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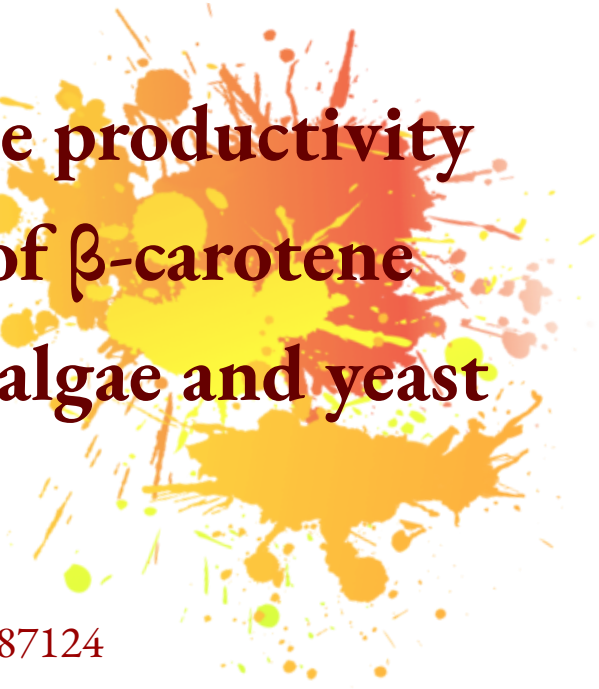
A realistic take on the productivity and sustainability of β-carotene biosynthesis in microalgae and yeast



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1. Abstract

Carotenoids are a class of natural pigment endowed with antioxidant, anti-inflammatory, immunity-enhancing and photoprotective properties, and thus are widely used in the food, cosmetic and pharmaceutical industries. One of the most desirable carotenoids is the precursor of vitamin A, β-carotene. In recent years there has been an increasing demand for natural β-carotene, due to the health risks associated with synthetic sources. Natural sources of β-carotene include vegetables, fruits and microorganisms. This review explores the potential of microalgae and yeast for β-carotene production, aiming to determine which organism is better suited as an industrial biofactory to meet the rising demand for natural carotenoids, specifically β-carotene. To this end, microalgae and yeast were comparatively analysed in terms of productivity and sustainability. It was found that yeast showed higher productivity relative to microalgae, whereas fermentation faced sustainability issues due to sugar-sourcing. Despite the higher potential for sustainability for the autotroph microalgae, the scale-up of β-carotene production was found to be costly and energy-intensive due to the lack of infrastructure. To optimise energy consumption and costs for sustainable β-carotene production, a combined approach using both microalgae and yeast was suggested. In major sugar-producing countries, yeast could be used for fermenting agricultural waste, while in those with limited agricultural production, microalgae cultivation using wastewater should be enhanced. Since genetic engineering can provide strains with improved productivity and optimised processes for sustainability, future research should focus on assessing the risks associated with the commercial use of genetically modified organisms (GMOs).

2. Introduction

Carotenoids are one of the most prominent colourants of nature, providing yellow, orange, red and purple hues to organisms like photosynthetic bacteria, algae, fungi, plants and animals (Maoka, 2020). In addition to their optical properties, they are highly important in biological processes such as photosynthesis, photoprotection, and serve as antioxidants quenching radical oxidative species (ROS) thereby preventing oxidative damage. Moreover, they are beneficial to our health due to their anti-inflammatory and immunity enhancing properties, and their ability to reduce cardiovascular and age-related disease risks (Razzak, 2024). Thus, carotenoids are utilised in the food industry as supplements and natural colourants with added nutritional value, in the cosmetic industry as pigments and UV protection, and in the pharmaceutical industry for their antibacterial and anticancer properties (Razzak, 2024). In fact, the global market of carotenoids is around USD 2.5 billion in 2024 and is predicted to reach USD 3.4 billion by 2029 (Global Carotenoids Market Report 2024: Booming Dietary Supplements and Food & Beverages Markets Drives Carotenoids Market Growth - A $ 3.4 Billion Market by 2029, accessed on 9 June 2024), showcasing the high demand and significance of carotenoids in our lives.

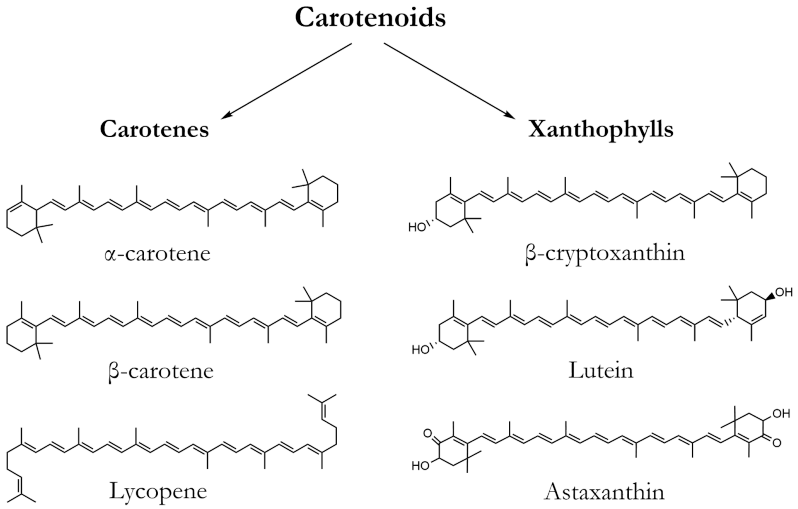

Figure 1. Classification of carotenoids based on their chemistry. Carotenoids are divided into primary carotenoids, carotenes like α-carotene, β-carotene and lycopene; and secondary carotenoids, xanthophylls such as β-cryptoxanthin, lutein, astaxanthin. The figure was made in ChemDraw.

Regarding their chemical structure, carotenoids are hydrophobic tetraterpenes that consist of 8 isoprene units (C_{40}-isoprenoids, Milani et al., 2017). They can be classified into two groups:

primary carotenoids called carotenes with a hydrocarbon chain and no functional group (e.g. α-carotene, β-carotene and lycopene), and secondary, oxygen-containing xanthophylls (e.g. β-cryptoxanthin, lutein, astaxanthin) (Britton et al., 2012). Figure 1. shows the classification and chemical structure of these compounds. β-carotene, a precursor of vitamin A, is one of the carotenoid molecules with the highest demand in recent years. Based on source, the market is divided into synthetic and natural. Natural production involves either the extraction of β-carotene from vegetable and fruit sources or the biosynthesis via microorganisms like microalgae, bacteria, and yeast (Ribeiro et al., 2011). In 2023, the natural segment reached 31.4% revenue share (Beta-carotene Market Size, Share & Growth Report, 2030 (grandviewresearch.com), accessed on 9 June 2024), which indicates a growing demand for natural β-carotene as synthetic production generates toxic by-products and poses health risks (Joshi et al., 2023). Moreover, natural β-carotene contains traces of other carotenoids, enhancing the nutritional value and health benefits of the final product.

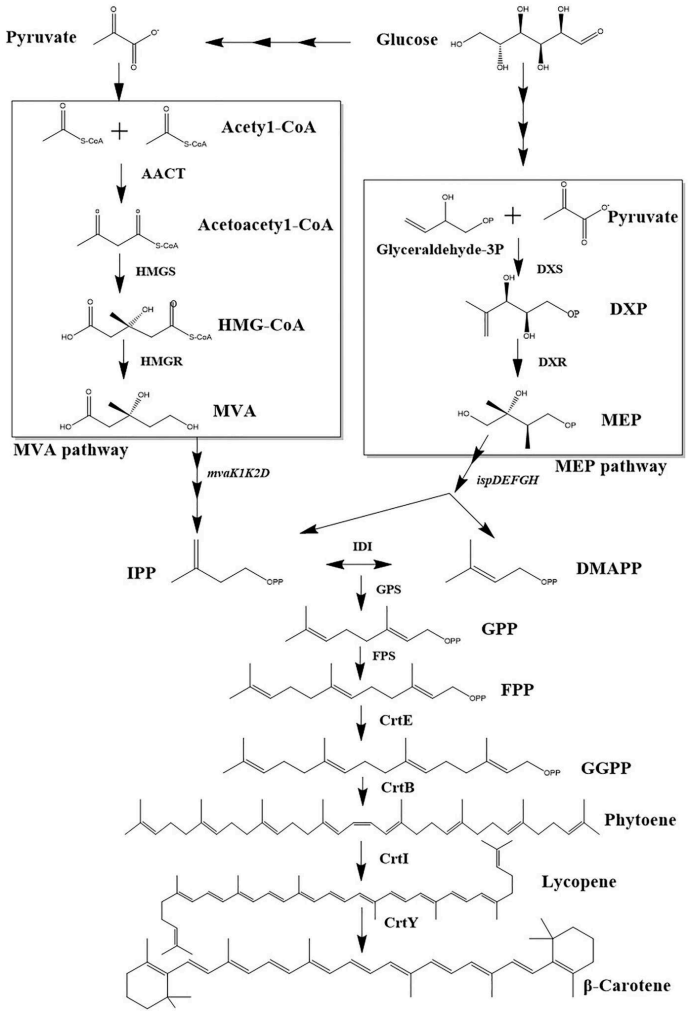

Figure 2. The biosynthesis of β-carotene via the 2C-methyl-D-erythritol-4-phosphate (MEP) and mevalonate (MVA) pathway. The MEP pathway is catalysed by the enzymes 1-deoxyxylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosph-ate reductoisomerase (DXR), which make 1-deoxy-D-xylulose-5-phosphate (DXP) and MEP. A group of enzymes, coded by the *ispDEFGH* genes, are required to synthesise isopentenyl pyrophosphate (IPP) and/or dimethylallyl diphosphate (DMAPP). The MVA pathway uses acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA synthetase (HMGS) and HMG-CoA reductase (HMGR) to produce MVA through the intermediates acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The gene cluster *mvaKIK2D* codes for the enzymes catalysing the stepwise conversion of MVA to IPP. Further, isopentenyl diphosphate isomerase (IDI) enables the transformation of IPP to DMAPP. The synthesis of β-carotene requires the enzymes geranyl pyrophosphate synthase (GPS), farnesyl pyrophosphate synthase (FPS), GGPP synthase (CrtE), phytoene synthase (CrtB), phytoene desaturase (CrtI) and lycopene cyclase (CrtY), and produces the intermediates geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), phytoene and lycopene. The figure was adapted from Wang et al., 2021.

In nature, β-carotene is synthesised by a complex enzymatic process, where the precursor isopentenyl pyrophosphate (IPP) is first derived via the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway in mostly prokaryotes, green alga and higher plants, while in eukaryotes like animals, plants, fungi and in archaea the mevalonate (MVA) pathway is used for its production (Figure 2.). In photosynthetic organisms like microalgae, the MEP pathway is located in plastids (Zhao et al., 2013) and requires certain precursors that are obtained via the combination of photosynthesis and glycolysis. The main carbon source, CO_2, is converted to glucose, after which it is broken down to pyruvate and glyceraldehyde-3P via glycolysis. They are subsequently converted to 1-deoxy-D-xylulose-5-phosphate (DXP) and eventually MEP using 1-deoxyxylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosph-ate reductoisomerase (DXR), which are the major rate-limiting enzymes in the MEP pathway (Cao et al., 2023). After that, MEP is converted to IPP and dimethylallyl diphosphate (DMAPP) in a stepwise manner (Wang et al., 2021). In eukaryotes like yeast, the MVA pathway occurs in the cytoplasm (Zhu et al., 2021), and it needs acetyl-CoA as a precursor. It is obtained from the main carbon source, glucose, via glycolysis and the citric acid cycle in the mitochondria. Using the enzymes acetoacetyl-CoA thiolase (AACT), 3-hydroxy-3-methylglutaryl-CoA synthetase (HMGS) and the rate-limiting HMG-CoA reductase (HMGR, Athanasakoglou and Kampranis, 2019, Liu et al., 2021), mevalonate

(MVA) is produced from the intermediates acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Eventually, a multi-step enzymatic catalytic process results in the formation of IPP, which is transformed into dimethylallyl diphosphate (DMAPP) by the enzyme isopentenyl diphosphate isomerase (IDI). The fusion of IPP with DMAPP is catalysed by geranyl pyrophosphate synthase (GPS), creating geranyl pyrophosphate (GPP). This intermediate merges with another molecule of IPP under the catalysis of farnesyl pyrophosphate synthase (FPS), and makes farnesyl pyrophosphate (FPP), a common precursor for different carotenes (Wang et al., 2021). In the next step, the addition of IPP to FPP makes geranylgeranyl pyrophosphate (GGPP), and is catalysed by the enzyme GGPP synthase (CrtE). The following step is said to be the most critical and rate-limiting in the biosynthesis of carotenoids (Cao et al., 2023, Cordero et al., 2011), where the fusion of two GGPPs results in the colourless phytoene, and is catalysed by phytoene synthase (CrtB). The desaturation of phytoene generates the red antioxidant lycopene, which cyclizes to form the orange end product, β-carotene. The enzymes catalysing the last two processes are phytoene desaturase (CrtI) and lycopene cyclase (CrtY, Wang et al., 2021).

Multiple microorganisms are naturally capable of the biosynthesis and accumulation of β-carotene, from which microalgae are favoured for commercial carotenoid production due to their active metabolism for carotenogenesis precursors, great storage capacity and fast growth (Joshi et al., 2023). The highest β-carotene content can be found in the genuses *Dunaliella* and *Chlorella* (Cao et al., 2023), with *Dunaliella salina* being one of the most utilised strains in industry. It is a unicellular autotroph capable of surviving high saline concentrations and defending against osmosis by modulating its internal glycerol content (Jin et al., 2003), and is able to accumulate β-carotene to protect against intense UV radiation. In fact, one way to induce and increase the carotenoid production in microalgae is to expose the cells to harsh environmental conditions like increased salinity, temperature and light (Razzak, 2024). However, microalgae are not the only natural β-carotene producers. A few strains of yeast from the genera *Phaffia, Rhodotorula* and *Sporobolomyces* have been identified as natural producers, which mainly synthesise β-carotene and astaxanthin (Park et al., 2007). An alternative way of carotenoid production is the genetic engineering of microorganisms. For example, using the tools of synthetic biology, the industrial workhorse *Saccharomyces cerevisiae* and the non-conventional, oleaginous *Yarrowia lipolytica* have been genetically modified to produce β-carotene and other carotenoids by the implementation of carotenogenic genes originating

from *Xanthophyllomyces dendrorhous*, a red yeast (Verwaal et al., 2007). In order to achieve higher yields and productivity, metabolic engineering of both producers and heterologous organisms have been explored in recent years. This usually entails metabolic flux engineering, storage capacity and membrane engineering among others (Eun and Lee, 2024).

Despite recent advancements in the genetic engineering of microalgae and their inherent potential for producing a wide range of carotenoids, our overall knowledge of industrially important strains remains limited (Stavridou et al., 2024). This lack of information complicates strain and genetic engineering, leading to numerous trials and errors, which make the improvement of industrial processes challenging. In contrast, yeast, particularly *S. cerevisiae*, has been utilised in biotechnology for decades and benefits from a well-established infrastructure and diverse product profile. However, the growth and maintenance of yeast pose sustainability issues, such as the sourcing of sugar. Given this context, we pose the question: Which organism, microalgae or yeast, is better suited as an industrial biofactory to meet the growing demand for natural carotenoids, specifically β-carotene?

To answer this question, a comparative analysis of β-carotene biosynthesis in microalgae and yeast is performed, providing a novel insight into the differences in productivity and the sustainability of the production process.

3. Differences in the productivity of microalgae and yeast

In 2022, the global carotenoid market was USD 1.9 billion (Carotenoids Market Share Projections: CAGR of 4.50% Envisions Market Size of $2.95 Billion by 2032, accessed on 22 June 2024), which would equal to 728 - 6910 tons of carotenoid, and 182 - 1728 tons of β-carotene sold globally. This amount represents the yearly demand for β-carotene, which needs to be satisfied by the chemical and biotechnological industry. Due to the inaccessibility of the most recent industrial reports, it was calculated based on a few assumptions. First, knowing that the synthetic segment dominated the global carotenoid market by 68.24% in 2022 (Carotenoids Market Share Projections: CAGR of 4.50% Envisions Market Size of $2.95 Billion by 2032, accessed on 22 June 2024), the market share of natural sources was calculated to be 31.76%. Next, the price of carotenoids was estimated to range between USD 250/kg and USD 2000/kg from synthetic sources, and between USD 350/kg and USD 7500/kg for carotenoids from natural sources (Global Carotenoids Market is Expected to Reach USD 3.59

Billion by 2025 : Fior Markets, accessed on 22 June 2024). This was used to calculate the total amount (tons) of carotenoids sold in 2022, assuming the price did not change much from 2017. Lastly, the amount of β-carotene sold was estimated by assuming that the market share of β-carotene remained 25% after 2021 (Global Carotenoids Market Size, Trends, Share, Forecast 2030, accessed on 22 June 2024). (See Appendix 1. for further details.)

The range of 182 - 1728 tons of β-carotene provides a perspective of the global demand that needs to be satisfied yearly. Currently, synthetic production of carotenoids contributes the majority to their global production (Saini and Keum, 2019). However, due to the health concerns raised by the toxic by-products, there is a desire to produce more natural β-carotene, from sources like vegetables or microorganisms (Joshi et al., 2023). The following section explores the cultivation of microalgae and yeast, their β-carotene productivity, and commonly used techniques for increasing β-carotene production.

3.1. β-carotene productivity in microalgae

Carotenoid production, especially on a large, industrial scale, consists of the cultivation of the microorganisms, the production of carotenoids in the desired concentration, and then the extraction of the products (Razzak, 2024). First, a high carotenoid-producing strain is selected or designed, which is then cultivated until the optimal growth conditions are reached. In the case of the microalgae like *Dunaliella salina*, it is usually cultivated in open tanks on a large- or limited-scale (Bogacz-Radomska and Harasym, 2018). Large-scale, extensive cultivation employs large open ponds (2,500,000 m^2), which are usually 0.5 m in depth, are unmixed and operate without any CO_2 supplementation (Del Campo et al., 2007). However, the β-carotene concentration in a cell suspension is considered to be low, 100 mg m^{-3} (Ben-Amotz, 2007). The limited cultivation of microalgae, coined intensive cultivation, operates in smaller ponds (3000 m^2) that are 0.2 m deep, are supplemented with additional CO_2, and are constantly mixed for aeration (Del Campo et al., 2007). The β-carotene productivity of intensive cultivation was reported to be 200 mg m^{-2} day^{-1} (Ben-Amotz, 2007). An alternative method is a closed cultivation system, where tubular, airborne, or planar photo-bioreactors are the most utilised. However, these methods are costly on a large scale (Pourkarimi et al., 2020). For example, Hejazi et al., 2004 used a continuous, two-phase, closed bioreactor on a lab-scale to produce β-carotene in *D. salina* with the productivity of 2.45 mg L^{-1} day^{-1}. These findings show that

scaling-up the β-carotene production in microalgae to industrial level gives rise to the challenges in the maintenance of productivity and the increase in costs.

In order to elevate the growth and productivity of microalgae, the nutrition supplied to the media (inorganic carbon source CO_2 and nitrogen) and the environmental factors (salinity, temperature, light) can be altered. Pourkarimi et al., 2020 investigated the factors affecting the growth and production of microalgae, and ultimately found that high salinity, nutrient starvation, increased light exposure and high temperature resulted in elevated β-carotene production in microalgae. On the other hand, these changes decreased the cell growth, thereby limiting the production in the long-run. As an example for the limited nutrient (nitrogen) conditions, de Souza Celente et al., 2023 found that *D. salina* cultivated on a small lab-scale, at low nitrogen concentrations (0.1 g L^{-1} KNO_3) had a biomass production of 67 mg L^{-1} day^{-1} and β-carotene productivity of 3.96 mg L^{-1} day^{-1}. On the contrary, the cells at high nitrogen concentrations produced a biomass of 108 mg L^{-1} day^{-1}, but a lower β-carotene productivity of 1.89 mg L^{-1} day^{-1}. In another study (Xu et al., 2018), the increase in light intensity was tested on *D. salina* (strain DF15), and at the highest intensity (1500 μmol photon m^{-2} s^{-1}) the cells produced β-carotene at 3.5 mg L^{-1} day^{-1}. In addition, no photoinhibition was observed when increasing the light intensity, in fact, higher growth rates were reported. This further supports the high tolerance and adaptability of microalgae to harsh environmental conditions. Table 1. summarises the above-mentioned findings.

Table 1. β-carotene productivity/content in microalgae, cultivated under different conditions. Abbreviations: DCW - dry cell weight; n.r. - not reported; sp. - species. * Ben-Amotz, 2004 reported 0.1 mg m^{-3} β-carotene concentration and it was converted for uniformity. ** Ben-Amotz, 2004 reported 200 mg m^{-2} day^{-1} β-carotene productivity, and knowing that the tank was 0.2 m deep, it was converted for uniformity. *** Cen et al., 2022 reported 4.34 mg g_{DCW}^{-1} β-carotene content after 9 days of fermentation, and it was converted for uniformity.

Strain	Method	β-carotene	Reference
Dunaliella sp.	large-scale, open tank, extensive cultivation	0.1 mg L^{-1} *	Ben-Amotz, 2007
Dunaliella sp.	limited, open tank, intensive cultivation	1 mg L^{-1} day^{-1} **	Ben-Amotz, 2007

Dunaliella salina	continuous, two-phase, closed bioreactor (lab-scale)	2.45 mg L^{-1} day^{-1}	Hejazi et al., 2004
Dunaliella salina	low nitrogen (Erlenmeyer flask, lab-scale)	3.96 mg L^{-1} day^{-1}	de Souza Celente et al., 2023
Dunaliella salina	high nitrogen (Erlenmeyer flask, lab-scale)	1.89 mg L^{-1} day^{-1}	de Souza Celente et al., 2024
Dunaliella salina (DF15)	high light intensity (Erlenmeyer flask, lab-scale)	3.5 mg L^{-1} day^{-1}	Xu et al., 2018
Phaeodactylum tricornutum	co-expression of DXS and CrtY (lab-scale)	0.48 mg g_{DCW}^{-1} day^{-1}***	Cen et al., 2022

While changing the environmental conditions and improving the cultivation have proven to increase the amount of carotenoids produced, the efficiency of the biosynthetic process was still limited by the inherent properties and metabolism of the organisms. Therefore, scientists have turned to synthetic biology to create a high-performance microalgal strain via metabolic engineering. For example, in a study conducted by Cen et al., 2022, two genes coding for two key enzymes were co-overexpressed in the carotenogenic diatom, *Phaeodactylum tricornutum*, in order to increase β-carotene production. One of them was 1-deoxy-D-xylulose-5-phosphate synthase (DXS), which is considered one of the bottlenecks in the production of the precursor IPP, while the other enzyme was lycopene cyclase (CrtY) that has a crucial role in converting lycopene to β-carotene. The β-carotene productivity of the genetically modified microalgal strain increased to 4.34 mg g^{-1} dry cell weight after 9 days of cultivation, while the biomass production of the cells was not affected, contrary to previous studies (Cen et al., 2022, Radakovits et al., 2011).

3.2. β-carotene productivity in yeast

The process of carotenoid production via fermentation is similar in essence to the production of microalgae: first a suitable organism is chosen or designed, carotenoids are synthesised via fermentation, and lastly they are obtained via extraction. However, significantly different

aspects and challenges need to be considered when cultivating yeast. The red yeast genus, *Rhodotorula* is one of the most researched organisms due to its ability to synthesise a high amount of carotenoids using the MVA pathway. It was also shown that approximately 70% of the total carotenoid content accumulated in these yeast cells is β-carotene (Sharma and Ghoshal, 2020). According to Ochoa-Viñals et al., 2024 the main factors influencing the production of carotenoids in *Rhodotorula* are medium composition, salinity, pH, light, temperature, agitation speed and fermentation mode.

Batch, fed-batch and continuous fermentation bioreactors are the most used, where the fed-batch mode has been reported as the most fruitful and suitable for carotenoid production in yeast. Fallahi et al., 2023 conducted a lab-scale, comparative analysis of batch and fed-batch fermentation of *Rhodotorula toruloides* in a bubble column reactor (BCR), and reported a 2.23 mg g_{cell}^{-1} h^{-1} β-carotene production rate in a fed-batch culture. After further optimization of feed flow rate and temperature, the final β-carotene production rate of 12.31 mg g_{cell}^{-1} h^{-1} was achieved. In comparison, the batch culture showed 5.98 mg g_{cell}^{-1} h^{-1} β-carotene productivity. The success of the fed-batch fermentation method is due to the timing of carotenoid synthesis, which begins in the late log phase and continues into the stationary phase. This allows for optimal conditions to be provided first for yeast growth and then for carotenoid production, similarly to a two-phase production method (Ochoa-Viñals et al., 2024). Additionally, Fallahi et al., 2023 emphasised the importance of appropriate aeration, which increases oxygen availability for catabolic pathways and the production of ATP and crucial intermediates that feed into the MVA pathway.

To achieve optimal growth and productivity, the composition of the medium and nutrients in the bioreactor can be altered. The most commonly used carbon sources are glucose and sucrose (Ochoa-Viñals et al., 2024), where a low carbon and nitrogen ratio (C/N) induces carotenoid production in yeast. For example, Sereti et al., 2023 achieved 2.59 mg L^{-1} total carotenoid content (approx. 75% β-carotene) under 39 hours by applying a low C/N ratio (80) during the cultivation of *Rhodosporidium kratochvilovae* red yeast. In addition to nutrient limitation, elongated light exposure has also a beneficial effect on carotenoid production, given the fact that these compounds accumulate in the yeast cell for photoprotection. As an example, Gao et al., 2024 showed that 12 hour per day light exposure resulted in a 1.98 times higher total carotenoid content (1.29 mg g^{-1}, over 92% β-carotene) than with dark cultivation (0.65 mg g^{-1}) of *Rhodosporidium toruloides* red yeast, in a 5 day period.

An alternative approach for carotenoid production is the design of high-performance carotenogenic yeast strains using genetic engineering. *Yarrowia lipolytica* and *Saccharomyces cerevisiae* are two of the most favoured yeast cell factories due to their safety, well-established genetic engineering tools and industrial scale-up (Guo et al., 2024). Despite not being natural producers, synthetic biology enabled the integration and overexpression of carotenogenic genes in these organisms, by building on the pre-existing lipid metabolic and MVA pathways (Kuzuyama, 2002). For example, Verwaal et al., 2007 introduced the genes *crtYB* (encoding the bifunctional phytoene synthase and lycopene cyclase), *crtI* (phytoene desaturase) and *tHMG1* (a truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene) from the red yeast *Xanthophyllomyces dendrorhous* to *Saccharomyces cerevisiae*. The combined overexpression of the homologous genes encoding CrtE (GGPP synthase) and the heterologous genes resulted in the β-carotene yield of 5.9 mg g^{-1} dry cell weight, produced over 3 days. Due to the high availability of acetyl-CoA in *Yarrowia lipolytica*, and its ability to accumulate and store intracellular lipids (Papanikolaou and Aggelis, 2010), Larroude et al. 2018 engineered this organism for the production of carotenoids. It was found that the overproduction of lipids, in combination with a heterologous carotenoid expression cassette, yielded 90 mg g^{-1} dry cell weight of β-carotene after 4 days of fed-batch fermentation. Following the successful construction of heterologous carotenogenic yeast strains, more metabolic engineering techniques were developed to enhance carotenoid production. These include metabolic flux engineering, spatial rearrangement of enzymes and storage capacity engineering (Eun and Lee, 2024). Table 2. summarises the above mentioned findings.

Table 2. β-carotene production in yeast on a lab-scale, cultivated under different conditions. Abbreviations: TC - total carotenoid, DCW - dry cell weight.

Strain	Method	Duration	β-carotene	Reference
Rhodotorula toruloides	batch bubble column reactor	1 hour	5.98 mg g_{DCW}^{-1}	Fallahi et al., 2023
Rhodotorula toruloides	fed-batch bubble column reactor	1 hour	2.23 mg g_{DCW}^{-1}	Fallahi et al., 2023
Rhodotorula toruloides	optimised fed-batch bubble column reactor	1 hour	12.31 mg g_{DCW}^{-1}	Fallahi et al., 2023

Rhodosporidium kratochvilovae	low C/N ratio (80)	39 hours	0.31 mg g_{DCW}^{-1} TC (75% β-carotene)	Sereti et al., 2023
Rhodosporidium toruloides	12 hour/day light exposure	120 hours	1.29 mg g_{DCW}^{-1} TC (92% β-carotene)	Gao et al., 2024
Rhodosporidium toruloides	dark cultivation	120 hours	0.65 mg g_{DCW}^{-1} TC (92% β-carotene)	Gao et al., 2024
Saccharomyces cerevisiae	transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*	72 hours	5.9 mg g_{DCW}^{-1}	Verwaal et al., 2007
Yarrowia lipolytica	transformation with a heterologous carotenoid expression cassette	96 hours	90 mg g_{DCW}^{-1}	Larroude et al. 2018

The β-carotene productivity of microalgae and yeast has been explored in this section by presenting several case studies. In microalgae, carotenoid production was enhanced by optimising environmental conditions such as salinity, light intensity, and nutrient levels, and by genetic engineering to overexpress key carotenogenic genes. The improvement of carotenoid production in yeast was achieved through optimised fermentation processes, adjusting medium composition and light exposure, and introducing carotenogenic genes into non-native producers. In comparison to microalgae, yeast showed higher β-carotene productivity, especially when genetically engineered or using optimised fed-batch fermentation methods. While microalgal cells are highly adaptable and capable of high β-carotene production under optimal conditions, they face challenges in scaling up and cost management. Thus, overall, yeast appears to be a more efficient and scalable option for β-carotene production compared to microalgae, benefiting from advanced fermentation techniques and genetic engineering enhancements.

4. Sustainability of carotenoid biosynthesis

To evaluate the potential and applicability of microalgae and yeast in the carotenoid industry, it is essential to assess the sustainability of their production processes. The following section provides an overview of the long-term aspects and challenges associated with each organism, by focusing on the environmental impact and production costs.

4.1. Sustainability of β-carotene production in microalgae

The environmental impact of carotenoid production via microalgae is commonly inspected using a life cycle assessment (LCA). This evaluates a number of production scenarios in order to find the least polluting one that still maintains the optimal growth and productivity of the organism. de Souza Celente et al., 2023 assessed the impact of media consumption by *Dunaliella salina* (DF15) to predict the best scenario of cultivation for the production of 1 kg β-carotene. It was found that the production involves high water and nutrient usage (especially the open tank cultivation), significant energy consumption and greenhouse gas emissions. Since the main source of energy for microalgae is inorganic carbon, CO_2 sequestration can help offset emissions. In addition, utilising alternative water sources, like wastewater or brine can provide alternative carbon sources and maintain high salinity, resulting in a negative carbon emission. In another study, Deprá et al., 2020 conducted a comparative LCA to assess the impact of synthetic and natural microalgal carotenoids, using 1 kg of pigment produced as the functional unit. The assessment showed that microalgal production required more energy consumption compared to synthetic production. This was due to the energy-intensive processes involved in bioreactor cultivation (including maintenance and lighting) and the downstream processing of carotenoids. Moreover, the high energy requirement accounted for the higher greenhouse gas (GHG) emissions too.

Although the cultivation of microalgae in refined bioreactors allows for fine-tuning of the environmental conditions, the scale-up of closed cultivation has been shown to be energy-intensive and costly (Pourkarimi et al., 2020). Despite this, the downstream processes are responsible for the majority of the costs. These processes include the time-consuming steps of harvesting, drying, and lysing the cells, followed by extraction using organic solvents like n-hexane or ethanol. An alternative extraction method employs green solvents, such as supercritical carbon dioxide ($scCO_2$), which is considered environmentally non-toxic (Razzak,

2024). Ludwig et al., 2021 found that although this method requires more energy compared to conventional extraction with organic solvents, its increased efficiency and the absence of solvent residues ultimately lead to lower costs. Following extraction, high-purity carotenoids can be obtained through purification techniques like column chromatography or high-performance liquid chromatography (HPLC, Razzak, 2024).

4.2. Sustainability of β-carotene production in yeast

The environmental impact and sustainability of biotechnological applications involving yeast are significantly influenced by the availability and composition of the cultivation medium. The most commonly used media include YPD medium (comprising yeast extract, peptone, and dextrose) and yeast nitrogen base (YNB) supplemented with glucose and amino acids. These primarily utilise pentoses, hexoses or disaccharides as the main carbon sources for the fermentation-based production of carotenoids, which are obtained from sugarcane and cereal grains (Kádár and Fonseca 2019). However, not all regions are suitable for the agricultural production of dedicated plant resources. Therefore, the large-scale production of carotenoids necessitates the transportation of feedstock from major sugar-producing and supplying countries, which include Brazil, India, Thailand, China, Pakistan, and Mexico (Aguilar-Rivera, 2022). The delayed availability of sugar leads to higher costs and contributes negatively to the environment through increased GHG emissions. Moreover, dedicating the plant feedstock to biotechnological production reduces the availability of resources, creating competition with the food industry. To increase the sustainability of the fermentation-based industrial production, the use of bio-waste and agricultural byproducts like wheat straw and corn stover have been suggested as alternative feedstock (Kádár and Fonseca 2019). For example, corn steep liquor (CSL), a by-product of corn wet milling (Zhou et al., 2022), was used by Fallahi et al., 2023 to produce β-carotene in the yeast *Rhodotorula toruloides*. Other studies used olive mill wastewater for bioremediation and carotenoid production (Hladnik et al., 2024), or orange and grape waste as carbon sources (Uğurlu et al., 2023). On the other hand, this method requires either the upstream processing of complex sugars or the genetic modification of yeast to enable the utilisation of polysaccharides (Usmani et al., 2020).

Similarly to microalgae, maintaining photobioreactors and extracting carotenoids incurs additional costs; however, the advantage of yeast lies in the already-developed infrastructure for large-scale production via fermentation.

In this section, the sustainability of large-scale carotenoid production using microalgae and yeast was evaluated. Microalgal production in open tank cultivation was found to be resource-intensive, particularly in terms of water and nutrients. Conversely, closed photobioreactors required significant energy, indirectly contributing to greenhouse gas emissions, and scaling up this method proved to be costly. Regarding carotenoid production via yeast, the need for an organic carbon source increased costs due to feedstock transportation, which also negatively impacted the environment. To reduce energy demands, the use of wastewater and agricultural by-products were suggested as alternative sources. For both microalgae and yeast, the extraction process was identified as the major cost factor.

5. Discussion

The health risks associated with the toxic by-products and intermediates of synthetic carotenoid production have led to the increase in demand for carotenoids from natural sources like plants, or from microorganisms like microalgae and yeast (Joshi et al., 2023). It has been shown that the high growth rate and productivity of microorganisms is highly beneficial for the production of biomolecules, especially compared to the slow-growth and weather-dependent farming of plants. Moreover, the β-carotene content of plants has been proven to be lower than for certain microorganisms. For example, Kyriakopoulou et al., 2015 found that to extract 1 kg of β-carotene, 10 kg of dried *D. salina* microalgae were needed, compared to 1250 kg of dried carrots. This demonstrates the higher potential of microorganisms for large-scale industrial production. Therefore, the properties of two microorganisms, microalgae and yeast, were assessed from a productivity and sustainability point of view to determine which organism is more suitable as a biofactory for industrial scale β-carotene production.

When comparing yeast and microalgae, yeast exhibited higher overall β-carotene productivity. However, the fermentation process for yeast raised sustainability issues due to high GHG emissions and increased costs from the transportation of sugar resources. Both yeast and microalgae cultivation in photobioreactors were energy-intensive. Microalgae, although environmentally beneficial due to their autotroph nature, faced additional challenges such as costly scale-up of closed cultivation owing to a lack of industrial infrastructure, and high water and land requirements for open tank cultivation, which negatively impacted sustainability. To address these issues and promote a zero-waste, sustainable approach, the use of alternative

sources like wastewater or agricultural waste for yeast fermentation or microalgae cultivation was suggested. Additionally, the downstream extraction process constituted the majority of the costs for both microorganisms. To enhance sustainability and reduce costs, the use of $scCO_2$ as a green solvent was proposed for the extraction of β-carotene.

In addition, a few safety concerns should be addressed regarding carotenoid production via microorganisms. Gressel et al., 2013 explored the environmental risks of large-scale open tank cultivation of microalgae, raising awareness to the inevitable event of accidental spillage of non-native algal species to nearby waters, thus destroying the balance in the aquatic ecosystems. Also, open cultivation was found to be susceptible to contamination by other organisms e.g. protozoa (Prieto et al., 2011), which creates competition for nutrients and lowers biomass production. During the assessment of productivity, it has been shown that the genetic engineering of both microalgal and yeast strains has the potential to optimise β-carotene biosynthesis and thereby boost productivity. However, the ethical concerns and safety regulations of genetically modified organisms (GMOs) need to be taken into consideration when designing a large-scale biotechnological production process. Thorough risk assessment should be conducted to prevent loss of biodiversity and environmental damage caused by the spillage of GMOs into the environment, which can be achieved by employing closed cultivation methods (Cao et al., 2023).

To evaluate the potential of microalgae and yeast for the biotechnological production of β-carotene, all the above mentioned findings need to be carefully considered. Regarding productivity, yeast appears to be a better candidate due to its higher production efficiency compared to microalgal strains, its scalability and already well-established infrastructure. It is this author's belief, however, that neither yeast, nor any other microorganism is capable of satisfying the estimated global yearly demand of 182 - 1728 tons of β-carotene alone, at its current production. While microorganisms have been shown to accumulate higher amounts of β-carotene than vegetable sources (e.g. carrots), they are not on par with production levels of the low-cost chemical synthesis. Therefore, it is suggested that both yeast and microalgal strains are further developed, and are utilised according to the availability of the required resources. For example, in major sugar-producing countries sustainable β-carotene production via yeast is more achievable, while in others the utilisation of wastewater for β-carotene production via microalgae should be employed to decrease costs. Moreover, even in countries with abundant feedstock plants, the change to agricultural and food waste could further increase the

sustainability of fermentation, while simultaneously contributing to the Sustainable Development Goal (SDG) of ending hunger and malnutrition by not competing for resources with the food industry. However, these promising alternatives entail additional challenges. Yeast cannot readily utilise polysaccharides, requiring either an energy-intensive upstream breakdown process or genetic modification to introduce enzymes capable of breaking down complex carbohydrates. Regarding the autotrophic microalgae, switching to mixotrophy or even heterotrophy generally decreases the growth rates and productivity (Chavoshi and Shariati, 2019, Diankristanti et al. 2024). Moreover, while genetic modification of microorganisms offers numerous possibilities for increasing productivity and sustainability, the commercial use of GMOs is hindered by strict policies and safety regulations.

6. Conclusion

Carotenoids are natural pigments that exhibit a range of colours including yellow, orange, red, and purple. Due to their optical, photoprotective, anti-inflammatory, antioxidant, antibacterial and anticancer properties they are highly desired compounds, and are widely utilised in the food, cosmetic and pharmaceutical industry. β-carotene, a major carotenoid and the precursor of vitamin A, is in particularly high demand. The majority of the demand is satisfied via synthetic production, however, due to health risks posed by the toxic by-products, the need for natural production is only increasing. Natural β-carotene is either extracted from plant sources or biosynthesized via microorganisms like yeast and microalgae. Owing to fast growth and higher productivity, microorganisms have shown an increasing potential for satisfying the demand for β-carotene. Therefore, this review explored the productivity and sustainability of β-carotene production via microorganisms, and aimed to answer the question: Which organism, microalgae or yeast, is better suited as an industrial biofactory to meet the growing demand for natural carotenoids, specifically β-carotene? When assessing productivity, it was found that yeast had a higher productivity compared to microalgal strains. However, despite its scalability and established infrastructure, the sustainability of yeast fermentation is compromised by the sourcing of sugar. On the other hand, although microalgae are not as productive as yeast, they offer significant potential due to their high adaptability and ability to sequester CO_2, making them a more sustainable choice in terms of nutrient sources. To reduce the energy and costs associated with the cultivation of both yeast and microalgae, the use of wastewater and agricultural waste was suggested. Further, due to the belief that no

microorganism can satisfy the global demand of β-carotene alone, the use of yeast was proposed in major sugar-producing countries and in those where agricultural waste can be utilised, while the simultaneous production via microalgae was suggested using wastewater. Future research should focus on overcoming challenges related to yeast fermentation using polysaccharides and the mixotrophic or heterotrophic cultivation of microalgae. Additionally, although extensive risk assessments and trials are necessary for the commercial use of GMOs, genetic engineering holds undeniable potential for enhancing productivity and sustainability. In conclusion, while yeast remains a more productive biofactory for the production of β-carotene, the simultaneous enhancement of carotenoid production in microalgae is crucial for achieving a more sustainable industrial process, particularly in countries with limited agricultural production.

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8. Appendix

Appendix 1. Calculation of the amount (tons) of carotenoids and β-carotene sold globally in 2022.

1. Calculation of the global market share of carotenoids from synthetic and natural sources

Global carotenoid market share in 2022: USD 1.9 billion
Synthetic segment: 68.24%, 0.6824 · USD 1.9 billion = USD 1.29656 billion
Natural segment: 31.76%, 0.3176 · USD 1.9 billion = USD 0.60344 billion

2. Calculation of the amount (tons) of synthetic and natural carotenoids sold globally

Synthetic carotenoids price: USD 250/kg - USD 2000/kg, it is assumed to remain constant over the years.
Minimum amount of synthetic carotenoids sold: USD 1.29656 billion / USD 2000/kg = 648.28 tons

Maximum amount of synthetic carotenoids sold: USD 1.29656 billion / USD 250/kg = 5186.24 tons

Natural carotenoids price: USD 350/kg - USD 7500/kg, it is assumed to remain constant over the years.
Minimum amount of natural carotenoids sold: USD 0.60344 billion / USD 7500/kg = 80.45866 tons
Minimum amount of natural carotenoids sold: USD 0.60344 billion / USD 350/kg = 1724.114286 tons

3. Calculation of the total amount (tons) of carotenoids sold globally

Minimum amount of total carotenoids sold: 648.28 tons + 80.45866 tons = 728.73866 tons
Maximum amount of total carotenoids sold: 5186.24 tons + 1724.114286 tons = 6910.354286 tons

4. Calculation of the amount of β-carotene sold globally

The market share of β-carotene was 25% of the global carotenoid market in 2021, it is assumed to remain constant over the years.
Minimum amount of β-carotene sold: 0.25 · 728.73866 tons = 182.184665 tons
Maximum amount of β-carotene sold: 0. 25 · 6910.354286 tons = 1727.588572 tons

9. Declaration on the use of AI

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