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Advances and trends for astaxanthin synthesis in *Phaffia rhodozyma*

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Abstract
Astaxanthin, a carotenoid endowed with potent antioxidant capacity, exhibits considerable application prospects in nutraceuticals, pharmaceuticals, and cosmetics. In contrast to the chemical synthesis method, the biosynthesis of astaxanthin is undoubtedly a greener and more environmentally friendly production approach. In this review, we comprehensively review the biosynthetic pathways and multiple strategies for astaxanthin synthesis in *Phaffia rhodozyma*. Some biotechnology advancements for increasing the yield of astaxanthin in *Phaffia rhodozyma* encompass mutagenesis breeding, genetic modification, and optimizing fermentation conditions, thereby opening up new avenues for its application in functional foods and feed. Nevertheless, the yield of product synthesis is constrained by the host metabolic stoichiometry. Besides breaking the threshold of astaxanthin production and alleviating the impact of astaxanthin accumulation on cell growth, a comprehensive comprehension of multiple interconnected metabolic pathways and complex regulatory mechanisms is indispensable for significantly enhancing astaxanthin production. This review presents some prospects of integrating digital concepts into astaxanthin production to aid in overcoming current challenges.

Keywords Astaxanthin, *Phaffia rhodozyma*, Fermentation, Antioxidants, Metabolic engineering

Introduction

Astaxanthin, a secondary carotenoid possessing strong antioxidant capacity, is a more potent antioxidant than Coenzyme Q10 or vitamin E, being 65 times more active than vitamin C [1]. It can eliminate nitrogen dioxide, sulfide, disulfide, etc. Moreover, it can alleviate lipid peroxidation and effectively inhibit the free radicals caused by lipid peroxidation. Simultaneously, astaxanthin possesses numerous physiological effects, such as inhibiting tumorigenesis, enhancing immunity, and eliminating free radicals in the body, etc. Moreover, astaxanthin also exhibits pharmacological effects in various diseases, has a favorable therapeutic outcome for skin cancer induced by ultraviolet light, and has a preventive and therapeutic impact on eye diseases caused by diabetes [2]. It has extensive application prospects in health care products, medicine, cosmetics, food additives, and aquaculture [3]. It is safe for human body and does not have the typical pro-oxidation effect of other carotenoids. Due to these benefits and applications, it has garnered significant global attention, and the demand and market size of astaxanthin has escalated rapidly. The global astaxanthin market is expected to increase from US $1.37 billion in 2020 to US $3.5 billion by 2026, at a compound annual growth rate of 16.8% [4]. Astaxanthin serves as an ideal pigment source in the aquaculture and food industry on account of its safety and stable light red hue.

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Currently, astaxanthin is primarily acquired through chemical synthesis and microbial fermentation. The chemical synthesis method is the predominant means for preparing astaxanthin in the market. However, chemical synthesis of astaxanthin is not only costly, but also generates numerous by-products with unclear structures and is environmentally unfriendly. Its products are mainly utilized as industrial raw materials. The regulation of chemical synthesis of astaxanthin worldwide is increasing strict, which promotes the extraction of natural astaxanthin gradually assume a dominant position. At present, the principal sources of microbial synthesis of natural astaxanthin are *Haematococcus pluvialis* and *Phaffia rhodozyma* (also known as *Xanthophyllomyces dendrorhous*) [5]. The different optical isomers of astaxanthin depend on the diverse microbial sources, the chemical synthesis method produces 3R, 3'S-astaxanthin, *H. pluvialis* produces 3 S,3'S-astaxanthin [6] and *P. rhodozyma* produces 3R,3'R-astaxanthin [7]. In addition, the bioactivity of the different isomers is slightly different. Among them, 3 S, 3'S-astaxanthin showed the highest antioxidant capacity, followed by 3R, 3'R-astaxanthin, and 3R, 3'S-astaxanthin showed lower antioxidant capacity. Studies based on model organisms have shown that the anti-aging activities of 3 S,3'S-astaxanthin and 3R,3'R-astaxanthin are slightly different [8]. Nevertheless, 3R,3'R-astaxanthin can significantly alleviate obesity and promote intestinal microbiota health [9]. These instances prove that both 3 S,3'S -astaxanthin and 3R,3'R -astaxanthin have excellent biological activity and potential application prospects. Though the content of astaxanthin in *P. rhodozyma* is lower than that in *H. pluvialis*, *P. rhodozyma* has the benefits of rapid self-propagation, simple culture conditions and media, and is not influenced by weather and climate. Furthermore, astaxanthin is the most important carotenoid in *P. rhodozyma*, accounting for 90% of the total carotenoid [10], and the production efficiency can be enhanced through industrial fermentation. From the perspective of astaxanthin composition, astaxanthin in *H. pluvialis* exists in free, monoester and diester forms, while astaxanthin in *P. rhodozyma* exists in free form, which is easier to purify and prepare. Moreover, *P. rhodozyma* possesses the pathway and enzyme system necessary for astaxanthin synthesis, which makes it easier to regulate and optimize the production process [11].

The regulation of astaxanthin biosynthesis and metabolism is primarily aimed at increasing the yield of astaxanthin, which can be accomplished by optimizing fermentation conditions, random mutagenesis and metabolic engineering [12]. Nevertheless, the yield of astaxanthin is still significantly lower than that of other fermentation products. The main cause is that traditional breeding and fermentation process optimization methods such as artificial mutagenesis and protoplast fusion are not reasonable, and the breeding efficiency of high-yielding strains of astaxanthin is low. The prerequisite for rational breeding is to deeply comprehend the metabolic pathway and regulatory mechanism of astaxanthin biosynthesis. Transcription factor (TF), as an essential regulatory protein, also plays an important role in activating or inhibiting the expression of its target genes [13]. The study of transcription factors will assist in understanding the regulatory mechanism of key genes in astaxanthin biosynthetic metabolic flow, and provide theoretical methods for exploring the regulatory pathways of similar metabolites. Furthermore, astaxanthin biosynthesis can be initiated by diverse nutritional and environmental conditions, thereby constantly endeavoring to maintain the global reduction-oxidation (REDOX) balance. During this process, the cells can safeguard themselves from conditions conducive to the emergence of severe intracellular oxidative stress [14]. In this review, we first comprehensively examine the pathways of astaxanthin biosynthesis in *P. rhodozyma*. Secondly, the transcription factors related to metabolic engineering and the factors that boost the production of astaxanthin in *P. rhodozyma* are elaborately introduced. Finally, the new technologies that can be employed to facilitate the production of high-yielding astaxanthin in *P. rhodozyma* are prospected.

Biosynthesis of astaxanthin in *P. rhodozyma*

The astaxanthin pathway in *P. rhodozyma* is well established [15], commencing with the conversion of carbon sources (glucose, xylose, etc.) to pyruvate. Subsequently, via the glycolytic pathway, the pyruvate dehydrogenase complex is transformed to acetyl-CoA, which is then oxidized to catalyze the subsequent reaction. Additionally, a portion of acetyl-CoA enters the tricarboxylic acid cycle (TCA), generating large quantities of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), which furnish energy and reducing power for subsequent substance conversion. The biosynthesis of astaxanthin in *P. rhodozyma* is accomplished through the mevalonate pathway, isoprene biosynthesis pathway, and carotenoid synthesis pathway. Acetyl-acetyl-CoA from glycolysis is initially synthesized by the condensation of two acetyl-CoA molecules through acetyl-CoA acetyltransferase (AACT), and acetyl-acetyl-CoA is further condensed with acetyl-acetyl-CoA molecules by hydroxymethylglutaryl-CoA synthase (HMGS) to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) [16]. Subsequently, the reduction step carries out by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) results in the formation of mevalonate. Thereupon, isopentenyl pyrophosphate (IPP) is generated through a three-step reaction mediated by mevalonate kinase (MK), phosphomevalonate kinase (PMK), and ultimately mevalonate diphosphodecarboxylase (MVD).

IPP is a significant constituent and common precursor for the endogenous synthesis of carotenoids, monoterpenes, sesquiterpenes, sterols, gibberellins, and other compounds in fungi [16]. Then it enters the isoprene biosynthesis pathway, where the formed IPP is isomerized to dimethylallyl pyrophosphate (DMAPP) by bifunctional IPP isomerase (idi) [9], and then condensation of the IPP and DMAPP produces through the mevalonate (MVA) pathway by geranyl pyrophosphate synthase (ERG20) to obtain geranyl pyrophosphate (GPP). Subsequently, it is converted to farnesyl pyrophosphate (FPP) under farnesyl diphosphate synthase (ERG20), and then geranylgeranyl pyrophosphate (GGPP) is synthesized by GGPP synthase (CrtE) [17, 18]. It then enters the carotenoid synthesis pathway, where GGPP is catalyzed by phytoene-β-carotene synthase (CrtYB) to produce the phytoene [19]. Subsequently, phytoene is converted to lycopene through the continuous action of phytoene desaturase (CrtI) [20, 21] Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *P. rhodozyma*. β-carotene is then formed from lycopene by lycopene cyclase (CrtYB) [21]. Subsequently, β-carotene is transformed to astaxanthin in astaxanthin synthetase (CrtS) along with cytochrome P450 reductase (CrtR) (Fig. 1) [22, 23]. The enzymes encoded by CrtYB and CrtS are bifunctional; the former catalyzes the formation of phytoene and lycopene, and the latter possesses oxygenase and hydroxylase activity that converts β-carotene to astaxanthin.

Enhancing astaxanthin production of *P. rhodozyma* through biotechnology

The application of biotechnology to increase astaxanthin production of *P. rhodozyma* is a hot research topic in biomedicine and food industry. At present, the main biotechnologies to increase astaxanthin yield of *P. rhodozyma* focus on mutagenesis breeding, genetic modification and fermentation process optimization (Fig. 2). Through mutagenesis, gene editing (such as overexpression of astaxanthin synthesis rate-limiting enzyme gene, knocking out marginal metabolism gene) and other techniques, several high-yield strains have been screened. The application of some frontier biotechnologies can also further understand the metabolic pathways and regulatory mechanisms of astaxanthin, and provide promising alternatives for astaxanthin industrial production.

Random mutagenesis

The most prevalent direct method to augment the astaxanthin production of *P. rhodozyma* is mutagenesis for

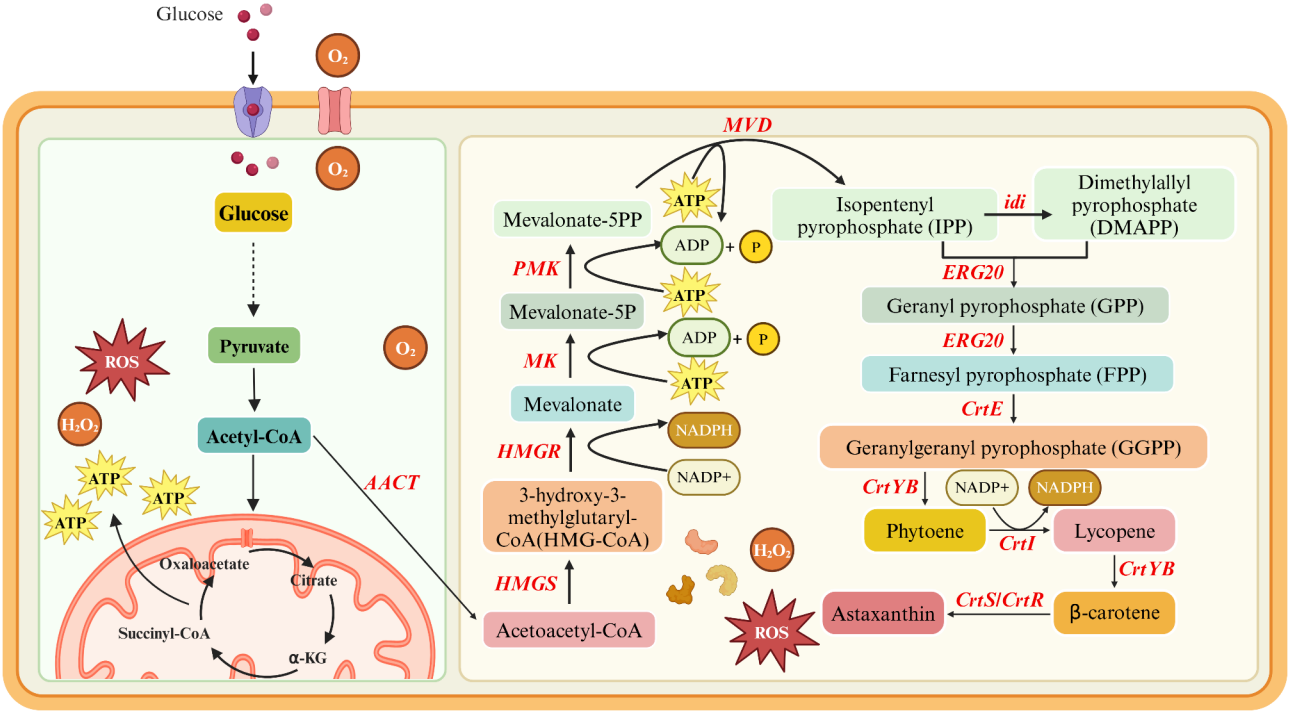

Fig. 1 Astaxanthin biosynthetic pathways in *P. rhodozyma*. Enzymes are as follows: (1) Mevalonate pathway: AACT, acetyl-CoA acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; MVD, mevalonate diphosphodecarboxylase. (2) Isoprenoids pathway: idi, isopentenyl pyrophosphate isomerase; ERG20, geranyl pyrophosphate synthase; CrtE, geranylgeranyl pyrophosphate synthase; CrtYB, phytoene-β-carotene synthase. (3) Carotenoids pathway: CrtI, phytoene desaturase; CrtYB, lycopene cyclase; CrtS, astaxanthin synthetase; CrtR, cytochrome P450 reductase. The Solid arrows indicate a single step reaction. The dashed arrows indicate multistep reactions

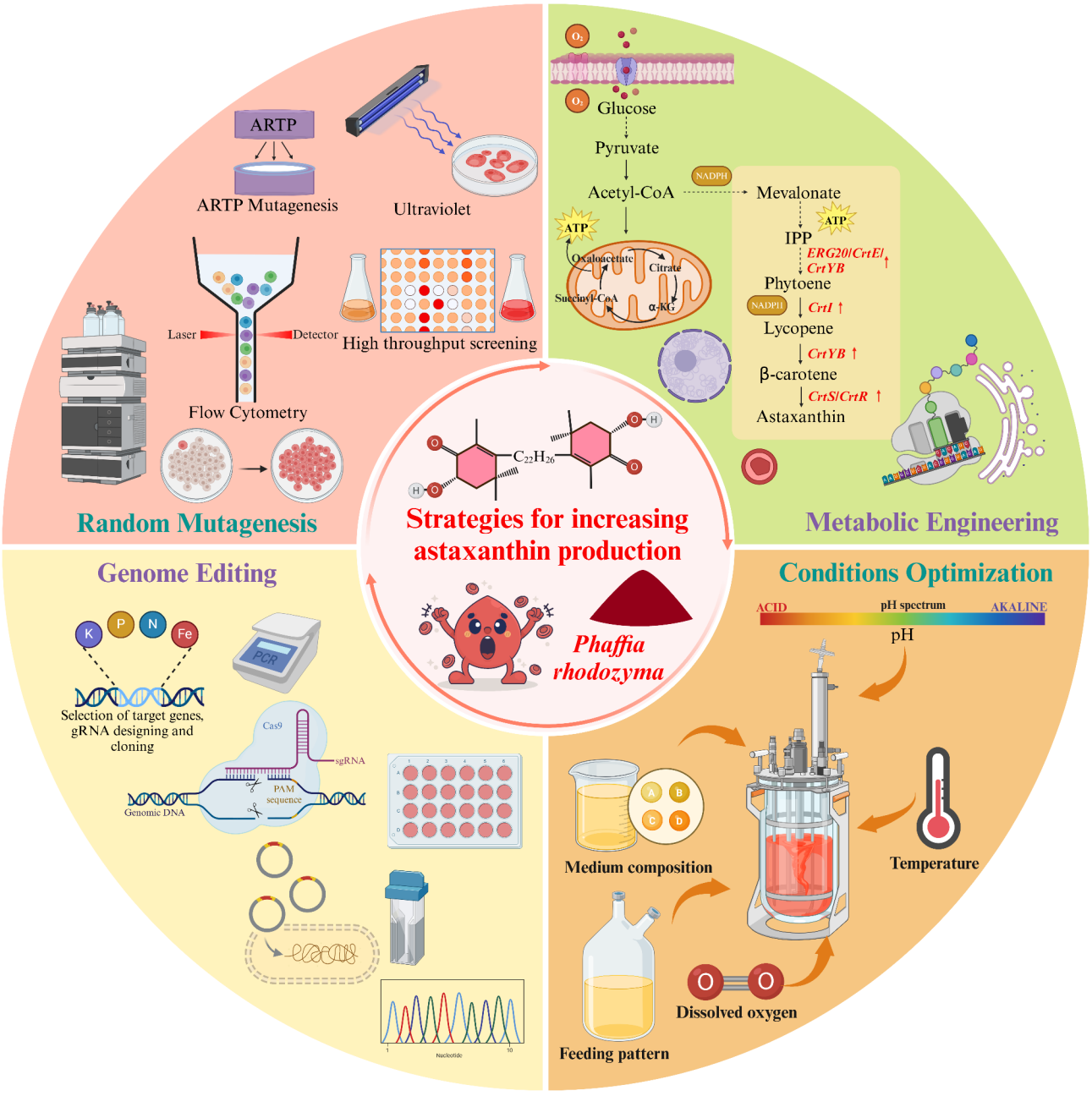

Fig. 2 An overview on different biotechnologies to enhance astaxanthin yield. (**A**) Random mutagenesis to enhance astaxanthin yield. (**B**) Using metabolic engineering to regulate astaxanthin metabolic pathways for enhancing astaxanthin yield. (**C**) Optimization of fermentation conditions to enhance astaxanthin yield. (**D**) Using genome editing to enhance astaxanthin yield

enhancing the content of the target product. These tactics involve subjecting yeast cells to physical and chemical mutagens [24–26], such as ultraviolet (UV) and gamma radiation, 1-methyl-3-nitro-1-nitroguanidine (NTG), benomyl, and diphenylamine (DPA), atmospheric and room temperature plasma (ARTP) mutagens, etc. (Table 1) [27]. Gamma-ray-mediated mutagenesis was utilized to boost astaxanthin production in *P. rhodozyma* and resulted in a 1.77-fold increase in astaxanthin [27]. ARTP mutagenesis of astaxanthin-producing Saccharomyces cerevisiae elevated the astaxanthin levels by 83% [28]. To acquire a superior mutagenesis effect, a combination of physical and chemical mutagenesis is frequently adopted. UV mutagenesis and EMS were employed for the mutagenesis of *H. pluvialis*, and mutant cells with astaxanthin production increased by 1.7 times compared to the wild type were obtained [29]. These mutants, obtained through random mutagenesis, are valuable tools for producing astaxanthin or carotenoid precursors. Since these strategies generate a

Table 1 Mutagenesis strategies for Astaxanthin production

Strains	Strategies	Fermentation scale	Production (mg/L)	Content (mg/g)	Fold change	Reference
S. cerevisiae SyBE_Sc2110M7	ARTP mutagenesis	5-L bioreactor	217.9	13.8	0.83	[28]
H. pluvialis DPA12-2	UV, Ethyl methane sulphonate (EMS), diphenylamine(DPA) mutagenesis	shake-flask 15 days	82.6	47.2	1.7	[29]
P. rhodozyma JH-82	γ-irradiation	shake-flask 8 days	2.6	0.16	1.77	[27]

Table 2 Metabolic engineering for Astaxanthin production

Strain	Strategies	Production (mg/L)	Content (mg/g)	Reference
Komagataella phaffii PP-L89-C	Optimization of the astaxanthin biosynthesis pathway, screening of different fusion enzymes, regulation of the NADPH synthesis, improvement of the precursor supply, and the optimization of fermentation parameters	716.13	16.88	[37]
P. rhodozyma DW6	Using two-stage pH strategies and an inferior substrate of sugarcane molasses as the sole carbon and nitrogen source	374.30	9.0	[38]
S. cerevisiae YM13-TS	Balance the expression of β-carotene hydroxylase and ketolase and spatial regulation through lipid droplet engineering	446.40	9.8	[39]
Yarrowia lipolytica YL17	Assemble the heterologous metabolic pathway using fusion enzymes CrtW-Z and target expression in subcellular organelles	858.00	16.7	[40]
H. pluvialis QLD	Establish an efficient nitrogen feeding strategy accompanied with high light induction	430.29	65.62	[41]

considerable number of mutant colonies, it is necessary to implement effective selection approaches to identify the optimal mutants. And due to their red color, the most common and simplest method is to visually screen for astaxanthin excess producers based on changes in color intensity. However, the background and color saturation due to interference from other intermediates make it challenging [30]. Using other screening methods, such as the employment of flow cytometry and near-infrared spectroscopy were used to screen astaxanthin high producers [31, 32]. However, the bottleneck of mutagenetic selection of high-yielding strains lies in the efficiency of mutagenesis and the genetic stability after mutagenesis. An et al. [28] recorded that EMS-induced mutation in *P. rhodozyma* resulted in unstable high-yield performance and a high frequency of recurrent mutation, up to 5–10%. Moreover, most mutant strains exhibited a decrease in growth rate or cell yield. Therefore, mutagenesis is an effective strategy for astaxanthin naturally producing strains, but the genetic stability and growth rate reduction caused by mutagenesis still needs to be considered. These methods effectively broke through the bottleneck of low yield of wild strains.

Metabolic engineering

At present, the enhancement of astaxanthin production through metabolic engineering is mainly based on the detection of carbon flow in the astaxanthin biosynthesis pathway, with the aim of maximizing the utilization of substrates and precursors and minimizing the production of undesired by-products [33, 34]. By uncovering the key enzymes and bottlenecks in the metabolic pathway and optimizing the expression level of related genes (Table 2), thereby increasing the storage of astaxanthin in cells and redirecting the carbon flux towards astaxanthin to achieve astaxanthin biosynthesis [35, 36]. For instance, increasing the synthesis rate of the products of a specific metabolic pathway, or reducing the reaction-limiting step in the metabolic pathway to divert the carbon flux to astaxanthin biosynthesis, or genetic modification can enhance the cell membrane permeability to promote the growth of *P. rhodozyma*.

Enhancement of astaxanthin precursor synthesis

The first approach is to boost astaxanthin production by strengthening astaxanthin precursor synthesis and weakening the competitive pathway. Generally, upregulation of the MVA or MEP pathways is the initial step to direct the carbon flow towards astaxanthin synthesis [42]. To remove metabolic obstacles in the synthesis pathway, overexpression of key genes and elimination of competing pathways are the most commonly adopted measures [43]. A variety of limiting responses exist in the carotenoid pathway. These restrictive responses can be precisely targeted through overexpression of genes in each pathway. Current metabolic engineering of *P. rhodozyma* focuses on overexpression of key genes such as HMGR, CrtE, CrtYB, and CrtS to enhance precursor synthesis [43]. To efficiently produce β-carotene and astaxanthin, phytoene-β-carotene synthase and lycopene cyclase (CrtYB) enzymes from *P. rhodozyma* were overexpressed in multiple hosts for pathway optimization [44, 45]. For

instance, in a medium-temperature and overproducing astaxanthin mutant strain of *P. rhodozyma*, Chi et al. [43] found that overexpression of CrtS would lead to upregulation of synthesis-related genes and an increase in astaxanthin production. However, by overcoming the limitations in the pathway, other reactions might become new bottlenecks, as their enzyme activity might not be capable of coping with higher flow rates. Moreover, according to numerous studies, there is a deficiency of research on metabolic regulatory mechanisms and the monocyclic and bicyclic synthesis pathways are jointly controlled by multiple genes and loci. Therefore, it is urgent to seek new methods for studying and regulating astaxanthin biosynthesis in order to increase astaxanthin production more effectively.

Weakening of competitive pathways

In addition to augmenting precursor supplementation, down-regulating the competitive pathway can also improve astaxanthin production efficiency. *P. rhodozyma* is beneficial for the development of high-yield astaxanthin producers as the pathway is established, an astaxanthin storage system exists, and highly active acetyl-CoA metabolism can be utilized [46]. Moreover, acetyl-CoA in *P. rhodozyma* is employed for the synthesis of fatty acids and terpenoids [47]. In *P. rhodozyma*, terpenoids are synthesized starting from three acetyl-group molecules through mevalonate (MVA), which can further synthesize carotenoids and sterols. For instance, squalene synthesis competes with β-carotene. Thus, downregulation of squalene synthase, SQS1, could raise β-carotene production from 453.9 mg/L to 797.1 mg/L [48]. Ergosterol synthesis will vie with astaxanthin for the intermediate metabolite of FPP. Therefore, the reduced flux of the competitive pathway in the mutant is conducive to astaxanthin synthesis, providing a potential route for the metabolic engineering of *P. rhodozyma* [15]. Simultaneously, ergosterol has also shown feedback inhibition on the MVA pathway. Deletion of CYP61 encoding the C-22 sterol desaturase related to ergosterol biosynthesis led to a significant 1.4-fold increase in astaxanthin synthesis [49]. Additionally, the ergosterol replacement pathway might exist in *P. rhodozyma*, so CYP61 does not negatively affect the growth of *P. rhodozyma*.

Balancing the cofactors NADPH and ATP

In addition to substrate, ensuring an adequate supply of cofactors is a crucial factor in maintaining cellular growth and enhancing overall transformation efficiency. The cofactors NADPH and ATP are also of vital importance for carotenoid biosynthesis. NAD(P)H, serving as the primary bioreducing equivalent, not only safeguards cells against oxidative stress but also elongates the carbon-carbon skeleton. Moreover, it acts as the principal limiting factor for fatty acid synthesis. Therefore, to enhance astaxanthin production, it is essential to balance the supply of cofactors. Central carbon metabolism plays a vital role in providing these cofactors. NADPH is mainly generated through the pentose phosphate pathway (PPP) and malic enzyme, while ATP is mainly produced through the NADH-initiated electron transport chain formed in the TCA cycle, thereby increasing the accumulation of carotenoid precursors and directing the carbon flux towards astaxanthin biosynthesis in *P. rhodozyma* [50]. Additionally, there exist alternative reactions capable of generating NADPH, including cytoplasmic and mitochondrial kinases that facilitate the ATP-driven conversion of NADH to NADPH. For instance, Zhao et al. [51] enhanced the NADPH supply by overexpressing NADH kinase in *Saccharomyces cerevisiae*, contributing to carotenoid biosynthesis in *S. cerevisiae*. Recent studies have also used coenzyme engineering (i.e. increasing NADPH levels) to increase astaxanthin production in *Komagataella phaffii* [37]. These indicate the potential of improved cofactor supply to increase astaxanthin production and could be further applied in *P. rhodozyma*.

Balancing the enzyme activities of the Crt gene family in the carotene biosynthesis pathway

In addition, because of the limitations of single genes for metabolic research, balancing the activity of the enzymes in the Crt gene family within the carotene biosynthesis pathway can facilitate the conversion of β-carotene to astaxanthin. CrtYB, CrtI, CrtS, and CrtR are several key enzymes that catalyze the transformation of β-carotene to astaxanthin. An imbalance in the expression of these enzymes can significantly impact astaxanthin synthesis. Therefore, balancing the metabolic flow by optimizing the copy numbers of CrtYB, CrtI, CrtS, and CrtR is the most direct approach. Studies have indicated that the phytoene-β-carotene synthase CrtYB is the limiting step in the biosynthesis of carotenoids and astaxanthin. Girard et al. [52] introduced multiple copies of CrtYB into PR-1-104, resulting in overexpression of the phytoene-β-carotene synthase and 1.5 times higher accumulation of β-carotene compared to the parental strain. Overexpression of CrtYB is essential for increasing carotenoid biosynthesis, but to overproduce astaxanthin, astaxanthin synthase (CrtS) must be overexpressed. Hence, Ledetzky et al. [53] produced a strain of *P. rhodozyma* containing three copies of CrtYB and one copy of CrtS, in which the astaxanthin biosynthesis increased to 70% of the total carotenoid production. The CrtI gene encodes the phytoene desaturase in *P. rhodozyma*, which is the second enzyme of the carotenoid biosynthesis pathway. The introduction of multiple copies of CrtI increased intracellular lycopene accumulation, but decreased concentrations of β-carotene and astaxanthin

were detected, and increased toluene and 3-hydroxy-3,4-didehydro-β, pedi-carotene-4-ona (HDCO) [52]. This indicated that overexpression of the phytoene desaturase in *P. rhodozyma* shifted the metabolic flow towards toluene biosynthesis. Therefore, the selection of the appropriate proportion of enzyme expression and the balance of metabolic flow has obvious effects on the increase of astaxanthin production.

Optimization of fermentation conditions

The biosynthetic pathway of astaxanthin in red yeast is also influenced by various culture conditions, such as medium composition, pH, dissolved oxygen, and feeding pattern. Therefore, astaxanthin biosynthesis can be controlled macroscopically by altering the fermentation conditions. Firstly, Johnson et al. [54] initially investigated the fermentation conditions of wild *P. rhodozyma* strains in order to enhance astaxanthin production. The results indicated that cellobiose was the most effective carbon source for astaxanthin accumulation, and the optimal culture temperature and pH values were 20–22 °C and 8.0, respectively. With further research, Zhou et al. [38] discovered that glucose or sucrose was the most favorable carbon source for promoting cell growth, although high concentrations of glucose inhibited astaxanthin production. Zhu et al. [55] demonstrated that the addition of ammonium nitrogen significantly increased astaxanthin production in *P. rhodozyma*, especially at the initial stage of the culture. When the ammonium sulfate supplemental level was 1 g/L, the astaxanthin content was the highest (9.06 mg/g), which was 64.4% higher than that of the control. Moreover, nitrogen restriction at the late growth stage of *P. rhodozyma* was beneficial for increasing astaxanthin production. Simultaneously, the addition of pH and some trace elements also enhanced astaxanthin yield. Schewe et al. [56] found that reducing the pH level in biological metabolism and increasing the concentrations of trace elements and vitamins were essential for astaxanthin concentration and purity. Astaxanthin production could also be increased to a certain extent by adding a single or combination of metabolic regulators, such as sodium citrate [57], soybean oil [58], glutamate [59], pyruvate, salicylic acid [60], penicillin, ethanol, and fluconazole [61]. Taken together, these findings have suggested that astaxanthin biosynthesis can be enhanced by altering the medium composition, including carbon sources, nitrogen sources, inorganic salts, and growth factors, with appropriate dosages and timings. In addition, it was found that astaxanthin biosynthesis is affected by various process conditions. As shown in Table 3, Zhou et al. [61] developed a two-stage pH fermentation strategy to decouple microbial growth and astaxanthin production of strain DW6 of *P. rhodozyma*, reducing the microbial metabolic burden and increasing astaxanthin production from 287.8 to 362.1 mg/L. Jiang et al. [61] succeeded in increasing the astaxanthin concentration of *P. rhodozyma* strain JMU-MVP14 to 414.1 mg/L through a pulsed feeding process, which was 200.2% higher than the yield obtained by batch fermentation. However, the optimization of these process conditions, including medium composition, pH, dissolved oxygen, light, feeding method, etc., can only improve the astaxanthin yield to a certain extent and provide a basic research direction for further studies, but cannot be used to confirm the specific molecular mechanism. In the future, intelligent bioreactor systems could also be applied to the industrial production of carotenoids, potentially using AI-driven algorithms to monitor and adjust parameters such as pH, temperature, and nutrient delivery in real time. Because of its short growth cycle and mature fermentation process, *P. rhodozyma* is more suitable for industrial scale-up. Therefore, appropriate optimization of fermentation process is an indispensable step to improve the performance of astaxanthin synthesis.

Genome editing

From the aspect of recombinant DNA technology, the transformation system of *P. rhodozyma* has been developed and optimized. To stabilize the integration in the genome and increase the frequency of integration, a portion of the ribosomal DNA of *P. rhodozyma* was introduced into the transformation vector. Under optimized

Table 3 Different fermentation processes of Astaxanthin from ***P. rhodozyma***

Strains	Production (mg/L)	Content (mg/g)	Strategies	References
DW6	374.3	9	Using two-stage pH strategies and an inferior substrate of sugarcane molasses as the sole carbon and nitrogen source	[38]
JMU-MVP14	414.1	-	EMS, using 0.5% hydrogen peroxide combined with UV irradiation to eliminate low producing strains and pulse-fed fermentation	[38]
JMU-ALE105	-	8.36	Using TiO_2 domestication and nitrogen source regulation through ammonium sulphate fed-batch fermentation	[62]
Y119	253.1	-	Using Jerusalem artichoke extract as carbon source through substrate-feedback fed-batch fermentation in a 3 L stirred-tank bioreactor	[63]

conditions, the conversion efficiency was 1000 transformants/μg plasmid DNA [64]. The recombination rate produced by homologous recombination using gene knockout and overexpression techniques was only 10^{-6}, and there were gene regulation errors [65], which indicated that the regulatory efficiency of exploring microbial metabolic pathways is inefficient and inaccurate. Considering the status of astaxanthin as a secondary metabolite, its synthesis process is more complex and is co-regulated by multiple genes and splice fragments [20]. Therefore, homologous recombination alone is rarely sufficient to elucidate the astaxanthin biosynthesis mechanism of *P. rhodozyma*. This challenge has prevented further increases in astaxanthin production. Fortunately, there have been significant advancements in CRISPR-Cas9 genome editing in recent years, including multi-site co-knockout, gene expression interference, single-base editing, and gene modification [66]. Hong et al. [67] thoroughly analyzed the results of CRISPR-Cas9 process-induced editing in *P. rhodozyma* by Sanger and Illumina sequencing and identified various patterns of DNA repair, such as DNA deletion, inter-chromosomal translocations, and targeted nucleotide substitution (point mutations), laying the foundation and providing evidence for the potential application of genome editing techniques in *P. rhodozyma*.

In the future, CRISPR-Cas9 can also be used to precisely modify astaxanthin biosynthesis pathways [68], and modifying the central pathway is an effective strategy to optimize the biosynthesis of terpenoids. Shukal et al. [69] improved the production of terpenoids by using the CRISPR-Cas9 system to knock out the competing branching pathway LDHA, thereby increasing levels of acetyl-CoA. In addition, using CRISPR-Cas9 technology to balance the MVA pathway and downstream astaxanthin biosynthesis pathway is also an effective strategy for cell reprogramming to improve metabolic pathways. Zhang et al. [70] successfully developed a synthetic platform for the natural product lycopene in *Pichia pastoris* by using CRISPR/Cpf1 technology. Kneip et al. [71] also used CRISPR-Cas9 to increase zeaxanthin content by 60% through functional double knockout of lycopene ε-cyclase (LCYE) and zeaxanthin epoxidase (ZEP). All these indicate that CRISPR has the ability to modify astaxanthin biosynthetic pathway accurately and efficiently and can be further applied in *P. rhodozyma*.

Extraction process of astaxanthin

It is well known that the extraction of fermentation products usually accounts for 30–50% of the entire production process. Therefore, in order to reduce production costs and improve production efficiency, the downstream production process of astaxanthin extraction is also particularly important for its industrial production. The extraction of astaxanthin mainly includes two aspects, namely, the cell wall breaking of *P. rhodozyma* and the extraction of astaxanthin. Astaxanthin is usually extracted with organic solvents (ethanol, etc.), and cell wall-breaking is usually treated by ultrasonic, chemical hydrolysis and other methods [72]. Because of the thick cell wall of *P. rhodozyma*, the extraction of astaxanthin is difficult, so the downstream production process problems mainly focus on this. At first, astaxanthin was extracted mainly by mechanical fracture and acetone, and the recovery rate of astaxanthin reached 85%. Recently, some greener extraction technologies have been developed to replace chemical methods [73], and the recovery rate of astaxanthin by supercritical liquid extraction (SFE) using sunflower oil as solvent can reach 87.42%, which is comparable to ethanol [74]. But its high cost has influenced the process. The recovery of astaxanthin can reach 98.3% by using supercritical carbon dioxide extraction and 20% ethanol as co-solvent [75]. Astaxanthin can also be extracted through using ionic liquid (ILs) extraction. Moreover, 1-ethyl-3-methylimidazolium ethylsulfate [Emim] [$EtSO_4$] is used as an ionic liquid to extract 70% astaxanthin from *H. pluvialis*, but it also has a high cost [76]. In summary, the downstream extraction of astaxanthin is also particularly important for its industrial production, and the efficient and green extraction methods are conducive to reducing costs and environmental impact.

Other frontier biotechnologies

Several other strategies, such as morphology and oxidative stress engineering, can also be utilized to increase astaxanthin production. For instance, morphology/membrane-related genes and oxidative stress-related genes can be deleted in Escherichia coli, enabling cells to become longer and larger. This will raise the level of intracellular reactive oxygen species and increase astaxanthin production in the future [77]. The combination of metabolic engineering with conventional mutagenesis is also an effective tactic for increasing astaxanthin production. Further ARTP mutagenesis of engineered astaxanthin yeast could enhance astaxanthin yield by 0.83 times [28]. The mutant AXG-13 was created through repeated mutagenesis using nitrosoguanidine and selection of crimson colonies on a plate using triazine. The mutant was transformed to overexpress the Hmg, CrtE, and CrtYB genes, thereby enhancing total carotenoid synthesis through overall synergy [12]. At present, there are some new genetic engineering strategies and biotechnological discoveries that can also be used to increase astaxanthin biosynthesis. For example, through rational methods (metabolomics analysis, etc.), the metabolic pathway and regulatory mechanism of astaxanthin can be further understood, and the efficiency of astaxanthin production

can be significantly improved. Yang et al. [78] found that the addition of sodium protovanadate and melatonin respectively increased astaxanthin production by 19.2% and 30.3% through metabolomics analysis. The application of transcriptomics and proteomics also provides new ideas for developing new targets for the synthesis of astaxanthin in order to gain a deeper understanding of the regulatory mechanisms of astaxanthin [79]. Yang et al. [62] regulated the nitrogen source $(NH_4)_2SO_4$ through fed-batch fermentation based on the pathway of nitrogen metabolism through transcriptomic analysis, so that the astaxanthin content reached 8.36 mg/g. Protein structure analysis can also be used to increase astaxanthin biosynthesis. In recent years, studies on key enzymes in astaxanthin biosynthesis have focused on enzymes from β-carotene to astaxanthin, and the use of Phyre2 tools provides an opportunity to predict the structure of these enzymes [80]. The corresponding enzyme can be modified by introducing a flexible linkers to achieve higher astaxanthin production [81].

Transcriptional regulation of astaxanthin biosynthesis

Astaxanthin biosynthesis is a complex process, and despite some research advances over the past five years, the transcription factor regulation mechanism of *P. rhodozyma* and other producers are still poorly understood. Further understanding of the metabolic pathway and regulatory mechanism of astaxanthin through the study of astaxanthin related transcription factors produced by *P. rhodozyma* can significantly improve the production efficiency of astaxanthin.

Sterol regulatory element binding protein Sre1 regulates the biosynthesis of carotenoids

The sterol regulatory element binding protein Sre1 is a transcription factor implicated in the regulation of sterols and carotenoids. It was initially discovered in the mammalian sterol regulatory element binding protein (SREBP) pathway [82]. Sterols directly control SREBP activity by regulating sterol metabolism. Additionally, it inhibited proteolysis when cytosterol levels were sufficient [83]. Recently, the Sre1 gene-encoded homologue of SREBP/Sre1 has also been identified in *P. rhodozyma* and shown to regulate the synthesis of carotenoids and sterols in the isoprene synthesis pathway [84]. The initial observation of *P. rhodozyma* overproduction of astaxanthin, a carotenoid, in ergosterol biosynthetic mutants suggested that yeast astaxanthin production can be regulated by the Sre1 pathway [85]. Gutiérrez et al. [85] conducted a comparative analysis between the SRE1-defective mutant and wild type strains, revealing that among the downregulated genes in the mutant strain, 8 genes were associated with mevalonate and sterol synthesis pathways: HMGS, HMGR, ERG6, ERG24, ERG25, and CYP5. These findings have partially explained reduced sterol production in Sre1-deficient mutants and provided support for constitutive activation of the SREBP pathway at basal oxygen and sterol levels. Furthermore, sre1 affects cytochrome P450 enzyme CrtR expression, however no significant difference was observed in carotene gene transcription levels between SRE1-deficient mutants and wild types. This suggested that while not specifically regulating carotene-producing genes' expression itself, Sre1 regulates carotene production through modulation of mevalonate pathway expression as well as genes related to carotenoid precursor synthesis within CrtR regulation context [84]. In summary, Sre1 plays a significant role in maintaining intracellular levels of both sterols and carotenoids, and holds important implications for understanding these metabolic pathways as well as enhancing astaxanthin production.

GATA transcription factor WCC regulates astaxanthin biosynthesis

The transcription factor white collar is a protein complex composed of two proteins, WC-1 and WC-2, known as the white-collar complex (WCC). Studies have shown that the lack of WC will significantly reduce the expression levels of CrtI and CrtS, and then reduce astaxanthin levels by 62%, affecting astaxanthin synthesis [86]. It impacts the production of astaxanthin through inducing the expression of phytoene desaturase CrtI and astaxanthin synthase CrtS in the carotenoid pathway [86]. In *Neurospora crassa*, WC primarily regulates the expression of GGPP synthase gene, phytoene-β-carotene synthase gene, and phytoene desaturase gene involved in mycelial carotene production, resulting in orange pigmentation and accumulation of carotenoids and other pigments [87]. Nevertheless, light induction is not required for WCC activity in *P. rhodozyma*; therefore, further investigation is needed to understand its correlation with nutrition. Both sterol regulatory element binding proteins Sre1 and WCC are involved in regulating astaxanthin synthesis genes [88]. Therefore, studying the correlation between WCC and Sre1 can provide a better understanding of their regulatory mechanism during this process and offer theoretical support for developing novel and efficient strains for astaxanthin biosynthesis.

Involvement of catabolic inhibitory transcription factor MIG1 affects astaxanthin biosynthesis

MIG1 acts as a metabolic suppressor that impacts astaxanthin biosynthesis by influencing the catabolic inhibition of glucose in *P. rhodozyma* [89, 90]. The regulation of glucose inhibition in *S. cerevisiae* was rather well comprehended, and the proteins involved have been extensively investigated [91]. This inhibition is mediated by a

metabolic suppressor encoded by the MIG1 gene, along with the co-repressor complex Cyc8-Tup1. Glucose inhibition mainly takes place at the transcriptional level. In the presence of glucose, the deletion of MIG1 upregulated CrtI, CrtYB, and CrtS transcripts [89]. Miao et al. [92] investigated the dysregulation of astaxanthin synthesis by glucose at the transcriptional level and the glucose signaling pathway level to clarify the underlying molecular mechanism. They established a red yeast strain MK19 with moderate temperature, high glucose tolerance, and overproduction of astaxanthin, and found that CreA is a transcription factor primarily responsible for inhibiting the genes needed to utilize fermentable carbon sources. CreA is strongly induced by high concentrations of glucose; it binds to DNA impacting carotene gene expression primarily through inhibiting lycopene to β-carotene conversion followed by β-carotene to astaxanthin steps within the carotenoid pathway. Conversely, low CreA gene expression leads to deinhibition of glucose metabolism leading to increase astaxanthin synthesis at high concentrations thereby increasing its production. Pamela et al. reported that the regulatory role of the co-inhibitory systems CYC8-TUP1 and MIG1 in the production of carotene has been confirmed in *P. rhodozyma* [90]. The loss of CYC8-TUP1 in *P. rhodozyma* eliminated the glucose-dependent inhibition of carotene production, leading to the upregulation of genes involved in carotenoid and carotenoid precursor synthesis [90]. This negative regulatory mechanism is crucial for maintaining intracellular carotene levels, and the absence of each transcription regulator can impact metabolic processes. Further investigation into CreA and its regulatory mechanisms will enhance our understanding of *P. rhodozyma* growth, development, and metabolic regulatory networks.

Damage response protein 1 (Dap1) affects the biosynthesis of carotenoids

The damage response protein 1 (Dap1) functions as a positive regulator that controls the activity of cytochrome P450 enzymes through protein-protein interactions in various organisms [93]. Dap1, which is involved in DNA damage response in *S. cerevisiae* [94], can bind to P450 enzymes and positively regulate sterol biosynthesis in *Schizosaccharomyces pombe* [93]. Cytochrome P450 is a large superfamily of enzymes that includes heme as a cofactor. It has monooxygenase function and is involved in a variety of cellular functions, including steroid biosynthesis and the detoxification of allogenic compounds [95]. CYP is typically a terminal oxidase in the electron transfer chain, and its function is to catalyze the oxidation of organic compounds. The general reaction of P450 involves binding organic substrates with molecular oxygen: $RH + O_2 + 2e^- + 2\ H^+ \rightarrow ROH + H_2O$ [96]. To date, three functionally characterized P450 species from *P. rhodozyma* have been identified, each involved in the biosynthesis of ergosterol or astaxanthin [97]. Martínez-Cárdenas et al. [98] demonstrated the interaction mechanism between CrtS and Dap1 in *P. rhodozyma*, elucidating how Dap1 regulates carotenoid production, particularly during the conversion of β-carotene to astaxanthin. This regulation occurred at the protein level by modulating CrtS activity and utilizing CrtR as an electron donor [99, 100]. Furthermore, Dap1 interacts primarily with three specific P450 enzymes, influencing both ergosterol and astaxanthin biosynthesis processes [101]. These findings significantly contribute to our understanding of the role played by P450 enzymes within *P. rhodozyma* biosynthetic pathway [102] while also providing novel insights for further research endeavors within related fields.

The role of stress-related transcription factors in astaxanthin synthesis

In fungi, multiple stress-related transcription factors exist that can respond to external environmental and cellular metabolic stresses, including high temperature, high salt concentration, intracellular toxic substances, and other stressors. These transcription factors regulate the expression of specific genes to facilitate fungal adaptation to diverse environmental conditions and enhance survival and product production. Stress-related transcription factors play a crucial role in the growth and development of fungi, making them highly significant for studying fungal adaptation mechanisms in response to environmental changes. In *P. rhodozyma*, multiple stress transcription factors are present that not only participate in oxidative stress responses but also contribute to cell wall biosynthesis, cell cycle regulation, and other pathways. When confronted with adverse environmental conditions, these transcription factors modulate gene expression and cellular metabolism enabling fungi to better adapt and survive external stresses while safeguarding themselves against metabolic perturbations. However, comprehending the intricate metabolic regulatory network mediated by these factors is a challenging task due to limited knowledge available on *P. rhodozyma* metabolic regulation.

Many stress-related transcription factors have been identified in *P. rhodozyma*, and the regulation of carotenoid levels is mediated by related transcription factors, including ROX1, SKN7, and YAP6. These transcription factors interact with the corepressor complex CYC8-TUP1 and participate in the regulation of gene expression [103]. Studies have demonstrated that binding of the repressor ROX1 to the corepressor complex CYC8-TUP1 prevented hypoxic gene expression under aerobic conditions and contributes to cellular and mitochondrial integrity [104]. Transcription factors SKN7 and YAP1 respond to cell REDOX stress through thioredoxin

in yeast. REDOX stress is a major factor influencing astaxanthin production in *P. rhodozyma*. By modulating the activity of transcription factors involved in cellular reducing capacity, such as NADPH and glutathione, astaxanthin production could be significantly enhanced [105]. These secondary metabolites may represent an adaptive response of yeast to its environment. Under unfavorable growth conditions, biosynthesis of transcription factors affects gene expression and consequently influences astaxanthin synthesis. The CYC8-TUP1 corepressor complex interacts with numerous transcription factors, making it a crucial component for regulating yeast's response to environmental cues through secondary metabolites beyond carbon catabolic inhibition; it may also play a role in regulating various other biological processes.

Factors that facilitate astaxanthin synthesis in *P. rhodozyma*

As the sole yeast or fungus capable of synthesizing astaxanthin, *P. rhodozyma* possesses a series of antioxidant defense mechanisms [106]. First of all, astaxanthin has high antioxidant capacity, which can help cells fight free radicals and oxidative stress, and protect cells from damage. Additionally, this yeast possesses a series of other genes involved in antioxidant defense mechanisms that are commonly found in all eukaryotes. These include mitochondrial manganese-dependent superoxide dismutase and relatively lower catalase activity compared to S. cerevisiae. *P. rhodozyma* might display a deficiency or have low cytoplasmic superoxide dismutase activity, thereby primarily relying on astaxanthin for antioxidant defense [107, 108]. Consequently, when confronted with stressful conditions, red yeast cells accumulate astaxanthin and acquire an intense reddish pink color, which typically inhibits cell growth. Typical stressful circumstances comprise: nutritional limitations (e.g., nitrogen or phosphate), the existence of toxic agents or mutations in genes related to respiration or nitrogen metabolism, etc [109, 110]. Since astaxanthin accumulates in cells is triggered by various stimuli, and the extent of such accumulation is somewhat proportional to the level of stress experienced by *P. rhodozyma* cells, it is imperative to comprehend the underlying mechanisms driving astaxanthin synthesis convergence for activating the antioxidant response in *P. rhodozyma*.

The intracellular accumulation of astaxanthin can be initiated by multiple distinct stress circumstances. Recently, Martínez-Cárdenas et al. [98] recently reported that in all cases, the onset of astaxanthin synthesis in *P. rhodozyma* was constantly triggered by intracellular events, namely the emergence of REDOX imbalances. It leads to oxidative stress, and eventually produces large amounts of astaxanthin to protect the cells. The NADH/NAD^+ ratio varied with nutritional and environmental conditions, making it crucial for cells to maintain REDOX equilibrium by balancing NADH production and oxidation [111]. The conditions that lead to astaxanthin synthesis and fermentation-induced REDOX imbalances can be categorized as follows (Fig. 3): (i) impairment of electron flow through the mitochondrial respiratory chain, such as molecular oxygen and genetic mutations. (ii) impairment of oxidative ADP-phosphorylation, potentially caused by nitrogen deficiency, phosphate deficiency, etc. (iii) conditions that lead to excessive NADH levels, including active re-assimilation of ethanol and high sugar concentration.

Impairment of Electron flow in the mitochondrial respiratory chain promotes astaxanthin synthesis

Molecular oxygen is a significant substrate for mitochondrial respiration and oxidative phosphorylation in all aerobic cells. Astaxanthin accumulation in red yeast cells usually occurred under aerobic conditions where the oxygen concentration (pO_2) in the medium was > 20% of the air saturation [112]. The expression of the carotene gene has consistently shown a positive association with the existence of O_2 in the growth medium [113]. The increase in pO_2 levels in yeast cultures is a common event that promotes astaxanthin synthesis and observed following depletion of key nutrients essential for cell growth (e.g., carbon, nitrogen, or phosphate). This event is associated with a decline in mitochondrial respiratory function and potentially higher NADH/NAD^+ ratios, thereby promoting astaxanthin production. This was anticipated to lead to augmented ROS generation and the requirement for additional antioxidant protection (Fig. 4) [98]. Numerous studies have demonstrated that the exposure of red yeast to diverse ROS, such as $O_2^{\bullet-}$, H_2O_2, or $^1O_2$, or ROS-producing compounds like TiO_2 promoted astaxanthin biosynthesis and enhanced the cellular content of carotenoids along with the relative abundance of lutein (i.e. oxygen-containing carotenoids). For instance, *P. rhodozyma* cultivated in the presence of 500 mg/L TiO_2 generates $O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$ while enhancing the carotenoid content of cells [108, 114] The mechanism of astaxanthin synthesis promoted by *P. rhodozyma* under TiO_2 stress was investigated through proteomic analysis [108]. The analysis suggested that TiO_2 promoted astaxanthin synthesis through a mechanism involving REDOX equilibrium, ribosome translation, and ion transmembrane transport. Nevertheless, in media containing growing cells, high pO_2 levels or rapid pO_2 elevation can induce cellular oxidative stress, leading to cells to be overwhelmed by exposure in high levels of O_2 or ROS experience and affected by non-specific damage [115]. Ultimately, this phenomenon may lead to growth arrest and eventual cell death. In fact, pO_2 > 20% plays

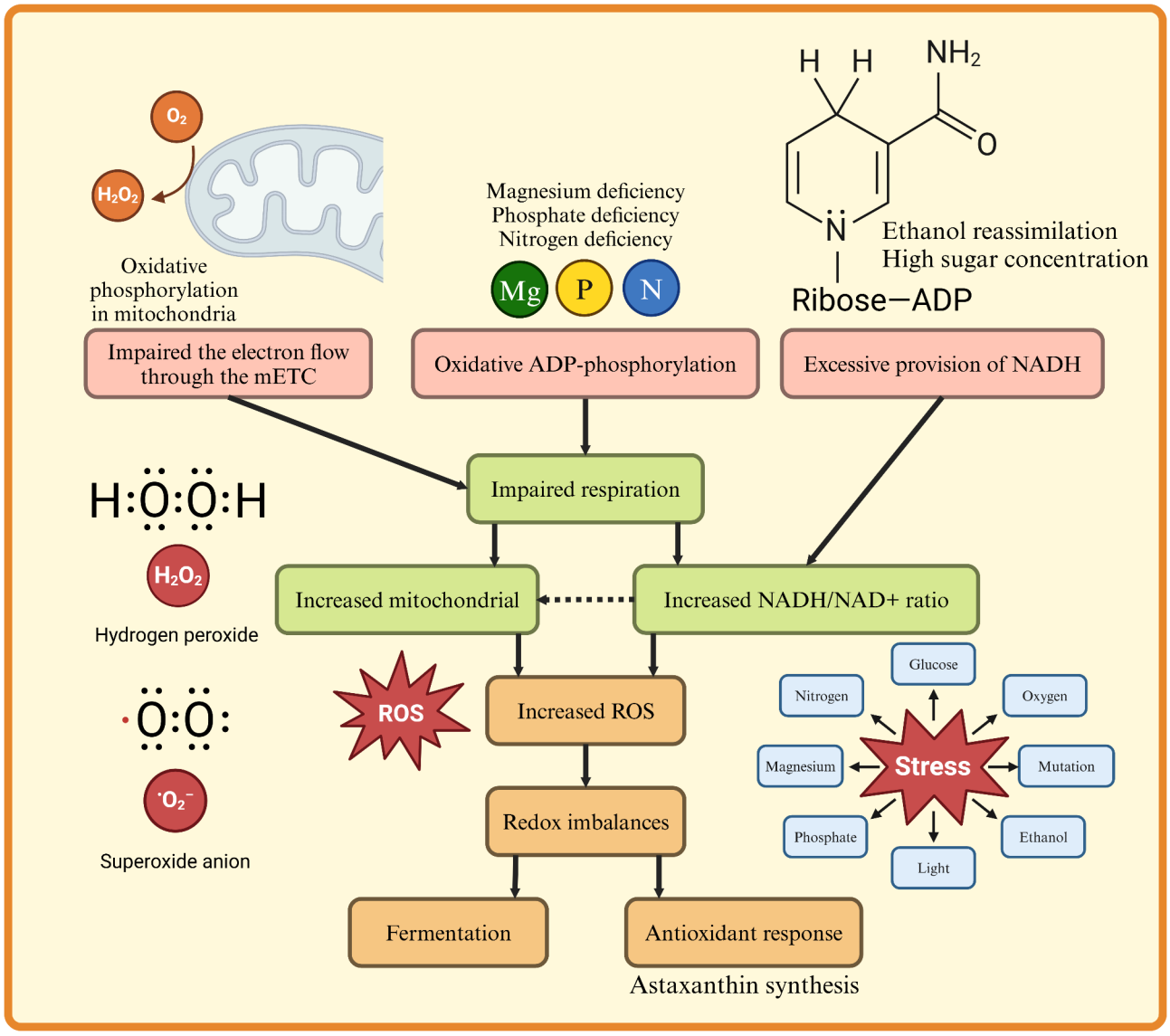

Fig. 3 Several different conditions to trigger the accumulation of astaxanthin. The induction of astaxanthin synthesis and fermentation either requires the occurrence of a REDOX imbalance can be categorized as follows: (1) Impairment of electron flow through the mitochondrial respiratory chain, such as hypoxia or genetic mutations through the mitochondrial electron transport chain (mETC). (2) Impairment of oxidative ADP-phosphorylation, potentially caused by nitrogen deficiency, phosphate deficiency, etc. (3) Conditions that lead to excessive NADH levels, including active re-assimilation of ethanol and high sugar concentration

a significant role in astaxanthin biosynthesis, however, excessive pO_2 or significant oxidation stress can easily inhibit or stop the growth of red yeast cells. Notably, growth inhibition or stagnation coincides with the accumulation of astaxanthin within cells [116].

Some mutants screened by mutagenesis isolation have higher astaxanthin content in their cells than their parent strains. Compared with their respective parent strains, almost all of these high-yielding astaxanthin mutant strains exhibited compromised respiration, slower growth rates, reduced colony formation, and lower yields when utilizing various carbon sources [117]. A distinguishing characteristic of many of these mutant strains is their ability to synthesize astaxanthin in a growth-dependent manner. Even during the early stages of growth in batch culture, when sugar levels were relatively high, astaxanthin synthesis appeared to be dysregulated [92, 118]. Mutant strains with distinct mutations that impact genes encoding components of the respiratory chain, TCA cycling, or nitrogen assimilation can be expected to predominantly exhibit growth-associated patterns in astaxanthin synthesis. Furthermore, any of these mutations can lead to the reoxidation of NADH and impairment in the reduction process of oxygen to water, consequently leading to an increased $NADH/NAD^+$ ratio and elevated pO_2 levels. This phenomenon consistently

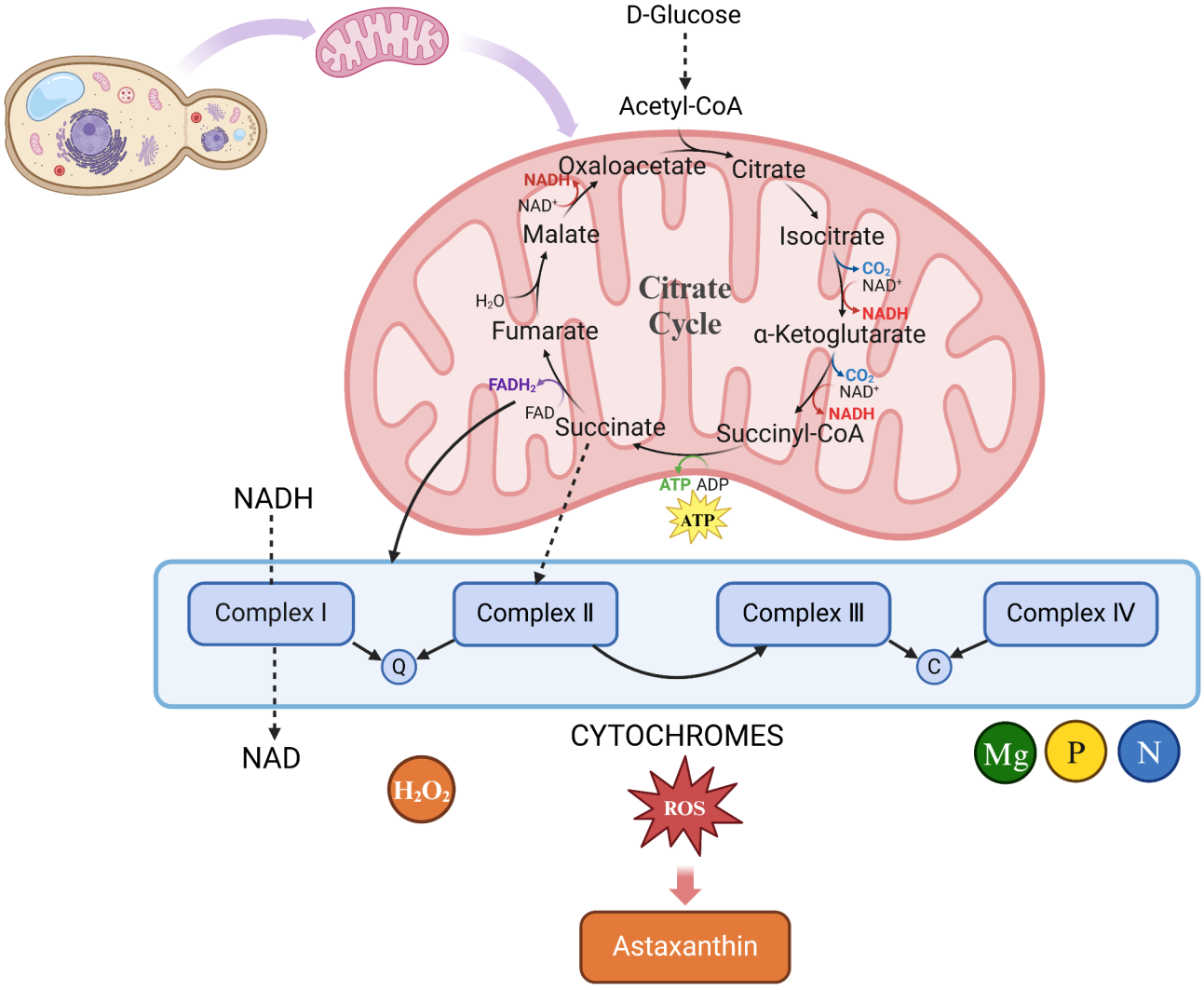

Fig. 4 Mechanism of NADH involved in energy metabolism. Through impairing the flow of electrons through the mETC or oxidative ADP-phosphorylation, the reduced state of NADH/NAD^+ pairs and increased pO_2 levels in the medium promote increased ROS production and therefore astaxanthin synthesis

promotes astaxanthin synthesis during the early growth stages of *P. rhodozyma*. For example, a mutation in the mitochondrial MTATP6 gene encoding subunit 6 of F0F1-ATP synthase in budding yeast may be potential for enhancing astaxanthin synthesis [119, 120].

Impairment of oxidative ADP-Phosphorylation promotes astaxanthin synthesis

The source and concentration of nitrogen in the medium play a critical role in cellular involvement in microbial carotenoid production [121]. In *P. rhodozyma*, the accumulation of intracellular astaxanthin and other carotenoids in *P. rhodozyma* frequently rises when it was cultivated under low nitrogen concentrations [112, 122]. During aerobic growth, the rapid transfer of respiratory electrons to oxygen leads to maintain the NADH/NAD^+ pairing in oxidative homeostasis (low NADH/NAD^+ ratio) in cells, thereby ensuring rapid accumulation of biomass [123]. Nevertheless, under nitrogen restriction, the restriction of oxidative phosphorylation of ADP will impact the aerobic reoxidation of NADH, leading to impairment of respiration, the reduced state of NADH/NAD^+ pairs and the increased pO_2 levels in the medium [124]. High pO_2 levels and a highly reduced REDOX cofactors created favorable conditions for cellular generation of $O_2^{\bullet-}$ /H_2O_2 [125, 126]. Consequently, as intracellular ROS levels increased, red yeast cells initiated astaxanthin accumulation in the cell. Therefore, the availability of carbon skeleton along with elevated pO_2 and NADH levels appear to be significant for initiating astaxanthin synthesis. However, nitrogen restriction exerts a global impact on inhibition of protein synthesis, leading to decreased expression levels of genes with ribosomal proteins, tRNA synthases, initiation and elongation

factors under nitrogen deficiency, and cell metabolism are decelerated [112, 127]. For instance, using a slowly metabolized amino acid as the sole nitrogen source for astaxanthin production increased astaxanthin production, but notably reduced the maximum specific growth rate and cell yield coefficient $Y_{X/S}$ [128].

Inorganic phosphates are indispensable nutrients necessary for the biosynthesis of adenine nucleotides, phospholipids, and metabolites employed in energy metabolism. On the contrary, magnesium is an essential metal indispensable for all biochemical reactions involving ATP, and plays a vital role in stabilizing the secondary and tertiary structure of DNA, thereby facilitating DNA replication and transcription [129]. Shortly after the occurrence of phosphates deficiency, the primary impact is observed on the phosphorylation of ATP and also lead to elevated pO_2 levels and NADH/NAD^+ ratios, consequently resulting in elevated production of ROS and astaxanthin. Limited magnesium also impacts oxidative phosphorylation and the function of the TCA cycle, resulting in elevated pO_2 levels and altered NADH/NAD^+ ratios, thereby leading to enhanced intracellular accumulation of carotenoids. Similarly, nutrient restriction is believed to adversely affect growth in cells. Phosphates and magnesium deficiency leads to impairment of protein and nucleic acid synthesis and slower growth rates due to insufficient energy supply for DNA/RNA synthesis, ultimately affecting the maximum biomass and growth rate [129, 130]. Therefore, it is very significant to balance the oxidative stress of cell growth and astaxanthin synthesis with appropriate nutrient restriction.

Conditions leading to excessive NADH promote astaxanthin synthesis

Various approaches for inducing astaxanthin accumulation in *P. rhodozyma* have been discussed in the preceding paragraphs. The mechanisms have primarily investigated involve impairing NADH reoxidation, specifically by disrupting electron flow through the mitochondrial electron transport chain (mETC) and oxidative ADP-phosphorylation, to facilitate astaxanthin accumulation. REDOX imbalances leading to astaxanthin synthesis and fermentation induction, as well as conditions leading to excessive NADH levels (e.g., active ethanol reassimilation and high sugar concentrations), are also considered. Ethanol production, a common outcome of glucose catabolic metabolism in *P. rhodozyma*, exhibits exponential increase under conditions of high sugar concentrations [131]. In such respiratory fermentation conditions, intracellular carotenoids levels are low and there is little or no pigment accumulation [132]. The presence of glucose is known to lead to relatively low expression of enzymes involved in carotenoid production [133]. However, when sugars are exhausted, *P. rhodozyma* employs ethanol as a carbon source, leading to an increase in the NADH/NAD + ratio, which triggers cellular oxidative stress and impacts astaxanthin synthesis [98]. It has been demonstrated that high sugar also impacts astaxanthin synthesis. In a previous article by Martinez-Cardenas, the growth phase of *P. rhodozyma* was investigated during which both sugar and pO_2 were elevated at high levels. This phase was characterized by rapid uptake of specific sugars, stable growth, and significant accumulation of astaxanthin cells [98]. Although transcription factors involved in glucose-dependent inhibition have been known to functionally regulate carotenoid production in *P. rhodozyma*, astaxanthin synthesis remains active under the under the conditions [89, 90]. Yeast can also cause excessive production of NADH under high sugar conditions, stimulate ROS production, and subsequently impact astaxanthin synthesis.

Outlook

Carotenoids are a class of terpenoids that are widely present in microorganisms. Besides astaxanthin, there are other bioactive carotenoids, such as torulene, torularodin and β-carotene, which also have antioxidant activities [134, 135]. In the past few decades, many yeasts belonging to different genera have been extensively studied as potentially effective producers of various carotenoids. Among them, the most common is *P. rhodozyma*. With the rapid development of synthetic biology, several model microorganisms, including *Escherichia coli*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, have also been engineered to produce carotenoids [34]. However, safety concerns regarding some of these microbial hosts, the instability of the engineered strains, as well as the long fermentation time also restrict further scale-up of this route [136]. In addition, some yeasts of the *Rhodotorula* genus, such as *Rhodotorula glutinis*, *Rhodotorula mucilaginosa* and *Rhodosporidiobolus colostri*, have also been found to have good biotechnological potential in the production of terpenoids [137–139]. Interestingly, recent studies have shown the existence of new *P. rhodozyma* strains isolated from nature, which may show a higher capacity to produce astaxanthin [140]. The biosynthesis of astaxanthin by *P. rhodozyma* has been investigated in detail, mainly due to several advantages such as simple culture conditions, broad substrate spectrum, and direct usability as a feed additive, thereby saving extraction costs. And there have been multiple advancements in genetic engineering and manipulation of metabolic pathways, presenting thrilling opportunities to enhance the yield of astaxanthin production. However, the metabolic network of *Phaffia rhodozyma* encompasses multiple interrelated metabolic pathways and regulatory mechanisms. The yield of product synthesis is limited by the metabolic stoichiometry of the host. Moreover, altering a

single gene or a few genes frequently has a limited effect as the overall metabolic balance and coordination are disrupted. While metabolic engineering modifications, such as gene deletion, over-expression, or attenuation, can enhance the experimental yield of astaxanthin, they cannot break the stoichiometric yield limit in *P. rhodozyma*. To significantly enhance the astaxanthin yield, a comprehensive understanding of the entire metabolic networks and coordinated manipulation of multiple genes or regulatory elements are necessary. Breaking the limitation can only be achieved by expanding the host metabolic network through the introduction of heterologous pathway genes. A high-quality cross-species metabolic network model (CSMN) and a quantitative heterologous pathway design algorithm (QHEPath) were developed to serve for the rational creation of cell factories [98]. Through systematic calculations using CSMN and QHEPath, the astaxanthin yield in *P. rhodozyma* will be improved by introducing appropriate heterologous reactions or predicting biologically plausible strategies based on carbon-conserving and energy-conserving.

Besides breaking through the yield threshold of astaxanthin, it is also very important to alleviate the impact of the massive accumulation of astaxanthin on cell growth. Previous studies have indicated that the accumulation of astaxanthin in *P. rhodozyma* might inhibit cell growth, such as multiple nutrient deficiencies, the presence of respiratory inhibitors, or certain specific genetic mutations and physical factors. Maintaining an optimum $NADH/NAD^+$ ratio is crucial for cell growth since alterations in the REDOX status of $NADH/NAD^+$ can considerably reconfigure cellular metabolism. Therefore, the $NADH/NAD^+$ pair should be considered as a bidirectional dynamic hub for the accumulation of astaxanthin and the coordination of cell metabolism that requires balance. In an era where digitization has become ubiquitous, integrating digital concepts into astaxanthin production can help overcome these challenges. In the future, machine learning algorithms will be used to simulate and calculate energy flow in *P. rhodozyma*. Highly accurate machine learning predictions from the metabolic network model will allow for precise adjustment of system compatibility among the core genes, pathways, and enzymes involved in endogenous or engineered astaxanthin synthesis mechanisms, based on genome-scale metabolic models. Optimization algorithms will be utilized to further enhance existing metabolic pathways, and effective modification targets will be discovered to increase astaxanthin synthesis efficiency. To better utilize the metabolic network model analysis tool for the design of metabolic pathways and metabolic engineering modification targets, a new tool called CAVE (a Cloud-based web tool for the Analysis and Visualization of metabolic pathways) has been developed [141]. It integrates functions such as model quality control modification, pathway calculation analysis, and pathway visualization, enabling balance between astaxanthin synthesis and cell metabolic in *P. rhodozyma*. Furthermore, potential modification targets can be identified more easily through visualization, facilitating the rapid construction of high-yield astaxanthin-producing *P. rhodozyma* strains.

Conclusions

Astaxanthin possesses a remarkable antioxidant capacity, we reviewed the advances and trends for astaxanthin synthesis in *Phaffia rhodozyma*, comprehending the biosynthetic pathway and stimulation strategies of astaxanthin in *P. rhodozyma* presents an exciting opportunity to enhance astaxanthin production. By employing metabolic network model analysis tool to balance the astaxanthin synthesis and cellular metabolism, the yield limit of astaxanthin can be overcome and potential modification targets can be identified more easily, which provides a new method for the rapid construction of *P. rhodozyma* strains with high production of astaxanthin.

Author contributions

Writing, original draft: J. J. Sun; Reviewing the manuscript: L.G and F.Y. All authors have read and agreed to the published version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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