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Improvement of the production of a red pigment in *Penicillium* sp. HSD07B synthesized during co-culture with *Candida tropicalis*

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ABSTRACT

Co-culture of *Penicillium* sp. HSD07B and *Candida tropicalis* resulted in the production of a red pigment consisting of six components as determined by TLC and HPLC. The pigment showed no acute toxicity in mice and was mot mutagenic in the Ames test. The pigment was stable between pH 2 and 10 and temperatures of 10–100 °C and exhibited good photo-stability and resistance to oxidization by hydrogen peroxide and reduction by Na_2SO_3. Glucose and ratio of *C. tropicalis* to strain HSD07B (w/w) in the inoculum were the important factors influencing production of the pigment. Under optimized conditions, a pigment yield of 2.75 and 7.7 g/l was obtained in a shake-flask and a 15 l bioreactor, respectively. Thus, co-culture of strain HSD07B and *C. tropicalis* is a promising way to produce a red pigment potentially useful for coloring applications.

1. Introduction

Synthetic and natural pigments are used extensively in the food, cosmetic and pharmaceutical industries (Mapari et al., 2005). Concerns over potential toxicity of some synthetic pigments have led to increased interest in pigments derived from natural sources (Downham and Collins, 2000). Traditionally natural pigments have been extracted from natural sources such as plant and insect tissues, but obtaining pigments through microbial fermentation is also possible. Some bacteria, yeasts, basidiomycetous fungi and microalgae are known to produce pigments (Arad and Yaron, 1992; Zhang et al., 2006; Davoli and Weber, 2002; Ginka et al., 2004; Mapari et al., 2008; Ogihara et al., 2001), but high costs and low productivity are significant bottlenecks for commercial production (Hejazi and Wijffels, 2004). Ascomycetous fungi of the genus *Monascus* have been used to produce a natural food colorant when grown on rice (Teng and Feldheim, 2001); however, *Monascus*-derived pigments contain citrinin, and the production of mycotoxin limits the use of *Monascus* as a producer of food colorants (Liu et al., 2005). Therefore, it is of interest to search for alternative pigment-producing organisms. In the present study, we found that during co-cultivation of *Penicillium* sp. HSD07B and *Candida tropicalis* a red pigment was produced that was not observed when the strains were cultured individually. Culture conditions were optimized to increase the production of the pigments and preliminary analyses of the composition and safety of the pigment were carried out.

2. Methods

2.1. Chemicals and microorganisms

All chemicals were of spectral or analytical grade unless otherwise stated. β-Carotene, lycopene and astaxanthin were obtained from Sigma. *Monascus* pigment and *Salmonella typhimurium* TA97, TA98, TA100 and TA102 were purchased from Zhengzhou Tianyu Co. Ltd. (China) and Shanghai Fuxiang Biotech. Co. Ltd. (China), respectively. *C. tropicalis* was obtained from the Laboratory of Applied and Environmental Microbiology, Henan Normal University, China.

2.2. Isolation and identification of strain HSD07B

During cultivation of *C. tropicalis* on Potato Dextrose Agar (PDA) medium, a large amount of red pigments appeared on a plate contaminated with a filamentous fungus. This fungus, designated strain HSD07B, was isolated and its morphological characteristics were determined as described by Raper and Thom (1949). Phylogenetic identification was carried out by sequencing of the D1/D2 domain of its 26S rRNA gene using the primers (NL-1: 5′-GCATATCAA TAAGCGGAGGAAAAG-3′; NL-4:5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett, 1998). Sequence alignments and calculation of sequence similarity were conducted using the Clustal X 2.0

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program (Chenna et al., 2003). A phylogenetic tree was constructed with the Mega 3.1 program using neighbor-joining method (Kumar et al., 2004). The sequence was deposited in Genbank under the Accession number HM367083.

2.3. Pigment production

Strain HSD07B and *C. tropicalis* were grown on PDA for 3 days at 30 °C before harvesting the conidia and yeast cells, respectively. Conidia suspension of strain HSD07B (3.7×10^7 conidia/ml) and cell suspension of *C. tropicalis* (4.1×10^7 cells/ml) were prepared in two 250 ml flasks each containing 100 ml sterile water. Liquid cultures of each strain in 150 ml of Potato Dextrose Broth (PDB, PDA without the agar) were initiated by inoculation with 0.5 ml of the respective suspensions, and co-cultures were prepared by simultaneous inoculation with 0.5 ml of each suspension. The cultures were grown at 30 °C in a rotary shaker at 150 rpm for 5 days. For cultivation on solid PDA medium, PDA plates were seeded with 0.2 ml of the individual suspensions or with 0.2 ml of both suspensions for co-cultivation. The plates were incubated at 30 °C for 3 days.

2.4. Preparation of red pigment and analysis

The culture medium was filtered using filter paper (Grade 1:11 μm, Whatman, UK), the cell-free filtrate was mixed with ethanol (filtrate: ethanol = 1:1.5) and the mixture was subjected to centrifugation at 2600g for 10 min. The supernatant was dried in a rotary evaporator at 50 °C and the crude pigment was mixed with 100 ml petroleum to remove hydrophobic substances. The remaining red pigment was dissolved in distilled water and analyzed by silica gel thin layer chromatograph (TLC) using 1-butanol:ethanol:water (3:5:2) as mobile phase. The pigment solution was also subjected to HPLC analysis. Twenty-five microliter aliquots were injected into a Kromasil ODS C-column (4.6 × 250 mm, 5 μm) with an auto-injector at 25 °C and fractions were eluted with 5% methanol in water at a flow rate of 2 ml/min. Eluates were monitored at 285 nm.

Monascus-derived pigment and the co-culture pigments were each dissolved in water and in ethanol, and their components were compared by the retention time after HPLC analysis. Solubility of the co-culture pigment in water and ethyl acetate was compared with that of red β-carotene, lycopene and astaxanthin. In addition, a preliminary analysis of a component (RP1) in the red pigment was conducted. RP1 was purified by column chromatography on silica gel 60 (0.040–0.063 mm, Merck) using 1-butanol, ethanol and water (4:6:1, v/v/v) as eluent at a flow rate of 1 ml/min. The collected RP1 (purity, 98%) was lyophilized, dissolved in acetonitrile and injected into a mass spectrometer (Waters-Micromass, Manchester, United Kingdom) controlled by MassLynx 4.0 software. The MS system was operated in the positive ESI mode using a linear 15–100% acetonitrile gradient in water over a 20 min period.

2.5. Characteristics of red pigment

Dried red pigment (1.0 g) was dissolved in 100 ml of water, glycerol, ethanol, acetone, acetic acid, 1-butanol, methanol, *n*-hexane, ether, petroleum ether and chloroform in order to investigate its solubility. The solubility was determined by the reduction in weight of the pigment after filtration with a filter paper (Grade 589: 2 μm, Whatman, UK) and the change of color value (CV) of the solvents. The effect of pH value on the color of pigment was studied by adjusting the pH of an aqueous solution of the pigment to 2, 3, 5, 7, 9, and 10 with 5 mol/l of NaOH or HCl, and the CV change of pigment solution was evaluated. The temperature stability of the pigment was tested by incubation at 10, 30, 50, 70, 90, and 100 °C for 1 h and measuring the CV. The effects of a 3-day exposure of the pigment, dissolved in 20 mM Na_2HPO_4–NaH_2PO_4 buffer (pH 7.0) at a concentration of 1.13 g/l–0.1 M sucrose, glucose, sodium benzoate, 0.01 M H_2O_2, Na_2SO_3, and 0.05 M $MgSO_4$, $CuSO_4$, $CaCl_2$, NaCl, KCl, and $MnSO_4$ on the CV of the pigment were determined by comparison of the CV of the solutions with the chemicals and that of the pigment buffer without any chemical. Light stability of the red pigment was determined by exposing the pigment solution to artificial light (1030 lux) for 7 and 15 days, and by irradiated with ultraviolet rays (15 W light and 50 cm distance) for 1.5 and 3 h.

2.6. Toxicity and mutagenicity studies

A mouse acute toxicity study was conducted according to the Evaluation Regulation of Food Safety in China (GB15193.13, 2003). Sixty mice of the Kunming species with an average body weight of 24 ± 2 g were divided into three groups containing 10 males and 10 females. One group served as control, and the other groups were fed 5000 and 15,000 mg of red pigment per kg of body weight, respectively. The mice were fed with normal granular feedstuff (Laboratory Animal Center of Henan Province, China) and supplied with water ad libidum, housed at 20–25 °C and observed for 14 days. Changes in movement, appetite and dejecta were recorded.

The Ames test was carried out according to the plate incorporation method (Ames et al., 1975). The pigment was tested against the *Salmonella enterica typhimurium* strains TA97, TA98, TA100 and TA102 at 10, 50, 100, 200 and 500 μg/plate with and without rat microsomal enzymatic activation fractions (S-9). Spontaneous reversion frequency was determined using distilled water, and 2-aminofluoren (2-AF) and NaN_3 were used as the positive mutagenicity controls. The test was performed in duplicate and the average counts of revertants were determined.

2.7. Optimization of culture parameters

The important factors influencing the production of red pigment were identified by the initial screening method of fractional factorial design (FFD). Six factors were chosen as independent variables and their levels are listed in Table 1. The effects of important factors on the production of pigment were studied by a central composite experimental design (CCD). Glucose and inoculum proportion (IP) of *C. tropicalis* to strain HSD07B (w/w, wet weight) were chosen as two independent variables and designated as X_1 and X_2; the yield of pigment was the dependent response and designated as Y. For predicting the mathematical relationship between the independent variables and dependant response, a second order polynomial function was fitted to the experimental results:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{12}X_1X_2 + b_{22}X_2^2$$

Table 1
Levels and significance of factors in fractional factorial experiment design.

Factor	Level		Coefficient	*t*-value	*p*-value
	−1	1			
A Inoculum quantity (%)	5%	10%	−0.02688	−0.59	0.570
B Inoculum proportion	2:1	1:2	0.42937	9.41	0.000**
C Rotation speed (rpm)	100	200	−0.03813	−0.84	0.425
D Glucose (g/l)	10	20	−0.15438	−3.38	0.008**
E Temperature (°C)	28	33	−0.00812	−0.18	0.863
F pH	4	6	0.01563	0.34	0.740

** Represents significance at 0.01 level.