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MINI-REVIEW

Research progress on carotenoid production by *Rhodosporidium toruloides*

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Abstract

Carotenoids are natural lipophilic pigments, which have been proven to provide significant health benefits to humans, relying on their capacity to efficiently scavenge singlet oxygen and peroxyl radicals as antioxidants. Strains belonging to the genus *Rhodosporidium* represent a heterogeneous group known for a number of phenotypic traits including accumulation of carotenoids and lipids and tolerance to heavy metals and oxidative stress. As a representative of these yeasts, *Rhodosporidium toruloides* naturally produces carotenoids with high antioxidant activity and grows on a wide variety of carbon sources. As a result, *R. toruloides* is a promising host for the efficient production of more value-added lipophilic compound carotenoids, e.g., torulene and torularhodin. This review provides a comprehensive summary of the research progress on carotenoid biosynthesis in *R. toruloides*, focusing on the understanding of biosynthetic pathways and the regulation of key enzymes and genes involved in the process. Moreover, the relationship between the accumulation of carotenoids and lipid biosynthesis, as well as the stress from diverse abiotic factors, has also been discussed for the first time. Finally, several feasible strategies have been proposed to promote carotenoid production by *R. toruloides*. It is possible that *R. toruloides* may become a critical strain in the production of carotenoids or high-value terpenoids by genetic technologies and optimal fermentation processes.

Key points

- *Biosynthetic pathway and its regulation of carotenoids in Rhodosporidium toruloides were concluded*
- *Stimulation of abiotic factors for carotenoid biosynthesis in R. toruloides was summarized*
- *Feasible strategies for increasing carotenoid production by R. toruloides were proposed*

Keywords *Rhodosporidium toruloides* · Carotenoids · Lipids · Abiotic factors

Introduction

According to the carotenoids database (http://carotenoiddb.jp.) up to March 2023, the molecular structures of 1204 natural carotenoids have been analyzed, which can be divided into C_{30}, C_{40}, C_{45}, and C_{50} isoprenoids by the number of carbon atoms within the carotenoid skeletons (Wang et al. 2007; Yabuzaki 2017). Carotenoids, which are widely distributed in plants, animals, and microorganisms, are a major class of functionally diverse pigments responsible for the yellow, orange, and red color in nature. Among microorganisms, some bacteria, algae, and fungi are well known to accumulate carotenoids as intracellular pigments (Ashokkumar et al. 2023; Maoka 2020). Fungal carotenoids are generally C_{40} or C_{40} derivatives, such as β-carotene, astaxanthin, neurosporaxanthin, torulene, and torularhodin (Rapoport et al. 2021) (Fig. 1). Their role in biological systems seems to be related to their activity as light-harvesting

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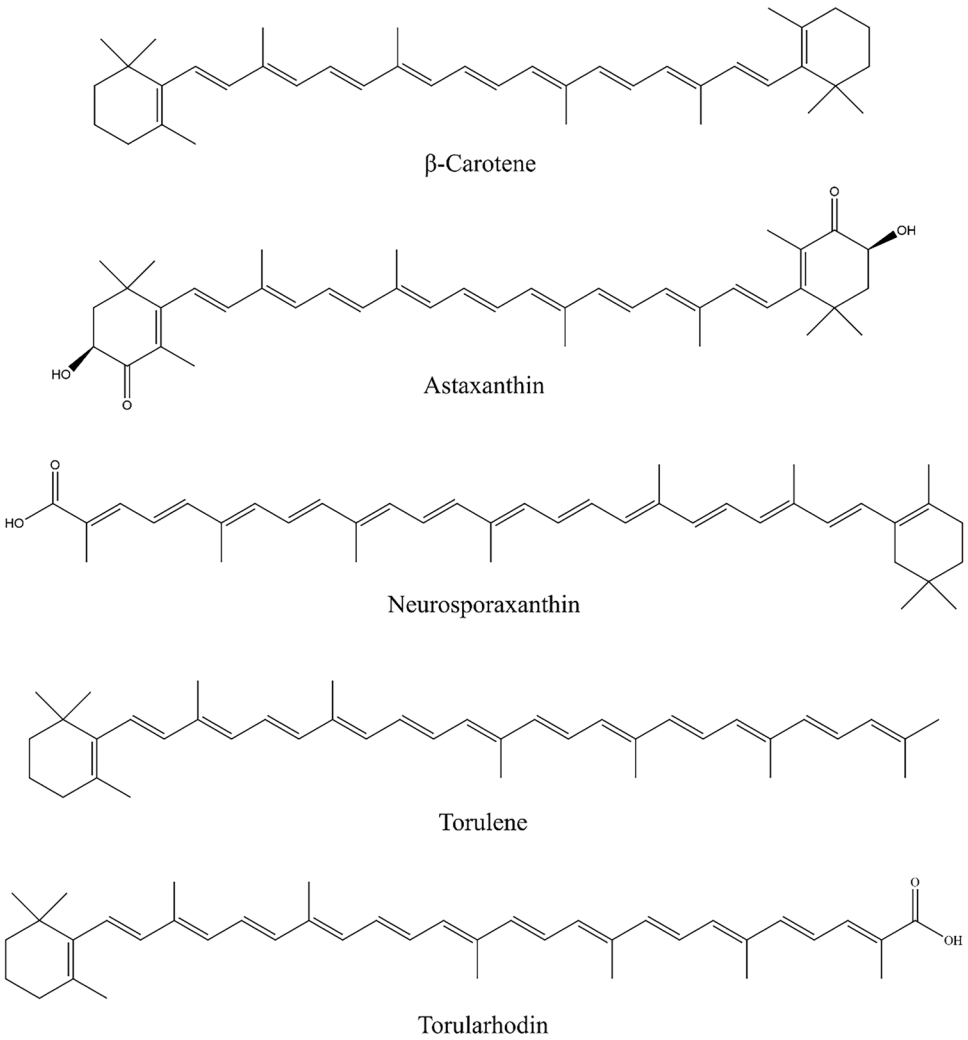


Fig. 1 Structural formulas of several different kinds of carotenoids

auxiliary pigments in photosynthetic organisms (Collini 2019; Simkin et al. 2022), antioxidant compounds active in protecting sensitive molecules from highly reactive oxygen form (González-Peña et al. 2023; Sandmann 2019), and precursors of vitamin A (Bohn et al. 2023; Roll Zimmer et al. 2022). In addition to their role in vitamin A biosynthesis, a number of attractive biological effects of carotenoids in vivo, for example, the prevention of oxidative damage (Rao et al. 2013), the suppression of tumor activity (Rowles and Erdman Jr 2020), and the modulation of the immune system (González-Peña et al. 2023; Pechinskii and Kuregyan 2014) have been discovered and well investigated, which expand their commercial applications and global market value.

Rhodosporidium toruloides, also named *Rhodotorula toruloides*, is a red, heterothallic, bipolar yeast belonging to the *Pucciniomycotina* (basidiomycetes). This red yeast was originally isolated in 1922 from the air in Dalian, China (Banno 1967). *R. toruloides* is an attractive strain with great potential for development and utilization in a variety of fields such as food (Rutz et al. 2016), chemicals (Polak and Jarosz-Wilkolazka 2012), and pharmaceuticals (Honda 2020) since it has been found to accumulate lipids and carotenoids in the natural conditions. Under certain conditions, this fungus can accumulate intracellular lipids of more than 70% of dry cell weight and a large number of various carotenoids in cellular lipid structures, including phospholipid membranes and lipid droplets, during the stationary phase of its growth (Li et al. 2007; Ma et al. 2019). Carotenoids produced by *R. toruloides* are mainly torulene, torularhodin, β-carotene, and a small amount of γ-carotene (Kot et al. 2023; Moliné et al. 2012). Studies have shown that torulene, as an active precursor of vitamin A, can promote the expression of intracellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in human prostate stromal cells (Du et al. 2017; Zoz et al. 2015). Torularhodin has a stronger scavenging efficiency of peroxide radical than β-carotene attributed to the two extra conjugated double bonds (Sakaki et al. 2002). Moreover, with the strong ability to biosynthesize lipid and carotenoids from low-cost carbon and nitrogen sources, such as agricultural

waste (Dai et al. 2019; Liu et al. 2020; Singh et al. 2018; Zeng et al. 2017) and industrial wastewater (Ling et al. 2013; Qi et al. 2020; Zhou et al. 2013), *R. toruloides* is regarded as a promising microbial cell factory for the production of various biochemicals, having broad prospects in industrial application. It has been shown that *R. toruloides* utilizes glucose, glycerol, xylose, and arabinose as carbon sources (Wiebe et al. 2012). Besides, it has high tolerance to the inhibitors produced by biomass hydrolysis and high growth rate in a high-density culture condition (Hu et al. 2009; Ma et al. 2019; Singh et al. 2020), which endow it with the ability to produce carotenoids efficiently. Despite the advantages mentioned above, its carotenoid biosynthesis mechanism and fermentation process conditions remain poorly documented largely because previous researches on this strain mainly focused on lipids biosynthesis. As a result, this strain has not been applied in the industrial production of carotenoids. In this work, the current status of molecular mechanism involved in carotenoid biosynthesis of *R. toruloides* is reviewed. Furthermore, carotenoid production by fermentation with *R. toruloides* as well as the influence of activity effectors have also been analyzed and summarized in order to promote its industrial application in carotenoid production.

Genes and enzymes responsible for the carotenoid production in *R. toruloides*

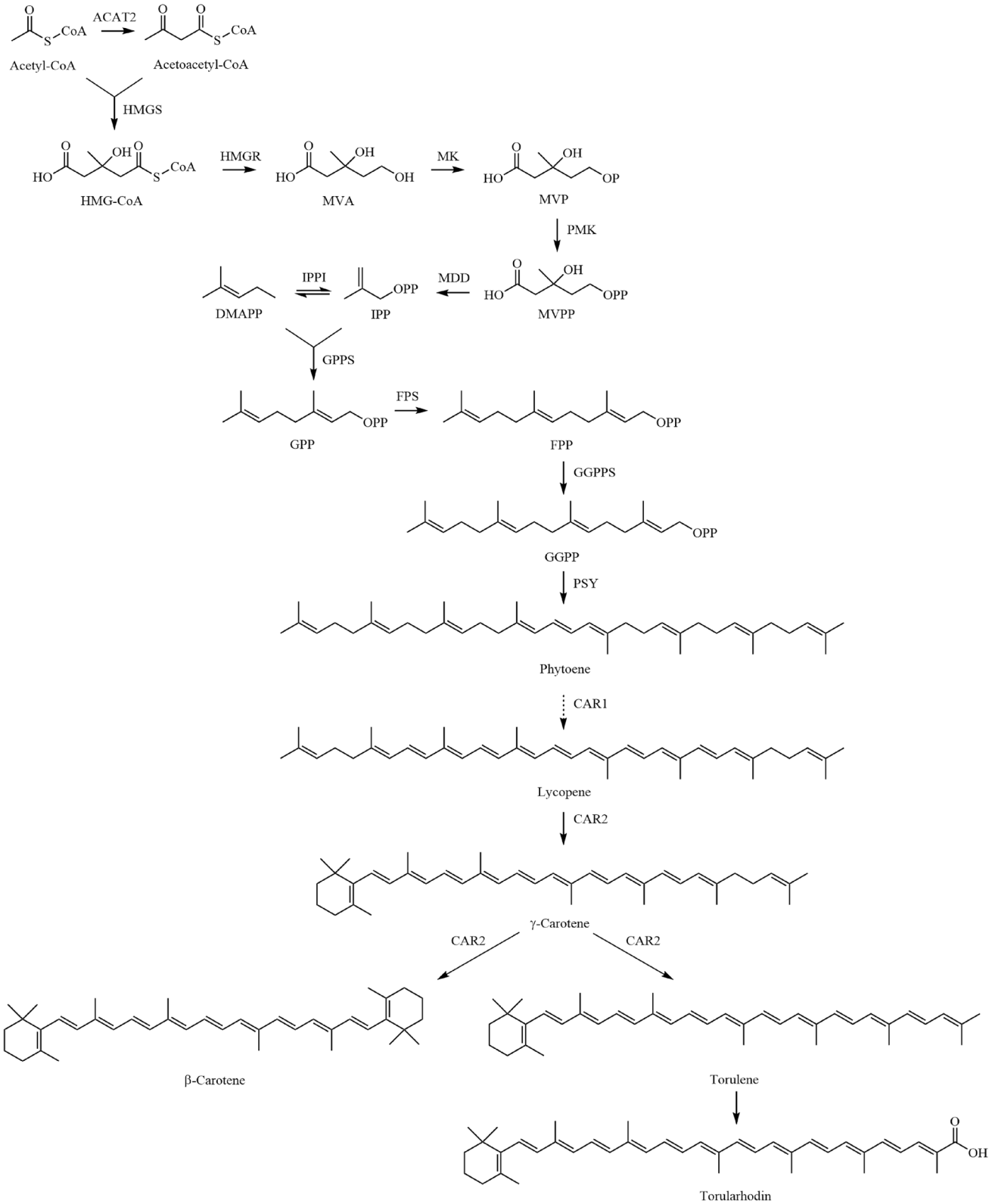
Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are common C_5 precursors for the biosynthesis of carotenoids and all other terpenoids. In vivo, these two substances are mainly derived from mevalonate (MVA) pathway or methyl-D-erythritol-4-phosphate (MEP) pathway (Chen et al. 2023; Vranova et al. 2013; Zingle et al. 2010). The MEP pathway mainly exists in Gram-negative bacteria and the plastids of green algae with pyruvate and glyceraldehyde-3-phosphate as precursors. The MVA pathway, which is commonly generated in the cytoplasm of mammals, eukaryotes, archaea, and Gram-positive cocci, begins with acetyl-CoA via intermediate MVA (Diner et al. 2018; Kuzuyama 2002; Lange et al. 2000; Ye et al. 2008). There are both MVA pathway and MEP pathway in higher plant cells, which are respectively located in cytoplasm and plastids (Vranova et al. 2013).

In *R. toruloides*, the C_5 carotenoids precursor IPP are biosynthesized through the MVA pathway, where the initial substrate acetyl-CoA is sequentially catalyzed by acetyl-CoA acetyltransferase 2 (ACAT2), 3-hydroxy-3-methylglutaryl CoA synthetase (HMGS), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), mevalonate kinase (MK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (MDD) with the corresponding intermediate metabolites acetoacetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), MVA, mevalonate 5-phosphate (MVP), and mevalonate-5-diphosphate (MVPP) (Miziorko 2011; Zhuang et al. 2019) (Fig. 2). DMAPP, isomerized from IPP by IPP isomerase (IPPI), is successively condensed with 3 molecules of IPP to form geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). The initial reaction for the production of phytoene, the first carotene of the pathway, is generated by condensing two molecules of GGPP catalyzed by phytoene synthase (PSY). The further conversion to lycopene involves four desaturation steps with the participation of phytoene dehydrogenase (CAR1). Both ends of lycopene can be cyclized by lycopene cyclase (CAR2) in succession, respectively yielding γ-carotene with one β-ionone ring and β-carotene with two β-ionone rings. Torulene, carrying 13 double bonds, is derived from γ-carotene through dehydrogenation catalyzed by CAR2 (Pham et al. 2021; Sandmann 1994) (Fig. 2). After that, torulene can be further converted to torularhodin by the introduction of oxygen groups, but the coding gene or enzyme responsible for this step is still unknown. Although several genes and corresponding enzymes in the carotenoid biosynthesis pathway of *R. toruloides* have been isolated and characterized (Table 1), there are few related studies on the gene level about a complex regulatory mechanism for carotenoid production, largely because genetic backgrounds of this naturally oleaginous species remain poorly documented (Li et al. 2022; Park et al. 2018; Pham et al. 2021).

Relationship between carotenoid and lipid accumulation in *R. toruloides*

Acetyl-CoA, the starting substance for the biosynthesis of IPP, could also flow to another metabolic pathways, such as the one concerned with the biosynthesis of fatty acids in *R. toruloides* (Zhu et al. 2012) (Fig. 3). Theoretically, the production of carotenoids in cells could be increased by enhancing the MVA pathway or weakening the lipids biosynthesis pathway to redirecting total acetyl-CoA flow for carotenogenesis. For example, the overexpression of endogenous *ACAT2* gene, responsible for the first rate-limiting enzyme of the MVA pathway, in *Rhodosporidium kratochvilovae* leads to the enhanced accumulation of carotenoids by 50.53% accompanied by a decrease in lipids content by 22.80% (Guo et al. 2021). It is suggested that overexpression of *ACAT2* gene not only promotes the entry of more acetyl-CoA into the MVA pathway but also enhances the transcription levels of *ACAT2*, *HMGR* (encoding 3-hydroxy-3-methylglutaryl-CoA reductase), and *MK* (encoding mevalonate kinase) genes in MVA pathway, which finally increases the content of carotenoids in the overexpressed strain.

However, due to the complex relationship between carotenoid and lipid accumulation in *R. toruloides*, in which


ACAT2
Acetyl-CoA
Acetoacetyl-CoA
HMGS
HMG-CoA
HMGR
MVA
MK
MVP
PMK
MVPP
MDD
IPPI
DMAPP
IPP
GPPS
GPP
FPS
FPP
GGPPS
GGPP
PSY
Phytoene
CAR1
Lycopene
CAR2
γ-Carotene
CAR2
CAR2
β-Carotene
Torulene
Torularhodin

◂**Fig. 2** Carotenoid biosynthesis in *Rhodosporidium toruloides*. Briefly, C_5 precursor IPP and its DMAPP were biosynthesized from acetyl-CoA via the MVA pathway with sequential intermediates acetoacetyl-CoA, HMG-CoA, MVA, MVP, and MVPP. Following that, the condensation of two molecules of GGPP, which is formatted by adding three molecules of IPP to DMAPP, produces the first carotene phytoene. Finally, phytoene is transformed into other carotenoids through dehydrogenation, cyclization, and hydroxylation. The enzyme responsible for the conversion is listed next to the reaction arrow. A solid arrow indicates a single catalytic step, whereas a dotted arrow means multiple steps. Abbreviations: ACAT2, acetyl-CoA acetyltransferase 2; CAR1, phytoene dehydrogenase; CAR2, lycopene cyclase; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; FPS, FPP synthase; GGPP, geranylgeranyl pyrophosphate; GGPPS, GGPP synthase; GPP, geranyl pyrophosphate; GPPS, GPP synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthetase; IPP, isopentenyl pyrophosphate; IPPI, IPP isomerase; MDD, mevalonate diphosphate decarboxylase; MK, mevalonate kinase; MVA, mevalonate; MVP, mevalonate 5-phosphate; MVPP, mevalonate-5-diphosphate; PMK, phosphomevalonate kinase; PSY, phytoene synthase

competition of precursor substance acetyl-CoA and mutual promotion of their biosynthesis coexist, the single regulation of one of these pathways may not achieve the ideal results in the massive carotenoid accumulation. By analyzing the carotenoid metabolic fluxes of wild-type and carotenoid-deficient mutants of *R. toruloides*, Wang et al. (2019) found that defects in the carotenoid biosynthetic pathway can also reduce the content of lipids. It means the block of carotenoid biosynthetic pathway did not lead to more flux of acetyl-CoA into the lipids biosynthetic pathway as expected but resulted in decreased lipid biosynthesis of the strain. Moreover, the accumulation of acetyl-CoA in carotenoid deficiency mutant eventually leads to the enhancement of the organic acids pathway. Generally, carotenoids could reduce the oxidation level in cells and prevent fatty acids from oxidative degradation, which speculated that the increase of intracellular oxidation level due to the lack of carotenoids may enhance the oxidative decomposition of fatty acids, which eventually reduces the content of lipids followed by the increased generation of succinic acid, glyoxylic acid, and malic acid through glyoxylate cycle (Demets et al. 2022; Tkacova et al. 2018).

It is found that there is a strong correlation between the accumulation of carotenoids and the production of fatty acids, especially in the unsaturated fat acid (UFA) oleic acid, which contributes to the increase of membrane fluidity and the formation of lipid body (Lee et al. 2014). The lipid body, as storage places of fat-soluble carotenoids, was conducive to the accumulation of carotenoids. Study on carotenoid and lipid metabolism of microalgae *Dunaliella bardawil* has shown that excessive β-carotene produced by *D. bardawil* cells was accumulated in triglyceride droplets. When triacylglycerol synthesis was blocked, the overproduction of β-carotene would also be inhibited. Interestingly, no up-regulation of key genes responsible for carotenoid biosynthesis was observed at the transcription or translation level during β-carotene overproduction, while acetyl-CoA carboxylase, a key regulator for acyl lipid biosynthesis, was increased (Rabbani et al. 1998). Similarly, a similar regulation mechanism was also found in *R. toruloides*, as some scholars have proved that there was a positive correlation between lipid accumulation and carotenoid formation in *R. toruloides* (Elfeky et al. 2020). Besides, it has been observed that exogenous supplementation of UFA can increase carotenoid contents, which is consistent with the conjecture that more carotenoids are needed to participate in maintaining the unsaturated state of UFA (Sun et al. 2016).

Lee et al. (2014) found that *R. toruloides* strain CBS 5490 with higher carotenoid production had a unique metabolic profile as compared to the other strains. In this strain, metabolites belonging to the TCA cycle and amino acids are produced in lower amounts which indicate that the lower production of the TCA cycle and amino acid metabolites promotes energy and metabolic flux to the carotenoid and lipid biosynthesis. On the one hand, the biosynthesis of carotenoids in *R. toruloides*, competing for the same substrate acetyl-CoA with lipid biosynthesis, may be enhanced by reducing the intensity of fatty acid biosynthesis or improving the activity of related enzymes in MVA pathway. Besides, enhanced catabolism of fatty acids may also partially increase the yield of carotenoids, whereby β-oxidation of fatty acids provides acetyl-CoA and energy for MVA pathway. On the other hand, lipid biosynthesis is beneficial to the accumulation and storage of carotenoids. Interestingly, the increase of the oxidative pressure in cells, attributed to the fatty acids, especially UFA, results in more carotenoid accumulation to resist. Considering their important physiological function in cells and key role in carotenoid storage, the strategy for the increase of carotenoid accumulation is not desirable or even counterproductive to completely block lipid biosynthesis, though lipids and carotenoids share the same precursor for their biosynthesis. Presently, it has been demonstrated that variable culture conditions, such as C/N ratios, pH, and light, influence carotenoid and lipid formation (Braunwald et al. 2013; Dias et al. 2016; Pham et al. 2020), and the genomes of several *R. toruloides* strains have been studied (Dinh et al. 2019; Sambles et al. 2017; Tiukova et al. 2019). The balance between lipid and carotenoid production, however, has not been accurately and fully elucidated. In order to increase carotenoid production, the genetic relationship between lipids and carotenoids should be thoroughly clarified.

Abiotic factors stimulate carotenoid biosynthesis in *R. toruloides*

In general, it is believed that the production of carotenoids, as a secondary metabolite, is not necessary for the growth of microorganisms in most case, and its biosynthesis is largely

Table 1 Genes related to carotenoid biosynthesis in *Rhodosporidium toruloides*

Genes	Enzymes	Strains of *Rhodosporidium toruloides*	GenBank accession numbers
HMGS	3-hydroxy 3-methylglutaryl CoA synthetase (HMGS)	NBRC 10032	LC384040.1
		ATCC 204091	AEVR02000010.1 (locus_tag: RTG_02094)
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)	NBRC 10032	LC384041.1
PMK	Phosphomevalonate kinase (PMK)	NBRC 10032	LC384042.1
MDD	Mevalonate diphosphate decarboxylase (MDD)	NBRC 10032	LC384043.1
IPPI	Isopentenyl pyrophosphate isomerase (IPPI)	NBRC 10032	LC384044.1
		NBRC 0880	LCTV02000008.1 (locus_tag: AAT19DRAFT_16203)
FPS	Farnesyl diphosphate synthase (FPS)	NBRC 10032	LC384045.1
		NP11	XM_016415341.1
		LAB-07	JAOWAH010000012.1 (locus_tag: OF846_004277)
GGPS	Geranylgeranyl pyrophosphate synthase (GGPPS)	NBRC 10032	LC384046.1
		NP11	XM_016416178.1
		LAB-07	JAOWAH010000001.1 (locus_tag: OF846_000220)
CAR2	Phytoene synthase (PSY)	NBRC 10032	LC384047.1
CAR2	Bifunctional phytoene synthase/lycopene cyclase (PSY/CAR2)	NP11	XM_016418270.1
CAR1	Phytoene dehydrogenase (CAR1)	NBRC 10032	LC384048.1
		NP11	XM_016418267.1

based on a protective mechanism generated by microorganism in response to adverse environmental stress (Yin et al. 2016; Zhao et al. 2021). Therefore, under the influence of certain external factors, such as light, high salt, and heavy metal, microorganism will accumulate carotenoids and other antioxidants to cope with cell damage. The production of carotenoids in *R. toruloides* also conforms to this regulatory mechanism.

Light

Carotenoids act as antioxidants that protect cells from light and other environmental stresses. Under the stress of light, *R. toruloides* would also increase the production of carotenoids, which is regarded as the effector of the photoprotective mechanism (Mata-Gomez et al. 2014). It is found that the light irradiation results in 70% higher carotenoid yield in *R. toruloides* (Pinheiro et al. 2020). It is displayed that the carotenoid content of *R. toruloides* cultured in light was higher than that cultured under dark conditions (Pham et al. 2020). Subsequently, it is demonstrated that the biosynthesis of carotenoids is regulated by irradiation at the transcriptional level. The transcription levels of carotenogenic genes *CAR1* and *CAR2* in cells cultured under light conditions increased by two and three times as much as those of cells cultured under dark conditions, respectively. In addition, a higher expression level of GGPP synthase (GGPPS), the first photoreactive enzyme in the carotenogenic pathway, was detected in light-cultured cells, which lead to a correlated increase of GGPP content and the stimulation of carotenoid biosynthesis. It is indicated that the carotenogenic genes *CAR1* and *CAR1* undergo a two-step transcriptional activation, which is potentially regulated by the blue light receptor cryptochrome DASH (CRY1) (Pham et al. 2021). It is not unique, increased carotenoid production of *Rhodotorula* genus and other fungi has been demonstrated in lighting culture (An and Johnson 1990; Garcia-Cortes et al. 2021; Linden et al. 1997; Zhang et al. 2014). As a consequence, strategies to explore the optimal light intensity or overexpression of CRY1 protein can be used to increase the carotenoid production of *R. toruloides*. However, the effect of light on carotenoid production is a complex network mechanism, which may involve a variety of regulatory pathways, and more studies are needed to further clarify this mechanism at gene level so as to optimize the production process of carotenoids in *R. toruloides*.

Salt

Salt or osmotic stress is one of the common abiotic stresses that affect the metabolism and growth of organisms. Salt stress could induce the change of carotenoid content or proportion in cells, which is a common phenomenon appearing in nature and probably due to that the strong antioxidant activity of these polyunsaturated pigment plays a key role in quenching oxidative stress induced by salt stress (Li et al. 2017; Sankari et al. 2019; Sayyad-Amin et al. 2016). The biosynthesis of carotenoids is one of the physiological responses of *R. toruloides* to hyperosmosis since this fungus produced more carotenoids in the presence of salt stress

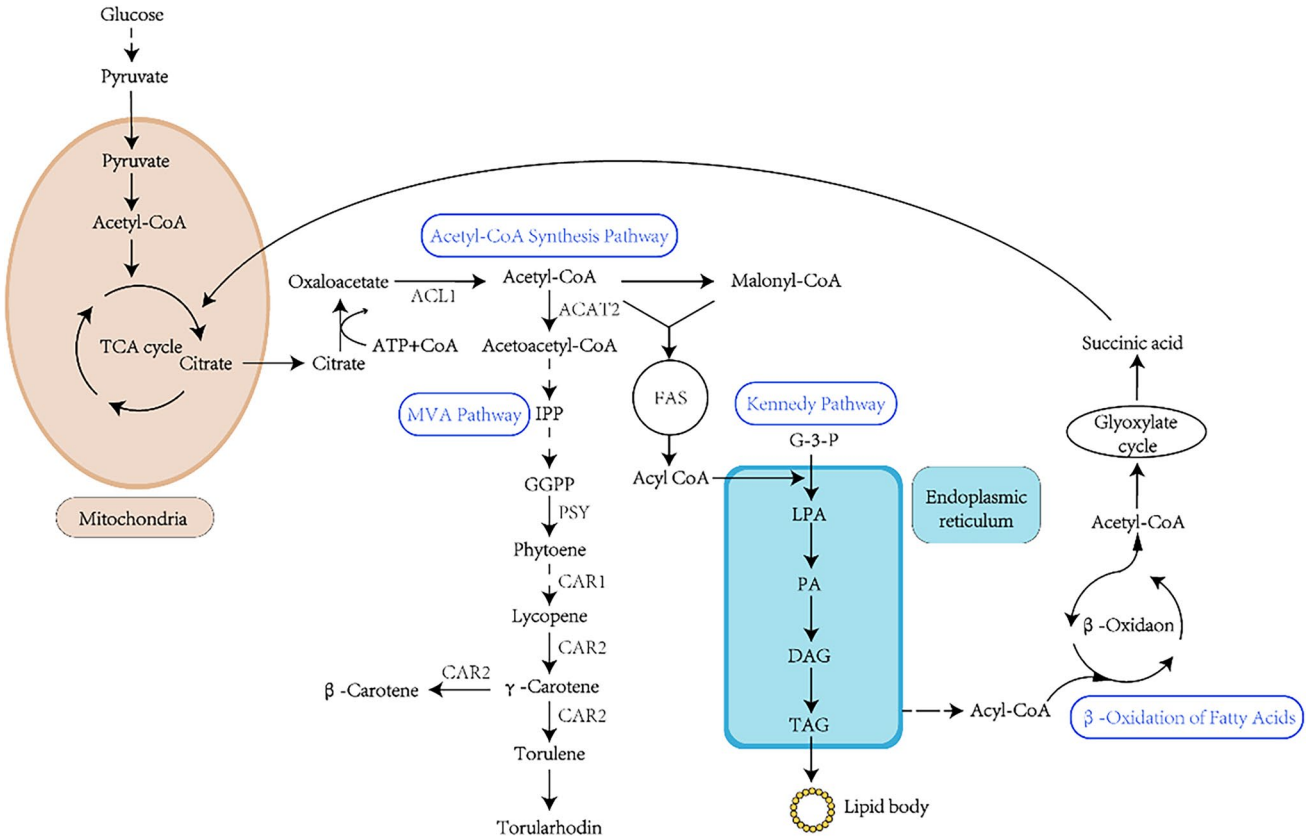


Fig. 3 Metabolic pathways related to lipid and carotenoid biosynthesis in *Rhodosporidium toruloides*. The acetyl-CoA synthesized by the TCA cycle ultimately flows to carotenoids and lipids through the MVA and Kennedy pathways respectively. Lipids can form lipid bodies for storing carotenoids, while the oxidative breakdown of lipids can form acetyl-CoA, which then enters the glyoxylate cycle to generate succinic acid and other substances. A solid arrow indicates a single catalytic step, whereas a broken arrow means multiple steps. Substrates and metabolites are connected by arrows, whereas the enzyme that catalyzes the conversion is indicated alongside the reaction arrow. Abbreviations: ACAT2, acetyl-CoA acetyltransferase 2; ACL1, citrate lyase; DAG, diacylglycerol; FAS, fatty acid synthase; G-3-P, glyceraldehyde-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; TAG, triacylglycerol

(Pinheiro et al. 2020). It is found that the combination of 0.1 M K^+ and 1.0 M Na^+ supplementation in *R. toruloides* cultures could enhance the productivity of β-carotene by 60% (Illarionov et al. 2021). Similarly, Singh et al. (2016) studied the carotenoid production of *R. toruloides* under different salt concentrations and showed that 1–10% NaCl stress can significantly increase the carotenoid production of *R. toruloides* (Singh et al. 2016). However, the molecular mechanism underlying the increased carotenoid biosynthesis in *R. toruloides* is not understood clearly. Transcriptome profiling of *Rhodotorula glutinis* and *Sporidiobolus pararoseus* reveals that salt stress efficiently promotes the expression of genes *CRTI* and *CRTYB*, which are responsible for the coding of phytoene dehydrogenase and lycopene cyclase similar to *CAR1* and *CAR2*, respectively (Li et al. 2019, 2021). Presumably, genes *CAR1* and *CAR2* in *R. toruloides* were also overexpressed in response to the elevation of ROS levels caused by the salt stress. As a result of the overexpression of carotenogenic genes, carotenoid production is increased to quench the excessive accumulation of ROS in the cell, which plays an indispensable role for the survival and physiological function of *R. toruloides*. Nevertheless, the molecular regulatory mechanism of massive carotenoid accumulation under salt stress conditions should be further studied and analyzed. To sum up, finding the optimal salt addition amount in culture or fermentation process is expected to further increase the carotenoid yield of *R. toruloides*. Because stress conditions including high salinity can inhibit cell growth, a two-stage culture strategy is commonly employed to resolve the conflict between cell growth and carotenoid production, with optimal growth conditions in the first stage used to obtain maximum biomass and the second stage used to accumulate carotenoids under various stress conditions (Sun et al. 2018).

Heavy metal ions

Generally, heavy metal ions can inhibit the growth of *R. toruloides* by increasing intracellular oxidation levels, and

the intensity of inhibition is positively correlated with the concentration of heavy metal ions (Cavelius et al. 2023; Zheng et al. 2021). However, studies have also shown that low concentration of Cd^{2+} (5.0 μmol/L) has no inhibitory effect on the growth of yeast but promotes it to some extent (Zhou et al. 2018). This promoting effect might be partially attributed to the stimulation of carotenoid biosynthesis. As a part of the non-enzymatic protection system of cells, carotenoids work together with the enzymatic protection system to maintain the balance of intracellular components and redox state when organisms are subjected to oxidative stress (Segal-Kischinevzky et al. 2022; Sies 1997). According to the experimental results, the biomass and carotenoid contents of *R. toruloides* reach the highest when the concentration of Cd^{2+} is 5.0 μmol/L; however, they gradually decreased with the subsequent increase of Cd^{2+} concentration. Currently, the mechanism of oxidative stress induced by heavy metals has not yet been fully understood, and related research mainly focuses on the cellular level. The yield of carotenoid in *R. toruloides* can be increased to some extent by adding low concentration of heavy metal ions, but attention should be paid to strictly control the concentration and treatment time. In addition, carotenoids and their derived products generated by this strategy must undergo heavy metal residue analysis and safety evaluation.

Optimization of *R. toruloides* fermentation for carotenoid production

In addition to the strategies of breeding of highly productive strains with genetic engineering technology and adding additional stress factors in culture, the optimization of fermentation process also plays a critical role in increasing yield and reducing cost in the industrial production of carotenoids.

Control of pH values

Each kind of microorganism has its optimal pH value for growth, and it is known that microbial biosynthesis of lipids and carotenoids is strongly dependent on the growth conditions, particularly medium pH (Ghazani and Marangoni 2022). Through the exploration of the optimal pH values for the carotenoid production by *R. toruloides*, it was found that the yeast biomass concentration and lipid content were maximum at pH 4.0 (5.90 g/L and 21.85% w/w, respectively), while the maximum carotenoid yield (63.37 μg/g) was obtained at pH 5.0 (Dias et al. 2016). As the optimal pH value for the growth of *R. toruloides* is different from that for the production of carotenoids, a two-stage pH control method has been adopted to achieve high carotenoid yield during culture (Dias et al. 2015). Briefly, the pH value of culture medium is adjusted to 4.0 for rapid cell growth and proliferation during the active growth period. Subsequently, cells are regulated at the optimal pH of 5.0 for carotenoid production. It is indicated that the two-stage pH control method could significantly improve the carotenoid yield of *R. toruloides* compared with that under a single pH condition. Therefore, balancing the relationship between cell mass accumulation and carotenoid production, as well as finding the optimal culture pH or utilizing step-by-step culture techniques may be a good strategy for industrial production of carotenoids by *R. toruloides*.

Control of carbon–nitrogen ratio

R. glutinis is a red yeast whose carotenoid biosynthesis is affected by the carbon-to-nitrogen (C/N) ratio of the cultural medium (Braunwald et al. 2013; Elfeky et al. 2019; Tkacova et al. 2017). Based on the experimental results, carotenoid formation is stimulated in this oleaginous yeast with a lower C/N ratio, while a higher C/N ratio prompts more lipid production than carotenoids (Kuttiraja et al. 2016; Saenge et al. 2011; Somashekar and Joseph 2000). A similar pattern is also observed in the culture of *R. toruloides* (Bao et al. 2019). As the C/N ratio increases, cell proliferation decreases due to the gradual depletion of nitrogen source. Nitrogen deficiency is known to promote lipid accumulation in several microorganisms (Adams et al. 2013; Li et al. 2014; Singh and Kumar 1992; Yang et al. 2016; Zhao et al. 2018), which may explain why a further increase in the C/N ratio does not lead to a further increase of carotenoid production of *R. toruloides* and instead shifts towards lipid synthesis by utilizing the available acetyl-CoA.

Moreover, studies have shown that cells tend to convert β-carotene into other carotenoids at high C/N ratios. For example, the ratio of β-carotene, torularhodin, and torulene of *R. toruloides* changes from 0.48:0.40:0.11 (C/N = 80) to 0.17:0.34:0.32 (C/N = 120) (Lopes et al. 2020). Torularhodin and torulene are less common carotenoids that cannot be generated in animals and plants, and only several yeasts and fungi can biosynthesize them naturally (Kot et al. 2018). Therefore, the production of torularhodin and torulene with an appropriate high C/N ratio may result in greater benefit than producing lipids with *R. toruloides*, especially in increasing the product diversity of carotenoids.

The regulatory mechanism by which the C/N ratio influences the biosynthesis of carotenoids and lipids in *R. toruloides* remains poorly understood due to insufficient gene-level investigation. The optimal C/N ratio for the highest carotenoid yield varied among the strains of *R. toruloides* and the types of carbon and nitrogen sources used. For example, a high carotenoid concentration (9.23 ± 0.97 mg/L) was obtained by *R. toruloides* NRRL Y1091 when using xylose as a carbon source with a C/N ratio of 50; however in

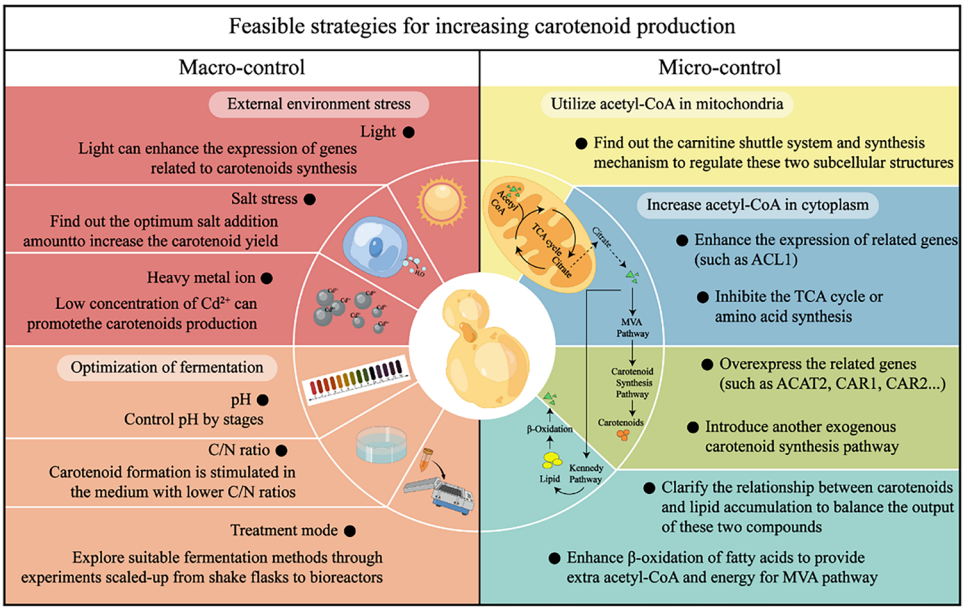


Fig. 4 Feasible strategies for increasing carotenoid production in *Rhodosporidium toruloides*. Enhancing carotenoid production can be achieved through two main approaches: macroscopically, the increased accumulation of carotenoids can be realized by applying external stress factors or optimizing fermentation conditions; microscopically, the production increase can be achieved by increasing the flow rate of acetyl-CoA, overexpressing genes related to carotenoid synthesis, and regulating the balance between lipid accumulation and carotenoid biosynthesis

a xylose medium with a C/N ratio of 25, this yeast accumulated more carotenoids (2.99 ± 0.66 mg/g) (Liu et al. 2020). Furthermore, varying C/N ratios can significantly impact not only the yield but also the composition of carotenoids. Consequently, it is crucial to explore the optimal C/N ratio based on the specific needs of the actual production.

Fermentation for carotenoid production by *R. toruloides*

The effects of different fermentation treatments on the carotenoid production of *R. toruloides* were studied by using *Camelina sativa* meal as raw material (Bertacchi et al. 2020, 2021). The results revealed that simultaneous saccharification and fermentation of *Camelina* meal hydrolysate resulted in a higher production of carotenoids (16 ± 1.9 mg/L) than separate hydrolysis and fermentation. Moreover, the study revealed that water-insoluble solids might trigger the production of carotenoids, which serve as scavenger molecules, to reduce the stress-inhibitory effect of these substances on cell growth (Bertacchi et al. 2020). Subsequently, the fermentation method was further optimized by separately adjusting enzymatic and biomass loadings (Bertacchi et al. 2021), which not only reduced materials and fermentation time but also provided a possibility for the efficient utilization of *Camelina* meal and future industrialization of *R. toruloides*.

R. toruloides can use diverse agricultural or chemical by-products for high-value product biosynthesis, and the choice of substrates plays a vital role in determining cell growth and product accumulation. Therefore, experimentally scaled-up investigations from shake flasks to bioreactors are necessary to determine appropriate fermentation conditions for industrial production by *R. toruloides*.

Conclusion and prospects

The biosynthesis of lipids in *R. toruloides* competes with the generation of carotenoids for the same substrate acetyl-CoA. However, lipid biosynthesis is beneficial to the formation of the lipid body and promotes the accumulation and storage of carotenoids. The interaction mechanism between the two pathways is still not well studied. In view of the above, the following ways for the regulation of the acetyl-CoA metabolic flow may be a potential strategy to increase carotenoid production by *R. toruloides*, while blocking lipid biosynthesis simply may not work as expected. First, it is crucial to increase the total flow of acetyl-CoA into these two biosynthetic pathways by either enhancing the expression of ACL1 (citrate lyase) or inhibiting the TCA cycle and amino acid biosynthesis within the acceptable range of cell generation. In addition, it is known that acetyl-CoA is mainly distributed in both mitochondria and cytoplasm of *R. toruloides*, while both the MVA and carotenoid biosynthetic pathways are located within the cytoplasm. Therefore, how to efficiently utilize acetyl-CoA in mitochondria and coordinate regulation of these two subcellular structures by finding out the carnitine shuttle system and synthesis mechanism in cells may also become an essential future research direction. Second, the enhancement of the MVA or carotenoid biosynthetic pathways can boost the utilization of acetyl-CoA in downstream carotenoid biosynthesis. This can be achieved by the overexpression of rate-limiting enzymes, such as ACAT2, CAR1, and CAR2. Third, the

introduction of a complete carotenoid biosynthetic pathway into other organelles of the cell can also be considered. The introduction of a complete MVA pathway in the peroxisome of yeast has already been achieved (Dusseaux et al. 2020). As more basic research is conducted on *R. toruloides*, this engineering transformation may also become achievable. Alternatively, introducing more efficient exogenous genes or building hybrid biological modules has been proposed to enhance the expression of endogenous genes. Furthermore, to promote the metabolic engineering of *R. toruloides*, developing a set of promoters with strong activity during the carotenoid-accumulation stage is critical.

The biosynthesis of carotenoids in *R. toruloides* functions as a mechanism for light protection, which can be enhanced by modifying the duration and intensity of light exposure. These modifications can promote the expression of genes involved in carotenoid biosynthesis. Additionally, a certain degree of salt stress or hyperosmosis can stimulate the accumulation of carotenoids in *R. toruloides*, and finding the optimal salt concentration can improve carotenoid production. Heavy metals can increase intracellular ROS levels (Bussche and Soares 2011), and carotenoid biosynthesis is one of the effective adaptation mechanisms for microbial cells to survive in these adverse environments (Bhosale and Gadre 2001). Heavy metals can affect the growth and carotenoid production of *R. toruloides* negatively. However, low concentration of Cd^{2+} can promote the growth of yeast and carotenoid production, while a downward trend of carotenoids was observed in continuously rising concentrations. On the basis of considering the residue problem, adding an appropriate amount of heavy metal ions can be tried to enhance carotenoid biosynthesis in cells.

It is vital to control the fermentation methods and conditions in the industrial production of microbial carotenoids. As carotenoids are secondary metabolites, the optimal pH value for cell growth may differ from that for this product biosynthesis in *R. toruloides*. To achieve efficient carotenoid accumulation, a staged pH control method can be adopted to balance and optimize conditions. The C/N ratio can impact the carotenoid yield and composition in *R. toruloides*. In general, carotenoid formation is stimulated in the medium with lower C/N ratios, while a higher C/N ratio directs acetyl-CoA towards lipid synthesis instead. Besides, yeast tends to convert β-carotene to other carotenoids under the condition of high C/N ratios. It is worth noting that toxic effects on yeast and treatment processes must also be considered when selecting fermentation substrates.

In this review, several feasible strategies to enhance carotenoid production of *R. toruloides* are summarized (Fig. 4). Obviously, *R. toruloides* is considered a cell factory for carotenoid production, but significant knowledge gaps need to be addressed before its large-scale implementation can be achieved. Specifically, the molecular mechanism of torularhodin biosynthesis remains largely unknown, which hinders genetic engineering efforts to achieve efficient industrial-level pigment production. Although several key enzyme genes have been elucidated in the carotenoid biosynthesis pathway of *R. toruloides*, there is still a lack of research on their molecular regulation mechanism. These limitations must be overcome before *R. toruloides* can be confidently deployed as an effective carotenoid cell factory. Furthermore, most of the researches on *R. toruloides* has focused on lipid accumulation, while its carotenoid synthesis has received less attention. The relationship between carotenoids and lipid accumulation is intricate and not yet fully understood, leading to difficulties in controlling their production separately. In addition, there is also a lack of molecular explanation regarding the increase of carotenoid production caused by external stress, and research on optimizing the fermentation process for carotenoid production is limited. Considering the remarkable capacity for carotenoid biosynthesis of *R. toruloides*, there is a high potential for its application in industrial production, provided that the above-mentioned challenges are addressed in future research. Currently, several molecular tools and omics techniques to engineer *R. toruloides* have already been developed (Park et al. 2018). These studies provide novel synthetic biology tools and rational metabolic engineering ideas to modify the carotenoid pathway of *R. toruloides*, which would promote its industrial application in the future.

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Data availability All data supporting the findings of this study are available within the paper.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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