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REVIEW PAPER

A comparison of metabolic engineering strategies applied in *Yarrowia lipolytica* for β-carotene production

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Abstract
With a better understanding of the health benefits of β-carotene, the precursor of vitamin A, as well as its coloring property, the need for this carotenoid has increased in various sectors. In order to meet the increasing demand efficiently, cheaply, and sustainably, interest in heterologous β-carotene production through metabolic engineering strategies has increased in recent years. In this context, although it is not a native producer of β-carotene, *Yarrowia lipolytica* yeast stands out due to its metabolic, physiological, and genomic properties. Successful results have been obtained by using a series of engineering strategies, including biosynthesis pathway engineering, morphological engineering, and fermentation engineering strategies, in the production of heterologous β-carotene from *Y. lipolytica*. However, these strategies have various strengths and weaknesses against each other, and there are also some points open to improvement. In this review, the engineering strategies that have been applied and have the potential to be applied for the production of β-carotene from *Y. lipolytica* have been examined in depth, including their advantages and disadvantages, and compared with each other. Moreover, a future perspective has been presented to increase the potential of using *Y. lipolytica* yeast as a cell factory in β-carotene production.

Keywords β-Carotene · *Yarrowia lipolytica* · Engineering strategies · Cell factory

1 Introduction

β-carotene ($C_{40}H_{56}$), a type of carotenoid belonging to the terpenoids group, which can also be called isoprenoids, is produced by many plants such as sweet potatoes and palm fruits [1], various macroalgae such as *Undaria pinnatifida* [2], various macrofungi such as *Suillus bovinus* and *Cantharellus cibarius* [3], as well as many microfungi, microalgae, and bacteria, and even naturally present in certain concentrations in certain animals, such as flamingo birds; depending on their diet [4], it is a precursor of vitamin A (retinol) that can be converted to vitamin A in intestinal epithelial cells when consumed by humans [5]. Since it is a vitamin A precursor, β-carotene plays a crucial role in maintaining eye health, especially in preventing night blindness [6]. Regardless of its pro-vitamin A property, β-carotene is recognized as a promising powerful antioxidant that can prevent cellular damage [7]. This feature provides protection to plants and animals that consume these plants against the devastating effects of oxygen radicals. Due to its antioxidant properties, it can also be utilized as a sunscreen to prevent photoaging of the skin and sunburn [8]. Moreover, β-carotene has been shown to have anticarcinogenic properties by Niranjana et al. [9] and immune system boosting by Wang et al. [10].

A liposoluble natural compound β-carotene is extensively utilized as an additive (E160a (ii)) in the industries such as food, textile, chemical, and cosmetics, due to its positive effects on health and its red-to-yellow color and accepted as GRAS (Generally Recognized as Safe) [10]. While the market price of β-carotene was US$ 442.1 million in 2018, it achieved

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approximately US$ 701.5 million in 2026 [6]. According to Ribeiro et al. [4], approximately 98% of commercially produced β-carotene is produced by physicochemical/chemical synthesis, while only approximately 2% is produced biologically. However, conventional methods such as extraction from a natural producer or chemical synthesis are not suitable to produce large quantities of β-carotene due to their low degree of purity, low efficiency, and high cost [11]. The demand to produce more sustainable, reliable, and low-cost β-carotene has enhanced the number of studies on alternative β-carotene obtaining methods. In this context, production of β-carotene by metabolic or morphological engineering of microorganisms provides promising results. Model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* are frequently utilized in metabolic engineering studies because much is known about their biology, and they are easy to grow and engineer. However, an unconventional yeast, *Yarrowia lipolytica*, is known to successfully produce carotenoids such as lycopene [12], β-carotene [13], and astaxanthin [11]. The GRAS status of *Y. lipolytica* yeast, the sequence of its genome, and the possibility of using different substrate sources in its growth allow the carotenoid production efficiency of this yeast to be enhanced by metabolic engineering.

In this review, metabolic engineering studies to produce β-carotene from *Y. lipolytica* were evaluated and it aimed to reveal the potential of this microorganism as a cell factory in large-scale β-carotene production. There are very few studies [14] examining this topic in the literature; however, the current study has the potential to contribute to the literature by examining in depth the metabolic engineering strategies used or having the potential to be used in the production of β-carotene from *Y. lipolytica* and comparing these strategies with each other. In addition, in this study, it is thought that comparing the strengths and weaknesses of native microbial β-carotene producers, as well as microorganisms that have the potential to be used as a chassis in heterologous β-carotene production, in light of current data will contribute to the literature.

2 The main pathways of β-carotene biosynthesis in eukaryotes

Most carotenoids, including C40 carotenoids like β-carotene, are derivatives of phytoene, a tetraterpenoid pigment, and the biosynthesis of these carotenoids begins with phytoene, which is produced by the condensation of two geranylgeranyl diphosphate (GGPP, C20) molecules, catalyzed by the phytoene synthase (*PSY*/*crtB*) enzyme [15]. Every carotenoid is produced utilizing two biosynthetic pathways that result in the genesis of dimethylallyl diphosphate (DMAPP, C5) and/or isopentenyl diphosphate (IPP, C5). Most eukaryotes, including yeasts, utilize the mevalonate (MVA) biosynthesis pathway in the synthesis of carotenoids, where DMAPP and IPP precursors arise, while many bacteria synthesize carotenoids using the 2C-methyl-D-erythritol 4-phosphate biosynthesis pathway (MEP) [16].

In the MVA biosynthetic pathway starting with acetyl coenzyme A (acetyl-CoA) (Fig. 1), IPP is produced under the catalysis of certain enzymes such as phosphomevalonate kinase (Pmk), mevalonate pyrophosphate decarboxylase (MvaD), mevalonate kinase (Mvk), 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase, *erg13*), and acetyl-CoA acetyltransferase (erg10p) [17, 18]. Firstly, acetyl-CoA is catalyzed by the enzyme erg10p and condensed to form acetoacetyl-CoA. Next, one more acetyl-CoA is condensed into acetoacetyl-CoA, which together results in the formation of 3-hydroxy-3-methyl-glutaryl-CoA (HMG), which is catalyzed with the enzyme HMG-CoA synthase. To obtain MVA, HMG is reduced with the enzyme HMG-CoA reductase (*HMGR1*). MVA is then phosphorylated first with the enzymes Mvk and Pmk, respectively. IPP is formed when phosphorylated MVA is decarboxylated by the enzyme MvaD [19]. The resulting IPP is isomerized to its isomer, DMAPP, with the enzyme IPP isomerase. Both DMAPP and IPP are used for the eventual production of lycopene by enzymes such as GGPP synthase (*GGS1*/*crtE*), PSY, and lycopene synthase (*CrtI*) [18, 20]. Condensation of IPP with DMAPP by the action of the GPP synthase enzyme leads to the production of geranyl diphosphate (GPP, C10). The combination of IPP with GPP causes the formation of farnesyl diphosphate (FPP, C15), while the incorporation of one more IPP unit results in the formation of GGPP. GGPP is a frequently used precursor for some plastid isoprenoid groups in eukaryotes [5]. A linear colorless phytoene is first synthesized by condensation of two GGPPs by PSY, which is involved in C40 carotenoid synthesis [16]. The resulting phytoene generally subjected a four-stage desaturation with the enzymes ξ-carotene desaturase and phytoene desaturase and two complementary isomerizations with the enzymes carotenoid isomerase and 15-cis-ζ-carotene isomerase, resulting in an enhancement in conjugated double bonds that convert colorless phytoene to bright pink colored lycopene [16]. Some carotenoids, including β-carotene, are generated from lycopene [21]. Gradual desaturations lead to intermediates with a conjugated double band number of 3 to 11; this creates dissimilar colors when the count of conjugated double bands exceeds 7. A β- or ε-ring is then incorporated into the chain ends of lycopene by reactions catalyzed by different lycopene cyclase enzymes to create α-, β-, γ-, or δ-carotene according to double-bond position of the ionone ring [16]. In the existence of lycopene cyclase crtL-e, joining together an ε-ring to lycopene yields δ-carotene. Next, the addition of a β-ring to the end of the δ-carotene results in of α-carotene. However, adding a β-ring to lycopene causes γ-carotene synthesis, while adding another β-ring to γ-carotene causes β-carotene synthesis [5, 22].

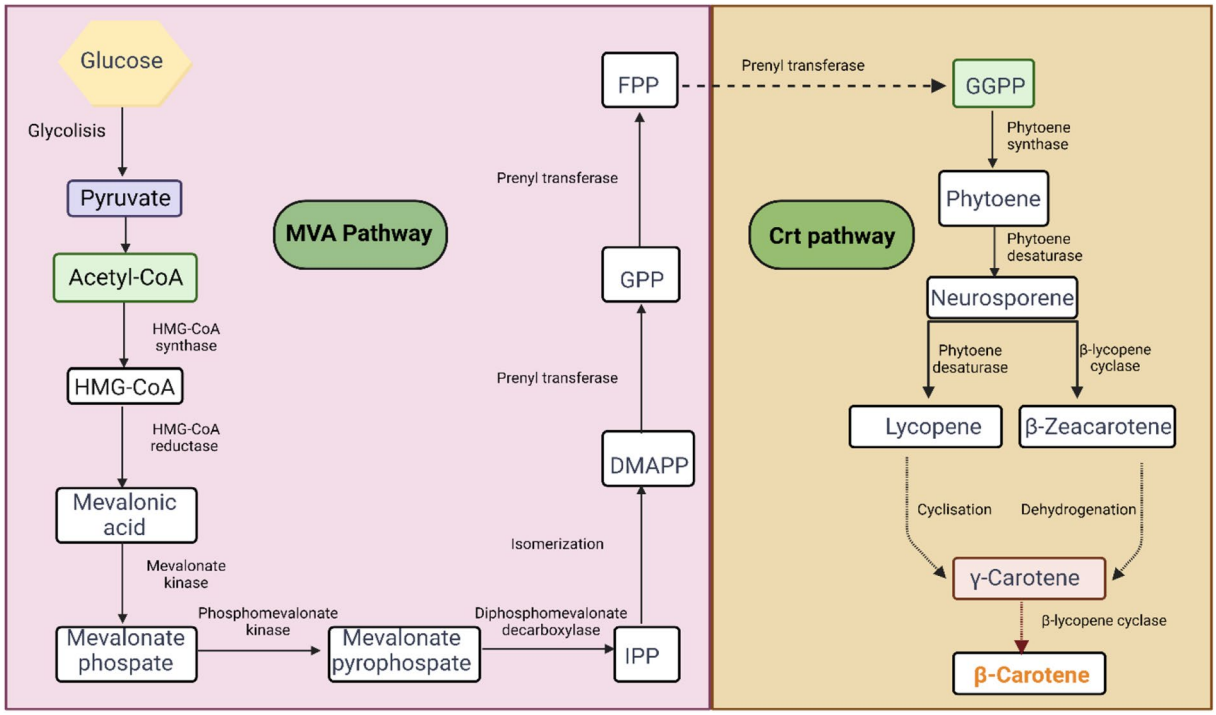


Fig. 1 Schematic representation of the metabolic pathways of *β*-carotene synthesized from the upstream mevalonate (MVA) and downstream carotenoid (Crt) biosynthesis pathways in the yeast cell. Acetyl-CoA: acetyl coenzyme A, HMG-CoA synthase: 3-hydroxy-3-methyl-glutaryl-CoA synthase, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate

3 Microbial *β*-carotene production with native producers

Conventional methods such as physicochemical extraction from plants and de novo chemical synthesis are unsuitable to produce large quantities of *β*-carotene due to their low purity, low efficiency, and high cost. These problems have led to an enhancement in the requisition to produce microbial carotenoids in recent years. The microbial production of carotenoids can be done in an eco-friendly way, regardless of seasons, climates, and regions, compared to chemical and physicochemical methods.

Carotenogenic microorganisms encompass various microorganisms including bacteria, yeasts, microalgae, and molds (Table 1) [23–32]. Among these microorganisms, especially *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*), *Blakeslea trispora*, *Dunalliela salina*, and *Haematococcus pluvialis* are considered eligible for large-scale production [5].

It has been shown that large quantities of carotenoids (*β*-carotene, astaxanthin, canthaxanthin, and others) can be produced from microalgae such as *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella zofingiensis*, *D. salina*, *Eustigmatos* cf. *polyphem*, and *H. pluvialis* [33, 34]. Among these microalgae, the most striking one in terms of *β*-carotene bioproduction is *D. salina* species, which is also utilized in commercial *β*-carotene production. It is reported that quantity of *β*-carotene synthesized by *D. salina* is approximately 138 mg/g [33], and *D. salina* can accumulate *β*-carotene up to 10% [35] and even 14% of dry cell weight (DCW) [36] in some extreme environmental conditions. It is thought that these amounts can be increased by subjecting *D. salina* to different nutritional and environmental conditions [36].

B. trispora, a mold that is also utilized in the commercial bioproduction of *β*-carotene, is one of the most notable molds for its capability to amass *β*-carotene. In two separate studies using different media, it has been shown that *B. trispora* produced *β*-carotene at concentrations of 643 mg/L [37] and 790 mg/L [38]. Although molds such as *Mucor circinelloides* and *Phycomyces blakesleeanus* are known to have the ability to accumulate *β*-carotene, their production efficiency is reported to be low compared to *B. trispora* [39].

The pigments liable for the red–orange color of some yeast cells can block certain wavelengths of light, preventing light from damaging the cell [5]. Red yeast genera such as *Rhodotorula*, *Phaffia*, and *Sporobolomyces* are capable of producing *β*-carotene [40]. Perrier et al. [41], in their work on 13 *Rhodotorula* strains that formed pink-red colonies, determined that all strains contained *β*-carotene between 5 and 75 µg/g DCW. However, in the relevant literature, it

Table 1 Some native microorganisms that produce β-carotene

Microorganisms	Substrate	Fermentation mode	β-carotene content	Strategy applied	References
Microalgae					
Dunalliela salina	Modified Rudic's culture medium supplemented with NaCl	Photobioreactor	10 mg/g dry biomass	Exposing the photobioreactor to two panels of flashing red and blue LEDs	[23]
Dunaliella bardawil	Mineral liquid medium	Air-fluidized bed photobioreactor	51.75 mg/L	Visible light (400–700 nm) and UV-A radiation (320–400 nm)	[24]
Vischeria stellata	BG-11 medium	Bubble column photobioreactor	33.52 mg/g DCW	Use of a bubble column photobioreactor irradiated at 300 µmol photons m^{-2} s^{-1}	[25]
Vischeria punctata	BG-11 medium	Bubble column photobioreactor	18.82 mg/g DCW	Use of a bubble column photobioreactor irradiated at 300 µmol photons m^{-2} s^{-1}	[25]
Eustigmatos magnus	BG-11 medium	Bubble column photobioreactor	13.31 mg/g DCW	Use of a bubble column photobioreactor irradiated at 300 µmol photons m^{-2} s^{-1}	[25]
Yeast					
Rhodotorula glutinis	Whey	Batch	45.68 mg/L	Use of several waste substrates and stress factors	[26]
Rhodotorula mucilaginosa	Potato extract with 5% NaCl	Batch	55.91 mg/L	Use of several waste substrates and stress factors	[26]
Rhodotorula graminis	Yeast extract, malt extract, peptone, glucose	Flask	303 µg/L	Production without illuminations	[27]
Sporobolomyces roseus	Yeast extract, malt extract, peptone, glucose	Flask	118 µg/L	Production without illuminations	[27]
Xanthophyllomyces dendrorhous	Pretreated whey	Flask	~8.4 µg/g yeast	Use of continuous illumination (white light)	[28]
Mold					
Blakeslea trispora	Corn steep liquor, glucose, casein acid hydrolysate, yeast extract, L-asparagine, KH_2PO_4, $MgSO_4.7H_2O$, Span 20, Tween 80, linoleic acid, butylated hydroxytoluene, kerosene, thiamine HCl	Flask	704.1 mg/L	Control of O_2 transfer rate	[29]
Mucor circinelloides	Modified Kendrick and Ratledge medium that contain diammonium tartrate	Batch	698.4 µg/g biomass	Use of light stress (continuous illumination)	[30]
Bacteria					
Serratia marcescens	Molasses	Flask	1.1 mg/L	Use of various agro-industrial by-products	[31]
Flavobacterium multivorum	Basal medium	Flask	7.85 µg/mL	Medium modifications (in terms of inorganic salts and urea)	[32]

DCW, dry cell weight; LED, light-emitting diode

has been reported that carotenoids are acquired from red colony-forming yeasts at concentrations ranging from 50 to 800 µg/g DCW [42, 43].

Different bacteria, such as *Flavobacterium multivorum*, *Serratia marcescens*, *Pantoea agglomerans,* and *Erwinia uredovora* species, are also capable of producing *β*-carotene [10]. Bhosale and Bernstein [32] reported that cultural manipulations based on the use of urea and sodium carbonate stimulated *β*-carotene production from *F. multivorum*, and as a result, this bacterium was able to produce 7.85 µg/mL *β*-carotene. They associated this with urea and sodium carbonate significantly inhibiting the formation of zeaxanthin by inhibiting the hydroxylase activity.

3.1 Comparison of different native microbial *β*-carotene producers

To be successful in the market, industrial-scale production of microbial carotenoids must be low cost, high yield, and environmentally friendly. In this context, microorganism groups have various advantages and disadvantages against each other in their suitability for industrial *β*-carotene production (Fig. 2).

Carotenoids such as lycopene and *β*-carotene are currently produced industrially, usually by *B. trispora* [4]. The bioproduction of carotenoids obtained from molds such as *B. trispora* is advantageous because of their high mycelial growth rate and their responsiveness to metabolic stimuli and inhibitors. In addition, the use of agro-industrial wastes as raw materials in production contributes to reducing environmental pollution as well as reducing production costs [35]. However, the fact that *B. trispora* can be unstable under fermentation conditions and as a result sometimes causes low-yield production limits its use in industrial production [6]. Some disadvantages of *B. trispora*, such as the need for shaking cultivation, lead to a decrease in *β*-carotene accumulation and make the industrial use of this microorganism difficult [1]. Additionally, the necessity of growing (+) and (–) sexual mating types of this mold together further complicates the production process [44].

Another important producer of *β*-carotene on an industrial scale is halophilic unicellular photosynthetic marine microalgae such as *D. salina* [45]. When exposed to extreme culture conditions such as high temperature, high light intensity, high salt concentration, and lack of nitrogen sources, *D. salina* is able to accumulate large amounts of *β*-carotene as droplets in the chloroplast stroma of its cells [46]. In addition, production from microalgae is advantageous in terms of carbon footprint compared to production from other microorganisms. Unlike other microorganisms, open pond systems or closed photo-bioreactors are commonly used in the cultivation of *β*-carotene from microalgae. However, a variety of problems can often arise with microalgae during the production and cultivation process, including water loss, lack of carbon source, other essential nutrients, and concerns about weather conditions [47]. Furthermore, the presence of chlorophyll in *D. salina* makes direct extraction of *β*-carotene difficult and reduces yield [46]. Long cultivation time, relatively low cell yield, risk of contamination by protozoa or bacteria, and high sensitivity to adverse weather conditions are other important factors that make it difficult to produce *β*-carotene from microalgae on an industrial scale [48].

Because yeasts are single-celled fungi with a portion to a high growth rate in cheap substrates that can consist of agro-industrial wastes, they have an advantage over microalgae, molds, and bacteria to produce more efficiently. The cost of media components is generally the determining factor in the economic sustainability of most biotechnological productions, and carbon and nitrogen sources can cause more than

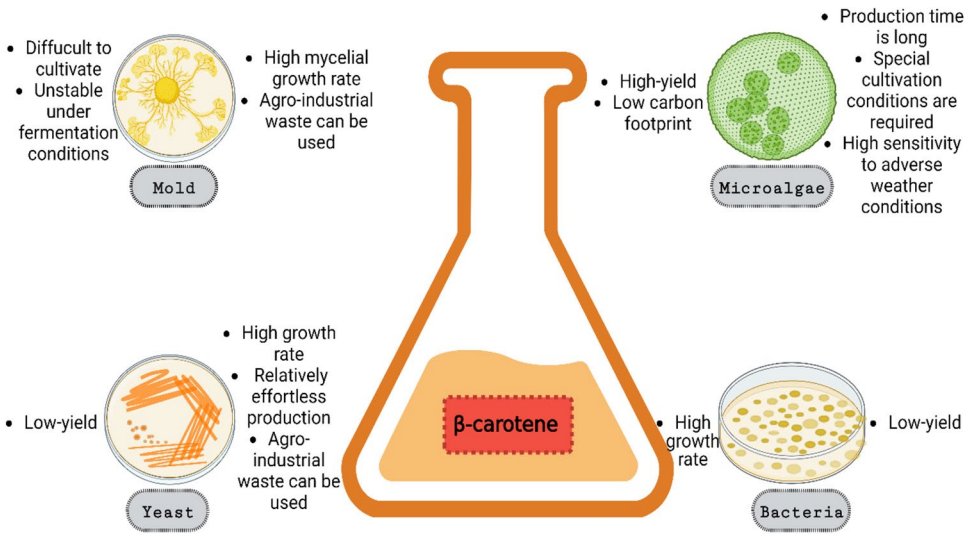


Fig. 2 Advantages and disadvantages of molds, microalgae, yeast, and bacteria in terms of suitability for microbial *β*-carotene production on an industrial scale

one-third of the entire cost of the bioprocess [48]. The fact that a wide variety of agricultural wastes and by-products such as sugar industry wastes (sugarcane/sugar beet bagasse and molasses), crude glycerin obtained from biodiesel production, and even spent brewer's yeast can be used as substrates in the production of β-carotene from yeasts, reduces costs and also contributes to waste management [44]. In addition, it is possible to further reduce the production cost by using the yeast biomass produced to obtain β-carotene and the β-glucan-rich yeast cell wall in animal nutrition, pharmaceutical industry, or cosmetic products. Considering issues such as yield of the β-carotene biosynthesis process, cost, and sustainability, red yeast species such as *Rhodotorula mucilaginosa* stand out [48]. Although the unit carotenoid yield of yeasts such as *R. mucilaginosa* is relatively low compared to *B. trispora* and *D. salina*, the yeasts have important advantages such as adaptability to various environmental conditions, high growth rate, shorter production time, and the relatively effortless obtaining of significant amounts of biomass with ordinary bioreactors [6, 48].

Compared to algae and plants, the utilization of bacteria species to produce β-carotene has many benefits, such as being comparatively reliable, having a fast metabolism, and having a short cycle. However, the poor viability and low final yield of these strains limit their use in commercial β-carotene production [10]. Among bacteria, there are some native carotenogenic species that produce β-carotene, such as *F. multivorum* [4]. However, although bacteria such as yeasts are suitable for large-scale production in fermenters due to their unicellular structure and high growth rate, native carotenogenic bacteria have a low potential for use in commercial β-carotene production, as their carotenoid content is generally low, and they generally do not produce important carotenoids such as β-carotene [49].

In summary, yeasts such as *R. mucilaginosa* have the potential to be used in industrial β-carotene production due to some important advantages they have against microorganisms such as *D. salina* and *B. trispora*, which are widely used in microbial β-carotene production. However, more efficient, and reliable production techniques are needed to meet the growing requisition for β-carotene. Optimizing heterologous β-carotene production via metabolic engineering seems to be the most promising way.

4 Potential of using *Y. lipolytica* as a biotechnological chassis in β-carotene production

In order to realize reliable production using metabolic engineering, several requirements must be met such as using an efficient host for production, harnessing metabolic pathways, selecting effective enzymes and genetic elements, choosing an efficient transformation technology, and preventing the formation of toxic by-products [50]. In selecting a suitable host for a heterologous biosynthesis pathway, characteristics such as ease of cloning, ease of culturing, and suitability of the host for new enzymes and compounds are important [51]. The β-carotene biosynthesis pathway has been introduced and engineered in microorganisms such as *E. coli*, and *S. cerevisiae* as well as hosts such as *Y. lipolytica* due to their existing genetic collections.

E. coli bacteria, which do not have the ability to naturally produce β-carotene, have some advantages such as rapid growth, well-known genetic structure, and easy metabolic engineering. In this context, many studies [52, 53] have been conducted using different metabolic engineering strategies for the production of heterologous β-carotene from *E. coli*. In one of these studies [54], the production of 3.2 g/L of heterologous β-carotene from *E. coli* was achieved. However, the β-carotene yield of *E. coli* still remains quite low, especially compared to *Y. lipolytica*. This is due to factors such as the low efficiency of the native MEP biosynthesis pathway for the supply of IPP and DMAPP and the inability of plant-derived enzymes such as P450s to be functionally expressed in *E. coli* [55]. Additionally, *E. coli* is not as suitable for use in factory food production as *S. cerevisiae* and *Y. lipolytica* [10]. These disadvantages of *E. coli* limit the use of this bacterium in the production of heterologous β-carotene on a commercial scale.

Yeasts are among the most used chassis species in synthetic biology, and some yeast species have well-developed techniques for culturing, cloning, and industrial scale-up, allowing them to be used as cell factories for the production of compounds such as β-carotene [56]. Among these yeast species, *S. cerevisiae* and *Y. lipolytica* stand out because they have mature genetic manipulation tools and are considered GRAS [57]. Both yeast species possess a natural MVA biosynthesis pathway with a high yield for the heterologous synthesis of isoprenoids, including β-carotene. In addition, their resistance to growing environments provides significant convenience for industrial-scale fermentations [14]. The system using *S. cerevisiae* as the host for the production of heterologous β-carotene is partially mature, but the production yield from this yeast is quite low compared to *Y. lipolytica* [10]. Disadvantages such as limited secretion limit the use of *S. cerevisiae* in the production of heterologous β-carotene [55]. Compared to *E. coli* and *S. cerevisiae*, *Y. lipolytica* has some unique advantages for producing heterologous β-carotene and is much more suitable for use in commercial production compared to these two microorganisms.

Although *Y. lipolytica*, a hemiascomycetous oleaginous yeast with a background in industrial practices, cannot naturally synthesize β-carotene, its metabolic, genomic, and physiological properties make it a potential excellent biotechnological chassis for metabolic engineering [50].

β-carotene, a hydrophobic molecule, accumulates in lipid bodies. Therefore, the oleaginous *Y. lipolytica* yeast is thought to be a more appropriate host for *β*-carotene production than non-oleaginous microorganisms such as *S. cerevisiae* or *E. coli* [13]. *Y. lipolytica* contains a sufficient amount of lipid bodies to store liposoluble pigments, as well as an excess of acetyl-CoA, which can be utilized as a precursor molecule in both lipid and *β*-carotene biosynthesis pathways [58]. Moreover, the genome of *Y. lipolytica* yeast has all the necessary genes in charge of obtaining GGPP [59]. *Y. lipolytica* can utilize various carbon sources such as fatty acids, glycerol, and alkanes via the active tricarboxylic acid cycle and pentose phosphate pathway [14]. The capability of *Y. lipolytica* to utilization multiple carbon sources like glucose, fructose, glycerol, and inexpensive hydrophobic substrates to grow and to have a high carbon flux toward acetyl-CoA are other features that increase the potential of this species to be utilized for heterologous *β*-carotene obtaining [60]. Concerns toward recombinant products still persist among a significant part of the public, whereas, in a study [61] to determine the safety of *β*-carotene acquired from engineered *Y. lipolytica*, recombinant *β*-carotene was shown to be as safe as its commercial counterparts (synthetic and natural).

Because of all these superior properties, *Y. lipolytica* is aforethought a very hopeful biotechnological chassis to produce heterologous *β*-carotene.

5 Metabolic engineering approaches applied to increase the production *β*-carotene in *Y. lipolytica*

Using genetic and metabolic engineering techniques, microbial carotenoid biosynthesis can be significantly improved. Carotenoid production in microorganisms can be increased by a wide variety of practices, such as optimization of biosynthetic pathways, insertion of foreign genes to increase flux in metabolic pathways, and adaptive laboratory evolution. Because the synthesis of all carotenoids relies on a common precursor, the carotenoid biosynthesis pathway is particularly can be manipulated with recombinant DNA tools. A wide variety of carotenoids can be synthesized by combining genes involved in carotenoid synthesis [5]. For example, it is possible to engineer non-carotenogenic microorganisms like *E. coli*, *S. cerevisiae*, *Candida utilis*, and *Y. lipolytica* to obtain conventional or new carotenoids following transformation with carotenoid biosynthesis genes derived from carotenogenic microorganisms [62]. Among these hosts, the highest productivity so far has been obtained from *Y. lipolytica*, as it exhibits the most favorable properties for *β*-carotene production [63, 64]. Therefore, some companies such as DuPont and Microbia prefer to use *Y. lipolytica* as the host to produce carotenoids [50].

To increase *β*-carotene production from *Y. lipolytica* so far, this host has been engineered using a variety of strategies. For this purpose, in addition to strategies to increase the amount of precursors and modulate lipid storage, various strategies such as biosynthetic pathway engineering, cofactor engineering, protein engineering, morphological modification, and fermentation optimization are frequently used (Fig. 3).

5.1 Increasing the amount of precursors

To enhance the synthesis of acetyl-CoA-derived compounds such as carotenoids, central carbon metabolism must be engineered to provide sufficient precursors [57]. Acetyl-CoA is an important node in central metabolism, and much research has focused on the metabolic engineering of acetyl-CoA metabolism for the production of valuable compounds such as carotenoids.

In *Y. lipolytica*, a significant fraction of cytosolic acetyl-CoA is produced by the cleavage of mitochondria-derived citrate into oxaloacetate and acetyl-CoA by ATP-citrate lyase [65]. Therefore, cytosolic acetyl-CoA level is possible by the continuous flow of citrate from the mitochondria. In a study [66] based on inhibiting the catalytic activity of isocitrate dehydrogenase to increase citrate efflux, lycopene content was significantly increased by overexpressing the *AMPD* gene (encoding AMP deaminase) in *Y. lipolytica*. To increase the cytosolic acetyl-CoA supply in *Y. lipolytica*, different strategies have also been tried. In one of these approaches [67], the phosphoketolase-phosphotransacetylase pathway was introduced to increase cytosolic acetyl-CoA supply and rechannel carbon to target products, and after modifying the endogenous pathway, an approximately 280-fold increase (with production of approximately 1 g/L) in *β*-ionone titer occurred compared to the baseline strain. Xu et al. [68], in *Y. lipolytica*, engineered five alternative cytosolic acetyl-CoA pathways, namely pyruvate-acetate, pyruvate-aldehyde, pyruvate formate lyase, acetyl-CoA shuttle, and non-oxidative pentose-phosphate, and investigated the effect of different pathways on lipid titer and oil content. As a result, they determined that the alternative cytoplasmic acetyl-CoA pathway has a very important function in lipid biosynthesis. In addition to increasing acetyl-CoA biosynthetic fluxes, an alternative way to ensure sufficient acetyl-CoA for the production of metabolites such as carotenoids is based on restricting acetyl-CoA flux to competing pathways such as the glyoxylate cycle in *Y. lipolytica*. In this context, deleting the *cit2* (encoding peroxisomal citrate synthase) and *mls1* (encoding malate synthase) genes to prevent acetyl-CoA consumption in the glyoxylate cycle may promote cytosolic acetyl-CoA accumulation. In fact, Huang et al. [69] reported that removing the *cit2* gene resulted in a two-thirds increase in acetyl-CoA concentration, promoting

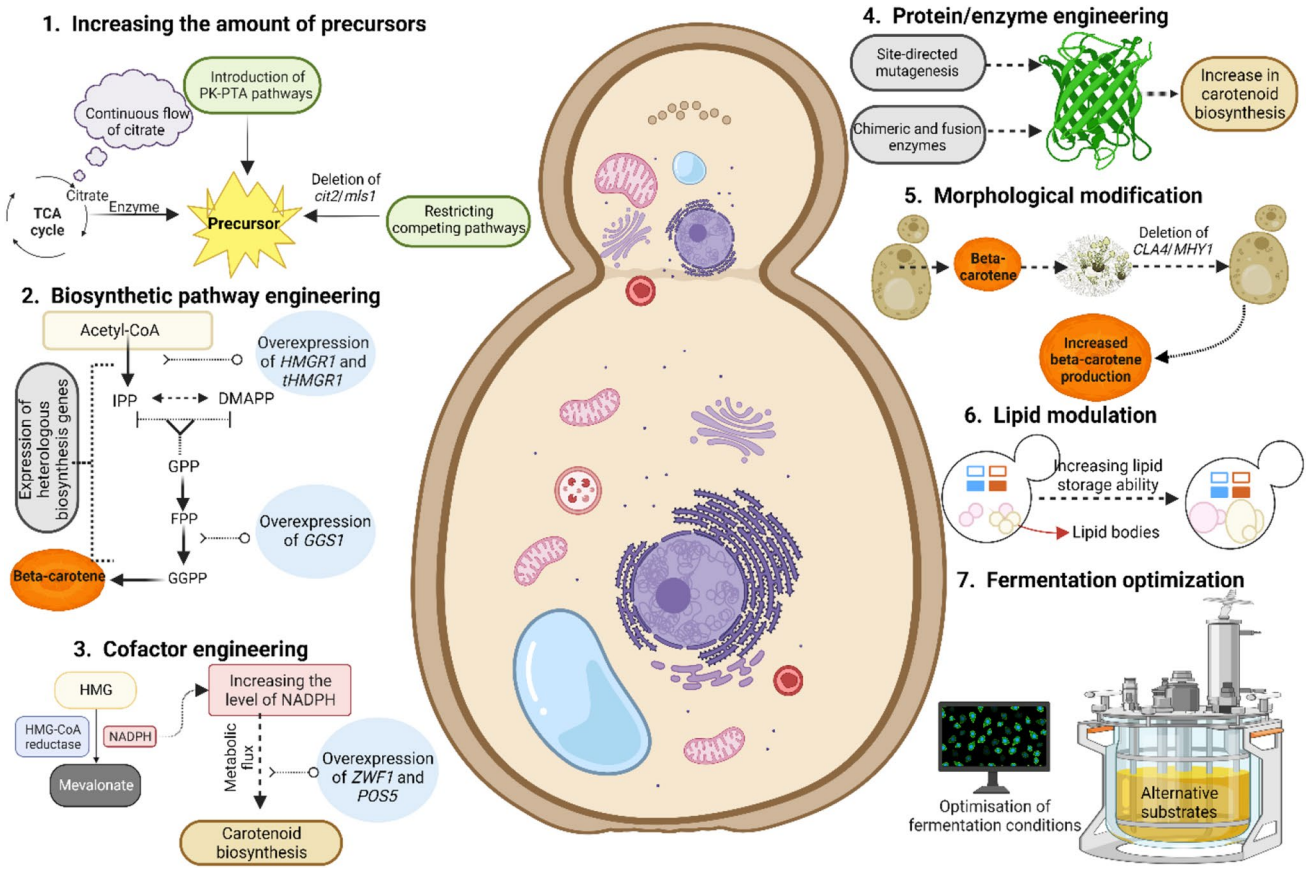


Fig. 3 Schematic representation of engineering strategies to increase *β*-carotene production in *Yarrowia lipolytica*. (1) Strategies to increase the amount of precursors. (2) Biosynthetic pathway engineering strategies. (3) Cofactor engineering strategies. (4) Protein and enzyme engineering strategies. (5) Morphological modification strategies. (6) Strategies for modulation of lipid storage. (7) Strategies for fermentation optimization and alternative substrate usage. PK-PTA: phosphoketolase-phosphotransacetylase, Acetyl-CoA: acetyl coenzyme A, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, GPP: geranyl diphosphate, FPP: farnesyl diphosphate, GGPP: geranylgeranyl diphosphate, HMG-CoA: 3-hydroxy-3-methyl-glutaryl-CoA

squalene production. In light of all these data, it can be said that increasing cytosolic acetyl-CoA flux is an effective strategy to increase *β*-carotene biosynthesis in *Y. lipolytica*.

5.2 Biosynthetic pathway engineering

As in other yeasts, the production of carotenoid precursors DMAPP and IPP from acetyl-CoA takes place in the MVA pathway in *Y. lipolytica* yeast [18]. Therefore, there must be sufficient flow in the MVA pathway [70]. The HMG-CoA reductase enzyme is the main rate limiter of the MVA pathway, and overexpression of *HMGR1*, which encodes this enzyme, is one of the frequently applied metabolic engineering strategies because it can direct the precursor to *β*-carotene synthesis. In this context, overexpression of intact or N-terminally truncated *HMGR1* (*tHMGR1*) can increase *β*-carotene production. Kildegaard et al. [71], in their study comparing *HMGR1* and *tHMGR1* variants, determined that native *HMGR1* was superior in terms of *β*-carotene production in *Y. lipolytica*.

DMAPP and IPP are converted to GPP in the presence of the enzyme GPP synthase, and then GPP is converted to FPP and GGPP. If engineered rightly, the high flux of acetyl-CoA in *Y. lipolytica* cell should result in the generation of more DMAPP and IPP followed by GGPP for carotenoid synthesis [50]. *Y. lipolytica* can synthesize GGPP via the natural MVA pathway, but to synthesize *β*-carotene from GGPP, heterologous genes encoding the enzymes phytoene synthase, lycopene cyclase and phytoene dehydrogenase involved in the downstream pathway must be inserted into its genome [72]. If *GGS1* (encoding GGPP synthase enzyme) activity is insufficient, the by-product squalene biosynthesis increases, and the metabolic flow toward *β*-carotene biosynthesis decreases. Therefore, accelerating the conversion of FPP to GGPP in *Y. lipolytica* cell by overexpression of the *GGS1* gene causes greater *β*-carotene production [11]. As a matter of fact, in the study performed by Zhang et al.

[11], β-carotene content increased due to the accelerated conversion of FPP to GGPP by overexpressing *GGS1* in *Y. lipolytica*. Another effective strategy for further production of DMAPP and IPP in *Y. lipolytica* cell is based on the upregulation of natural MVA pathway genes and the downregulation of side fluxes, for example toward squalene. To achieve this, heterologous β-carotene biosynthesis genes (*crtE*, *crtYB*, and *crtI*) need to be expressed in *Y. lipolytica* cell [73].

In different previous works, it was reported that overexpression of *GGS1*, *carRP* (encoding phytoene synthase/lycopene cyclase enzyme), and *carB* (encoding phytoene dehydrogenase enzyme) genes led to more β-carotene synthesis (Table 2). In the first study on the synthesis of β-carotene from engineered *Y. lipolytica* reported by Gao et al. [74], at a concentration of 2.22 mg/g DCW β-carotene was obtained from *Y. lipolytica* by expressing the *carRP* and *carB* (from *M. circinelloides*) and *GGS1* genes. In another study Gao et al. [13], found that overexpression of MVA pathway genes led to a 46.9% increase in the amount of β-carotene. In the study by Kildegaard et al. [71], 453.9 mg/L of β-carotene was obtained from the best strain by integrating the *crtYB* and *crtI* genes (from *X. dendrorhous*) into *Y. lipolytica* strains and optimizing *HMGR1* and *GGS1*/*crtE*. Additional downregulation of the competitor enzyme squalene synthase SQS1 resulted in an enhancement in the β-carotene titer of approximately 797 mg/L. In a study carried out by Larroude et al. [59] using a combinatorial strategy based on Golden Gate assembly for the optimization of promoter–gene pairs of the heterologous Crt pathway, several lipids overproducing strains were integrated with multiple copies of the biosynthetic genes (*carB*, *carRP*, and *GGS1*) and the best strain synthesized 6.5 g/L titer of

Table 2 β-carotene production from genetically engineered *Yarrowia lipolytica*

Applied engineering strategy	Carbon source	Fermentation mode	β-carotene content	References
Expression of *carRP* and *carB* (from *Mucor circinelloides*) and *GGS1* genes	Glucose	Flask	2.2 mg/g DCW	[74]
Expression of *carS* (from *Schizochytrium* sp.) and *GGS1* genes	Glucose	Flask	0.41 mg/g DCW	[76]
Overexpression of *carRP* and *carB* (from *M. circinelloides*), *tHMGR1*, *GGS1*, *erg8*, *erg10*, *erg12*, *erg13*, *erg19*, *erg20,* and *IDI1* genes	Glucose	Fed-batch	4 g/L	[13]
Integrating the *crtYB* and *crtI* (from *X. dendrorhous*) genes and optimizing *HMGR1* and *GGS1*/*crtE* genes	Glucose	Microtiter plate	453.9 mg/L	[71]
Overexpression of *carRP* and *carB* (from *M. circinelloides*), *GGS1,* and *tHMGR1* genes	Glucose and glycerol (used separately, no difference in titer and yield)	Fed-batch	6.5 g/L	[59]
Overexpression of *carRP* and *carB* (from *M. circinelloides*) genes	Glucose	Flask	75 mg/L	[77]
Overexpression of *carRP* and *carB* (from *M. circinelloides*) and *GGS1* genes	Glucose	Flask	50.1 mg/L	[78]
Overexpression of *carB* and *carRA*, *tHMG,* and *GGS1* genes	Glucose	Fed-batch	2.01 g/L	[79]
Overexpression of *carB* and *carRA* (from *Blakeslea trispora*), *tHMGR1*, *GGS1, erg13,* and *Hxk* genes	Glucose	Fed-batch	2.4 g/L	[60]
Overexpression of *carRP* and *carB* (from *M. circinelloides*), *GGS1*, *HMGR1,* and *erg13* genes	Glucose	Fed-batch	4.5 g/L	[11]
Overexpression of *carB*, *carRA*, *GGS1*, *tHMG,* and *DID2* genes	Glucose	Fed-batch	~2.6 g/L	[80]
Overexpression of *carB* and *carRA* (from *B. trispora*), *tHMGR1*, and *GGS1* genes	Glucose	Fed-batch	1.7 g/L	[58]
Overexpression of *carRP* and *carB* (from *M. circinelloides*), *AtoB*, *erg13*, *HMGR1*, *erg8*, *erg12*, *erg19*, *erg20*, *IDI*, *GGS1*, and VHb genes and deletion of *CLA4*, and *MHY1* genes	Glucose	Fed-batch	7.6 g/L	[63]
Overexpression of *carRP* and *carB* (from *M. circinelloides*), *erg12*, *erg20*, *tHMGR1*, *crtE*, *IDI*, *CK,* and *IPK* genes	Glucose	Fed-batch	39.5 g/L	[64]

DCW, dry cell weight

β-carotene. Non-homologous end-joining control DNA repair at *Y. lipolytica*, making targeted integrations complicated. CRISPR/Cas9 system has been engineered to utilize a guide RNA sequence to cause a double-stranded break at a particular locus to overcome this restriction and enable more suitable targeted modifications. This double-stranded break can later be repaired utilizing donor DNA. By producing an indel in the lack of donor DNA, the rapid knockout phenotype is established, thus allowing rapid and successful engineering of *Y. lipolytica* [75]. In a study carried out by Zhang et al. [11], *carB* and *carRP* genes (from *M. circinelloides*) were inserted into the *Y. lipolytica* genome using the CRISPR/Cas9 strategy to generate a *Y. lipolytica* starter platform to acquire *β*-carotene. The insertion of the *carB*, and *carRP* genes to the selected *Y. lipolytica* strain caused the heterologous *β*-carotene synthesis. In addition, *β*-carotene production was effectively enhanced (approximately 408 mg/L, 24-fold enhancement compared to parental strain) by enhancing copy numbers of these genes (*carB* and *carRP*) and by overexpressing the genes *GGS1*, *erg13*, and *HMGR1*, which are genes associated with the MVA biosynthesis pathway. In addition to genetic engineering, the researchers were able to obtain 4.5 g/L *β*-carotene in the production they carried out in the bioreactor, with the optimization of the culture (Table 2) [76–80].

The MVA pathway is the most important pathway of isoprenoid production in yeasts, but among the various challenges in isoprenoid synthesis, producing sufficient amounts of IPP and DMAPP is generally considered to be one of the primary bottlenecks. The use of native pathways may lead to an inefficient IPP and DMAPP acquisition rate because it requires numerous enzymatic steps and cofactor regeneration and competes with the central carbon metabolism [81, 82]. For these reasons, the natural MVA pathway may be insufficient to produce the precursors required for isoprenoid biosynthesis on a large scale. In addition to offering advantages such as being less complex and separated from the central carbon metabolism compared to the MVA pathway, the heterologous pathway called the Isopentenol Utilization Pathway (IUP) requires fewer enzymatic steps for the synthesis of IPP and DMAPP and only ATP as cofactor; compared to the MVA pathway it has the capacity to increase IPP and DMAPP levels by 15.7 times [83]; and this may increase carotenoid production. In another study by Ma et al. [64], researchers introduced the IUP in *Y. lipolytica* through the expression of Isopentenyl Phosphate Kinase (IPK) and Choline Kinase (CK) and observed that β-carotene content increased by 23%. All these results show that biosynthetic pathway engineering is a highly effective strategy to increase *β*-carotene production.

5.3 Cofactor engineering

The key cofactor in carotenoid synthesis in yeast is NADPH. NADPH acts as a cofactor in the reduction of HMG to MVA by HMG-CoA reductase. Therefore, by controlling the NADPH level, it is possible to direct the metabolic flux toward carotenoid biosynthesis. To increase NADPH supply, cytosolic NADPH pathways consisting of different enzymes such as glucose-6-phosphate dehydrogenase (*ZWF1*), mannitol dehydrogenase (*MnDH1* and *MnDH2*), NADP-specific isocitrate dehydrogenase (*IDP2*), NADP-specific glutamate dehydrogenase (*GDH3*), 6-phosphogluconate dehydrogenase (*GND2*), succinate semialdehyde dehydrogenase (*UGA2*), and malic enzyme (*MAE1*) have been examined [56]. It was determined that the enzyme mannitol dehydrogenase (*MnDH2*) was successful in increasing squalene production [84]. In another study [85], overexpressing *ZWF1* and *POS5* (encoding NADH kinase enzyme) genes separately in carotenoid-producing recombinant *S. cerevisiae* resulted in an increase in *β*-carotene yield. Researchers also reported that overexpression of *POS5* was more effective than overexpression of *ZWF1*. In conclusion, it can be said that increasing NADPH supply has the potential to increase *β*-carotene synthesis in *Y. lipolytica*, but further research is needed on this topic.

5.4 Protein and enzyme engineering

The results obtained in some studies conducted in recent years indicate that protein and enzyme engineering approaches may be effective in order to increase the synthesis efficiency of carotenoids. Various approaches have been used for this purpose, such as combining proteins that catalyze successive biocatalytic steps or coupling enzymes to supporting protein partners. Site-directed mutagenesis technique can be used to improve enzyme stability and substrate specificity. As a matter of fact, in the study carried out by Ma et al. [64] to eliminate the substrate inhibition effect of lycopene cyclase through protein engineering using this technique, a *CarRP* variant was obtained in which lycopene substrate inhibition could be successfully eliminated without a decrease in enzymatic activity, and a significant increase in the amount of *β*-carotene was achieved by expressing this variant.

It has been reported that *β*-carotene production efficiency increases with the engineering of chimeric and fusion enzymes. Spatial clustering of enzymes has been shown to be an effective approach to optimize metabolite transfer between enzymes in synthetic metabolic pathways, and among the methods used to promote colocalization, enzyme fusion stands out for being easy and effective. In their study investigating the metabolic consequences of the spatial rearrangement of the catalytic domains of *X. dendrorhous*

carotenoid enzymes produced in *S. cerevisiae*, Rabeharindranto et al. [86] reported that the β-carotene production of *S. cerevisiae* approximately doubled with the expression of a tridomain enzyme fusion protein covering *CrtB*, *CrtI*, and *CrtY*.

These results indicate that protein and enzyme engineering techniques, such as site-directed mutagenesis and fusion of enzymatic domains, can increase β-carotene production efficiency. However, further research is required to elucidate the effect of these techniques on *Y. lipolytica*.

5.5 Morphological modification

In recent years, different strategies have also begun to be implemented to obtain more β-carotene from the engineered *Y. lipolytica*. In one of these [63], 7.6 g/L β-carotene was acquired by the metabolic engineering and morphological modification of *Y. lipolytica*. This yeast is a dimorphic species capable of transitioning from yeast to mycelium under stressful conditions. However, the mycelial form is not suitable for large-scale production and thus for utilization in industrial fermentations [63]. First, the researchers engineered strains of *Y. lipolytica* that synthesized β-carotene through the integration of multiple copies of 13 genes involved in the biosynthesis pathway of β-carotene. However, increasing the quantity of β-carotene led to the replacement of the oval-shaped yeast form with pseudohyphae or hyphae. This indicates that β-carotene accumulation in *Y. lipolytica* cell causes a morphological transition. Thereupon, the *Y. lipolytica* strains were morphologically modified by deletion of the *CLA4* and *MHY1* genes (key genes in pathways controlling morphological transition) in order to convert the mycelium back to the oval-shaped, and this approach resulted in a 139% enhancement in β-carotene production. As in this example, new strategies to be developed as a result of elucidating the morphological characteristics of *Y. lipolytica* seem to be a promising approach to increasing β-carotene production.

5.6 Lipid storage modulation

As mentioned previously, lipophilic terpenoids such as β-carotene are often stored in hydrophobic areas such as membranes or lipid bodies because they are difficult to dissolve in the hydrophilic cytoplasmic environment [87]. Further enhancing the already good lipid synthesis and storage ability of lipoyeast *Y. lipolytica* through metabolic modulation can increase β-carotene production. The most commonly applied strategy to achieve this is to increase the intracellular lipid content, as this will allow more carotenoid accumulation. As a matter of fact, Matthäus et al. [88] reported that lycopene accumulation in *Y. lipolytica* is associated with increased lipid content. These results were supported by the demonstration by Larroude et al. [59] that there was a positive correlation between β-carotene and lipid contents in an engineered obese *Y. lipolytica* strain that overproduced lipids and that increasing the lipid accumulation capacity increased the β-carotene titer. Luo et al. [83] increased de novo lipid synthesis through overexpression of *ACC1* (acetyl-CoA carboxylase) and *DGA1* (diacylglycerol acyltransferase) genes in *Y. lipolytica* and observed that the accumulation of precursors such as GPP, FPP, and GGPP increased significantly, resulting in an increase in lycopene accumulation. In a few studies, a different approach was applied and fatty acids such as oleic acid and palmitoleic acid were added to the medium and it was shown that this stimulates the production of β-carotene [89]. As a result, significant gains in β-carotene production can be achieved with strategies to increase the lipid synthesis and storage ability of *Y. lipolytica* yeast.

5.7 Fermentation optimization and alternative substrate usage

Another effective strategy that can be applied to increase β-carotene production yield is based on the optimization of fermentation conditions. Lipid accumulation in the cell is related to the enhancement of carotenoid content, and a higher carbon/nitrogen ratio in the fermentation medium in which cells are developed is known to promote lipid and carotenoid accumulation in the cell [90]. Indeed, Gao et al. [13] determined that using a 3:1.5 (wt/wt) carbon/nitrogen ratio in the cultivation of engineered *Y. lipolytica*, they produced higher yields (increased from 29.12 to 49 mg/g DCW) when compared to those using a 2:3 (wt/wt) carbon/nitrogen ratio. Glucose is generally preferred as the carbon source to produce β-carotene from the engineered *Y. lipolytica* (Table 2). In this context, various studies have attempted to increase the glucose utilization capacity of *Y. lipolytica*. For example, Qiang et al. [60] overexpressed the *Hxk* gene, which encodes the hexokinase enzyme that catalyzes the phosphorylation of glucose in the first step of glycolysis. In their study where they examined the effect of hexokinase on glucose consumption and β-carotene accumulation, they determined that increased hexokinase activity increased the accelerated glucose utilization rate and β-carotene content by 98% (increased to 4.63 mg/g DCW). However, there is a need to use cheap carbon sources for sustainable production. In this context, studies on determining the potential of alternative substrate sources such as glycerol, sugars other than glucose, different lipids, acetate, and lignocellulosic materials in bioprocesses are increasing day by day. For example, Larroude et al. [59] utilized glucose and glycerol (as a cheaper carbon source) in the cultivation of engineered *Y. lipolytica* and observed no significant difference in β-carotene production titer and yield. However, *Y. lipolytica*

cannot naturally metabolize xylose, the main pentose found in lignocellulosic materials, whereas its cheapness and widespread availability in nature can make the use of xylose as a substrate in bioprocesses very advantageous. For this reason, various metabolic engineering studies have been carried out to ensure that *Y. lipolytica* can metabolize xylose. In one of these studies [91], overexpression of genes encoding heterologous xylose reductase, xylitol dehydrogenase, and endogenous xylulokinase was found to enable engineered *Y. lipolytica* to grow efficiently on xylose.

Many yeast fermentations result in low biomass yields due to the Crabtree effect [92]. In order to prevent this, besides controlling the glucose concentration in the environment continuously, it is necessary to work in an aerobic environment. This is also pertaining to the efficiency of obtaining β-carotene from *Y. lipolytica*. Relatively, Lv et al. [79] observed that the amount of β-carotene obtained from engineered *Y. lipolytica* increased approximately 1.28 times with DO-stat (dissolved oxygen feedback control) fed-batch fermentation in proportion to that obtained with fed-batch fermentation.

Based on these results, it is possible to say that β-carotene can be produced cheaper and in higher amounts from engineered *Y. lipolytica* strains, with the data obtained from different fermentation optimization studies and alternative substrate utilization strategies in future.

6 Conclusions and future perspectives

With a better understanding of the health benefits of β-carotene, as well as its coloring properties, the demand for this product has increased in recent years. In parallel, research on low-cost and sustainable β-carotene production has increased to meet this need. As a result, it became possible to produce heterologous β-carotene at high efficiency through metabolic and genetic engineering applications. The metabolic, genomic, and physiological properties of the oleaginous yeast *Y. lipolytica* are more suitable for β-carotene production than *E. coli* and *S. cerevisiae*, which are conventionally used in metabolic engineering. The current review reveals different engineering strategies used to increase heterologous β-carotene production from *Y. lipolytica*.

There are studies reporting that directing metabolic flux to cytosolic acetyl-CoA increases β-carotene production, but the production and regulation of cytosolic acetyl-CoA have not yet been fully elucidated. It has been shown in many studies that biosynthetic pathway engineering is more effective in increasing the amount of β-carotene. Increasing cofactor supply has the potential to increase β-carotene synthesis in *Y. lipolytica*, but the topic needs to be addressed in more detail. It has been reported that the engineering of chimeric and fusion enzymes causes an increase in β-carotene production efficiency in different microorganisms. However, the effects of these strategies on *Y. lipolytica* need to be examined. Strategies to increase the lipid storage ability of *Y. lipolytica* are also effective in increasing the amount of β-carotene. Bringing *Y. lipolytica* the ability to successfully metabolize alternative substrates such as xylose by using various strategies is very promising in reducing the cost of β-carotene. In addition, studies carried out in recent years show that new strategies such as morphology engineering are also successful in increasing β-carotene production in *Y. lipolytica*. As a result of these strategies, although great progress has been made in the β-carotene production efficiency of *Y. lipolytica*, there are still some bottlenecks.

The most important limitation to engineering applications for the assembly and regulation of metabolic flows is that there is still not enough basic knowledge about the metabolic and physiological properties of *Y. lipolytica*. Additionally, more information is needed on the expression of heterologous enzymes and the balancing of non-native pathways in *Y. lipolytica*. Expression of heterologous enzymes can cause a metabolic burden, leading to an imbalance of metabolic fluxes and the formation of toxic intermediates. The increasing complexity of new pathways introduced into *Y. lipolytica* may also cause flow imbalance, resulting in decreased efficiency. For these reasons, the effects of all these applications on the reliable, efficient, and low-cost β-carotene production from *Y. lipolytica* need to be investigated in detail and precisely with further studies, using approaches such as metabolomics, genomics, transcriptomics, and proteomics.

As a result, although very successful results have been obtained with the various strategies implemented so far, it is thought that exciting results will be obtained in β-carotene biosynthesis from *Y. lipolytica* by better elucidating the metabolic and physiological properties of *Y. lipolytica*, developing effective genetic tools and applying different engineering strategies.

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Declarations

Conflict of interest None of the authors of this work have any conflict of interest.

Ethical approval Neither ethical approval nor informed consent was required for this study.

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