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REVIEW

Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance

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Abstract Carotenoids represent a group of valuable molecules for the pharmaceutical, chemical, food and feed industries, not only because they can act as vitamin A precursors, but also for their coloring, antioxidant and possible tumor-inhibiting activity. Animals cannot synthesize carotenoids, and these pigments must therefore be added to the feeds of farmed species. The synthesis of different natural commercially important carotenoids (β-carotene, torulene, torularhodin and astaxanthin) by several yeast species belonging to the genera *Rhodotorula* and *Phaffia* has led to consider these microorganisms as a potential pigment sources. In this review, we discuss the biosynthesis, factors affecting carotenogenesis in *Rhodotorula* and *Phaffia* strains, strategies for improving the production properties of the strains and directions for potential utility of carotenoid-synthesizing yeast as a alternative source of natural carotenoid pigments.

Keywords Carotenogenesis · *Rhodotorula* species · *Phaffia rhodozyma* · Carotenoid pigments

Introduction

Carotenoids are a group of over 600 molecules which can be found in most life forms and fulfil diverse functions, ranging from their original evolutionary role as photosynthetic or light-quenching pigments to antioxidants, precursors of vitamin A, or pigments involved in the visual attraction of animals such as flower pollinators or mating partners [1]. Several microorganisms, including bacteria [2, 3], algae [4, 5], molds [6, 7] and yeasts of the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Phaffia* [8–31], are able to produce carotenoids naturally. The structure of carotenoids is derived from phytoene [32]. The majority are hydrocarbons of 40 carbon atoms which contain two terminal ring systems joined by a chain of conjugated double bonds or polyene system. Two groups have been singled out as the most important: the carotenes which are composed of only carbon and hydrogen; and the xanthophylls, which are oxygenated derivatives. In the latter, oxygen can be present as OH groups (as in zeaxanthin), or as oxy-groups (as in canthaxanthin); or in a combination of both (as in astaxanthin).

Carotenoids are of importance in animals and humans, including enhancement of the immune response, conversion to vitamin A and the scavenging of oxygen radicals [33–38]. Epidemiological evidence and experimental results suggest that dietary carotenoids inhibit the onset of many diseases in which free radicals are thought to play a role in initiation, such as arteriosclerosis, cataracts, multiple sclerosis and cancer [34, 39–44].

Animals cannot synthesize carotenoids, and these pigments must therefore be added to the feeds of farmed species, including aquacultured salmon [45–50]. The color of the meat of salmon and trout is an essential demand for customers [39], and for this reason the aquaculture industry requires substantial amounts of carotenoids as animal feed additives per year. Humans are exposed to carotenoids through their diet. This exposure results from carotenoids present in vegetables and fruits as well as from animal products rich in carotenoids. The latter products might be additionally enriched in these components by specific feed additives.

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In conclusion, carotenoids represent a group of valuable molecules for the pharmaceutical, chemical, food and feed industries, not only because they can act as vitamin A precursors, but also for their coloring, antioxidant and possible tumor-inhibiting activity. The scrutiny and negative assessment of synthetic food dyes by the modern consumer, have given rise to a strong interest in natural coloring alternatives. Despite the availability of a variety of natural and synthetic carotenoids, there is currently renewed interest in microbial sources [1, 51–53]. The growing scientific evidence that these carotenoid pigments may have potential benefits in human and animal health has led to an increased commercial interest in the search for alternative natural sources. Biological sources of carotenoids receive major focus nowadays because of the stringent rules and regulations applied to chemically synthesized/purified pigments. Compared with the extraction from vegetables [54] or chemical synthesis [55], the microbial production of carotenoids is of paramount interest, mainly because of the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin [56], and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate sources.

The synthesis of different natural commercially important carotenoids (β-carotene, torulene, torularhodin and astaxanthin) by several yeast species belonging to the genera *Rhodotorula* and *Phaffia*, has led to consider these microorganisms as potential pigment sources. Yeasts are more convenient than algae or molds for large-scale production in fermenters, due to their unicellular nature and high growth rate. This review focuses on research works related to this field, published over the past 15 years.

Biosynthesis of carotenoids by yeasts of the genera *Rhodotorula* and *Phaffia*

Possible biosynthetic pathways for carotenoid formation

In order to discuss the response to cultural and environmental stimulants of carotenoid production, it is essential to briefly describe carotenoid biosynthesis, since the activity and quantity of the biosynthetic enzymes are known to significantly influence stimulant activity. In 1964, Simpson et al. [57], and later Goodwin [58, 59] reviewed the general pathways for carotenoid synthesis by yeasts and concluded that carotenoid biosynthetic pathways commonly involve three steps: (1) The conversion of acetyl-CoA to 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA) is catalyzed by HMG-CoA synthase. HMG-CoA is then converted into a C_6 compound, mevalonic acid (MVA), which is the first specific precursor of the terpenoid biosynthetic route. MVA is further converted into isopentenyl pyrophosphate (IPP) by a series of reactions involving phosphorylation by MVA kinase followed by decarboxylation; (2) IPP is isomerized to dimethylallyl pyrophosphate (DMAPP) with the sequential addition of three IPP molecules to DMAPP. These reactions are catalyzed by prenyl transferase to yield the C_{20} compound geranyl geranyl pyrophosphate (GGPP). Condensation of two molecules of GGPP leading to phytoene (the first C_{40} carotene of the pathway), which undergoes desaturation to form lycopene; (3) As lycopene is an all-*trans* compound, the isomerization of the first or second double-bond of the phytoene must occur at the same stage in the desaturation process [59]. Lycopene acts as precursor of cyclic carotenoids and undergoes a number of metabolic reactions (e.g. cyclization) to form β-carotene, γ-carotene, torulene, torularhodin and astaxanthin. γ-Carotene is the major branch point and acts as the precursor for β-carotene and torulene. Hydroxylation and oxidation of torulene by mixed function oxidase leads to the formation of torularhodin. In 1976, Andrewes et al. [60] suggested the first scheme for astaxanthin biosynthesis, leading through many steps and intermediates including lycopene, β-carotene and echinenone to astaxanthin. Later, other intermediates were detected including β-zeacarotene [61], 3,3′-dihydroxy-β,γ-carotene-4,4′-dione (DCD) and torulene [62], which indicated an alternative pathway through β-zeacarotene → torulene → 3-hydroxy-3′,4′-didehydro-βϕ-caroten-4-one (HDCO) → DCD to *trans*-astaxanthin. Above described carotenoid biosynthetic pathways in yeasts are schematically shown in Fig. 1.

Profile of carotenoids

The yeast species of *Rhodotorula* and *Phaffia* are well known carotenoid producers. The major carotenoid pigments produced by the yeasts *Rhodotorula* are β-carotene, torulene (3′,4′-didehydro-β-ψ-carotene), and torularhodin (3′,4′-didehydro-β-ψ-caroten-16′-oic acid), in various proportions [9–11, 15, 16, 18, 21, 23, 24, 63–67] and astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) by *Phaffia rhodozyma* [20, 22, 28, 31, 68–77]. The relative share of the three major carotenoid pigments in the total carotenoids is highly variable, and depends on differences between strains of the same species and is strongly influenced by the cultivation conditions. γ-Carotene (β-ψ-carotene) was shown to contribute 11–15% of the total carotenoids in some strains of *R. glutinis* [57, 65, 78] and *R. graminis* [12]. The concentrations of individual pigments (percentage of total carotenoids) in carotenoids synthesized from *Rhodotorula* strains are shown in Table 1.

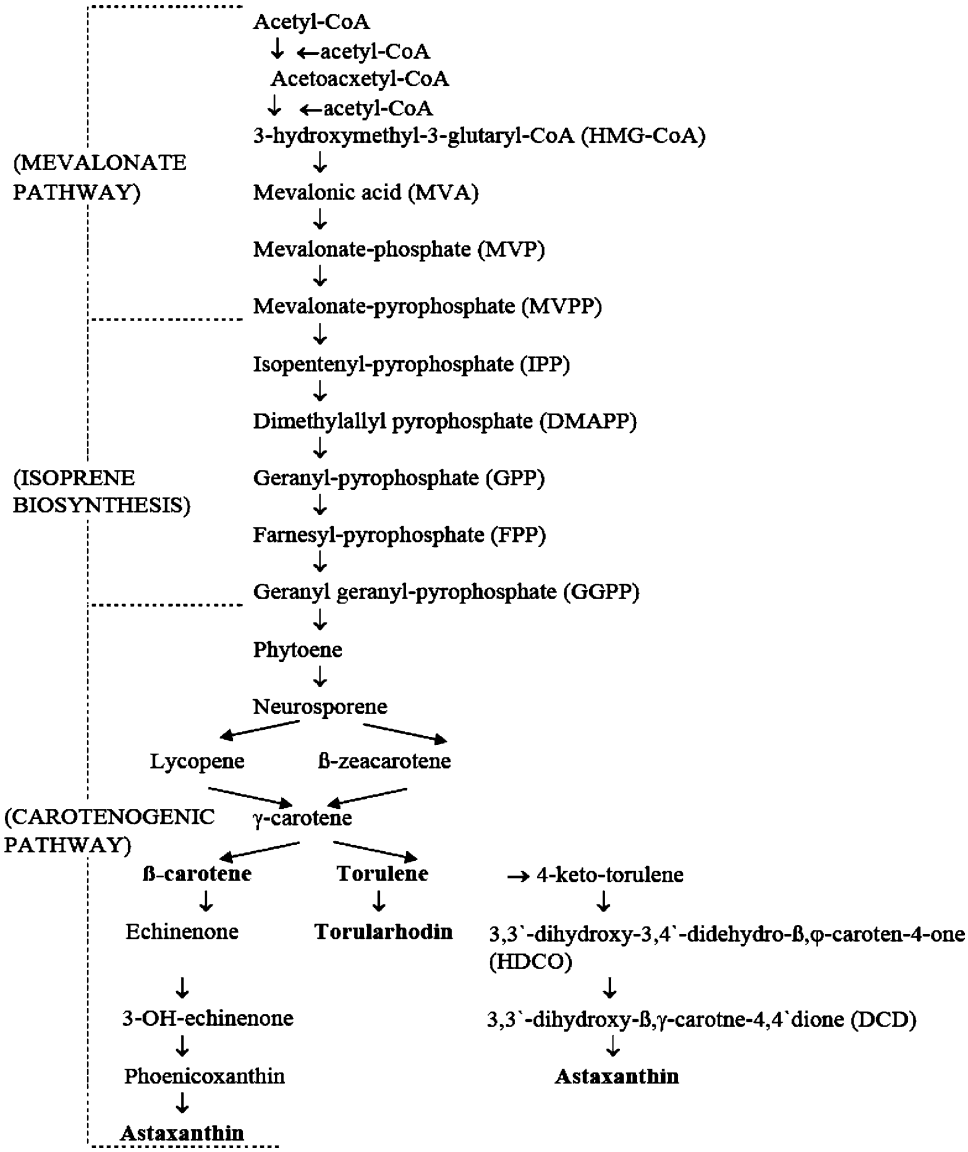

Fig. 1 Biosynthetic pathways from acetil-CoA to β-carotene, torulene and torularhodin in *Rhodotorula* species and astaxanthin in *P. rhodozyma*/*X. dendrorhous*

Substrates for production of carotenoids by yeasts and methods of cultivation

Carotenoid pigments accumulation in most yeasts starts in the late logarithmic phase and continues in the stationary phase [79], and the presence of a suitable carbon source is important for carotenoid biosynthesis during the non-growth phase. Yeasts can synthesize carotenoids when cultivated in synthetic medium, containing various refined carbon sources, such as glucose [9, 22, 23, 25, 31, 80–86], xylose [74], cellobiose [85], sucrose [87, 88], glycerol [71] and sorbitol [85]. Studies on carotenogenesis have led to a growing interest in using natural substrates as carbon sources: grape juice [72, 89]; grape must [11, 63]; peat extract and peat hydrolysate [20, 21, 77, 90]; date juice of Yucca fillifera [76]; hydrolyzed mustard waste isolates [28]; hemicellulosic hydrolysates of eucalyptus globules wood [68, 75]; hydrolyzed mung bean waste flour [91]; sugar cane juice [69, 73, 78]; sugar cane and sugar-beet molasses [8, 10, 18, 92, 93]; corn syrup [18, 64]; corn hydrolysate [94, 95]; milk whey [15, 16, 24, 30, 67, 92]. In recent years, raw materials and by-products of agro-industrial origin have been proposed as low-cost alternative carbohydrate sources for microbial metabolite production, with the view of also minimizing environmental and energetic problems related to their disposal [96].

Rhodotorula species and *Xanthophyllomyces dendrorhous* (formerly *P. rhodozyma*) have potential commercial value as a dietary sources of natural carotenoids (β-carotene, torulene, torularhodin and astaxanthin); however, the high cost of production limits the use of these yeasts. Production cost could be reduced using less expensive substrates (different agro-industrial raw materials), as well as increasing yields of these pigments by optimizing the

Table 1 Concentrations of individual pigments in carotenoids synthesized from *Rhodotorula* strains grown on different substrates as a carbon sources

Rodotorula species and microbial associations	Carbon source	Fermentation process	Carotenoid pigments (% of total carotenoids)				References
			β-carotene	Torulene	Torularhodin	γ-carotene	
R. glutinis 32	Glucose	Batch	80.0	17.0	2.3	–	[9]
R. mucilaginosa CRUB 0064	Glucose	Batch	10.8	5.7	83.4	–	[18]
R. glutinis 48-23 T	Glucose	Batch	27.4	30.2	26.3	13.7	[57]
R. graminis DBVPG 7021	Glucose	Batch	50.3	22.7	11.6	15.4	[12]
R. lactosa BKM-1264	Whey	Batch	19.1	11.3	69.9	–	[30]
R. glutinis 22P + *L. helveticus* 12A	Whey ultrafiltrate	Batch	16.3	8.5	67.9	–	[15]
R. rubra GED5 + *K. lactis* MP 11	Whey ultrafiltrate	Batch	31.6	6.4	52.8	–	[67]
R.rubra GED5 + *L. casei* Ha4	Whey ultrafiltrate	Batch	46.6	10.7	36.9	–	[16]
R. rubra GED8 + (*L. bulgaricus* 2-11 + *S. thermophilus* 15HA)	Whey ultrafiltrate	Batch	50.0	12.3	35.2	–	[24]
R. glutinis 32	Sugar cane molasses	Fed-batch	47.4	49.2	3.1	–	[10]
R. glurinis 32	Sugar cane molasses supplemented with yeast extract	Fed-batch	87.0	10.0	3.0	–	[10]
R. glutinis DBVPG 3853	Beet molasses	Batch	3.1	9.6	80.7	–	[11]
R. glutinis DBVPG 3853	Grape must	Batch	25.5	7.9	65.3		[11]
R. glutinis DBVPC 3853	Concentrated grape must	Batch	17.9	7.0	73.0	–	[63]
R. glutinis DBVPG 3853 + *D. castellii* DBVPG 3503	Corn syrup	Fed-batch	12.0	14.0	74.0	–	[64]
R. rubra	Sugar cane juice	Batch	13.0	50.0	35.0	2.0	[78]
R. glutinis CCT 2186	Sugar cane juice	Batch	36.0	40.0	5.0	17.0	[78]
R. glutinis ATCC 26085	Glucose	Batch	39.0	48.0	2.0	11.0	[65]

culture conditions. An alternative for utilization of some natural substrates for production of carotenoids by *Rhodotorula* species is the method of cocultivation. A widespread natural substrate is milk whey containing lactose as a carbon source. Carotenoid synthesis by lactose-negative yeasts (*R. glutinis*, *R. rubra* strains) in whey ultrafiltrate can be accomplished: by enzymatic hydrolysis of lactose to assimilable carbon sources (glucose, galactose) thus providing the method of co-cultivation with lactose-positive yeasts (*Kluyveromyces lactis*), producers of β-galactosidase [67] or by creating conditions under which lactose is transformed into carbon sources (glucose, galactose, lactic acid) easily assimilated by the yeast when they were grown in association with homofermentative lactic acid bacteria or yogurt starter culture [15, 16, 24]. The maximum carotenoid yields for the microbial associations [*R. rubra* + *K. lactis*; *R. glutinis* + *Lactobacillus helveticus*; *R. rubra* + *L. casei*; *R. rubra* + (*L. bulgaricus* + *Streptococcus thermophillus*)] were, as follows: 10.20, 8.10, 12.12, 13.09 mg/l, respectively. These yields are about five times higher than that of a lactose-positive strain *R. lactosa* cultivated in whey reported in literature [30]. *R. glutinis–Debaryomyces castellii* co-cultures was produced (5.4 mg carotenoids/l) about three times the amount of total carotenoids formed by the red yeast cultured alone in low hydrolyzed corn syrup (LHCS) [64]. The author concluded that oligosaccharides and dextrins of LHCS could be profitably utilized for pigment production by *R. glutinis* after hydrolysis to maltose and glucose by the extracellular amylolytic enzymes produced by *D. castellii* DBVPC 3503 in co-cultures.

The traditional batch production system has the disadvantage of inducing the Crabtree effect (characterized by the synthesis of ethanol and organic acids as fermentation products) [97], due to high concentrations of initial sugars, diminishing pigment and biomass yield. Above 12 g/l carbon concentration, the carotenoid yield by *P. rhodozyma* began to diminish and the Crabtree effect was observed [98]. The strategy for solving this problem is the fed-batch culture. Maximum astaxanthin production (23.81 mg/l) by *P. rhodozyma* was achieved in fed-batch fermentation with constant pH = 6.0, 4.8 times greater that the one obtained in a batch culture (4.96 mg/l) and the biomass concentration (39.0 g/l) was 5.3 times higher than that in the batch culture [76]. The maximum astaxanthin concentration by *X. dendrorhous* at fed-batch fermentation with pH-shift control strategy reached 39.47 mg/l, and was higher by 20.2 and 9.0% than that of the batch and fed-batch fermen-

tation, respectively, with constant pH = 5.0 [82]. However, the maximal cell density at fed-batch fermentation with pH-shift control was 17.42 g dry cells/l, and was lower by 2.0% than that of fed-batch fermentation with constant pH = 5.0. As a result of the two stage fed-batch culture *P. rhodozyma*, cell and astaxanthin concentrations reached 33.6 g/l and 16.0 mg/l, respectively, which were higher when compared with batch culture [99]. The final specific astaxanthin concentration (mg/g dry wt of cells) in the second stage was ca. threefold higher than that in the first stage and 1.5-fold higher than that in the dissolved oxygen-controlled batch culture, indicating that the astaxanthin production was enhanced mush more in the second stage than in the first stage. The astaxanthin production was enhanced by a high initial C/N ratio in the medium (second stage), whereas a lower C/N ratio was suitable for cell growth (first stage). A significant increase (54.9%) in astaxanthin production by *X. dendrorhous* was achieved in pulse fed-batch process when compared with batch process [70]. The astaxanthin concentration was 33.91 mg/l in pulse fed-batch when compared with 30.21 mg/l in constant glucose fed-batch and 21.89 mg/l in batch fermentation. In contrast with this strain producing high yields of biomass and astaxanthin in pulse fed-batch process [70], another strain of *P. rhodozyma* demonstrated high astaxanthin-synthesizing activity during continuous fed-batch process [73]. The utilization of continuous feeding showed to be the most efficient feeding method in fed-batch processes, as it did not lead to a reduction in the cellular astaxanthin concentration, as observed in the pulsed feeding. In the pulsed and continuous fed-batch processes, a cellular astaxanthin concentration of 0.303 mg/g biomass and 0.387 mg/g biomass, an astaxanthin concentration of 5.69 and 7.44 mg/l, a biomass concentration of 18.7 and 19.3 g/l were obtained, respectively. High total carotenoid production of 52.4 mg/l by *P. rhodozyma* was obtained using constant fed-batch fermentation [95].

Fed-batch co-cultures *R. glutinis–D. castellii* gave a volumetric production of 8.2 mg total carotenoid/l, about 150% of that observed in batch co-cultures and biomass concentration of 9.8 g/l which was about two times higher when compared with batch fermentation [64]. The fed-batch technique maximized the specific growth rate of *R. glutinis*, resulted in higher biomass and minimized substrate inhibition of pigment formation [10]. Feeding molasses in the fed-batch mode led to increased biomass by 4.4- and 7-fold in double- and triple-strength feed, respectively when compared with 12.2 g/l biomass in batch fermentation. *R. glutinis* also produced a very high carotenoid concentration for double- and triple-strength feed supplement (71.0 and 185.0 mg/l, respectively), and was higher 2- and 3.7-fold of that observed in batch fermentation.

Strategies for improvement of carotenoid-synthesizing strains

Mutagenesis is an alternative to classical strain improvement in the optimization of carotenoid production. Mutagenic treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG), UV light, antimycin, ethyl-methane sulfonate, γ-irradiation, high hydrostatic pressure have been used successfully to isolate various strains with enhanced carotenoid-producing activity [62, 66, 83, 85, 86, 100–105].

Vijayalaksmi et al. [86] reported that the UV mutant *R. gracilis* (Orange 1) has shown 1.8 times higher carotenoid-synthesizing activity than that of the parent strain (0.91 mg carotenoids/g dry cells and 0.54 mg carotenoids/g dry cells, respectively) and the relative share of β-carotene in the total carotenoids was 60%. The yellow colored mutant 32 was also obtained by UV mutagenesis of the pink yeast *R. glutinis* and produced a large quantity of total carotenoids (2.9 mg/g dry cells), which was 24-fold higher accumulation of total carotenoids compared with the wild-type [100]. Mutant 32 produced 120-fold more β-carotene (2.05 mg/g dry cells) than the parent culture in a much shorter time (36 h), which was 82% (w/w) of the total carotenoid content. Mutant 32 produced a minute quantity of torularhodin and probably had an affected oxidase activity. However, there was no direct correlation between the decrease in torularhodin and the increase in β-carotene content, because the increase in β-carotene was several-fold greater [100]. Similarly, Frengova et al. [66] also reported that the mutant *R. rubra* 56–13 was with enhanced carotenoid-synthesizing activity (3.4 times) for synthesizing total carotenoids and β-carotene (8.3 times) and was obtained by NTG mutagenesis. The mutant manifested carotenoid-synthesizing activity of 0.95 mg carotenoids/g dry cells and the relative share of β-carotene was 71%. The mutant produced insignificant amount of torularhodin, which can be related to affected oxydase activity. Later, Wang et al. [105] after the treatments of five repeated cycles by high hydrostatic pressure of 300 MPa, the mutant *R. glutinis* RG6p was obtained, β-carotene production of which reached 10.01 mg/l, increased by 57.89% compared with 6.34 mg/l from parent strain.

A fivefold increase in β-carotene accumulation was reported for yellow mutant *P. rhodozyma* 2-171-1 which was obtained after ethyl-methane sulfonate mutagenesis of dark red strain *P. rhodozyma* [101]. The authors suggested that this mutant is likely to be blocked in the oxidase step and therefore unable to perform the conversion of β-carotene to echinenone and latter to astaxanthin. Later, these authors reported that the UV-mutant *P. rhodozyma* PG 104 produced 46-fold more β-carotene (92% of total carotenoids) than the parent culture (2% of total carotenoids) and maximum β-carotene yields were 1.08 mg/g dry cells and

9.95 mg/l [106]. Using NTG mutagenesis two different strains of carotenoid accumulating *X. dendrourhous* mutants JH1 and JH2 were also isolated [102]. Astaxanthin-overproducing mutant JH1 produced 4.03 mg astaxanthin/g dry cells, and this value was about 15-fold higher than that of wild-type. β-Carotene-producing mutant JH2 produced 0.27 mg β-carotene/g dry cells, and this was fourfolds increase from that of wild-type. Later, these authors reported that the mutant *X. dendrourhous* JH1 produced maximum astaxanthin concentration of 36.06 mg/l and 5.7 mg/g dry cells under optimized cultivation conditions [83]. The carotenoid production of the mutant *P. rhodozyma* (isolated by NTG mutagenesis) increased up to 6.4 mg/l and 1.2 mg/g dry cells, whereas for the wild-type the maximum carotenoid yields were 2.7 mg/l and 0.39 mg/g dry cells [104]. Astaxanthin-overproducing mutant *P. rhodozyma* 11-36-489 (also isolated by NTG mutagenesis) produced 2.2 mg astaxanthin/g dry cells and this value was about 10-fold higher than that of wild-type [107]. Previously, An et al. [62] reported that one of the antimycin mutant (*P. rhodozyma* ant-1) and a nitrosoguanidine derivate of ant-1 (*P. rhodozyma* ant-1-4) produced considerably more astaxanthin than the parent strain (ant-1 mutant had 0.8–0.9 mg/g; ant-1-4 mutant had 2.0–2.5 mg/g and parent strain had 0.3–0.45 mg/g). The physiology of the antimycin isolates and the known specificity of antimycin for cytochrome *b* in the respiratory chain suggests that alteration of cytochrome *b* or cytochrome P-450 components involved in oxygenation and desaturation of carotenes in mitochondria are affected, which results in increased astaxanthin production. To isolate a carotenoid-hyperproducing yeast, *P. rhodozyma* 2A2 N was treated by low-dose gamma irradiation below 10 kGy and mutant 3A4-8 was obtained [85]. It produced 3.3 mg carotenoids/g dry cells, 50% higher carotenoid content than that of the unirradiated strain (antimycin NTG-induced mutant 2A2 N). Gamma irradiation produces oxygen radicals generated by radiolysis of water [108] and could induce mutation of *P. rhodozyma* through a chromosomal rearrangement [109]. A primary function of carotenoids in *P. rhodozyma* is to protect cells against singlet oxygen and these compounds have been demonstrated to quench singlet oxygen [110]. Schroeder and Johnson [111] also suggested that singlet oxygen might induce carotenoid synthesis in *P. rhodozyma* by gene activation. Oxygen radicals have been known to cause changes in the molecular properties of proteins as well as enzyme activities [112]. Therefore, Sun et al. [85] suggested that oxygen radicals generated by gamma irradiation might modify the pathway in astaxanthin biosynthesis of *P. rhodozyma* and cause an increase in carotenoid production of the mutant 3A4-8 isolated by gamma irradiation. In a recent study, mutant *X. dendrourhous* G276 (isolated by NTG mutagenesis) produced about 2.0 mg carotenoids/g dry cells and 8.0 mg/l of carotenoids; in comparison, the parent strain—0.66 mg/g dry cells and 4.5 mg/l [103]. Fleno et.al. [113] reported that the mutant *P. rhodozyma* CBS 215-88 (isolated by EMS- and UV-treatment) produced high amounts of carotenoids and astaxanthin—43.4 mg/l and 1.36 mg/g and 29.9 mg/l and 1.18 mg/g, respectively. Significantly higher yields of astaxanthin (561.0 mg/l and 7.2 mg/g dry cells) were produced by mutant *P. rhodozyma* UBX-AX, obtained by chemical and UV mutagenesis [94].

One possibility for the improvement of the metabolic productivity of an organism is genetic modification. This strategy can be successful when an increase of the flux through a pathway is achieved by, e.g., the overproduction of the rate-limiting enzyme, an increase of precursors, or the modification of the regulatory properties of enzymes. In the carotenogenic yeasts, mevalonate synthesis, which is an early step in terpenoid biosynthesis, is a key point of regulation of the carotenoid biosynthetic pathway. In fact, addition of mevalonate to a culture of *X. dendrourhous* stimulated both astaxanthin and total carotenoid biosynthesis four times (from 0.18 to 0.76 mg/g and from 0.27 to 1.1 mg/g dry cells, respectively) [114]. This indicates that the conversion of HMG-CoA to mevalonate by HMG-CoA reductase is a potential bottleneck on the road to modified strains with higher astaxanthin content. Like carotenoids, ergosterol is an isoprenoid and it is biosynthetically related to them by a common prenyl lipid precursor, FPP. Astaxanthin production by *P. rhodozyma* strain was enhanced (1.3-fold) when sgualene synthase phenoxypropylamine-type inhibitor for sterol biosynthesis was added to the medium [115]. The isolation and characteristic of the carotenogenic genes of yeasts facilitates the study of the effect of their overexpression on carotenoid biosynthesis. Verdoes et al. [116] described the use of recombinant DNA technology for metabolic engineering of the astaxanthin biosynthetic pathway in *X. dendrourhous*. In several transformants containing multiple copies of the phytoene synthase-lycopene cyclase-encoding gene (*crtYB*), the total carotenoid content was higher (with 82%) than in the control strain. This increase was mainly due to an increase of the β-carotene and echinenone content (with 270%), whereas the total content of astaxanthin was unaffected or even lower. Alternatively, in recent years, several food-grade non-pigmented yeasts (*Saccharomyces cerevisiae*, *Candida utilis*) have been engineered in order to obtain strains possessing the ability to produce selected carotenoids [117, 118]. Identification of genes of enzymes from the astaxanthin biosynthetic pathway and their expression in a non-carotenogenic heterologous host have led to the overproduction of β-carotene [118]. Verwaal et al. [118] have been investigated the possibility of the use of *S. cerevisiae* as a host for efficient β-carotene production by successive transformation with carotenogenic genes (*crtYB* which encodes a bifunctional phytoene synthase and lycopene

cyclase; *crtI*, phytoene desaturase; *crtE*, heterologous GGPP synthase; *tHMGI*, HMG-CoA reductase) from *X. dendrourhous*. Like *X. dendrourhous*, *S. cerevisiae* is able to produce FPP and converts it into GGPP, the basic building block of carotenoids. *S. cerevisiae*, the industrially important conventional yeast, cannot produce any carotenoid, while is sintesizes ergosterol from FPP by a sterol biosynthetic pathway. Conversion of FPP into GGPP is catalyzed by GGPP synthase encoded by *BTS1* gene in *S. cerevisiae*. The authors have succeeded in the construction of a strain, producing a high level of β-carotene (5.9 mg/g dry cells). These experiments demonstrate that entire biosynthetic pathways can be introduced into new host cells through recombinant DNA technology. In addition, these results demonstrate that carotenoids can be produced in organisms that do not normally produce carotenoids. β-Carotene and astaxanthin have also been produced by *C. utilis* engineered through the introduction of carotenogenic genes from the *Agrobacterium* [117].

Factors affecting carotenogenesis in the yeasts *Rhodotorula* and *Phaffia*

Light

Carotenogenesis in many organisms is regulated by light. However, the intensity and protocol of illumination varies with the microorganism. Irrespective of whether increases or decreases in illumination time and/or intensity lead to improvements in carotenoid yield, there are two aspects to the theory of photoinduction. The first is that improvemens of the volumetric production of carotenoid (mg/l) are generally associated directly with improved growth of the microorganism [51]. Thus, the effect of light on growth of the microorganism plays an important role in establishing the authentic role of white-light illumination as a stimulant of carotenoid production. The second aspect to be considered is that increases in the cellular accumulation (mg/g) of carotenoid are associated with the increased activity of enzymes involved in carotenoid biosynthesis. In this case, it is important to assess the levels of biosynthetic enzymes, which in turn will establish the role of white-light illumination as a stimulant.

The amount and intensity of light tolerated by *Rhodotorula* species or strains vary, as it was observed that *R. minuta* can tolerate up to 5,000 Lx [119], whereas a mutant of *R. glutinis* displayed poor growth on exposure of 1,000 Lx [80]. In order to study the role of biosynthetic enzymes in carotenoid production in *R. minuta* under light, Tada et al. [119] used mevinolin, which is a highly specific competitive inhibitor of HMG-CoA reductase. It was observed that the photoinduced production of carotenoids was competitively inhibited by mevinolin. The concentration of inhibiter required to completely suppress carotenoid production depends on the light dose given to the cells. However, the relationship between inhibition and mevinolin concentration was almost identical regardless of the light dose. These results suggested that the activity of enzymes involved in the formation of HMG-CoA might not be affected by light. When an adequate amount of mevalonate was added to a growth medium containing sufficient mevinolin to completely inhibit photoinduction of carotenoid production, the same quantity of carotenoids was produced as in the absence of mevinolin. These results show that one or more photoinducible enzymes, such as HMG-CoA reductase, may be present in the carotenogenic pathway beyond mevalonate. In previous studies, Tada and Shiroishi [27] reported that the carotenoid content of resting cells and of cells that had been cultured at 0°C did not increase even with illumination. These results indicate that the carotenoid production in response to light is related to biochemical reactions dependent on nutrient and temperature, but not to changes in precursors due to a photochemical reaction. The cells exposed to light at 0°C were able to produce a small amount of carotenoid under subsequent incubation in the dark at 26°C. These results suggest that the promotion of carotenoid biosynthesis in *R. minuta* by light occurs in two phases: the first involves a photochemical reaction independent of temperature and the second involves biochemical reactions independent of light. This control mechanism of carotenoid production by light in *R. minuta* is similar to that of photoinduced carotenogenesis in fungi and bacteria. However, in *R. minuta*, the rate of carotenoid production and the final carotenoid content are controlled by the light intensity, but not by the light dose [27]. Sakaki et al. [120] reported that torularhodin production of *R. glutinis* can be increased (to 180% under light irradiation compared with in the dark) at a cost of growth following exposure to weak white light. On the other hand, the production of β-carotene was also increased by light irradiation, but the increase was only 14%. However, the negative effect on growth of the yeast can be overcome by manipulating growth conditions, as reported by Bhosale and Gadre [80] wheren exposure of a β-carotene-producing mutant of *R. glutinis* to white light in the late exponential growth phase resulted in a 58% increase in β-carotene production with a concurrent decrease in torulene.

In *X. dendrorhous*, astaxanthin production was also proposed to be photo-inducible [89]. It was observed that the astaxanthin concentration was almost constant in cells grown in the dark but high levels of astaxanthin were present under strong light illumination. The stimulation was of a transient nature; however, and a reduction in the total pigment content was observed when the cultures were returned to the dark. Maximum astaxanthin production was obtained

when cultures were continuously illuminated. An and Johnson [121] reported a decrease in β-carotene content on exposure to white light in *X. dendrorhous*. However, exposure to light led to stimulate total carotenoid production (mainly astaxanthin) and had a negative effect on growth. Similar improvements in the volumetric levels of carotenoids were also reported in another strain of *X. dendrorhous* [122]. The illumination influences not only total carotenoid concentrations, but also carotenoid profile and biomass concentration. The sixth strains studied by the author were not affected by the light in the same way. However, all strains synthesize higher amounts of total carotenoids in the light than in the dark. Among the studied strains, ATCC 24,288 grown in the light produced more carotenoid and astaxanthin, 2.45 mg/l and 2.13 mg/l, respectively—the values obtained in the dark were 1.72 and 1.38 mg/l, respectively.

Temperature

Temperature is another important factor affecting the performance of cells and product formation. It brings about changes in many biosynthetic pathways, including carotenoid biosynthesis. The effect of temperature depends on the species specificity of the microorganism and often manifests itself in quantity variations of synthesized carotenoids [20, 21, 61, 72, 86, 92]. With some yeast species, there have been noticed different proportions of the individual pigments (torulene, torularhodin, β-carotene, astaxanthin), which form the synthesized carotenoids [11, 57, 80, 87, 123–125]. It has been confirmed that the change in the relative concentration of individual pigments, in relation to the total carotenoids synthesized by *R. glutinis* cultivated at 5 and 25°C, depends on the system`s thermosensitivity. The latter is responsible for the dehydration of γ-carotene to torulene. The temperature effect is controlled by the concentrations of β-carotene synthetase and torulene synthetase [125]. The study of the biosynthetic pattern of carotenoid formation by *R. glutinis* 48-23T cultivated at 5 and 25°C in glucose medium prove that: at 25°C the culture synthesizes the pigments β-carotene, torularhodin and torulene in concentrations of about 30% of the total carotenoid; at 5°C the synthesized carotenoids are represented mainly by β-carotene (64%) and significantly less by torulene and torularhodin [57]. A biosynthetic study showed that γ-carotene acts as the branch point of carotenoid synthesis. Subsequent dehydrogenation and decarboxylation leading to torulene synthesis is known to be temperature dependent since the respective enzymes are less active at lower temperature compared with the activity of β-carotene synthetase. Presumably, this was the reason for increased β-carotene accumulation (90% of total carotenoid) by mutant *R. glutinis* at 20°C and a decrease (71% of total carotenoid) as the incubation temperature was increased to 30°C [80]. The above-reported results come close to the results reported by other authors [11, 87, 124], who have studied the temperature effect on the carotenoid-forming ability of yeast. The lower temperatures exert favorable influence on the synthesis of β-carotene by *R. glutinis* co-cultivated with *L. helveticus*, at 20°C, the concentration of β-carotene was 19.0% of the total carotenoids compared to 9.6% at 35°C. The increased temperature led to higher concentration of torularhodin (78.3%) in relation to the total carotenoid pigments concentration, while torulene and β-carotene concentrations decreased [124]. Buzzini and Martini [11] also reported that the lower temperatures (25°C) seemed to favor synthesis of β-carotene and torulene, whereas higher temperatures (35°C) positively influenced torularhodin synthesis by *R. glutinis*.

Phaffia rhodozyma cultivated at 20°C synthesized carotenoids with prevailing astaxanthin content (85%) and small β-carotene (10%). At 30°C the synthesized carotenoids are represented by torularhodin (60%), torulene (30%) and β-carotene (5%) [87]. The effect of nicotine and diphenylamine on astaxanthin biosynthesis in moderately psychrophilic *X. dendrorhous* was studied under standard and low temperature conditions, 22 and 4°C, respectively [123]. It was found that 10 mM nicotine inhibits the cyclization of lycopene. The oxidation of β-carotene was irreversibly inhibited by 10 μM diphenylamine, while the dehydrogenation of phytoene was reversibly inhibited by 60 μM diphenylamine. The simultaneous exposure to low temperature (4°C) overcomes the inhibition of β-carotene oxidation at low diphenylamine concentration and bring about interconversion of β-carotene to astaxanthin.

Aeration

Carotenogenesis is an aerobic process and the air flow rate in the yeast culture is an essential factor to assimilate the substrate as well as for growth rate, cell mass and carotenoid synthesis. The effect of aeration is dependent on the species of the microorganism. The reported optimal values of air flow rate and agitation are in range 0.5–1.9 l/l min and 180–900 rpm, respectively, for carotenogenesis in yeasts *Rhodotorula* and *Phaffia* [10, 15–17, 22, 24, 31, 64, 67, 71, 73, 76, 77, 80–82, 126]. The effect of aeration frequently manifests itself in quantitative variation of the synthesized carotenoids registered by some authors [21, 65, 72, 92]. Moreover, other authors have recorded changes in the composition of individual pigments making up the total carotenoids [30, 76, 126, 127]. The aeration of the mixed culture (*R. rubra* + *L. casei*) influenced not only the amount of carotenoids produced, but also the composition of individual pigments making up the total carotenoids. Increasing the air flow rate the relative proportion of β-carotene

increased from 42.0 to 60.0%, the proportion of torularhodin decreased from 44.0 to 29.0%, while the proportion of torulene changed only slightly (9.5–11.0%) [126]. Similar tendency was observed by Zalashko [30] for *R. lactosa*, but the changes registered were slight—by increasing the air flow rate the relative proportion of β-carotene and torulene increased from 18.25 to 21.5% and from 10.3 to 14.6%, respectively, the proportion of torularhodin decreased from 71.5 to 63.9%. In contrast with Zalashko [30] and Simova et al.[126], Sakaki et al.[127] reported that the increase in content of torularhodin produced by *R. glutinis* was related to elevation of the dissolved oxygen concentration. Oxygen supply in sufficient quantities stimulates efficient astaxanthin synthesis by *P. rhodozyma* [76]. Low oxygen causes biomass formation of yellowish color, product of the β-carotene accumulation; therefore, under these conditions the β-carotene hydroxilase it is not very efficient.

Metal ions and salts

Several divalent cations (Ba, Fe, Mg, Ca, Zn and Co) have been demonstrated to act as stimulants for growth of *R. glutinis* [128]. Later Buzzini et al. [12] reported that trace elements have been shown to exert a selective influence on the carotenoid profile in *R. graminis*—Al^{3+} and Zn^{2+} had a stimulatory effect on β-carotene and γ-carotene synthesis, while Zn^{2+} and Mn^{2+} had a inhibitory effect on torulene and torularhodin synthesis. The observed effect of trace elements on the biosynthesis of specific carotenoids in red yeasts may be explained by hypothesizing a possible activation or inhibition mechanism by selected metal ions on specific carotenogenic enzymes, in particular, on specific desaturases involved in carotenoid biosynthesis. In a recent study, calcium, zink and ferrous salts were shown to have a stimulatory effect on volumetric production as well as cellular accumulation of carotenoids from the yeast *R. glutinis* [100]. Divalent cation salts increased the total carotenoid content (mg/l) about two times. It can be assumed that this positive response was due to a stimulatory effect of cations on carotenoid-synthesizing enzymes, as reported earlier [58], or to the generation of active oxygen radicalcals in the culture broth. In contrast, the addition of manganese salt in the presence of generators of oxygen radicals had an inhibitory effect on carotenoid formation in *X. dendrorhous* since manganese acts as a scavenger [129]; however, this effect could be concentration dependent as manganese is also known to act as a cofactor for enzymes involved in carotenoid biosynthesis and thus enhances carotenoid accumulation at certain concentrations [58]. Astaxanthin content was decreased significantly at >1 mg/l $FeCl_3$ and growth of *P. rhodozyma* was poor at an $FeCl_3$ concentration of <0.1–1.0 mg/l [130]. Carotenoid production decreased in yeast with increasing Mn^{2+} concentration (0–10 mg/l) when succinate was used as the sole C source, but not when growth took place in the presence of glucose. The week oxygen radical scavengers Zn^{2+} and Cu^{2+} had no effect on carotenoid production by *P. rhodozyma* [130], whereas Cu^{2+} below 3.2 μM increased the astaxanthin content of cells *P. rhodozyma* but at the expense of a slightly decreased growth [131]. In yeast, there are at least two intracellular enzyme systems requiring copper: cytochrome-*c*-oxidase and superoxide dismutase [132]. These enzymes are probably related to the increased astaxanthin production seen in concentrations of Cu^{2+} below 3.2 μM. Copper deficit decreases the activity of antioxidant enzyme Cu,Zn-superoxide dismutase, as reported previously [133] and may induce oxidative stress and astaxanthin synthesis because of diminished antioxidant defences. In contrast, iron below 1 μM decreased both the growth and astaxanthin content of cells *P. rhodozyma* [131].

Solvents and chemical or natural agents

The supplementation of ethanol (10 g/l) or acetic acid (5 g/l) was reported to stimulate cell mass accumulation and astaxanthin formation in fed-batch culture of *P. rhodozyma*—the astaxanthin concentrations of 45.62 mg/l and 43.87 mg/l were obtained, respectively, which were about 25% higher than that without ethanol or acetic acid [134]. High cell mass concentration (30 g/l) was obtained by glucose fed-batch culture with pH-stat, and the ethanol feeding was performed based on DO-stat [135]. Therefore, the astaxanthin content in cells reached 0.72 mg/g, which was 2.2-fold higher than that without ethanol feeding. Gu et al. [136] also reported increased carotenoid production (from 1.65 mg carotenoids/g cells to 2.65 mg carotenoids/g cells) upon addition of 0.2% (v/v) ethanol to cultures of the yeast *X. dendrrohous*. Detailed studies revealed that ethanol activates oxidative metabolism with induction of HMG-CoA reductase, which in turn enhances carotenoid production. Later Hoshino et al. [137] isolated a DNA sequence coding for enzymes involved in a carotenoid biosynthetic pathway that includes HMG-CoA synthase and reductase. Strains of *P. rhodozyma* transformed by such DNAs schowed increased astaxanthin-synthesizing activity.

The β-carotene content in cells *R. glutinis* increased up to 35% when phenol was added to culture medium at 500 ppm [138]. The ratio of torularhodin decreased with increasing phenol concentration, while torulene content was almost constant. Flores-Cotera et al. [14] reported that supplementation of citrate in the medium at levels of 28 mM or higher notably increased the final carotenoid concentration (from 3.2 to 4.5 mg/l) and carotenoid content (from 1.1 to 1.7 mg/g) in cells *P. rhodozyma*. However, dry weight and growth yield decreased with increasing citrate. Increased carotenoid synthesis at low ammonium or phos-

phate levels, and stimulation by citrate were both paralleled by decreased protein synthesis. This suggested that restriction of protein synthesis could play an important role in carotenoid synthesis by *P. rhodozyma* [14]. The astaxanthin production by *X. dendrorhous* was enhanced most significantly with double feeding of 10 mmol/l H_2O_2 at 0 and 24 h, reaching a cellular content of 1.30 mg/g cells and a volumetric yield of 10.4 mg/l, which were 83 and 65% higher, respectively, than those of the control (0.71 mg/g cells and 6.3 mg/l) [139]. The intracecellular catalase activity was also increased after H_2O_2 treatment. No enhancement of the astaxanthin biosynthesis was attained when H_2O_2 was added to the yeast culture together with a sufficient amount of exogenous catalase. The authors suggested that astaxanthin biosynthesis in *X. dendrorhous* can be stimulated by H_2O_2 as an antioxidative response. In other article [19] these authors reported that the addition of 9% *n*-hexadecane to the liquid medium for growth of *P. rhodozyma* leads to increase of carotenoid yield by 58% (14.5 vs. 9.2 mg/l in the control) and oxygen transfer rate by 90%.

Aksu and Eren [92] reported that the supplementation of cotton seed oil in the culture medium for growth of *R. mucilaginosa* resulted in an increased production of total carotenoids. The yeast produced 57.6 mg/l carotenoid with cotton seed oil, while 39.5 mg/l carotenoid was formed without the activators. The carotenoids yields in the presence of tomato juice at 2.6 ml/l, groundnut oil at 1.0 ml/l, vitamin B_2 at 3.5 ml/l or vitamin B_1 at 2.2 ml/l in a fermentation medium for growth, *Rhodotorula* strain were 34.36, 17.28, 11.27 and 8.3% higher than that in the control culture, respectively [88]. Kim et al. [103] reported that the addition of plant extracts such as *Perilla frutescens* (final concentration, 5%) in a culture medium for growth of *X. dendrourhous* enhanced the pigment production to 32 mg/l and reduced the cultivation time by 2 days.

Similarly, different fungal elecitors also stimulate astaxanthin formation in *X. dendrourhous* strains [140, 141]. *X. dendrourhous* strains exposed to fungal concentrate extract (prepared from *Epicoccum nigrum*—plant pathogen) enhanced astaxanthin up to approximately 40% per unit dry cell weight in two strains [140]. Interestingly, the fungal extract restored astaxanthin biosynthesis in non-astaxanthin-producing mutants previously isolated, including the albino and the β-carotene mutant. The authors suggested that oxidizing agents produced from the metabolic activities of *E. nigrum* could stimulate astaxanthin production of *X. dendrourhous*. The plant pathogens produced oxidative enzymes that could lead to plant cell walls degradation, resulting in generating reactive oxygen species including peroxyl radicals and singlet oxygen, which could enhance astaxanthin yield of *P. rhodozyma*, as reported earlier [111, 142]. In addition, it is known that *E. nigrum* synthesizes secondary metabolites including isoprenoids and certain compounds or intermediates, which could affect carotenoid formation. Three fungal elecitors prepared from *R. rubra*, *R. glutinis* and *Mucor mucedo* have shown stimulating effects on the growth, total carotenoids and astaxanthin formation by *X. dendrourhous* [141]. Among the fungal elecitors tested, the *R. glutinis* elecitor concentration of 30 mg/l stimulated the highest astaxanthin yield with a 91% increase compared with the control.

In conclusion, the reported by some researchers values of cell mass and carotenoid concentrations produced by *Rhodotorula* species and *P. rhodozyma*/or *X. dendrorhous* at different cultural conditions and stimulants using batch or fed-batch processes are summarized in Tables 2 and 3. The highest total carotenoid concentrations of 129.0 and 183.0 mg/l were produced by *R. glutinis* mutant 32 cells grown in glucose-rich synthetic medium [9] and sugar-cane molasses [10], respectively (Table 2). Under fed-batch fermentation in sugar-cane molasses, β-carotene was the major carotenoid pigment produced (87% of total carotenoids), with a minor quantity of torulene (10% of total carotenoids), followed by torularhodin (3% of total carotenoids) [10]. As shown in Table 3, the highest astaxanthin concentration of 516.0 mg/l at fed-batch process in 20 l fermentor was produced by *P. rhodozyma* mutant UBV-AX cells grown in hydrolyzed corn syrup [94]. In order to commercially produce natural carotenoids by microorganism, scale-up process is essential. *P. rhodozyma* 2A2 N also synthesized high quantities total carotenoids (52.4 mg/l) when it was cultivated in natural substrate (corn starch hydrolysate) as carbon source using lab-scale fed-batch fermentation [95]. Similarly, significant amount of astaxanthin (52.32 mg/l) at batch process in 10^3 fermentor was produced by *X. dendrorhous* ZJUT003 cells grown in glucose-rich synthetic medium [31]. The obtained results of maximum astaxanthin concentrations of 58.77 and 52.32 mg/l at batch fermentation in the 50 l fermentor and 10 m^3 fermentor, respectively demonstrated that scale-up was effective. The best strains *R. glutinis* mutant 32, *P. rhodozyma* mutant UBV-AX and *X. dendrorhous* ZJUT003 appear very promising for biotechnological production of natural carotenoids (β-carotene, astaxanthin).

Currently the carotenoid-synthesizing yeast is marketed in a fine powder form [e.g. Aquasta® is Astaxanthin Partners Ltd`s (USA) trade name for its inactivated dried yeast product made from *P. rhodozyma* and Ecotone® Phaffia Astaxanthin (spray dried *P. rhodozyma* product manufactured by Archer Daniels Midland Co (USA)] as a natural source of astaxanthin, protein and other nutrients and utilized in many countries (European Union, Canada, USA) as an ingredient in salmonid feed. They are manufactured by natural fermentation in a carefully controlled environment thus effectively obtaining a product with a high percentage of free astaxanthin (minimum 8.0 mg astaxanthin/g

Table 2 Comparison of cell mass and carotenoid production by *Rodotorula* species grown on different refined and nonrefined substrates as a carbon sources

Rodotorula species and microbial associations	Carbon source	Fermentation process	Cell mass (g/l)	Total carotenoids (mg/g dry cells)	Total carotenoids (mg/l culture fluid)	References
R. gracilis CFR 1 AU	Glucose	Batch	2.4	26.0	62.4	[25]
R. glutinis 32	Glucose	Batch	23.9	5.4	129.0	[9]
R. glutinis CCT 2186	Sugar cane juice	Batch	6.7	0.197	1.32	[78]
R. rubra	Sugar cane juice	Batch	4.4	0.427	1.88	[78]
R. rubra	Peat extracts	Batch	4.8	1.26	6.03	[21]
R. glutinis KCTC	Sugar cane molasses	Batch	11.7	0.295	3.46	[93]
R. glutinis TISTR	Hydrolyzed mung bean waste flour	Batch	10.35	0.345	3.48	[91]
R. glutinis DBVPG 3853	Grape must	Batch	6.30	1.1	6.90	[63]
R. mucilaginosa CRUB 0195	Corn syrup	Batch	10.60	0.156	1.66	[18]
R. glutinis 32	Sugar cane molasses	Fed-batch	78.0	2.36	183.0	[10]
R. mucilaginosa NRRL-2502	Sugar-beet molasses	Batch	4.2	21.20	89.0	[92]
R. mucilaginosa NRRR-2502	Whey	Batch	2.4	29.2	70.0	[92]
R.glutinis DBVPG 3853 + *D. castellii* DBVPG 3503	Corn syrup	Fed-bach	15.3	0.535	8.20	[64]
R. glutinis 22P + *L.helveticus* 12A	Whey ultrafiltrate	Batch	30.2	0.268	8.10	[15]
R. rubra GED5 + *K. lactis* MP11	Whey ultrafiltrate	Batch	24.3	0.421	10.20	[67]
R. rubra GED2 + *L.casei* Ha1	Whey ultrafiltrate	Batch	27.0	0.448	12.10	[16]
R. rubra GED2 + (*L.bulgaricus* 2-11 + *S. thermophilus* 15 HA)	Whey ultrafiltrate	Batch	26.0	0.503	13.09	[24]

Aquasta® product and minimum 5.5 mg astaxanthin/g Ecotone® product).

Carotenoid-synthesizing yeasts—directions for their use

Because of the biological role of the carotenoids as vitamin A precursors in humans and animals [1, 143] and owing to their antioxidant properties and suspected activity in preventing some forms of cancer as well [44, 144, 145], carotenoid pigments represent a group of most valuable molecules for industrial applications. The pharmaceutical, chemical, feed and food industries have shown increased interest in the use of carotenoids, mainly as provitamin A, but also as natural food and feed colorants [1, 53, 56]. Accordingly, the red yeast *P. rhodozyma* is currently used for the production of astaxanthin, an important carotenoid pigment that can be exploite in aquaculture to give an appealing pink color to the flesh of farmed salmonid fish, and it also helps to impart a desirable golden color to the egg yolk and flesh of poultry. Salmon farming is an industry that is growing and gradually replacing the world's wild salmon fisheries [146]. The most expensive ingredient in salmonid feeds is astaxanthin, and though the actual revenues are privately held, it has been estimated that the market for astaxanthin in >US $100 milion per year [147]. In 1977, Johnson et al. [148] first reported that *P. rhodozyma* could be a source of astaxanthin for salmonids raised in aquaculture.

One limitation impacting the industrial utility of *P. rhodozyma/X. dendrourhous* or *Rhodotorula* species has been hindered absorption of carotenoids, due to the yeast`s thick cell wall. The biotechnology industry has developed different means of pigment liberation by the yeast including optimization of drying conditions, mechanical breakage, microwave treatment and enzyme treatment [149–155]. When disrupted cells *P. rhodozyma*, without cell walls, are added to the diets of animals, astaxanthin is readily absorbed from the gut; it effectively colors the flesh of pen-reared salmonids [148], and also helps impart a desirable golden color to the egg yolk and flesh of poultry [156]. The yeast also contains a high level of unsatured fat, protein and vitamins that contribute to good growth of animals [157]. These attributes enhance the potential utility of *P. rhodozyma* as a source of astaxanthin in animal diets. Akiba et al. [158] also reported that a fracturing yeasts *P. rhodozyma* allowed higher amounts of astaxanthin to be available for pigmentation of egg yolk in white leghorn hens, so less yeast was required to achieve a similar level of yolk color score. Later, these authors demonstrated that the cell wall fractured *Phaffia* yeast containing high concentrations of astaxanthin can be a useful source of astaxanthin for the modification of meat color, thus meeting consumer preferences in relation to the qualities of poultry meat [159]. The

Table 3 Comparison of cell mass and astaxanthin production by *Phaffia rhodoxyma*/*Xanthophyllomyces dendrorhous* strains grown on different refined or nonrefined substrates as a carbon sources

Strain	Carbon source	Fermentation process	Cell mass (g/l)	Astaxanthin (mg/g dry cells)	Astaxanthin (mg/l culture fluid)	References
P. rhodozyma PR 190	Glycerol	Batch	18.7	1.8 2.1[a]	33.7 43.1[a]	[71]
P. rhodozyma 7B 12	Glucose	Batch	7.71	1.0	7.71	[22]
X. dendrorhous JH1	Glucose	Batch	7.10	5.07	36.06	[83]
P. rhodozyma 3A4 - 8	Glucose	Batch	4.0	3.3[a]	13.2[a]	[85]
P. rhodozyma 3A4 - 8	Cellobiose	Batch	4.4	3.5[a]	15.4[a]	[85]
P. rhodozyma 3A4 - 8	Sorbitol	Batch	5.3	2.8[a]	14.8[a]	[85]
X. dendrorhous ZJUT 46	Glucose	Fed-batch	17.42	2.26	39.47	[82]
X. dendrorhous TISTR 5370	Glucose	Batch	20.58	2.57	52.36	[31]
P. rhodozyma CBS 215-88	Molasses	Fed-batch	24.0	1.18 1.36[a]	29.9 43.4[a]	[113]
P. rhodozyma UBV-AX	Hydrolyzed corn syrup	Fed-batch	79.0	7.2	561.0	[94]
P. rhodozyma	Sugar-cane juice	Batch	9.2	1.9	17.5	[69]
P. rhodozyma ATCC 24202	Sugar-cane juice	Fed-batch	19.35	0.384	7.44	[73]
X. dendrorhous 2A2 N	Sugar-beet molasses	Fed-batch	36.0	1.11[a]	40.0[a]	[8]
X. dendrorhous TISTR 5730	Hydrolyzed mustard waste isolate	Batch	19.6	1.31	25.8	[28]
X. dendrorhous ATCC 24228	Eucaliptus hydrolysates	Batch	10.0	0.174 0.214[a]	1.74 2.14[a]	[68]
P. rhodozyma NRRL Y-17268	Eucaliptus hydrolysates	Batch	23.2	0.448 0.550[a]	10.4 12.9[a]	[75]
P. rhodozyma ATCC 24202	Peat hydrolysate	Continuous	4.95	0.544	2.69	[90]
P. rhodozyma UCD-FST 484	Grape juice	Batch	38.0	0.300	11.4	[72]
P. rhodozyma 25-2	Date juice of Yucca fillifera	Fed-batch	39.0	0.618	23.81	[76]
P. rhodozyma 2A2 N	Corn hydralysate	Fed-batch	32.0	1.64[a]	52.40[a]	[95]

[a] Total carotenoid content (mg/g or mg/l)

effect was heightened by supplementing fractured yeasts in the broiler feed in proportion to dietary astaxanthin concentration. Similarly An et al. [149] concluded that a astaxanthin in yeast (*X. dendrorhous*) prepared by spray drying and flat-roller milling was well absorbed by laying hens and was successfully used as a pigmentation agent in animals. Specifically, when spray-dried and milled yeast was supplied in the feed (40 mg astaxanthin/kg feed), astaxanthin was successfully absorbed (1,500 ng/ml blood and 1,100 ng/g skin) by laying hens. Extrusion temperature did not affect utilization of dietary astaxanthin or rainbow trout flesh color significantly, but cell wall disruption of red yeast (*X. dendrorhous*) cells was critical to optimize carotenoid utilization [155]. Increasing the degree of enzymatic cell wall dispurtion increased flesh astaxanthin concentrations from 2.2 to 6.7 mg/kg, redness values from 5.5 to 10.7, yellowness values from 11.7 to 16.7 and astaxanthin retentions in the muscle from 3.7 to 17.4% [155]. A formulation of *P. rhodozyma* cells blended with ethoxyquin, lecithin and safflower oil prior to drying also increased astaxanthin deposition in salmonid fish flesh and rainbow trout flesh when supplied in feed as an additive [94]. Whyte and Sherry [160] and Fleno et al. [113] observed that the astaxanthin concentration was not significantly different between fish (Atlantic salmon) fed diet supplemented with spray-dried ruptured cells *P. rhodozyma* and those fed the synthetic astaxanthin diet. The astaxanthin-rich oil produced by a process for extracting carotenoids from *P. rhodozyma* biomass, was directly used as an additive to salmonid fish feed [161]. Biovailability testing of said additive, showed that, advantageously, this astaxanthin-rich oil has a higher biovailability than that of a commercially available synthetic product (Carophyl Pink®, from Hoffman La-Roche) and a much higher biovailability than that of dried *P. rhodozyma* cells. Recently, an interest in astaxanthin has been increased, because it also has a strong antioxidant effect. Antioxidant activity of astaxanthin was 10 times higher than that of β-carotene and 100 times higher than that of tocopherol [162]. An et al. [163] suggested that astaxanthin from *X. dendrorhous* could be used as an antioxidant as well as a colorant for broiler chickens. The concentrations of chemically synthesized astaxanthin (45 mg/kg

feed) and biological astaxanthin (spray-dried cells of the red yeast—22.5 mg/kg feed) were set to give similar levels of pigmentation. Lightness value was not significantly affected by astaxanthin but redness and yellowness values were significantly increased. Absorption and accumulation of biological astaxanthin were higher thah those of chemical astaxanthin, probably because of the high contents of lipids in the yeast (17%). Lipid peroxide formation in skin was significantly decreased by astaxanthin. The peroxide production in chickens fed chemical astaxanthin was markedly lowered compared to biological astaxanthin [163]. Previously, Nakano et al. [164] reported that a *P. rhodozyma*-producing astaxanthin should have a reducing effect on oxidized oil-induced oxidative stress in rainbow trout. The levels of serum transaminase (glutamic-pyruvic transaminase and glutamic-oxaloacetic transaminase) activities and of lipid peroxides in fish fed oxidized oil were significantly higher that those of the control fish fed non-oxidized oil. However, the supply of freeze-dried red yeast preparation considerably decreased both enzyme activities and lipid peroxides level. Furthermore, the serum lipid (triglycerides, total cholesterol and phospholipids) concentrations were also significantly decreased. Especially, the serum triglyceride level of fish fed the red yeast was as low as that of the control. Rapta et al. [165] found that Zn^{2+} ions induced changes in yeasts (*R. glutinis* and *R. rubra*) leading to more efficient scavenging and antioxidant capacities compared with Ni^{2+} ions, and antioxidants (carotenoids) present in yeast's walls showed higher ability to scavenge free radicals than those from inside the cells. Later, the in vivo antioxidant and protective effects of astaxanthin isolated from *X. dendrorhous* against ethanol-induced gastric mucosal injury were established in animal models, especially rats [166, 167]. Oral administration of astaxanthin showed significant protection against ethanol-induced gastric lesion and inhibited elevation of the lipid peroxide levels in gastric mucosa. In addition, pretreatment with astaxanthin resulted in a significant increase in the activities of radical scavenging enzymes such as superoxide dismutase, catalase and glutathione peroxidase. A histologic examination clearly indicated that the acute gastric mucosal lesion induced by ethanol nearly disappeared after pretreatment with astaxanthin [166]. Chemopreventive and anticarcinogenic effects of carotenoids by *Rhodotorula* on the development of preneoplastic lesions during *N*-nitrosodiethylamine (DEN)-induced hepatocarcinogenesis in female Wistar strain rats were also studied [168]. Spray-dried yeast *R. glutinis* (containing carotenoid pigments torulene, torularhodin and β-carotene in proportion 58:33:2) showed significant effect on the prevention of liver tumor development. However, *R. glutinis* effects were relatively more significant in groups where *R. glutinis* was administered after DEN treatment, suggesting that *R. glutinis* is quite effective in the prevention of liver tumor development especially when administered after DEN treatment, indicating possible protective effects at the promotional stages. Similarly, It et al. [169] also found that astaxanthin by *P. rhodozyma* may prevent breast cancer.

Conclusions

Carotenoids are playing an ever-increasing role in human health in the developed world. The nutritional value of carotenoids, such as a α- and β-carotene, β-cryptoxanthin and astaxanthin has been known for many years, and their antioxidant properties and their efficiency in the prevention of certain human diseases have also been claimed. Consenquently, interest in these compounds from a nutritional aspect has increased substantially and a multimillion dollar market has been established in the last 20 years. However, it is also a well-established fact that chemical synthesis is fulfilling most of this demand. The high commercial demand for many carotenoids have long been met by chemical synthetic technology. However, some of the by-products resulting from such chemical processes may have undesirable side effects on consumption. For this reason, the production of carotenoids from microbial sources has been the focus of extensive research. Furthermore, the microbial production of carotenoids could also be of interest because of the problems of seasonal and geographic variability in the production and marketing of colorants of plant origin. In the current literature, yeasts *Rhodotorula* (producing β-carotene, torulene and torularhodin as the major carotenoids pigments) and *Phaffia* (producing mainly astaxanthin) are described as a potential source of carotenoids with medical or industrial interest. Recently, the industrial interest has increased also towards the valuable orange-red carotenoids, like torularhodin and torulene for which, at present, no cheap commercially exploitable plant sources are known. The high cost involved in the practical implementation of modern technologies is the major limiting factor for scale-up of fermentation processes for carotenoid production. There is a need to improve fermentation strategies such that the intracellular accumulation of carotenoid from yeast is feasible on an industrial scale. Manipulation of external and cultural stimulants, as detailed in this review, will allow carotenoid production to be scaled-up for commercialization. Furthermore, various statistical methods for optimizing the amount of stimulant and different approaches for improving the production properties of the yeast strains, combined with locating inexpensive sources for microbial metabolite production will allow carotenoid biosynthesis by yeasts to become more efficient and more economical. As a matter of fact, in all developed countries agro-industrial by-products represent a

low cost source of non-refined sugars, which could profitably be converted into a pool of chemical compounds with relevant applications in food and pharmaceutical industries via the biotechnological route.

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