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Metabolic engineering of *Yarrowia lipolytica* for terpenoids production: advances and perspectives

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ABSTRACT

Terpenoids are a large family of natural products with diversified structures and functions that are widely used in the food, pharmaceutical, cosmetic, and agricultural fields. However, the traditional methods of terpenoids production such as plant extraction and chemical synthesis are inefficient due to the complex processes, high energy consumption, and low yields. With progress in metabolic engineering and synthetic biology, microbial cell factories provide an interesting alternative for the sustainable production of terpenoids. The non-conventional yeast, *Yarrowia lipolytica*, is a promising host for terpenoid biosynthesis due to its inherent mevalonate pathway, high fluxes of acetyl-CoA and NADPH, and the naturally hydrophobic microenvironment. In this review, we highlight progress in the engineering of *Y. lipolytica* as terpenoid biomanufacturing factories, describing the different terpenoid biosynthetic pathways and summarizing various metabolic engineering strategies, including progress in genetic manipulation, dynamic regulation, organelle engineering, and terpene synthase variants.

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KEYWORDS

Yarrowia lipolytica; terpenoid; terpene synthase; metabolic engineering; fine-tuning; mevalonate pathway; microbial production



Introduction

Terpenoids are a large group of natural products, differing in both structure and function, and are found in a variety of plants, microbes, and insects [1,2]. Various terpenoids are extensively used in foods, pharmaceuticals, cosmetics, and agricultural products [3]. However, the extraction of terpenoids from plants is challenging because of low yields and purity, as well as the huge consumption of resources. On the other hand, due to their complex structure, the chemical synthesis of terpenoids is inherently difficult and expensive [4]. The production of terpenoids by microbial fermentation using renewable resources therefore provides an interesting alternative strategy to meet the increasing demands for terpenoids.

Model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* have been extensively used for terpenoids biosynthesis. However, the low efficiency of the native 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway for isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) supply [5] and the

inability to functionally express key plant enzymes such as P450s [6] has limited terpenoids biosynthesis in *E. coli*. Additionally, the risk of bacterial toxins and acetate inhibition also limits the use of *E. coli* as a biological host for industrial fermentation [7]. *S. cerevisiae* is more suitable than *E. coli* for the production of value-added terpenoids because its mevalonate (MVA) pathway and redox systems offers high potential for terpenoids production and skeleton modification [5,8,9]. However, the use of *S. cerevisiae* also has disadvantages, including limited secretion, relatively slow growth rates, and the Crabtree Effect, which increases the flux toward fermentation metabolism and ethanol production at the expense of metabolic flux toward acetyl-CoA [10–12].

Recently, *Yarrowia lipolytica*, an oleaginous yeast, is being adopted as a promising chassis microbe for the production of lipid-derived compounds. In addition, *Y. lipolytica* is also known for its ability to express and secrete homologous or heterologous enzymes, and is therefore frequently used as a host for the production of recombinant proteins [13,14]. Moreover, with the

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development of metabolic engineering and synthetic biology tools, it has also been investigated for the biosynthesis of industrial chemicals, including organic acids [15], sugar alcohols [16,17], lipids [18], functional polyunsaturated fatty acids [19], fatty acid-derived fuels, and oleochemicals [20,21]. In addition, *Y. lipolytica* possesses an active tricarboxylic acid cycle (TCA cycle) and a pentose phosphate pathway (PPP), and it supports high fluxes of acetyl-CoA, NADPH, and the ATP supply by metabolizing a variety of carbon sources [22]. Meanwhile, some strains of *Y. lipolytica* have been approved by the FDA and obtained the generally recognized as safe (GRAS) status [23], making them suitable hosts for the production of food additives, pharmaceuticals and cosmetics. These characteristics make *Y. lipolytica* an ideal cell factory in comparison to *E. coli* and *S. cerevisiae* for the biosynthesis of some natural products, including terpenoids and polyketides [24–26].

Recent studies compared the terpenoids production in *Y. lipolytica* and other hosts such as *S. cerevisiae* [27], summarized several engineering strategies used for the construction of platform strains for the overproduction of mono-, sesqui-, tri-, and tetraterpenoids [28], and clarified the main challenges for terpenoid production in *Y. lipolytica* [29]. In this review, we focus on progress within the last five years in the metabolic engineering of *Y. lipolytica* as a microbial cell factory for the biosynthesis of terpenoids. Specifically, the terpenoids biosynthetic pathways and the different engineering strategies for terpenoids biosynthesis are discussed in detail. This review also focuses on perspectives for further improving terpenoids bioproduction in *Y. lipolytica* through analyzing the achievements, challenges and cutting-edge technologies generated in metabolic engineering and synthetic biology.

Terpenoids biosynthetic pathways

All terpenoids are derived from IPP and its enzymatic tautomer DMAPP, both of which are 5-carbon building blocks. There are two biosynthetic pathways for IPP and DMAPP: the MVA and MEP pathways [30]. Generally, eukaryotes (except for plants, which possess both MVA and MEP pathways), archaea and a few bacteria convert acetyl-CoAs to IPP and DMAPP through the MVA pathway, while prokaryotes produce IPP and DMAPP by the MEP pathway. In *Y. lipolytica*, IPP and DMAPP are synthesized through the MVA pathway (Figure 1(A)). Specifically, two acetyl-CoA molecules are condensed into acetoacetyl-CoA in a reaction catalyzed by acetyl-CoA acetyltransferase (ERG10). There are two genes

encoding for acetyl-CoA acetyltransferase in *Y. lipolytica*, which are also termed *ACOAA1* and *ACOAA2*. The acetoacetyl-CoA and acetyl-CoA are condensed to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) under the facilitation of HMG-CoA synthase (ERG13). Then, HMG-CoA is reduced to mevalonate by NADPH dependent HMG-CoA reductase (HMGR). Subsequently, mevalonate is phosphorylated to form 5-diphosphomevalonate by mevalonate kinase (ERG12) and phosphomevalonate kinase (ERG8) with the consumption of ATPs. Subsequently, 5-diphosphomevalonate is decarboxylated by mevalonate diphosphate decarboxylase (ERG19) to form IPP, which is then isomerized by IPP isomerase (IDI) and converted into DMAPP [27].

Terpenoids are classified by the number of carbons in the backbone structures and include monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetraterpenoids referring to products with 10, 15, 20, 30, and 40 carbons, respectively [31]. The coupling chemistry used to link the isoprenoid precursors determines the structural diversity of natural terpenoids. For example, DMAPP and IPP complexes can generate the 10-carbon block geranyl diphosphate (GPP), and GPP can sequentially condense with other IPP molecules to generate longer carbon scaffolds such as farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), and geranyl-farnesyl diphosphate (GFPP). Following this, terpene synthases with various functions including glycosyl transfer, transmethylation, or oxidation, decorates the scaffold producing structurally diverse terpenoids. The decorations of representative terpenoids are shown in Figure 1(B).

Engineering strategies for terpenoids production

Y. lipolytica can be engineered to synthesize various valuable terpenoids by the introduction of heterologous terpene synthases. In the last few years, researchers have applied a variety of strategies to improve terpenoid(s) production in *Y. lipolytica*. The main products, metabolic engineering targets, fermentation conditions, and levels are summarized in Table 1. The strategies adopted in these publications are described in the following sections.

Engineering the substrate utilization

Y. lipolytica can utilize different hydrophilic and hydrophobic carbon sources, such as glucose, fructose, mannose, glycerol, fatty acids, and alkanes [76–78], and some of these substrates have been adopted for terpenoids biosynthesis in *Y. lipolytica* [33,43,45,68].

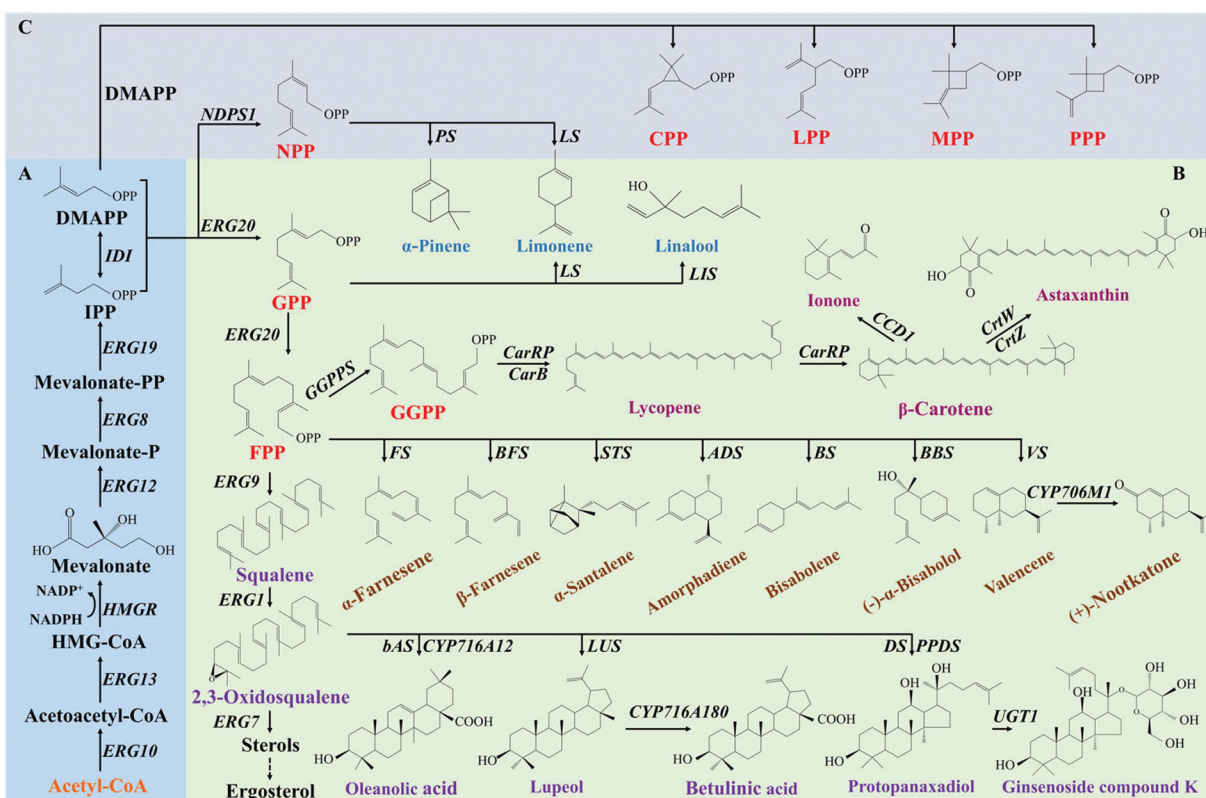


Figure 1. The biosynthetic pathways of terpenoids. (A) MVA pathway. (B) The decoration process of representative terpenes. (C) Noncanonical precursors derived from DMAPPs. Metabolites: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; NPP, neryl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; CPP, chrysanthemyl diphosphate; LPP, lavandulyl diphosphate; MPP, maconellyl diphosphate; PPP, planococyl diphosphate. Enzymes: *ERG10*, acetyl-CoA acetyltransferase; *ERG13*, HMG-CoA synthase; *HMGR*, HMG-CoA reductase; *ERG12*, mevalonate kinase; *ERG8*, phosphomevalonate kinase; *ERG19*, mevalonate diphosphate decarboxylase; *IDI*, IPP isomerase; *ERG20*, farnesyl pyrophosphate synthetase; *ERG9*, squalene synthase; *ERG1*, squalene epoxidase; *ERG7*, lanosterol synthase; *NDPS1*, neryl diphosphate synthase; *PS*, pinene synthase; *LS*, limonene synthase; *LIS*, linalool synthase; *FS*, α -farnesene synthase; *BFS*, β -farnesene synthase; *STS*, α -santalene synthase; *ADS*, amorphadiene synthase; *BS*, bisabolene synthase; *BBS*, ($-$)- α -bisabolol synthase; *VS*, valencene synthase; *CYP706M1*, (+)-nootkatone synthase; *bAS*, β -amyryn synthase; *CYP716A12*, oleanolic acid synthase; *LUS*, lupeol synthase; *CYP716A180*, betulinic acid synthase; *DS*, dammarenediol-II synthase; *PPDS*, protopanaxadiol synthase; *UGT1*, UDP-glucose glucosyltransferase; *CCD1*, carotenoid cleavage dioxygenase; *GGPPS*, geranylgeranyl diphosphate synthase; *CarRP*, phytoene synthase/lycopene cyclase; *CarB*, phytoene dehydrogenase; *CrtW*, β -carotene ketolase; *CrtZ*, β -carotene hydrolase.

However, *Y. lipolytica* does not naturally metabolize xylose, which is an economically interesting feedstock for chemical production because of its abundance in lignocellulose hydrolysates [79]. Several efforts have been made to modify *Y. lipolytica* in order to allow utilization of xylose as a substrate. Expression of a combination of genes that encode xylose reductase, xylitol dehydrogenase, and xylulose kinase allows the engineered strains to grow efficiently on xylose [80,81]. Similar strategies have been applied to the production of isoprenoids in *Y. lipolytica*. Yao et al. [35] introduced *SsXR* and *SsXDH* from *Scheffersomyces stipitis* to a *Y. lipolytica* strain with limonene synthetic modules. After overexpressing endogenous *XKS*, *HMGR*, and *ERG12*, the limonene production from the lignocellulosic feedstock

by the recombinant strains increased significantly, reaching 20.57 mg/L after 72 h when 50% of the detoxified hydrolysate was employed. After introducing the xylose utilization pathway, Wu et al. [49] used directed evolution to further improve xylose consumption and eliminate growth instability in the xylose medium. Then, with a series of metabolic engineering strategies to improve the xylose metabolism rate and enhance protopanaxadiol production, the resulting strains demonstrated highly efficient protopanaxadiol production from xylose, with a titer of 300.63 mg/L being achieved through fed-batch fermentation in a 5-L bioreactor (Figure 2(A)).

In addition to engineering *Y. lipolytica* for xylose utilization, researchers have also attempted to improve the

Table 1. Summary of metabolic engineering strategies for improving terpenoids biosynthesis in *Y. lipolytica*, and information of representative terpenes with the highest titers reported in *E. coli* and *S. cerevisiae*.

Products	Strains	Engineering strategies	Medium/ Carbon source	Fermentation	Titer	References
Monoterpenoids						
Limonene	<i>Y. lipolytica</i> Po1f	HMGR, ERG12, tSINDP51, tArLS	YPD Glucose	Flask Batch	23.56 mg/L	[32]
Limonene	<i>Y. lipolytica</i> Po1f	HMGR, ERG12, tSINDP51, tArLS	YPG Pyruvate	1.5-L fermenter Fed-batch	165.3 mg/L	[33]
Limonene	<i>Y. lipolytica</i> Po1g	Δku70, HMGR, I-MsLS, d-CILS	YPDM Glycerol	5-L fermenter Fed-batch	L-limonene 11.705 mg/L D-limonene 11.088 mg/L	[34]
Limonene	<i>Y. lipolytica</i> ST6512	ACL1, SeACS, HMGR, ERG12, IDI, ERG20 ^{8BW-N119W} , P _{ERG11} -ERG9, P _{FLS}	YPD Glucose	Glass tube Batch	35.9 mg/L	[28]
Limonene	<i>Y. lipolytica</i> Po1f	SsXR, SsXDH, XK, HMGR, ERG12, tSINDP51, tArLS,	YP Glucose	Flask Batch	20.57 mg/L	[35]
Linalool	<i>Y. lipolytica</i> Po1f	HMGR, IDI, ERG20 ^{8BW-N119W} , AaLS	YP Lignocellulose hydrolysate	Flask Batch	6.96 mg/L	[36]
α-Pinene	<i>Y. lipolytica</i> Po1f	AMPD, HMGR, ERG12, ERG8, tSINDP51, P _{PS} , P _{PPS}	YP Pyruvate	Flask Batch	36.1 mg/L	[37]
Limonene	<i>E. coli</i> BL21 (DE3)	atoB, ScERG13, tScHMG1, ScERG12, ScERG8, ScERG19, IDI, tAgGPPS, tMsLS	M9 Glycerol	3.1-L fermenter Fed-batch	3.63 g/L	[38]
Limonene	<i>S. cerevisiae</i> EGY48	EfmVaS, EfmVaE, ERG12, ERG8, ERG19, IDI, ERG20 ^{N127W} , CILS Compartmentalization	SD Galactose	Flask Fed-batch	2.58 g/L	[39]
Sesquiterpenoids						
(+)-Nootkatone	<i>Y. lipolytica</i> ATCC 201249	tScHMG1, ERG20, CnVS, CrCYP706M1-tAtCPR1	YPD Glucose	Flask Batch	0.9782 mg/L	[40]
α-Santalene	<i>Y. lipolytica</i> ATCC 201249	ScHMG1, ERG8, CIST5	YPD Glucose	5-L fermenter Fed-batch	27.92 mg/L	[41]
Valencene	<i>Y. lipolytica</i> ATCC 201249	tScHMG1, ERG20, CnVS, CrCYP706M1-tAtCPR1	YPD Glucose	Flask Batch	22.8 mg/L	[40]
Valencene	<i>Y. lipolytica</i> ST6512	ACL1, SeACS, HMGR, ERG12, IDI, ERG20, P _{ERG11} -ERG9, CnVS	YPD Glucose	Glass tube Batch	113.9 mg/L	[28]
Amorphadiene	<i>Y. lipolytica</i> Po1g	Supplementation of cerulenin	Complete synthetic media Glucose	Flask Batch	171.5 mg/L	[42]
α-Farnesene	<i>Y. lipolytica</i> Po1h	tScHMG1, IDI, MdFS-linker-ERG20	PM Glucose	1.5-L fermenter Batch	259.98 mg/L	[43]
α-Farnesene	<i>Y. lipolytica</i> Po1f	Ecat08, ERG13, BpHMGR, ERG12, ERG8, ERG19, IDI, GPPS, MdFS-linker-ERG20	YPD Fructose	1-L fermenter Fed-batch	25.55 g/L	[44]
β-Farnesene	<i>Y. lipolytica</i> ST6512	ACL1, SeACS, HMGR, ERG12, IDI, ERG20, AaBFS	YPD Glucose	Glass tube Batch	955 mg/L	[28]
Bisabolene	<i>Y. lipolytica</i> Po1g	Δku70, HMGR, GcABC-G1, α-AgBS, β-ZoBS, γ-HaBS	YP with Mg ²⁺ WCO	Flask Batch	α-bisabolene 973.1 mg/L β-bisabolene 68.2 mg/L γ-bisabolene 20.2 mg/L	[45]
(-)-α-Bisabolol	<i>Y. lipolytica</i> Po1f	Δku70, tHMGR, ERG20, P _{ERG9_50bp} -ERG9, P _{OTT} , MIBBS	YPD Glucose	Flask Batch	364.23 mg/L	[46]
β-Farnesene	<i>E. coli</i> BL21 (DE3)	atoB, ScERG13, tScHMG1, ScERG12, ScERG8, ScERG19, IDI, ispA, AaFS	2 × YT Glycerol	7-L fermenter Batch	8.74 g/L	[47]
β-Farnesene					130 g/L	[48]

(continued)

Table 1. Continued.

Products	Strains	Engineering strategies	Medium/ Carbon source	Fermentation	Titer	References
Triterpenoids						
Protopanaxadiol	<i>S. cerevisiae</i> Y227	$\Delta gal80$, $\Delta hxt3$, $\Delta dit1$, $\Delta ndt80$, Δho , $\Delta gal2$, $\Delta adh5$, $\Delta ald4$, $\Delta ald6$, $\Delta hrh2$, $\Delta ygr250c$, $\Delta bdh1$, $\Delta adh1$, $GAL4$, $GAL4^*$, $ERG10$, $ERG13$, $ERG13^*$, $tHMG1$, $ERG12$, $ERG8$, $ERG19$, $ERG20$, P_{MET3} - $ERG9$, $PDR3$, $BjHMGs$, $CaTHL$, AoS , $ACS2$, $ZmPDC$, $DzeutE$, $LmPK$, $CRPTA$, $SpHMGr$	Mineral salt Cane sirup	0.5-L fermenter Fed-batch		
	<i>Y. lipolytica</i> ATCC 201249	$\Delta ku70$, $\Delta pox1-3$, Tx , $SsXR$, $SsXDH$, XKS , TKL , TAL , $tHMGR$, $ERG20$, $ERG9$, $PgDS$, $PgPPDS$ - $linker$ - $AVATR1$	YPX Xylose	5-L fermenter Fed-batch	300.63 mg/L	[49]
Ginsenoside compound K	<i>Y. lipolytica</i> ATCC 201249	$tHMGR$, $ERG20$, $ERG9$, $PgDS$, $PgPPDS$ - $linker$ - $AVATR1$, $PgUGT1$	YPD Glucose	5-L fermenter Fed-batch	161.8 mg/L	[50]
2,3-Oxidosqualene	<i>Y. lipolytica</i> ST6512	$ACL1$, $SeACS$, $HMGR$, $ERG12$, IDI , $ERG20$, $ERG9$, $ERG1$, $P_{ERG7-50bp}$ - $ERG7$	YPD Glucose	Deepwell plate Batch	22 mg/L	[28]
Oleanolic acid	<i>Y. lipolytica</i> ATCC 201249	$tHMGR$, $ERG20$, $ERG9$, $GgbAS$, $MtCYP716A12$ - $linker$ - $AVATR1$	YPD Glucose	5-L fermenter Fed-batch	540.7 mg/L	[51]
Lupeol	<i>Y. lipolytica</i> ATCC 201249	$\Delta pah1$, $\Delta dgk1$, $HMGR$, $ERG9$, $ERG1$, $OLE1$, $RcLUS$	YPD Glucose	Flask Batch	411.72 mg/L	[52]
Betulinic acid	<i>Y. lipolytica</i> ATCC 201249	$tHMGR$, $ERG9$, $AtLUP1$, $MtCYP716A12$ - $AtCPR1$	YPG Glycerol	Flask Batch	26.53 mg/L	[53]
Betulinic acid	<i>Y. lipolytica</i> ATCC 201249	$\Delta ku70$, $\Delta ku80$, $MFEI$, $HMGR$, $ERG9$, $ERG1$, $LjCPR$, $BPLO$, $RcLUS$	YPD Glucose	Flask Batch	204.89 mg/L	[54]
Squalene	<i>Y. lipolytica</i> Pot1f	$ACL1$, $SeACS^{641P}$, $HMGR$	YPD Glucose	Flask Batch	10 mg/g DCW	[55]
Squalene	<i>Y. lipolytica</i> ST6512	$ACL1$, $SeACS$, $HMGR$, $ERG12$, IDI , $ERG20$, $ERG9$, $P_{ERG7-50bp}$ - $ERG7$	YPD Glucose	Deepwell plate Batch	402.4 mg/L	[28]
Squalene	<i>Y. lipolytica</i> Pot1g	$MndH2$, $HMGR$, $ERG9$	Minimal media Glucose	Flask Fed-batch	502.75 mg/L	[56]
Squalene	<i>E. coli</i> DH5 α	$SpmvaK1$, $SpmvaD$, $SpmvaK2$, IDI , $EfmvaE$, $EfmvaS$, $ispA$, $IScERG9$, tsr	2 \times YT Glycerol	Flask Batch	612 mg/L	[57]
Squalene	<i>S. cerevisiae</i> Y2805	$tHMG1$, $EcispA$ Supplementation of terbinafine	YPD Glucose	5-L fermenter Fed-batch	2.011 g/L	[58]
Tetraterpenoids						
Lycopene	<i>Y. lipolytica</i> Pot1f	$PaCrtE$, $PaCrtB$, $PaCrtI$	Designed-medium	1.5-L fermenter Fed-batch	242 mg/L	[59]
Lycopene	<i>Y. lipolytica</i> Pot1f	$flux$ balance analysis $HMGR$, $EGR8$, $ERG19$, $PaCrtB$, $PaCrtE$, $PaCrtI$	YPD Glucose	1-L fermenter Fed-batch	213 mg/L	[60]
Lycopene	<i>Y. lipolytica</i> ATCC 201249	$ScERG20$, $ScERG8$, $ScIDI$, $POT1$, $EnCrtE$, $EnCrtB$, $EnCrtI$	SC Glucose	Flask Batch	259 mg/L	[61]
Lycopene	<i>Y. lipolytica</i> Pot1f	$AMPD$, $PaCrtE$, $PaCrtB$, $PaCrtI$	MM Glucose	5-L fermenter Fed-batch	745 mg/L	[62]
Lycopene	<i>Y. lipolytica</i> Pot1f	$AtHPK$, $ScCHK$, $ERG20$, $PvIDI$, $LpCrtE$, $LpCrtB$, $LpCrtI$	YNB Glucose Palmitic acid Isoprenol	3-L fermenter Batch	4.2 g/L	[63]
β -Carotene	<i>Y. lipolytica</i> Pot1f	$\Delta ku70$, $\Delta ku80$, $GGPPS$, $SsCarS$	YPD	Flask Batch	0.41 mg/g DCW	[64]
β -Carotene	<i>Y. lipolytica</i> Pot1f		YPD Glucose	1.5-L fermenter Fed-batch	4.00 g/L	[65]

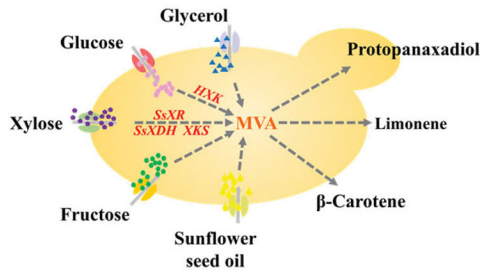
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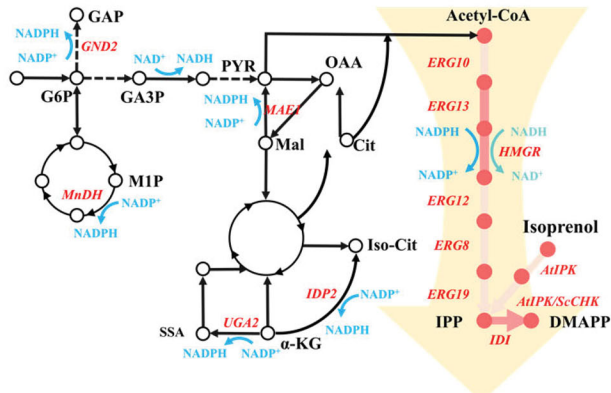
Products	Strains	Engineering strategies	Medium/ Carbon source	Fermentation	Titer	References
β -Carotene	<i>Y. lipolytica</i> Po1d	$\Delta ku70$, $\Delta pox3$ -6, $\Delta lip1$, $\Delta YALI0F30987g$, <i>ERG10</i> , <i>ERG13</i> , <i>tHMGR</i> , <i>ERG12</i> , <i>ERG8</i> , <i>ERG19</i> , <i>IDI</i> , <i>ERG20</i> , <i>GGPPS</i> , <i>McCarRP</i> , <i>McCarB</i>	YPD	5-L fermenter Fed-batch	6.5 g/L	[66]
β -Carotene	<i>Y. lipolytica</i> ST6512	$\Delta pox1$ -6, $\Delta tgl4$, <i>DGA2</i> , <i>GPD1</i> , <i>tHMGR</i> , <i>GGPPS</i> , <i>McCarRP</i> , <i>McCarB</i>	Glucose	Deepwell plate Batch	164 mg/L	[28]
β -Carotene	<i>Y. lipolytica</i> Po1f	<i>ACL1</i> , <i>SeACS</i> , <i>tHMGR</i> , <i>ERG12</i> , <i>IDI</i> , <i>GGPPS</i> , <i>P_{ERG1T}</i> , <i>ERG9</i> , <i>XdCrTYB</i> , <i>XdCrI</i>	Glucose	5-L fermenter Fed-batch	4.5 g/L	[67]
β -Carotene	<i>Y. lipolytica</i> Po1f	Δmfe , $\Delta Pox2$ -3, $\Delta lip1$ <i>ERG13</i> , <i>tHMGR</i> , <i>GGPPS</i> , <i>McCarRP</i> , <i>McCarB</i>	Glucose	50-L fermenter Fed-batch	2.4 g/L	[68]
Astaxanthin	<i>Y. lipolytica</i> GB20	$\Delta ku70$, Δsnf , <i>HXK</i> , <i>ERG13</i> , <i>tHMGR</i> , <i>GGPPS</i> , <i>BtCarRA</i> , <i>BtCarB</i>	Glucose	24-well plates	54.6 mg/L	[69]
Astaxanthin	<i>Y. lipolytica</i> GB20	<i>tHMGR</i> , <i>XdCrE</i> , <i>XdCrTYB</i> , <i>XdCrI</i> , <i>P_{ScrtW}</i> , <i>PoCrTZ</i> , <i>P_{ERG9-500p}</i> - <i>ERG9</i>	Glucose	1-L fermenter Fed-batch	285 mg/L	[70]
α -Ionone	<i>Y. lipolytica</i> CLIB138	<i>HpNphT7</i> , <i>ERG13</i> , <i>tHMGR</i> , <i>ERG12</i> , <i>ERG8</i> , <i>ERG19</i> , <i>HpIDI</i> , <i>GPPS</i> , <i>ERG20</i> - <i>GGPPS</i> , <i>McCarRP</i> *, <i>McCarB</i> , <i>LsLYe</i> *, <i>OfCCDI</i>	Glucose	5-L fermenter Fed-batch	408 mg/L	[71]
β -Ionone	<i>Y. lipolytica</i> CLIB138	<i>SsNphT7</i> , <i>ERG13</i> , <i>tHMGR</i> , <i>ERG12</i> , <i>ERG8</i> , <i>ERG9</i> , <i>HpIDI</i> , <i>GPPS</i> , <i>ERG20</i> - <i>GGPPS</i> , <i>McCarRP</i> - <i>McCarB</i> , <i>OfCCDI</i>	Glucose	2-L fermenter Fed-batch	380 mg/L	[72]
β -Ionone	<i>Y. lipolytica</i> Po1f	$\Delta ku70$, $\Delta ku80$, $\Delta pox3$, $\Delta lip1$, $\Delta DI7$, <i>ERG10</i> , <i>ERG13</i> , <i>tHMGR</i> , <i>ERG12</i> , <i>ERG8</i> , <i>ERG19</i> , <i>IDI</i> , <i>ERG20</i> , <i>GGPPS</i> , <i>BbPK</i> , <i>BsPTA</i> , <i>McCarB</i> , <i>McCarRP</i> , <i>PhCCDI</i>	Glucose	3-L fermenter Fed-batch	0.98 g/L	[73]
β -Carotene	<i>E. coli</i> BL21 (DE3)	<i>ScERG12</i> , <i>ScERG8</i> , <i>ScERG19</i> , <i>ScDI</i> , <i>EfmvaE</i> , <i>EfmvaS</i> , <i>Bsdxs</i> , <i>Bsfni</i> , <i>AgGGPS2</i> , <i>EhCrE</i> , <i>EhCrB</i> , <i>EhCrI</i> , <i>EhCrY</i>	Mineral salt with beef extract Glucose	5-L fermenter Fed-batch	3.2 g/L	[74]
β -Carotene	<i>S. cerevisiae</i> D452-2	$\Delta ald6$, <i>SsXYL1</i> , <i>SsXYL2</i> <i>XKS1</i> , <i>XdCrTYB</i> , <i>XdCrI</i> , <i>XdCrE</i> *	Glycerol Verduyn medium Xylose	3-L fermenter Fed-batch	772.8 mg/L	[75]

" Δ " refers to gene knockouts; "*" refers to gene with mutant; "-" refers to protein fusion, "*P_{xx-XX}*" refers to genes driven by specific promoters. The uppercase and lowercase letters before some genes refer to the source of the gene. The information about terpenes produced in *E. coli* and *S. cerevisiae* are marked with blue shading.

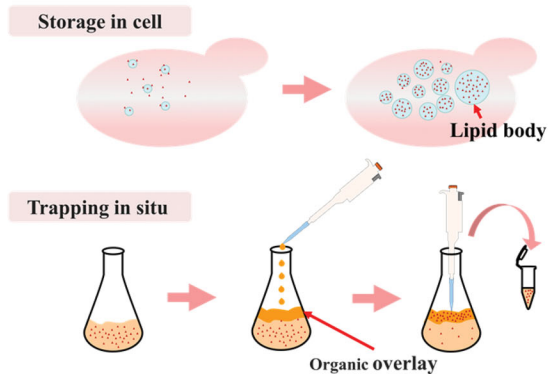
A. Engineering the substrate utilization



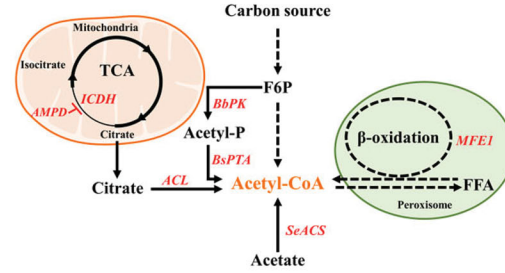
C. Overexpressing the genes in MVA pathway and engineering cofactor supply



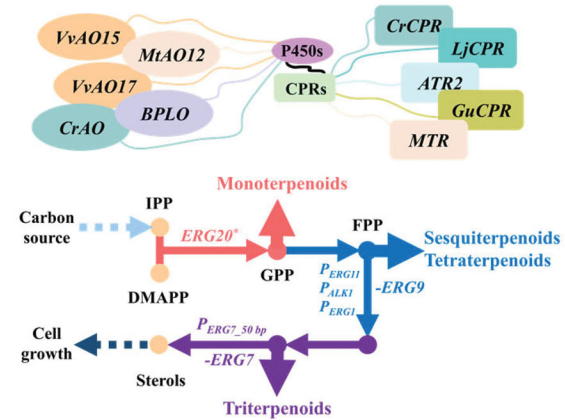
E. Modulating hydrophobicity for terpenoid accumulation



B. Increasing cytosolic acetyl-CoA supply



D. Fine-tuning the expression of key enzymes



F. Engineering based on metabolomics analysis

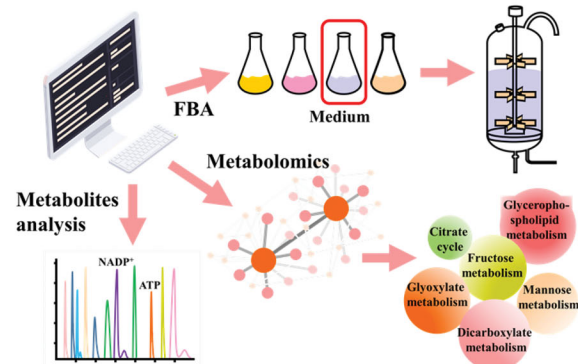


Figure 2. Overview of the engineering strategies for terpenoid production in *Y. lipolytica*. (A) Engineering substrate utilization to minimize production costs. *HXK*, hexokinase; *SsXR*, xylose reductase from *Scheffersomyces stipitis*; *SsXDH*, xylitol dehydrogenase from *Scheffersomyces stipitis*; *XKS*, xylulose kinase. (B) Increasing cytosolic acetyl-CoA supply by introducing heterologous pathways or redirecting native central carbon metabolism. *AMPD*, AMP deaminase; *ICDH*, isocitrate dehydrogenase; *BbPK*, phosphoketolase from *Bifidobacterium bifidum*; *BsPTA*, phosphotransacetylase from *Bacillus subtilis*; *ACL*, ATP citrate lyase; *SeACS*, acetyl-CoA synthetase from *Salmonella enterica*; *MFE1*, multifunctional β -oxidation enzyme. (C) Overexpressing the genes in MVA pathway and engineering cofactor supply. *GND2*, 6-phosphogluconate dehydrogenase; *MnDH*, mannitol dehydrogenase; *MAE1*, malic enzyme; *UGA2*, succinate semialdehyde dehydrogenase; *IDP2*, cytosolic NADP^+ -specific isocitrate dehydrogenase; *AtIPK*, isopentenyl phosphate kinase from *Arabidopsis thaliana*; *ScCHK*, bifunctional choline kinase/ethanolamine kinase from *Saccharomyces cerevisiae*. (D) Fine-tuning the expression of key enzymes: combinatorial optimization of P450s-CPRs among species and constructing self-sufficient P450s by fusion with CPRs; protein engineering to improve the specificity and activity of enzymes; relieving side pathway competition. (E) Modulating hydrophobicity for terpenoid accumulation: increasing intracellular lipids by genetic manipulation or feeding with hydrophobic substrates and *in situ* trapping with organic overlay. (F) Engineering based on metabolomics analysis: modeling-guided fermentation optimization and metabolic engineering. FBA: flux balance analysis.

glucose utilization capacities of strains. Overexpression of the hexokinase gene (*HXK*) in *Y. lipolytica* was found to improve the yield of both biomass and lipids [82]. Qiang et al. [68] investigated the effect of hexokinase on glucose consumption and β -carotene accumulation by overexpressing *HXK*. The increased hexokinase activity resulted in a higher glucose-6-phosphate content, accelerated glucose utilization rate, and improved β -carotene content by 98%, to 4.63 mg/g DCW (Figure 2(A)).

Increasing cytosolic acetyl-CoA supply

Adequate acetyl-CoA supply is crucial for terpenoids production. *Y. lipolytica* possesses only one acetyl-CoA synthase that is similar to the acetyl-CoA synthetase (ScACS2) of *S. cerevisiae*, and most of the cytosolic acetyl-CoA is produced by the action of ATP citrate lyase (ACL). The ACL cleaves citrate derived from the mitochondria to oxaloacetate and acetyl-CoA under conditions of nutrient limitation [83]. Different approaches have been used to improve the supply of cytosolic acetyl-CoA, including introducing heterologous pathways, as well as redirecting the native central carbon metabolism (Figure 2(B)).

Lu et al. [73] introduced the phosphoketolase-phosphotransacetylase (PK-PTA) pathway to enhance the cytosolic acetyl-CoA supply and re-channel carbon into the target products. After the introduction of the *BbPK-BsPTA* pathway and modification of the endogenous pathway, the titer of β -ionone was 0.98 g/L in the 3-L fed batch bioreactor, a staggering 280-fold improvement compared with the starting strain. To enhance the carbon flux from pyruvate toward the production of acetyl-CoA, Huang et al. [55] overexpressed the heterogenous acetyl-CoA synthetase gene from *Salmonella enterica* (*SeACS*) and the native ATP citrate lyase gene (*ACL1*) in an *HMGR*-overexpressing strain. The squalene production of the resulting strain was improved 16.4-fold compared to the control strain. The squalene production ultimately reached 10 mg/g DCW with 10 mM citrate supplemented in shake flasks fermentation.

In *Y. lipolytica*, continuous efflux of citrate from the mitochondria is required to maintain the level of cytosolic acetyl-CoA. The citrate efflux is the result of the downregulation of isocitrate dehydrogenase, which is localized in mitochondria and catalyzes citrate to isocitrate in an AMP-dependent manner. Zhang et al. [62] overexpressed an AMP deaminase-encoding gene (*AMPD*) in *Y. lipolytica* to inhibit the catalytic activity of isocitrate dehydrogenase and enhance the citrate efflux to the cytosol. The lycopene content in the engineered

strains was significantly improved, achieving 46–60 mg/g DCW.

As an oleaginous microorganism, *Y. lipolytica* is capable of accumulating abundant intracellular lipids from acetyl-CoA using a pathway that competes with terpenoids biosynthesis. To direct the carbon flux toward terpenoid production, Jin et al. [54] engineered the acetyl-CoA generation by reinforcing the fatty acid β -oxidation pathway. Combined with other modules such as heterogenous CYP/CPR and a reinforced MVA pathway, overexpression of the multifunctional fatty acid β -oxidation gene (*MFE1*) further increased squalene accumulation 1.72-fold, ultimately achieving a total titer of 204.89 mg/L for triterpenoids in shake flask fermentation.

Overexpressing MVA pathway genes

The MVA pathway, which converts acetyl-CoA into IPP and its isomer DMAPP represents the core, upstream part of the terpenoid biosynthetic pathway, while the downstream pathway involves decoration, including the conversion of IPP and DMAPP into diverse functional terpenoids [27].

In many organisms, HMG-CoA reductase (HMGR) is the major rate-limiting enzyme of the MVA pathway. Several metabolic engineering strategies are applied to overcome this “bottleneck” and improve the biosynthesis of terpenoids (Figure 2(C)). HMGR is an endoplasmic reticulum (ER)-resident protein that contains an N-terminal membrane anchor and a C-terminal catalytic domain [84]. Overexpression of intact or N-terminal-truncated HMGR enhances the production of diverse terpenoids [28]. In earlier studies, an N-terminal-truncated HMGR was thought more effective, because the N-terminal truncation can prevent self-degradation of HMGR and make it more stable in the cytoplasm. However, Kildegaard et al. [69] compared the performance between the full-length and truncated HMGR variants and found that the native HMGR is superior for β -carotene production in *Y. lipolytica*. Overexpression of the full-length *HMGR* gene in a limonene-producing strain increased the yield of D-limonene and L-limonene by nearly 10-fold, reaching 0.256 mg/L and 0.316 mg/L, respectively [34].

IPP isomerase (IDI), which is responsible for the conversion between IPP and DMAPP and plays a crucial role in the flux distribution of GPP and FPP, is commonly overexpressed to enhance the biosynthesis of terpenoids (Figure 2(C)). Yang et al. [43] reported that IDI overexpression increased the titer of α -farnesene to 57.08 mg/L, nearly 2.67-fold higher than that produced

by the wild-type strain. In addition to overexpressing *PvIDI* from *Pseudodescherichia vulneris*, Luo et al. [63] introduced a two-step isoprenol utilization pathway (*AtIPK-ScCHK*) in *Y. lipolytica* to enhance the supply of IPP and DMAPP. This chimeric *AtIPK-ScCHK* pathway elevated the total levels of IPP and DMAPP by 15.7-fold compared to the MVA pathway alone, which further improved the lycopene content to approximately 120 mg/g DCW.

In addition to *HMGR* and *IDI*, overexpression of other genes in the MVA pathway has also been found to be helpful in directing the MVA flux toward terpenoid production. Zhao et al. [45] found that overexpression of *ERG10* (*ACOAT1* or *ACOAT2*), *ERG13*, *ERG12*, *ERG8*, and *ERG19* individually could improve the bisabolene production compared to controls expressing only the bisabolene synthases. Although the effect of *ERG13* seems little on boosting bisabolene production, it became more significant for β -carotene production in the context of *HMGR* being co-overexpressed. Qiang et al. [68] found that overexpressing *ERG13* elevated the content of β -carotene by 181% compared to the parent strain which harboring overexpressed *tHMGR*, and the recombinant strain with two copies of *ERG13* performed better, which was 259% higher than parental strain. Therefore, the genes involved in the MVA pathway are usually considered as engineering targets for enhancing the production of terpenoids (Figure 2(C)).

Fine-tuning the expression of key enzymes

In contrast to the upstream MVA pathway, the downstream terpenoid synthesis pathway is more complex, because it involves heterogenous terpene synthases, various precursors, and different modification steps. In order to improve synthesis efficiency and the yield of products, some strategies have been applied to fine-tune the expression of key enzymes (Figure 2(D)).

Most natural plant terpene synthases are members of the cytochrome P450 superfamily (P450s or CYPs), that couples with suitable NADPH-cytochrome P450 reductases (CPRs) in order to maintain their catalytic activities. However, there is no P450s-CPRs system associated with terpenoids in *Y. lipolytica*. Consequently, it is necessary to co-express heterologous P450s and their redox partners for terpenoid production in *Y. lipolytica*. Although individual expression of P450s and CPRs showed better effects than fused expression of these two proteins in Jin's studies [54], under certain circumstances, co-expression of individual P450s and CPRs may have little effect on the engineering because of the poor coupling between them [53]. By contrast,

constructing self-sufficient P450s by fusing P450s and their redox partner CPRs proved a more effective solution. The fusion of P450s and CPRs accelerated the effective electron transfer from NADPH to the P450s and improved the turnover rate of the enzymes [85]. This strategy has been widely used to improve terpenoids production in *Y. lipolytica*, including protopanaxadiol, valencene, ginsenoside compound K, and oleanolic acid [40,49–51].

In some cases, introduction of an artificial chimeric pathway from different species works more effectively than an intact pathway from the single heterologous species. Therefore, combinatorial optimization by screening alternative steps or isoenzymes among species plays an important role in enhancing the productivity of exogenous pathways [86]. In order to augment the yield of betulinic acid, 25 combinations of P450s and CPRs were screened, and the results showed that the combination of P450s from *Betula platyphylla* and CPRs from *Medicago truncatula* contributed the best catalytic effects on the production of betulinic acid (32.33 ± 4.34 mg/L) [54].

In addition, rational protein engineering is also an effective strategy to improve the specificity and activity of enzymes. Some enzymes, such as *ERG20*, are promiscuous and can catalyze the production of a wide range of natural and non-natural substrates. The enzyme *ERG20* functions as both a GPP synthase (GPPS) and FPP synthase (FPPS), which causes difficulty in separating the two synthases during monoterpenoids biosynthesis. To increase the monoterpenoids titer, a double mutant *ERG20^{88W-N119W}* was employed to improve the GPP pool in *Y. lipolytica*, resulting in higher linalool or limonene titers significantly compared to the original strain [28,36]. Meanwhile, protein fusion is a convenient strategy for enhancing the local intermediates concentration and enzyme catalytic efficiency, which is helpful to efficiently redirect the metabolic flux to the product [87]. For example, FPP is the immediate precursor in the synthesis of α -farnesene. Co-expression of *ERG20* and α -farnesene synthase with a linker is beneficial to improve the catalytic efficiency of α -farnesene synthase on FPP, thereby preventing FPP from entering branched metabolic pathways, such as the biosynthesis of farnesol and squalene [44].

Deleting the key genes in the ergosterol (a component of the cell membrane) biosynthetic pathway leads to serious growth defects [88]. To maintain cell growth and improve the production of target terpenoids, it is feasible to fine-tune the expression of critical genes by promoter engineering. Squalene is a native terpenoid in *Y. lipolytica* and is the first committed product after

FPP in the ergosterol biosynthetic pathway. Squalene synthase is responsible for the synthesis of squalene, encoded by *ERG9*, which is the key node for competition with FPP for target terpenoid synthesis. To increase the supply of FPP for the biosynthesis of the terpenoids of interest, Kildegaard et al. [69] truncated the native promoter of *ERG9* or replaced it with P_{ERG1} or P_{ERG11} , which are repressed by ergosterol, and the resulting strains showed a 2-2.5-fold increase in the β -carotene titer compared to the strain with the native *ERG9* promoter. Meanwhile, Gao et al. [65] substituted the *ERG9* promoter with P_{ALK1} , which is repressed by glucose and glycerol [89], and the squalene content decreased by 57.2% in the recombinant strain, resulting in increased lycopene accumulation. Similar tactics have been applied to downregulate the expression of *ERG7* (encoding lanosterol synthase) when triterpenoids are regarded as targets such as squalene or 2,3-oxidosqualene [28].

Engineering the cofactor supply

The levels of intracellular cofactors determine the metabolic flux, because cofactors are involved in enzymatic reactions and regulate the chemical equilibrium state. Therefore, the metabolic flux can be directed toward the target biosynthesis by regulating the cellular cofactor levels.

Cellular NADPH, the essential reducing factor for protecting cells from oxidative stress, has been reported as the major rate-limiting factor for fatty acids synthesis in *Y. lipolytica* [90]. As mentioned above, NADPH dependent HMGR is considered a rate-limiting enzyme and plays an important role in terpenoid biosynthesis. The heterogenous CYP/CPR system for terpenoid biosynthesis also is NADPH dependent. In *Y. lipolytica*, it has been demonstrated that increasing the supply of NADPH is more effective for the production of betulinic acid than increasing the supply of NADH [54]. Recently, to augment the NADPH pool, a collection of endogenous auxiliary cytosolic NADPH pathways including 6-phosphogluconate dehydrogenase (GND2), mannitol dehydrogenase (MnDH1 or MnDH2), malic enzyme (MAE1), cytosolic NADP⁺-specific isocitrate dehydrogenase (IDP2), and succinate semialdehyde dehydrogenase (UGA2) has been tested and investigated (Figure 2(C)). The results showed that mannitol dehydrogenase (MnDH2) demonstrated the best improvement in squalene production [56,91].

In *Y. lipolytica*, excess NADH will accumulate in cells when oxygen is limited, which leads to the formation of mannitol, and the amount of mannitol can be

promoted by fed-batch fermentation [92,93]. Thus, the cofactor NADH may be more abundant than NADPH under certain conditions in *Y. lipolytica*. Liu et al. [44] have constructed libraries of strains by integrating a chimeric NADH-dependent MVA pathway including *Ecatob*, *ERG13* and NADH dependent *BpHMGR* into chromosome randomly *via* non-homologous end-joining (NHEJ) to obtain a mevalonate overproduction strain. In this strain, the α -farnesene synthesis pathway was enhanced by fusing *FS* with *ERG20* as well as overexpressing *ERG12*, *IDI*, *ERG8*, *ERG19* and *GPPS*, and a titer of 25.55 g/L α -farnesene was achieved in fed-batch fermentation under the conditions of pH 5.5 with a 1.5 vvm air flux.

Modulating hydrophobicity for terpenoid accumulation

Most long-chain terpenoids, such as carotenoids, are fat-soluble due to their inherent hydrophobic characteristics and they are difficult to dissolve in the hydrophilic cytoplasmic environment. Therefore, excess isoprenoids are usually stored within intracellular hydrophobic compartments, such as membranes or lipid bodies [94]. It is well known that *Y. lipolytica* shows a strong ability to synthesize and accumulate lipids, and its intracellular lipid content can be further increased through metabolic modulation, which provides an ideal environment for terpenoid storage [65,90,95]. It has been previously demonstrated that lycopene accumulation is related with increased lipid content, and that nitrogen limitation is conducive to the accumulation of both lipids and carotenoids [96] (Figure 2(E)). Based on these studies, Larroude et al. [66] remodeled a lipid-overproducing strain (*DGA2*, *GPD1*, Δ *pox1-6*, and Δ *tg14*) with the carotenoid expression cassette, and the generated strain accumulated higher amounts of β -carotene, 1.93 times more than a wild type strain with the same carotenoid expression cassette.

In *Y. lipolytica*, intracellular lipids can be synthesized in two ways: the *de novo* and *ex novo* pathways. In the *de novo* synthesis pathway, hydrophilic substrates are required to produce acetyl-CoA, and the acetyl-CoAs are condensed to synthesize acyl-CoA and lipids, while the *ex novo* synthesis pathway requires the hydrolysis of hydrophobic substrates to produce either fatty acids or glycerol, which are then transported into cells for reassembly as lipids [18]. Lipid synthesis through an *ex novo* pathway can be activated by feeding *Y. lipolytica* with exogenous fatty acids. Luo et al. [63] enhanced lipid synthesis separately by overexpressing both *ACC1* and *DGA1* or supplementing palmitic acid and

compared the effects of these two tactics on the yield of lycopene. The addition of palmitic acid to strains resulted in a lycopene titer of 594.6 mg/L, much higher compared with the controls (less than 400 mg/L). The titer of lycopene in *ACC1-DGA1*-overexpressing strain was 905.9 mg/L, which was elevated more than 2 folds. These results illustrated the importance of providing an intercellular hydrophobic microenvironment for the sequestration and accumulation of long-chain terpenoids.

Unlike carotenoids, some terpenoids are not suitable for intracellular accumulation due to their characteristics, such as cytotoxicity, evaporability, or secretion. To enhance terpenoid production, it is helpful to artificially provide an extracellular hydrophobic environment for product enrichment and storage. A liquid two-phase solvent extraction system that had previously been proved helpful in improving cell viability [97], has been widely used to enrich products and avoid loss from vaporization during fermentation, especially for volatile terpenoids, such as limonene, linalool, and β -ionone (Figure 2(E)). Cao et al. [32] found that the limonene yield was associated with the amount of dodecane when pyruvic acid was used as the auxiliary carbon source. Meanwhile, it has been shown that an excess of organic overlay could not indefinitely improve the trapping efficiency for products, and the optimum volume of the overlay should be determined according to the actual fermentation process [34]. Recently, Zhang et al. [52] developed a strategy to enhance the product efflux from the cell to the lipophilic solvent by modifying the membrane components. The resultant strain showed an increased unsaturated fatty acid content and an improved production of lupeol with a 33.2-fold increase compared with the original strain.

Engineering based on metabolomics analysis

Genome-scale metabolic models embody significant advantages of systems biology because such approaches can predict globally the biosynthesis of interesting metabolites, facilitating an understanding of the metabolic networks and guiding rational metabolic engineering (Figure 2(F)). Nambou et al. [59] combined flux balance analysis (FBA) and Plackett-Burman design to screen compounds suitable for lycopene production in *Y. lipolytica*. An efficient medium was designed based on the variables screened from the factors predicted by FBA and employed for fed-batch cultivation in a 1.5-L fermenter. As a result, a lycopene titer of 242 mg/L was achieved, increased by 92% compared to the FBA-independent media. Zhao et al. [98] investigated the

metabolome of *Y. lipolytica* with expression of the heterologous lycopene biosynthesis cassette *PaCrtE-PaCrtB-PaCrtI*. The comprehensive analysis showed that lycopene biosynthesis in *Y. lipolytica* has a significant impact on carbon metabolism, including the metabolism of fructose, mannose, glyoxylate, dicarboxylate, and glycerophospholipid, as well as on the citrate acid cycle. Meanwhile, lycopene production also affected the biosynthesis and metabolism of hydrophobic amino acids such as valine, leucine, and isoleucine.

In later work, researchers [99] analyzed the role of metabolites such as nucleotides and coenzymes in lycopene-producing and control strains using optimized capillary electrophoresis. The concentrations of ATP and NADP^+ changed remarkably between the strains, indicating that these two metabolites may be closely linked with lycopene production. Czajka et al. [71] quantified the physiological responses and temporal changes in the metabolism of an engineered *Y. lipolytica* strain with the production of α -ionone during fermentation. The results of dynamic labeling showed a measurable ATP shortage during the phase of α -ionone production, suggesting that electron transport and oxidative phosphorylation may be bottlenecks in energy supply and strain performance. In conclusion, these findings offer insights into the metabolic dynamics of terpenoid biosynthesis in *Y. lipolytica* and provides guidance for rational metabolic engineering.

Conclusion and future perspective

Great progress has been made in the metabolic engineering of *Y. lipolytica*, and several products synthesized in this host have been successfully implemented in commercial production. However, reported terpenoid titers in *Y. lipolytica* are often in the $\text{mg}\cdot\text{L}^{-1}$ range, and gram-scale production has only been reported for a few intensively engineered strains. Meanwhile, the titers of some representative terpenoids, such as limonene, farnesene and squalene, are still much lower in *Y. lipolytica* than in *E. coli* or *S. cerevisiae* (Table 1), and also remain well below the concentrations of endogenous molecules such as lipids, citric acid, α -ketoglutarate, succinic acid, and erythritol. This suggests that the potential of *Y. lipolytica* as a cell factory for terpenoids production has not yet been fully exploited, and there are still challenges that limit the terpenoid biosynthesis in *Y. lipolytica*. Some of the main reasons may include insufficient genetic tools, lack of basic knowledge and dynamic regulatory elements, as well as underdeveloped compartmentalization engineering in *Y. lipolytica*.

Firstly, the lack of molecular tools for genetic manipulation limits harnessing the full potential for chemical biosynthesis in *Y. lipolytica* compared to *E. coli* and *S. cerevisiae*. In *Y. lipolytica*, non-homologous end-joining (NHEJ) is the predominant route for repairing DNA double strand breaks (DSB), while homology-mediated DNA repair is not effective, thereby limiting the application of many synthetic biology tools that depend on homologous recombination (HR) [100]. It has also been shown that disruption of the NHEJ genes, such as *KU70*, is helpful to increase the HR rate. However, strains with disrupted *KU70* show undesirable growth phenotypes and are more sensitive to UV light, which are unfavorable characteristics for the construction of robust host strains [101]. Recently, this problem has been mitigated by the CRISPR/Cas9-based gene editing systems, but more tools are still needed to allow rapid, multiplexed strain engineering in *Y. lipolytica* [102].

Secondly, the poor basic knowledge of *Y. lipolytica* metabolism and physiology also limits the rational engineering of strains. For example, the production and regulation of cytosolic acetyl-CoA has not yet been unraveled in detail. This is especially the case for the regulatory mechanisms underlying the multiple acetyl-CoA generation and consumption pathways, as well as for the mechanisms governing acetyl-CoA translocation between subcellular compartments [29]. Although different engineering strategies have been applied to direct the flux of cytosolic acetyl-CoA toward terpenoid biosynthesis, this has not yet been wholly successful. Markham et al. [26] have investigated and demonstrated the utility of the pyruvate bypass pathway for improving the formation of acetyl-CoA and malonyl-CoA, the key precursors of polyketide triacetic acid lactone. However, the strategy has not yet been reported to help increase terpenoid production in *Y. lipolytica*. Meanwhile, several promising alternative routes for improved cytosolic acetyl-CoA formation in *S. cerevisiae* have not yet been tested in *Y. lipolytica*; including the pyruvate oxidase-phosphotransacetylase (PO-PTA) pathway [12], pyruvate decarboxylase-acetylating acetaldehyde dehydrogenase (PDC-A-ALD) pathway and the pyruvate-formate lyase-formate dehydrogenase (PFL-FDH) pathway [103]. Therefore, more specific and detailed knowledge is needed to guide rational metabolic engineering with *Y. lipolytica*, particularly regarding the ensembling of metabolic fluxes and their regulation.

Thirdly, engineering terpenoids biosynthesis requires a variety of heterologous enzymes. The introduction of these heterologous enzymes can impose a metabolic

burden, and will result in flux imbalance, toxic intermediates accumulation, and genetic instability, which in turn limits yield. Dynamic metabolic engineering (DME) is a rapidly developing field that has been applied widely in both *E. coli* and *S. cerevisiae*. It allows the cell to autonomously adjust its flux in response to its external and internal metabolic state through the design of genetically encoded metabolic control systems [104]. DME is helpful in improving pathway production and can be further modified and utilized to prevent degenerated and abortive production phenotypes during long-term cultivation and scale-up [105]. However, a dynamic system relies on precise regulation of its elements, such as metabolite-responsive transcription factors, responsive promoters, and riboswitches, which are particularly rare and interesting. Exploring such elements and applying them in *Y. lipolytica* will provide exciting clues for improving terpenoids production.

Fourthly, compartmentalization into different subcellular organelles can limit interference between different cellular processes and provide suitable environments and sufficient precursors or enzymes for complex biosynthetic pathways. Peroxisomes are the main sites where β -oxidation of fatty acid takes place, creating a pool of acetyl-CoA that can be utilized by heterologous pathways. Recent studies demonstrated the positive effects of peroxisome compartmentalization on monoterpenoid yields in *S. cerevisiae*, suggesting that the peroxisome is a promising subcellular factory for terpenoid production [39]. Theoretically, peroxisome compartmentalization in *Y. lipolytica* should offer potential for terpenoids production, as the strains can synthesize and accumulate large amounts of lipids and provide more acetyl-CoA through β -oxidation. However, these findings still need to be verified in *Y. lipolytica*.

Finally, terpenoids decoration is completed mainly by terpene synthases, which are a large group of related enzymes involved in primary and secondary metabolism [106]. Although many terpene synthases have been identified and characterized, the full repertoire of these enzymes is not yet completely known [2,107]. The range of terpene synthases that are currently used for the construction of biosynthetic pathways in *Y. lipolytica* is limited to 21 common candidates that lead to poor product variety compared with the reported 80 000 different terpenoids [2], and a low titer in products (Table 1). Thus, further mining and directed evolution of terpene synthases from other genomes suitable for use in *Y. lipolytica* may result in more efficient and diverse biosynthetic pathways. Alternatively, as a result of an irregular coupling reaction, such as

cyclobutylation, branching, or cyclopropanation, DMAPP can be converted to neryl diphosphate (NPP) [108], DMAPP and IPP molecules can be fused to produce different carbon skeletons, such as chrysanthemyl diphosphate (CPP), lavandulyl diphosphate (LPP), macronellyl diphosphate (MPP), or planococyl diphosphate (PPP), resulting in further diversification of the terpene structure [2] (Figure 1(C)). Therefore identifying specific enzymes that catalyze conversion of these uncommon substrates may also help to further diversify the family of terpenoids.

In conclusion, more background knowledge, genetic tools, engineering strategies and efficient enzymes are needed to exploit the full potential of *Y. lipolytica* as a platform strain for terpenoids biosynthesis. We envision that the insights from physiological studies of *Y. lipolytica*, tools developed by synthetic biology and systems biology will provide exciting novel avenues for research into the biosynthesis of terpenoids in *Y. lipolytica*.

Disclosure statement

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