Determination of optimal culture medium conditions for mass production of *Beauveria bassiana* clone EF 46

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| **ABSTRACT:**The issue of food safety has become a major concern due to the use of chemical pesticides, which have deleterious effects on human health, the environment and biodiversity. Biological pest control, which uses entomopathogenic agents, is an alternative that is more respectful of the health of living beings and the environment. This study aimed to determine the optimal culture medium parameters for the indigenous strain *Beauveria bassiana* clone EF 46, aiming to facilitate the development of biopesticides. Potato Dextrose Agar (PDA) medium was used to evaluate the influence of physical (temperature, photoperiod, pH) and nutritional (carbon and nitrogen sources) parameters on the sporulation of *B. bassiana*. Concurrently, rice husk supplemented with different types of starch flours was used to determine the best substrate for solid-state fermentation. This study revealed that the optimal physical parameters for sporulation were: a temperature of 25 °C, an alkaline medium with a pH of 9, and a 12 h/12 h light/dark photoperiod. Regarding nutritional parameters, fulvic acid and baker's yeast were identified as the best carbon and nitrogen sources, respectively. Concerning the culture substrate for solid-state fermentation, yellow corn flour proved to be the best supplement for rice husk. |

***Keywords:*** *Entomopathogenic fungus, Beauveria bassiana, sporulation factors, food safety*

1. INTRODUCTION

For several decades, synthetic pesticides have been employed by farmers to protect their crops. However, these products are toxic and undermine the efficacy of natural control methods. Indeed, the damage inflicted upon ecosystems (loss of biodiversity, disruption of ecological services), environmental contamination, and health risks compromise the sustainability of this approach (Malaj *et al*., 2014 ; Van der Sluijs *et al.,* 2015 ; Mostafalou and Abdollahi, 2017).

Furthermore, numerous crop insect pests have developed resistance to certain chemical insecticides. To date, 43 active ingredients have been reported in a global database, documenting 204 cases of resistance (Nascimento *et al*., 2016 ; Mota-Sanchez and Wise, 2017 ; Okuma *et al*., 2018 ; APRD, 2021). Among these active ingredients are flubendiamide, chlorantraniliprole, methomyl, thiodicarb, permethrin, chlorpyrifos, zeta-cypermethrin, deltamethrin, triflumuron, and spinetoram (Gutiérrez-Moreno *et al.,* 2019). These findings urgently advocate for a transition towards crop protection strategies that are effective, sustainable, and respectful of the environment and human health.

Biological control through the use of entomopathogenic agents, such as fungi and bacteria, presents an alternative to chemical insecticides. Over 750 species of entomopathogenic microorganisms have been identified (Aktar *et al.,* 2009 ; Sandhu *et al.,* 2012). Several studies have confirmed that the fungus *Beauveria bassiana* is pathogenic to various insects, including weevils, the coffee stem borer beetle, the palm weevil, the cabbage white butterfly, *Helicoverpa armigera*, and *Maruca vitrata*, with mortality rates varying considerably, ranging from 29% to 100% (Khorrami *et al*., 2018 ; Akutse *et al*., 2019 ; Sutanto *et al.,* 2021). For the large-scale application of this fungus, it is necessary to determine the optimal conditions for mass multiplication, with the goal of designing biopesticides to effectively control crop insect pests.

2. material and methods

2.1 Fungal material

The *Beauveria bassiana* strain clone EF 46 used in this study was isolated from a dead larva of *Spodoptera frugiperda* (Lepidoptera, Noctuidae). The strain appeared white with a cottony texture and slightly raised relief in early growth stages, becoming powdery after three to four weeks of culture (**Fig. 1**). The reverse side of the plate was yellowish. Its growth was slow on standard Potato Dextrose Agar (PDA) medium, reaching an average diameter of 6 cm after 21 days.



**Fig. 1** Macroscopic aspect of the strain *Beauveria bassiana* clone EF 46

2.2 Synthetic and solid-state fermentation culture media

For experiments investigating the influence of pH, photoperiod, temperature, and carbon and nitrogen sources, Potato Dextrose Agar (PDA) medium was used. This medium consists of 20 g of potato flakes, 20 g of glucose, and 16 g of agar dissolved in 1000 mL of distilled water. The pH was adjusted according to the specific requirements of the different tests performed. For the selection of the best supplement to the rice husk-based culture substrate, flours from certain cereals or tubers were used, namely wheat, corn, sorghum, millet, cassava, and rice flour.

**2.3 Fungal Strain Preparation**

A volume of 500 µL of a conidial suspension (1 x 107 spores/mL) was spread onto PDA medium in 8 cm diameter plastic Petri dishes. The Petri dishes were then incubated at 25 °C for 10 days to obtain mycelial mats. These mats were subsequently cut into round agar plugs using a 5 mm diameter cork borer.

**2.4 Influence of pH on Sporulation**

To evaluate the effect of pH, PDA culture media were prepared and their pH adjusted to five distinct levels: 5, 6, 7, 8, and 9. Adjustment was performed by controlled addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH). After pH adjustment, the media were sterilized by autoclaving at 121 °C and 1 bar for 15 min. Once sterilization was complete, the media were poured into sterile 8 cm diameter Petri dishes. After solidification, a mycelial disc of *B. bassiana* (5 mm diameter), obtained from a pure culture, was placed centrally on each plate. The cultures were then incubated at a constant temperature of 25 °C for 21 days. Three replicates were performed for each tested pH level.

**2.5 Influence of Photoperiod on Sporulation**

The study of photoperiod influence on sporulation was conducted under three distinct light regimes: continuous light exposure (24 /0 h), alternating periods of light and darkness (12/12 h), and total darkness (0/24 h). PDA medium was prepared, sterilized, and poured into Petri dishes following the standard protocol described previously. After solidification, each plate was centrally inoculated with a 5 mm mycelial disc of B. bassiana. Incubation took place at 25 °C for 21 days under the respective photoperiodic conditions defined for each treatment. Three independent replicates were conducted for each light regime.

**2.6 Influence of Temperature**

The study of the temperature effect involved four thermal levels: 20 °C, 25 °C, 30 °C, and 35 °C. PDA culture medium was prepared, sterilized, and distributed into Petri dishes according to the standard methodology. Inoculation was performed as described for the previous experiments, using a 5 mm mycelial disc. The inoculated Petri dishes were then placed in incubation for 21 days at the specific temperatures under investigation. Three replicates were also performed for each thermal level.

**2.7 Influence of Carbon Source**

Five (5) carbon sources were tested: glucose, sucrose, humic extract, fulvic acid, and humic acid. In this test, potato served as the sole nitrogen source. Thus, each culture medium consisted of 20 g or 20 mL of a single carbon source, 20 g of potato flakes, 20 g of agar, and 1000 mL of distilled water. The pH level was adjusted using an electric pH meter before sterilization by autoclaving at 121 °C. The sterilized media were distributed into 8 cm diameter Petri dishes. After solidification, a 5 mm disc of Beauveria bassiana culture was placed in the center of each plate and incubated for twenty (20) days at 25 °C.

**2.8 Influence of Nitrogen Source**

To select the best nitrogen source promoting good growth and sporulation, four (4) nitrogen sources were tested: housefly larvae meal (MD), peptone, yeast extract (EXT\_LEV), and baker's yeast (LEVURE). Glucose was used as the carbon source. Thus, each culture medium consisted of 20 g of a single nitrogen source, 20 g of glucose, 20 g of agar, and 1000 mL of distilled water. The pH level was adjusted using an electric pH meter before sterilization by autoclaving at 121 °C. The media were sterilized and then distributed into 8 cm diameter Petri dishes.

**2.9 Influence of Solid-State Fermentation Substrate Composition on Sporulation**

To reduce the production cost of *B. bassiana*, rice husk was used as an alternative substrate to polished rice grains. However, since the growth and sporulation of *B. bassiana* are very low, or even absent, on rice husk alone (Heviefo *et al.,* 2019), starch flour was added to the culture substrate to address this issue (Mishra *et al.,* 2016). Six (06) flours were tested to select the best one : wheat, corn, cassava, rice, millet, and sorghum flour. For each type of flour, a 20% flour slurry was prepared, and a 25 mL volume was transferred into a 250 mL Erlenmeyer flask. Subsequently, 50 g of rice husk was added to the Erlenmeyer flasks containing the slurry. The mixture was combined using a spatula and then plugged with cotton and aluminum foil. The constituted substrates were then autoclaved at 121 °C under 1 bar pressure for 20 minutes. After cooling, the flasks were inoculated with 2.5 mL of a spore suspension containing 1 x 108 spores/mL. The flasks were manually homogenized and then incubated at 25 °C for 21 days. Each treatment was performed in triplicate.

**2.10 Data Collection**

After the incubation period for the Petri dishes, 15 mL of distilled water containing 0.02% Tween 80 was poured into each dish. The surface of each plate was then scraped with a spatula, and the resulting suspension was filtered through three layers of sieve material to retain debris. For the solid-state fermentation, a volume of 100 mL of sterile distilled water was added to the substrates to facilitate conidia detachment. The resulting filtrates were manually swirled and then filtered through three layers of No 8 coffee filter paper to retain debris. Using a Malassez hemocytometer and a light microscope, spores were counted, and the spore concentration was calculated according to the formula by **Heviefo *et al.* (2019)** :

$$C=Mean (X1 +X2)×10^{4}×10^{d}$$

Where X1 = sum of spores (conidia) counted in the five squares of the first side, X2 = sum of spores on the second side; C = final concentration of the solution (number of spores per mL of the solution); Mean = average count of the two sides over 5 squares; 104 = extrapolation factor, and d = dilution factor (number of times the solution was diluted).

**2.11 Statistical Analysis**

Analysis of variance (ANOVA) was performed to better interpret the results, along with various statistical tests. Statistical analyses were conducted using Xlstat software, version 2019. Data were expressed as means with standard deviations. Normality and homoscedasticity (homogeneity of variances) of the experimental data were verified using the Shapiro-Wilk test. After confirming normality and homoscedasticity, a one-way analysis of variance (ANOVA) was performed at the 5% significance level (α= 0.05). In case of significant differences, Tukey's HSD post-hoc test was conducted to separate treatment means into homogeneous groups.

3. results and discussion

**3.1 Results**

**3.1.1. Effect of pH on Sporulation**

The results concerning the influence of pH on sporulation are presented in **Fig. 2.** A progressive increase in conidial density was observed with increasing alkalinity of the medium. Maximum sporulation was obtained on alkaline media at pH 9 and pH 8. The mean spore concentrations were 7 x 108 spores/mL and 4.77 x 108 spores/mL, respectively. Conversely, the lowest sporulation rates were observed in media with acidic pH (5 and 6). No significant difference was observed between these two acidic pH levels (5 and 6).

**Fig. 2.** Influence of the pH of the culture medium on *B. bassiana* sporulation. Vertical bars illustrate standard deviation of means. Values with different letters at the top of the bar indicate a significant difference (\*\* P < 0.001) determined by the Tukey HSD test.

**3.1.2 Effect of Photoperiod on Sporulation**

**Fig. 3** presents the effect of photoperiod on the sporulation of *B. bassiana*. The highest spore concentration (3.9 x 107 conidia/mL) was observed in Petri dishes subjected to the alternating light/dark regime of 12h/12h (light/darkness). Conversely, the lowest spore concentration was observed when Petri dishes were subjected to constant light regime (24 hours), with a mean spore concentration of 3.07 x 107 conidia/mL.

**Fig. 3**. Influence of photoperiod on *B. bassiana* sporulation. Vertical bars illustrate the standard deviation of means. Values with different letters at the top of the bar indicate a significant difference (\*\* P < 0.001) determined by the Tukey HSD test.

**3.1.3 Effect of Temperature on Sporulation**

**Fig. 4** shows a positive relationship between temperature and fungal sporulation within the range of 20 to 25 °C. In this thermal range, a progressive increase in conidia production was observed, indicating a favorable physiological response to rising temperature. Maximum sporulation was determined at 25 °C with a spore production of 3.83 x 108 spores/mL. This maximum value suggests that 25 °C constitutes the most favorable temperature for the metabolic activity and reproduction of this fungal strain. However, beyond 25 °C, a significant reduction in sporulation was observed at 30 °C and became more pronounced at 35 °C, where the minimum sporulation value (5.67 x 104 spores/mL) was recorded.

**Fig. 4.** Influence of incubation temperature on *B. bassiana* sporulation. Vertical bars illustrate the standard deviation of means. Values with different letters at the top of the bar indicate a significant difference (\*\* P < 0.001) determined by the Tukey HSD test.

**3.1.4 Effect of Nitrogen Source on Sporulation**

All studied nitrogen sources induced positive sporulation of *B. bassiana*. However, the intensity of sporulation varied from one source to another, with rates ranging from 2 x 106 to 1 x 107 spores/mL (**Fig. 5**). Maximum sporulation was induced by baker’s yeast, with a concentration of 1.05 x 107 conidia/mL. This was followed by housefly meal, with a concentration of 8.5 x 106 conidia/mL. Peptone induced the lowest conidial production (3.19 x 106 conidia/mL).

**Fig. 5.** Influence of nitrogen source on *B. bassiana* sporulation. The vertical bars illustrate the standard deviation of means. ET-LEUVRE : yeast extract; MD: larval meal of the domestic fly. Values with different letters at the top of the bar indicate a significant difference (\*\* P < 0.001) determined by the Tukey HSD test.

**3.1.5 Effect of Carbon Source on Sporulation**

The results presented in **Fig. 6** showed that fulvic acid and humic extract promoted abundant sporulation, with mean values of 1.4 x 109 and 1.3 x 109 spores/mL, respectively. These were followed by humic acid with a value of 6.8 \times 10^8 spores/mL. As for the lowest sporulation values, they were obtained with sucrose and glucose, with respective values of 3.65 x 108 and 4.65 x 108 spores/mL. Glucose and sucrose, although permitting sporulation, produced fewer spores compared to the tested humic substances. Sucrose was associated with the lowest spore production, with a mean value of 3.65 x 108 spores/mL.

**Fig. 6.**  Influence of carbon source on *B. bassiana* sporulation. Vertical bars illustrate standard deviation from means. Fulvic acid (FA) ; humic acid (HA) ; humic extract (HE). Values with different letters at the top of the bar indicate a significant difference (\*\* P < 0.001) determined by the Tukey HSD test.

**3.1.6 Effect of Culture Substrate on Sporulation**

The results showed that all tested substrates allowed the fungal strain to develop correctly. However (**Fig.7**), conidial yields varied from one substrate to another (**Fig. 8**). The highest conidial concentrations were obtained with the substrate consisting of rice husk and corn flour, with a yield of 2.26 x 107 conidia/mL. This substrate was followed by the substrate composed of rice husk and sorghum (5.69 x 107 conidia/mL). These flours were followed by cassava (4.87 x 107 conidia/mL) and wheat (4 x 107 conidia/mL). The lowest conidial concentrations were obtained with rice (2.47 x 107 conidia/mL) and millet (2.25 x 107 conidia/mL) flours.



**Fig. 7.** Development of the strain according to the type of flour added to the substrate. **(a)** rice flour; **(b)** corn flour; **(c)** millet flour; **(d)** sorghum flour; **(e)** cassava flour; **(f)** wheat flour.

**Fig. 8.** Influence of the type of flour on sporulation. The vertical bars illustrate the standard deviation of the means. Values with different letters at the top of the bar indicate a significant difference (\*\* P < 0.001) determined by the Tukey HSD test.

3.2 Discussion

Medium pH is a major regulator of fungal cellular processes (Padmavathi *et al.,* 2003). Although *B. bassiana* is often described as preferring slightly acidic to neutral conditions for growth (Karthikeyan *et al*., 2008), the present study revealed optimal sporulation (7 x 108 spores/ml) at an alkaline pH of 9, contrasting with significantly lower production in acidic media (pH 5-6). These results align with those of Tamires *et al*. (2022), who obtained high yields (7.2 x 108 and 6.8 x 108 spores/ml for *B. bassiana* ESALQ 171 at pH 7 and 8, respectively). However, they diverge from the observations of Castellanos and Pedro (2010), who reported maximum sporulation (11.4 x 108 spores/mL) at more acidic pH values (4.4 - 4.5). The preference for alkaline pH (8-9) in the current study suggests optimal functioning of enzymatic and transport systems related to sporulation under these conditions for the studied strain, potentially indicating an effective adaptation mechanism to alkaline environments, possibly linked to its origin or exploitable for specific formulations (Gao *et al.,* 2009).

Light, through its intensity, wavelength, and photoperiod, regulates fungal growth and reproduction (Corrochano, 2019). The study showed that *B. bassiana* sporulated best under an alternating regimen of 12 hours of light and 12 hours of darkness (12L:12D), producing 3.9 x 107 conidia/ml. Continuous light (24L:0D) was the least favorable (3.07 x 107 conidia/ml). This is consistent with the work of Mahdieh *et al*. (2013) on *Pyricularia oryzae*, where alternating light/darkness (16L:8D) promoted sporulation compared to continuous light. This requirement for alternation could be linked to circadian regulation and the activation of signaling pathways by light-dark transitions (Tisch and Schmoll, 2010). Indeed, light perceived by the White-Collar Complex (WCC) synchronizes the internal clock with the external day/night cycle, aligning conidiation (Dunlap and Loros, 2004; 2007). It is important to note that continuous light can often suppress or strongly attenuate these sporulation rhythms, as was the case in this study, highlighting the crucial role of light/dark alternation.

Temperature directly affects enzyme kinetics, overall metabolism, and membrane fluidity, impacting growth and sporulation (De Ligne *et al.,* 2019; Nanjundaswamy and Okeke, 2020). The study observed an increase in conidia production between 20°C and 25°C, peaking at 3.83 x 108 spores/ml at 25°C. This optimal temperature is consistent with data from Moldovan *et al.* (2022) for another *B. bassiana* strain (9.78 x 107 conidia/cm² at 25°C). Beyond the optimum, high temperatures induce thermal stress, diverting resources towards survival mechanisms (e.g., production of Heat Shock Proteins, HSPs) and inhibiting sporulation (Guan *et al*., 2024). This was evidenced in the current study by a significant reduction in sporulation at 30°C and a drastic drop at 35°C (5.67 x 104 spores/ml), illustrating the existence of cardinal temperatures (minimum, optimum, maximum) specific to each fungus (Ugine *et al*., 2013).

The carbon source provides energy and precursors for biosynthesis. Its nature influences the quantity and quality of spores (Maldonado-Blanco *et al*., 2016). The study revealed that complex organic carbon sources, fulvic acid (1.4 x 109 spores/ml) and humic extract (1.3 x 109 spores/ml), induced significantly higher sporulation than simple sugars like glucose (4.65 x 108 spores/ml) or sucrose (3.65 x108 spores/ml). Humic acid also yielded good results (6.8 x 108 spores/ml), which is consistent with the stimulatory effect of commercial humic substances on Metarhizium reported by Majchrowska-Safaryan *et al*. (2024). The superior performance of fulvic and humic extracts (derived from animal manure composts) suggests they provide, beyond carbon, essential micronutrients (N, P, K, Ca, Fe, S, Zn, Mg, Mn, etc.), considered fundamental as basal salts for inducing fungal biomass (Jackson *et al*., 1997; Biekre *et al.,* 2018).

Nitrogen is crucial for proteins, nucleic acids, and the cell wall. Complex organic sources are often preferred as they can supply amino acids and vitamins (Jaronski, 2010). The study showed that baker's yeast (1.05 x 107 conidia/ml) was the best-performing nitrogen source, followed by housefly larvae meal (8.5 x 106 conidia/ml). Yeast extract and peptone, conventionally used, were less effective, with peptone yielding the lowest output (3.19 x 106 conidia/ml). This could be explained by *B. bassiana*'s ability to utilize diverse nitrogen sources such as sodium nitrate, potassium nitrate, DAP, cotton meal, or corn meal (Luo *et al*., 2023) and by the nutritional richness of baker's yeast and insect meal in proteins, lipids, ash, and fiber (Idriss *et al*., 2021; Valesca *et al.,* 2024), potentially being more complete or better assimilated than peptone by this strain.

The use of solid substrates like rice husk often requires supplementation to overcome nutritional deficiencies. The study evaluated the addition of different starch flours to rice husk. Corn flour yielded the highest production (6.26 x 107 conidia/mL), followed by sorghum (5.69 x 107 conidia/mL). Cassava and wheat flours gave intermediate results, while rice and millet flours were the least effective (2.47 and 2.25 x 107 conidia/ml, respectively). These findings confirm the benefit of enriching rice husk (Mishra *et al*., 2016; Song *et al.,* 2019; Aminudin *et al.,* 2023) and demonstrate the variable efficacy of supplements. The superiority of corn and sorghum could be due to a more balanced nutritional profile (Alhadj *et al.,* 2023), a more optimal C/N ratio (Mishra *et al.,* 2016), or better substrate structure/aeration (Barrena *et al.,* 2017). The fact that flours other than rice flour improve a rice-based substrate suggests benefits from nutritional diversification. This approach using agricultural by-products and simple flours represents a potentially cost-effective method for enhancing *B. bassiana* production (Arnau *et al.,* 2019).

4. Conclusion

Based on the findings of this study, the results obtained showed that the Beauveria bassiana strain requires specific conditions for its mass production. Regarding the environmental factors studied, the values required for optimal development were as follows: a temperature of 25 °C during the incubation period, an alkaline pH of 9, and an alternating 12/12 h light/dark cycle. Concerning nutritional factors, corn flour, followed by sorghum flour, were identified as the best additives to the culture substrate (rice husk). Yeast, followed by housefly meal, emerged as the best nitrogen sources, while humic substances, particularly fulvic acid, proved to be the best carbon sources. In summary, conidia production at small and medium scales varies depending on key parameters including the substrate used, pH, temperature, photoperiod, aeration (substrate structure), and various additives, among others. Optimal conditions must be evaluated for each entomopathogenic fungal species, and potentially even for each specific strain.

**DISCLAIMER (ARTIFICIAL INTELLIGENCE)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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