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Alleviation of metabolic bottleneck by combinatorial engineering enhanced astaxanthin synthesis in *Saccharomyces cerevisiae*

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ABSTRACT

Highly efficient biosynthesis of the commercially valuable carotenoid astaxanthin by microbial cells is an attractive alternative to chemical synthesis and microalgae extraction. With the goal of enhancing heterologous astaxanthin production in *Saccharomyces cerevisiae*, metabolic engineering and protein engineering were integrated to improve both the expression and activity of rate-limiting enzymes. Firstly, to increase the supply of β-carotene as a key precursor for astaxanthin, a positive mutant of GGPP synthase (CrtE03M) was overexpressed together with three other rate-limiting enzymes tHMG1, CrtI and CrtYB. Subsequently, to accelerate the conversion of β-carotene to astaxanthin, a color screening system was developed and adopted for directed evolution of β-carotene ketolase (OBKT), generating a triple mutant OBKTM (H165R/V264D/F298Y) with 2.4-fold improved activity. After adjusting copy numbers of the above-mentioned rate-limiting enzymes to further balance the metabolic flux, a diploid strain YastD-01 was generated by mating two astaxanthin-producing haploid strains carrying the same carotenogenic pathway. Finally, further overexpression of OCrtZ and OBKTM in YastD-01 resulted in accumulation of 8.10 mg/g DCW (47.18 mg/l) of (3*S*, 3′*S*)-astaxanthin in shake-flask cultures. This combinatorial strategy might be also applicable for alleviation of metabolic bottleneck in biosynthesis of other value-added products, especially colored metabolites.

1. Introduction

As a member of the carotenoids family, astaxanthin has emerged as a commercially valuable molecule with protective potential in humans due to its antioxidative, anti-inflammatory, anticancer feature [1]. Its wide applications in nutraceutical, pharmaceutical, and cosmetic industries, as well as in aquaculture lead to a fast growing world market [2]. Considering the limited productivity of astaxanthin via extraction from natural producers such as *Haematococcus pluvislis* and the biosafety issues of chemical synthesis [3], microbial production of astaxanthin via metabolic engineering becomes an attractive alternative [4,5]. GRAS (generally regarded as safe) nature, well-characterized genetic background, superior stress tolerance and excellent fermentation properties make *S. cerevisiae* a promising microbial chassis for fermentative production of astaxanthin [6]. In our previous work, we have significantly enhanced astaxanthin production in *S. cerevisiae* via introduction of codon-optimized CrtZ and BKT from *H. pluvislis*, giving an astaxanthin yield of 4.70 mg/ g DCW in shake-flask cultures [7]. The astaxanthin configuration was later confirmed to be optically pure (3*S*, 3′*S*) as in *H. pluvislis* (Fig. S1), which is generally deemed to have much higher antioxidant activity than the other two isomers [8]. In contrast, *Xanthophyllomyces dendrorhous* produces pure (3*R*, 3′*R*) astaxanthin whereas the chemically synthetic astaxanthin is a mixture of all three isomers (3*R*, 3′*R*; 3*S*, 3′*S*; and 3*R*, 3′*S*).

Despite its desirable configuration, the astaxanthin titer was still not satisfactory. Analysis of fermentation products implied shortage in the supply of precursors such as β-carotene. Therefore, elevation of precursor flux might be an efficient approach to further enhancement of astaxanthin. Astaxanthin biosynthesis pathway in *S. cerevisiae* constitutes of the endogenous mevalonate (MVA) pathway and the exogenous carotenogenic pathway containing phytoene synthase (CrtB or CrtYB), phytoene desaturase (CrtI), lycopene cyclase (CrtY or CrtYB), β-carotene ketolase (CrtW or BKT) and β-carotene hydroxylase (CrtZ) (Fig. 1) [9]. As the basic building block of carotenoids biosynthesis, GGPP synthesis catalyzed by GGPP synthase (CrtE) represents the first

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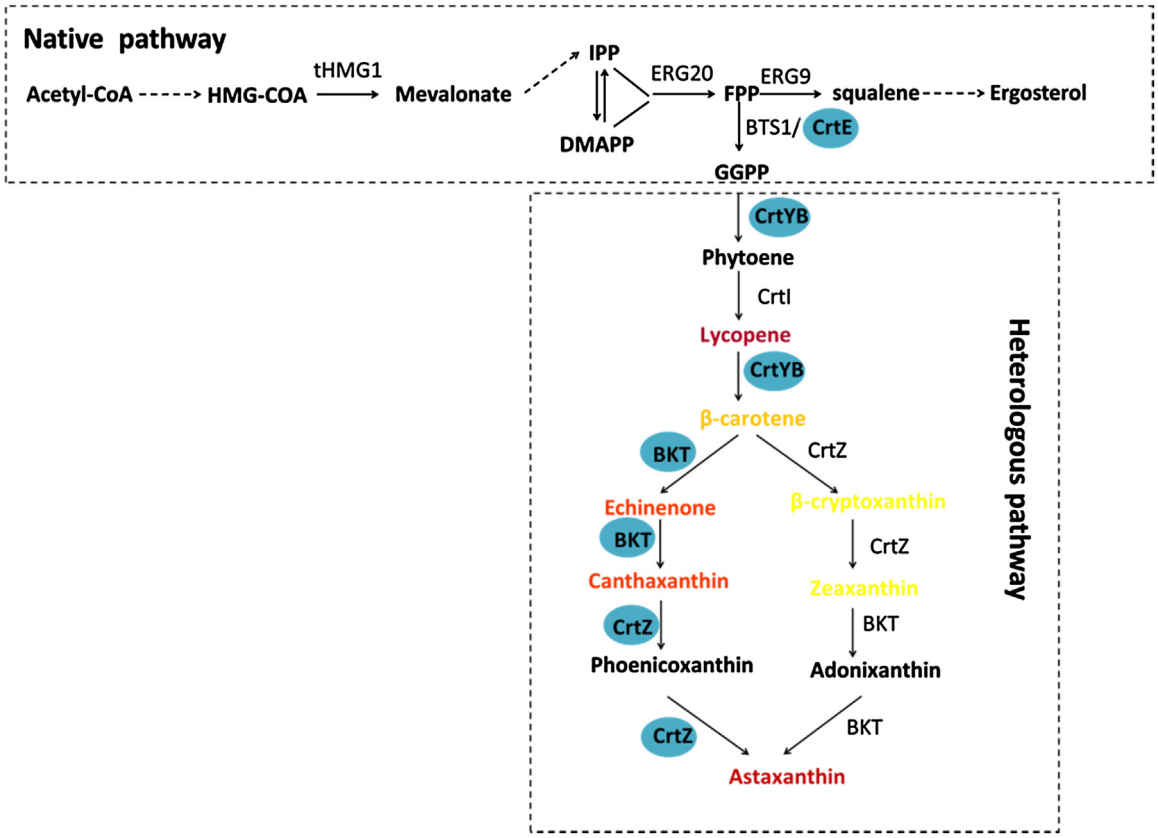

Fig. 1. Overview of astaxanthin biosynthesis pathway in *S. cerevisiae*. tHMG1: Truncated HMG-CoA reductase; ERG20: Farnesyl diphosphate (FPP) synthase; ERG9: Squalene synthase; BST1 (CrtE): Geranylgeranyl diphosphate synthase; CrtYB: Phytoene synthase/lycopene cyclase; CrtI: Phytoene desaturase; CrtZ: β-carotene hydroxylase; BKT: β-carotene ketolase. The heterologous genes *crtE*, *crtI*, *crtYB* are from *X. dendrorhous*, while *crtZ* and *bkt* are from *H. pluvialis*. Dashed arrows indicate multiple reaction steps. The rate-limiting enzyme were highlighted with blue ellipses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

committed step to divert the flow of intracellular FPP from other competing products such as dolichol, ubiquinone and sterols to the targeted carotenoids [10]. To enhance GGPP supply, overexpression and directed evolution of CrtE has been demonstrated as an efficient strategy. By creating and integrating the positive CrtE mutant CrtE03M into the lycopene biosynthesis pathway, a 2.2-fold enhancement of lycopene accumulation was achieved in *S. cerevisiae* [11]. Meanwhile, the significant reduction of squalene accumulation suggested that CrtE03M indeed rerouted the flux of FPP from the competing pathway to the target metabolite. As the next step of GGPP synthesis and the first step in carotenoid synthesis, phytoene synthase (encoded by the dual-functional enzyme CrtYB from *X. dendrorhous*) is another rate-controlling enzyme in the carotenogenic pathway, the overexpression of which has successfully stimulated carotenogenesis [12]. In addition, as a major rate-limiting enzyme in the MVA pathway, overexpression of 3-hydroxymethyl-3-glutaryl coenzyme A reductase (HMG-CoA reductase) and its truncated form (tHMG1) has been adopted as a common and efficient strategy to enhance isoprenoids production in yeast [13,14]. Taken together, overexpression of the above-mentioned rate-limiting enzymes might improve the β-carotene supply and thus contribute to further enhancement of astaxanthin synthesis.

Aside from precursor supply, the reactions catalyzed by β-carotene hydroxylase and β-carotene ketolase constitute the rate-limiting steps in the lower part of astaxanthin biosynthetic pathway. Protein engineering is an efficient strategy for improving enzyme catalytic activity. For both enzymes, there is so far no available crystal structure and the catalytic mechanism remains unclear [15]. Moreover, the substrate specificity of β-carotene ketolase and β-carotene hydroxylase differs among different organisms, yielding different metabolite intermediates during conversion of β-carotene to astaxanthin [16]. We have determined the substrate preference of CrtZ and BKT from *H. pluvialis* in a previous study, and found that β-carotene was first ketolated by BKT to echinenone and canthaxanthin, and then hydroxylated to form astaxanthin [7]. Since β-carotene grants the accumulating colonies yellow color while accumulation of echinenone and canthaxanthin results in red-colored colonies, it should be possible to develop a color-based high throughput screening method for directed evolution of BKT in yeast. Directed evolution of CrtW from *Paracoccus* sp. strain N81106 [17] and *Sphingomonas* sp. DC18 [18] employing similar color-based screening systems has successfully improved astaxanthin synthesis in *Escherichia coli*. Therefore, it is highly possible that the astaxanthin yield in *S. cerevisiae* could be enhanced by creation and adoption of a BKT mutant screened based on the color change of the colonies.

The efficiency of biosynthesis is not only determined by the activity of single rate-limiting enzymes but more importantly also by their balanced expression. In our earlier work, we have observed reduction in biomass upon codon optimization and overexpression of CrtZ and BKT, despite the improved astaxanthin yield [7]. Therefore, there is an implicit requirement for avoiding cellular burden caused by excessive protein expression and accumulation of cytotoxic intermediates [19,20]. In order to achieve the balance between cell growth and metabolite accumulation, the copy numbers of key pathway enzymes should be carefully adjusted [21].