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# Optimization of the medium composition of a biphasic production system for mycelial growth and spore production of *Aschersonia placenta* using response surface methodology

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ABSTRACT

The culture media for mycelial growth and sporulation of the entomopathogenic fungus *Aschersonia placenta* were optimized using the response surface method (RSM). Interactions of medium components and the optimization of a biphasic production system were studied using Box–Behnken design (BBD) with three levels of three variables. Experimentation confirmed that the model developed based on RSM and BBD successfully predicted mycelia production ($R^2$ = 0.9336) and conidia production ($R^2$ = 0.9532). In the first phase, mycelial dry weight was highest (2.14 ± 0.17 g per 100 ml of culture, mean ± SE) when the concentrations (g/l) of glucose, vitamin $B_6$, and $MgSO_4{\cdot}7H_2O$ were 31.4, 11.5, and 0.64, respectively. In the second phase, conidia production was highest (9.31 ± 0.48 × $10^7$ spores per $cm^2$) after 18 d of cultivation in the medium containing 33.8 g/l of millet, 1.11 g/l of $KH_2PO_4$, and 0.37 g/l of $MgSO_4$. Mycelial and conidial yields were 3.6- and 10-fold greater, respectively, with the optimized media than with the non-optimized basal media. The results indicate that RSM and BBD methods are effective for increasing the production of *A. placenta* mycelia and conidia.



## 1. Introduction

Entomopathogenic fungi in the genus *Aschersonia* have been recognized as important biological control agents against whiteflies (Aleyrodidae) and scale insects (Coccidae) (Fransen, 1990; Meekes et al., 2002) and can cause spectacular epizootics in whitefly and scale populations in the tropics and subtropics (Evans and Hywel-Jones, 1990). In an early example of classical biological control, *Aschersonia aleyrodes* was used against whiteflies in Florida citrus groves in the early 1900s (Berger, 1921). Previous research has indicated that *A. aleyrodes* can infect the nymphal instars of the greenhouse whitefly but cannot infect eggs and only sporadically infects adults (Fransen, 1990). *A. aleyrodes* can persist on leaf surfaces for long periods (Fransen, 1995; Meekes et al., 2000) and has a high tolerance to low relative humidity (Fransen, 1990). Recent studies have shown that the extracts of secondary metabolites from *Aschersonia* spp. are cytotoxic to insect cells but not to mammalian cells (Watts et al., 2003). These results suggest that *Aschersonia* spp. are not hazardous to humans and that some species can be potentially used as biological control agents against insect pests. Our preliminary results indicate that another *Aschersonia* species, *Aschersonia placenta*, has substantial potential as a biological control agent of whiteflies in China (unpublished data). The current study concerns the optimization of *A. placenta* mycelia and conidia production.

Fungal spores of some biological control agents have been regarded as potential alternatives to agrochemicals because they can often be easily produced and may be adapted to survive unfavorable conditions. For some fungal species, however, spore production methods have not been well developed. Moreover, the commercial use of spores for biological control depends on the determination of the optimal conditions for their large-scale production. Media components significantly affect spore properties (including yield, biological control efficacy, desiccation tolerance, and persistence) of the entomopathogenic fungi *Paecilomyces fumosoroseus*, *Beauveria bassiana*, and *A. aleyrodis* (Cliquet and Jackson, 2005; Safavi et al., 2007; Zhu et al., 2008; Chong-Rodríguez et al., 2011). Because the optimal media components differ among species and sometimes even among strains of the some species, the nutritional composition of the production medium must be optimized for each individual organism.

Industrial production of conidia involves three systems (solid, liquid, and biphasic); in the biphasic system, the fungus is cultivated under submerged conditions before being transferred to a solid or semi-solid medium to sporulate (Jenkins and Goettel, 1997; Bartlett and Jaronski, 1998; Kassa et al., 2008). Biphasic liquid–solid fermentation combines the benefits of both liquid and solid fermentation and is used mainly for hyphomycetous

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fungi, e.g., *Metarhizium flavoviride* Gams & Rozsypal and *Beauveria bassiana* (Balsamo) Vuillemin (Jenkins and Goette, 1997; Vega et al., 2003). Sporogenesis is more rapid, convenient, and economical in the biphasic system than in a single-phase system (Anellis et al., 1972). A semi-solid medium is much better than a liquid medium for the sporulation of the entomopathogenic fungus *A. placenta* (Ibrahim et al., 1993). The aim of this study was to optimize the media compositions of the biphasic production system for production of *A. placenta* conidia.

Optimization of media components by classical methods, in which one factor is changed while other factors are kept constant, is tedious, time consuming, and expensive, especially when many variables must be considered (Prasad et al., 2005; Cai, 2008; Ghosh and Hallenbeck, 2010). Response surface methodology (RSM) combines mathematical and statistical techniques for designing experiments, building models, identifying effective factors, studying interactions, and searching for optimal conditions; it thereby eliminates the limitations of single-factor optimization (Meyers and Montgomery, 2002; Bandaru et al., 2006; Tabandeh et al., 2008; Wang, 2009; Tan et al., 2010). Hence, the present study applied RSM to identify the optimal media components of the liquid–solid fermentation system in order to enhance *A. placenta* mycelial growth in the first stage and enhance sporulation in the second stage. In addition, we used Box–Behnken design (BBD) to study interactions of the important components and to identify their optimal levels. BBD has been used previously to optimize the physical production of entomopathogenic fungi (Feng et al., 1994; Shih et al., 2007; Dong et al., 2009) but it has not been used for *A. placenta* or other *Aschersonia* spp.

## 2. Materials and methods

### 2.1. Fungal strain, inoculum preparation, and culture conditions

Strain FJSM was selected from among 43 *A. placenta* laboratory strains based on previous mortality assays against the whitefly *Bemisia tabaci* (unpublished data). This strain was originally isolated from *Aleurotrachelus camelliae* Kuwana in a *Camellia oleifera* Abel orchard (Fujian, China) in 2010. Strain FJSM was stored in glycerol at −80 °C. For preparation of inoculum, *A. placenta* FJSM was initially grown on potato dextrose agar (PDA) in Petri dishes at 25 °C for 20 d. Sterile water containing 0.05% (v/v) Tween 80 was then added to the cultures, which were agitated to dislodge conidia, and the liquid with conidia was collected and used to prepare a conidial suspension. The conidial concentration was determined with a haemocytometer, and suspensions containing $1 \times 10^8$ conidia $ml^{-1}$ were prepared and used to inoculate 250-ml flasks containing liquid media for production of mycelia in the first stage of the biphasic system; the liquid media are described in Section 2.3. The flasks were incubated at 25 °C on a shaker at 160 rpm. After 5 d, mycelia were recovered and used to inoculate 9-cm-diameter dishes containing 30 ml of agar media for production of conidia in the second stage of the biphasic system; the agar media are also described in Section 2.3. For inoculation of the dishes, the mycelia obtained from one flask were used to inoculate one dish; 1 g (wet weight) of mycelia was added per dish. After 20 days at 25 °C, the conidia produced in each dish were enumerated.

### 2.2. Assessment of mycelial and conidia production

The mycelial biomass produced in the liquid media was expressed as dry mycelial mass (g) per 100 ml of culture medium. The mycelial dry weight was determined by passing the liquid culture through filter paper and drying the collected mycelia to constant weight at 60 °C. The number of conidia produced in dishes containing 30 ml of agar medium was determined by dislodging the conidia (by adding 0.05% (v/v) Tween 80 and shaking the dishes) and collecting the conidia in the liquid. Conidia concentration was determined with a haemocytometer, and conidial production was expressed as number of conidia per $cm^2$ of agar.

### 2.3. Experimental design and statistical analyses

#### 2.3.1. Preliminary experiments: The effects of carbon, nitrogen, and other components on production of mycelia and conidia by A. placenta FJSM

To study the effect of carbon source and nitrogen source on production of *A. placenta* FJSM mycelia and conidia, various carbon sources (glucose, fructose, galactose, sucrose, lactose, maltose, soluble starch, chitin, mannitol, sorbitol, corn flour, wheat flour, millet, and potato) and nitrogen sources (peptone, tryptone, yeast powder, yeast extract, beef extract, casein, soybean meal, $NaNO_3$, $NH_4NO_3$, urea, and $(NH_4)_2SO_4$) were tested individually while keeping other components of Czapek medium at a constant level. Trace elements (consisting of $ZnSO_4$, $CuSO_4$, $MnSO_4$, $CoCl_2{\cdot}6H_2O$, and $Na_2B_4O_7{\cdot}10H_2O$) and vitamins (covering $B_1$, $B_2$, $B_4$, $B_5$, $B_6$, C, PP, and folacin) were also evaluated for their suitability for the production of *A. placenta* FJSM mycelia and conidia.

We first tested the effect of different carbon sources on the production of mycelia and conidia. Production of mycelia and conidia was highest with the addition of glucose for mycelia production and millet for conidia production: the yields were 0.62 ± 0.05 g of biomass dry wt/flask and $2.51 \pm 0.13 \times 10^7$ conidia/$cm^2$. With respect to nitrogen sources, production of mycelia and conidia was highest when either casein or tryptone was used as the nitrogen source: the yields were 1.33 ± 0.11 g of biomass dry wt/flask and $1.38 \pm 0.12 \times 10^7$ conidia/$cm^2$, respectively. The series of experiments indicated that the major variables affecting the production of mycelia and conidia were glucose, casein, $ZnSO_4$, vitamin $B_6$, millet, and tryptone (data not shown). Thus, these components were investigated for further optimization of the media.

#### 2.3.2. Plackett–Burman design

The Plackett–Burman design (PBD) is a powerful mathematical approach for investigating and evaluating the effect of parameters (Singh et al., 2011), and it has been widely used to optimize biological processes (Naveena et al., 2005; Yuan et al., 2008; Singh et al., 2011). The variables obtained in the preliminary experiments were screened using the PBD to identify those variables that most affected production of mycelia and conidia. Each independent variable was set at two levels, −1 for a low level and +1 for a high level. These levels were selected based on the results of the preliminary experiments (see Section 2.3.1) and on published data (Prakash et al., 2008a). The experimental design details and significance of regression coefficients are given in Tables 1–2b.

#### 2.3.3. The steepest ascent experiment

The steepest ascent method uses the magnitude and sign of linear effects to determine the direction toward predictive higher responses (Chen et al., 2002). The path begins at the center of the current design space and stretches well outside the design space. A sequence of equally spaced locations along the path is then selected to form a set of experiments. The steepest ascent was adopted to obtain the largest response area by stepwise decreasing or increasing the concentrations of variables, which ensures the validity and correctness of direction (Lin et al., 2007). If the optimal region falls outside of the current design space, the method can be used to determine the next set of experiments (Chen et al., 2002). The experimental design details are shown in Table 3. This steepest ascent experiment involved five steps as indicated by the numbers 1–5 following the plus sign in Table 3. The paths of glucose,