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ORIGINAL ARTICLE

Production of astaxanthin by a mutant strain of *Phaffia rhodozyma* and optimization of culture conditions using response surface methodology

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Abstract To improve astaxanthin production in yeast, *Phaffia rhodozyma* 2.1557 was subjected to mutagenesis. A genetically stable mutant, YZUXHONG686, was isolated through the screening of combined carotenes biosynthesis inhibitors, and demonstrated a high yield of astaxanthin. Sequential methodology was employed to optimize the astaxanthin production of mutant stain YZUXHONG686 and minimize the cost of production. A screening test was first conducted using a Plackett-Burman design and variables with statistically significant effects on astaxanthin biosynthesis were identified. These significant factors were optimized by central composite designed experiments and response surface methodology. Finally, an initial value of pH 5.09 as well as sucrose and dried corn steep liquor powder concentrations of 49.32 g/L and 4.77 g/L, respectively, were found to give an optimal final carotenes concentration of 53.68±1.72 mg/L. The astaxanthin yield was 2.56±0.68 mg/g dry biomass under the optimized conditions.

Keywords Astaxanthin · Carotene · *Phaffia rhodozyma* · Plackett-Burman design · Response surface methodology

Introduction

Astaxanthin (3, 3′-dihydroxy-β, β′-carotene-4, 4′-dione) is an abundant carotene pigment of great interest to animal nutrition and human health. Traditionally, astaxanthin is used as a source of pigment for cultured fish and shellfish (Nakano et al. 1995). It can be produced by chemical or biotechnological methods. However, astaxanthin from natural sources is preferable to the synthetic material because of increasingly strict safety regulations concerning the use of chemicals as food additives, and the poor absorption of the synthetic form compared with forms obtained from biological sources. Biotechnological production of astaxanthin from the red yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) or the microalga *Haematococcus pluvialis* has advantages over its chemical synthesis or extraction from crustaceans (Andrewes et al. 1976; Johnson 2003; Sandesh Kamath et al. 2008; Rodríguez-Sáiz et al. 2010; Schmidt et al. 2011). *P. rhodozyma* is an excellent astaxanthin-producing yeast and a promising producer of dietary astaxanthin by fermentation process. However, commercial *P. rhodozyma* fermentation for astaxanthin production remains hampered by relatively low productivity, compared with other chemical and biological processes. Many approaches have been employed for strain improvement, but strain instability and the tendency of mutants to produce less astaxanthin are significant problems (Schmidt et al. 2011). Therefore, the development of yeast strains that are rich in astaxanthin would be of significant commercial value.

To develop efficient fermentation conditions, certain parameters should be optimized to reduce their impact on the economy and practicability of the process. Carbon, nitrogen and phosphorus have been identified as the major nutrients, and medium pH and culture temperature as the major conditions that affect astaxanthin production by *P. rhodozyma.* Achieving optimal fermentation conditions for production is

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crucial to the industrialization of astaxanthin production, while reducing the fermentation costs would also facilitate industrial application. Because astaxanthin production is dependent on many culture factors, the use of statistical design methods is more effective and reliable than the one-factor-at-a-time approach to identify and optimize major factors (Johnson and Lewis 1979; Fang and Cheng 1993; Meyer and du Preez 1994; Parajó et al. 1998; Ramírez et al. 2001; Liu and Wu 2007; Ni et al. 2007; de la Fuente et al. 2010; Ning et al. 2012; Bhatt et al. 2013).

In this study, *P. rhodozyma* CGMCC 2.1557 was selected as the original strain of astaxanthin production. After mutagenesis of the strain by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and gamma irradiation, an astaxanthin-hyperproducing mutant resistant to three metabolic inhibitors was obtained and named YZUXHONG686. Furthermore, the optimal conditions for the mutant YZUXHONG686 to achieve maximum astaxanthin accumulation were established using Plackett-Burman design, central composite design (CCD) and response surface methodology (RSM).

Materials and methods

Chemicals

Astaxanthin (>98 %), lithium chloride (LiCl, >99 %), and corn steep liquor (CSL, 40 % solids content) were purchased from Sigma-Aldrich (St. Louis, MO). 2-Deoxy-D-glucose and β-ionone were purchased from Fluka (Buchs, Switzerland). NTG was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Yeast extract (YE) was purchased from Oxoid (Basingstoke UK) (total nitrogen 10.0–12.5 %; amino nitrogen 5.1 %), and YE of industrial grade used in experimental designs was from Angel Yeast (Yichang, China) (total nitrogen 10.9 %, amino nitrogen 5.0 %). Peptone, yeast juice, beef extract and other chemicals of analytical grade were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Methanol and acetonitrile of HPLC grade were used for determination of astaxanthin. Dried corn steep liquor power (DCSLP) of industrial grade was purchased from Henghui Starch Sugar Co. (Xuzhou, China).

Strains and growth conditions

Phaffia rhodozyma CGMCC 2.1557 obtained from Institute of Microbiology of Chinese Academy of Sciences was used as the parental strain. The medium for routine liquid culture was composed of $(NH_4)_2SO_4$ 3.0 g, $MgSO_4 \cdot 7H_2O$ 1.5 g, KH_2PO_4 1.5 g, glucose 35 g, YE 1.5 g, and CSL 10 g (per liter), with the pH adjusted to 5.25. Agar was added at 2 % when needed. The liquid culture was maintained in 250 mL shake-flasks, each filled with 30 mL medium, on a rotary shaker at 170 rpm and 23 °C. Each of the flasks was inoculated with 10 % starter culture broth, prepared by preculture of the yeast in shake-flask cultures for 48 h. The medium pH value was not adjusted during the process, and yeasts were grown for different numbers of days depending on the experiment.

Mutagenesis

UV mutagenesis was carried out using a UV lamp (254 nm) at a power of 30 W, which was switched on and allowed to preheat for 30 min before use. Cells of *P. rhodozyma* were washed twice in 0.9 % (w/v) NaCl and resuspended in the same solution. Strain suspension (6 mL; about 10^7 cells/mL) was transferred to a plate and exposed for 3 min (93 % kill rate). A distance of 20 cm was maintained between the plate and the UV light. Cells were grown for 18 h in the dark and then plated on medium with LiCl.

NTG mutagenesis was performed by washing freshly grown cells in 100 mM sodium citrate buffer (pH 5.5) and resuspending in the same buffer. NTG was then added to the suspension at final concentrations ranging from 1.14 to 2.84 mM (83 to 99.9 % kill rate, respectively), and the cells were incubated for 30 min at 30 °C, then washed twice with sodium citrate buffer. Surviving cells were inoculated into a liquid medium for overnight growth before being plated on selective media.

Gamma irradiation treatment was used to isolate mutants according to the method of Sun et al. (2004). Cells were harvested and suspended in 0.9 % (w/v) NaCl and washed twice. The suspension (OD_{660nm} reaching 2.0–2.5) was transferred to sterile tubes and irradiated at room temperature using a ^{60}Co gamma ray irradiator, with dosages of 4.25–5 kGy (90–99 % kill rate). Irradiated cells were grown overnight and then plated on selective media.

Isolation of resistant mutants

After mutagenization, the surviving cells were spread on dishes with selective medium. Selective medium was composed of basal medium agar plus one metabolic inhibitor such as β-ionone, diphenylamine (DPA) or 2-deoxy-D-glucose. Pinkish colonies of the parental strain became white when grown in the presence of some metabolic inhibitors, while the mutants maintained their color in the presence of the same metabolic inhibitors, allowing ready screening of the mutants. After 7–10 days of incubation at 25 °C, colonies appearing with deep-red color were transferred onto slants and subcultured weekly at 25 °C until mutants were found to be stable and evaluated in shake-flasks. The mutant generation procedure was shown in Fig. 1.

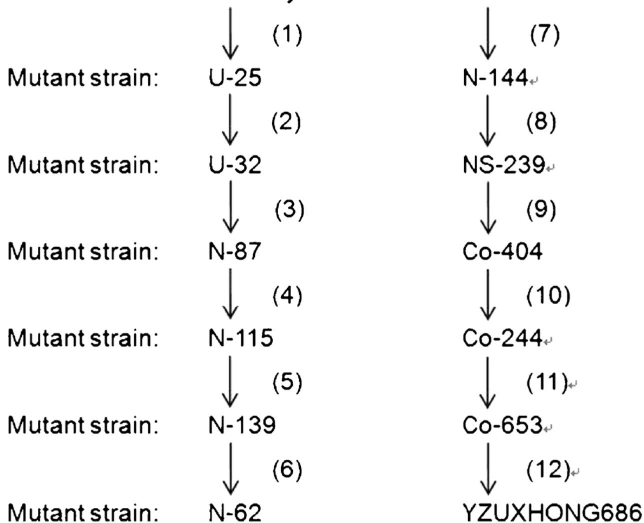

Fig. 1 Strategies employed to obtain high-yielding mutant strains of *Phaffia rhodozyma*. (*1*) UV for 3 min+47 mM LiCl; (*2*) UV for 3 min+71 mM LiCl; (*3*)1.70 mM NTG+2.60 mM β-ionone; (*4*) 2.27 mM NTG+2.60 mM β-ionone; (*5*) 2.84 mM NTG+3.12 mM β-ionone; (*6*) 1.70 mM NTG+4.87 mM 2-deoxy-D-glucose; (*7*) 1.14 mM NTG+71 μM DPA; (*8*) natural isolation; (*9*) 4.25 kGy γ-ray+3.12 mM β-ionone; (*10*) 4.75 kGy γ-ray+6.09 mM 2-deoxy-D-glucose; (*11*) 4.75 kGy γ-ray+83 μM DPA; (*12*) 5 kGy γ-ray+95 μM DPA

Carotene extraction and analysis

Cell growth was determined by drying biomass at 105 °C until constant weight, and expressed as grams per liter. The modified dimethylsulfoxide (DMSO) method of Sedmak et al. (1990) was used to analyze carotene production. Briefly, 1 mL yeast culture was sampled and centrifuged, and the supernatant was removed. Then DMSO, acetone, 20 % (w/v) NaCl and petroleum ether were added serially to the pellet and vortexed for 5 min. The upper petroleum ether layer containing carotenes was separated by centrifugation for 2 min. The absorbance of the final sample solutions was measured at 474 nm using a UV/visible spectrophotometer (Amersham Biosciences, Ultrospec 6300 pro, Little Chalfont , UK). Yield of carotenes was calculated using the 1 % extinction coefficient=1,600 and the following formula (dry biomass was replaced by 1 mL yeast culture for carotenes concentration measurement, mg/L):

$$\text{Carotenes}(\text{mg/g dry biomass}) = \frac{(mL\ of\ petrol) \times A_{474} \times 100}{16 \times (dry\ biomass)} \quad (1)$$

Analysis of astaxanthin content was carried out by reversed phase HPLC (Agilent 1200) with a diode array detector and a 6460Triple Quad LC/MS system. The sample solution was injected through a 20 μL loop and separated on a DIONEX C_{18} column (5 μm, 4.6 mm×150 mm). Isocratic elution was performed with methanol-acetonitrile (90:10, v/v) as the mobile phase, at a flow rate of 1.0 mL/min, and the detection wavelength was 474 nm (Rubinstein et al. 1998). DMSO was added to 20 mg samples of lyophilized cells. Acetone, 20 % (w/v) NaCl and petroleum ether were added serially with vortexing. The extracts were condensed with a vacuum rotary evaporator and diluted with mobile phase.

Single factor design experiments

When CSL, YE and $(NH_4)_2SO_4$ were used as nitrogen sources, a mutant obtained via mutagenesis was grown in a basic medium (g/L) [CSL 10, YE 1.5, $(NH_4)_2SO_4$ 3.0, $MgSO_4 \cdot 7H_2O$ 1.5, KH_2PO_4 1.5] that was supplemented with various carbon sources including arabinose, glucose, sucrose, mannitol, fructose, sorbitol, maltose, glycerin and ethyl alcohol at an equimolar carbon level equivalent to 0.4. When sucrose was employed as a carbon source, the mutant was grown in a basal medium (g/L) (sucrose 33.3, $MgSO_4 \cdot 7H_2O$ 1.5, KH_2PO_4 1.5) that was supplemented with various organic nitrogen sources. CSL, YE, dried yeast powder, beef extract, yeast juice, peptone and DCSLP were used individually at a nitrogen concentration equivalent to 0.45 %. After optimal carbon and organic nitrogen sources were chosen, the mutant was grown in a basal medium (g/L) (sucrose 33.3, $MgSO_4 \cdot 7H_2O$ 1.5, KH_2PO_4 1.5, DCSLP 6.5) that was supplemented with various inorganic nitrogen sources. NH_4Cl, urea, KNO_3, NH_4NO_3, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$ were used individually at an equimolar nitrogen level equivalent to 0.21. Yeasts were grown in 30 mL culture medium at 23 °C for 8 days. For all analyses, samples were processed in duplicate or triplicate for each experiment, and averages are presented.

Statistical experimental design and data analysis

An initial screening test was conducted on all six components of the basal medium and culture conditions using a Plackett-Burman experimental design. Each variable was tested at two levels, namely a low level denoted by (−1) and a high level denoted by (1) as listed in Table 1. Therefore, a total of 12 runs were required for the eight test variables, plus three dummy variables (C, F, I). The actual values of variables were temperature (A, 20 and 25 °C), initial pH (B, 5 and 6), $(NH_4)_2SO_4$ (D, 3 and 4 g/L), KH_2PO_4 (E, 1.5 and 2 g/L), $MgSO_4 \cdot 7H_2O$ (G, 1.5 and 2 g/L), sucrose (H, 33 and 40 g/L), YE (J, 1.5 and 2 g/L, Angel Yeast) and DCSLP (K, 4 and 6 g/L). Based on the results of the Plackett-Burman design, CCD (for the experiments) and RSM (for identifying the optimal levels) were performed in order to determine the optimum conditions for carotene production. The effects of three factors (initial pH, sucrose and DCSLP) on the production of carotenes were studied at five experimental levels (−1.6818, −1, 0, 1, 1.6818) in a total of 20 runs (Table 2). Experimental results were fitted to a predictive quadratic

Table 1 Plackett-Burman design for screening of process variables

Run	A	B	C	D	E	F	G	H	I	J	K	Carotenes concentration (mg/L)
1	1	−1	1	−1	−1	−1	1	1	1	−1	1	32.40±0.37
2	1	1	−1	1	−1	−1	−1	1	1	1	−1	30.48±0.72
3	−1	1	1	−1	1	−1	−1	−1	1	1	1	34.88±0.30
4	1	−1	1	1	−1	1	−1	−1	−1	1	1	28.35±1.16
5	1	1	−1	1	1	−1	1	−1	−1	−1	1	29.98±0.98
6	1	1	1	−1	1	1	−1	1	−1	−1	−1	32.34±0.22
7	−1	1	1	1	−1	1	1	−1	1	−1	−1	41.06±0.56
8	−1	−1	1	1	1	−1	1	1	−1	1	−1	44.71±1.04
9	−1	−1	−1	1	1	1	−1	1	1	−1	1	44.37±0.99
10	1	−1	−1	−1	1	1	1	−1	1	1	−1	33.54±0.65
11	−1	1	−1	−1	−1	1	1	1	−1	1	1	38.96±1.76
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	42.05±0.35

polynomial equation as the correlation between the response variable and the independent variables:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

where Y is the predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients; β_{11}, β_{22}, β_{33}, quadratic coefficients; and β_{12}, β_{13}, β_{23} are interactive coefficients. Analysis for variance (ANOVA) and the model coefficients were computed with SAS9.1 software, and two-dimensional contour plots were constructed for visual observation of the maximum response trend using the MATLAB R2007b software package. Because astaxanthin synthesis was only partially associated with biomass growth and still occurred even after growth stopped and sugars were exhausted (Domínguez-Bocanegra and Torres-Muñoz 2004), the objective of statistical experimental design was to optimize culture conditions for the carotene production and not necessarily for cell growth. We measured and presented only those carotenes concentrations that are equivalent or proportional to the astaxanthin yield according to the method of Liu and Wu (2007), and all runs were performed in triplicate or quintuplicate.

Table 2 Experimental variables and levels in central composite design (CCD)

Run	Factor			Y (mg/L)	
	X_1	X_2 (g/L)	X_3 (g/L)	Observed value	Predicted value
1	4.7	47.0	4.0	49.62±1.74	49.23
2	4.7	47.0	6.0	48.85±1.84	47.95
3	4.7	53.0	4.0	51.63±1.07	50.23
4	4.7	53.0	6.0	48.34±0.27	47.83
5	5.3	47.0	4.0	53.79±1.47	54.41
6	5.3	47.0	6.0	51.09±0.28	52.61
7	5.3	53.0	4.0	49.56±1.49	50.57
8	5.3	53.0	6.0	47.17±0.55	47.65
9	4.5	50.0	5.0	46.72±2.31	48.69
10	5.5	50.0	5.0	54.98±2.20	52.89
11	5.0	45.0	5.0	52.55±0.86	52.12
12	5.0	55.0	5.0	48.46±1.27	48.79
13	5.0	50.0	3.3	51.65±1.43	51.82
14	5.0	50.0	6.7	48.58±1.75	48.29
15	5.0	50.0	5.0	54.33±0.69	56.59
16	5.0	50.0	5.0	58.09±1.57	56.59
17	5.0	50.0	5.0	56.87±0.57	56.59
18	5.0	50.0	5.0	56.62±0.83	56.59
19	5.0	50.0	5.0	57.75±1.29	56.59
20	5.0	50.0	5.0	55.84±1.15	56.59

Results and discussion

Mutagenesis and isolation of astaxanthin-hyperproducing mutants

The parental strain showed lower astaxanthin production after 5 days of growth. Attempts to isolate the highly pigmented variants were not successful after screening several thousand colonies following UV exposure. This observation is in agreement with the findings of An et al. (1989). By extensive NTG mutagenesis and screening on plates containing three metabolic inhibitors, the astaxanthin content of a mutant (NS-239) reached 1.13±0.15 mg/g (Table 3). During the course of the screening, more than 800 resistant colonies could be picked in the sixth mutation cycle. In order to reduce the work involved in the flask evaluation stage and eliminate unstable mutants, resistant colonies were transferred to slants without inhibitor and sub-cultured weekly. Most of resistant mutants lost their deep-red color after about six subcultures. Through irradiation

Table 3 Evaluation of mutants derived from *P. rhodozyma* 2.1557 during successive mutations. Values followed by the same lower case letter differ significantly

Strain	Dry biomass (g/L)	Carotenes concentration (mg/L)	Carotenes mg/g dry biomass	Astaxanthin content (mg/g)
Parental strain	4.92±0.29 f	1.54±0.05 g	0.32±0.02 f	0.13±0.01 g
U-25	6.25±0.25 e	3.70±0.13 f	0.59±0.04 ef	0.25±0.05 f
U-32	7.58±0.14 d	5.98±0.06 e	0.79±0.01 e	0.35±0.04 e
N-87	8.08±0.38 d	15.57±0.33 d	1.93±0.12 d	0.87±0.06 d
NS-239	8.83±0.14 c	20.53±0.71 c	2.33±0.10 c	1.13±0.15 c
Co-404	12.25±0.50 a	32.83±1.04 b	2.69±0.19 b	1.33±0.03 b
YZUXHONG686	10.25±0.43 b	41.13±1.63 a	4.02±0.34 a	2.24±0.13 a

with a dosage of 5 kGy on Co-653, a colony with a deep-red pigment named *P. rhodozyma* YZUXHONG686 (CCTCC: No. M208262; Patent No. ZL200910029952.5, State Intellectual Property Office, PR China), was finally selected for astaxanthin production. The mutant produced a remarkably high astaxanthin yield (2.24±0.13 mg/g dry biomass), about 17.23 times higher than that of the parent strain *P. rhodozyma* 2.1557 (Table 3). But cell yields of the higher astaxanthin-containing mutants decreased with increasing astaxanthin content. Such a result was also observed by Meyer et al. (1993) and might indicate that carotene production retards cell growth.

Strain improvement has been achieved conventionally through random mutation and selection methods that might cause desired physiological changes in cellular metabolism. Random mutation methods require no prior knowledge of the molecular biology and physiology of the microorganisms being manipulated (Rubinstein et al. 1998). However, random screening requires time-consuming procedures to identify superior isolates among a mutagen-treated population. Selection of biochemical mutants resistant to a toxic analogue of the desired metabolite is one of the most common rational screening approaches (Liu et al. 2003). There have been many studies on strain improvement of *P. rhodozyma* using antimycin A, NTG, UV, β-ionone, ^{60}Co, DPA, H_2O_2, 2-deoxy-D-glucose, and duroquinone (An et al. 1989; Lewis et al. 1990; Meyer et al. 1993; Schroeder and Johnson 1993; Chumpolkulwong et al. 1997; Bon et al. 1997; Sun et al. 2004). A genetically stable mutant with high yield of astaxanthin (2.51 mg/g) was isolated using low-energy ion beam implantation (Liu et al. 2008). Although An et al. (1989) reported that UV light yielded no highly pigmented mutants, LiCl as a co-mutagen has co-operative effect with UV treatment according to conventional mutagenesis experience. But most of the UV-generated mutants were pale and the few small and deep-red colonies obtained on LiCl selective medium lost their color after being subcultured once to a fresh slant. NTG is a mutagenic agent that induces point mutations in a DNA molecule by a G–C transition to A–T. In the presence of NTG, native sequences of affected genes are changed and their related products are modified structurally, causing inactivation of functional proteins. Gamma irradiation produces oxygen radicals and could induce mutation of microorganisms through a chromosomal rearrangement (Sun et al. 2004). In a preliminary screening experiment to find deeper red colonies as higher astaxanthin producers, over a dozen enhanced astaxanthin producers were obtained after NTG and ^{60}Co mutagenic treatment. However, purified strains still often reverted at high frequency, and we tried to develop selection procedures for astaxanthin overproduction. Some of the resistant mutants, which could be expected to have carotenogenic enzymes altered in quality and/or quantity, could possibly result in improved astaxanthin yield. Several of the inhibitors tested, including 2-deoxy-D-glucose, β-ionone, and DPA, changed the color of colonies distinctly, indicating a change in carotene composition. Because excessive levels of glucose and semi-anaerobic conditions decreased carotenes formation markedly, the nonmetabolizable D-glucose analogue 2-deoxy-D-glucose can inhibit the growth of microorganisms and modulate enzyme synthesis, resistance to it has been used as a criterion to select mutants showing increased yield of astaxanthin (An et al. 1989; Liu et al. 2003). In *P. rhodozyma*, carotenes arise via the mevalonate pathway, where astaxanthin biosynthesis pathway is: acetyl-CoA→mavalonate→geranylgeranyl pyrophosphate→phytoene→lycopene→β-carotene→echinenone→3-hydroxyechinenone→phoenicoxanthin→astaxanthin (Schmidt et al. 2011). DPA was used to inhibit astaxanthin biosynthesis, but not cell growth, resulting in white colony formation. DPA is likely to block astaxanthin biosynthesis by inhibiting the phytoene desaturase reaction in *P. rhodozyma* (Chumpolkulwong et al. 1997). β-Ionone, being an end ring analog of β-carotene, is likely to block the astaxanthin pathway after β-carotene formation, by competing with β-carotene for oxygenation at the C-3 and/or C-4 position. Mutants resistant to β-ionone have also been found to produce more astaxanthin (Lewis et al. 1990; Fang and Cheng 1993). By screening successively, mutants developed an enhanced degree of tolerance to mutagenesis and inhibitors (Fig. 1). The number of selectable isolates increased to 1,265 after the tenth mutation cycle.

Astaxanthin represents the majority of total carotenes, followed by phoenicoxanthin, 3-hydroxyechinenone, echinenone and β-carotene in *P. rhodozyma* (Schmidt et al. 2011). The total carotenes concentration of the parental strain was 1.54±0.05 mg/L, with the following distribution: 58.12 % astaxanthin, 4.67 % phoenicoxanthin, 11.36 % 3-hydroxyechinenone, 3.79 % echinenone and 22.06 % other carotenes biosynthetic intermediates. In comparison with the parental strain, the carotenes concentration of the mutant was 41.13±1.63 mg/L, with the following distribution: 75.36 % astaxanthin, 1.41 % phoenicoxanthin, 2.24 % 3-hydroxyechinenone, 0.31 % echinenone and 20.68 % other carotenes biosynthetic intermediates. An increased ratio of astaxanthin was associated with a decreased ratio of phoenicoxanthin, 3-hydroxyechinenone and echinenone in mutant YZUXHONG686. Research showed that an increase in lycopene cyclase activity will shift the carotene pathway in the direction of astaxanthin. Moreover, several monoketolated precursors, such as echinenone, 3-hydroxyechinenone and phoenicoxanthin, accumulate and can add up to about 50 % of the bicyclic ketocarotenoids. So these precursors are intermediates and potential substrates of astaxanthin synthase, increased levels of this enzyme could convert them all to astaxanthin (Schmidt et al. 2011). Differences in composition of carotenes observed between the parental strain and the mutant may be explained by speculation that mutation might result in a change in the activity of carotenogenic enzymes.

This report described a selection procedure that was effective for isolating strains of *P. rhodozyma* with significantly higher content of astaxanthin. To examine the stability of the mutants, the stability of the level of astaxanthin was measured from YZUXHONG686 of different grades. Mutant YZUXHONG686 was subcultured 18 times, and the yield of astaxanthin was almost constant.

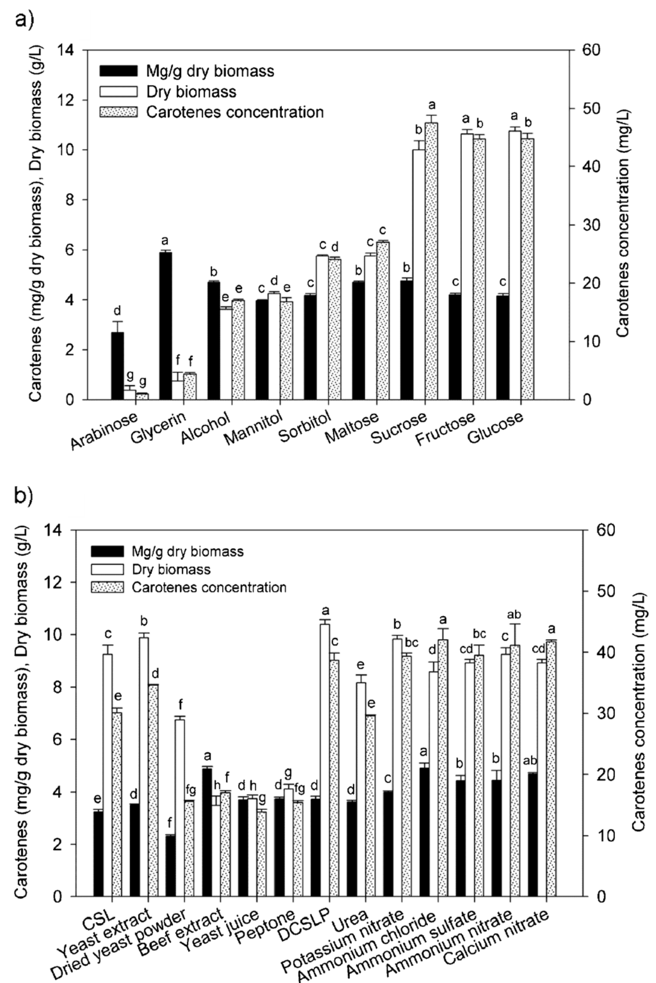

Fig. 2 Effects of **a** carbon and **b** nitrogen sources on cell growth and the production of carotenes by *P. rhodozyma* YZUXHONG686. **a** Corn steep liquor (CSL), yeast extract (YE) and $(NH_4)_2SO_4$ were used as nitrogen sources. **b** Sucrose was employed as a carbon source. Different letters above *bars* mean that each value is significantly different by ANOVA test at $P<0.05$

Effects of carbon and nitrogen sources on production of carotenes

Glucose supported higher cell growth of *P. rhodozyma* YZUXHONG686 than any other carbon source tested. The mutant utilized sucrose, fructose and glucose efficiently as carbon sources for carotenes production (Fig. 2a). Sucrose displayed the highest increase in carotenes concentration (47.55±1.24 mg/L). This phenomenon was similar with that observed for *X. dendrorhous* SKKU 0107 showing the superiority of sucrose over fructose as carbon source (Park et al. 2008). *P. rhodozyma* degrades sucrose to glucose and fructose, and assimilates glucose faster than fructose (An et al. 2001). Glucose and fructose provide both the major energy source for cell metabolism and the carbon element for biosynthesis of biomolecules. Among the nitrogen sources tested, DCSLP as a by-product of corn starch extract, exhibited the highest dry biomass of 10.38±0.18 g/L and carotenes production of 38.69±1.17 mg/L. Amino nitrogen as well as vitamins and mineral salts in the DCSLP, may contribute to the great enhancement of carotenogenic enzymes activity. Dry biomass and carotenes production on the different ammonium salts were similar (Fig. 2b). The use of sucrose and DCSLP could substantially lower the substrate-cost and improve carotenes production, and will be used in subsequent experiments.

Optimization of culture conditions

A Plackett–Burman screening design was used to evaluate eight factors that had a higher impact on the response variable. Factors having a confidence level greater than 95 % ($P>F\leq 0.05$) were considered to have a significant effect on carotene yield and were selected for further study. Incubation temperature with $P>F$ of 0.0007 was found to be the most influential factor, followed by initial pH (0.0219), and DCSLP (0.0326)

and sucrose (0.0451) medium content. The coefficient of determination (R^2) of the model had a value of 0.9892, which indicated that the model could explain up to 98.92 % variation of the data. Of the four significant variables identified, incubation temperature, initial pH and DCSLP exerted a negative influence, while sucrose exerted a positive influence on carotenes yield.

Temperature was the main influential factor for astaxanthin production. *P. rhodozyma* is a moderately psychrophilic yeast with a temperature range of growth from 0 to 27 °C. Depending on the (mutant) strain used, the optimal temperature for maximal astaxanthin production and cell growth usually lies between 18 and 22 °C (Schmidt et al. 2011). However, mutant YZUXHONG686 showed virtually no growth at 27 °C, and dry biomass and carotenes concentration reached 5.63±0.18 g/L and 22.14±0.20 mg/L at 25 °C, respectively. The mutant YZUXHONG686 produced dry biomass and carotenes concentration of 10.66±0.19 g/L and 45.21±1.33 mg/L at 21 °C, and 10.09±0.16 g/L and 42.18±1.56 mg/L at 23 °C. Because of the relative longer fermentation time the higher temperature is optimal, especially in the industrial setting (Liu et al. 2008). A temperature of 23 °C was fixed and $CaCl_2 \cdot 2H_2O$ was added to the medium as described by An et al. (1996). To take into account interactions between temperature and other variables, a repeated Plackett–Burman experiment proved that variables including initial pH, DCSLP and sucrose had significant effects on carotenes production.

Based on the above screening test, the three significant variables in the CCD experiments were set in the ranges shown in Table 2, while the other medium components that had been tested as non-significant were fixed at zero level. The model *F*-value of 10.28 with the low probability value of Probe > *F* with 0.0006 from the ANOVA of production of carotenes implied that this model was significant. The ANOVA also indicated that the model terms of X_1, X_2, X_3, X_1^2, X_2^2, and X_3^2 could significantly affect carotenes concentration (Table 4). However, interactive effects of X_1X_2, X_1X_3, and X_2X_3 were not significant, as judged by a *P* value above 0.05. The regression equation obtained from ANOVA

Table 4 Analysis of variance for the response of carotenes production

X_i	β_i	SEb	MS	*F*	*P*
	−1,185.08				
X_1	234.30	50.51	62.28	21.52	0.0005
X_2	24.06	5.05	65.69	22.69	0.0004
X_3	21.94	4.51	68.51	23.67	0.0003
X_1^2	−23.01	5.05	60.14	20.78	0.0005
X_2^2	−0.24	0.05	67.57	23.34	0.0003
X_3^2	−2.30	0.45	76.01	26.26	0.0002
Error	13	37.63	2.89		
Total	19	258.92			

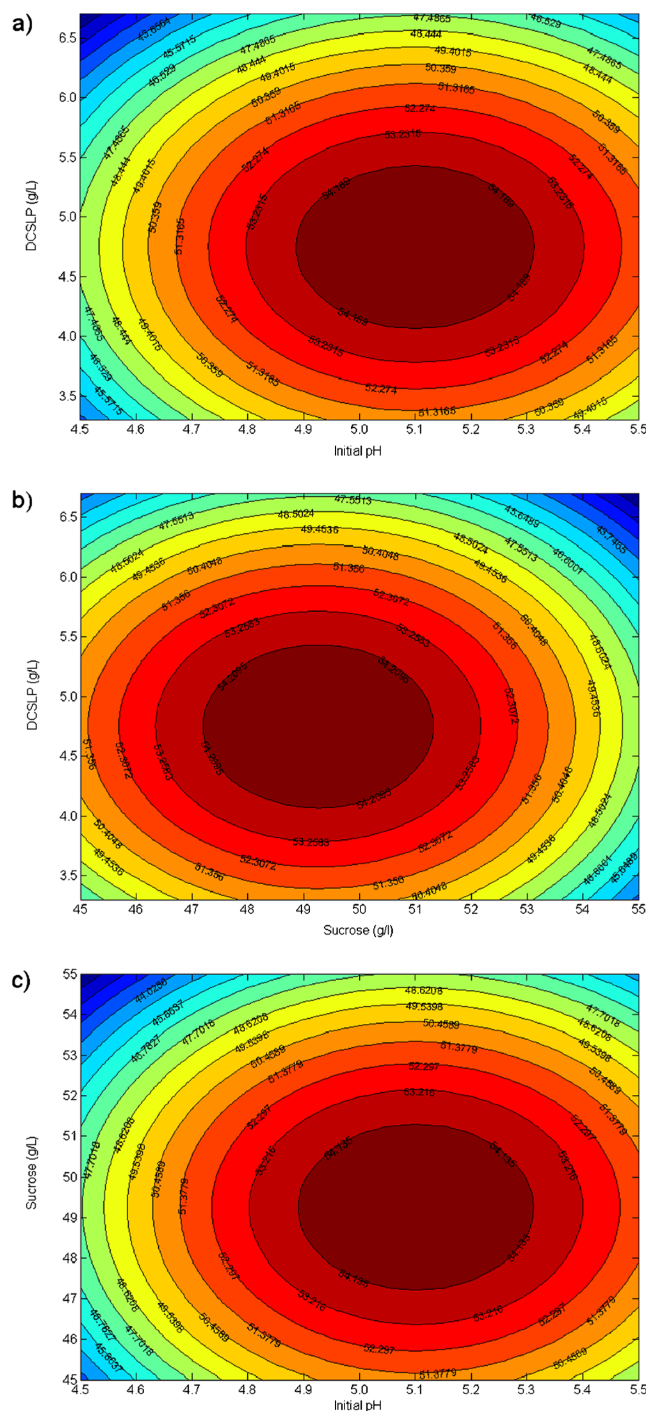

Fig. 3a–c Two-dimensional response surfaces displaying the relative effect of two variables on the production of carotenes (mg/L) by *P. rhodozyma* YZUXHONG686. Interaction between initial pH and **a** dried corn steep liquor power (DCSLP), **b** sucrose and DCSLP, and **c** initial pH and sucrose

indicated that the multiple correlation coefficient of R^2 was 0.9025. The value of the adjusted determination coefficient (Adj. R^2=0.8547) was high, arguing for the significance of this model. By applying multiple regression analysis to the experimental data, the following second-order polynomial

equation giving the carotenes concentration (Y) as a function of pH (X_1), sucrose (X_2) and DCSLP (X_3) was obtained:

$$Y = -1185.08 + 234.30X_1 + 24.06X_2 + 21.94X_3 - 23.01X_1^2 - 0.24X_2^2 - 2.30X_3^2 \quad (3)$$

Two-dimensional contour plots were generated and analyzed using MATLAB software for determination of the optimal variable levels. Figure 3 showed the two-dimensional contour plots, with two variables varying within the experimental range and the third variable fixed at its optimal level. From these plots, the optimal values of the independent variables could be observed and the interaction between pairs of independent variables could be readily understood. All plots are convex, containing the maximum response inside the design boundary.

The optimal parameters for carotenes production were optimized numerically according to a canonical analysis. The results predicted by the model suggested that maximum carotenes production could be achieved with supplementations of sucrose and DCSLP of 49.32 g/L and 4.77 g/L, with the initial pH set at 5.09 and other variables fixed at zero level. A repeat experiment using *P. rhodozyma* YZUXHONG686 was conducted to attest the validity of the optimal conditions. The mutant produced an astaxanthin content of 2.56± 0.68 mg/g dry biomass under the optimized conditions. The maximal carotenes concentration obtained was 53.68± 1.72 mg/L, and the proportion of astaxanthin, phoenicoxanthin, 3-hydroxyechinenone, echinenone and other carotenes biosynthetic intermediates was 78.04 %, 3.81 %, 5.47 %, 1.63 % and 11.05 %. The proportion of astaxanthin and three monoketolated precursors had a significantly increased ratio, from 77.94 to 88.95 %, under optimal conditions compared with that in originally basic conditions. This observation was in good agreement with previous studies that an appropriate C/N ratio could enhance enzyme activity for the β-carotene conversion to astaxanthin (Vustin et al. 2004). The improvement of astaxanthin production by optimization was presumed to be the result of an appropriate C/N ratio (Liu and Wu 2007). An increase in sucrose concentration (from 33.3 to 49.32 g/L) and a decrease in DCSLP concentration (from 6.5 to 4.77 g/L) implied an increase of carbon/nitrogen ratio in optimal medium. Most previous studies have suggested that a high C/N ratio may favor the biosynthesis of carotenes in *P. rhodozyma*. Vustin et al. (2004) revealed that a marked decrease in cell growth was concomitant with an increase in the astaxanthin content of the cell as the C/N ratio of the medium was increased in *P. rhodozyma* cultures. They then suggested that abundant nitrogen in the medium might enhance cell growth but suppress the enzymes activity for the β-carotene conversion to astaxanthin. In this study, the mutant showed a high carotenes production in the pH range of 4.5–5.5. The optimum pH for a particular strain will depend mainly on the interaction between variables, both physical and nutritional.

Through a series of screenings and optimization, the obtained mutant YZUXHONG686 gave higher yields of carotenes and astaxanthin. However, under these conditions, yields of carotenes and astaxanthin were relatively low compared with the value of 115 mg/g dry biomass and 350 mg/L (4.1 mg/g) by *Blakeslea trispora* strain in flask (Nanou and Roukas 2011) and by *P. rhodozyma* strain in semi-industrial (de la Fuente et al. 2010) fermentation, respectively. Facing these challenges, further efforts such as the induction of ultraviolet light, will be made to promote astaxanthin yield (de la Fuente et al. 2010; Ning et al. 2012).

Conclusion

The results presented above show that, after mutagenesis using combined UV, NTG, and gamma irradiation treatments, selection of biochemical mutants resistant to some metabolic inhibitors was a more efficient method for improvement of astaxanthin production. A mutant strain of *P. rhodozyma* YZUXHONG686, with astaxanthin yield improved more than 17-fold compared to the parental strain, was obtained by consecutive mutagenesis treatments in this study. The application of RSM resulted in an enhancement of astaxanthin yield. Thus the present work has shown that statistical experimental designs can be used as valuable and dependable tools for the optimization of pigment yield from mutants. *P. rhodozyma* YZUXHONG686 can accumulate astaxanthin at a high level and therefore has significant commercial potential.

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