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Improved hypocrellin A production in *Shiraia bambusicola* by light-dark shift

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ABSTRACT

Hypocrellin A (HA) is a major bioactive perylenequinone from the fruiting body of *Shiraia bambusicola* used for the treatment of skin diseases and developed as a photodynamic therapy (PDT) agent against cancers and viruses. The mycelial culture of *S. bambusicola* under dark is a biotechnological alternative for HA production but with low yield. In this study, light and dark conditions were investigated to develop effective elicitation on HA production in the cultures. Our results showed the constant light at 200 lx stimulated HA production without any growth retardation of mycelia. A light/dark shift (24: 24 h) not only increased HA content in mycelia by 65%, but stimulated HA release into the medium with the highest total HA production 181.67 mg/L on day 8, about 73% increase over the dark control. Moreover, light/dark shifting induced the formation of smaller and more compact fungal pellets, suggesting a new effective strategy for large-scale production of HA in mycelium cultures. The light/dark shift up-regulated the expression levels of two reactive oxygen species (ROS) related genes including superoxide-generating NADPH oxidase (*Nox*) and cytochrome *c* peroxidase (*CCP*), and induced the generation of ROS. With the treatment of vitamin C, we found that ROS was involved in the up-regulated expression of key biosynthetical genes for hypocrellins and improved HA production. These results provide a basis for understanding the influence of light/dark shift on fungal metabolism and the application of a novel strategy for enhancing HA production in submerged *Shiraia* cultures.

1. Introduction

Hypocrellins, perylenequinone pigments are extracted from fruiting-body of pathogenic fungus *Shiraia bambusicola* to bamboo, which has been used in traditional Chinese medicine for treating rheumatic pain, stomachache, vitiligo and psoriasis [1]. Due to its wide absorption band and extremely high light-induced singlet oxygen ($^1O_2$) generation, hypocrellin A (HA) has received widely interest in photodynamic therapy (PDT) on skin diseases and becomes a new type of non-porphyrin photosensitizer for the treatment of cancers and viruses [2,3]. Because of the limitation in the supply of wild fruiting body, mycelium culture of *S. bambusicola* is becoming a promising alternative for HA production. HA production could reach 2.02 mg/g dry solid substrate in the solid-state cultures of *Shiraia* sp. SUPER-H168 [4] and relatively lower values (about 10–40 mg/L) in submerged cultures [5]. Many strategies have been applied to enhance HA production in *S. bambusicola* cultures, including the alternation of medium components [6], addition of surfactant [7], ultrasound stimulation [8] and fungal elicitation [9]. To our knowledge, the mycelia of *S. bambusicola* were cultured in the dark in all above mentioned reports. However, it was reported that the stroma of *S. bambusicola* developed at the bamboo shoot apex in wild environment [10]. The frequency of the fungal occurrence was higher in the edge of bamboo forest than that in the forest interior [11]. Those reports imply a possible light-dependent regulation of the fungal growth and development.

Light is a very important environmental signal for fungal growth, development and metabolism. The physiological and developmental responses of fungi to light have been well reviewed previously [12,13]. As for the fungal secondary metabolites induced by light and dark conditions, it was reported that the dark culture is more profitable for metabolite accumulation of some soil and endophytic fungi. The red pigment of *Monascus purpureus* reached maximum in darkness but lower under white light [14]. The ochratoxin A produced by *Aspergillus carbonarius* and *A. westerdijkiae* were higher in darkness than that under light condition (445–740 nm) [15]. In the dark, *A. nidulans* also accumulated more toxin sterigmatocystin [16]. However, the effects of light on fungal metabolite accumulation were also investigated by several researchers. Schmidt-Heydt et al. reported that *Penicillium verrucosum* increased the production of ochratoxin by 5 folds in constant light than that in darkness [17]. In *A. nidulans*, the constant white light could induce the expression of some secondary metabolism genes significantly and initiated the accumulation of terrequinone A and emericellamide [18]. The works on light and dark shift mainly focused on fungal growth and development. It was found that a 5: 19 h light/dark

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shift (10–13 W/m^2) could enhance conidiation (6.7 × 10^7 per colony) of *A. nidulans* [19]. Rumbolz et al. reported that a 58-h darkness and 30-h light cycle was beneficial to the generation of sporangia in *Plasmopara viticola* [20]. However, the changes of secondary metabolites induced by light/dark shift were seldom reported. Zhang et al. reported that a light/dark condition (0–60 h light and 60–84 h dark) could stimulate fungal polyketide production of *Halorosellinia* sp. [21]. As the biosynthesis of fungal hypocrellins proceeds via a polyketide pathway [22], we wish to explore the effects of light and dark conditions on HA production of *S. bambusicola*. In our previous work, we found the stain could be elicited by the lower intensity ultrasound [8] and the nonionic surfactant Triton X-100 [23]. The studies on the effects of lights on HA biosynthesis in mycelium cultures may help us understand the mechanism of abiotic elicitation on the fungal metabolism and apply a novel eliciting strategy for HA production.

2. Materials and Methods

2.1. Strains, Media and Culture Conditions

The strain *S. bambusicola* S8 was isolated from bamboo (*Brachystachyum densiflorum*) shoots in our previous work [8] and reserved in China General Microbiological Culture Collection Center as CGMCC 3984, Beijing, China. The stain was stored on a potato dextrose agar (PDA) slant at 4 °C and initially grown on PDA medium in a Petri dish at 28 °C for 6 d. The spore suspension (4 mL of 10^7 spores/mL) was inoculated into 1000-mL Erlenmeyer flasks containing 200 mL modified liquid medium (100 g/L potato, 20 g/L starch, 4 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L $CaCO_3$ and 0.01 g/L VB_1, pH 6.3). The culture was incubated at 150 rpm at 28 °C for 1 d as a seed culture for the subsequent experiments. The seed culture (10%, v/v) was poured into a 150-mL flask containing 50 mL of the same liquid medium at 150 rpm and 28 °C for 8–10 d.

2.2. Light Irradiation

The light source used in this study was light-emitting diode (LED) XYC-T5001 lamps with length of 0.9 m and intensity of 18 W/m^2 (Xiaoyecao Photoelectric Technology Co., Ltd., Shenzhen, China). A light box (1 × 1 × 0.8 m) was installed on the shaking table and equipped with 4 light lamps in which parallel to each other (20 cm apart) to regulate different light conditions. For irradiation, the flasks (150 mL) containing 50 mL of medium were put into the light box. The light intensity was measured by a digital lux meter (LX-1332, Custom Corporation, Tokyo, Japan) and adjusted to 200, 400, 600 and 800 lx by changing the distance between lamps and flasks. The light/dark shift time (0, 12, 24 and 48 h) was adjusted by controlling the switch of light on schedule.

2.3. Residual Sugar, pH and Morphology Analysis

The residual sugar in cultures was measured by anthrone-sulfuric acid method [24], and the pH of cultural broth was measured by pH Meter (FE20, Metteler Toledo, Zurich). The fungal pellets were observed using a stereoscopic microscope (SMZ1000, Nikon, Japan) and photographed by an external camera (Coolpix S4, Nikon, Japan) at the different cultivation time (4, 5, 6 and 8 d). Pellet diameters were calculated by 50 objects in a sample for each determination at different days of growth (day 1–10) [25].

2.4. Extraction and Analytical Methods of HA

The HA extraction and quantification were based on the method described in our previous report [8]. HA content was determined by the reverse-phase Agilent 1260 HPLC system equipped with the Agilent HC-C18 column (250 × 4.6 mm dimension) (Agilent Co., Wilmington, USA). The sample was eluted by constant mobile phase (acetonitrile: water at 65: 35, v/v) for 20 min with a flow rate of 1 mL/min at 465 nm. HA was quantified with the standard (Chinese National Compound Library, Shanghai, China). Total HA production refers to the sum of intracellular and extracellular HA.

2.5. Detection of Reactive Oxygen Species (ROS) Generation and Activities of the Antioxidant Enzymes

The ROS generation in hyphal cells was detected by 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime Biotechnology, Jiangsu, China) [26]. After 4 day-old culture, fungal mycelium was pre-treated with 10 μM DCFH-DA for 1 h. The fluorescence in mycelia was observed using a fluorescent Olympus CKX41 microscope (Tokyo, Japan) with excitation wavelength at 485 nm and emission wavelength at 528 nm. The O_2^- and hydrogen peroxide content in mycelium were assayed as previously described [27]. The activities of the antioxidant enzymes including superoxide dismutase (SOD) and catalase (CAT) were measured by Enzyme Activity Assay Kit (Beyotime Biotechnology, Jiangsu, China).

2.6. Quantitative Real-Time PCR

Total RNA was extracted from frozen mycelia using RNAprep pure Plant Kit (Tiangen, Beijing, China) on the basis of manufacturer's instruction. The cDNA was obtained using the reverse transcriptase (Fermentas, Burlington, Canada). Primers of target genes and internal reference gene (18S ribosomal RNA) were designed using Primer Premier 5 (Primier, California, USA) (Supplementary Table S1). The qRT-PCR condition was performed with FastStart Universal SYBR Green Master (Roche, Switzerland) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, USA). The cycling parameters were set at 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 15 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression levels from cycle threshold values.

2.7. Statistical Analysis

All the following light or light/dark shift-induced responses were measured in the shake-flask cultures. All treatments consisted of triplicate independent repeats (ten flasks per replicate). Student's *t*-test was applied for experimental results regarding the effect of light/dark shift on ROS generation and expression levels of key genes. Experimental results regarding the effects on fungal growth and HA production were analyzed with SAS 9.2 (North Carolina, USA) by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. All the data are normally distributed and variances are homogeneous. Data are represented as mean ± standard deviation (SD). The p value of < 0.05 was considered statistically significant.

3. Results

3.1. Effect of Light on Fungal Growth and HA Production

The fungal biomass and HA production in the cultures under dark and constant light were compared in Fig. 1. Although the strong irradiation (600–800 lx) resulted in a decrease in fungal biomass by 4–16%, mycelial HA by 37–47% and the released HA in cultural broth by 33–55%, respectively (Fig. 1A, B, C), the weak irradiation (200, 400 lx) showed no growth retardation and positive effects on HA biosynthesis. The enhanced HA production reached to 117.58 mg/L at 200 lx, which is 28% higher than that of dark control (Fig. 1D).

Subsequently, influence of light pulse on fungal growth and HA production was investigated. The different light/dark shift (12: 12, 24: 24 and 48: 48 h) at 200 lx did not cause any significant inhibition on fungal growth (Fig. 2A). However, morphology of the fungal pellets was