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# Study on the relationship between intracellular metabolites and astaxanthin accumulation during *Phaffia rhodozyma* fermentation

Anfeng Xiao [a,b,c], Xinglong Jiang [a], Hui Ni [a,b,c], Qiuming Yang [a,b,c], Huinong Cai [a,b,c,*]

[a] College of Food and Biological Engineering, Jimei University, Xiamen 361021, China
[b] Fujian Provincial Key Laboratory of Food Microbiology and Enzyme Engineering, Xiamen, Fujian Province 361021, China
[c] The Research Center of Food Biotechnology, Xiamen 361021, China

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ABSTRACT

*Background:* To study the relationship between intracellular anabolism and astaxanthin production, the influence of intracellular protein and fatty acids on astaxanthin production by four mutant *Phaffia rhodozyma* strains and their variations was investigated in this research.
*Results:* First, the content of astaxanthin in cells showed a reverse fluctuation in contrast to that of protein during the whole fermentation process. Moreover, compared with the three other strains, the astaxanthin-overproducing mutant strain of the yeast *P. rhodozyma*, called JMU-MVP14, had the highest specific productivity of astaxanthin as 6.8 mg/g, whereas its intracellular protein and fatty acid contents were the lowest. In addition, as a kind of sugar metabolic product, ethanol was only produced by *P. rhodozyma* JMU-VDL668 and JMU-7B12 during fermentation.
*Conclusions:* The results indicated that the accumulation of ethanol, intracellular protein, and fatty acids had competition effects on astaxanthin synthesis. This condition may explain why the *P. rhodozyma* strains JMU-VDL668 and JMU-7B12 achieved relatively lower astaxanthin production (1.7 and 1.2 mg/L) than the other two strains JMU-MVP14 and JMU-17W (20.4 and 3.9 mg/L).



## 1. Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione; $C_{40}H_{52}O_4$) is one of the most important natural pigments widely used in salmon and trout aquaculture. This pigment can serve as a protective agent against oxidative damage to cells in vivo [1,2,3] and is also used as a nutraceutical and medicinal ingredient against diseases, such as cancer, diabetes, and other related aspects [1,4,5]. Based on its various biological functions, astaxanthin has significant economic value and a growing global commercial market.

The astaxanthin-producing *Phaffia rhodozyma* has great industrial potential for natural astaxanthin production with such advantages as fast breeding, short growth cycle, and mature fermentation process [6,7,8]. However, the production of natural astaxanthin cannot meet the market demand, but a large majority of the commercial supply (approximately 97%) is synthetic astaxanthin [9]. The reason for the limited market share may be the considerable price difference: synthetic astaxanthin has a market price of approximately $2000 per kilogram, whereas the natural product is more than $7000 per kilogram [9]. Although attempts have been made to reduce the production costs of natural astaxanthin, including such approaches as strain improvement [10,11,12], the use of low-cost raw materials [8,13], selection of additives [14], and optimization of fermentation conditions [15,16], biotechnological astaxanthin production that can result in cost-competitive processes by such technologies still requires a significant amount of work.

Astaxanthin is an isoprenoid belonging to the mevalonate pathway in *P. rhodozyma* [9,17]. Carotenoid biosynthesis is inevitably affected by the primary and other secondary metabolisms, such as protein, fatty acids, and ethanol. The synthesis of protein and fatty acids, which are important cell components, as well as that of astaxanthin requires numerous carbon sources, energy (ATP), NADPH, and acetyl-CoA [18,19,20]. Thus, competition for these factors exists among protein, fatty acids, and astaxanthin synthesis and may thus encourage astaxanthin accumulation in cells, whereas protein and fatty acid synthesis were inhibited to a certain extent [21,22,23]. In the process of glucose catabolism, ethanol will be produced during yeast fermentation if dissolved oxygen is insufficient or if glucose concentration is extremely high. Ethanol synthesis could consume the limited carbon source in medium, and previous studies showed that ethanol metabolism during fermentation could be important in astaxanthin synthesis [24,25].

* Corresponding author.
*E-mail address:* huinongcai@163.com (H. Cai).
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To improve astaxanthin production, many researchers recently focused on the metabolic engineering and genetic levels of biosynthetic astaxanthin [11,26,27,28,29]. Clearly and effectively manipulating the major factors could regulate astaxanthin biosynthesis and aid in the design of the optimal carbon source allocation scheme to establish effective means of inducing a significant increase in astaxanthin content in strains of *P. rhodozyma*. However, astaxanthin biosynthesis is a complex process, the regulatory mechanisms of which remain to be clearly identified. The metabolic behavior differs for various yeast strains, such that controversial reports exist on whether carotenoid synthesis in *P. rhodozyma* will be growth-associated [30] or will continue after cell growth retardation [15,20,31]. Thus, the dynamic variation of intracellular metabolites and astaxanthin accumulation during *P. rhodozyma* fermentation should be studied.

This work aims to explore the astaxanthin synthesis mechanism by analyzing the relationship of the astaxanthin biosynthetic pathway with the anabolic process in *P. rhodozyma*, as well as to identify an economic and effective route for improving astaxanthin production by detecting the changes in intracellular metabolites. Consequently, an astaxanthin overproducing mutant *P. rhodozyma* strain, JMU-MVP14, along with the three other strains called JMU-VDL668, JMU-7B12, and JMU-17W, was employed in this research. Cell growth, astaxanthin synthesis, ethanol accumulation, and changes in intracellular protein and fatty acid contents were examined.

## 2. Materials and methods

### 2.1. Yeast strain

*P. rhodozyma* strains JMU-MVP14, JMU-VDL668, JMU-17W, and JMU-7B12 were used in the experiments. *P. rhodozyma* JMU-VDL668, JMU-7B12, and JMU-17W strains in our laboratory originated from *P. rhodozyma* Past-1 (generously provided by professor Ulf Stahl, Berlin Industrial College, Germany). The astaxanthin overproducing mutant *P. rhodozyma* JMU-MVP14 was established through ethyl methylsulfonate mutagenesis from JMU-VDL668. These strains were stored in 15% (v/v) glycerol at -70°C.

### 2.2. Media

The medium for inoculum culture and fermentation was composed of the following (per liter): glucose, 20 g; $(NH_4)_2SO_4$, 5 g; $KH_2PO_4$, 2 g; $MgSO_4 \times 7H_2O$, 1 g; $CaCl_2 \times H_2O$, 0.01 g; $H_3BO_3$, 0.00267 g; $CuSO_4 \times 5H_2O$, 0.0008 g; KI, 0.00027 g; $MnCl_2 \times 4H_2O$, 0.00267 g; $Na_2MoO_4 \times 2H_2O$, 0.00107 g; $ZnSO_4 \times 7H_2O$, 0.012 g; $CoCl_2 \times 6H_2O$, 0.0008 g; $FeSO_4 \times 7H_2O$, 0.008 g; pantothenic acid, 0.00267 g; biotin, 0.00013 g; inositol, 0.06667 g; nicotinic acid, 0.00267 g; $H_2NC_6H_4COOH$, 0.00053 g; Vitamin $B_6$, 0.00267 g; and Vitamin $B_1$, 0.00267 g.

### 2.3. Culture conditions

The strains were activated by inoculation into a 250 mL flask containing 30 mL of inoculum media and cultured on a rotary shaker at 22°C and 190 r/min for 48 h. Inoculums were transferred to 150 mL fermentation medium in a 500 mL flask at a ratio of 3% (v/v). Fermentation was performed at 22°C and 190 r/min for 120 h. The initial pH was 6.0. All the shake flask experiments were performed at least in quadruplicate.

### 2.4. Biomass detection

For dry weight determinations, samples of 2.5 mL were centrifuged for 5 min at 3500 r/min, and cells were washed twice with distilled water. The washed cells were dried to constant weight at 105°C.

### 2.5. Residual sugar concentration determination

Samples of 2.5 mL were centrifuged for 5 min at 3500 r/min, and supernatants were collected to measure residual sugars. Then, 1 mL of supernatant was mixed with 3 mL of 3,5-dinitrosalicylic acid and then cooled rapidly after a boiling water bath of 15 min. Absorbance was detected at 520 nm.

### 2.6. Carotenoid extraction and astaxanthin detection

Carotenoid was extracted by dimethysulfoxide method: 2.5 mL of samples were centrifuged for 5 min at 3500 r/min, and the cells were washed twice with distilled water. After decanting the water, the cells were then treated with 2 mL of dimethylsulfoxide at 75°C. Next, 5 mL of ethanol was used to extract carotenoid. Astaxanthin concentration was detected at a wavelength of 474 nm [32].

### 2.7. Ethanol analysis

Ethanol was analyzed using VARIAN GC3800 gas–liquid chromatography with a flame ionization detector (FID). Chromatography was equipped with HP-INNOWAX column (Agilent, diameter: 0.25 mm, length: 30 m, Classification Codes: 19091N-133, Sequence numbers: USB220566H) and the temperature program of the column ranged from 120°C to 240°C at 3°C/min. The temperatures of the FID and injector were 300°C and 220°C, respectively. The nitrogen (99.999%) flow rate was 1.5 mL/min.

### 2.8. Protein analysis

For protein analyses, 2.5 mL of each sample was centrifuged for 5 min at 3500 r/min and washed once with distilled water. The washed cells (samples of 2.5 mL each) were resuspended in 0.5 mL of 1 N NaOH and disrupted for 15 min in a bath at 92°C. Once cooled, the samples were diluted to 2.5 mL with distilled water and were analyzed by Coomassie brilliant blue method (detected at the wavelength of 595 nm) with bovine serum albumin (Sigma Chemical Co.) as standard [21].

### 2.9. Fatty acid extraction and detection

Samples of 2.5 mL each were centrifuged for 5 min at 3500 r/min and washed once with distilled water. The washed cells were broken with 4 mol/L of HCl solution in a boiling water bath. The fatty acids were extracted by $CHCl_3$:$CH_3OH$ (1:1) and analyzed by the vanillin dyeing method [33].

## 3. Results

Four *P. rhodozyma* strains were cultured in a chemically defined medium with the original glucose concentration of 20 g/L. Fig. 1 shows a comparison of biomass, volumetric productivity of astaxanthin, residual sugar concentration, and ethanol content among different strains.

In the lag phase, an inconspicuous change in biomass, sugar, and astaxanthin contents can be seen in Fig. 1. Fast growth synchronized with fast sugar consumption was observed from 24 h to 60 h during the experiment, followed by the stable ferment period after 60 h (Fig. 1a and b). The biomass of *P. rhodozyma* JMU-17W strain reached a maximum of 8.2 g/L at 60 h, followed by a decreasing trend of growth upon sugar exhaustion. JMU-VDL668 and JMU-7B12 had similar biomass contents at 3.7 and 3.5 g/L, respectively. Sugar consumption slowed down, and final residual sugar concentrations were 5.6 and 9.6 g/L, separately. A negative drop in sugar was observed during the whole fermentation of the JMU-MVP14 strain, which obtained the lowest biomass content at only 2.4 g/L.