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ORIGINAL PAPER

# Reduction of fatty acid flux results in enhancement of astaxanthin synthesis in a mutant strain of *Phaffia rhodozyma*

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**Abstract** A moderate-temperature mutant strain of the yeast *Phaffia rhodozyma*, termed MK19, was selected by 1-methyl-3-nitro-1-nitrosoguanidine (NTG) and Co60 mutagenesis. MK19 displayed fast cell growth and elevated astaxanthin content at 25°C, whereas optimal temperature for growth and astaxanthin synthesis of wild-type *P. rhodozyma* was 17–21°C. Optimized astaxanthin yield for MK19 after 4 days culture in shaking flask at 25°C, determined by response surface methodology, was 25.8 mg/l, which was 17-fold higher than that of the wild-type. MK19 was tolerant of high initial concentration of glucose (>100 g/l) in optimized medium. Total fatty acid content of MK19 was much lower than that of the wild-type. Acetyl-CoA is a common precursor of fatty acid and terpenoid biosynthesis, and it is possible that decreased fatty acid synthesis results in transfer of acetyl-CoA to the carotenoid biosynthetic pathway. Our results indicate that astaxanthin content is negatively correlated with fatty acid content in *P. rhodozyma*. Nutrient analysis showed that MK19 cells are enriched in lysine, vitamin E, and other rare nutrients, and have potential application as fish food without nutritional supplementation. This moderate-temperature mutant strain is a promising candidate for economical industrial-scale production.

**Keywords** *Phaffia rhodozyma* · Moderate-temperature strain · Astaxanthin · Fatty acid · Response surface methodology

## Introduction

Astaxanthin is an orange-red pigment widely distributed in organisms ranging from algae and microbes (which are capable of synthesizing the compound) to higher animals such as rainbow trout, salmon, and flamingo (which obtain the molecule from their environment but do not synthesize it de novo). Astaxanthin is in high demand because it imparts a distinctive orange-red coloration to fishery products such as salmon, and thereby increases customer appeal. The pigment also displays a strong anti-oxidant property against reactive oxygen species (ROS), thereby supporting the immunological system and protecting cells from ageing and from diseases such as cancer, senile dementia, and Parkinson's Disease [7, 11, 16, 26].

*Phaffia rhodozyma* (sexual form, *Xanthophyllomyces dendrorhous*) is a carotenoid-producing yeast with astaxanthin as the primary carotenoid pigment, which has great industrial potential for astaxanthin fermentation. *P. rhodozyma* was initially isolated from exudates of trees in Japan and the West Coast of North America. Unlike other known carotenoid-containing yeast species, it is a strong fermenter of sugars, including D-glucose, sucrose, and raffinose. This is an important property with regard to industrial production. Astaxanthin synthesis by

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*P. rhodozyma* is suppressed by high glucose concentration [12, 19–21], and by high temperature. The optimal temperature range for both cell growth and pigment synthesis is 17–21°C [7, 11, 12], as found in the native environment.

The low content of astaxanthin in wild-type *P. rhodozyma*, 30–200 μg/g, is a critical issue in industrial astaxanthin production. Experimental strains that can tolerate high (50–60 g/l) initial glucose concentration have been reported. Several studies have focused on nutrients in culture medium (and environmental factors) that increase astaxanthin or biomass yield [9].

The astaxanthin metabolic pathway in *P. rhodozyma* was elucidated many years ago. Astaxanthin and ergosterol belong to the class of terpenoids, and share a conserved mevalonate pathway divergent from that of farnesyl diphosphate (FPP). Acetyl-CoA is a substrate of both fatty acid and terpenoids [1]. The ergosterol and fatty acid pathways are closely interdependent with the carotenoid pathway through a complex regulatory feedback system.

All carotenoids are highly lipophilic and water-insoluble, and lipids have been shown to accelerate the reaction velocity of carotenoid synthesis by removing them from membrane. Rabbani et al. [18] reported that blocked synthesis of triacylglycerol inhibited production of $\beta$-carotene in the unicellular alga *Dunaliella bardawil*.

In the present study, an astaxanthin-overproducing mutant *P. rhodozyma* strain, termed MK19, with astaxanthin content >1,300 μg/g (dry cells), was selected by NTG and Co60 mutagenesis. MK19 was able to grow at high glucose concentration, and produce astaxanthin at temperatures up to 25°C. Synthesis profiles of astaxanthin, fatty acid, and ergosterol were compared between the mutant and wild-type, in order to clarify the relationships among these pathways. An optimizing technique termed "response surface methodology" was successfully applied to reduce work load and production cost, decrease the frequency of adding substances during fermentation, simplify process strategy, and reduce the energy requirement for large-scale astaxanthin production.

## Materials and methods

### Strains and culture conditions

Wild-type *P. rhodozyma* strain (JCM9042) was purchased from the Institute of Physical and Chemical Research (RIKEN), Japan. JCM9042 was mutagenized by NTG and Co60 methods as described by Sun et al. [23], and a crimson-colored mutant strain termed MK19 was selected in our laboratory. Both strains were maintained on potato dextrose agar (PDA) slants at 4°C.

### Seed culture

Seed medium, composed of (per liter) glucose 40.0 g, yeast extract 4.0 g, urea 2.4 g, potassium dihydrogen phosphate 2.0 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g, was adjusted to pH 6.0, and sterilized by autoclaving at 115°C for 25 min. Urea was sterilized by filtering. All experiments were performed in shaking flask culture, with 25 ml volume of liquid culture medium in each 250-ml flask.

Cells were transferred from 4°C slants to fresh slants for 72 h at 21–25°C. Loopfuls of lawn were inoculated to seed medium, and incubated at 25°C for 72 h. To make the starter culture, 5% of pre-incubation broth was inoculated for another 36 h.

### Flask fermentation

Media used for maximum glucose tolerance test were the same as seed medium, except that glucose concentration varied (40, 110, and 160 g/l). After glucose concentration was established, fermentation culture was incubated at 21 or 25°C for 115 h on a rotary shaker at 210 rpm. Samples were extracted and tested at intervals of $\sim$24 h.

Media for fractional factorial design $2^{6-2}$ and central composite design were prepared in accordance with the statistical experimental design (Table 1). The overall production period for fermentation culture was 4 days on a rotary shaker at 210 rpm at 25°C, unless specified otherwise. All experiments were performed in duplicate or triplicate.

Metabolic analysis medium was used for measurement of astaxanthin, ergosterol, and fatty acid content in wild-type and mutant strains. This medium contained 110 g glucose, 1.8 g urea, 2.0 g potassium dihydrogen phosphate, 0.5 g magnesium sulfate, 0.1 g sodium chloride, and 0.1 g calcium chloride per liter, sterilized by autoclaving at 115°C for 25 min. Vitamins (all in μg/l) were: biotin 20.0, calcium pantothenate 2,000, lipoic acid 2, inositol 10,000, nicotinamide 400, aminobenzoic acid 200, B6 400, riboflavin 200, and thiamine 400. Trace elements (all in μg/l) were: boracic acid 500, copper sulfate 40, potassium iodide 100, ferric sulfate 200, manganese sulfate 400, sodium molybdate 200, zinc sulfate 400. Urea, vitamins, and trace elements were sterilized by filtering. Culture temperature

**Table 1** Code levels for variables of factorial $2^{6-2}$ design

| | Independent variables (% w/v) | | | | | |
|---|---|---|---|---|---|---|
| | Glucose | Yeast extract | Urea | $KH_2PO_4$ | $MgSO_4 \cdot 7H_2O$ | pH |
| −1 | 5 | 0.2 | 0.12 | 0.05 | 0.01 | 4 |
| +1 | 11 | 0.8 | 0.36 | 0.25 | 0.09 | 6 |

was set at 21°C, according to the optimal temperature of wild-type strain.

### Cell mass

Cell concentration was determined based on optical density at 600 nm, or dry cell weight. To obtain dry weight, 35 ml of broth was centrifuged at 12,000 rpm, rinsed with distilled water, and dried at 85°C until constant weight.

### Response surface methodology and statistical analysis

Factorial $2^{6-2}$ design leading to 16 sets of tests was performed to determine the significant factors in astaxanthin fermentation [4, 13, 17]. Natural (real) values and code level of variables investigated in this study are shown in Table 1. $x_i$ was the code value of the independent variable with corresponding real value $\varepsilon$. Their relationship was expressed by:

$$x_i = (\varepsilon_i - \varepsilon_0)/\triangle\varepsilon \quad (1)$$

$\varepsilon_0$ was the real value of the center point; $\triangle\varepsilon$ was the step length of the real value of the independent variable.

To further optimize astaxanthin yield based on the most important factors, a second-order central composite design with five coded levels was performed. The model was expressed by the equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum\sum \beta_{ij} x i x_j \quad (2)$$

Dependent or response variable Y = astaxanthin yield; $\beta$ = regression coefficient; $x_i$ = code value of the independent variable. Statistical significance was determined by $F$ value, and $p$ value. Analysis of variance (ANOVA) was performed by SPSS (Statistical Program for Social Sciences). Polynomial regression of experimental data, maximum astaxanthin yield, and plotting were performed by MATLAB7.1.

### Astaxanthin and ergosterol measurement

Culture solution (1.0 ml) was centrifuged at 12,000 rpm for 1 min, and washed with distilled water. The resulting pellets were mixed with 200 µl dimethyl sulfoxide preheated to 70°C [2], stirred thoroughly, and the mixture was kept at 70°C for 20 min in a water bath. Broken cells were extracted with methanol/dichloromethane (3:1), shaken, and centrifuged at 2,000 rpm. Pigments in the supernatant were transferred to another tube, and the process was repeated until pellets showed no red color. Astaxanthin and ergosterol were analyzed quantitatively by high-performance liquid chromatography (HPLC) on C18 column (250 × 4.6 mm; 5 µm, Chuangxintongheng, Beijing) at 40°C with flow rate 1.0 ml/min, with detection wavelength 476 nm for astaxanthin and 280 nm for ergosterol, respectively. The mobile phase consisted of methanol 80%/dichloromethane 20%. Astaxanthin and ergosterol were identified based on retention time in comparison to control standard (Sigma).

### Detection of fatty acid in cells

Fatty acids were extracted as described by Sukhija et al. [24], with internal standard nonadecanoic acid (19:0) 4 mg/ml in hexane or petroleum ether (PE). Fatty acids were analyzed using Agilent 6890 gas–liquid chromatograph with FID detector. Conditions: DB23 J&W Scientific column (diameter: 0.25 mm; length: 60 m) (Agilent), temperature programmed from 180 to 230°C at 3°C/min. Gas flow: carrier nitrogen 45 ml/min; air 450 ml/min. Fatty acids were identified by retention times in comparison to internal standards. Total fatty acids (mg/g dry sample) were calculated as follows:

$$\frac{(\text{total area under peaks}) - (\text{area under int std})}{\text{area of int std}} \times \frac{4\,\text{mg}}{\text{dry wt of sample (g)}}.$$

### Cell nutritional analysis

MK19 cells were dried using a Spray Dryer (Labplant SD-50, UK), and nutrients were analyzed by the Feed Safety and Bio-availability Evaluation Center, Ministry of Agriculture, Beijing, China.

## Results

### Moderate-temperature mutant strain

A mutant strain was selected by NTG and Co60 mutagenesis, and inoculated at room temperature (25–26°C) for 15 days. Under this condition, surviving colonies were likely to be high-temperature-tolerant mutant strains. A deeply pigmented colony was selected and termed MK19. This mutant strain was maintained at 4°C, and a sample was deposited at China General Microbiological Culture Collection Center (No. CGMCC3445).

### Optimal temperature, and initial glucose concentration design

In order to decrease the frequency of adding substances during fermentation, to simplify process strategy, and to reduce energy requirement for large-scale astaxanthin production, we determined the temperature and carbon source concentration leading to optimal growth of mutant

MK19 cells. Optimal temperature for growth of the wild-type strain was 17–21°C, consistent with previous reports. Astaxanthin content of the wild-type at 25°C (0.42 mg/l) was much lower than at 21°C (1.36 mg/l). In striking contrast, MK19 colonies cultured for 115 h at 25°C showed no significant difference in cell growth (Fig. 1a) or astaxanthin content (Fig. 1b) compared to cells cultured at 21°C. To test the effects of glucose concentration, wild-type and MK19 cells were cultured at 25°C with initial glucose concentration 40, 110, or 160 g/l, and harvested at 45 or 115 h. Compared to wild-type, MK19 grew well at high glucose concentration, and had higher astaxanthin content (Fig. 2). Astaxanthin yield of MK19 increased for initial glucose concentrations up to 110 g/l, but not 160 g/l. The biomass of MK19 was ~30% higher than that of the wild-type. MK19 was clearly resistant to glucose catabolic repression.

## Fractional factorial design $2^{6-2}$

Fractional factorial design $2^{6-2}$ was employed to determine the independent variable having the greatest effect on astaxanthin yield. Experimental design and results are shown in Table 2, and ANOVA analysis by SPSS is shown in Table 3. The first-order model fit well to the experimental data, as indicated by the $R^2$ value of 0.853. Important factors were glucose, yeast extract, and urea ($p < 0.01$, Table 3), as well as the interaction between glucose and urea, corresponding to C/N ratio. High astaxanthin yield was obtained with high glucose concentration (110 g/l) and low urea (Table 2, runs 13, 14), consistent with a previous report that high C/N promotes astaxanthin synthesis [28]. This is the first report of a mutant strain of *P. rhodozyma* tolerant of high initial glucose concentration at room

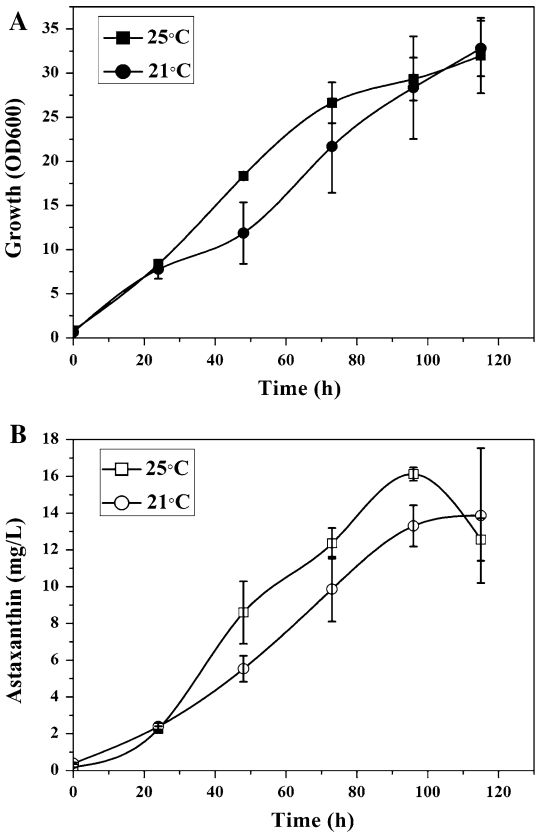


**Fig. 1** Growth curve (**a**) and astaxanthin content (**b**) of mutant strain MK19 at 21°C and 25°C, in media containing 110 g/l of glucose. Experiments were performed in triplicate

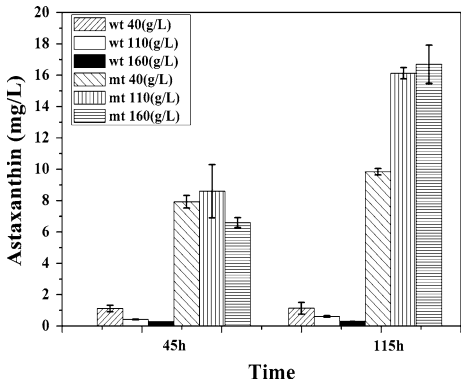


**Fig. 2** Astaxanthin content of MK19 (*mt*), compared to wild-type (*wt*), in media with various glucose concentrations, cultured for 115 h at 25°C. Sampling at 45 and 115 h. Experiments were performed in triplicate

**Table 2** Factorial design and results for astaxanthin yield

| | Code levels ($x_i$) | | | | | | Astaxanthin (Y) |
|---|---|---|---|---|---|---|---|
| | Glucose | Yeast extract | Urea | $KH_2PO_4$ | $MgSO_4 \cdot 7H_2O$ | pH | mg/l |
| 1 | −1 | −1 | −1 | −1 | −1 | −1 | 8.02 |
| 2 | −1 | −1 | −1 | −1 | +1 | +1 | 7.12 |
| 3 | −1 | −1 | +1 | +1 | −1 | +1 | 9.97 |
| 4 | −1 | −1 | +1 | +1 | +1 | −1 | 7.26 |
| 5 | −1 | +1 | −1 | +1 | −1 | +1 | 11.86 |
| 6 | −1 | +1 | −1 | +1 | +1 | −1 | 11.39 |
| 7 | −1 | +1 | +1 | −1 | −1 | −1 | 10.69 |
| 8 | −1 | +1 | +1 | −1 | +1 | +1 | 10.69 |
| 9 | +1 | −1 | −1 | +1 | −1 | −1 | 10.23 |
| 10 | +1 | −1 | −1 | +1 | +1 | +1 | 11.43 |
| 11 | +1 | −1 | +1 | −1 | −1 | +1 | 7.85 |
| 12 | +1 | −1 | +1 | −1 | +1 | −1 | 6.13 |
| 13 | +1 | +1 | −1 | −1 | −1 | +1 | 17.15 |
| 14 | +1 | +1 | −1 | −1 | +1 | −1 | 18.43 |
| 15 | +1 | +1 | +1 | +1 | −1 | −1 | 13.3 |
| 16 | +1 | +1 | +1 | +1 | +1 | +1 | 9.38 |

All experiments were performed in duplicate

**Table 3** ANOVA for response of dependent variables in astaxanthin production

| Factor | Sum of square | *df* | Mean square | *F* ratio | *p* value |
|---|---|---|---|---|---|
| $x_1$ | 27.629 | 1 | 27.629 | 12.421 | 0.002 |
| $x_2$ | 146.030 | 1 | 146.030 | 65.651 | 0.000 |
| $x_3$ | 41.968 | 1 | 41.968 | 18.868 | 0.000 |
| $x_4$ | 3.714E-02 | 1 | 3.714E-02 | 0.017 | 0.898 |
| $x_5$ | 6.544E-02 | 1 | 6.544E-02 | 0.029 | 0.865 |
| $x_6$ | 3.366 | 1 | 3.366 | 1.513 | 0.232 |
| $x_1x_2$ | 8.658 | 1 | 8.658 | 3.892 | 0.061 |
| $x_2x_3$ | 6.370 | 1 | 6.370 | 2.864 | 0.105 |
| $x_1x_3$ | 50.571 | 1 | 50.571 | 22.735 | 0.000 |
| Error | 48.936 | 22 | 2.224 | | |
| Total | 3,955.576 | 32 | | | |

*R* squared = 0.853; *df* degree of freedom

temperature. $KH_2PO_4$, $MgSO_4$, pH, and their interactions, had little impact on astaxanthin yield.

## Central composite design

Central composite design (CCD) with two independent variables, glucose ($x_1$), and yeast extract ($x_2$), was applied to determine the optimal conditions for astaxanthin fermentation by mutant strain MK19, and for maximal glucose tolerance. Natural (real) values, code levels, and results are shown in Table 4. Other factors of medium were added economically. Sixteen sets of tests were performed: $2^2$ full factorial, four star points ($\pm 1.63$), and eight central points.

Regression analysis was performed by MATLAB 7.1. *F* value and *p* value were 249.532 and 1.03476e-010, respectively. The model was statistically significant at 0.01 level of significance with $R^2 = 0.9891$, adjusted $R^2 = 0.9851$, indicating that $\sim$98.51% of variability in the response can be explained by the second-order polynomial prediction equation.

$$Y = 24.75 - 3.14x_2 - 5.14x_1^2 - 5.30x_2^2 - 2.45x_1x_2 \qquad (3)$$

Coefficients of factors were statistically significant, except for $x_1$ (Table 5).

The fitted response for the above regression model is plotted in Fig. 3. The response surface plot has a maximum when $x_1 = 0.0747$ and $x_{2=} -0.3130$, corresponding to real values = glucose 102.99 g/l and yeast extract 8.435 g/l. The predicted maximal astaxanthin yield was 25.24 mg/l. The optimal astaxanthin fermentation medium predicted by the model was (per liter): glucose 102.99 g, yeast extract 8.435 g, urea 0.12 g, $KH_2PO_4$ 2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, pH 6.0. To verify predicted optimal yield, 25.8 mg/l astaxanthin was obtained with the predicted formula, which was consistent with the predicted yield by the quadratic polynomial.

**Table 4** Experimental design and results of central composite design (CCD)

| Run | $x_1$ (glucose %) | $x_2$ (yeast extract %) | Astaxanthin yield (mg/l) |
|---|---|---|---|
| 1 | 1 (14) | 1 (1.5) | 8.10 |
| 2 | 1 (14) | −1 (0.5) | 20.64 |
| 3 | −1 (6) | 1 (1.5) | 13.57 |
| 4 | −1 (6) | −1 (0.5) | 16.31 |
| 5 | 1.63 (16.52) | 0 (1) | 11.55 |
| 6 | −1.63 (3.48) | 0 (1) | 10.17 |
| 7 | 0 (10) | 1.63 (1.85) | 6.12 |
| 8 | 0 (10) | −1.63 (0.185) | 14.68 |
| 9 | 0 (10) | 0 (1) | 24.48 |
| 10 | 0 (10) | 0 (1) | 24.74 |
| 11 | 0 (10) | 0 (1) | 23.60 |
| 12 | 0 (10) | 0 (1) | 25.02 |
| 13 | 0 (10) | 0 (1) | 24.33 |
| 14 | 0 (10) | 0 (1) | 24.77 |
| 15 | 0 (10) | 0 (1) | 24.68 |
| 16 | 0 (10) | 0 (1) | 25.04 |

**Table 5** *T* test of coefficients in quadratic model

| Factor | Coefficient | *T* | *p*-value |
|---|---|---|---|
| $x_1$ | 0.12 | 0.4143 | 0.6874 |
| $x_2$ | −3.15 | −11.199 | 0.0000* |
| $x_1^2$ | −5.13 | −21.2469 | 0.0000* |
| $x_2^2$ | −5.30 | −21.964 | 0.0000* |
| $x_1x_2$ | −2.45 | −5.7302 | 0.0001* |

*Statistically significant at 99% confidence level. Intercept: 24.75

## Astaxanthin, ergosterol, and fatty acid content of cells

It is possible that precursor molecules such as acetyl-CoA and FPP are transferred from the fatty acid and ergosterol pathways to the carotenoid pathway, and the increased content of astaxanthin in MK19 may be derived from both of these pathways. To test this hypothesis, wild-type and MK19 cells were cultured in metabolic analysis medium, and astaxanthin, ergosterol, and fatty acids were extracted and analyzed.

FPP is the direct precursor of astaxanthin, and of the ergosterol biosynthetic pathway. Astaxanthin content of MK19 (1,300 μg/g) was 8.7-fold higher than that of the wild-type (150 μg/g) (Fig. 4a). This excessive astaxanthin is presumably not derived solely from the ergosterol pathway, since ergosterol content was not significantly

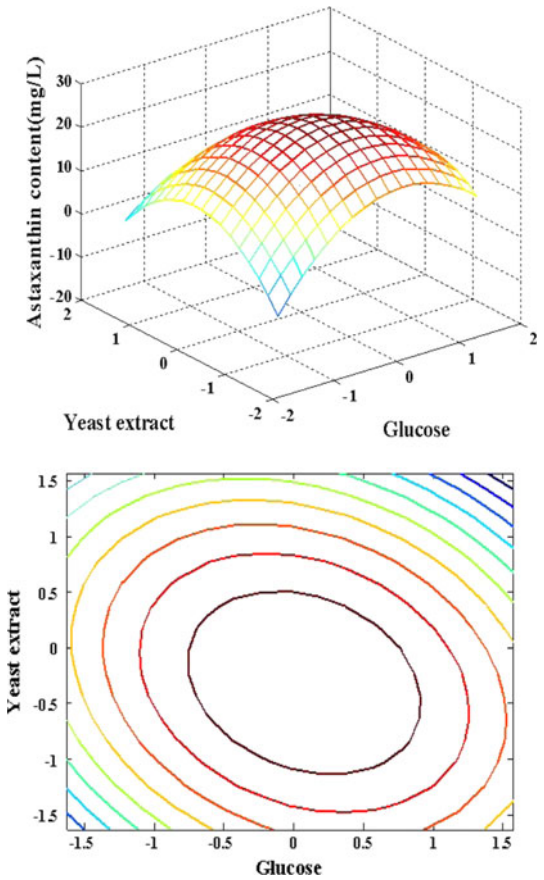


**Fig. 3** Three-dimensional response surface plot of central composite design (CCD) experiment (*top*), and contour plot of calculated response surface (*bottom*). See text for details

different in wild-type vs. MK19 ($p = 0.19$) (Fig. 4b). Ergosterol is an essential component in cell membrane, whose synthesis curve parallels that of cell growth. Accumulation of ergosterol continued until the cessation of cell growth. Cell growth slowed at 48 h for wild-type, but remained fast until 72 h for MK19 (Suppl. Fig. 1).

The major fatty acids in *P. rhodozyma* are oleic acid (18:1) and linoleic acid (18:2) (Table 6). Total fatty acid content was higher for wild-type (198.04 mg/g at 48 h; 179.51 mg/g at 72 h) than for MK19 (128.60 mg/g at 48 h; 135.13 mg/g at 72 h) (Fig. 4c). A decrease of fatty acid may lead to the accumulation of acetyl-CoA, a common precursor of fatty acid and astaxanthin biosynthesis, with consequent transfer of acetyl-CoA to astaxanthin metabolic flux in MK19. This is a possible explanation of the increased astaxanthin content in MK19.

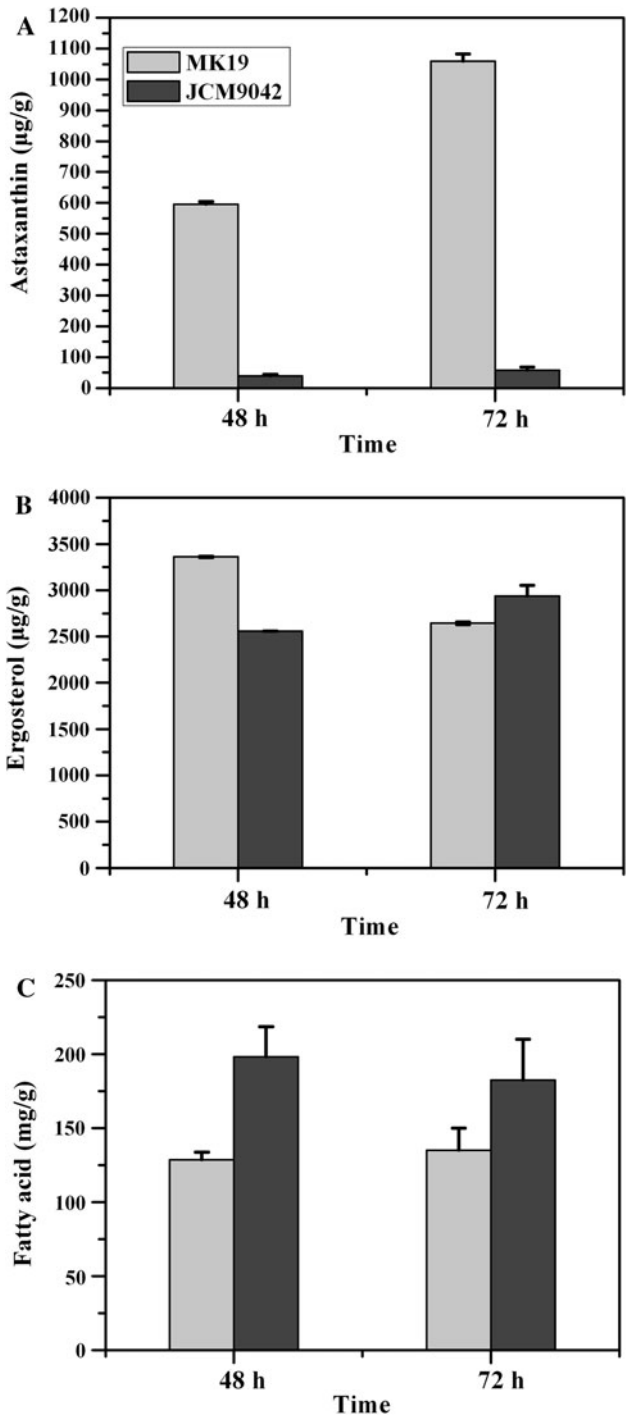


**Fig. 4** Comparison of astaxanthin, ergosterol, and fatty acid content in wild-type versus MK19. Experiments were performed in triplicate

## Discussion

Astaxanthin (3,3′-dihydroxy-β-,β-carotene-4,4′dione) is an important pigment used in the aquaculture industry, and also a strong antioxidant. A recent review described details of biosynthesis, factors affecting carotenogenesis, and strategies for enhancing production in *Rhodotorula* and *Phaffia* strains [5]. It appears that the major obstacle to industrial application of these yeasts is the high cost of production. *P. rhodozyma* is a prospective candidate strain for industrial-scale astaxanthin production. It yields free

**Table 6** Fatty acid composition of wild-type (wt) *P. rhodozyma* and mutant strain (mt) MK19

| Fatty acid (%) | 24 h | | 48 h | | 72 h | |
|---|---|---|---|---|---|---|
| | mt | wt | mt | wt | mt | wt |
| C10:0 | 0.08 | 0.25 | 0.09 | 0.03 | 0.09 | 0.04 |
| C12:0 | 0.09 | 0.35 | Trace | Trace | Trace | 0.04 |
| C14:0 | 0.27 | 0.38 | 0.23 | 0.33 | 0.83 | 0.27 |
| C16:0 | 17.34 | 15.19 | 16.63 | 17.25 | 19.85 | 15.94 |
| C16:1 | 0.75 | 0.97 | 0.51 | 0.68 | 0.54 | 0.68 |
| C18:0 | 11.11 | 8.69 | 9.72 | 5.28 | 7.70 | 4.76 |
| C18:1n7 | 32.82 | 38.57 | 39.80 | 49.64 | 46.00 | 49.70 |
| C18:2 | 29.49 | 30.01 | 27.70 | 25.09 | 23.55 | 26.48 |
| C18:3n3 | 5.84 | 4.66 | 4.78 | 1.45 | 1.94 | 1.43 |
| C20:0 | 0.48 | 0.43 | Trace | Trace | Trace | 0.48 |
| C20:1 | 0.57 | 0.75 | Trace | Trace | Trace | 0.29 |
| C20:2 | Trace | Trace | Trace | Trace | Trace | 0.50 |
| C20:4 | 0.13 | 0.89 | Trace | Trace | Trace | Trace |
| C22:0 | 0.47 | 0.45 | 0.55 | 0.51 | 0.44 | 0.49 |
| C22:5 | 0.63 | 1.03 | Trace | Trace | Trace | 0.96 |

Values shown are percentage of total fatty acids. Experiments were performed in triplicate

astaxanthin or monoester-astaxanthin, which differ from the diester-astaxanthin produced by *Haematococcus pluvialis*. Cell growth and astaxanthin yield of *P. rhodozyma* are typically inhibited by high glucose concentration and by high temperature [6, 12, 21]. Fed-batch processes may be useful [10, 14, 27] when growth and/or metabolite production are inhibited at high substrate concentration, as a result of substrate inhibition, end-product inhibition, or catabolite repression [8, 22].

A novel population of the biotechnologically important yeast *Xanthophyllomyces dendrorhous* (the sexual stage of *P. rhodozyma*) was recently isolated for the first time in the southern hemisphere (Argentina) [3]. Results of the present study contribute to our understanding of biological and biochemical variations of *P. rhodozyma,* which are relevant to further development of strains with industrially desirable properties.

Astaxanthin synthesis in microbial strains typically requires low temperature (17–21°C), which presents another obstacle to industrial production [19]. Mutant strain MK19 grows well and synthesizes astaxanthin at higher temperatures, up to 25°C, which could reduce energy required for refrigeration during industrial fermentation, and thereby reduce production costs. MK19 also provides a useful experimental model for further studies of temperature effects on astaxanthin synthesis.

Yeasts and other fungi often display competition for acetyl-CoA between terpenoids and fatty acid, or competition for FPP between astaxanthin and ergosterol. Lipids have been shown to accelerate formation velocity of carotenoids by removing them from membranes, due to their highly lipophilic property. We found that wild-type *P. rhodozyma* and MK19 show no significant difference in ergosterol content, whereas total fatty acid content is lower in MK19 than in wild-type. This could lead to accumulation of acetyl-CoA, NADPH, and ATP in MK19, which are then transferred to astaxanthin flux.

Results described here indicate a negative correlation of astaxanthin level with fatty acid biosynthesis in *P. rhodozyma*, which is further supported by the finding that suppression of fatty acid synthesis in wild-type by treatment with the metabolic inhibitor triclosan led to increased astaxanthin (unpublished data). These results are in striking contrast to those from a study of *Dunaliella salina*, in which oleic acid (18:1) level showed a strong positive correlation with carotene content [15].

We observed a negative correlation of carotenoid level with fatty acid biosynthesis in *P. rhodozyma*. Total fatty acid content was greatly reduced (44–70 mg/g) in MK19 compared to wild-type, whereas astaxanthin content was >1,000 μg/g higher. Metabolic engineering to reduce fatty acid level appears to be a promising strategy for increasing synthesis of astaxanthin or other carotenoid pigments in MK19, and analogous mutant strains in other species.

We applied response surface methodology to determine optimal composition of fermentation medium, and to reach maximal glucose tolerance for MK19. Astaxanthin synthesis and cell growth of MK19 with >100 g/l glucose were similar to those at lower glucose concentration. Nutrient analysis performed by the Ministry of Agriculture Feed Safety and Bio-availability Evaluation Center, Beijing, China, showed that MK19 is enriched in several rare nutrients such as lysine and vitamin E (Suppl. Table 1). Therefore, MK19 also has potential application as fish food without nutritional supplementation [25]. Studies on large-scale fermentation of MK19, and differences in expression of key genes between wild-type and MK19, are in progress in our laboratory.

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