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# A nonconventional two-stage fermentation system for the production of aerial conidia of entomopathogenic fungi utilizing surface tension

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aerial conidia, *Beauveria bassiana*, fermentation, mass production, mycopesticides.

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## Abstract

**Aim:** To describe a new approach in which production of conidia of an entomopathogenic fungus takes place on the surface of an unstirred shallow liquid culture kept in nonabsorbent wells distributed in plastic sheets resembling a honeycomb.

**Methods and Results:** First, liquid incubation time and medium composition for production of *Beauveria bassiana* aerial conidia were optimized. Wells inoculated with Sabouraud dextrose yeast extract produced $2{\cdot}2 \times 10^8$ conidia per $cm^2$ of liquid surface following 5 days of incubation. Finally, tests were carried out in a prototype comprised of stacked plastic sheets in a cylindrical container. Conidia production on liquid culture surface varied from 1·2 to $1{\cdot}6 \times 10^9$ conidia per ml of fermented broth. Germination rates and insect activity towards *Tenebrio molitor* larvae were not negatively affected when compared to conidia produced on solid medium.

**Conclusions:** The two-stage fermentation process here described, based on a simple nonabsorbent inert support, has potential for the application in the production of aerial conidia of *B. bassiana* and other fungi.

**Significance and Impact of the Study:** Aerial conidia are the most extensive propagule type used in commercial mycopesticides, traditionally produced by solid-state fermentation (SSF). The industrial applications and other important benefits of the two-stage fermentation process here described may overcome some hurdles inherent to SSF aiming for the production of aerial conidia. Additionally, production consistency is increased by the use of chemically defined medium, and the better control of the environmental conditions could allow for more reproducible industrial batches.

## Introduction

Invertebrate pathogenic fungi have been considered as candidates for integrated pest management in several crops. The ability of some hypocrealean species to readily grow and reproduce, either on insect hosts or on alternative substrates, is one of the main characteristics which makes them potential biological control agents. This characteristic is particularly important when a large quantity of infective propagules is needed, such as in inundative strategies or sequential applications. Mass production of the active ingredient at competitive cost can be one of the bottlenecks to their success as commercial products (Jenkins and Grzywacz 2000; Roberts and St. Leger 2004; Glare *et al.* 2012). Moreover, fermentation processes must yield high concentrations of vigorous and persistent propagules (Jackson *et al.* 1997; Faria *et al.* 2015).

Most of the commercial mycopesticides available in the market worldwide are produced by either solid-state fermentation (SSF) or liquid-state fermentation (LSF), and the resulting fungal propagule type depends on the nature and conditions of the fermentation process.

Solid-state fermentation can be defined as the microbial development on solid substrates in the absence of free water, where the substrate has the ability to absorb water and support the microbial growth and metabolism (Pandey 2003; Thomas *et al.* 2013; Cruz-Quiroz *et al.* 2015). Aerial conidia are produced using both SSF and biphasic fermentation (LSF followed by SSF) techniques. Conidia are used as the main propagule type in commercial products (Faria and Wraight 2007; Li *et al.* 2010). However, long incubation times, labour-intensive processes and contamination are commonly some of the drawbacks of SSF (Roberts and St. Leger 2004; Jackson *et al.* 2010). In contrast, LSF more commonly yields mycelia and blastospores (thin-walled single cells formed by budding), but under certain conditions, submerged conidia and microsclerotia may also be produced (Bidochka *et al.* 1987; Jenkins and Goettel 1997; Jackson and Jaronski 2009; Kobori *et al.* 2015). Liquid-state fermentation provides better automation of processes and scale-up capabilities to rapidly produce a great amount of cells under controlled nutritional and environmental conditions. On the other hand, tolerance to harsh industrial and environmental conditions, such as desiccation after harvesting, stability under unrefrigerated storage conditions or field persistence is lower than that observed for aerial conidia (Hegedus *et al.* 1992; Jenkins and Prior 1993; Roberts and St. Leger 2004).

Interestingly, the production of aerial conidia on the surface of the liquid medium has been much less discussed and explored. In this system, conidiation occurs at the liquid-air interface on an unstirred shallow liquid culture or fermented broth (FB). There were published studies in the 1970s and 1980s (Kybal and Vlcek 1976; Samsinakova *et al.* 1981) and this approach was also used in commercial-scale production, as briefly reported by Jenkins and Goettel (1997). Difficulties in managing high volumes of liquid culture distributed on flat surfaces, the large space requirements of such systems and the need for careful handling to prevent contamination have reduced interest in this technique. Some efforts have been made to improve this fungal production approach, mainly by the use of absorbent supports which do not contribute nutritionally to fungal growth. Absorbent material such as paper (Gouli *et al.* 2014) or cloth (Jenkins and Lomer 1994; Higuchi *et al.* 1997; Ooijkaas *et al.* 2000; Shanley *et al.* 2009; Ugine *et al.* 2013) are soaked in a FB rich in vegetative cells which then serves as a structure to anchor vegetative cells already produced by LSF, resulting in the production of conidia on the liquid–air interface. Despite solving some of the hurdles associated with the traditional SSF on grains, the lack of uniformity of conidial production and anchoring structure means that harvesting from the absorbent supports adds complexity. Herein, we propose a nonconventional two-stage system for aerial conidia production of insect pathogenic fungi on a nonabsorbent inert support resembling a honeycomb structure and based on the concept of surface tension of liquids.

## Materials and methods

### Fungal strain and inoculum

*Beauveria bassiana* strain J21, originally isolated from a surface-sterilized maize leaf and maintained in long-term storage (−80°C) in the BPRC Culture Collection at Bio-Protection Research Centre in Lincoln University, New Zealand, was used as a model. The strain was grown on potato dextrose agar medium (PDA, Difco®) for 8–10 days at 26 ± 0·5°C and 14:10 h (L/D) of photoperiod. Subsequently, conidia were scraped from the medium surface with a spatula into 10 ml of sterile distilled water with 0·05% (v/v) of Tween 80 (Polysorbate 80, Sigma® Chemical). Conidial concentration was measured using a Neubauer haemocytometer in a light microscope (×400 magnification) and a standardized suspension ($1 \times 10^8$ conidia per ml, >95% germination) was used to inoculate the liquid media.

### Influence of liquid medium composition and incubation time on the production of conidia of *Beauveria bassiana* at the liquid–air interface

A set of experiments were conducted to evaluate the ability of the fungus to produce conidia on the surface of FB after liquid fermentation, with or without the addition of nutrients. At this stage, plates containing 2% water agar (Difco) were used as an inert support for fungal conidiation on FB surface.

Firstly, 250-ml Erlenmeyer flasks containing 99 ml of different liquid media were tested for conidial production on the surface of FBs. Potato dextrose liquid medium (PD; protein-poor medium), Sabouraud dextrose liquid medium (10 g peptone and 40 g glucose per litre of distilled water—SD; protein-rich medium) and Sabouraud dextrose liquid medium+yeast extract (YE) 1% w/w (Sabouraud dextrose yeast (SDY); protein-very rich medium) were autoclaved and inoculated with 1 ml of the conidial suspension, providing a final concentration of $1 \times 10^6$ conidia per ml. Flasks were placed in an orbital shaker incubator at 26 ± 0·5°C and 250 rev $min^{-1}$. After 2 days of incubation, 2 ml samples of $FB_{PD}$, $FB_{SD}$ and $FB_{SDY}$ were transferred to Eppendorf tubes and 1% (w/w) of yeast extract (YE) was added (or not) to each of the treatments. Tubes were vortexed for 1 min and three aliquots (100 $\mu$l) of treatments (liquid

medium × additional YE) were gently placed on the water agar plates with the aid of a micropipette and 200 μl wide-bore plastic pipette tips. Plates were kept opened for approximately 15 min until air-dried or absorption of the liquid by the agar medium was complete. The entire experiment was repeated on a different date.

Next, 250-ml Erlenmeyer flasks containing 99 ml of autoclaved SD liquid medium were inoculated with a conidial suspension, as above. Flasks, media volume and growth conditions were the same as described above. After 2, 3 or 4 days of incubation in the orbital shaker, 2 ml samples of $FB_{SD}$ from the same flasks were transferred to Eppendorf tubes and 1% (w/w) of corn starch ($FB_{SD}$+CS), wheat flour ($FB_{SD}$+WF), soybean flour ($FB_{SD}$+SF) or YE ($FB_{SD}$+YE) was added. Tubes were vortexed for 1 min and three aliquots (100 μl) of each treatment (liquid incubation time × additional nutrient) were placed on the water agar plates following methodology described above. The FB with no additional nutrient was used as control ($FB_{SD}$) and the entire experiment was repeated on a different date.

Conidia production per colony on water agar was measured for both experiments after 5 days of incubation at 26 ± 0·5°C and 14:10 h (L/D) of photoperiod. Water agar discs containing the whole colony were cut from the plates and washed in 10 ml of distilled water with 0·05% (v/v) of Tween 80 in 50-ml Falcon tubes. A spatula was used to remove all fungal propagules attached on disc surface. The suspension was vortexed for 1 min and conidial concentrations were counted microscopically (×400 magnification) using a Neubauer haemocytometer.

### Comparison of a nonabsorbent inert material as a structure for conidia production of *Beauveria bassiana* with water agar

Erlenmeyer flasks containing SD liquid medium were inoculated with the conidial suspension (1 ml) and placed in an orbital shaker incubator for 2 days under the same conditions described above. Subsequently, YE (1% w/w) was added in some treatments to the FB and vortexed for 1 min. Sheets of a nonabsorbent clear plastic containing two wells were sterilized by immersion in sodium hypochlorite 5% (10 min) and alcohol 70% (5 min), rinsed in sterile water twice (2 min) and air-dried in the laminar flow. The nonabsorbent material tested as a structure in this two-stage fermentation process contained small shallow wells (5 mm deep), which hold up to 200 μl of liquid each, even when tilted or turned upside down. The structure was primarily designed and developed to capture fluids in 100% recyclable plastic meat trays (Pact Group, Richmond, Australia), in substitution of the environmentally unfriendly polystyrene trays used by most supermarkets. Each well (approximately 0·43 $cm^2$) is triangular in shape with rounded corners and slightly curved on the sides and bottom (Fig. 1). Triangles are organized side by side in a reverse position sequence, producing an optimized square shape. To maintain a high moisture environment during fungal conidiation, sheet pieces were placed on the water agar surface in Petri dishes. Pieces of sheet were filled with the 2-day-old FB ($FB_{SD;}$ 100 μl per well) for each of treatment (with or without additional YE). One hundred-microlitre aliquots of both broths were also placed on the surface of water agar as positive controls. The area covered by a 100-μl droplet on the agar surface (2 $cm^2$) was approximately 2·3 times larger than the liquid-air surface on the two-well sheet piece. Plates were incubated for 2, 3 or 5 days at 26 ± 0·5°C and 14:10 h (L/D) of photoperiod. Three replicates were made for each of the treatments (harvesting time × additional YE), on water agar and plastic sheet, and the entire experiment was repeated one more time on a different date.

In a second experiment, the influence of the FB volume per well in the production of conidia was also determined. Two-well sheet pieces were filled with 75, 100, 125, 150 and 175 μl per well of a 2-day-old $FB_{SDY}$ and placed into water agar plates. Plates were incubated for 5 days at 26 ± 0·5°C and 14:10 h (L/D) of photoperiod. Three replicates were made for each of the treatments (broth volume) and the entire experiment was repeated one more time on a different date.

Conidia from colonies on water agar were harvested as already described. Conidia from wells were also washed in 10 ml of distilled water with 0·05% (v/v) of Tween 80 in 50-ml Falcon tubes. The biomass of both mycelium and conidia was easily removed from the wells by stirring

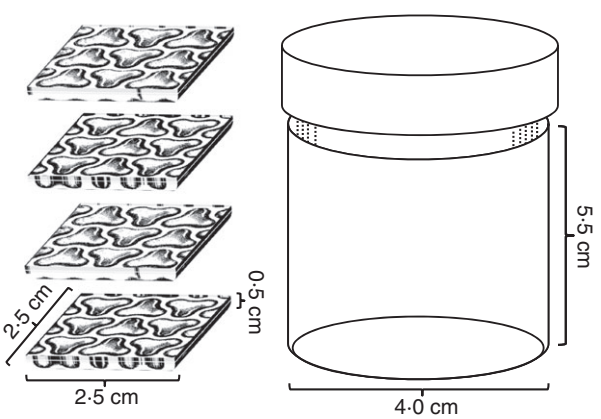


**Figure 1** Graphical illustration of the inert support (plastic sheets containing shallow wells) and containers used as a laboratory prototype of the two-stage conidia production system.

the plastic pieces in water and a needle was used when necessary. Suspensions were vortexed for 1 min and conidia concentration were measured in a Neubauer haemocytometer using a light microscope (×400 magnification).

### Conidia production of *Beauveria bassiana* in a two-stage fermentation system and virulence against *Tenebrio molitor*

The small laboratory prototype consisted of same-sized layers of the previously described nonabsorbent plastic sheets stacked inside a plastic container, as illustrated in Fig. 1. Wells of a layer were arranged mismatched from those of the upper layer. The triangular shape and curved bottom of the wells prevented any leakage of liquid (by maintaining liquid surface tension) and allowed air flow throughout the pile. The rigid plastic sheet served as a framework and no additional structure was necessary for assembling.

Firstly, Erlenmeyer flasks containing SDY were inoculated with the conidial suspension and placed in an orbital shaker incubator for 2 days under the same conditions described previously. Yeast extract (1% w/w), acid hydrolysed casein (HC, 1% w/w) (Hy-case® Amino; Sigma-Aldrich) and fresh *T. molitor* extract (TM; 1% w/w) were added to the FB and vortexed for 1 min. TM was prepared by grinding 5 g of last-instar larvae in a mortar with 25 ml of distilled water. The final extract was passed through a cloth and autoclaved.

For experimental purposes we used four square sheet layers ($h$ = 0·5 cm and 6·25 $cm^2$), each one with nine wells (approximately 3·9 $cm^2$ of total liquid surface area), stacked in the cylindrical transparent plastic container ($h$ = 5·5 cm and Ø = 4·0 cm). Sheets were previously sterilized as described above and containers were autoclaved. All the wells of a pile were filled with the FB (100 $\mu$l per well) for each of the treatments (protein source), totalling 3·6 ml of broth per container. The FB with no additional nutrient was used as the control. Although headspace and the side space between the pile and the container wall supported a volume of air, containers were opened every 24 h for gas exchange. Containers were kept in an incubator for 5 days at 26 ± 0·5°C and 14:10 h (L/D) of photoperiod. Three replicates were made for each of the treatments and the entire experiment was repeated on a different date.

For harvesting of conidia, containers were filled with 20 ml of distilled water with 0·05% (v/v) of Tween 80 and conidia were washed by stirring for 2 min and a needle was used to remove the fungal biomass when necessary. The concentration of conidia in suspension was measured in a Neubauer haemocytometer. Additionally, the quality of the harvested conidia in all treatments was determined by direct count of germinated conidia using compound microscope and by bioassays against *T. molitor*. In the first case, a 20 $\mu$l droplet of the suspension of each container (diluted to 2 × $10^5$ conidia per ml) was placed on PDA plates and incubated at 26 ± 0·5°C. A sample of 300 cells was counted after both 16 and 22 h of incubation and the number of germinated and ungerminated conidia was scored. Conidia were considered as germinated when the size of the germ-tube was two times longer than the diameter of an ungerminated conidium.

Insect activity was determined by the mortality of third-instar larvae inoculated with 1 × $10^8$ conidia per ml suspensions from the containers. Groups of 30 larvae were inoculated by immersing insects in the conidial suspension for 30 s, totalling 180 insects per treatment in both experiments. Groups of untreated insects (dipped in water) and treated insects with conidia from a 10-day-old culture on PDA were also included in the bioassays as treatments. After air drying, insect groups were transferred to Petri dishes (16 mm deep) with 15 g of oat flour and kept at 26 ± 0·5°C. Insect mortality was measured daily after the 2nd day and up to the 12th day. Cadavers were removed and maintained for a further 4–5 days in moisture chambers to encourage fungal outgrowth and sporulation. Fungal identification was confirmed after sporulation on the cadavers through microscopic observation.

### Statistical analyses

For the sake of comparison, the production of conidia was adjusted to 1 $cm^2$ for either water agar surfaces or the nonabsorbent support. Conidia production data from all experiments were analysed by generalized linear models (GLM) of mixed effects, attributing to the variable response a normal distribution (link identity) and submitted to a one-way or two-way ANOVA. Percentages of germinated conidia from different media were submitted to a one-way ANOVA using a logistic regression model using a binomial distribution. GLM selection was previously performed to choose the best model to fit proportional data (Moral *et al.* 2017). In cases where the observed variability was higher or lower than the assumed variance by theoretical distribution (overdispersion), a heterogeneity factor was applied (quasi-likelihood model). When fixed effects and their interactions were significant according to the $F$-test, mean values were statistically separated by Tukey's HSD test at $P < 0·05$. Experiments in time were treated as a random effect (block), and removed from the models when represented as a minor component of total variance. Survival times ($ST_{75}$) of *T. molitor* larvae were estimated for the different *B. bassiana* conidia batches produced in the two-stage fermentation

system by Kaplan–Meier. Survival curves were compared by LogRank test at 5% of probability. All analyses were performed using R statistical software (R Development Core Team 2006).

## Results

### Influence of liquid incubation time and medium composition on the production of conidia of *Beauveria bassiana* on the liquid–air interface

The liquid medium composition had a significant effect on conidial production during the second stage on water agar surface ($F$ = 8469; d.f. = 5, 30; $P$ = 2·2e-16). Protein-rich liquid media promoted higher conidiation than protein-poor liquid media. Conidial production on water agar from aliquots of $FB_{PD}$ was 59·1 and 76·1% lower than that seen for $FB_{SD}$ and $FB_{SDY}$, respectively. The addition of YE in $FB_{PD}$ and $FB_{SD}$ after liquid fermentation increased 2·03 and 1·46 times the number of conidia produced on drop surface respectively. However, this increase was not observed for the protein-rich medium, $FB_{SDY}$ (Fig. 2).

There was a significant interaction between medium composition and liquid culture incubation time ($F$ = 17 389; d.f. = 8, 75; $P$ = 2·2e-16). Significant differences were seen for the main effect of time ($F$ = 41 153; d.f. = 2, 83; $P$ = 2·2e-16), where production of conidia on the liquid–air interface was inversely correlated with the FB age for protein-poor treatments. The number of conidia produced on water agar from aliquots of 3-day-old liquid culture treated with corn starch ($FB_{SD}$+CS), wheat flour ($FB_{SD}$+WF) or without additional nutrients ($FB_{SD}$) decreased by 59·1, 43·5 and 59·5%, respectively, when compared to the 2-day-old broths of the same treatments (Table 1). Regardless the FB age, no significant decrease in conidia production was noted for broths amended with YE and soybean flour (protein-rich treatments). The addition of nutrients to the FB after liquid fermentation showed differences in the average number of conidia produced on drop surface on water agar ($F$ = 76 032; d.f. = 4, 85; $P$ = 2·2e-16). For all wells containing FB, the production was significantly higher when increasing amounts of a protein was added, shown by comparing WF (14% protein), SF (50% protein) or YE (70% protein) (Table 1). The number of conidia on $FB_{SD}$+YE or $FB_{SD}$+SF was around two times higher than $FB_{SD}$ for 2-day-old cultures and more than five times higher for 4-day-old cultures.

### Comparison of a nonabsorbent inert material as a structure for conidia production of *Beauveria bassiana* with water agar

Significant interaction was seen between conidiation and incubation time using the nonabsorbent inert support ($F$ = 28 073; d.f. = 6, 60; $P$ = 2·2e-16). The number of conidia increased significantly with the increase in the incubation time ($F$ = 192 502; d.f. = 2, 66; $P$ = 2·2e-16). Conidia production per $cm^2$ of area on water agar surface was 7·2 and 4·5 times higher after 5 days of incubation than that on harvest after only 2 days of incubation, for $FB_{SD}$ and $FB_{SD}$+YE respectively. These differences were even more evident in wells, reaching up to 50 times more for the same treatments (Table 2). Significant differences were seen for the main effect of inert support used in the second stage ($F$ = 877 463; d.f. = 3, 68; $P$ = 2·2e-16), where production of conidia per $cm^2$ was higher on water

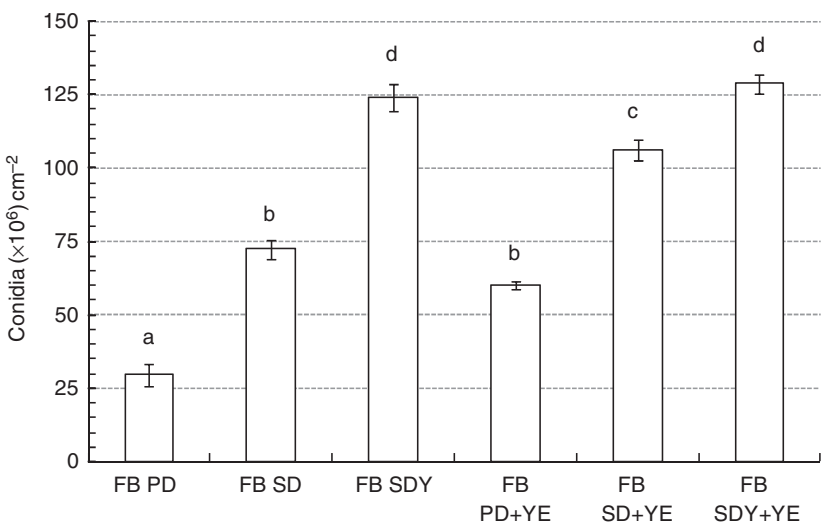


**Figure 2** Mean number (±SE) of *Beauveria bassiana* conidia produced on different fermented broth surfaces (per $cm^2$) after 5 days of incubation (26 ± 0·5°C) using water agar (2%) as support. Means followed by the same letter did not differ significantly ($P$ > 0·05).

**Table 1** Mean number (±SE) of *Beauveria bassiana* conidia ($\times 10^6$ per $cm^2$) produced on fermented broth (FB) surface after 5 days of incubation using water agar (2%) as support (26 ± 0·5°C)

| | Inoculation material | | | | | |
|---|---|---|---|---|---|---|
| Treatments* | 2-day-old broth | | 3-day-old broth | | 4-day-old broth | |
| $FB_{SD}$ | 56·27 ± 3·80 | Aa† | 22·75 ± 3·06 | Ba | 18·88 ± 2·33 | Ba |
| $FB_{SD}$+CS | 54·96 ± 3·97 | Aa | 22·46 ± 1·56 | Ba | 23·92 ± 1·26 | Ba |
| $FB_{SD}$+WF | 80·33 ± 2·57 | Ab | 45·42 ± 2·29 | Bb | 42·67 ± 4·18 | Bb |
| $FB_{SD}$+SF | 110·58 ± 5·57 | Ac | 106·67 ± 3·69 | Ac | 102·63 ± 2·62 | Ac |
| $FB_{SD}$+YE | 108·83 ± 1·64 | Ac | 114·21 ± 2·68 | Ac | 114·00 ± 2·05 | Ad |

*$FB_{SD}$, Sabouraud dextrose liquid medium; CS, corn starch; WF, wheat flour; SF, soy flour; YE, yeast extract.
†Means followed by the same letter in a column (lowercase) or in a row (uppercase) did not differ significantly ($P > 0·05$).

agar surface than in wells after 2 days of incubation, regardless of the addition of YE. For longer periods of incubation (3 and 5 days), conidial production in wells increased faster than on water agar surface and FB on inert support, with or without addition of YE, yielded between 1·5 and 2 times more conidia per $cm^2$ than on water agar (Table 2).

The volume of FB in each well had an influence on conidia production ($F = 60\,763$; d.f. = 4, 25; $P = 0·023$), but significant differences were only observed between 75 and 125 $\mu$l per well, where the number of conidia was 30% lower in the former (Fig. 3).

### Conidia production of *Beauveria bassiana* in a two-stage fermentation system and virulence against *Tenebrio molitor*

Significant differences were observed on the number of viable conidia produced in the laboratory prototype 16 h postinoculation ($F = 41·24$; d.f. = 4, 25; $P = 2·2e\text{-}16$) and 22 h ($F = 40·53$; d.f. = 4, 25; $P = 0·00026$). Germination of conidia from PDA plates was slight lower and slower than of those produced by the two-stage process. The addition of protein sources to the FB just prior to inoculation of wells had no influence on overall conidial germination rates. Similarly, no differences on conidia production were noted when additional protein were incorporated to $FB_{SDY}$ ($F = 260\,166$; d.f. = 3, 20; $P = 0·210$) (Table 3).

The comparison of survival curves indicated differences among the treatments ($\chi^2 = 266·3$; d.f. = 5; $P = 0$). Since insect mortality did not reach 50% in most of the treatments, the time to reach 25% mortality was therefore calculated. Survival times ($ST_{75}$) of *T. molitor* larvae for conidia produced in the laboratory prototype varied between 6·66 and 8·92 days, similar to the value estimated for conidia produced on PDA plates (7·21 days). Pairwise comparisons showed that addition of TM extract in the FB increased fungal virulence when compared to the $FB_{SDY}$ ($\chi^2 = 19·7$; d.f. = 1; $P = 9·01e\text{-}6$) with $ST_{75}$ being reduced from 8·92 to 6·66 days (Table 3). A marginal difference was also detected when YE was added to the FB ($\chi^2 = 4·6$; d.f. = 1; $P = 0·031$), but no difference was observed when HC was used ($\chi^2 = 2·1$; d.f. = 1; $P = 0·146$). More than 86% of the cadavers of all fungal-treated insects were colonized by the fungus and showed profuse conidiation on insect surface.

## Discussion

We described the application of a nonconventional two-stage fermentation system for production of aerial conidia

**Table 2** Mean number (±SE) of *Beauveria bassiana* conidia ($\times 10^6$ per $cm^2$) produced on 2-day-old fermented broth (FB) surface using water agar (2%) (WA) and a nonabsorbent plastic well as supports (26 ± 0·5°C)

| | Incubation time | | | | | |
|---|---|---|---|---|---|---|
| Treatments* | Harvested at day 2 | | Harvested at day 3 | | Harvested at day 5 | |
| $FB_{SD}$/WA | 10·71 ± 1·23 | Ab† | 40·46 ± 1·66 | Bc | 76·92 ± 1·27 | Cd |
| $FB_{SD}$+YE/WA | 23·13 ± 2·06 | Aa | 60·00 ± 2·84 | Bb | 103·58 ± 0·99 | Cc |
| $FB_{SD}$/well | 2·50 ± 0·32 | Ab | 72·27 ± 2·96 | Bb | 122·98 ± 7·89 | Cb |
| $FB_{SD}$+YE/well | 6·88 ± 1·47 | Ab | 93·15 ± 9·34 | Ba | 219·11 ± 7·39 | Ca |

*$FB_{SD}$, sabouraud dextrose fermented broth; WA, water agar; YE, yeast extract.
†Means followed by the same letter in a column (lowercase) or in a row (uppercase) did not differ significantly ($P > 0·05$).

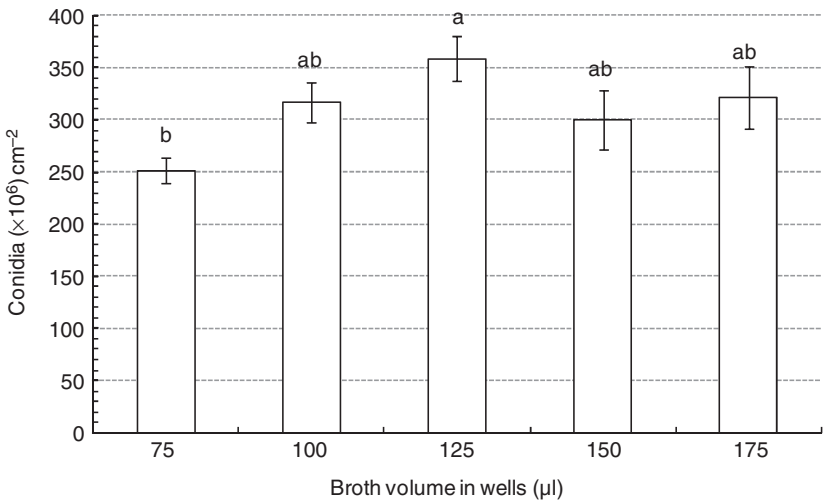


**Figure 3** Mean number (±SE) of *Beauveria bassiana* conidia produced on the inert support (per $cm^2$ of liquid surface) filled with different volumes of fermented broth ($FB_{SDY}$) after 5 days of incubation (26 ± 0·5°C). Means followed by the same letter did not differ significantly ($P > 0·05$).

**Table 3** Number, viability and virulence against *Tenebrio molitor* larvae of *Beauveria bassiana* conidia produced in the laboratory prototype after 5 days of incubation (26 ± 0·5°C)

| Treatments | No. conidia*·† ($\times 10^9$) | | Conidia germination (%)† 16 h | | 22 h | | Survival time (days)‡ *n* | $ST_{75}$ | CI | Cadavers showing Conidiation (%)§ |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | – | | – | | – | | 180 | ND | ND | 0·0 |
| PDA | ND | | 83·6 ± 3·66 | a | 96·2 ± 1·41 | a | 180 | 7·21 | 6·97–7·45 | 86·5 |
| $FB_{SDY}$ | 4·25 ± 0·374 | a | 98·7 ± 0·31 | b | 99·3 ± 0·31 | ab | 180 | 8·92 | 8·64–9·20 | 93·1 |
| $FB_{SDY}$+HC | 5·62 ± 0·584 | a | 99·0 ± 0·29 | b | 99·1 ± 0·31 | b | 180 | 7·70 | 7·41–7·99 | 87·3 |
| $FB_{SDY}$+YE | 4·86 ± 0·436 | a | 99·3 ± 0·31 | b | 98·9 ± 0·31 | b | 180 | 7·46 | 7·17–7·75 | 93·8 |
| $FB_{SDY}$+TM | 4·70 ± 0·442 | a | 98·8 ± 0·21 | b | 99·2 ± 0·31 | b | 180 | 6·66 | 6·39–6·93 | 93·5 |

*Number of conidia per container (3·6 ml of fermented broth).
†Means followed by the same letter in a column did not differ significantly ($P > 0·05$).
‡$ST_{75}$, time to insect mortality reach 25%; CI, confidence interval; ND, not determined.
§Colonized cadavers × 100/total cadavers.

deploying a nonabsorbent support material. The structure is based on small shallow wells specially shaped to hold liquids by surface tension on a rigid plastic sheet. The stack of sheet layers produces a tridimensional structure which holds at least a third of its volume in liquid, homogenously distributed. We demonstrated that conidia can be readily produced on FB surface kept inside the wells and yield can be improved by changing medium composition.

One of the major advantages of the two-stage systems is the possibility of managing the main abiotic and nutritional parameters during the process. Firstly, in two-stage systems the metabolic heat generated mainly during the vegetative growth can be easily controlled by the equipment in the liquid fermentation stage, even for large volumes of medium. Moreover, the arrangement of the fungal biomass in overlaid layers, resembling a tray bioreactor and facilitating gas exchange and temperature control during the conidiation stage, is another advantage of this approach. Conversely, metabolic heat generation by fungal respiration during growth in ordinary solid fermentation on grains, for example, leads to a temperature increase and low levels of oxygen availability in high volume bioreactors, affecting the overall fungal metabolism, growth and conidiation (Dorta and Arcas 1998; Arzumanov *et al.* 2005; Kang *et al.* 2005; Ye *et al.* 2006). Also, the uncoloured plastic of the sheets and container used in this novel approach allows better light exposure, an important parameter for the conidiation of some fungal species, such as *Metarhizium rileyi* (formerly *Nomuraea rileyi*) (Glare 1987) and *Isaria fumosorosea* (Sanchez-Murillo *et al.* 2004), or for the production of conidia with increased stress tolerance (Rangel *et al.* 2011).

Media composition and incubation time in the liquid fermentation stage greatly influenced the final conidial yield, as also shown by Mascarin *et al.* (2018). Our experiments on water agar showed that the availability of a protein source is crucial for conidia production. For instance, the number of conidia per volume of the 2-day-

old FB amended with YE ($FB_{SD}$+YE) was twice as high as the remaining treatments. The importance of a protein source for spore production was reported by Holdom and Van de Klashorst (1986) for *M. rileyi* and later by Kassa *et al.* (2008) for *M. anisopliae* and *B. bassiana* and by Silva *et al.* (2018) for *Isaria javanica* and *B. bassiana* in two-stage production processes. Interestingly, our results also showed that conidial yield on water agar increases as the FB age decreases. Although low aeration in old liquid cultures caused by fast mycelial growth may lead to cell autolysis (Roberts and St. Leger 2004), the lower conidial production on the liquid surface is probably due to a depletion of key nutrients in motionless liquid fermentation, as suggested by Kassa *et al.* (2008). On the other hand, no decrease in conidial production in different incubation times was observed for broths amended with either YE or soy flour, revealing that protein depletion in broths during the exponential vegetative growth leads to subsequent lower yield in a solid substrate. As such, the number of conidia on the broth surface was not increased when a very rich protein medium was used.

Conidial production on the liquid–air interface in this two-stage system is directly related to the surface area of the liquid and also the availability of nutrients during the second stage of the process. Although optimal liquid–air interface and distribution of the fungal biomass can be achieved on water agar (100 $\mu$l of FB covered an area of 2 $cm^2$), the number of conidia harvested per $cm^2$ in wells using our biphasic system was between 1·5 and 2 times higher than that observed on water agar for the same surface area. This was because a higher volume of broth kept in each well provided all nutrients for a better conidiation, since an available protein source proved to be important at this stage. In contrast, the thin layer of broth spread over a larger area on the water agar seemed to be insufficient to provide a satisfactory nutrition for the fungus growth and reproduction. Additionally, the bowed liquid surface in the wells produced by surface tension increase the liquid–air interface, leading to an optimal productivity when compared to flat liquid surfaces. On the other hand, the volume of broth used on water agar was half of that used to fill both wells; therefore production per volume of FB was lower in the wells. In spite of that, the production of large volumes of FB by liquid fermentation is not a limiting factor in industrial systems and the benefits of a better nutrition on conidial yield and easier handling may surpass this disadvantage.

The availability of nutrients and other compounds during fungal conidiogenisis can also influence the biological activity of conidia and their tolerance to abiotic stress factors (Daoust and Roberts 1983; Lane and Trinci 1991; Ibrahim *et al.* 2002). The manipulation of the culture medium to increase conidia germination speed and their tolerance to environmental conditions was also suggested by Rangel *et al.* (2004). In our study, the composition of the media had some influence on the biological activity of the conidia harvested from the wells, as observed with the addition of TM extract in the FB, although the addition of HC and YE showed no increase in insecticidal activity.

In the preliminary experiment in which two well sheet pieces filled with differing amounts of broth were kept on water agar in Petri dishes, the number of conidia produced per well (or 100 $\mu$l FB surface) was around 1–1·5 $\times$ $10^8$ conidia for protein-rich treatments. Production of conidia per well in the laboratory prototype was very similar to that of these flasks, suggesting that temperature and humidity inside the containers were similar to Petri dishes with water agar. Recent advances in LSF have shown that concentrations of 1 $\times$ $10^9$ blastospores per ml of *B. bassiana* can be produced in 3 days under laboratory conditions (Mascarin *et al.* 2015). Although the production of conidia by this fungus is strain dependent, the same concentration of this infective cell was achieved in our system after a relatively short time (7 days). Likewise, conidia production of *B. bassiana* in SSF can reach 1 $\times$ $10^{10}$ conidia per gram of dry substrate, as recently reviewed by Lopez-Perez *et al.* (2015), however, yield is usually lower under industrial conditions and varies between 2 and 4 $\times$ $10^9$ conidia per gram of wet rice grain (40–45% of moisture content) in a whole process taking 12–14 days.

It may seem counter-intuitive to add nutrients in a process designed to reduce costs, but the production of robust conidia is a trade-off between the cost of production and the quality of the product. We demonstrate that more conidia can be produced through selective nutrient additions. Further optimization would be needed for industrial-scale production.

Apart from the industrial costs, another important issue in the mass production of micro-organisms is related to the generation of large amounts of solid and/or liquid waste. Concerns about the waste from SSF and LSF processes have not been extensively discussed. Even though both processes may use industrial or agricultural wastes as a substrate for the fungal growth, the liquid effluent from LSF and large amounts of solid waste from SSF after active ingredient segregation may require special treatment before being discarded. The latter is particularly important in the industrial production of fungal biopesticides where hundreds of tons of solid waste, usually grains such as rice or barley, may be generated monthly. On the other hand, the novel two-stage approach on inert support does not produce significant industrial waste as in SSF, since plastic sheets have the potential to be reused, and the liquid phase can be

gradually evaporated during the second production stage and posterior drying process. One major issue with SSF is that any expansion of scale-up simply replicates the system used, with little space saving, where stacking plastic trays used in these experiments is more space conserved.

Other important benefits of the system proposed are the feasibility of automation and scale-up, and a relative low labour requirement. Equipment from food and pharmaceutical industries can be easily adapted to fill the sheets with broth and arrange them into the containers under semisterile conditions, since the structure can support its own weight. Downstream processing can also be automatized, including drying and harvesting processes. High-quality vigorous conidia with low contamination levels can be obtained at the end of the process. Automation and short time of production could greatly reduce the cost of the final product. This two-stage fermentation process, based on a simple nonabsorbent inert support here described, has a potential for application in the production of aerial conidia of *B. bassiana* and other fungi. Production consistency is increased by the use of chemically defined medium and the better control of the environmental conditions could allow for more reproducible industrial batches.

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## Conflict of Interest

No conflict of interest declared.

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