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Influence of nutrient, pH and dissolved oxygen on the production of *Metarhizium flavoviride* Mf189 blastospores in submerged batch culture

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Abstract

The influence of different parameters on the sporulation of *Metarhizium flavoviride* was studied during submerged cultures in shake flasks and in 5 l bioreactors. The screening in shake flasks of several carbon and nitrogen sources allowed the definition of an optimal medium, based on sucrose and brewer's yeast with a C/N ratio of 1.6. With this medium, a production of 5.4×10^8 blastospores per ml (Bspores ml^{-1}) was obtained after 169 h of cultivation. The influence of pH and pO_2 was independently studied in 5 l working volume bioreactors using the optimal medium. The best production was obtained with pH and pO_2 regulated respectively to 7 and 100%. Finally, when the culture was grown under optimized conditions, the blastospores concentration increased 16-fold, with 1.1×10^9 Bspores ml^{-1} obtained after 144 h cultivation. This represents a gain of productivity of about 4.8 times.

Keywords: Metarhizium flavoviride; Submerged culture; Blastospore production; Dissolved oxygen influence

1. Introduction

Concerns about the negative effects of chemical pesticides have lead to emphasis on alternate strategies for pest control. There is a worldwide resurgence of interest in the use of entomopathogenic fungi as microbial control agents [1–3] and a significant advance in development of myco-insecticides in the future is expected with recent innovations [4–6]. The improvement of potential control agents often depends on an adequate mass-production method for producing the infective propagules on a suitably large scale [7].

The entomopathogenic hyphomycete fungi are often easy to grow on cheap media in submerged culture on a large scale. Depending upon strain, medium and culture parameters, the fungal biomass increases by vegetative growth forming either hyphal filament, often with copious branching, or various forms of flocs and pellets of mycelia [8]. Most fungal isolates are also able to form single cells by schizolytic separation at septa or by mechanical fragmentation of hyphae or can also be produced from hyphae by yeast-like budding [9,10]. Even though hyphal cells, mentioned as hyphal bodies or blastospores, are usually the only type of propagules produced in liquid fermentation, several studies have reported the formation of conidiospores, microscopically identical to aerial conidia [11–13]. Submerged propagules are usually less resistant to environmental stresses than aerial conidia, but they can be produced economically in deep tank fermenters [7]. In contrast, solid state fermentation used to produce aerial conidia of entomopathogenic hyphomycetes has been mainly performed on a cottage industry base [14,15].

No universal factor is known for eliciting fungus sporulation. Both nutrients and physico-chemical parameters of

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the liquid culture can have a huge incidence on fungal growth, sporulation, stability and virulence of submerged propagules [6,12,16]. Moreover, the influence of most of these parameters varied according to the fungal isolate [17].

The entomopathogenic hyphomycete, *Metarhizium anisopliae* var. *acridum*, previously described as *M. flavoviride* has been reported as one of the promising species for development as a biological control agent against locusts and grasshoppers [15,18–20]. The production of submerged conidia by *M. flavoviride* was performed by Jenkins and Prior [13]. Studies on blastospore production have also been devoted to locust and grasshopper control [6,17].

This paper reports studies on the influence of nitrogen sources and main culture parameters for assessing blastospore yields in submerged fermentation of the isolate IMI 330189 of *M. anisopliae* var. *acridum* (Mf189). This isolate was used because of its infectivity towards locusts and grasshoppers [21]. Firstly, the medium composition and C/N ratio were optimized both in shake flasks and in a bioreactor. Secondly, the influence of pH and dissolved oxygen was studied during batch culture. Blastospore production was then carried out by using these optimized parameters.

2. Materials and methods

2.1. Microorganism

Strain Mf189 of *M. flavoviride* (IMI 330189) was isolated in Niger from *Ornithacris cravoisi* Finot (Orthoptera: Acrididae) in 1988 [19]. The original strain was stored at +4 °C on slant PDA medium (20% potato infusion, 2% glucose and 1.5% agar).

3. Culture conditions

3.1. Inoculum preparation

The spores used were produced in Roux flasks containing PDA medium. After 7 days of incubation at 28 °C, the surface of the medium was scraped using 0.1% (v/v) of Tween 80 solution. All experiments were carried out with an inoculation rate of 10^5 spores ml^{-1}.

3.2. Culture in shake flasks

Cultures in 500 ml baffled Erlenmeyer flasks, containing 200 ml of each liquid medium were performed at 28 °C on a rotary shaker (120 rpm).

3.3. Culture in a bioreactor

Cultures were carried out in a 7 l fermentor vessel (Applikon, Netherlands) containing 5 l of defined medium (0.5% sucrose, 2% brewer's yeast). The pH was regulated with 2.5N NaOH and 2.5N H_2SO_4 solutions and the temperature was set at 28 °C. By means of three motorised valves (air/N_2/O_2) the dissolved oxygen concentration was regulated at different set-point values in a range between 0 and 100% saturation. Mainly for high dissolved oxygen concentration, the agitation speed was increased up to 250 rpm to maintain saturation during culture.

3.4. Determination of blastospore yield

Blastospores were counted with a haemocytometer. The Colony Forming Unit (CFU) procedure was used for the measurement of the blastospore viability. Serial dilutions up to 10^{-5} in distilled water with 0.1% (v/v) Tween 80 were carried out and 500 μl of each dilution were plated onto PDA medium. This measurement was carried out in triplicate for each dilution in order to take into account the standard deviation. After 6 days incubation at 28 °C of the Petri dishes, the colonies were counted and results expressed in CFU ml^{-1}.

3.5. Analyses of carbohydrates by HPLC

Samples were centrifuged at 20,000 × *g* for 15 min. The supernatants were filtered through a 0.45 μm membrane and the different carbohydrates were separated with an Aminex Ion Exclusion HPX-87 H column in HPLC (Merck L-600). The mobile phase was 5 mmol l^{-1} sulphuric acid and the flow rate was 0.6 ml min^{-1}. Detection was performed by refractometry (RI detector 8110 Bischoff).

4. Results

Screening of nutrient medium for the production of blastospores in shake flasks.

The production of blastospores was first tested in baffled flasks in order to define a culture medium. For the screening of the different liquid media, sucrose and both organic and mineral nitrogenous sources were tested. According to the results obtained, the carbon/nitrogen ratio was optimised.

4.1. Choice of nitrogen source

Sucrose was tested with different nitrogen sources: yeast extract (Organotechnie® 19512), and two brewer's yeast coming from different suppliers. The first brewer's yeast came from an European supplier (Organotechnie® 11102), the second one from USA (Blue Ridge Brewery, Charlottesville, Virginia). Malt extract addition was also tested in the medium composition and in the same way a mineral nitrogenous source: potassium nitrate (Table 1).

All the media permit the production of propagules with a very low amount of mycelium, except media 5 and 6. Indeed, with a mineral nitrogen source, mycelium production is observed without blastospore formation. The evolution of