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Culture medium improvement for *Isaria fumosorosea* submerged conidia production

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ABSTRACT

Submerged conidia and blastospores of the entomopathogenic fungus *Isaria fumosorosea* are produced in several liquid culture media. However, yields and the ecological fitness of these propagules vary according to culture media composition. In most culture media, hyphae, blastospores and submerged conidia are white but we found that in some media they develop a brown pigmentation. A dark pigment was extracted from brown-pigmented propagules and analyzed by IR spectroscopy. Adsorption bands coincided to those characteristics of melanins.

Hadamard's matrices were employed in order to increase submerged conidia yields and brown pigmentation of fungal propagules. Media containing 20–30 mg/l of $FeSO_4 \cdot 7H_2O$ and 6–12 mg/l of $CuSO_4 \cdot 5H_2O$ allowed reaching the highest pigmentation (9 in a hedonic scale). A maximal concentration of submerged conidia of 1.0 ($\pm$1.2) $\times$ 10^{12} cell/l was achieved after 120 h of liquid culture in a improved culture medium, containing 25 ml/l of Polyethylene glycol (MW 200), substance which enhanced submerged conidia production, reducing free mycelia or mycelial pellets formation. In the improved medium, it was estimated that more than 60% of produced biomass corresponded to submerged conidia and blastospores, while in other media, mycelia were the main product (80–97%).

1. Introduction

Isaria fumosorosea (Wize) (formerly *Paecilomyces fumosoroseus*) is a fungal biocontrol agent with potential for controlling several insect pests [1]. When grown on solid media it produces differential asexual conidia, which are genetically stable [2] and resistant to adverse environmental conditions, like other Hyphomycetes. Aerial conidia of *I. fumosorosea* have a typical brown color [3] attributed to melanins, pigments which may contribute to fungal spores protection from solar radiation, particularly from UV light incidence, high temperatures, desiccation, enzymatic lysis and fungicides [4]. In submerged culture, *I. fumosorosea* produces usually non-pigmented propagules which include freely dispersed mycelia, mycelial pellets, short hyphae, blastospores and submerged conidia [5–7]. Blastospores and submerged conidia are infective propagules, but they are lesser resistant to environmental stresses than aerial conidia [6]. Cell wall composition [8–10], osmolites [11,12] and pigments like melanins may contribute to the resistance of the fungal spores to abiotic stresses.

One approach to improve fungal biopesticides is to enhance the ecological fitness of aerial conidia, submerged conidia and/or blastospores by physiological manipulation [13], through modifications of both growth conditions and culture media. Historically, one-at-a-time strategy has been one of the most popular choices for improving fungal medium composition [6,10,14–16]. The rationale behind of this strategy is keeping the concentration of all culture medium components constant except one. Nevertheless, the technique has some major flaws; for example the optimum can be completely missed, and a relatively large number of experiments is needed [17].

In this study we assessed the critical factors involved in submerged conidia yield of *I. fumosorosea*. We also identified which media components are important for pigmentation of these propagules and we report the improvement of the culture medium through the use of Hadamard's matrices. These are most commonly employed for identifying important factors of a system and optimizing medium compositions and growth conditions, both in closed and open improvement strategies [17,18]. The improved medium resulted in high concentration of brown-pigmented submerged conidia, scarce production of blastospores and disperse-short-mycelium. Melanin was identified in brown-pigmented fungal propagules.

2. Materials and methods

2.1. Microorganism and growth conditions

A stock culture of the *I. fumosorosea* Pfrd strain obtained from the culture collection (a public-access culture collection) of the

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Centro Nacional de Referencia de Control Biológico (DGSV-SAGARPA, Mexico) was maintained in slant tubes, using SDY medium [65 g/l Sabouraud dextrose agar (Difco) supplemented with 10 g/l yeast extract (Difco)]. The fungus was grown on SDY-Petri dishes for 14 days at 27 °C with a 12:12 light:dark photoperiod; dishes were sealed with parafilm from inoculum up to day 7, then the seal was removed to allow dry-out in order to stimulate sporulation. Aerial fresh conidia were harvested in sterile 0.05% Tween 80 and the suspension, containing 1×10^8 conidia/ml, was stored in 5-ml cryogenic tubes at −40 °C. This spore suspension was utilized as inoculum for flask cultures.

2.2. Submerged culture

Erlenmeyer flasks (125 ml) containing 30 ml of designed medium were inoculated with 0.5 ml of the 1×10^8 conidia/ml suspension stored in the cryogenic tubes. Flasks were incubated at 28 °C and 150 rpm for 5 days.

2.3. Analytical methods

Total biomass was determined by gravimetric analysis after filtration of the sample through pre-weighed nitrocellulose filters (45 mm diameter, pore size 0.45 μm), rinsing twice with 10 ml distilled water aliquots and drying at 95 °C to constant weight. Biomass of blastospores and submerged conidia was estimated in a similar way after filtering a sample aliquot through gauze to remove mycelia.

Blastospores and submerged conidia were counted in a hemacytometer using a light microscope (400× magnification). A propagule was considered as blastospore when a monocellular hyphal body presenting an oblong to cylindrical shape was observed and as submerged conidia when the morphological characteristics were close to those of aerial conidiospores [19]. The estimated average value of number of blastospores and submerged conidia per gram of them was 1.2×10^{11} cell/g_{cell}.

Color was measured by comparing the color of the biomass retained in nitrocellulose filters (45 mm diameter, pore size 0.45 μm) and rinsed twice with 10 ml distilled water aliquots, with the Munsell color chart (10R), choosing 9 possibilities (8/1, 8/2, 8/3, 8/4, 7/6, 7/4, 6/3, 5/3 and 5/1), where 8/1 correspond to white and 5/1 to the darkest brown color. To each one of these colors a numerical value (1–9) was assigned in order to develop further mathematical and statistical analysis.

Extraction and purification of pigments from the fungal biomass were performed according the procedure for plant melanins described by Sava et al. [20] with minor modifications. First, the fungal biomass was treated with 2 M NaOH, pH 10.5, for 36 h. Thereafter, the mixture was centrifuged at 4000 × *g* for 15 min and the supernatant was acidified with 2 M HCl to pH 2.5, incubated for 2 h at room temperature, and centrifuged at 4000 × *g* for 15 min. The precipitate was purified by acid hydrolysis using 6 M HCl at 100 °C for 2 h to remove carbohydrates and proteins and treated with ethyl acetate to wash away lipids. The precipitate was then dried at room temperature, re-dissolved in 2 M NaOH and centrifuged at 4000 × *g* for 15 min. The supernatant was precipitated by the addition of 1 M HCl, washed with distilled water and lyophilized. This procedure yielded about 1.1 mg of pigment per g of pigmented fungal mass.

Pigment was mixed with KBr (1:100, w/w), and the mixture was ground in a mortar and pestle until uniform color was achieved. Aliquots were pressed into 5-mm pellets and characterized with IR spectroscopy in a FTIR Nicolet spectrophotometer, Protege 460 ESP model (Nicolet Co., Madison, WI).

Histological identification of melanins was performed using Fontana-Masson-melanin-specific stain. Aliquots of 10 ml of brown and white cultures were centrifuged at 4000 × *g* for 15 min to remove supernatant. Precipitate fungal biomass was washed twice with 10 ml of water. Then, biomass was resuspended in 10 ml of water and 200 ml of stain solution were added, maintained at 62 °C for 15 min, and observed in a light microscope (400× magnification).

2.4. Experimental design

Hadamard's matrices with seven parameters ($k = 7$; $N = 8$) varying between a high and a low level were used to assess the influence of mineral culture medium components on color development of propagules and blastospores and submerged conidia production in order to improve the culture medium. These matrices have only −1 and +1 values, and are constructed with an equal number of +1 and −1 by column. Each column represents one variation factor (k) of the study and each line, one experiment (N). For a given column, +1 and −1 are the two chosen levels for the corresponding factor. From a mathematical point of view, these matrices are orthogonal, which guarantees the non-confusion of facts in the estimate of the factors'.

The responses are polynomial models represented as

$$Y_i = b_0 + \sum b_i X_i \quad (1)$$

with Y_i = response; b_0 = mean of the response; b_i = coefficient; X_i = factors studied.

The response mean value is

$$b_0 = \frac{Y_1 + Y_2 + \cdots + Y_N}{N} \quad (2)$$

The b_i coefficients to be determined are given by

$$b_i = \frac{X_i' Y_i}{N} \quad (3)$$

where X_i' correspond to the transposed matrix.

A medium containing (per liter) 50 g of glucose, 1.84 g of NH_4NO_3, 0.39 g of KH_2PO_4, 1.42 g of $Na_2HPO_4{\cdot}12H_2O$, 0.60 g of $MgSO_4{\cdot}7H_2O$, 0.1 mg of KCl, 2 mg of $MnSO_4{\cdot}H_2O$, 10 mg of $CoCl_2{\cdot}6H_2O$, 2 mg of $Na_2MoO_4{\cdot}2H_2O$, 20 mg of $ZnSO_4{\cdot}7H_2O$, 3 mg of $CuSO_4{\cdot}5H_2O$, 2 mg of $FeSO_4{\cdot}7H_2O$ and 60 mg of EDTA (disodium salt) was used as basal medium.

The aim of the first experimental design (EP1) was to evaluate the effect of salts (except NH_4NO_3, $ZnSO_4{\cdot}7H_2O$ and EDTA) at +1 or −1 level. In the second experimental plan (EP2); the influence of Cu^{2+}, Fe^{2+}, Mg^{2+}, K^+ (as chloride), and Mn^{2+} and Mo^{6+} at the new concentrations were assessed. Additionally, KH_2PO_4 was used as the sole phosphate source and evaluated as a new factor, excluding Na_2HPO_4. The third plan (EP3) was similar to the two preceding ones, the effect of new concentrations of phosphate, K^+ (as chloride), Cu^{2+}, Fe^{2+} and the inclusion of Ca^{2+}, Polyethylene Glycol (PEG) MW 200 (Purchased from Sigma) and EDTA in the culture medium were assessed. Final adjustments to estimate the optimal PEG concentration were made by one-at-a-time procedure assaying different concentrations of the compound. In these experiments, mean comparisons were conducted using the Tukey–Kramer adjustment [21] for multiple comparisons; letters indicating statistical significance or similarity were obtained using *NCSS 2000* software.

In all experiments pH was adjusted to 5.5 before sterilization. Two replicate flasks were used for each trial. All experiments were repeated at least twice.

3. Results and discussion

The maximal concentration of submerged conidia in the basal medium was $2(\pm1) \times 10^8$ cell/ml and blastospores were just around $2(\pm1) \times 10^7$ cell/ml. The biomass had pale brown color [22]. In the