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Review

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# Biosynthetic Pathway of Carotenoids in *Rhodotorula* and Strategies for Enhanced Their Production

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*Rhodotorula* is a group of pigment-producing yeasts well known for its intracellular biosynthesis of carotenoids such as β-carotene, γ-carotene, torulene and torularhodin. The great potential of carotenoids in applications in food and feed as well as in health products and cosmetics has generated a market value expected to reach over $2.0 billion by 2022. Due to growing public concern over food safety, the demand for natural carotenoids is rising, and this trend significantly encourages the use of microbial fermentation for natural carotenoid production. This review covers the biological properties of carotenoids and the most recent findings on the carotenoid biosynthetic pathway, as well as strategies for the metabolic engineering methods for the enhancement of carotenoid production by *Rhodotorula*. The practical approaches to improving carotenoid yields, which have been facilitated by advancements in strain work as well as the optimization of media and fermentation conditions, were summarized respectively.

**Keywords:** *Rhodotorula*, carotenoids, biosynthetic pathway, strategies

## Introduction

*Rhodotorula* is a genus of unicellular pigmented yeasts, part of the phylum Basidiomycota, family Cryptococcaceae, subfamily Rhodotorulodae [1]. According to the latest records of Mycobank (www.mycobank.org), there are 164 species in the genus *Rhodotorula*, including *R. glutinis*, *R. toruloides*, *R. mucilaginosa*, *R. graminis* and other varieties (Fig. 1). The cells of these *Rhodotorula* strains are polyphyletic in shape, appearing as subglobose, ovoid, ellipsoidal and elongated. The asexual reproduction of *Rhodotorula* is usually undertaken via multilateral and polar budding, whereas the sexual reproduction life cycle has occasionally occurred in the form of pseudohyphae in some strains [2].

*Rhodotorula* species are ubiquitous saprophytic yeasts and they can present in habitats with broad geographical varieties, *e.g.*, from the equator to the poles and from land to the ocean. Strains in *Rhodotorula* are able to grow on different substrates in wide-ranging ecological conditions, such as air, soil, and manure, as well as in the bodies of animals, plants, and some lower organisms [3]. *Rhodotorula* is one of the dominant yeast flora in nutrient-poor aquatic environments, where they are actually reported to account for about 50% of the yeast population in seawater and fresh water [4].

One of the most notable characteristics of *Rhodotorula* is the formation of natural carotenoid biosynthesis. Enriched with pigments, *Rhodotorula* biomass alone can also serve as a high quality, single-cell protein source for utilization as a valuable feed additive.

Carotenoids have long been recognized worldwide as food additives and nutritional supplements, thanks to their valuable biological functionalities, such as antioxidative effects, immune response enhancement, preventive effects to cardiovascular disease, eye diseases and cancer, respectively [5, 6]. Nowadays, as a popular food additive, carotenoids are used broadly in the EU (listed as additive E160a), the US, Australia and New Zealand (listed as 160a) and other countries or regions, respectively. Statistically, the global market for carotenoids reached $1.5 billion in 2017 and should reach $2.0 billion by 2022, at a compound annual growth rate (CAGR) of 5.7% for the period of 2017–

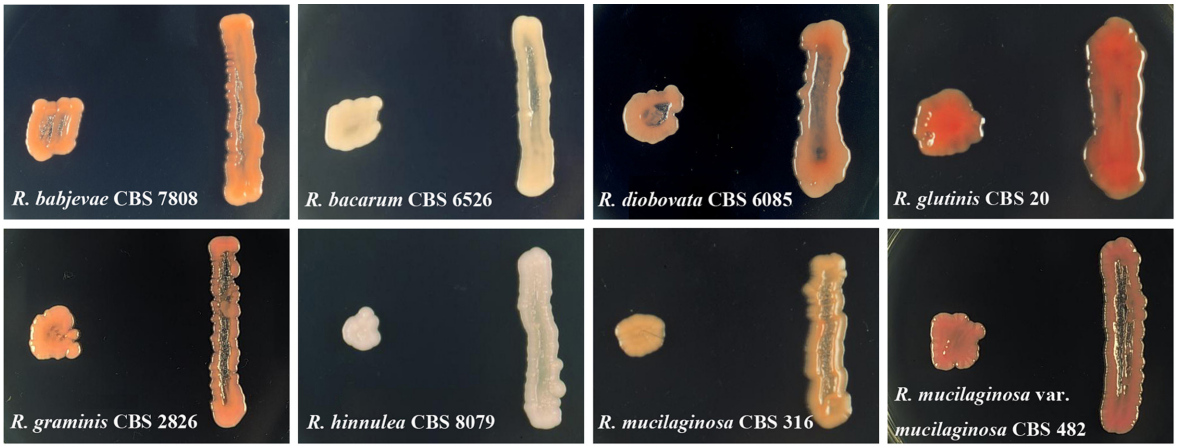


**Fig. 1.** Colony morphology of different strains of *Rhodotorula*.
The data were collected from the CBS strain database. All the other strains were type strains except *R. mucilaginosa* var. *mucilaginosa* CBS 482.

2022 [7]. Currently, more than 90% of commercial carotenoids are produced by chemical synthesis. Superior to chemical synthesis, the yeast-based production of carotenoids is natural and organic, entitled with a panel of extra advantages such as short production cycle time, environment-friendly usage, and ease of scale-up by manipulation through an engineered fermentation process. As one of the famous groups of carotenoid producers, strains of *Rhodotorula* may play important roles in the production of natural carotenoids in the future. In this study the biosynthetic pathway of carotenoids by *Rhodotorula* was reviewed and the strategies for carotenoid production were discussed.

## Biosynthetic Pathway of Carotenoids in *Rhodotorula*

The biosynthetic pathway of carotenoids has been studied in a panel of *Rhodotorula* species. Buzzini *et al*. [8] indicated that *R. minuta*, *R. glutinis*, *R. graminis*, *R. mucilaginosa* and *Rhodotorula* sp. nov. possess analogical carotenoid profiles, with β-carotene, γ-carotene, torulene, and torularhodin representing the principal carotenoids in all these species. Villoutreix [9] also showed that a similar pigment profile was found in *R. mucilaginosa*. These approaches allowed the conclusion that the genus *Rhodotorula* possesses an identical or conserved carotenoid biosynthetic pathway (Fig. 2) [10, 11]. Carotenoid biosynthesis is known to follow the consecutive condensations of isoprenoid units into phytoene, the first colorless carotenoid in the pathway. The phytoene is continuously dehydrogenated and the conjugated double bond is extended until the formation of neurosporene, and subsequently, lycopene. There are two independent cyclization routes leading to two branches with either γ-carotene or torulene as the immediate products, which may further be converted to β-carotene and torularhodin respectively. Fig. 2 shows the most representative steps and products in the carotenoid biosynthesis of *Rhodotorula*.

## Principal Carotenoids and Their Physiology of *Rhodotorula*

### β-Carotene and γ-Carotene

β-Carotene is one of the most well-known pigments having been widely used in medicines, health products, food additives, cosmetics, feed additives, and many products in other industries. It has also been approved as a food and feed additive for its dual functions in nutritive use and coloring in more than 50 countries and regions. Structurally, β-carotene is a fat-soluble, orange-yellow carotenoid with 11 conjugated double bonds and 2 retinyl groups (β-ionone ring). It is this large number of double bonds in its polyene chain and rings that makes it prone to be oxidized by free radicals. And this property endows it with antioxidant activity as well as enables its broad uses in food and feed applications [12]. For medical and or heath purpose use, β-carotene has been prescribed orally for the prevention of cancer, tumors and cardiovascular disease. Among the pigments in *Rhodotorula* species, β-carotene accounts for about 70% of total carotenoids [13].

γ-Carotene is the isomer of β-carotene, structurally containing 11 conjugated double bonds, 1 non-conjugated double bond and 1 retinyl group. Functionally, γ-carotene has vitamin A activity (though less than β-carotene) thanks

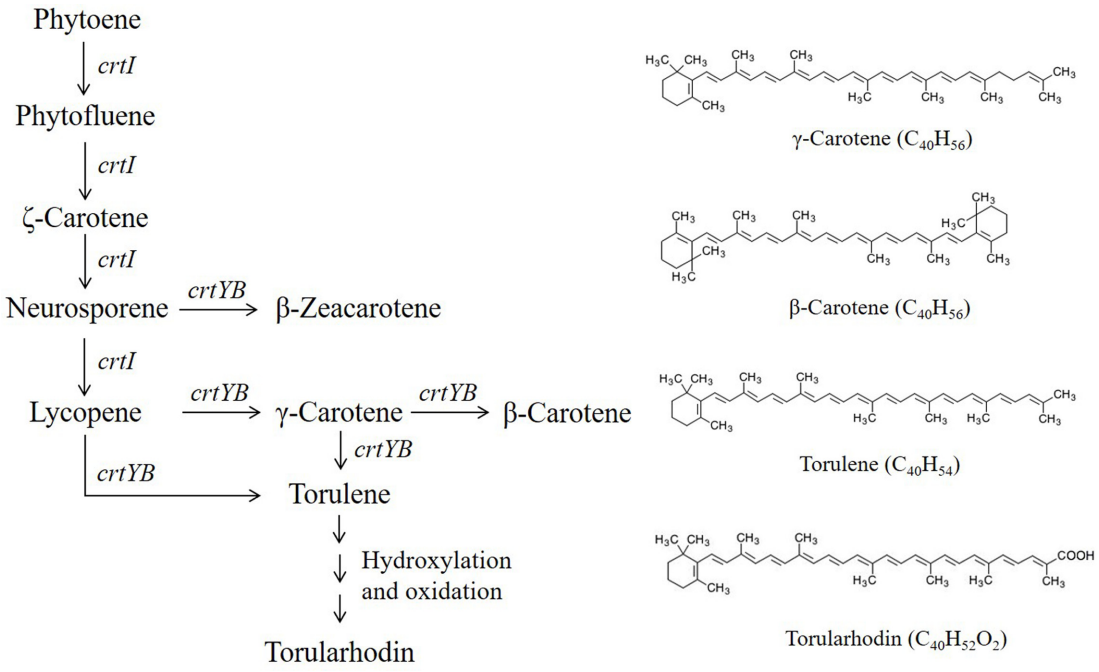


**Fig. 2.** Biosynthetic pathway of carotenoids in *Rhodotorula*.
*crtI*: phytoene desaturase encoding gene; *crtYB*: bifunctional lycopene cyclase/phytoene synthase encoding gene.

to its single retinyl group [14]. It is formed via cyclization of lycopene through the enzymatic reaction facilitated by lycopene ε-cyclase.

**Torulene and Torularhodin**

As an acid pigment, torularhodin was first isolated from the genus *Rhodotorula* as early as the 1930s [15]. Subsequently, in 1946, Bonner *et al*. [16] announced the finding of torulene from *R. rubra* and they were able to overproduce torulene (amounting to 76% of the total carotenoids) by applying a mutant of *R. rubra* using quantitative chromatographic resolution. Interestingly it was not until the 1990s that torulene and torularhodin were regarded as potentially valuable substances [17]. Hence the number of works describing the properties of the two compounds increased quickly in the last decade.

Torulene is derived from the subtraction of 2H from γ-carotene, with the formation of an extra double bond in 13C (*i.e*., 13 double bonds), whereas torularhodin is formed from torulene by the substitution of one methyl group with one carboxyl group (that is, 14 double bonds). Since the two carotenoids contain the built-in β-ionone ring structure, the backbone of vitamin A, both of them can be the potential precursors of vitamin A. Compared to β-carotene, torulene and torularhodin have shown stronger antioxidant activity, thanks to the existence of the extra conjugated double bonds in 13C [18]. The extra double bond also endowed torularhodin with stronger capacity for scavenging hydrogen peroxide radicals as well as stronger resistance to the substrate degradation caused by singlet oxygen, compared to that of β-carotene, respectively [19]. It was also documented that high-torularhodin-production mutant can reduce the susceptibility to oxidative damage induced by active oxygen species [20].

In addition to antioxidant activity, in vivo anti-cancerous properties of the two carotenoids have also been demonstrated. In the anti-cancer supplementation experiments, compared to lycopene, both torulene and torularhodin performed much more significant inhibition of the growth of prostate cancer in nude mice by the induction of apoptosis of tumor cells [21]. Furtherly, it was confirmed that both of them facilitated protective activity on human prostate stromal cells from oxidative stress damage [22]. Meanwhile, the anti-microbial activity of the two carotenoids had also been confirmed, which revealed their potential usage on infection prevention particularly in implanted medical products and preparations that require natural antimicrobials (Kot *et al.*, 2018) [11]. Lastly, torularhodin is one of the few carotenoids with carboxylic acid function [19].

## Strategies for Enhanced Production of Carotenoids by *Rhodotorula*

### Exploration of Native Carotenoid-Producing Strains

To satisfy the increasing demand for natural carotenoids, the development and utilization of high-yield carotenoid strains has become a research hotspot. So far *Phaffia rhodozyma* is the most popular red yeast used in the large-scale production of carotenoids, *e.g.*, astaxanthin. Next to it, *Rhodotorula* may present as another red yeast that can be explored for production of carotenoids, particularly the production of mixtures of carotenoids at higher amounts. A wild strain of *R. mucilaginosa* CRUB 0138, an isolate from high-altitude Patagonian Lake Toncek, was found to produce carotenoids at a level of 234 ± 7 μg/g after 4 d incubation when the initial glucose concentration was adjusted to 1% [23]. Decent carotenoid yield at 33.2 mg/g was achieved by *R. glutinis*, a wild strain from refinery wastewater, when whey lactose-containing medium was applied [24]. A red yeast *Rhodotorula* sp. KF-104 of plant origin (isolated from vegetative parts of vine) was found to produce a mixture of carotenoids, including β-carotene, γ-carotene, torulene and torularhodin, respectively [25].

### Improvement of Carotenoid-Synthesizing by Mutagenesis

Mutagenesis is a classical way for elevating the yield of carotenoids from wild strains, and the methods for mutation include UV radiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), γ-irradiation, ethyl-methane sulfonate (EMS) and high hydrostatic pressure. Bhosale *et al*. [26] obtained a mutant 32 (2,048 μg/g) of a wild strain *R. glutinis* NCIM 3353 by UV radiation, which led to a yield increase of β-carotene in mutant 32 by 120-fold, as compared to that in the parent strain. Similarly, Wang *et al*. [27] was able to increase the yield of β-carotene by 57.89% in mutant strain RG6p, by using five repeated cycles of high hydrostatic pressure (HHP) mutation of the parent isolate *R. glutinis* GR6. Significant carotenoid yield improvement in *R. mucilaginosa* RM-1 was also documented by mutagenesis manipulation using N+ implantation of 10 keV and $2.0 \times 10^{14}$ ion/cm$^2$ [28]. Promising yield benefit (3-fold increase) was also achieved regarding carotenoid biosynthesis by the combined mutagenesis approaches, through a serial of UV, EMS and NTG mutagenesis [29]. In addition, the effects of different mutagenesis methods on the production of pigment from *R. glutinis* was evaluated, and it was found that mutagens could significantly increase the production of pigments, with UV irradiation as the better mutagenesis choice, compared to that of sodium azide (SA) [30].

### Optimization Approaches on Cultivation Conditions for the Production of Carotenoids

The biosynthesis and accumulation of carotenoids are subjected to regulation by light, more specifically by photoinduction, which can improve carotenoid yields by promoting growth and cell density as well as by elevating the activity of enzymes involved in carotenoid biosynthesis. And this was well exemplified in *R. glutinis* by the work of Zhang *et al*. [31] that reported the irradiation caused significant biomass improvement and carotenoid production enhancement. In general, light-induced oxidative or radiation damage can limit the growth of microbes in some species, particularly those microorganisms that lack the proper intracellular photoprotective substances. Thanks to the endogenous carotenoid-empowered protection against light-induced damage, it has become possible for a panel of non-phototrophic yeasts and bacteria to grow and colonize in the natural world [32]. The work of Sakaki *et al*. [20] even reported the weak-white-light-facilitated yield improvement of carotenoids, in which a torularhodin increase of 180% was documented for *R. glutinis*. Simultaneously, the production of β-carotene in *R. glutinis* was also increased by 14%. Taken together, the author suggested that torularhodin may play a key role in the protection against oxidative damage caused by light irradiation. Practically, LED-derived irradiations of different colors could simply promote the growth of *R. glutinis*, among which irradiation by red LEDs induced the highest β-carotene production, followed in order by blue, green and white LEDs, respectively [33].

Temperature is another important factor affecting carotenoid biosynthesis. It can affect the propagation of carotenoid producers in general and influence the production of single carotenoid and carotenoid ratios along the biosynthesis pathway in specific. Hayman *et al*. [34] reported that the relative concentrations of individual carotenoids produced by *R. glutinis* were different when cultivated at 4°C and 25 °C, respectively. Similar results were observed in another strain of *R. glutinis*, 48-23T, in which the composition of carotenoids varied when different culture temperatures were applied. It was found that at 25°C the synthesized β-carotene, torulin and torulaihodin accounted for about 30% of the total carotenoids, respectively, whereas at 5°C, the proportion of β-carotene increased to 64%, by contrast the content of the other two carotenoids decreased significantly [35]. Buzzini and Martini [36] also reported that in *R. glutinis* the lower temperature (at 25°C) favored the synthesis of β-carotene and torulene whereas the higher temperature (30~35°C) is more suitable for the production of torularhodin. For *R. glutinis* it was even found that the

optimal temperature of growth was different with that for carotenoid biosynthesis [37]. In this report the mutant 32 could grow well at optimum temperature of 30°C; however, the optimum temperature for the production of β-carotene was 20°C and its yield decreased with further reduction of the incubation temperature.

Many metal ions ($Ba^{2+}$, $Fe^{2+}$, $Mg^{2+}$, $Ca^{2+}$, $Zn^{2+}$ and $Co^{2+}$) were found capable to affect carotenogenesis in *Rhodotorula*, possibly because the activity of enzymes in the biosynthesis pathway were regulated, *e.g.*, activated or inhibited, by those cations aforementioned, just as exemplified in desaturases in particular [38]. Buzzini *et al*. [39] reported that metal ions could selectively affect the carotenoid profile of *R. graminis* DBVPG 7021, and it was found in the research that $Al^{3+}$ and $Zn^{2+}$ promoted the production of β-carotene and γ-carotene, whereas $Zn^{2+}$ and $Mn^{2+}$ inhibited the production of torulene and torularhodin. In addition, the composing of exogenous solvents, chemicals or natural agents may affect the carotenoids in varied ways. Kim *et al*. [40] reported that the β-carotene content by *R. glutinis* increased by 35% when phenol was added to culture media at 500 ppm, and in contrast a decrease in torularhodin was observed when the phenol concentration was elevated. Diphenylamine, a widely used inhibitor, was also used to modify the carotenoid profile [41]. Diphenylamine was manipulated to block the sequential de-saturation reactions by inhibiting phytoene synthase, so that the accumulation of phytoene and other substances was enabled [41]. Squina and Mercadante [42] reported that the addition of 5 μmol of diphenylamine to the culture of *R. rubra* allowed more carotenoids to be produced in the broth, and interestingly in the experiment a significant reduction in torularhodin/torulene ratio was identified in the cases for both *R. rubra* and *R. glutinis*. It is noteworthy that for both strains, the accumulation of β-carotene was enabled when further supplementation of diphenylamine (to a higher concentration at 10 μmol) was conducted.

As a group of secondary metabolites, most of the carotenoid biosynthesis initiated in the late logarithmic phase of yeasts near full growth was achieved, and their accumulation kept increasing during the stationary phase [43]. Nutrition wise, the producers can assimilate and

**Table 1.** Carotenoid productivities under varied conditions by different strains of *Rhodotorula*.

| Strategy | Strain | Method/Scale | Carotenoid | Control | | Treatment | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | | | mg/g[a] | mg/l[a] | mg/g[a] | mg/l[a] | |
| Mutagenesis | | | | | | | | |
| | *R. glutinis* NCIM 3353 | UV radiation | Total carotenoids | 0.12 | 2.2 | 2.9 | 33 | [26] |
| | *R. rubra* GED8 | NTG | Total carotenoids | 0.187 | 2.67 | 0.64 | 8.12 | [46] |
| | *R. glutinis* RG6 | High hydrostatic pressure(HHP) | β-carotene | - | 6.34 | - | 10.01 | [27] |
| | *R. glutinis* NR-98 | Ultra high pressure (UHP)\ low energy nitrogen ion | β-carotene | - | 6.03 | - | 17.36 | [46] |
| | *R. acheniorum* | UV, ethymethanesurfonate (EMS), and nitrosoguanidine (NTG) | β-carotene | 2.31 | 40.60 | 10.69 | 262.12 | [47] |
| Inducers | | | | | | | | |
| | *R. glutinis* | White light | β-carotene | 3.6[b] | - | 4.2[b] | - | [48] |
| | | | Torulene | 29.3[b] | - | 32.2[b] | - | |
| | | | Torularhodin | 7.9[b] | - | 14.2[b] | - | |
| | *R. glutinis* RY-8 | Thiamine | β-carotene | 48.1 | 250.1 | 52.9 | 280.4 | [49] |
| | | Riboflavin | | 51.8 | 300.4 | 53.0 | 323.3 | |
| | | Soybean oil | | 51.4 | 303.3 | 57.2 | 366.1 | |
| | | Tomato juice | | 50.6 | 288.4 | 56.1 | 342.2 | |
| Low-cost substrates | | | | | | | | |
| | *R. glutinis* CCY20-2-26 | Whey | β-carotene | 0.48 | 17.93 | 1.03 | 45.68 | [50] |
| | *R. glutinis* ATCC 4054 | Rice bran | β-carotene | 1.23[c] | - | 3.20[c] | - | [51] |
| | *R. glutinis* MT-5 | Waste chicken feathers | Total carotenoids | 5.76 | 60 | 6.47 | 92 | [52] |
| | *R. mucilaginosa* CCY20-7-31 | Potato medium | β-carotene | 0.16 | 4.31 | 1.86 | 55.91 | [50] |
| | *R. mucilaginosa* NRRL-2502 | Cotton seed oil | Total carotenoids | - | 39.5 | - | 57.6 | [53] |
| | *R. aurantiaca* | Waste glycerol | β-carotene | 0.34 | - | 1.08 | - | [54] |

[a]Carotenoid content (mg/g cells dry weight or mg/l culture fluid); [b]Carotenoid content (mg/100 g cells dry weight); [c]Carotenoid content (mg/kg rice bran).

metabolize diversified carbon sources, such as monosaccharides, disaccharides and polysaccharides, organic acids and alcohols, and they can also rapidly utilize simple nitrogen sources (ammonium salt, nitrate, urea and amino acids) and complex mixtures (beef extract, yeast extract, malt extract, tryptone, etc.) [44]. For carotenoid production by *Rhodotorula* fermentation, the economical way through cost reduction is to use agro-industrial raw materials and by-products instead of using defined components as found in commercial media [36]. Table 1 summarized the examples regarding the production of carotenoids using low-cost raw materials. As shown, the production of carotenoids was affected by the choice of media components, such as the carbon and nitrogen sources, and the proportion of minerals and other components. Potential gains in yield can be attained simply by media optimization.

## Enhancing Carotenoid Production by Genetic Engineering and Metabolic Engineering Manipulations

### Use of Genomic Tools to Characterize Carotenoid Biosynthesis in *Rhodotorula*

With the advancing of genetic engineering and metabolic engineering approaches, the construction of high-yield carotenoid strains as well as the maneuvering of the techniques for large-scale carotenoid production have become feasible. For *Xanthophyllomyces dendrorhous* (*P. rhodozyma*), a panel of genes encoding the key enzymes in the carotenoid pathway had been cloned and characterized with the astaxanthin biosynthesis pathway well illustrated [55]. However, so far there has been very limited progress for *Rhodotorula* spp. in this regard. This could be partially due to the limitation on available genomic data as well as the lack of functional annotation of the key genes, which impedes metabolic engineering manipulations aimed at the improvement of carotenoid production. Nevertheless, whole genome sequences of a few *Rhodotorula* spp. strains have been identified, and the establishing of the relevant bioinformatic data may benefit future research on aspects of the regulation of carotenoid biosynthesis, the pursuit of yield improvement, and the manipulation of relevant genes encoding other useful products, respectively (Table 2).

The progress at the genetic level of *Rhodotorula* has been mostly focused on the identification of genes encoding the key enzymes and their distribution patterns along the carotenoid gene clusters. The whole genome of *R. mucilaginosa* RIT389 was sequenced with the identification of a genomic region associated with carotenoid, in which three genes encoding phytoene synthase (*crtB*), lycopene cyclase (*crtY*), and phytoene desaturase (*crtI*) were found closely located, whereas the gene encoding geranyl pyrophosphate synthase was located apart at a separate contig. The two other key genes, *crtX* and *crtBY*, that

**Table 2.** Comparative analysis on genome data of strains of *Rhodotorula*.

| Organism | Accession No. | Size (Mb) | GC% | Scaffolds | Gene | Characteristics | References |
|---|---|---|---|---|---|---|---|
| *R. toruloides* NP11 | ALAU00000000 | 20.22 | 62 | 94 | 8,171 | Triacylglycerol-producing | [56] |
| *R. toruloides* CGMCC 2.1609 | LKER00000000 | 33.39 | 61.9 | 365 | 9,820 | Inulinase activity | [57] |
| *R. glutinis* ATCC 204091 | AEVR00000000 | 20.48 | 61.9 | 29 | 3,359 | Lipids (>50% of its biomass) and antioxidant production | [58] |
| *R. graminis* WP1 | JTAO00000000 | 21.03 | 67.76 | 26 | 7,283 | Improves plant vigor, ferments both pentoses and hexoses, and degrades fermentation inhibitors | [59] |
| *R. mucilaginosa* RIT389 | NIUW00000000 | 19.66 | 60.28 | 250 | 7,065 | Isolated from the chewing stick (*Distemonanthus benthamianus*), and genomic regions containing the key genes for carotenoid production | [60] |
| *R. mucilaginosa* C2.5t1 | JWTJ00000000 | 19.98 | 60.50 | 1,034 | 6,413 | Isolated from cacao seeds (*Theobroma cacao* L) in Cameroon, produces high carotenoid levels when grown in glycerol-containing media | [61] |
| *R. taiwanensis* MD1149 | PJQD00000000 | 19.58 | 61.69 | 181 | 7,122 | Resistant to acid (pH 2.3) and gamma radiation (66 Gy/h) | [62] |
| *R. kratochvilovae* strain LS11 | PQDI00000000 | 22.56 | 66.6 | 62 | 7,642 | Antagonistic activity against several plant pathogens | [63] |

encoded carotenoid oxygenase and phytoene synthase/lycopene cyclase, respectively, were found located in close proximity and convergently transcribed in all the species in *Rhodotorula* (except for *R. mucilaginosa*) [60]. On the base of the genome sequence of *R. mucilaginosa* C2.5t1, a set of genes involved in carotenogenesis were identified. Subsequent quantitative PCR showed that genes coding for 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (*HMG1*) and mevalonate kinase (*ERG12*) were induced at the end of the exponential growth phase. However, no clear trend of induction was observed for phytoene synthase/lycopene cyclase (*CAR2*) and phytoene dehydrogenase (*CAR1*) encoding genes. It seems that the gene induction aforementioned was transient and occurred just at the beginning of carotenoid production, and somehow downstream-wise the expression level of *CAR* genes does not correlate with the amount of carotenoids produced [10].

**Metabolic Engineering for Carotenoid Production in Non-Carotenogenic Bacteria and Yeasts**

*E. coli* has been one of the most described microorganisms engineered to produce the exogenous carotenoids [64]. The cDNA of *crtI*, GGPP synthase (*crtE*) and *crtYB* genes from *Erwinia uredovora* were heterologously expressed in *E. coli*, showing lycopene accumulation in its transformants [65]. In addition, the *crt* genes derived from *E. uredobora* or *E. herbicola* were successfully used for the de novo biosynthesis of lycopene, β-carotene and zeaxanthin in *E. coli* [66]. In *E. coli* the innate apparatuses of the 2-C-methyl-*D*-erythritol 4-phosphate (MEP) pathway prepare isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which serve as the precursors to enter the carotenoid biosynthesis pathway. IPP and DMAPP condense to form geranyl-pyrophosphate (GPP), which then synthesizes the precursor farnesyl-pyrophosphate (FPP) of β-carotene. The β-carotene can be synthesized by introducing genes *crtE*, *crtB*, *crtI* and *crtY* into *E. coli*. The expression of β-carotene in *E. coli* is shown in Table 3. The documented works were mainly focused on: (1) introducing β-carotene synthesis genes from different sources; (2) overexpressing synthase in the MEP pathway; (3) introducing mevalonate (MVA) pathway from eukaryotes into *E. coli* to increase IPP and FPP production; (4) reducing gene expression in competitive pathways; (5) adjusting ATP synthesis pathway, pentose phosphate pathway and TCA cycle to increase ATP and NADPH production.

Some yeasts, such as *S. cerevisiae* [72], and *Candida utilis* [73] have also been used to produce exogenous carotenoids through metabolic engineering manipulation. These yeasts are considered as food-grade organisms and have a mature genetic transformation system. They have been able to successfully produce carotenoids by insertion of carotenogenic genes or metabolic pathways from *E. uredovora*, *X. dendrorhous* or *Agrobacterium aurantiacum*. Although wild-type *S. cerevisiae* cannot produce carotenoids, it does synthesize geranylgeranyl diphosphate (GGPP) which is an important precursor of carotenoid synthesis. In practice, two key genes from *X. dendrorhous*, *crtYB* and *ctrI*, were integrated into the genomic DNA of *S. cerevisiae* strain to complete the intact carotenoid biosynthesis pathway, and the manipulations ended up with successful expression of β-carotene [74]. Similarly, *C. utilis* does possess their potential precursors, but does not have the complete apparatuses for carotenoid biosynthesis. Through introduction of the three exogenous carotenoid genes, *crtE*, *crtB*, and *crtI*, a *C. utilis* strain producing 1.1 mg of pure lycopene per g (dry weight) of cells was obtained [75]. For the food yeast *Candida utilis*, manipulations of metabolic engineering were able bring about a seven-fold increase regarding lycopene production [76]. In addition, *Pichia pastoris* was also used to produce exogenous carotenoids through metabolic engineering manipulation. Araya-Garay *et al*. [77] designed and constructed two plasmids, pGAPZA-

**Table 3.** Heterologous expression of β-carotene biosynthetic genes in *E. coli*.

| Strategy | Maximal yield | References |
|---|---|---|
| Engineered *E. coli* with a synthetic *crt* operon constructed to produce β-carotene | 390 mg/l | [64] |
| Recombinant *E. coli* with engineered whole MVA pathways as well as harboring genes for β-carotene synthesis | 663 mg/l | [67] |
| Recombinant *E. coli* harboring an engineered isoprenoid precursor pathway with mevalonate addition | 503 mg/l | [68] |
| IPP and DMAPP supply can be increased significantly through the introduction of foreign MVA pathway into *E. coli* | 464 mg/l | [69] |
| Improving the IPP and GPP concentration in the cell to increase β-carotene production, the optimized MEP pathway and hybrid MVA pathway have been introduced and co-expressed in an engineered *E. coli* strain | 3.2 g/l | [70] |
| Combined engineering of MEP, β-carotene synthesis and central metabolic modules, a genetically stable *E. coli* strain was obtained which exhibited 74-fold yield increase over the wild type | 2.1 g/l | [71] |

EBI* and pGAPZA-EBI*L*, which were integrated into *P. pastoris* genomic DNA, and the clones Pp-EBI and Pp-EBIL were used successfully for the production of lycopene or β-carotene, respectively.

It is noteworthy that the establishment of the aforementioned metabolic engineering processes of carotenoid biosynthesis are of great help in the manipulation toward the higher production of carotenoids by the engineered strains of choice. Actually, metabolic engineering techniques are becoming practical methods applied to *Rhodotorula* for the expression of exogenous carotenoids of interest as well as for the enhancement of the biosynthesis of endogenous carotenoid in specific. Abbott *et al*. [78] evaluated the feasibility of genetic engineering for different red yeasts by using common plasmids and transformation methods. The results showed that the success of transformation depends on the species and exogenous genes, which may be related to the G+C DNA content of several species. Consistent with this assumption, Liu *et al*. [79] reported that the codon optimization was the key to *Agrobacterium* tumefaciens-mediated transformation in *R. toruloides*. Pi *et al*. [80] transformed β-carotene biosynthesis genes (*crtI*, *crtE*, *crtYB* and *tHMG1* from *X. dendrorhous* and *Kluyveromyces marxianus*) into *R. glutinis* genome. The transformant P4-10-9-63Y-14b produced β-carotene (27.13 ± 0.66 mg/g) 7-fold higher than the wild type. It is reasonable to expect that sophisticated metabolic engineering methods would allow the construction of suitable strains for the large-scale production of carotenoids of importance.

In conclusion, carotenoids not only serve as a class of excellent colorants, but also present as a group of natural products used for multi-dimensional applications. They have been widely used in food, medicine, health products cosmetics, and animal feed additives. The growing public concern on food safety urges the supply of carotenoids with natural origin, which prioritizes microbial carotenoid fermentation over other options, despite that the former not being the most cost-effective approach. *Rhodotorula* is well known for its potency in carotenoid production as well as for its feasibility to be used for carotenoid fermentation thanks to the short cycle time and the low cultivation cost. Systematic approaches have been made to enhance carotenoid production by *Rhodotorula*, utilizing advances in the strain work through natural breeding or mutagenesis, optimization of media and fermentation conditions, and metabolic engineering approaches on yield improvement as well as the attempts on the heterologous expression. It is reasonable to believe that the advent of elaborate strategies for mass carotenoid production by *Rhodotorula* are not far, given the availability of genetic and metabolic engineering potential and the maturity of advanced yeast-based industrial fermentation manufacturing systems.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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