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

Wang Hailei [a,*], Ren Zhifang [a], Li Ping [a], Gu Yanchang [a], Liu Guosheng [a], Yao Jianming [b]

[a] College of Life Sciences, Henan Normal University, Xinxiang 453007, China
[b] Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, Hefei 230031, China

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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

* Corresponding author. Address: Jianshe Road 46#, Xinxiang, China. Tel.: +86 3733326759; fax: +86 3733326916.
E-mail addresses: whl@htu.cn, whl@henannu.edu.cn (W. Hailei).

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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.

where Y is the predicted response; X_1 and X_2 are the code forms of the input variables; b_0 is a constant; b_1 and b_2 are the linear coefficients; b_{11} and b_{22} are the quadratic coefficients; b_{12} is the cross-product coefficient.

2.8. Red pigment production in 15 l bioreactor

Half a milliliter of conidium and cell suspensions of the two fungi were separately inoculated into 200 ml of PDB in 500 ml flasks and grown for 3 days at 30 °C in a rotary shaker at 150 rpm. Two-hundred milliliter of mycelial suspension (2 g dry mycelia/100 ml of suspension) of strain HSD07B and 200 ml of yeast suspension (2 g dry cells/100 ml of suspension) were added to in the 15 l fermenter (Shanghai Gaoji Bioengineering Co. Ltd., China) containing 10 l of PDB. The temperature of the bioreactor was maintained at 29 ± 1 °C. Dissolved oxygen (DO), monitored by an on-line DO surveymeter, was maintained at 6.2 mg/l during 0–6 h, and reduced to 3.9 mg/l starting at 7 h by adjusting the aeration and rotary speed of stirrer.

2.9. Analytical methods

Morphological characteristics of strain HSD07B were viewed with bio-microscope. CV of pigment solution was defined as the optical density value and determined at the maximum absorption wavelength of water solution of the red pigment, 505 nm, and the concentration of red pigment was calculated according to the regression equation:

$$y = 1.4491x + 0.0130\,(R^2 = 0.9998)$$

where y is the concentration of red pigment (g/l); x is CV of pigment solution at 505 nm and pH 7.

RR of the red pigment was defined as the ratio of CV after treating (CV_a) to that before treating (CV_b), and it was calculated as follows:

$$RR\,(\%) = \frac{CV_a}{CV_b} \times 100$$

Minitab 15 (Minitab Inc., USA) was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. The quality of the fit of the polynomial model was expressed by the determination coefficient R^2, and its statistical significance was validated by an F-test; the significance of the regression coefficients was tested by a t-test.

3. Results and discussion

3.1. Isolation and identification of microorganism

The phenomenon of red pigment production in a mixed culture was discovered when a red pigment appeared in a culture of *C. tropicalis* contaminated with another fungus. This contaminant, designated HSD07B, was isolated and characterized. The strain grew well at 37 °C, but hardly at 5 °C. After 7-day culture at 25 °C, the diameters of colonies on 25% Glycerol Nitrate Agar, Malt Extract Agar and Czapek-Dox Agar were 32–35, 30–38 and 15–22 mm, respectively. Strain HSD07B produced white and floccose mycelia. The conidia were olivaceous or gray olivaceous and a clear exudate appeared on day 3. The penicillus of strain HSD07B was a biverticillate symmetric branching system and at the top of the branches, catenulate conidiophores occurred. The presence of a broom-like penicillus is a fundamental character of some species of *Penicillium* (Raper and Thom, 1949). Based on the analysis of a 586-bp sequence from the 26S rRNA gene (Fig. 1), strain HSD07B is related to *Penicillium marneffei* (AB363759), *Penicillium purpurogenum* (EF087978), *Penicillium aculeatum* (PAU15467) and *Penicillium funiculosum* (HM017065).

3.2. Red pigment production

When *C. tropicalis* and *Penicillium* sp. HSDO7B were separately cultured in liquid medium, no red pigment production was observed even after 10 days of cultivation. When the two fungi were cultured together, red pigment started to appear after 16 h and 0.8 g/l of red pigment was produced after 24 h of cultivation. On solid media, red pigment production also only occurred when both strains were present simultaneously (Supplemental material 1).

3.3. Components and characteristics of red pigment

The HPLC analysis showed that the red pigment produced by co-culture was a mixture of six components (Supplemental material 2), and TLC (Fig. 2) indicated that RP1-4 were the main substances of the pigment. The red pigment was compared with some other red pigments described in literature (Britton, 1995; Lian et al., 2007), and it differed from fat-soluble β-Carotene, lycopene and astaxanthin in consideration of its water-solubility. Twelve components existed in the ethanol solution of *Monascus* pigment according to the analysis of HPLC, and the retention times

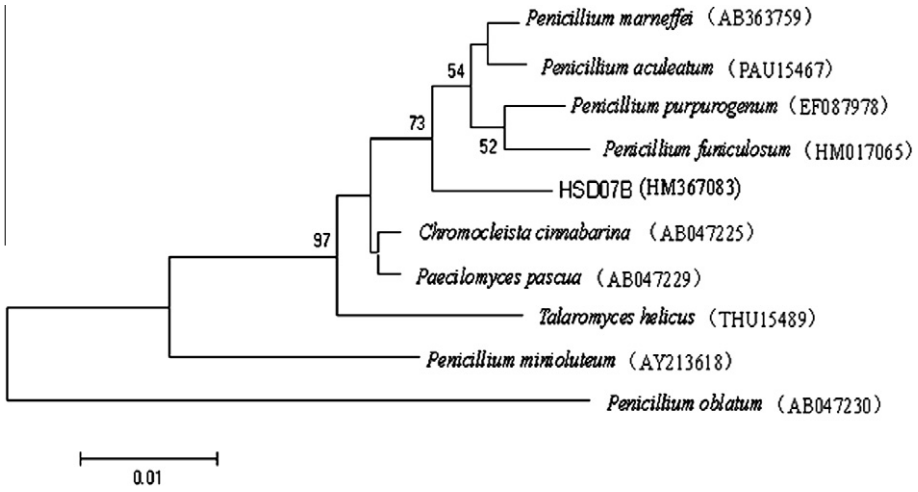

Fig. 1. Neighbor-joining analysis tree of strain HSD07B 26S rRNA gene sequence. *Confidence values above 50% obtained from 1000-replicate bootstrap are indicated at the branch nodes. The scale bar indicates the number of base substitutions per site.

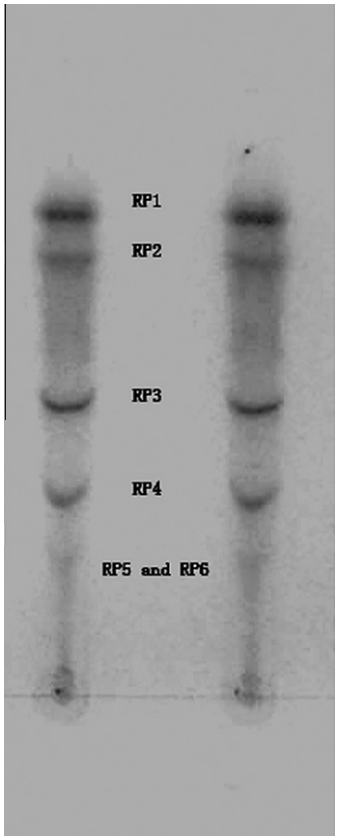

Fig. 2. TLC analysis of the components of the red pigment produced by co-culture.

of five water-soluble components (2.34, 2.49, 3.08, 4.69 and 6.53 min) in the aqueous solution were different from those of RP1-6 (1.82, 2.18, 2.77, 3.60, 4.50 and 5.15 min) in both ethanol and aqueous solution under the same operating conditions, indicating the components of the two pigments were dissimilar in the number and molecular structure. In addition, molecular mass of RP1 (Supplemental material 3), 436.2, was distinct from that of red pigment reported before such as 12-carboxyl-monascorubramine, monascorubrin and helminthosporin (Mapari et al., 2009, 2010), suggesting that the co-culture pigment probably contained the novel natural compound.

The red pigment was soluble in water, ethanol, acetic acid, acetone, glycerol, 1-butanol and methanol, but insoluble in ether, chloroform and non-polar solvents such as petroleum ether and *n*-hexane. The pH had no significant effect on the color of the pigment; however, maximum CV was observed at pH 7 (Table 2). The pigment was stable at 10–100 °C and thus did not show the thermal lability of some other natural pigments (Cai and Corke, 2000; Fatahi et al., 2009).

As shown in Table 2, most metal ions did not influence the CV of the pigment. Cu^{2+} reduced the color of the pigment solution by 18.2% after 3 days. In the presence of the food preservative, sodium benzoate, the RR of the red pigment on day 3 was still 97.4%. Although some natural pigments are oxidized or reduced by chemical oxidizing and reducing agents (Zhang et al., 2006), no significant effect of H_2O_2 and Na_2SO_3 on the CV of red pigment was observed .

The RR of the pigment solution in the dark did not change even after 30 days; however, there was a slight decline of RR when the pigment solution was exposed to indoor natural light for 7 days and by day 15, about 20% of CV had been lost. Under ultraviolet light, a 10.1% and 16.7% reduction in the CVs was observed after 1.5 and 3 h, respectively.

Table 2
Effects of pH value, temperature and reagents on the color value of pigment solution.

pH value, temperature and reagents	CVb	CVa	RR (%)
pH 2	0.77	0.74	96.1
pH 3	0.77	0.74	96.1
pH 5	0.77	0.76	98.7
pH 7	0.77	0.78	101.3
pH 9	0.77	0.76	98.7
pH 10	0.77	0.74	96.1
10 °C	0.77	0.76	98.7
30 °C	0.77	0.77	100.0
50 °C	0.77	0.77	100.0
70 °C	0.77	0.75	97.4
90 °C	0.77	0.73	94.8
100 °C	0.77	0.73	94.8
Sucrose (0.1 M)	0.77	0.68	88.3
Glucose (0.1 M)	0.77	0.67	87.0
$MgSO_4$ (0.05 M)	0.77	0.69	89.6
$CuSO_4$ (0.05 M)	0.77	0.63	81.8
$CaCl_2$ (0.05 M)	0.77	0.67	87.0
NaCl (0.05 M)	0.77	0.67	87.0
KC1 (0.05 M)	0.77	0.67	87.0
$MnSO_4$ (0.05 M)	0.77	0.69	89.6
Sodium benzoate (0.1 M)	0.77	0.75	97.4
H_2O_2 (0.01 M)	0.77	0.65	84.4
Na_2SO_3 (0.01 M)	0.77	0.68	88.3

3.4. Mouse acute toxicity and mutagenicity tests

The main objective of an acute toxicity trial is to measure the half lethal dose of the tested substance (Lorke, 1983). In this test, the mice were observed for 2 weeks after orally taking the pigment. During this period, no mortalities were recorded. Therefore, the pigment can be classified as a nontoxic substance according to the general toxicity standard (GB15193.13, 2003). Furthermore, the results of Ames assay are negative, suggesting that the pigment is not mutagenic (Table 3). These data suggest that the red pigment could be potentially acceptable for food applications, although more thorough testing will be required.

3.5. Fractional factorial experimental design and response surface analysis

Table 4 shows the design and results of the FFD experiment, and statistical analysis indicated that glucose and IP were the important factors affecting the yield of red pigment. A *t*-test was conducted and the *p*-values (0.000 for IP and 0.008 for glucose) of glucose and IP were less than 0.01, suggesting that they were statistically significant at the 0.01 level (Table 1). The two significant

Table 3
Colony number of revertants in the Ames test.

Group	Average number of revertants/plate							
	TA97		TA93		TA100		TAI02	
	S-9(−)	S-9(+)	S-9(−)	S-9(+)	S-9(−)	S-9(+)	S-9(−)	S-9(+)
SRG	102	110	40	47	106	117	202	205
10 μg	100	99	33	42	105	108	198	203
50 μg	113	111	35	39	112	120	203	210
100 μg	120	115	41	38	115	119	212	215
200 μg	117	119	45	48	107	114	200	198
500 μg	115	109	42	36	121	118	215	220
PCG	–	922*	–	1983*	2980#	1596*	–	250*

SRG: spontaneous reversion group; PCG: positive control group.
S-9(+): with microsomal activation; S-9(−): without microsomal activation.
* 2-AF (10.0 μg/plate).
# NaN_3 (1.5 μg/plate).

Table 4
Experiment design and results of fractional factorial experiment.

Trial no.	A	B	C	D	E	F	Red pigment (g/l)
1	1	−1	−1	1	1	1	0.45
2	1	1	1	−1	−1	−1	1.70
3	−1	−1	1	1	−1	−1	1.30
4	−1	−1	−1	−1	1	−1	0.53
5	−1	−1	1	1	1	−1	1.68
6	1	1	−1	−1	1	1	0.65
7	−1	−1	−1	−1	−1	−1	0.74
8	1	1	1	1	1	1	1.25
9	−1	−1	1	−1	1	1	1.87
10	−1	−1	1	−1	−1	1	1.28
11	1	1	1	−1	1	−1	0.98
12	1	1	−1	−1	−1	1	0.72
13	1	1	1	1	−1	1	1.54
14	1	1	−1	1	1	−1	0.69
15	1	1	−1	1	−1	−1	0.42
16	−1	−1	−1	1	−1	1	0.53

Table 5
The variables and the experimental results of CCD.

Trial no.	X_1		X_2		Red pigment (g/l)
	Code value	Real value (g/l)	Code value	Real value	
1	−1	10	1	1.5	1.10
2	0	15	0	1	2.70
3	1	20	−1	0.5	0.43
4	0	15	0	1	2.66
5	1.414	22	0	1	0.41
6	0	15	0	1	2.75
7	0	15	0	1	2.87
8	0	15	−1.414	0.3	0.15
9	−1.414	8	0	1	1.04
10	1	20	1	1.5	0.75
11	0	15	0	1	2.78
12	0	15	1.414	1.7	0.66
13	−1	10	−1	0.3	0.22

factors identified by FFD were considered for parameter optimization in the next stage.

Response surface analysis methodology is used extensively in optimizing the performance and improving the design of many products (Li and Jia, 2008; Chen et al., 2010), and in the current study, the most popular class of second order design, CCD, was used for the response surface analysis. The independent variables, X_1 (glucose) and X_2 (IP), were coded according to the following equations:

$$X_1 = \frac{Z_1 - 15}{5};\ X_2 = \frac{Z_2 - 1}{0.5}$$

Z_i (i = 1 and 2) is the actual value for X_i (i = 1 and 2).

The variables and the experimental results of CCD are shown in Table 5. After data processing using Minitab 15 software, the quadratic polynomial was regressed and the function equation of Y (red pigment) could be expressed as follows:

$$Y = 2.752 - 0.123X_1 + 0.234X_2 - 0.996X_1^2 - 0.153X_1X_2 - 1.16X_2^2 \quad (1)$$

Table 6
Estimated regression coefficients for Eq. (1).

Model	b_0	b_1	b_2	b_{11}	b_{12}	b_{22}
Coefficient	2.752	−0.123	0.234	−0.996	−0.153	−1.156
SE-Coe.	0.063	0.050	0.050	0.054	0.071	0.054
t-value	43.55	−2.45	4.68	−18.58	−2.16	−21.57
p-value	0.000**	0.044*	0.002**	0.000**	0.068	0.000**

* Represents significance at 0.05 level.
** Represents significance at 0.01 level.

Table 6 shows the significance analysis of regression term for the quadratic response surface model. The linear and quadratic terms of the model are highly significant with p-values less than 0.05, but the interaction term is not significant (p-value, 0.068), indicating the selected factors are critical to the production of pigment but independent of each other.

The ANOVA of the quadratic regression model demonstrates the model is significant, and the low p-value demonstrates the model has a high correlation with the experimental data. The goodness of the fit of the model was checked by determination coefficient (R^2). In this case, R^2 of the quadratic response surface model, 0.991, proved that the model fitted the experimental results well. The fitted quadratic polynomial equation was expressed as 3-D response surface diagram and contour plot (Fig. 3) in order to visualize the relationship between the yield of red pigment, glucose and IP. Optimum parameters can be obtained by solving the second order polynomial regression Eq. (1). The corresponding maximum yield of pigment (2.76 g/l) is obtained when the coded values of glucose and IP are −0.14 and 0.11, respectively. Under the optimal conditions, confirmatory experiments were carried out and the experiment value of pigment yield, 2.75 ± 0.31 g/l (n = 6), was in

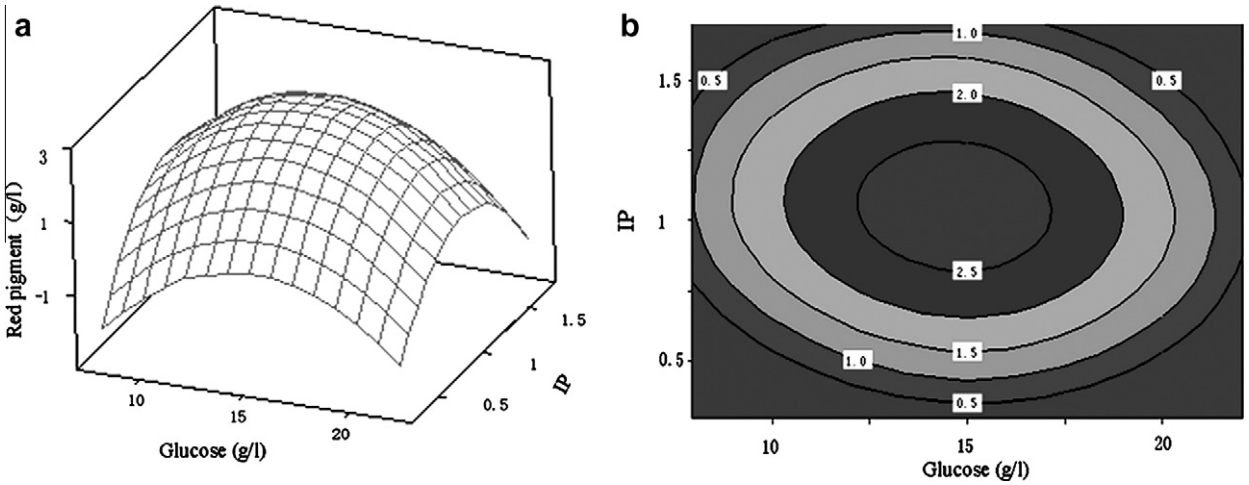

Fig. 3. 3-D response surface diagram and contour plot to visualize the relationship between red pigment, glucose and inoculum proportion. (a) 3-D response surface diagram; (b) contour plot.

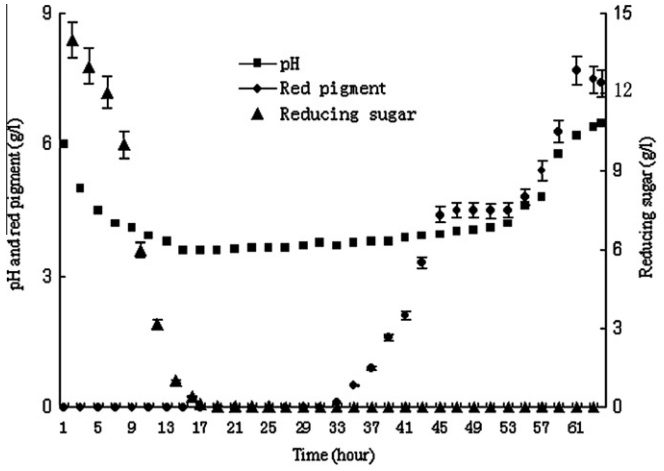

Fig. 4. Time courses of pH value, red pigment and reducing sugar in the 15 l bioreactor.

agreement with the predicted value, and this indicated that the model could predict the production of pigment accurately. Furthermore, it also proved that parameter optimization using statistically based experimental designs was effective, and the yield of pigment was increased 3.4 times in comparison with that obtained under the initial liquid fermentation conditions.

3.6. Production of red pigment in 15 l bioreactor

Changes in pH and red pigment and reducing sugar concentrations in the 15 l bioreactor are illustrated in Fig. 4. Reducing sugar decreased quickly and reached 0.03 g/l after 16 h. The pH decreased during the early phase of fermentation (1–16 h), suggesting that some organic acids were produced. Red pigment appeared in the bioreactor after 33 h, and the pigment concentration reached 7.70 g/l after 62 h, which was about 2.8 times higher than that obtained in the shake-flask.

4. Conclusions

In co-culture, *Pencillium* sp. HSD07B and *C. tropicalis* produced a stable and apparently non-toxic red pigment, and after culture optimization, a pigment yield of 2.75 and 7.7 g/l was obtained in a shake-flask and a 15 l bioreactor, respectively. These observations suggest that the production of a natural red pigment by the co-culture is feasible; however, further toxicological studies will need to be performed, and the molecular structure of the compounds in the pigment will have to be determined before applications in the food industry can be considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.01.040.

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