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Building Synthetic Yeast Factories to Produce Fat-soluble Antioxidants

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Fat-soluble antioxidants play a vital role in protecting the body against oxidative stress and damage. The rapid advancements in metabolic engineering and synthetic biology have offered a promising avenue for economically producing fat-soluble antioxidants by engineering microbial chassis. This review provides an overview of the recent progress in engineering yeast microbial factories to produce three main groups of lipophilic antioxidants: carotenoids, vitamin E, and stilbenoids. In addition to discussing the classic strategies employed to improve precursor availability and alleviate carbon flux competition, this review delves deeper into the innovative approaches focusing on enzyme engineering, product sequestration, subcellular compartmentalization, multistage fermentation, and morphology engineering. We conclude the review by highlighting the prospects of microbial engineering for lipophilic antioxidant production.

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Introduction

Fat-soluble antioxidants are crucial for safeguarding lipid-rich structures against oxidative damage and reducing the risk of chronic diseases. These antioxidants are indispensable components of a balanced diet and find diverse applications across various industries, including food, cosmetics, nutraceuticals, pharmaceuticals, and animal feed. Traditionally, the extraction of valuable compounds like lipophilic antioxidants has heavily relied on natural sources or chemical synthesis. However, the production of these antioxidants from natural sources, such as plants, often faces significant challenges. Fluctuations in both content and effectiveness pose obstacles associated with limited yield and costly cultivation practices. Additionally, extraction and purification expenses add to the difficulties, along with concerns regarding sustainability. Chemical synthesis processes, on the other hand, come with their own set of drawbacks. They are associated with adverse environmental impacts and encounter inherent difficulties in achieving the desired stereochemistry and regiochemistry for complex structures. Restricted yields further complicate matters, and adherence to stringent safety and regulatory standards is necessary before marketing these compounds as dietary supplements or cosmetic additives. The rapid advancements in metabolic engineering and synthetic biology have paved the way for scalable, controllable, and sustainable microbial production systems, presenting cost-effective biomanufacturing processes. This review specifically focuses on the progress made in engineering yeasts as chassis organisms for the production of three essential families of lipophilic antioxidants: carotenoids, vitamin E, and stilbenoids. These antioxidants rely on the mevalonate (MVA) pathway, the shikimate pathway, or a combination thereof to access precursor molecules. The review will highlight recent innovative strategies, including enzyme engineering, product sequestration, subcellular compartmentation, multistage fermentation, and morphology engineering, all aimed at enhancing the production of these valuable fat-soluble antioxidants.

Biosynthesis of carotenoids and vitamin A

Carotenoids are lipophilic terpenoid metabolites that act as pigments, producing vibrant yellow, orange, and red colors. They can be classified into two groups: carotenes,

which are hydrocarbon-based (such as α-carotene, β-carotene, and lycopene), and xanthophylls, which are more polar due to the presence of oxygen functional groups (such as lutein and zeaxanthin) (Figure 1a). Specially in human, carotenoids cannot be synthesized internally, and therefore their uptake relies only on dietary sources. Certain carotenoids have the ability to undergo specific enzymatic conversions in the intestine, resulting in the synthesis of vitamin A, which is crucial for vision, cellular communication, immune responses, growth, development, and reproductive system health [1]. There are two sources of vitamin A in the diet: (1) pre-formed forms, which include retinol, retinal, retinoic acid, and retinyl esters; and (2) provitamin A carotenoids, such as β-carotene, β-cryptoxanthin, and α-carotene. In addition, there are non-provitamin A carotenoids, such as lycopene, lutein, zeaxanthin, and astaxanthin. They have been associated with a diverse array of health benefits, largely attributed to their antioxidant properties.

The biosynthesis of carotenoids involves two modules: the upstream isoprene synthesis module, which converts a carbon source to geranylgeranyl pyrophosphate (GGPP), and the downstream heterologous module responsible for carotenoid synthesis (Figure 1a). In the upstream module, acetyl-CoA (acetyl coenzyme A) undergoes condensation through the MVA pathway, resulting in the production of 5-C isomers, namely isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These molecules are then sequentially condensed by farnesyl pyrophosphate synthetase (FPPS) and geranylgeranyl pyrophosphate/diphosphate synthase (GGPPS) to form GGPP. GGPP undergoes sequential conversions catalyzed by phytoene synthase (CarRP or CrtYB), phytoene desaturase (encoded by CarB or CrtI), and lycopene cyclase (LCY). LCY exists in two forms: lycopene β-cyclase (LCYB, CarRP, or CrtYB) and lycopene ε-cyclase (LCYE). The utilization of LCYB or LCYE, either individually or in combination, determines the production of β-carotene, ε-carotene, or α-carotene, respectively. Among these, β-carotene and α-carotene are the most produced carotene molecules. The former serves as the precursor for retinal, retinol, zeaxanthin, and astaxanthin in commercial products, whereas the latter serves as the precursor for the commercial product lutein.

Biosynthesis of vitamin E

Vitamin E, or tocochromanols, is a group of lipid-soluble compounds consisting of four tocopherols and four tocotrienols (α, β, γ, and δ forms) with methyl substitutions on the phenolic ring (Figure 1b). These compounds have an amphipathic structure, with a polar chromanol head group derived from homogentisic acid (HGA) and a lipophilic isoprenoid-based hydrocarbon tail obtained from either GGPP for tocotrienols or phytyl-pyrophosphate for tocopherols [2]. Tocotrienols differ from tocopherols in their unsaturated isoprenoid side chain, which contains three trans double bonds, potentially aiding their penetration into tissues with saturated fatty layers like the brain and liver [3]. Natural vitamin E is exclusively synthesized by photosynthetic organisms such as plants and algae, primarily located in the chloroplast envelope and stored in the stroma [4].

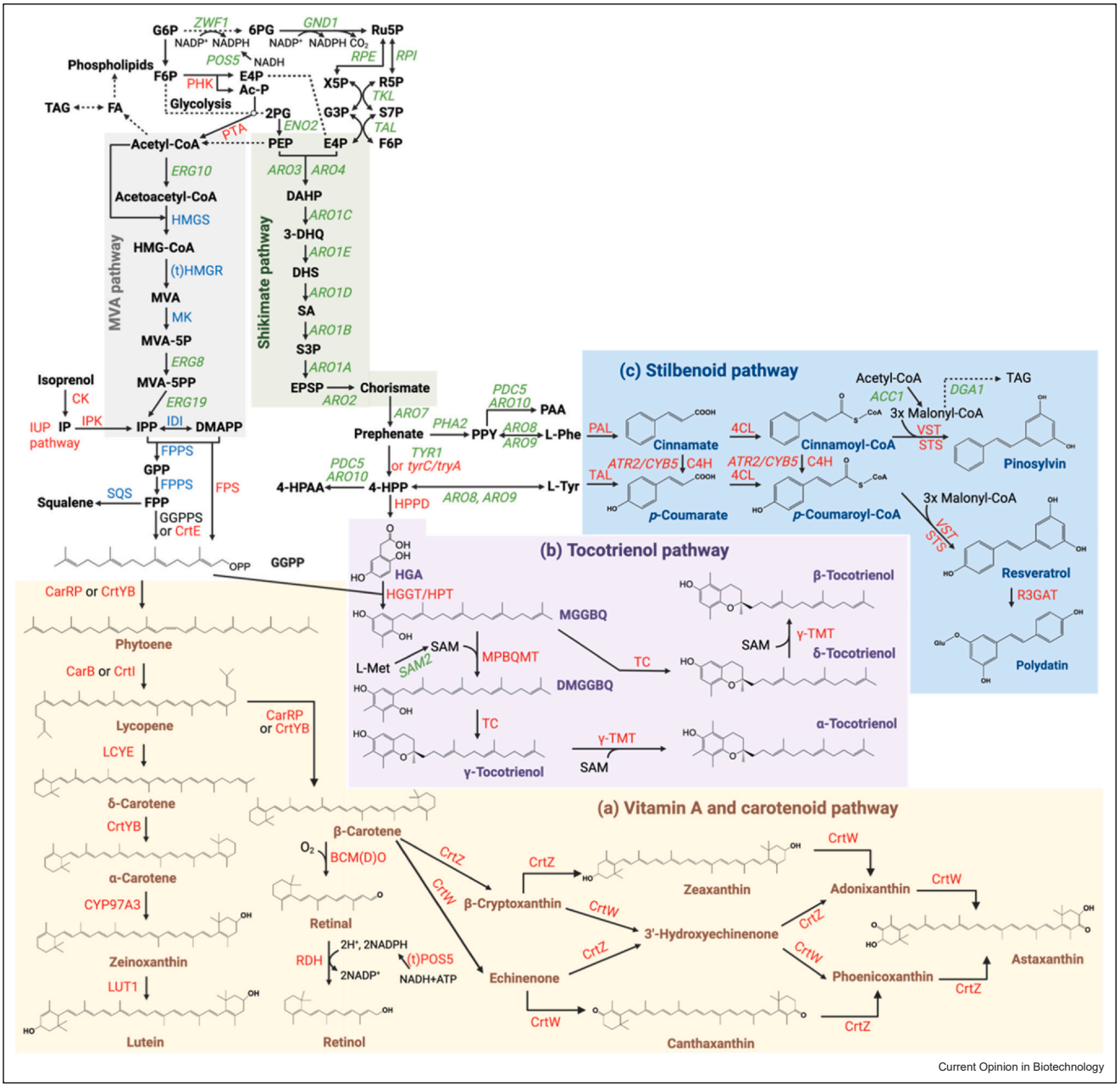
Vitamin E biosynthesis involves two essential precursors: GGPP and HGA (Figure 1b). The shikimate pathway intermediate, 4-hydroxyphenylpyruvate, is converted to HGA via the action of 4-hydroxyphenylpyruvate dioxygenase (HPPD). In the downstream module, the process begins with the condensation of GGPP and HGA, facilitated by either homogentisate geranylgeranyl transferase (HGGT) or homogentisate phytyltransferase (HPT), forming 2-methyl-6-geranylgeranyl benzoquinol (MGGBQ). Subsequently, MGGBQ undergoes methylation and/or cyclization by three essential enzymes: 2-methyl-6-phytylbenzoquinol methyltransferase (MPBQMT), tocopherol cyclase (TC), and γ-tocopherol methyltransferase (γ-TMT). The specific presence and sequence of these enzymatic reactions result in the synthesis of tocotrienols in the δ, γ, α, and β forms.

Biosynthesis of stilbenoids

Stilbenoids and flavonoids are two related families of plant-derived secondary metabolites with known health benefits [5]. Stilbenoids, hydroxylated derivatives of stilbenes, have a C6-C2-C6 chemical structure, with resveratrol being the most common example found in grapes, berries, and peanuts (Figure 1c). Flavonoids, characterized by a three-ring skeleton (C6-C3-C6), are more diverse due to various substitutions and modifications, leading to subgroups like anthocyanins, flavanols, flavanones, and more [6]. Stilbenoids, with their lipophilic aromatic ring structure, are typically more soluble in organic solvents, rendering them more fat-soluble. On the other hand, flavonoids are generally regarded as water-soluble due to their possession of multiple hydroxyl groups and other polar functional groups in their chemical structures. Several strain engineering strategies can be applied interchangeably to enhance the production of both stilbenoids and flavonoids.

In the stilbenoid pathway, L-Tyr and L-Phe, derived from the shikimate pathway, undergo sequential reactions catalyzed by Tyr/Phe ammonia lyase (TAL/PAL) and 4-coumarate-CoA ligase (4CL) to be converted into *p*-coumaroyl-CoA and cinnamoyl-CoA, respectively (Figure 1c). Each of these CoA molecules can react with three malonyl-CoA molecules, catalyzed by resveratrol synthase (VST), also known as stilbene synthase (STS), to form resveratrol or pinosylvin. Additionally, to

Figure 1



Biosynthetic pathway of fat-soluble antioxidants in yeast (*S. cerevisiae*). **(a)** Vitamin A and carotenoid pathway, **(b)** tocotrienol pathway, and **(c)** stilbenoid pathway. Black arrows represent pathways, double-headed arrows represent reversible reactions, and dashed arrows represent multiple enzymatic steps. Abbreviations of compounds: G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; Ru5P, ribulose 5-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; G3P, glyceraldehyde 3-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; PEP, phosphoenolpyruvate; 2-PG, 2-phosphoglycerate; Ac-P, acetyl phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA-5P, mevalonate 5-phosphate; MVA-5PP, mevalonate 5-pyrophosphate; IP, isopentenyl monophosphate; GPP, geranyl diphosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; 3-DHQ, 3-dehydroquinic acid; DHS, dehydroshikimate; SA, shikimic acid; S3P, shikimate 3-phosphate; EPSP, 5-enolpyruvylshikimate-3-phosphate; 4-HPP, 4-hydroxyphenylpyruvate; 4-HPAA, 4-hydroxyphenylacetaldehyde; PPY, phenylpyruvate; PAA; L-Phe, L-phenylalanine; L-Tyr, L-tyrosine; L-Met, L-methionine; DMGGBQ, 2,3-dimethyl-5-geranylgeranyl-benzoquinone; SAM, S-adenosyl-L-methionine. Genes and abbreviations of encoded endogenous enzymes: green italic labels represent genes, while blue labels represent native enzymes, as discussed in the cited references: *ZWF1*, glucose-6-phosphate dehydrogenase; *GND1*, 6-phosphogluconate dehydrogenase; *RPE*, ribulose 5-phosphate 3-epimerase; *RPI*, ribose 5-phosphate isomerase; *TKL*, transketolase; *TAL*, transaldolase; *ENO2*, phosphopyruvate hydratase; *ERG10*, acetyl-CoA acetyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; tHMGR, (truncated) HMG-CoA reductase; MK, mevalonate kinase, *ERG8*, phosphomevalonate kinase; *ERG19*, mevalonate pyrophosphate decarboxylase; IDI, isopentenyl-pyrophosphate delta isomerase; SQS, squalene synthase; GGPPS, GGPP

synthase; *ARO3/ARO4*, DAHP synthase; *ARO1*, pentafunctional protein; *ARO1A*, EPSP synthase; *ARO1B*, SA kinase; *ARO1C*, 3-DHQ synthase; *ARO1D*, SA dehydrogenase; *ARO1E*, 3-DHQ dehydratase; *ARO2*, chorismate synthase; *ARO7*, chorismate mutase; *ARO8/ARO9*, aromatic amino acid transaminase; *ARO10*, phenylpyruvate decarboxylase; *PDC5*, pyruvate decarboxylase; *PHA2*, prephenate dehydratase; *TYR1*, cyclohexadienyl dehydrogenase, *POS5*, NADH kinase**;** *ACC1*, acetyl-CoA carboxylase; *DGA1*, diacylglycerol acyltransferase. Genes and abbreviations of heterologous enzymes: red labels represent heterologous genes or enzymes, as discussed in the cited references: MvaE, acetyl-CoA acetyltransferase/HMG-CoA reductase; MvaS, HMG-CoA synthase; CK, choline kinase; IPK, isopentenyl phosphate kinase; PHK, phosphoketolase; PTA, phosphotransacetylase; phosphotransacetylase FPS, farnesyl pyrophosphate synthase; CrtE, geranylgeranyl pyrophosphate synthase; *tyrC/tyrA*, feedback inhibition-insensitive enzyme cyclohexadienyl dehydrogenase; CarRP/CrtYB, bi-functional phytoene synthase/lycopene β-cyclase; CarB/CrtI, phytoene dehydrogenase; CYP97A3, carotene β-ring hydroxylase; LUT1, carotene ε-ring hydroxylase; BCM(D)O, β-carotene 15, 15'-mono(di)oxygenase; RDH, retinol dehydrogenase; (t)POS5, (truncated) NADH kinase; *ATR2*, P450 reductase; *CYB5*, cytochrome b5; GGPP, geranylgeranyl pyrophosphate; GGPPS, geranylgeranyl pyrophosphate/diphosphate synthase.

hydroxylate cinnamate to *p*-coumarate and cinnamoyl-CoA to *p*-coumaroyl-CoA, cytochrome P450 cinnamate 4-hydroxylase (C4H) from *A. thaliana* and *Silybum marianum* can be introduced. In particular, the heterologous expression of C4H requires the concurrent expression of a heterologous cytochrome P450 reductase (encoded by *ATR2* in *A. thaliana*) [7]. Additionally, the overexpression of endogenous cytochrome b5 (encoded by *CYB5*) has been shown to enhance the conversion process [8,9].

Manipulating the upstream precursor module to enhance the flux

In yeast hosts, efforts to enhance carotenoid and vitamin E production commonly focus on increasing the upstream flux in the native MVA pathway. Additionally, both vitamin E biosynthesis and stilbenoid biosynthesis depend on the shikimate pathway, which supplies the precursor compounds necessary for their synthesis (Figure 1). Table 1 provides a summary of the key enzymes that are frequently manipulated in these processes.

Furthermore, there are several noteworthy genes and pathways that are related to cofactor availability, making them worthy of further discussion. As the production of one molecule of IPP or DMAPP via the MVA pathway requires two molecules of nicotinamide adenine dinucleotide phosphate (NADPH) and three molecules of ATP, key genes such as *ZWF1* (encoding glucose 6-phosphate dehydrogenase), *POS5* (encoding NADH kinase), and *GND1* (encoding phosphogluconate dehydrogenase) have been overexpressed to increase NADPH availability [25] (Figure 1). Luo et al. introduced a two-step isopentenol utilization pathway (IUP) to convert isoprenol in feed to IPP in *Y. lipolytica* [26]. This IUP is believed to operate free of yeast native regulations and only utilizes two molecules of ATP, not requiring NADPH per IPP synthesized. By implementing precise isoprenol supplementation timing and suitable palmitic acid concentrations to boost the formation of lipid bodies, this method significantly amplifies IPP and DMAPP levels by an impressive 15.7-fold, consequently leading to lycopene synthesis reaching 4.2 g/L in a batch bioreactor. Notably, Clomburg et al. leveraged Claisen condensation reactions to convert common carbon sources to prenol via acetyl-CoA in *E. coli*, which could potentially be applied in yeasts to achieve complete *de novo* biosynthesis without the addition of isoprenol [27].

Tackling the unique challenges in the downstream heterologous module

Each class of antioxidants presents unique challenges in their downstream module, distinct from those encountered in the upstream module. Merely placing heterologous enzymes downstream of robust promoters does not guarantee satisfactory production levels. The production process faces challenges such as inadequate matching of substrate preferences among enzymes, imbalanced expression levels and activities, and substrate and product inhibitions, leading to the accumulation of intermediate compounds and reduced pathway efficiency. Additionally, the production of lipophilic compounds encounters unique obstacles arising from their hydrophobic nature, which obstructs subcellular and cell membranes, ultimately impeding host growth. Furthermore, it has been observed that the expression destinations of enzymes do not align effectively within a heterologous host, particularly when the compartment-targeting sequences are not easily discernible. The following five subsections delve into an in-depth exploration of innovative strategies recently developed to tackle these challenges, while Table 2 summarizes the strategies implemented along with their corresponding fermentation outcomes.

Enzyme matching, protein engineering, and metabolite channeling

Combining enzymes from different sources has proven to be an effective strategy for microbial production of nonnative products. These enzymes have not been coevolved from the same source in nature, yet their combination in a heterologous host can unexpectedly enhance production compared with cloning enzymes from a single source. Enzymes from different sources may have distinct substrate preferences, leading to intermediate and side-product accumulation. Astaxanthin biosynthesis exemplifies the conversion of β-carotene through two hydroxylation steps and two ketolation steps, catalyzed by β-carotene hydroxylase (CrtZ) and

Table 1

Summary of the genetic manipulations that are frequently performed to improve the upstream flux involved in the synthesis of carotenoids, vitamin E, and stilbenoids.

	Proteins or genes	Encoded enzymes	Notes
The MVA pathway (carotenoids and vitamin E)	HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase	Overexpression of endogenous gene
	HMGS	3-hydroxy-3-methylglutaryl-coenzyme A synthase	
	FPPS	farnesyl pyrophosphate synthetase	
	IDI	Isopentenyl diphosphate isomerase	
	MK	mevalonate kinase	Overexpression of endogenous genes; feedback-resistant variants discovered [10].
	HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase	N-terminal truncated HMGR (tHMGR) is often used in *S. cerevisiae* to counter the feedback inhibition posed by FPP [11], while the nontruncated version is more effective in *Y. lipolytica* [12,13].
	MvaE	acetyl-CoA acetyltransferase/HMG-CoA reductase	From *Enterococcus faecalis*; an alternative shortcut for the first three steps of the MVA pathway in *S. cerevisiae* and *Y. lipolytica* [14–17]
	MvaS	HMG-CoA synthase	
	SQS	squalene synthase	Expression can be carefully reduced through promoter truncation [12] or swapping [18].
	GGPPS	geranylgeranyl diphosphate synthetase	Overexpressing the endogenous one or introducing a heterologous copy from carotenoid producers like *Xanthophyllomyces dendrorhous* (e.g. CrtE and its mutant CrtE03M) [19,20]
	FPS^{F112A}	farnesyl diphosphate synthase	From *Gallus gallus*; directly synthesize GGPP from IPP and DAMPP, reducing the direct competition with FPP [21].
The shikimate pathway (vitamin E and stilbenoids)	$ARO4^{K229L}$	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase	Tyr-insensitive mutant
	$ARO7^{G141S}$	chorismate mutase	Tyr-insensitive mutant
	$ARO3^{K222L}$	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase	Phe-insensitive mutant
	TKL	transketolase	Overexpression of endogenous gene [8]
	TAL	transaldolase	
	ENO2	phosphopyruvate hydratase	
	ARO1	pentafunctional arom protein	
	ARO2	chorismate synthase	
	TYR1	cyclohexadienyl dehydrogenase	Overexpression of endogenous gene [21]
	tyrC		Tyrosine-insensitive variant from *Zymomonas mobilis* [22]
	tyrA		Tyrosine-insensitive variant from *E. coli* [23]
	ARO10	phenylpyruvate decarboxylase	Knockout targets; blocking the aromatic amino acid degradation
	PDC5	pyruvate decarboxylase	
	SAM2	*S*-adenosylmethionine (SAM) synthetase	Enhancing SAM accumulation and resulting in a 10–20% increase in the yield of methylated tocotrienols (γ/β-tocotrienol and α-tocotrienol) compared with δ-tocotrienol [23]
	ACC1 $ACC1^{S659A,S1157A}$	acetyl-CoA carboxylase	The double mutant can effectively prevent phosphorylation and subsequent degradation [24].
	PHK	phosphoketolase and	PHK from *Bifidobacterium bifidum* or *Clostridium acetobutylicum*, converts fructose 6-phosphate into E4P and acetyl phosphate (Ac-P), and PTA from *Bacillus subtilis* converts Ac-P into acetyl-CoA [8,22].
	PTA	phosphotransacetylase	
	DGA1	diacylglycerol acyltransferase	knockout target; increasing malonyl-CoA supply for stilbenoid

GGPPS, geranylgeranyl pyrophosphate/diphosphate synthase.

β-carotene ketolase (CrtW), respectively. The production of astaxanthin is greatly affected by the sequence of these reactions, as the accumulation of different intermediates can significantly reduce its yield. Screening of nine CrtZ and eight CrtW enzymes from various organisms in *S. cerevisiae* identified the combination of CrtW from *Brevundimonas vesicularis* DC263 and CrtZ from *Alcaligenes* sp. strain PC-1 as the most effective, resulting in high astaxanthin yield with minimal intermediate accumulation [43]. In stilbenoid biosynthesis, two TAL enzymes, HaTAL from *Herpetosiphon aurantiacus* and FjTAL from *Flavobacterium johnsoniae*, were compared for coexpression in *Y. lipolytica* with 4CL from *A. thaliana* and VST from *Vitis vinifera*. FjTAL showed

Table 2

Selected production strains with their fermentation outcomes and the corresponding engineering strategies employed.

Strategy collection	Fine-tuning copy number (Cop); promoter swapping/engineering (Pro); enzyme matching or engineering (Enz); metabolite channeling (Cha); engineering storage (Sto); two-phase extraction (Ext); engineering secretion (Sec); relocating to organelles (Org); multistage fermentation (Mul); adjusting C-to-N ratio (C/N); engineering morphology (Mor)															
Antioxidant group	Target product	Host	Titer/yield in flasks	Titer/yield in bioreactors	Fermentation mode	Engineering Strategies											Ref
						Cop	Pro	Enz	Cha	Sto	Ext	Sec	Org	Mul	C/N	Mor	Others	
Vitamin E	δ-Tocotrienol	*Sc*	3.26 mg/L	-	Batch	✓		✓	✓		✓	✓	✓	✓				[21]
	δ-Tocotrienol	*Sc*	241.7 mg/L 63.65 mg/g DCW	-	Batch	✓	✓		✓		✓	✓		✓				[28]
	Tocotrienols (α-, β-, γ-, and δ)	*Sc*	ND	320 mg/L 7.6 mg/g DCW	Fed-batch	✓	✓	✓	✓				✓	✓				[23]
Vitamin A	Retinal Retinol	*Sc*	581.38 mg/L 371.70 mg/L	2094 mg/L 1256 mg/L	Fed-batch				✓		✓			✓			Xylose as substrate	[29]
	Retinol	*Sc*	443.43 mg/L	2479.34 mg/L	Fed-batch	✓		✓	✓					✓			Fe^{2+} optimization	[30]
Other carotenoids	Lutein	*Sc*	19.92 mg/L 4.53 mg/g DCW	-	Batch	✓	✓	✓	✓				✓	✓				[31]
	Lycopene	*Sc*	70.5 mg/g DCW	2.73 g/L 73.3 mg/g DCW	Fed-batch		✓		✓	✓								[32]
	Lycopene	*Yl*	8.02 g/L	17.6 g/L 313 mg/g DCW	Fed-batch			✓	✓	✓					✓		IUP	[33]
	β-carotene		7.5 g/L 360.8 mg/g DCW	39.5 g/L 494 mg/g DCW														
	Astaxanthin	*Yl*	858 mg/L 16.7 mg/g DCW	-	Fed-batch			✓	✓				✓		✓			[34]
	Lycopene	*Yl*	1.6 g/L	4.2 g/L	Batch						✓						IUP	[26]
	Astaxanthin	*Yl*	119.8 mg/L	3.3 g/L	Fed-batch	✓		✓	✓									[35]
	Zeaxanthin	*Yl*	775.3 mg/L	-	Batch	✓		✓	✓								Oxidoreductase RFNR1	[17]
	Astaxanthin	*Sc*	47.18 mg/L 8.10 mg/g DCW	-	Batch	✓		✓									Directed evolution; Fe^{2+} optimization	[36]
	Lycopene	*Sc*	ND	2.3 g/L	Fed-Batch			✓	✓									[37]
	β-carotene	*Sc*	149.8 mg/L (cytosolic)	-	Batch					✓	✓						10.1 mg/L in secretion	[38]
	Astaxanthin	*Sc*	44.56 mg/L	235 mg/L	Fed-batch									✓			Directed coevolution	[39]
	β-carotene	*Yl*	57.4 mg/g DCW	7.6 g/L 159 mg/g DCW	Fed-batch	✓										✓		[40]
Stilbenoids	Resveratrol	*Sc*	1155 mg/L	4.1 g/L	Fed-batch	✓								✓				[7]
	Polydatin	*Sc*	241 mg/L	545 mg/L 27.83 mg/g DCW	Fed-batch		✓	✓										[41]
	Resveratrol	*Yl*	-	12.4 g/L	Fed-batch	✓	✓							✓	✓			[42]
	Resveratrol	*Yl*	819.1 mg/L	22.5 g/L 0.28 g/g DCW	Fed-batch	✓	✓		✓						✓	✓		[8]

Table 2 (*continued*)

Strategy collection	Fine-tuning copy number (Cop); promoter swapping/engineering (Pro); enzyme matching or engineering (Enz); metabolite channeling (Cha); engineering storage (Sto); two-phase extraction (Ext); engineering secretion (Sec); relocating to organelles (Org); multistage fermentation (Mul); adjusting C-to-N ratio (C/N); engineering morphology (Mor)																	
Antioxidant group	Target product	Host	Titer/yield in flasks	Titer/yield in bioreactors	Fermentation mode	Engineering Strategies												Ref
						Cop	Pro	Enz	Cha	Sto	Ext	Sec	Org	Mul	C/N	Mor	Others	
	Polydatin	*Yl*	-	6.88 g/L	Fed-batch	✓			✓								Degradation blocking	[22]
	Resveratrol	*Rt*	125.2 mg/L	-	Batch		✓											[9]

Abbreviations: Host: *Sc*, *Saccharomyces cerevisiae*; *Yl*, *Yarrowia lipolytica*; *Rt*, *Rhodosporidium toruloides*. Others: IUP, isopentenyl phosphate pathway; Ref., reference.

over 40% higher resveratrol production compared with HaTAL [42]. Another comparison was made between feedback-insensitive mutants of Aro4 and Aro7 from *S. cerevisiae* and their counterparts in *Y. lipolytica*, revealing that the latter exhibited greater potential for improvement. This was evident in the sequential increases in resveratrol levels achieved by gradually increasing the copy number of the heterologous pathway from one to six when the *Y. lipolytica* version was used. The final strain produced 12.4 g/L of resveratrol in a fed-batch fermentation. The distinguishable colors of many carotenoids make high throughput screening for desired mutants straightforward. Through directed evolution, $CrtW^{H165R/V264D/F298Y}$ was identified to enhance canthaxanthin and astaxanthin production in *S. cerevisiae* [36]. Structure-guided protein engineering aided in resolving substrate inhibition of LCY with $CarRP^{Y27R}$, resulting in a yield of 39.5 g/L β-carotene and complete removal of substrate inhibition in *Y. lipolytica* [33]. In the case of δ-tocotrienol production, the design of 16 truncated TC (tTC) mutants using Rosetta Cartesian_ddg resulted in the most effective mutant, TC^{N331P}, which increased δ-tocotrienol titer by 83% [21].

Metabolite channeling improves conversion efficiency by preventing intermediate diffusion. Han et al. constructed fusion enzymes and found that among the three enzymes, HPPD, HGGT, and tTC, HGGT-tTC fusion outperformed other fusion pairs and the fusion with all three enzymes for δ-tocotrienol production [21]. Zhu et al. implemented a modular enzyme assembly using short peptides (i.e., anchoring (RIAD) and docking and dimerization domain (RIDD)) to form an enzyme complex of CrtW and CrtZ [35]. The 2:1 binding stoichiometry between RIDD and RIAD worked better when RIDD was added to CrtW, suggesting CrtW as a potential rate-limiting step. The final strain, with 20 copies of the two genes integrated into the genome of *Y. lipolitica*, achieved the highest reported level to date: 3.3 g/L with 71.9% astaxanthin among total carotenoids in a fed-batch condition. The RIDD/RIAD peptide pair was also applied to complex IDI and CrtE, connecting the upstream and downstream modules and enhancing lycopene production in *S. cerevisiae* [37]. Additionally, synthetic scaffolds (Src homology 3 (SH3), Postsynaptic density 95, Disc large 1, and Zonular occludens 1 (PDZ)and GTPase-binding domain (GBD)) were tested for assembling HPPD and the HGGT-tTC fusion complex, with SH3 ligand and domain proving most effective in accelerating metabolite channeling, increasing δ-tocotrienol titer by 1.56-fold [21]. In the case of stilbenoid production, the fusion of STS and 4CL was also employed to enhance resveratrol production in *Y. lipolytica* and *Rhodotorula toruloides* [8,9]. Interestingly, Liu et al. tested four rigid and four flexible linkers and found that the rigid linker E3AK (EAAAK) increased production by 40% [8]. This finding is intriguing as flexible linkers are typically used to enable independent functioning of the partner

enzymes. The optimized distance between STS and 4CL, facilitated by the rigid linker, proved critical in this specific context.

Engineering storage, extraction, and secretion of lipophilic compounds

The hydrophobic nature of lipophilic compounds presents a challenge to their production, as they can obstruct subcellular and cell membranes, hindering host growth. Enhancing membrane and lipid body formation presents a promising strategy, albeit a complex one, due to the competition with the MVA pathway. The MVA pathway shares acetyl-CoA as a precursor for the biosynthesis of fatty acids (FAs), phospholipids, and triacylglycerol (TAG). Balancing the carbon flux among these pathways is crucial to ensure sufficient encapsulation without depleting acetyl-CoA from the MVA pathway. Ma et al. focused on FA synthesis and TAG production in *S. cerevisiae*, modifying the fatty acyl composition of TAG to regulate lipid body size. This strategy increased lycopene content by 25%, reaching a record-high level of 2.37 g/L in fed-batch fermentation in *S. cerevisiae* [32]. *Y. lipolytica* naturally possesses adequate TAG accumulation capacity for sequestering lipophilic compounds, and therefore, adjusting the C/N ratio in the fermentation medium can modulate the crosstalk between these pathways and lead to a balanced flux distribution between the two nodes [33]. A potential drawback of this strategy arises when the formation of lipid bodies is insufficient during the initial stage, impairing effective product sequestration and resulting in a significant deceleration in growth, even at low production levels. To mitigate the toxic effects of membrane blockage, exploring extracellular exportation of lipophilic targets has been considered. Biphasic extraction fermentation, employing organic extractants such as dodecane or olive oil, has shown promise in enhancing the production of vitamin A [29,30] and vitamin E [21,44]. To maximize extraction efficiency without compromising cell growth, it is imperative to identify the suitable type of organic extractant and determine the organic/aqueous phase ratio customized for the specific product and host. This is essential as the extraction mechanism involves the modulation of cell membrane fluidity and permeability to facilitate the release of the desired product. Additionally, incorporating specialized transporters has proven effective in facilitating secretion and production of hydrophobic chemicals. Overexpressing ATP-binding cassette transporters such as Pdr, Yol075, and Snq2p has been reported to enable the export of β-carotene [38] and vitamin E [28,44] in *S. cerevisiae*.

A strategy to convert resveratrol into the more water-soluble polydatin via glycosylation at the C3 hydroxyl position has been tested. Polydatin offers improved stability and biological activities. The conversion was achieved in *S. cerevisiae* through heterologous expression of resveratrol 3-*O*-glycosyltransferase (R3GAT) from *Polygonum cuspidatum* [41]. In a subsequent study [22], a *Y. lipolytica* strain was modified to incorporate four heterologous enzymes: TAL and 4CL from *Rhodotorula glutinis* and *A. thaliana*, respectively, VST from *V. vinifera*, and R3GAT. The strain was further optimized by increasing the upstream flux, fine-tuning gene expression, and adjusting the copy number of rate-limiting genes. Particularly, two glucosidase-encoding genes were deleted to prevent polydatin hydrolysis. This optimized strain achieved a remarkable production of 6.88 g/L polydatin in a batch process, with all 100 g/L glucose fed at the beginning. This represents the highest reported titer for *de novo* synthesis of polydatin in microbial cells.

Relocation to subcellular organelles

Colocalization of enzymes in the cytoplasmic biosynthetic pathways offers advantages by sharing precursors and cofactors with central metabolism. However, this can lead to competition between endogenous metabolism and the targeted pathway. To address this issue and enhance pathway efficiency, enzyme colocalization to other cellular compartments serves as a complementary metabolic engineering strategy. In a specific instance of astaxanthin production in *Y. lipolytica*, augmenting the number of pathway enzymes was insufficient to address the issue of intermediate accumulation. This raised the possibility of β-carotene sequestration within lipid bodies, thereby creating a challenge for cytosolically expressed astaxanthin pathway enzymes to access it. Ma et al. designed a fusion protein (CrtW-CrtZ) and targeted it to lipid droplets using an oleosin-based protein-location tag. This approach resulted in a 1.62-fold increase compared with cytosolic expression [34]. Interestingly, among the three heterologous proteins involved in β-carotene biosynthesis, CarB, and CarRP were localized in the endoplasmic reticulum (ER), while GGPPS cloned from *Sulfolobus acidocaldarius* remained in the cytosol. Leveraging the knowledge that TAG synthesis occurs in the ER and the observation of peroxisomes as storage compartments for squalene in *S. cerevisiae* [45], the CrtW-CrtZ fusion protein was targeted to these two organelles individually and simultaneously with lipid body targeting. Anchoring the enzymes to all three compartments resulted in the highest reported astaxanthin production of 858 mg/L and 16.7 mg/g dry cell weight (DCW) in shake flask fed-batch fermentation in *Y. lipolytica* [34].

Heterologous production of vitamin E faces a unique challenge due to the absence of suitable subcellular membranes for expressing chloroplast-derived enzymes. In plants, MPBQMT, TC, and γ-TMT enzymes are located in the inner membrane of chloroplasts with transit peptides, which may hinder proper protein

folding in yeast cells. Removing 51, 47, and 40 amino acids from the N-termini of MPBQMT, TC, and γ-TMT, respectively, has been shown to increase the accumulation of their respective products by 900%, 56%, and 67%. Combining all three truncated enzymes resulted in a 2.2-fold increase in total tocotrienol levels compared with strains expressing the full-length versions [23]. Online platforms such as TargetP-2.0 [46] and Alphafold 2 [47] can be utilized to predict signal peptides and assess overall protein folding, respectively. Constructing a fusion with a fluorescent potein offers convenience in examining the expression location of heterologous enzymes. In the case of constructing the tocotrienol biosynthetic pathway in *S. cerevisiae*, HGGT expression was undetectable when fused with enhanced green fluorescent protein (EGFP) as a reporter. Subsequent tests involving HPT/HGGT isoenzymes from 12 plants, the alga *Chlamydomonas reinhardtii*, and the cyanobacteria *Synechocystis* revealed successful expression only with the codon-optimized HPT from *Synechocystis* sp. PCC6803. This enabled the complete production of all four forms of tocotrienol at 320 mg/L [23]. In another study, it was found that both HGGT and HGGT-tTC complex were located in the ER, while HPPD was situated in the cytoplasm [21].

Multiple-stage fermentation

Decoupling cellular growth from product synthesis is a promising strategy to address the conflict between these processes. In *S. cerevisiae*, galactose-inducible (GAL) promoters regulated by Gal4 and Gal80 have been widely used for gene expression control [48]. Zhou et al. [49] created a temperature-responsive system by deleting Gal80 and introducing a temperature-sensitive Gal4 mutant, Gal4M9, obtained through directed evolution. This cold-shock temperature control system effectively balances high cell densities and accumulation of toxic target products. Two-stage high-cell-density fermentations, implemented for astaxanthin [39] and tocotrienols [23], involving a temperature shift from 30°C to 24°C during the mid-late logarithmic growth phase, resulted in 235 mg/L astaxanthin and the highest reported production of 320 mg/L tocotrienols. Moreover, to overcome competition between difunctional enzymes, such as LCY, a temporospatial pathway control system combining dynamic regulation and enzyme colocalization was developed for lutein production, yielding 595.3 μg/L in *S. cerevisiae* [50]. Recently, a dual-signal hierarchical dynamic regulation in response to glucose depletion and culture temperature was developed. This strategy effectively separates cell growth, δ-carotene formation, and δ-carotene conversion into three distinct stages, resulting in the highest reported lutein titer of 19.92 mg/L in heterologous production systems [31]. The utilization of glucose depletion and/or temperature as input signals in this strategy offers the advantage of being pathway-independent, making it applicable to various pathways in *S. cerevisiae*. However, the transcription mechanisms responsive to catabolite repression, such as the *S. cerevisiae* GAL regulon, in many nonconventional hosts, still remain unclear. Furthermore, the practical implementation of precise temperature control on a large industrial fermentation scale may pose significant challenges.

Morphology surveillance and engineering

It was interestingly observed that *Y. lipolytica* displays a dimorphic feature, existing in both ovoid yeast and filamentous forms. While the mycelial morphology provides resistance to harsh environments, it is unfavorable for industrial processes due to its impact on medium viscosity, rheology, and mass/oxygen transfer efficiency. By controlling dissolved oxygen, pH, and C/N ratio, the majority of resveratrol-producing *Y. lipolytica* cells were maintained in the yeast form, resulting in a record-high titer of 22.5 g/L in a 5-L fed-batch fermentation [8]. A similar transition from yeast to pseudomycelia and hyphae was observed in *Y. lipolytica* strains producing β-carotene as production levels increased [40]. By deleting serine/threonine-protein kinase (CLA4) in the mitogen-activated protein kinase pathway and C_2H_2-type zinc finger transcription factor (MHY1) in the cAMP protein kinase A pathway, hyphal growth was completely abolished, leading to a 139% increase in β-carotene titer without impacting growth rate. The final strain achieved a β-carotene production of 7.6 g/L with a content of 159 mg/g DCW in a fed-batch process, consistently maintaining the yeast form throughout fermentation.

Conclusion and future perspectives

High-level microbial production of lipophilic antioxidants clearly faces unique challenges such as cellular toxicity, limited intracellular storage space, and mismatched enzyme expression location. In addition to the aforementioned strategies, we believe that emerging technologies that could tackle these challenges and further advance the field can be developed in three future aspects. Firstly, a comprehensive characterization of inducible transcription machinery suitable for other nonconventional yeasts is needed. Oleaginous yeasts like *Y. lipolytica* and *R. toruloides* present attractive host options due to their storage capacity to sequester hydrophobic products, higher malonyl-CoA levels, and lack of Crabtree effect. However, inducible systems to decouple production from growth are lacking in these hosts. Secondly, the unique antioxidant activities can be harnessed to screen for nonobvious mutations that enhance production. This approach mirrors the strategies employed in native hosts, where treatments like TiO_2 and H_2O_2 are utilized to induce carotenoid production as a response to oxidative stress [51]. In heterologous hosts, the creation of mutant libraries can be facilitated using

techniques like clustered regularly interspaced short palindromic repeats (CRISPR)/Cas or transposon-mediated genome-wide mutagenesis, which are comparatively easier to implement compared with native producers. Lastly, it is essential to thoroughly evaluate the robustness and stability of a strain, even if it exhibits high production performance in flasks or bench-top bioreactors, considering the potential adverse effects of cellular stress caused by lipophilic compound production. Different integration loci are associated with varying levels of stability, and a locus that proves stable for one pathway may present a different stability scenario when applied to a different set of genes. Rigorous assessment, including deep sequencing [52], is necessary to identify mutation hotspots within the pathway and its surrounding regions. It is crucial to re-evaluate the strain's stability after recoding the mutation hotspots or relocating the pathway to a different locus. This thorough assessment is of utmost importance to ensure the suitability of the strain for future industry-scale production. While this review emphasizes the production of three classes of lipophilic antioxidants by yeasts, it is worth noting that several of the aforementioned strategies, including enzyme matching and engineering, metabolite channeling, multistage production, and morphology engineering, can be adapted and applied to other products or chassis organisms.

CRediT authorship contribution statement

Yuxin Zhao: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Zhanyi Yao**: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Vedika Desai**: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Dan Chen**: Investigation. **Zengyi Shao**: Conceptualization, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

None.

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- • of special interest
- •• of outstanding interest.

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