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Review

Yeast Carotenoids: Cost-Effective Fermentation Strategies for Health Care Applications

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Abstract: Carotenoid production from oleaginous red yeast has been considered as a safe alternative to chemically synthesized carotenoids commonly used in the food industry, since plant-based carotenoids are expensive and an irregular source for obtaining pigments. This is a summative review on the factors affecting carotenoid production, cost-effective production strategies using various inexpensive feedstock, metabolic engineering, and strain improvisation. The review specially highlights the various potential applications of carotenoids as anti-microbial, anti-viral, antioxidant, anti-cancerous, anti-malarial agents, etc. The importance of such natural and easily available resources for prevention, evasion, or cure of emerging diseases and their plausible nutraceutical effect demands exhaustive research in this area.

Keywords: yeast carotenoids; low-cost substrate; fermentation; healthcare

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1. Introduction

Carotenoids are ubiquitously found in plants, algae, bacteria, yeast, and fungi and are easily identified due to their vibrant yellow-orange color. Their antioxidant property and lucrative color have drawn the attention of researchers and industries for manufacturing a wide range of healthcare, food, or feed products [1,2]. According to a current report titled "Global Carotenoid Market–Growth, Trends, and Forecast (2018–2023)" by 2023, the international market for carotenoid production was predicted to reach USD 2 billion [3]. Out of the several highly valued carotenoid components, β-carotene alone captured USD 233 million in the global market in 2010 and is growing with an annual rate of 3.6% [4]. Astaxanthin and lutein, found in flowers, fruits, and some microorganisms, are also of high economic value. Plant-based carotenoids often vary in their content and productivity depending on the climatic changes and are also dependent on arable land, leading to limited availability or higher production costs [5,6]. Alternately, optimized production of microbial carotenoids as per need and economy is possible via large scale fermentation in bioreactors [7–9]. Fat-soluble carotenoids produced by red yeast (e.g., *Rhodosporidium*, *Phaffia*, *Rhodotorula*, *Sporobolomyces*, etc.) as secondary metabolites, are economical, especially because these yeast strains grow on a vast plethora of carbon sources, such as those obtained from waste feedstock e.g., agro-waste, mill effluent, whey/pineapple cannery waste–water, etc. [10–12].

Agro-industrial waste is a rich source for carbon, nitrogen, including minerals/salts necessary for microbial metabolism, slashing down production cost and mitigating environmental problems resulting from such wastes. This leads to the establishment of successful biorefinery, having good ROI (return on investment). Here we discuss the yeast-carotenoid production on inexpensive feedstock and carotenogenesis factors for cost-effective production. The expansive range of applications of carotenoids for biomedical purposes in the form of antioxidant, anti-viral, anti-microbial, anti-cancerous agents, etc. have also been comprehended, to highlight their potential use as natural therapeutic molecules.

2. Microbial Carotenoids: Natural Sources, Classification, and Properties

Carotenoid pigments are hydro-carbons which contain 40 carbon atoms along with two terminal rings [13]. These are the tetraterpenoids (C_{40}) consisting of eight isoprene units linked in order that the molecules are linear as well as symmetrical, with its order reversed in the center. General examples of carotenoids include α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, torulene, and torularhodin. Carotenoids can be classified as: carotenes and xanthophylls, the former (α-carotene, β-carotene, torulene, lycopene) being pure hydrocarbons while the latter (lutein, zeaxanthin, violaxanthin, and neoxanthin) are oxygenated hydrocarbon derivatives [14,15]. The hydrocarbons contain eight isoprenoid units of five-Cs, linked by conjugated double bonds, which confer multiple geometrical isomers as cis/trans molecules. Mostly the all-trans configuration is stable [16,17]. Double bonds act as chromophores that absorb white light and emit in yellow, orange, and red range to provide coloration [18,19]. The most vital property of carotenoids is as efficient "quenchers" of reactive oxygen species (ROS) and endows defensive health properties when consumed as food or nutraceuticals. Some of them, namely β-carotene, and to a lesser extent α-carotene and β-cryptoxanthin, may be transformed into vitamin A, hence they are classified as "provitamin A". These applications contribute immensely toward its importance in various sectors e.g., pharmaceutical, food, feed, and also in chemical or cosmetics industry [1].

Since carotenoids are widely found in innumerable sources, including plants (vegetables, fruits, cereals e.g., maize, etc.), animals (crustaceans, marine fish, insects, etc.), algae (all green and blue-green algae), non-photosynthetic microbes (bacteria, fungi, yeast), it is pertinent to identify the most dependable and sustainable source for continued supply and economic production, especially in the form of nutraceuticals or food/feed additives [7]. Carotenoids can be duly extracted from vegetables/fruits or flowers, but this requires large production areas/arable land leading to increased production costs. Since plant-based carotenoids are unstable, variable in composition and heavily dependent on unpredictable climate conditions [20], alternate green and sustainable sources are being explored, especially microbial sources for obtaining economical and high quality compounds via optimized fermentation and down streaming protocols. Re-utilization of agricultural by-products have also been exploited as a resource for carotenoid production; however, it does not fall under the purview of this topic and will not be discussed further. Microbial sources of some industrially important carotenoids (Table 1 [21–32]) indicates their ubiquitous prevalence. Depending on yield and cost of production, yeast is eminent for its use as a source of protein and easily cultivable on inexpensive waste feedstock [33].

Table 1. Table showing various sources for microbial carotenoids.

Microorganism	Name	Type of Carotenoids	Reference
Cyanobacteria	*Anabaena variabilis*	canthaxanthin	[21]
Cyanobacteria	*Aphanizomenon flos-aquae*	canthaxanthin	[21]
Bacteria	*Mycobacterium brevicaie*	canthaxanthin	[21]
Bacteria	*Mycobacterium lacticola*	astaxanthin	[21]
Bacteria	*Streptomyces chrestomyceticus*	canthaxanthin,	[21]
Algae	*Chlorella pyrenoidosa*	lutein	[21]
Algae	*Dictycoccus cinnabarinus*	canthaxanthin	[21]
Algae	*Dunaliella salina*	β-carotene	[22]
Algae	*Dunaliella tertiolecta*	β-carotene	[23]
Algae	*Haematococcus pluvialis*	astaxanthin	[24]
Algae	*Spongiococcum excetricum*	lutein	[21]
Microalgae	*Chlorella vulgaris*	lutein	[25]
Microalgae	*Chlorella saccharophila*	Zeaxanthin	[26]
Microalgae	*Chlorella vulgaris*	astaxanthin	[27]
Microalgae	*Haematococcus pluvialis*	astaxanthin	[27]
Yeast	*Blakeslea trispora*	lycopene	[21]
Yeast	*Xanthophyllomyces dendrorhous*	astaxanthin	[28]
Yeast	*Rhodotorula mucilaginosa*	β-carotene and torularhodin	[29]
Yeast	*Sporobolomyces roseus*	β-carotene, torulene, torularhodin	[30]
Yeast	*Phaffia rhodozyma*	astaxanthin and β-carotene	[31]
Yeast	*Rhodosporidium* sp.	torulene and β-carotene	[21]
Yeast	*Rhodotorula glutinis*	β-carotene, torulene, torularhodin	[30]
Yeast	*Rhodotorula graminis*	torulene and β-carotene	[21]
Yeast	*Sporidiobolus salmonicolor*	β-carotene	[32]

3. Carotenoid Production via Optimized Fermentation

Yeasts are favored over plants or other microorganisms for production of carotenoids due to the following characteristics:

i. Faster growth rate to produce high cell densities with high content of product. ii. Cell cultures can be scaled up easily without any need for an arable land in controllable manner as compared to plant based carotenoids. iii. Capability to use various inexpensive and renewable substrates such as lignocellulosic hydrolysates, organic industrial waste, vegetable mandi waste etc. which makes yeasts reasonable. iv. Optimal growth at low pH is advantageous in reducing bacterial growth, together contributing toward a sustainable process development for strategic industrial applications [34]. Due to robust process conditions, such as, good growth by utilizing variety of carbons sources, low pH, and a broad range of temperature, high cell density and high content of fatty acid and carotenoids could be achieved by oleaginous yeast strains making it economically feasible for process development of future industrial applications [35].

3.1. Factors Affecting Microbial Growth and Carotenoid Production

3.1.1. Temperature

The incubation temperature is the main factor for biomass and carotenoid production which depends on the type of microorganism. The most favorable temperature for biomass and carotenoids production observed in *Rhodotorula* sp. RY1801 was 28 °C, with about 987 µg/L carotenoid concentration [36]. Other studies also suggested that finest temperature for maximum biomass and carotenoid production was about ~28 °C–30 °C. Maximum biomass as well as carotenoid production was observed at 29 °C for *Rhodotorula glutinis* [37] in monoculture and 30 °C in co-culture with lactic acid bacteria [38]. Malisorn and Suntornsuk [37] optimized carotenoid and biomass production at 29° and 30 °C as the maximum production temperature for *Rhodotorula glutinis*. Vijayalakshmi et al. (2001) decreased the incubation temperature of *R. gracilis* from 32 °C to 24 °C and reported significant increase in product formation from 148 to 622 µg/100 g dry cell weight. Temperature directly influenced the enzyme activities in the carotenogenic pathways thus warranting its optimization by regulating the enzyme activity and concentration of the reactions they catalyze [39,40]; although, depending on the strain, environmental parameters, and medium composition this effect varies.

3.1.2. pH of Culture Medium

pH of the medium is an extremely significant factor which affects the microbial growth along with the type of pigment produced. The influence of pH of the culture medium on biomass growth and carotenoid production in *Rhodotorula* sp. RY1801 was evaluated by Zhao et al. [36] and the optimal initial pH observed was 5.0. But there was no difference in the biomass and carotenoid concentrations at pH 6.0 and 7.0. Latha et al. [41] reported although the cellular biomass of *R. glutinis* increased when the pH of culture medium was increased to 7.5 from initial 5.5, the maximum carotenoid production was supported by pH 5.5. Other study also coincided with the results with maximum production of β-carotene by *Rhodotorula acheniorum* at pH 5.5 [42]. Increasing the pH from 5–7 improved carotenoid production from 3.31–3.93 mg/L, which also reflected upon the increase in other factors that enhance the biomass production simultaneously; although 6.0 was taken as optimal pH [43]. It was suggested that alkaline pH acted as a stressor and alters metabolic rates and nutrient absorption resulting in inducing cellular glucose metabolism genes and therefore enhanced polysaccharide synthesis instead of carotenoids [43]. Under a more acidic pH (~4.0), the growth of the organism is retarded but the carotenoid concentration is high, suggesting that at low pH, yeast is compelled to synthesize carotenoids [44].

3.1.3. Carbon Source

Carbon sources, such as, glucose, fructose, maltose, lactose, galactose, etc. have variable effects in different yeast strains [45]. Some basidiomycete yeast strains, especially oleaginous

ones, e.g., *Rhodosporidium* and *Phaffia*, grow on various sugars available in hydrolysates of lignino-cellulosic waste matter (wood pulp, corn syrup, wheat straw, peels of vegetables/fruits), waste water, etc. [38,46–48]. They accumulate and store hydrocarbon-rich fats as primary metabolite during early log phase and carotenoids as their secondary metabolite during the late stationary phase of growth by utilizing diverse carbon sources [49,50]. Wild yeast strains utilize xylose, glucose [51], waste extract (inedible parts of fruits and vegetables) [46], acetate [52], hydrolysates [53], whey [54,55], starch [56,57], industrial waste waters [58–60] for the synthesis of metabolites. They are relatively tolerant to many forms of stress, including osmotic stress [46] and toxic radicals present in hydrolysates [61,62].

The availability of carbon source present in the medium affects the production of biomass and other metabolites during fermentation [63]. Glucose is the most widely used carbon source for good biomass production and the other preferred C source is glycerol; xylose and other sugar alcohols being lesser preferred C sources [64,65]. A dual stage fed-batch fermentation conducted at 25 g/L glucose concentration during lag and early log phases and switched to 5 g/L during late log and the stationary phases enhanced carotenoid (astaxanthin) production to about 109% [66].

3.1.4. Nitrogen Source

Nitrogen sources including yeast extract, peptone, calcium nitrate, sodium nitrate, beef extract, malt extract, urea, ammonium phosphate, and ammonium sulphate have been successfully used for cultivating yeast for carotenoid production [41,67,68]; whereas other reports mentioned the use of a mixture of ammonium sulfate, potassium nitrate with beef extract for maximum growth and carotenoid production [69]. The studies indicated that 1% yeast extract, and peptone were better nitrogen sources and resulted in the production of 5.7 mg/L and 4.7 mg/L carotenoids respectively as compared to ammonium sulphate and beef extract with 3.8 and 3.6 mg/L carotenoid production. Baraka et al. [67] also suggested that yeast extract at 0.75% concentration was a better nitrogen source for production of total carotenoids (381.15 µg/g), as compared with ammonium sulphate at the same concentration. Latha et al. (2005) also reported that casein acid hydrolysate and yeast extract stimulated carotenoid production in *R. glutinis* [41]. Enhanced growth rate at 2 g/L ammonium sulphate concentration was used for cultivating *R glutinis* [70]. For *P. rhodozyma*, the optimal nitrogen source was a mixture of 13.11% $(NH_4)_2SO_4$, 22.82% KNO_3, and 64.07% beef extract (containing 6% nitrogen) for good astaxanthin production (6.4 mg/L). Nitrogen starvation induced astaxanthin production effectively [71,72].

3.1.5. Aeration Rate

Aeration rate influences cell growth, biomass, and carotenoid production by improving mass transfer of oxygen and other nutrients to the aerobic microbial cells. The effect of the aeration rate on specific growth and total carotenoid production by the yeast showed that both growth and carotenoid production increased considerably when the aeration rate was increased from 0.0 to 2.4 vvm. It was higher than the values obtained from the un-aerated cultivation medium [70]. Simova et al. [73] reported that the yeast strains require more intensive aeration for maximal cellular carotenoid synthesis.

3.1.6. Light

Light is another important factor for producing microbial carotenoids; as, it stimulates carotenogenesis which is a photoprotective mechanism to inhibit the cells from the damaging impact of radiations [74]. Studies show that carotenoid production is affected positively by white light (395–530 nm), depending on the type of the strain [4]. An illuminated phase changed the intensity of the pigment and enhanced carotenoid concentration from 170 µg/g in dark to 228 µg/g dry weight in light. Yen and Zhang [75] reported that the productivity of β-carotene increased from 14.69 µg/g to 24.6 µg/g, in batch reactor where it was cultivated under two white LED (light emitting diode) lamps. Blue light resulted in enhanced carotenoid accumulation in *Colletotrichum gloeosporioides* (fungus),

which did not appear under dark conditions or when cultured in red light. When the fungal filaments were irradiated with blue light of intensity 6.5 micromol $\times$ m $^{-2}$ $\times$ s $^{-1}$, the carotenoid content increased with irradiation time and reached to a peak after 5 days to 71.8 microg/g [76]. Studies also indicated that high light intensities are lethal to the cells [77].

3.1.7. Carbon/Nitrogen Ratio

Carbon–nitrogen (C/N) content affected the growth and carotenoid production in yeast strains [4]. For carotenoid production, a lower C/N ratio (20:1) was preferred by *R toruloides* and *R glutinis*, as compared to lipid biosynthesis, where the C/N ratio above 30:1 was required [50,78,79]. C/N ratios above 50:1 decreased pigment production since the acetyl-CoA flux diverted toward fatty acid biosynthesis instead of mevalonate synthesis for carotenoid production. Braunwald et al. (2013) reported that C/N ratio above 70 to 120, when C was glucose, did not elevate the lipid production in R. glutinis, but had a positive effect on carotenoid synthesis [80].

In another experiment with a dual-stage fed-batch culture, lower C/N ratio during the early growth stages promoted biomass production. At late log phase, astaxanthin production (16.0 mg/L) was stimulated using a higher C/N ratio. Stoichiometric analysis showed that under a high C/N ratio, protein biosynthesis was repressed, resulting in decreased NADPH levels required for anabolism, thereby enhancing carotenoid biosynthesis [81].

3.1.8. Sonication

Sonication has a positive effect on enzyme activity and microbial processes [82]. Ultrasound-induced enhancement of carotenoid production using wild strain of *P. rhodozyma* MTCC 7536X and *X. dendrorhous* culture was reported by Batghare et al. [82]. The media composition and fermentation conditions were optimized using statistical methods in a wild strain of *P. rhodozyma*. Sonication at 33 kHz considerably enhanced the astaxanthin production by about 27%. Sound waves caused micromixing of substrates, reduced substrate inhibition and might have induced beneficial conformational changes in intracellular enzymes.

3.1.9. Chemical Supplements

Metal salts of Co, Mg, Ba, Fe, Ca, Zn, etc. stimulated carotenoid production in *R. glutinis*, whereas trace elements present in the medium, influence carotenoid profile in another yeast, *R. graminis* [83,84]. It was observed that Zn^{2+} and Al^{3+} stimulated γ- and β -carotene production, but Zn^{2+} plus Mn^{2+} inhibited torularhodin and torulene production, probably because ions were involved in catalysis of some carotene-biosynthesis pathway [4,40]. Few other chemical supplements, e.g., solvents/natural agents stimulated carotenogenesis, including ethanol (10 g/L) or acetic acid (5 g/L) [85,86]. The carotenoid content was reported to increase from 1.65 mg carotenoids g^{-1} cells to 2.65 mg carotenoids g^{-1} cells, in yeast *X. dendrorhous* due to addition of 0.2% (v/v) ethanol to the fermentation medium [66].

3.1.10. Fermentation Modes

Bioreactors are of various types and their modes of operation vary with products and microbes. They offer advantages such as optimal integration of parameters viz., temperature, pH, aeration, agitation, nutrient supply, etc. to ensure higher productivity with economical production.

During batch fermentation, a limited supply of nutrients is provided leading to lower investment costs, and the process does not require much control and is accomplished by unskilled labor. Batch fermentation has the advantages of low investment costs, simple control and operations, and easy-to-maintain complete sterilization. When all carbohydrate is consumed during the stationary phase, the maximum amount of product is formed [51]. The major disadvantage of batch culture is the deficiency of carbon and nitrogen sources, which, once depleted or utilized, stalls the growth and product formation [87]. The biomass

produced is not maximum, but this fermentation is good for production of secondary metabolites, since they are formed during the late log or stationary growth phase, when growth is almost stalled. To increase the biomass content, fed batch fermentation may be adopted, where, catabolite repression is prevented by intermittent feeding of the substrate. If the substrate has an inhibitory effect, intermittent addition improves the productivity of the fermentation. However, there is a high risk of contamination due to long cultivation periods and periodic handling. Larger reactor volumes require higher initial investment but can certainly promise good productivity upon accurate optimization [88]. In fed-batch culture, generally two metabolic phases are observed: (i) growth phase, and (ii) product accumulation phase. Phase (i) occurs when all nutrients are available in the medium, and carbon sources (i.e., sugars) are consumed. The biomass grows to a concentration where the process can be continued by limiting substrate concentration. Phase (ii) is activated by nutrient depletion, mostly carbon, and the C/N ratio falls, leading to the accumulation of carotenoids [89]. However, high cell density cultures in *R. toruloides* and *Cryptococcus curvatus* using fed-batch fermentation have yielded good lipid production instead of carotenoid production, because the cells do not enter a stationary phase due to multiple feeding [90].

Several expenditures are foreseen for microbial carotenoids such as cost of the feedstock, labor cost, expenditures including operation cost, and other downstreaming costs. However, the total production cost may be best reduced by utilizing inexpensive substrates and supplemented via valuable by-products. The oleaginous yeast strains which can grow and produce lipids and carotenoids on low-cost substrates such as glycerol, agricultural waste, wastewaters, etc. should be identified from the environment and employed for carotenoid production. Waste sector is a globally disorganized sector lacking accurate data [91]. It is estimated that India alone generates around 50 tons of vegetable and fruits waste per annum [92–94]. Waste can be recycled after hydrolysis for channelizing to fermentation units in the form of readily available, inexpensive feedstock. Recently, various cheap raw materials and waste hydrolysates have been explored for economical production of microbial products, and sustainable management of waste concomitantly.

4. Metabolic Engineering for Improvised Production

Carotenoids being precursors of vitamin A are important for malnourished people or underdeveloped countries where they can be provided as diet supplements, or as fortified foods and feed. Biotechnological manipulations for preparation of carotenoid-based products and their marketing, has been taken up seriously by the research community, for adequate nourishment primarily in cases where rice is the staple food.

Increased carotenoid production may be achieved by either modifying the bioprocess or metabolically engineering the strain to steer the carbon flux toward formation of this secondary metabolite. Metabolic engineering may be also used for ease of intracellular product recovery without transforming or destroying their properties [95]. Product downstreaming is a tedious, time consuming, and cost-intensive procedure in case of yeast-carotenoids and requires the use of ultrasonic waves or extremely high pressure and solvents. Hyper producing strains thus solve the problem of high production costs and ease out the downstreaming process. Chemical (antimycin A, ethyl methyl sulfone, etc.) or radiation-mediated (gamma or UV rays) mutagenesis have been employed as the simplest tool for strain improvement by enhancing pigment formation [96–98]. The carotenoids are protective antioxidants, incorporated in the cell membrane of yeast as lipid soluble compounds [76,99] and hence exposure to radiation or chemical stress pushes the cell to produce more carotenoids for protection. The disadvantage of this type of random mutagenesis is that the mutants are unstable and in some cases site-directed mutagenesis also fails to yield desirable mutants to overproduce one specific carotenoid component [100]. Modification of biochemical or metabolic pathways leading to product formation was the other alternative solution to this problem and was readily adapted by researchers along with the use of recombinant DNA technology [4,101]. This technique has several

advantages, including the production of carotenogenic pathways in rapidly multiplying non-carotenogenic strains [7,102]. The first step to metabolic engineering is selection of a host microorganism, which is then improvised through site directed mutagenesis for manipulating the existing metabolism toward production of desirable metabolites [103]. However, the key challenge is to have stable strains that can be easily selected over the wild type. Fast growing strains having a known life cycle and optimized fermentation pattern are employed for cloning suitable genes for carotenogenesis. Metabolic engineering of yeast strains, such as *Pichia pastoris* [104], *Yarrowia lipolytica* [7], *Sacharomyces cerevisiae* [105–107], and *Candida utilis* [108,109] have been widely reported for producing various compounds of high value, e.g., β -carotene, astaxanthin, lycopene, etc. Carotenogenic genes (phytoene synthase and phyotene desaturase) from other microbes, i.e., *Xanthophyllomyces dendrorhus*, *Erwinia uredovora*, *Agrobacterium aurantiacum* have been cloned in suitable host yeast strains. The modified *S. cerevisiae* strain was used as a carrier for the external carotenoid genes having the desired physiology and regulatory networks. These yeast models are therefore of high value in food industries and they are considered as safe hosts by USFDA [104], as opposed to bacterial strains e.g., *Escherichia coli*.

Araya-Garay et al. [104] constructed two plasmids by inserting genes encoding lycopene and β -carotene using *Pichia pastoris* as the host and successfully obtained 1.141 µg and 339 µg per gram dry biomass of lycopene and β -carotene, respectively. *Pichia pastoris* can grow in organic materials, increasing the choice of inexpensive feedstock for the industry. *Yarrowia lipolytica* has immense potential as an expression platform for production of several high-value products, e.g., carotenoids, omega fatty acids, enzymes, etc. since the past couple of decades [110] because it can generate acetyl-CoA along with other precursors of the mevalonate pathway, in ample quantities [111]. Carotenoid biosynthesis requires sufficient carbon flux toward mevalonate pathway, by disrupting the β-oxidation or integrating many copies of HMG reductase gene (HMG1) and geranylgeranyl diphosphate synthase gene (GGS1). During carotenoid production, the two C5 precursors (IPP and DMAPP) areengineered to produce C40 metabolites, via C10-C20 intermediates. High levels of β-carotene (6.5 g β-carotene/L) stored in lipid droplets within engineered *Y. lipolytica* ob-CHC^{TEF}C^{TEF} was achieved under optimized fed-batch fermentation conditions [111,112]. Multiple copies of key genes in the β-carotene pathway were expressed in host cells to increase β-carotene production [113,114]. About 46–60 mg/g dry cell weight of lycopene and 159 mg/g DCW of β-carotene was produced by multiplying the copy number of related genes in *Y. lipolytica* [112,115].

Since the accumulation of carotenoids occurs mostly in the lipid layer of the cell membranes, it induces a toxic effect for cells, their yield is delimited. This requires proper channelizing of these storage molecules to resolve the toxicity issue in *S. cerevisiae* and *Y. lipolytica* [111,116], and in this direction, glycosylation has been suggested to enhance carotenoid solubility, such that they can accumulate within the cytoplasm [117,118]. Downstreaming and recovery process using hydrophilic solvents is facilitated due to glycosylation [118]. However, for complete conversion to the glycosylated form, the glycosyl donor supply may be optimized.

Despite the limitations in understanding of the total cellular metabolism, assistance of "deep" machine learning has been instrumental to understand cellular processes of engineered cells for optimizing fermentation processes. This tool has been applied for accurately optimizing efficient β-carotene gene expression in a recombinant *S. cerevisiae* strain [119]. The need for newer and better technologies has been felt for increasing the yield and productivity of secondary metabolites from yeast, including carotenoids. Innovations in this area is an emerging and lucrative area for yeast biotechnological research.

5. Innovations in Fermentation for Higher Productivity

Evolution of biotechnological processes for microbial carotenoid production has seen an upsurge in recent years, owing to their applicability in healthcare products. Several

patents for microbial carotenoids have been registered worldwide, for the fermentation, extraction, or entire production process (Table 2 and [120–125]). Novel engineered or mutated yeast cell lines have been constructed using recent techniques of genetic manipulations to improvise the existing carotenogenic pathways, or for easy extraction of the product.

Table 2. Table showing the various types of patents filed for carotenoids reflecting their importance for industrial applications.

Patent No	Title	Inventor (s)	Company	Ref.
US8288149B2	Production of carotenoids in oleaginous yeast and fungi	Richard B. Bailey, Kevin T. Madden, Joshua Trueheart	NA	[120]
US8846374B2	Carotenoid production in a recombinant oleaginous yeast	Pamela L. Sharpe, Rick W. Ye, Quinn Qun Zhu	EI Du Pont de Nemours and Co	[121]
EP 3 839 056 A1	Astaxanthin over-producing strains of *Phaffia rhodozyma*	Shemesh, Paz, Cohen, Tzafra, Lifshitz, Yael, Khutorian, Marina, Harari, Yaniv	NextFerm Technologies Ltd. 2069208 Yokneam Illit (IL) EP 3 839 056 A1	[122]
382/DEL/2001	A process for the production of carotenoid from microbial source using wheat bran extract	Govindaswamy Vijayalakshmi, Vasudeva Vanajahshi	COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH	[123]
EP2192191A4	Method for production of carotenoid	Uchizawa, Shotaro; Hideyuki Dohi; Shimizu, Kentaro; Ishizaki, Tomoyuki; Takahashi, Toshiyuki	NIPPON OIL CORPORATION	[124]
US-5310554-A	A high Purity Beta carotene	Haigh W Geoffrey	Natural Carotene Corp	[125]

Genetic manipulations have seen a new era with the discovery of "artificial" nucleases to cut DNA near a predetermined site. Insertion of desired genes into the genome was made further possible via the discovery of homing endonucleases to recognize ZFNs (zinc finger nucleases), and TALENS (transcription activator-like effector nucleases) [7]. The type II bacterial clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (CRISPR-Cas9) system is also used popularly for genome-editing in several eukaryotes. They were programmed for targeting specific gene sequences to edit them at specified sites via NHEJ (non-homologous DNA end joining) or HR (homologous recombination) of DNA repair mechanism. CRISPR-Cas9 system was employed for several applications using various yeast strains, e.g., *Candida albicans* [126], *Schizosaccharomyces pombe* [127], *Saccharomyces cerevisiae* [128], oleaginous yeast strains [34], *Kluyveromyces* sp. [129]. Homology-integrated CRISPR-Cas9 system is an upcoming tool for constructing metabolically and morphologically modified yeast mutants. Another important and effective means for engineering metabolic pathway in *Y. lipolytica*, after the CRISPR-Cas9 system is the use of self-replicating YaliBricks vectors [130] and piggyBac transposon system [131]. The piggyBac transposon platform was designed for constructing a mutant library and for genome editing in *Y. lipolytica* [131]. Several new tools and mutant libraries are being constructed for further research in *Y. lipolytica* to accelerate the production of a wide range of secondary metabolites using the new engineered strains. Although there are many upcoming tools including ZFNs and TALENs that can be used for site-directed genome editing, challenges in protein designing, synthesis or during validation remain as bottlenecks for regular application [132].

6. Applications in Health Care

6.1. Antioxidant Property

The main cause of diseases such as cancer, cardio-vascular disease, ophthalmic diseases, and neuro-degenerative diseases are because of the free oxygen radicals which move in human body. Carotenoids act as antioxidants and hunts down free oxygen radicals from the body. Taking carotenoids complex supplementation for 8 weeks alleviated oxidative stress in trial cases taken from target populations of healthy subjects, athletes, and pregnant women. Data have shown that dietary supplementation of carotenoids has huge potential for disorders/diseases relating to oxidative stress [133]. The doses of carotenoids complex ranged from low (<20 mg) to high (>50 mg) and carotenoids were given in the form of capsules and fruits/vegetables/juice servings. Levels of FRAP (Fe^{3+} reducing ability of plasma) and ORAC (O radical absorbance capacity) in treatment and control groups were mainly used for their study [133]. The antioxidant property of carotenoids also helps in decreasing the neurological disorders and level of diabetes [2,134].

6.2. Anti-Inflammatory Property

The type of carotenoids, which contain oxygen in its structure, for example, fucoxanthin and astaxanthin can suppress the cytokines IL-6, TNF-α, and IL-1β expression and serve as pro and anti-inflammatory compounds [1,6,135]. Other carotenoids e.g., β-carotene and lycopene components are known to quench the singlet oxygen but also inhibit peroxidation of lipid, resulting in anti-inflammatory activity. It was found that pretreatment with lutein (50 μM) inhibited the expression of nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor-α, interleukin-1β, and nitric oxide production by quenching ROS (reactive oxygen species). It slowed neuroinflammation in LPS-activated BV-2 microglial cells by inhibition and activating necessary proteins suggesting that a nutritional preventive strategy may be applicable in inflammation-related neurodegenerative disorders [135].

6.3. Antibacterial Property

Antibacterial activity of glucosidal carotenoids from the yeast *R. mucilaginosa* AY-01 toward antibiotic resistant bacteria isolated from the porcine semen, was elucidated using paper disc diffusion assay and monitoring the size of clear-zone around the discs. Natural carotenoids may be potentially used as antibacterial agents against both antibiotic susceptible and resistant strains and may be added to medical/surgical materials, apart from dietary supplements. The carotenoid contents of *Rhodotorula glutinis* M29 strain (1.07 mg L^{-1}) showed antibacterial activity against a range of bacteria, e.g., *Staphylococcus aureus*, *Bacillus subtilis Bacillus cereus*, *Salmonella enteritidis*, and *Escherichia coli* at 10^3 CFU/mL [136,137].

6.4. Property against Ophthalmic Infections

Vitamin A plays a critical role in human eye since it is a component of rhodopsin—the biological pigment present in the rods of retina—which facilitates the proficient transfer of energy from photos of light to electro-chemical signals. Insufficiency of vitamin A causes vision problems, including, night blindness and this disease can be prevented through the consumption of carotenoid supplements in appropriate amount in the form of dietary supplements [133]. The oxygen-containing carotenoids, e.g., lutein and zeaxanthin found in the macular region of the retina are responsible for sharp and complete vision and work as filters for blue light from screens as well as scavenge the free radical from retina [6,138,139]. Studies on mice model with zeaxanthin supplementation showed shielding of retinoids from blue (405 nm) low energy laser light treatment, which otherwise resulted in aberrant retinaldehyde isomers in the retina. In addition, they are also helpful in the prevention of cataract in eyes and macular degeneration caused due to ageing [133].

6.5. Anti-CVD (Cardiovascular Diseases) Activity

The different experiments conducted in vitro as well as on animals, have proved that the carotenoid diminishes the inflammation as well as oxidative stress by promoting normal cellular activity or metabolism. It has been seen that a diet rich in carotenoid when given to the patients suffering from cardiovascular diseases has helped in reducing the intensity of the disease [140,141].

6.6. Anti-Cancerous Properties

Several experiments have proven the anti-cancer properties of carotenoids. It has been observed that carotenoids arrest the cell cycle that is coupled with down regulation of cyclin D1, cyclin D2, CDK4, and CDK6 expression. Type of carotenoids, for example, β-cryptoxanthin as well as lycopene are found to suppress the NF-κB signaling pathway that is helpful against lung and prostate cancer [142]. The anti-angiogenic activity of β-carotene has been discovered recently, which helps to discontinue the development of new blood vessels that is habitually seen in cancer-causing tumors [143]. Anti-breast cancer and anti-ovarian cancer properties of yeast-carotenoids produced via a cost-effective and environment friendly technique were evaluated [144]. The yeast carotenoid extract composed of three main pigments- β-carotene, torulene, and torularhodin exhibited anti-breast cancer activities in vitro while being bio-

compatible in normal cells. Interestingly, the carotenoid extract showed better cytotoxicity in MDA-MB-231 cells (IC50 = 7.82 µg/mL) (triple negative breast cancer cells) than MCF7 (ER + PR + HER2 cells) where IC50 was 29.11 µg/mL, suggesting strategic applications of yeast carotenoids in formulation of cancer drugs.

6.7. Neurodegenerative Diseases

Increased levels of oxidative stress in the nervous system caused a number of neurodegenerative diseases, for example, Alzheimer's disease, Parkinson's disease, and Huntington's disease etc. [1,6,135]. Some of these diseases are due to the inability of Ca^{2+} to mediate in cell signaling, but carotenoids, for example, β-carotene, astaxanthin, and lycopene are involved in the transportation of Ca^{2+} ions in the brain. It has been reported that carotenoids are able to reduce the risk of diseases associated with the nervous system, which is caused due to the improper signaling in nervous system and this malfunction can be reduced through the uptake of carotenoids in the form of dietary supplements [145].

6.8. Ultra Violet Radiation

The therapeutic property of carotenoids also shows the protection from ultraviolet light reported in various studies. In a study conducted by Thirumalaiarasu and Rajeswari [146], the sun screening property for carotenoid of *R. mucilaginosa* YM was determined as SPF of 3.138. The SPF value of YM strain was in the range of marine microbes, which experienced continuous environmental stress. In the wide range of carotenoids, β-carotene, astaxanthin, canthaxanthin mainly showed photo defensive properties. It has been reported that lycopene and β- carotene helps to reduce the red patches of skin as well as other skin damage caused by the UV rays [138] and in this way it works as soothing agent for skin against the UV rays coming from sun as well.

6.9. Antimalarial Property

Carotenoids find use in various applications as drug and food additives and are known to be useful as antioxidants. Recently, carotenoids have shown antimalarial activity against *Plasmodium falciparum* (malaria parasite) [147]. For this study, *Plasmodium falciparum*, which causes malaria (a prevalent disease in tropical countries), was chosen as target parasite for analysis of antimalarial activity of extracted yeast carotenoids using in vitro growth inhibition assay. The extracted carotenoid was non-toxic to RBCs and HepG2 cells, but active against malaria parasite 3D7 strain of *P. falciparum.* Results suggested that the extracted carotenoid from isolated yeast strain *R. glutinis* was able to kill 96.9% parasite as compared to standard β-carotene, in which the percentage of killing was 99.59% [147].

6.10. Anti-Viral Properties

Carotenoids combat various diseases including those caused by viruses, since they are effective antioxidants that prepare the body to combat viruses and related symptoms. The World Health Organization recommended people to maintain a strong immunity with dietary intake of fruits and vegetables, during the pandemic COVID-19. The main reason was that an inverse correlation was reported between CRP and IL-6 (markers of inflammation), and β-carotene in infants and patients with acute respiratory infections. Diets rich in carotenoids were thus proposed to be suitable for COVID-19 and other viral diseases in alleviating their symptoms [148]. Marine algal-based carotenoids, namely fucoxanthin and siphonaxan, were predicted as useful weapons used for the prevention and treatment of COVID-19 [148]. In silico tests performed with Siphonaxanthin, extracted from *Codium fragile*, possessed high antiviral activity (IC50 of 87.4 µM) against SARS-CoV-2 pseudovirus and prevented viral entry. Lycopene given to human patients suffering from COPD at about 30–90 mg/d resulted in decreased neoplastic lesions and provided protection against cholesterol accumulation in lungs. Lycopene and astaxanthin also decreased allergic inflammations in the lung by reducing Th2 cytokine (IL-4 and IL-5) response [149]. β-carotene showed inhibition against pro-inflammatory mediators (IL-1β,

prostaglandin E2 (PGE2), COX-2, iNOS, TNF-α, etc.), by ceasing NF-κB activation [150]. Microbial carotenoids thus pave the way for natural, affordable formulations that promise strong immunity building during prevalent viral diseases and pandemics.

7. Conclusions

Human beings can obtain carotenoids from various sources for combating a wide plethora of diseases, but unfortunately, cannot synthesize them in their body. Red oleaginous yeast has attracted interest because of its ability to converge several beneficial and environmentally befitting aspects for developing a high value, non-toxic, and bio-medically important products [136,144,151,152]. Recent studies have explored applications of red yeast in various areas such as drug synthesis, health-care, food and feed industries, etc. [1,4,6,138,153]; however, there are very few microbial, especially yeast carotenoids in the market (Table 3). The primary purpose of the review is to highlight the need for industrial production of yeast carotenoids enabled by using low-cost agro-industrial feedstock, optimized fermentation conditions and engineered cellular metabolism. It is believed that engineering the cell morphology and metabolism holds great potentials for improving substrate uptake and further bioconversion in yeast. Drug resistance to important drugs has led to scarcity of alternate effective therapies for anti-bacterial, anti-viral, and aggressive breast cancers. This kind of upsurging health problems have opened avenues for natural, affordable, nontoxic products for mitigation of issues on preliminary first line treatment. Dietary intake and supplementation of such products is necessary and hence the dire need for cost-effective production of carotenoids at larger scale stands unchallenged.

Table 3. Table showing the commercialized yeast carotenoids with their applications.

Company	Compound	Source/Strain	Applications	Web Links
Angel Yeast Company (China)	β-carotene	Red yeasts (*Monascus spp.*)	Large-scale production of carotenoid compounds from red yeasts for widespread pharmaceutical and nutraceutical and cosmetic applications	en.angelyeast.com (accessed on 11 December 2022)
Kemin Industries Inc., California, USA	Cryptoxanthin	Yeast *(Rhodotorula taiwanensis)*	Carotenoid derivatives from having potential applications in food, nutraceutical, pharmaceutical, and cosmetic sectors	www.kemin.com (accessed on 11 December 2022)
NextFerm Technologies Ltd.	Astaxanthin	Yeast (*Phaffia rhodozyma)*	Astaxanthin is the strongest naturally occurring antioxidant and is considered the best among radical scavengers	www.nextferm.com (accessed on 11 December 2022)
Lycored	Carotenoids	Fungi *(Blakeslea trispora*)	A range of carotenoids find applications in food, nutraceutical, pharmaceutical, and cosmetic sectors.	www.lycored.com (accessed on 11 December 2022)
Made-In-China	Astaxanthin	Algae(*Haematococcus pluvialis)*	Astaxanthin is the strongest naturally occurring antioxidant and is considered the best among radical scavengers	www.made- in-china.com (accessed on 11 December 2022)
Lycored	Carotenoids	Algae(*Haematococcus pluvialis)*	A range of carotenoids find applications in food, nutraceutical, pharmaceutical, and cosmetic sectors.	www.lycored.com (accessed on 11 December 2022)
Biolifescience	Astaxanthin	Algae *(Haematococcus pluvialis)*	The natural carotenoid, astaxanthin, is the strongest naturally occurring antioxidant and is considered the best among radical scavengers	www.biolifesciences.com (accessed on 11 December 2022)
Allied Biotech Corporation (Taiwan)	Beta carotene, beta -opo-8- carotenal, lycopene, canthaxanthin, lutein	Algae *(Dunaliella salina)*	Natural Beta Carotene Powder 20% (Extract). This product naturally produces high level of carotenoids to help increase survival in harsh conditions	www.altratene.com/en (accessed on 11 December 2022)

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