

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



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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).

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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].

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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; $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 2 g; $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 1 g; $\text{CaCl}_2 \times \text{H}_2\text{O}$, 0.01 g; H_3BO_3 , 0.00267 g; $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 0.0008 g; KI, 0.00027 g; $\text{MnCl}_2 \times 4\text{H}_2\text{O}$, 0.00267 g; $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$, 0.00107 g; $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 0.012 g; $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, 0.0008 g; $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, 0.008 g; pantothenic acid, 0.00267 g; biotin, 0.00013 g; inositol, 0.06667 g; nicotinic acid, 0.00267 g; $\text{H}_2\text{NC}_6\text{H}_4\text{COOH}$, 0.00053 g; Vitamin B₆, 0.00267 g; and Vitamin B₁, 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 dimethylsulfoxide 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 $3^{\circ}\text{C}/\text{min}$. 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 $\text{CHCl}_3:\text{CH}_3\text{OH}$ (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.

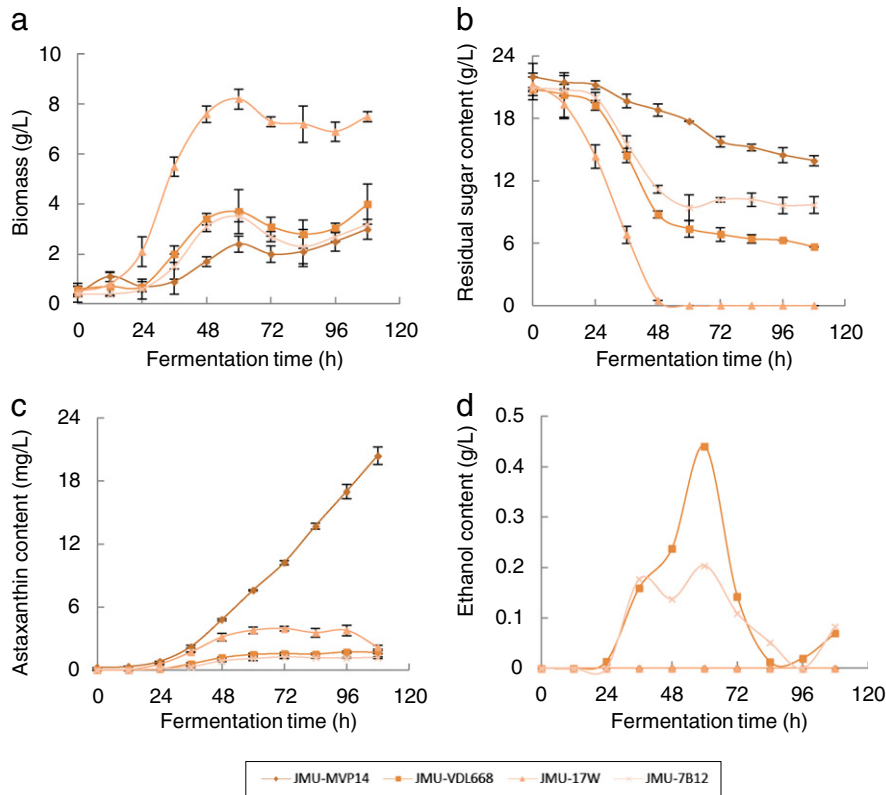


Fig. 1. Development of culture parameters for different *P. rhodozyma* strains. Culture conditions: 190 r/min, 22°C, pH 6.0. (a) Biomass; (b) reducing sugars content; (c) astaxanthin concentration; (d) ethanol content.

Fig. 1c shows that the accumulation of astaxanthin was linked with biomass during batch cultures of *P. rhodozyma* JMU-VDL668, JMU-17W, and JMU-7B12. Once the stable phase was reached, the rise of astaxanthin leveled off. However, when fermented with *P. rhodozyma* JMU-MVP14 strain, the increasing trend of biomass and astaxanthin remained consistent before 60 h, after which the astaxanthin content exhibited linear growth after cell growth retardation. As an astaxanthin overproducing mutant strain, JMU-MVP14 reached a volumetric astaxanthin productivity of 20.4 mg/L, which was significantly higher than that of the other three strains. The highest biomass of the JMU-17W strain encouraged astaxanthin accumulation in the medium up to 3.9 mg/L. The astaxanthin production of JMU-VDL668 and JMU-7B12 was only 1.7 and 1.2 mg/L, respectively.

Related research shows that aerobic fermentation in the presence of a fully aerobic culture and high sugar level conditions can result in reduced biomass yield and the formation of fermentation products [34]. Ethanol concentration was detected in this work, and ethanol accumulation only occurred in medium when fermented by *P. rhodozyma* JMU-VDL668 and JMU-7B12 (Fig. 1d). The rapid increase in ethanol concurred with rapid growth when sugar was available in the culture medium. The ethanol content of JMU-VDL668 reached a maximum of 440.8 mg/L at 60 h, whereas the highest ethanol concentration of JMU-7B12 was 203.7 mg/L. However, after reaching the peak at 60 h, ethanol concentration was depleted along with the slow increase in both biomass and astaxanthin concentration (Fig. 1a and c). Meanwhile, the biomass contents of the two strains were at a relatively low level compared with *P. rhodozyma* JMU-17W, and their astaxanthin concentrations were significantly lower than JMU-MVP14. For *P. rhodozyma* strain JMU-MVP14, sugar absorption and utilization were slower than those of the other three strains, which resulted in low biomass. This condition may be attributed to the fact that the Crabtree effect during batch fermentation processes with high initial

glucose concentration results in overflow metabolism and accumulation of ethanol, which cause significant reductions in biomass and astaxanthin production [15,34].

To analyze the relationship between growth anabolic processes and astaxanthin production, protein and fatty acids were also detected. Fig. 2 shows the dynamic changes in protein and fatty acids, as well as astaxanthin content, in cells of the four strains considered in this study.

The variation of the intracellular metabolite content of *P. rhodozyma* strain JMU-MVP14 is illustrated in Fig. 2a. The protein content in cells changed sharply in the earlier stage of fermentation and then reduced quickly after reaching the peak of approximately 139.0 mg/g at 36 h. The value was maintained at approximately 20.0 mg/g in the stable phase. The change in fatty acid content in cells was similar to that of protein but did not exhibit synchronism. Meanwhile, fatty acid content exhibited an increase similar to that of astaxanthin after reaching the minimum value at 36 h. In the stable phase, the fatty acid content was 110.4 mg/g on average. The astaxanthin content in cells exhibited a slight decrease within the first 12 h then underwent rapid growth. This trend was different from the change in protein. The specific productivity of astaxanthin reached a maximum of 6.8 mg/g at 96 h.

Different results are shown in Fig. 2b. The protein and fatty acid concentrations increased with astaxanthin content in cells but decreased slightly at the beginning of the fast growth phase. Thereafter, a progressive drop in intracellular fatty acid and protein contents occurred with astaxanthin accumulation. Fatty acid content in cells then increased again, similar to the change in astaxanthin concentration, finally reaching 136.4 mg/g on average. Meanwhile, protein content was stable at approximately 28.2 mg/g. The specific productivity of astaxanthin was merely 0.6 mg/g, which was significantly lower than that of JMU-MVP14.

In the whole growth process, the intracellular protein and astaxanthin contents of *P. rhodozyma* JMU-17W changed in the same

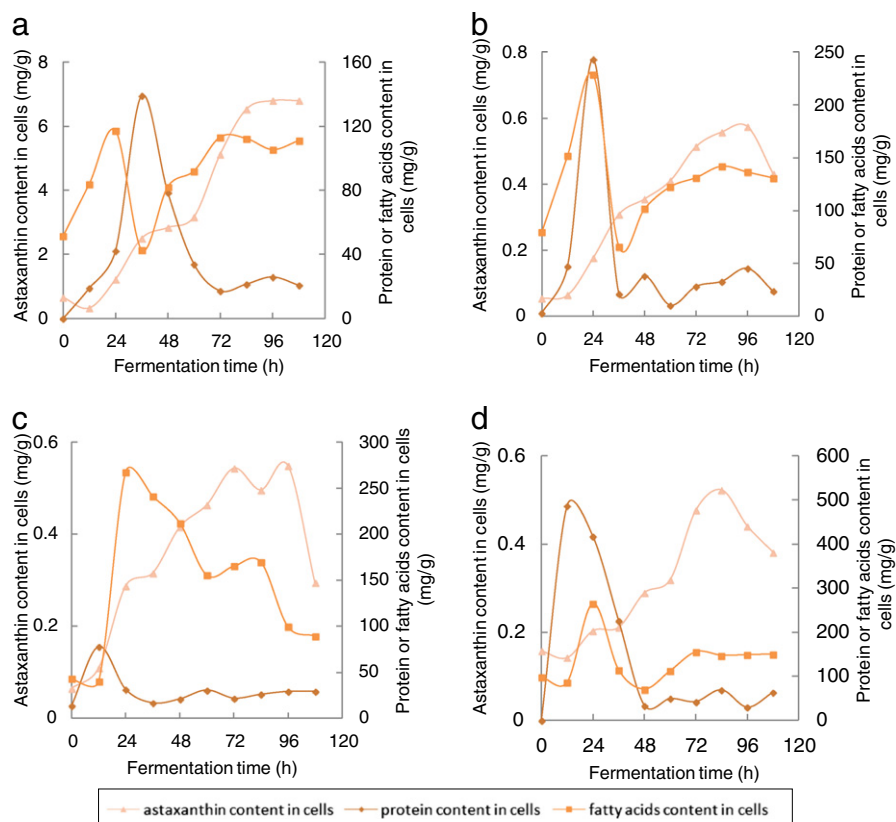


Fig. 2. Relationship between astaxanthin production and anabolism processes of different *P. rhodozyma* strains. Culture conditions: 190 r/min, 22°C, pH 6.0. (a) JMU-MVP14; (b) JMU-VDL668; (c) JMU-17W; (d) JMU-7B12.

manner as those of JMU-MVP14 and JMU-VDL668 (Fig. 2c). Fatty acid content in cells rapidly increased and reached values close to 268.0 mg/g, followed by a rapid increase in astaxanthin content. However, in the decline phase, significantly reduced fatty acid and astaxanthin contents were observed mainly because of the absence of sugar, which resulted in a decrease in biomass. The final intracellular protein content was approximately 25.0 mg/g, whereas astaxanthin content in cells reached a maximum of 0.6 mg/g, which exhibited no difference from that of JMU-VDL668.

The intracellular protein and fatty acid contents of *P. rhodozyma* JMU-7B12 rapidly increased and leveled off at values of approximately 47.8 and 150.0 mg/g, respectively. These values were significantly higher than those of the other three strains (Fig. 2d). The astaxanthin productivity of the four strains was the lowest during fermentation at only 0.5 mg/g.

4. Discussion and conclusion

A metabolic network consists of the Embden–Meyerhof–Parnas pathway, the pentose phosphate pathway, the TCA cycle, and the mevalonate pathway in which astaxanthin is present in *P. rhodozyma* [35]. Thus, the entry of carbon sources into these alternative pathways may have a significant effect on the astaxanthin synthesis, which is somehow regulated. For example, Flores-Cotera et al. [21] reported that when citrate is supplemented in the medium at levels of 28 mM or higher, the increase in carotenoid concentration in cells was parallel to the decrease in protein synthesis. In this study, the relationship between astaxanthin synthesis and intense anabolic processes was analyzed by comparing the changes in intracellular protein and fatty acid contents of four *P. rhodozyma* strains.

The primary metabolism is generally active at the beginning of cultivation because of the optimum allocation of factors in the cell anabolic process. Vustin et al. [22] found that the intense anabolic processes coupled with active culture growth during the first 24 h inhibited carotenoid synthesis. A similar case was found in this study in that intracellular protein content increased significantly while astaxanthin content decreased slightly in the lag phase (see Fig. 1b and Fig. 2). A large number of intracellular metabolites may have accumulated for the rapid schizogamy of the upcoming exponential phase, which have considerably affected the quantity of carbon, ATP, and NADPH channeled into protein synthesis. Thus, the carbon flux toward astaxanthin was reduced, thus resulting in slow astaxanthin synthesis. Biomass rapidly increased in the exponential phase (Fig. 1a). The intracellular protein content sharply decreased, whereas the astaxanthin content in cells presented a growth trend (Fig. 2). This finding may be attributed to the rapid growth of cells, which cause a change in the culture environment change, such that astaxanthin accumulated as in response to environmental stress [36,37]. Previous studies have showed that astaxanthin synthesis in *P. rhodozyma* required a large amount of ATP and acetyl-CoA, which were used as precursor substances for carotenoid synthesis [27,38]. Meanwhile, acetyl-CoA is an important intermediate that connects primary and secondary metabolism. Thus, when the carbon demands for protein synthesis drop, ATP and acetyl-CoA, as key factors, switch the excess carbon source from TCA respiratory to astaxanthin and fatty acid synthesis [21,39]. Fig. 2 shows that astaxanthin production continued after the cessation of cell growth, and intracellular protein content reached a stable level.

The results also showed that the astaxanthin overproducing strain JMU-MVP14, which was established through EMS mutagenesis from JMU-VDL668, had the lowest protein content (approximately

20.0 mg/g) compared with the other three strains. In contrast, *P. rhodozyma* JMU-7B12 obtained the highest intracellular protein content, which reached 47.8 mg/g, but had the lowest astaxanthin concentration of only 1.2 mg/L. These data suggested that a certain degree of protein synthesis inhibition could increase astaxanthin production. Therefore, selecting an amino acid-deficient strain through breeding methods [40] or adopting a metabolic regulation method to inhibit protein synthesis [21,22,24] can be an effective method to improve astaxanthin yield.

P. rhodozyma is known as a kind of oleaginous yeast and can accumulate a large quantity of lipids [23]. Thus, as an essential component of the cell membrane, a large number of fatty acids should be accumulated to meet the needs of cell growth. When the change in intracellular fatty acid content was studied, the trend was found to be similar to that of protein in the early phase of fermentation with drastic increases and decreases (Fig. 2). In addition, some other interesting phenomena could be observed. Fatty acid content in cells exhibited a slight increasing trend, whereas intracellular protein content reached a relative stable value. In particular, the increasing trends of *P. rhodozyma* JMU-MVP14 and JMU-VDL668 were evident (Fig. 2). Fatty acid and astaxanthin biosyntheses in *P. rhodozyma* are known to share several common precursors, including the requirement for ATP, NADPH, molecular oxygen, and acetyl-CoA [19]. The TCA cycle is the sole source of ATP. Thus, when protein synthesis is inhibited, the redundant ATP and NADPH are used for other metabolic pathways, such as those for fatty acid and carotenoid syntheses.

This study compared the four *P. rhodozyma* strains and then found another link between fatty acids and astaxanthin. The *P. rhodozyma* of JMU-MVP14 had the lowest intracellular fatty acid content, whereas the specific productivity of astaxanthin was 6.8 mg/g (Fig. 2). When compared with the other three *P. rhodozyma* strains, the reduced fatty acid metabolic pathways may be another important reason for the high astaxanthin yield of *P. rhodozyma* JMU-MVP14. This finding suggested that the decrease in fatty acid biosynthesis, which led to precursor accumulation and transferred to the astaxanthin pathway, resulted in the enhancement of astaxanthin [41].

With the examination of different *P. rhodozyma* strains in this research, astaxanthin content in cells was correlated with intracellular protein and fatty acid content. Despite its preliminary characteristics, astaxanthin biosynthesis in *P. rhodozyma* was closely linked with anabolic processes. The results indicated that intracellular protein changed significantly throughout the fermentation process, exhibiting an increase in the lag phase, a decrease in the exponential phase, and a leveling off in the stationary phase. The changes in fatty acid content exhibited a trend similar to that of protein, with the exception of a slight up regulation in the stationary phase. The accumulation of astaxanthin had competitive effects on intracellular protein and fatty acid synthesis, such that the changes in astaxanthin content and that in intracellular protein and fatty acid contents were opposite. As one of the four astaxanthin-producing strains selected in our laboratory, JMU-MVP14 possessed low protein and fatty acid contents but high astaxanthin yield (6.8 mg/g). Compared with other astaxanthin-producing *P. rhodozyma* strains, ethanol accumulated in the fermentation process of JMU-VDL668. JMU-17W had the highest biomass of 7.5 g/L, which was more than twice that of the other three strains. Moreover, this strain consumed a large amount of sugar. JMU-7B12 obtained the highest protein and fatty acid contents in cells (47.8 and 150.0 mg/g, respectively), whereas astaxanthin production was the lowest (1.2 mg/L), and ethanol accumulated in the medium. These results suggested that in the follow-up work, artificial mutation, gene engineering, auxotroph strain screening, and other means will effectively enhance astaxanthin production in *P. rhodozyma* by reducing the metabolic flows of protein and fatty acid syntheses.

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Conflict of interest

None.

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