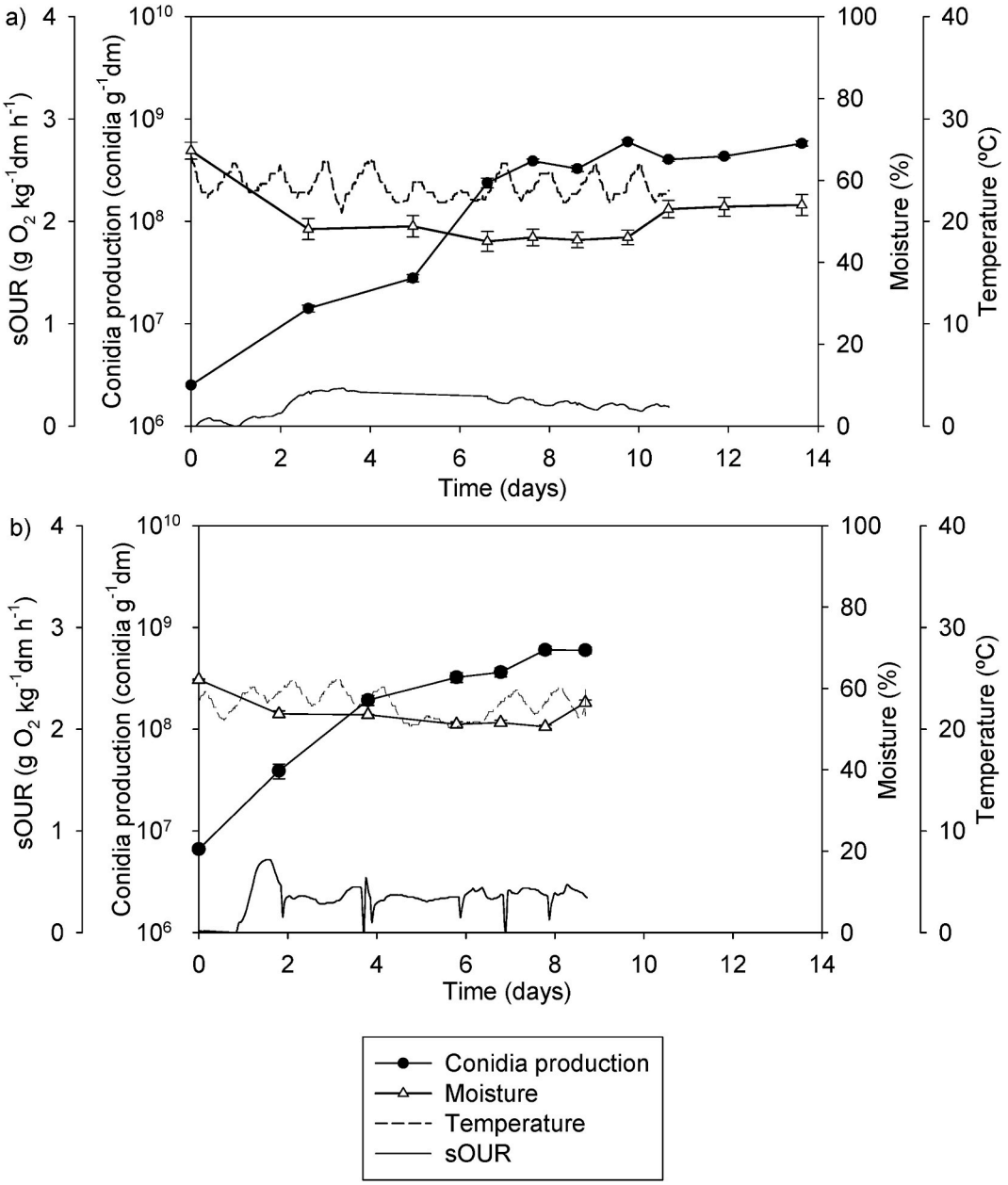

Fig. 1. SSF process parameters evolution in rice husk *Beauveria bassiana* SSF batch strategy. a) 1.5 L reactor and b) 22 L reactor.

S-shaped curve generated when plotting the dosages against time of death is transformed into a straight line by converting the response to a probit scale and the dosage to the log scale. Level of response can be obtained by going up the probit scale on the y-axis and reading across to where the slope of the line intersects the dosage scale on the x-axis, which then provides the dosage needed to produce that response.

Statistical difference between samples was analysed by means of a one-way ANOVA ($p < 0.05$ confidence) followed by the Tukey test for separation of means, using Minitab 17 (Minitab Ltd) software. Mean values, sample sizes and standard deviations were provided for the analysis. Power of the performed test was checked by means of alpha (0.05:1.000). No transformations were performed.

3. Results and discussion

3.1. Conidia production scale-up: from 1.5 L to 22 L

Successful batch scaling was achieved using both substrates. Fig. 1 shows process profiles from rice husk SSF, corresponding to 1.5 L (Figs. 1a) and 22 L (Fig. 1b) batch reactors whereas Fig. 2 shows same profiles for beer draff SSF batches, 1.5 L (Figs. 2a) and 22 L (Fig. 2b). In terms of conidia production, rice husk reactors presented similar values (around 6.0×10^8 conidia g^{-1}dm) once stabilised, although conidia production rise in 1.5 L reactors was observed 1–2 days later than in 22 L. Conidia production on beer draff was higher at both scales, being close to 1.5×10^9 conidia g^{-1}dm at 1.5 L and of 2.5×10^9 conidia g^{-1}dm at 22 L. Rice husk conidia values were similar to those previously observed in Sala et al. (2020) working with 0.5 L reactors and using the same strain. In contrast, beer draff values were far superior to conidia concentrations achieved in 0.5 L reactors using the same *B. bassiana*

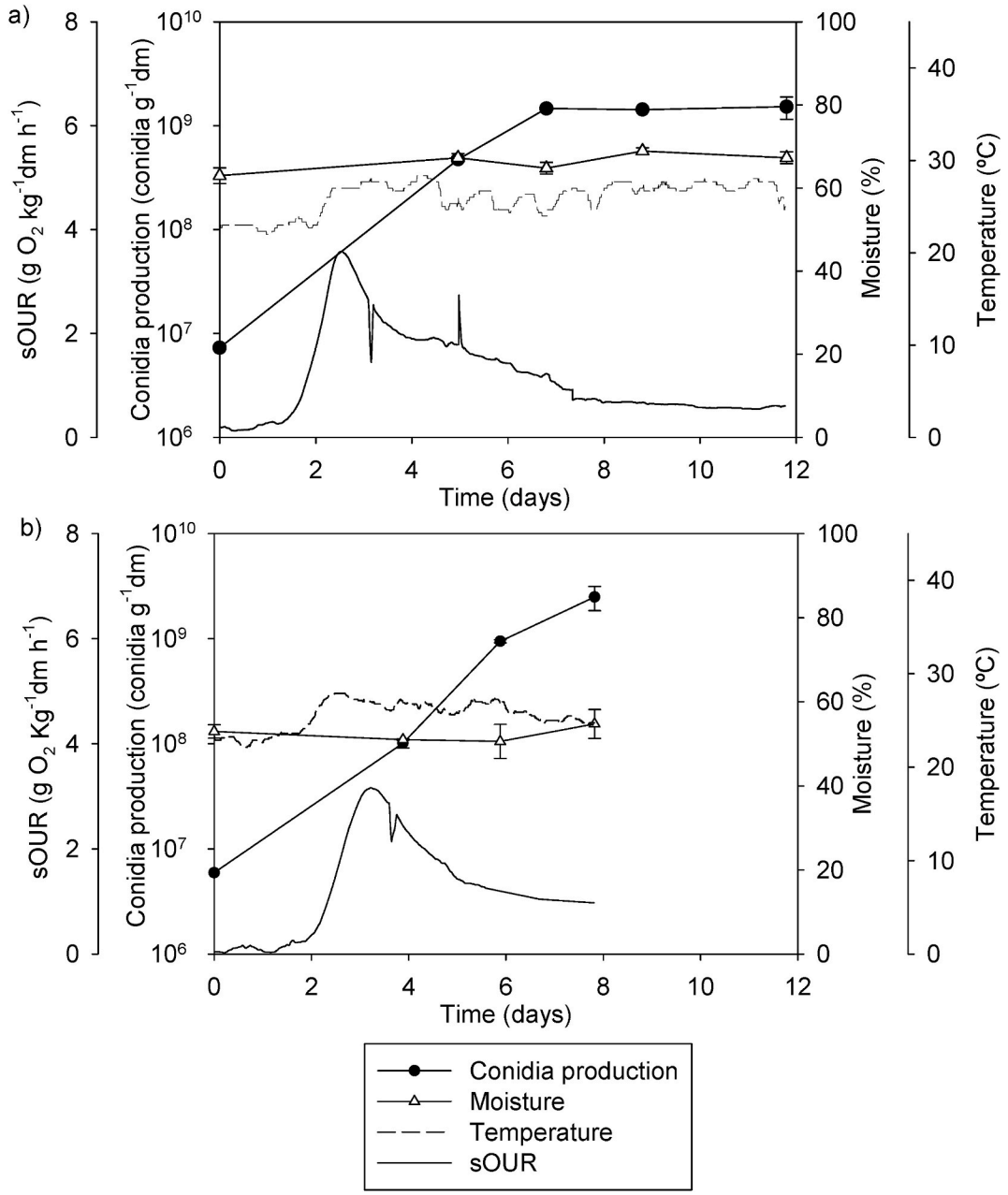

Fig. 2. SSF process parameters evolution in beer draff *B. bassiana* SSF batch strategy. a) 1.5 L reactor and b) 22 L reactor 40/60 w/w beer draff/wood chips.

strain (Sala et al., 2021b), where their values were lower than 1.0×10^9 conidia g^{-1}dm. Differences in conidia production could have been caused by differences in AFP, reaching higher values in this work due to porosity adjustment to 80% (this work) vs 66% (previous work). Working with rice husk in 0.5 L reactors, Mishra et al. (2016) obtained similar conidia production comparing with the same reactor scale also using *B. bassiana*. No other work was found on the use of beer draff as substrate to produce *B. bassiana* conidia, although it has been used with other fungi such as *Aspegillus niger* (Llimós et al., 2020).

As expected, sOUR profiles were different for both substrates, but little differences were found between reactor volumes when using the same substrate. When working with rice husk, respiration profiles were similar, showing higher oxygen consumption maximums at 22 L. sOUR values at 1.5 L scale never surpassed 0.5 gO_2 kg^{-1} dm h^{-1}, whereas at 22 L the respiration profile rose up closer to 0.8–0.9 g O_2 kg^{-1} dm h^{-1}. When using beer draff, both respiration profiles reached their maximum at values between 3 and 3.5 gO_2 kg^{-1} dm h^{-1}. Higher values of sOUR are related to high amount of easily biodegradable organic matter content in beer draff as also indicated by the total sugar content. Therefore, the two substrates present different biodegradability levels according to the relation between oxygen consumption and organic matter content (Barrena et al., 2011).

Temperature profiles for both substrates were also obtained, and are presented in Fig. S1. No temperature differences were observed in rice husk, reaching values ranging from 20 to 25 °C throughout the reactor bed, corresponding to ambient temperature. However, when working with beer draff, differences between reactor areas were observed, reaching temperatures close to 34 °C at the center of the packed bed at the time of maximum biological activity, and presenting differences between center and wall temperatures between 4 to 8 °C depending on biological activity. Thus, when working with high biodegradability substrates, SSF processes in packed beds can present problems related to heat transfer and bed-packing at industrial or pilot scale, that might negatively affect conidia production (Krishania et al., 2018). At both reactor volumes, beer draff conidia production was much higher than rice husk, mostly due to the superior biodegradability of the substrate. As temperature affects *B. bassiana* growth and sporulation, biodegradability must always be considered when scaling its production by SSF. Regardless of lower conidia production when comparing to beer draff (as seen when comparing Figs. 1 and 2), the use of rice husk as substrate presents no issues related to energy transfer, at least up to a scale of 22 L, confirming its feasibility as *B. bassiana* SSF substrate.

Despite successful upscaling, beer draff tests at 22 L presented some difficulties that highlighted the importance of air filled porosity (AFP) in the scale-up process. Different combinations of substrate and bulking agent were tested. A 70/30 w/w (beer draff/wood chips) proportion was tested both at 1.5 L and 22 L scales (data not shown) not achieving conidia production in the 22 L bioreactor. Mean temperatures higher than 40 °C were achieved, while sOUR reached values close to 8 g O_2 kg^{-1}dm. This behaviour was similar to the one presented when working with *T. harzianum* using same reactor configuration (Sala et al., 2021a). The use of 40/60 w/w beer draff/wood chips ratio produced successful results as shown in Fig. 2b. Changes in beer draff/wood chips ratio between scales, rising bulking agent quantity in 22 L bioreactor, caused a decrease in generated heat and bed temperature. This decrease was at the cost of biodegradability loses (bulking agent biodegradability is almost null at process conditions) to enhance final conidia productivity. In that sense, AFP adjustment helped at enhancing process conditions for fungal conidia production, being key for the success of beer draff SSF scale-up. This behaviour seems to be independent of the fungi used, as it was both necessary for *B. bassiana* (this work) and *T. harzianum* (Sala et al., 2021a).

Fig. 3 shows mean values and standard deviations for conidia production (a), moisture (b) and pH (c) at different heights in the reactor bed for both rice husk and beer draff 22 L reactors. Values of the parameters correspond to the final product obtained after each SSF. No significant differences in most of the analyses of the three parameters were observed for both substrates. These results indicate possible independence from reactor height for all analysed parameters, being especially relevant for conidia production due to radial temperature differences observed in the beer draff reactor.

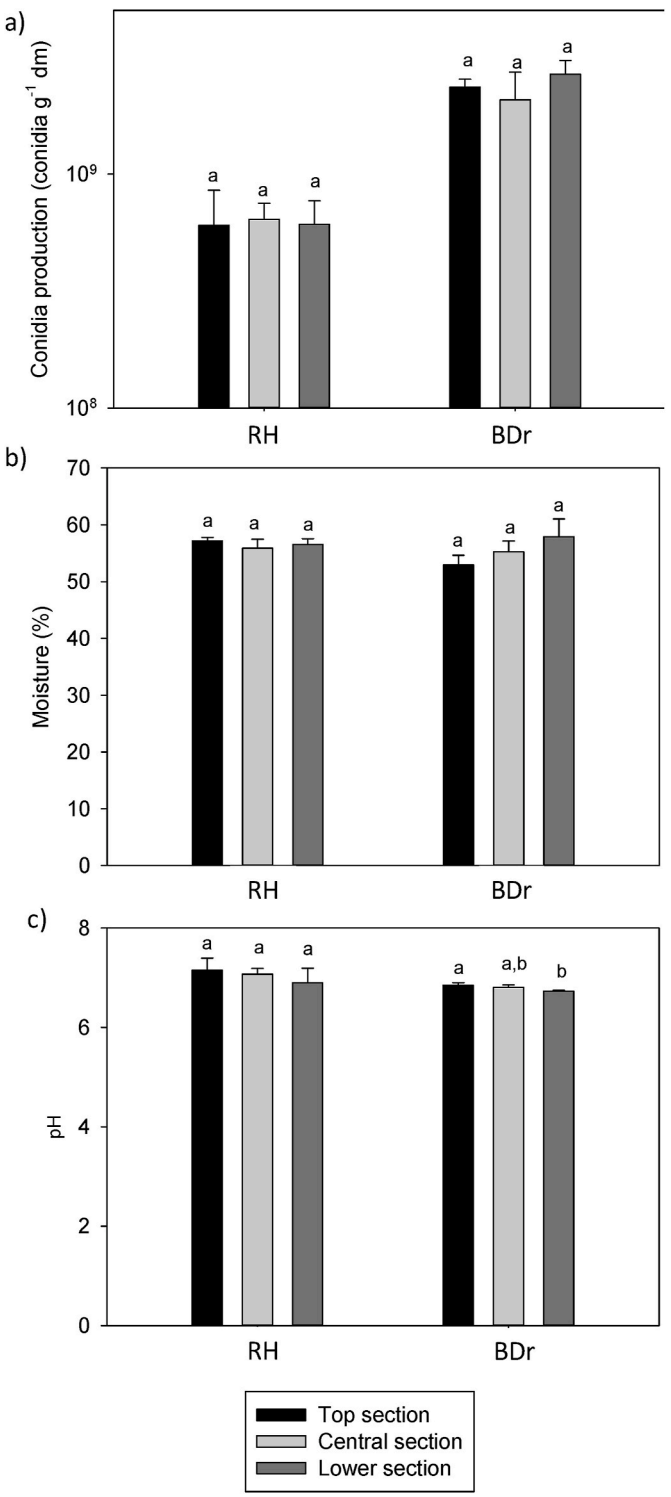

Fig. 3. Mean values and standard deviations of samples collected at different reactor heights at the end of all *B. bassiana* 22 L SSF batches. First column corresponds to rice husk fermentations and second column to beer draff. a) conidia production, b) moisture and c) pH. Statistical differences between areas of the same reactor and substrate are indicated in lowercase letters.

3.2. Dose-response test using T. molitor larvae

Virulence against the model insect *T. molitor* of the conidia produced during the up-scaled SSF process with *B. bassiana* CECT strain was compared to a reference strain GHA (KVL). Firstly, a dose-response test using conidia obtained from agar media plates was performed. Fig. S2 presents mortality of *T. molitor* exposed to different conidial concentrations of *B. bassiana*.

When working with the CECT strain (BB in Fig. S2 a and c x-axes), larval mortalities were generally very low for most of the tested concentrations below 10^8 conidia mL^{-1}. In contrast, the positive control treatments using *B. bassiana* GHA (KVL, C+ in Fig. S2 a and c x-axes) produced relatively high mortality in a dose-dependent manner, ranging from 20 to 90% mortality between the two tested concentrations (10^5 and 10^7 conidia mL^{-1}). Mortality in the lowest KVL strain concentration was similar to mortality produced by the highest concentration of the CECT strain. The proportion of mycosis development was also low for CECT, showing similar values as in lower concentration applied of the KVL strain. The only exception was at the high concentration of KVL, which reached up to 83% mycosis. Seid et al. (2019) achieved similar values using different *B. bassiana* isolates against *T. molitor* and similar concentrations. Additionally, 10^8 conidia mL^{-1} treatment was the only one presenting statistically significant differences with the rest of the tested concentrations.

As shown in Table 1, differences in germination rates between the two *B. bassiana* strains were observed, being 10% lower in the CECT strain in comparison to KVL when cultivated on agar media. However, reduced germination rate is unlikely to account for the large difference in infection levels between strains, as seen in Fig. S2a. Similarly, Maistrou et al. (2020) found that low virulent strains of *Beauveria* spp. were characterized by relatively low germination rate at 18 h incubation although at 24 h these strains showed same germination as the virulent strains. We did not test germination for >24 h, but potentially CECT could show higher germination rates by longer incubation. However, slow germination is expected to be a trait not conducive for causing successful host mortality (Maistrou et al., 2020), hence this is not a desirable trait in a biological control agent.

3.3. Dose-response test using T. molitor adults

Adults of *T. molitor* were also tested as hosts for the two *B. bassiana* strains.

Contrary to the larval stage, total mortality caused by CECT strain in adult *T. molitor* increased with increasing conidia concentration, starting at 40.7% for 10^5 conidia mL^{-1} and rising to 61 and 83% for 10^7 and 10^8 conidia mL^{-1}, respectively. This increment was also observed for level of mycosis, reaching its maximum (nearly 60% of the total dead population) when using highest tested concentration. Virulence was still not comparable to that of strain KVL, which achieved 100% dead adults at 10^7 conidia mL^{-1}. Similar mortality to that caused by CECT strain at 10^7 conidia mL^{-1} (around 80%) was reached at 10^5 conidia mL^{-1} for KVL. Observed levels of mycosis (around 50%) were similar between the highest CECT concentration (10^8 conidia mL^{-1}) and the lowest concentration of KVL (10^5 conidia mL^{-1}), but mycosis level was the highest for the high concentration of KVL, being close to 80% of the total dead population (Fig. S2c).

Lower virulence of CECT strain is further evident from the survivorship curves (Fig. S2d). Although the main mortality effects occurred at day 4 for both 10^8 conidia mL^{-1} of CECT and 10^7 conidia mL^{-1} of KVL, the curves reach different percentages of survivors. At day 8, all individuals were dead in the KVL treatment, whereas approximately 40% remained alive after the same period in the treatment with CECT. These differences can partially be explained by reduced germination rate, but are also reflecting reduced ability of CECT to infect *T. molitor* adults compared to KVL. Others have also found variation in infectivity between *Beauveria* spp. strains against adults of *T. molitor* (Maistrou et al., 2020).

The current results confirm other studies, whichhave reported the adult stage of *T. molitor* to be more susceptible to *B. bassiana* infection in comparison to larvae (Rodríguez-Gómez et al., 2009; Seid et al., 2019). Differences in structure of the cuticle between larvae and adults, as well as possible shedding of attached conidia on the cuticle after larval ecdysis (Hajek and Leger, 1994; Vestergaard et al., 1999) might be the principal mechanisms causing differences in susceptibility.

The CECT strain showed more pronounced virulence against the adult stage of *T. molitor* compared to the larval stage at the highest tested concentrations, increasing in a concentration dependant manner and confirming dose-dependent virulence.

3.4. Comparative virulence test of fermented conidia

To compare *B. bassiana* conidia virulence of fermented samples from the different substrates used in SSF (RH and BDr), a virulence test using adult *T. molitor* was performed at two concentrations. Assay results are presented in Fig. 4. Conidia from fermented samples CECT (SSF) were compared with conidia from agar plates of the strains KVL (P) and CECT (P). Both strains were tested at a concentration of 10^6 conidia mL^{-1} (low concentration) and a concentration of 5×10^7 conidia mL^{-1} (high concentration) for this comparative test with conidia of the samples listed in Table 1.

Interestingly, differences in total mortality were observed depending on the substrate used to produce fungal conidia (Fig. 4a). At the high concentration, mortality caused by conidia from beer draff SSF was higher than when using conidia produced using rice husk as substrate (SSF-BDr1-H sample vs SSF-RH-H sample). No differences were observed when comparing the low concentration for the two substrates, as little mortality with very low mycosis was obtained with all treatments at low concentration. Variation in virulence between conidia from different fermented samples can also be attributed to different conidia germination rates. Obtained values were of 72–77% for conidia produced in beer draff samples and even lower for conidia from rice husk, reaching 63–67%. Although both substrates have been successful at producing *B. bassiana* conidia using a batch strategy, germination rates of conidia were higher when using beer draff compared to rice husk (Table 1). Consequently, higher virulence of conidia from beer draff samples could be expected. Despite this, there were no significant differences between observed mycosis in the high concentration samples, regardless of the substrate used (Fig. 4a).

Conidia in all SSF samples were generally causing lower mortality than what is usually reported in virulence assays with *B. bassiana*, which is often 90–100% at high concentrations (10^7–10^8 conidia mL^{-1}) (Keyser et al., 2014; Barta, 2018; Seid et al., 2019). High germination rates are mandatory to ensure proper infection of the insect in controlled experiments. In this study, all germination rates were calculated at 24 h of incubation. However, the optimal germination time can vary depending on temperature and media (Maistrou et al., 2020). With the low percentages observed, and also considering growth and conidiation time of the CECT strain (presenting a latency phase between 1 and 2 days as shown in Figs. 1 and 2), it would be advisable to perform conidia germination tests at 36–48 h instead of 24 h. Other works have used this timeframe to calculate germination rates (Keyser et al., 2016).

Conidia quality loss was observed when comparing any sample to its correspondent agar plate control, which could also be expected from the germination test results in Table 1. These losses in viability were possibly caused by a sub-optimal downstream and conservation process. In fact, the downstream process was simple, consisting only of filtration and centrifugation steps with no formulation involved. These steps allowed conidia separation from almost all the solid material. As of conservation when lyophilising, conidia must be dried to a water content below 9% w/w or equal or to water activity equal to or lower than 0.3% to optimize shelf-life and maintain viability (Jaronski and Jackson, 2012; Moore et al., 1996). Drying and anaerobic packaging are required

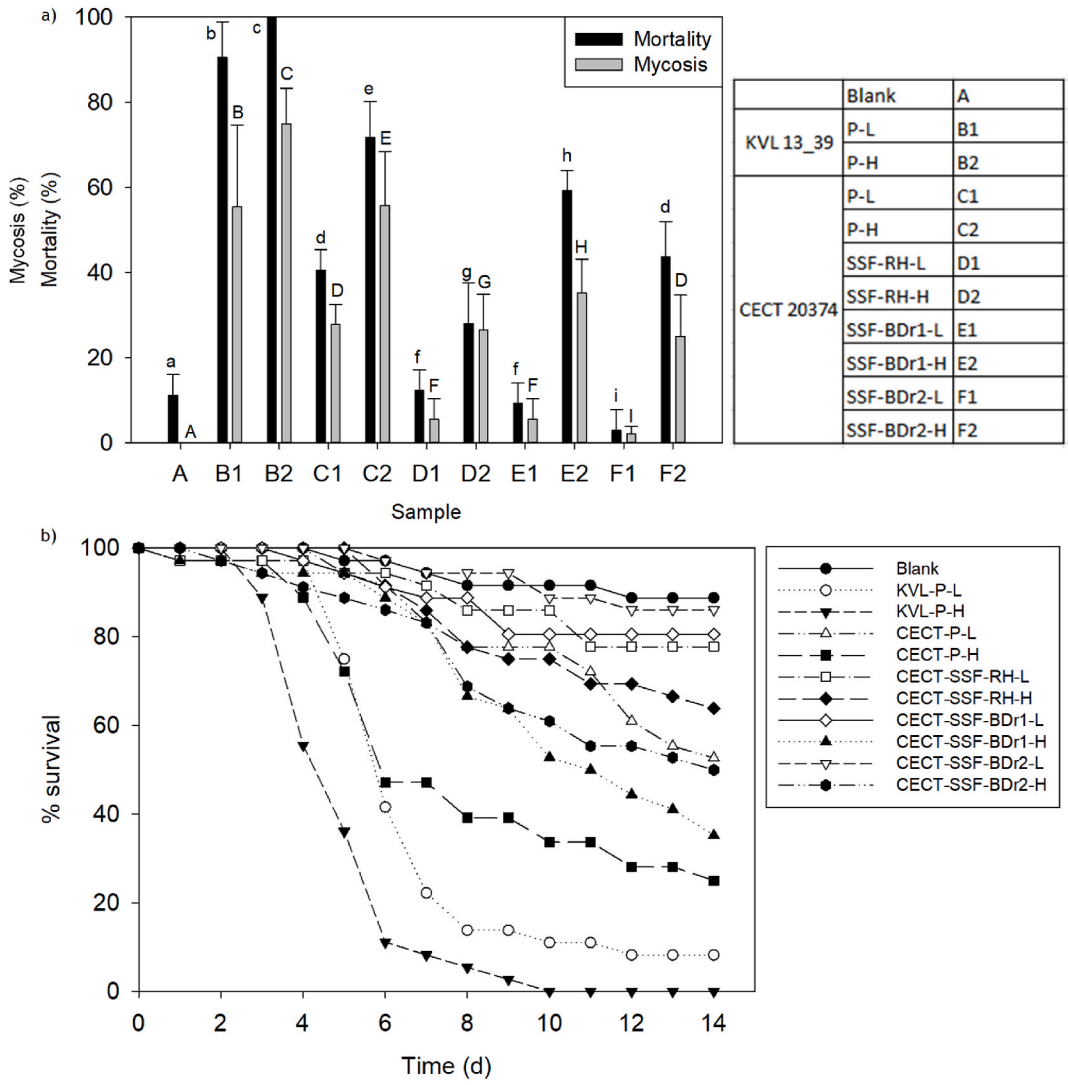

Fig. 4. Adult *T. molitor* virulence test results a) total mortality and mycosis and b) survival evolution. P indicates plate samples (KVL or CECT strains), SSF indicates fermentation samples (CECT strain), RH corresponds to rice husk and BDr to beer draff. Low concentration (L) corresponds to 10^6 conidia mL^{-1}, high concentration (H) corresponds to 5×10^7 conidia mL^{-1}. Statistical differences in 6a are indicated in lowercase letters for total mortality and in uppercase letters for mycosis.

to maximize the storage period (Chen et al., 2009; Faria and Wraight, 2007). However, despite using recommended conservation methods, possible quality loses might still be attributed to sample conservation. Formulation is a crucial aspect of any biopesticide product development, highly affecting both viability and infectivity of the active ingredient (Mascarin and Jaronski, 2016; Brar et al., 2006). As such, both conidia extraction, separation and formulation should be improved to obtain maximal conidia virulence.

When comparing treatments in the survivorship curves (Fig. 4b), mortality rates for CECT strain were higher and occurred earlier for conidia produced on agar plates when comparing to all fermentation samples. When working with agar plate samples at 5×10^7 conidia mL^{-1}, most reduction in survival occurred between days 3–6, while in fermentation samples SSF-BDr1-H (E2) and SSF-BDr2-H (F2), the main reduction in survival occurred between days 5 and 10, exemplifying virulence loss from plate to fermentation samples or the need of a larger lag phase for fermented conidia. Formulation improvement should be directed not only at increasing total mortality but also reducing the culture lag phase, as seen for the same strain with conidia from agar plates. However, when comparing samples from two different beer draff fermentations (treatments E and F in Fig. 4a) at the same conidia concentration, there were no significant differences between mortality or mycoses frequencies, demonstrating repeatability between fermentations and showing the robustness of the SSF process using the CECT strain.

LT_{50} values corresponding to all treatments with higher than 50% mortality are presented in Table 2. Only one fermented sample, SSF-BDr1-H (E2), caused more than 50% mortality in insects exposed, while samples obtained from rice husk fermentation did not reach 50% mortality. Statistical difference between LT_{50} values confirm higher

Table 2
Mean time to death for 50% of adult *Tenebrio molitor* (LT_{50} values) exposed to *Beauveria bassiana* conidia of different strain and/or production.

Sample	LT_{50} (d)
KVL-H	4.4 ± 0.4^a
KVL-L	5.8 ± 0.3^b
CECT-P-H	7.2 ± 0.6^c
BDr 22 L CECT–SSF–H	10.7 ± 0.5^d

LT_{50}: lethal time 50; BDr: beer draff; L: low concentration (10^6 conidia mL^{-1}); H: high concentration (5×10^7 conidia mL^{-1}); P: plate; SSF: solid-sate fermentation. Statistical significance between tests is shown in superscript.

virulence of KVL compared to CECT, while also exemplifying virulence loss from plate to fermentation samples in CECT strain. Aside from formulation and conidia conservation, differences in virulence between agar plate and fermented samples suggest the fermentation process and/or recuperation and conservation steps as a possible cause of viability losses. Differences in relevant parameters (such as temperature), being much easier to maintain close to optimum values when working with plates, could be a reason of viability losses. Improvements in fermentation with the aim of maximizing virulence maintenance and not only conidia production should be considered, including the use of substrates similar to beer draff, as it allowed both higher conidia production and higher virulence in comparison to rice husk.

4. Conclusions

Successful upscaling of *B. bassiana* conidia production using two substrates of different biodegradability was achieved. Substrate biodegradability effect was highlighted, playing a relevant role in heat generation throughout the packed-bed, consequently affecting the final conidia production. AFP relevance in fungal conidia production scaling when working with packed-bed configuration was also demonstrated. Under adequate conditions, higher conidia productions were achieved using beer draff (higher biodegradability substrate).

Virulence effect of the 22 L SSF reactor products using rice husk or beer draff as substrates and strain CECT was tested against *T. molitor* adult stage, causing mortality against this insect in laboratory assays. Differences in virulence depending on the substrate were detected, beer draff being the most effective. The results emphasize the importance of maintaining product virulence after fermentation. Differences observed between plate and fermented samples also suggest fermentation process, subsequent extraction and conservation steps as possible explanations for quality losses, highlighting the need to optimize them to maximize virulence maintenance.

Credit author statement

Arnau Sala: Conceptualization, Investigation, Visualization. Writing – Original draft & editing; Raquel Barrena: Conceptualization, Supervision, Writing – review & editing; Nicolai V. Meyling: Conceptualization, Supervision, Writing – review & editing; Adriana Artola: Conceptualization, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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