Engineering of *Yarrowia lipolytica* for terpenoid production

Jonathan Asmund Arnesen, Irina Borodina *

The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet 220, 2800, Kgs. Lyngby, Denmark

**A B S T R A C T**

Terpenoids are a group of chemicals of great importance for human health and prosperity. Terpenoids can be used for human and animal nutrition, treating diseases, enhancing agricultural output, biofuels, fragrances, cosmetics, and flavouring. However, due to the rapid depletion of global natural resources and manufacturing practices relying on unsustainable petrochemical synthesis, there is a need for economic alternatives to supply the world’s demand for these essential chemicals. Microbial biosynthesis offers the means to develop scalable and sustainable bioprocesses for terpenoid production. In particular, the non-conventional yeast *Yarrowia lipolytica* demonstrates excellent potential as a chassis for terpenoid production due to its amenability to industrial production scale-up, genetic engineering, and high accumulation of terpenoid precursors. This review aims to illustrate the scientific progress in developing *Y. lipolytica* terpenoid cell factories, focusing on metabolic engineering approaches for strain improvement and cultivation optimization.

1. Introduction

Terpenoids or isoprenoids represent a vast and structurally diverse class of small molecules involved in specialized and general metabolism across the entire kingdom of life (Ashour et al., 2010; Gershenzon and Dudareva, 2007). A unifying feature for all terpenoids is their genesis from 5 carbon (C5) units. The classification of terpenoids is based on the number of C5-units comprising the basic scaffolds: hemiterpenes (C10), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), sesquiterpenes (C30), and tetraterpenes or carotenoids (C40) (Ashour et al., 2010; Sato, 2013). In addition, the isoprene scaffolds may undergo re-arrangement and other modifications, creating numerous diverse structures. Many terpenoids hold value as potent pharma- and nutraceuticals, biofuels, or as flavor, colour, or cosmetic agents (Tetali, 2018). But harvesting terpenoids from natural resources or manufacturing by chemical synthesis is often economically disadvantageous or unsustainable (Idris and Mohd Nadzir, 2021; Pateraki et al., 2017). Therefore, considerable attention has been applied to the producing terpenoids via engineered microbes. The common chassis organisms are *Escherichia coli* and *Saccharomyces cerevisiae* since they are easy to cultivate and engineer, and much is known about their biology (Moser and Pichler, 2019). Recently, the oleaginous yeast *Yarrowia lipolytica* has been intensely researched for its terpenoid production capabilities. This yeast offers benefits like broad substrate utilization, sequenced genomes, and available genetic engineering toolkits, with several strains having achieved GRAS-status (Darvishi et al., 2018; Dujon et al., 2004; Holkenbrink et al., 2018; Magnan et al., 2016; Turck et al., 2019). This review aims to showcase both established and nascent strategies for improving terpenoid production in *Y. lipolytica*.

2. Terpenoid biosynthesis in *Y. lipolytica*

Terpenoid biosynthesis in *Y. lipolytica* occurs via the cytosolic mevalonate (MVA) pathway starting from the condensation of two acetyl-CoA units into acetoacetyl-CoA catalyzed by the acetyl-CoA acetyltransferase (ERG10p) (Cao et al., 2016; Ma et al., 2019; Miziorko, 2011). The 3-hydroxy-3-methylglutaryl-CoA synthase (ERG13p) converts acetoacetyl-CoA and another acetyl-CoA unit forming 3-hydroxy-3-methylglutaryl-CoA (HMGC-CoA), which is then reduced by the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGP) into mevalonate. Subsequently, mevalonate is phosphorylated by the mevalonate kinase (ERG12p) and the phosphomevalonate kinase (ERG8p), forming mevalonate-5-diphosphate, which in turn is decarboxylated by the mevalonate diphosphate decarboxylase (ERG19p) into isopentenyl diphosphate (IPP). The isopentenyl diphosphate isomerase (IDIP) can reversibly convert IPP to its isomer dimethylallyl diphosphate (DMAPP). Further enzymatic condensation of IPP and DMAPP results in the formation of phosphorylated isoprene units such as C10 geranyl diposphate (GPP), C15 farnesyl diposphate (FP), and C20 geranylgeranyl diposphate (GGPP) that serve as precursors for other terpenoids. Interestingly, a recent report suggested the presence of the methylylthritol phosphate (MEP) pathway in *Y. lipolytica* based on liquid chromatography mass-spectrometry and C13C-analysis of...
Various terpenoids have been produced in *Y. lipolytica* with many examples for mono-, sesqui-, tri-, and tetraterpenoids, and a few instances of di- and hemiterpenoids and apocarotenoids (Table 1). This includes flavor and fragrance additives such as the monoterpoids limonene, α-pinene, and linalool, and the sesquiterpoid valencene, (+)-nootkatone (Cao et al., 2017; Cheng et al., 2019; Guo et al., 2018; Wei et al., 2021). Likewise, the sesquiterpene biofuel candidates α- and β-farnesene have been produced at 25.6 and 22.8 g/L, respectively, while the potential pharmaceutical sesquiterpene α-humulene was produced at 3.2 g/L (Guo et al., 2021; Y. Liu et al., 2019; T. Shi et al., 2021). *Y. lipolytica* has also been engineered to produce medicinal triterpenoids like oleanolic acid, protopanaxadiol, ginsenoside K, and the cosmetic ingredient squalene (Gao et al., 2017b; D. Li et al., 2020; Li et al., 2019; Wu et al., 2020). Even plant hormones like the sesquiterpoid abscisic acid and giberellin diterpenoids (GAs) usable for agriculture have been produced in this yeast (Arnesen et al., 2022; Kildegaard et al., 2021). While *Y. lipolytica* shows general potential for terpenoid production, its carotenoid production capabilities are perhaps the most striking. Indeed, the cultivation of an engineered *Y. lipolytica* strain recently resulted in 39.5 g/L and 494 mg/g DCW β-carotene. These production metrics exceed what has been reported in scientific and patent literature on β-carotene production by recombinant and native microbes (Costa Perez et al., 2017; M. Liu et al., 2021).

### 3. Mevalonate pathway engineering

Engineering of the mevalonate pathway for terpenoid overproduction often involves the upregulation of MVA-pathway genes. In particular, the reduction of HMG-CoA to mevalonate catalyzed by HMGp is a popular target for upregulation (Ashour et al., 2010; Polakowski et al., 1998). This tendency is highlighted by the preponderance of the surveyed literature, which uses HMGp-upregulation as a terpenoid overproduction strategy (Table 1). The model yeast *S. cerevisiae* contains two HMGG genes encoding SchHMGIp and SchHMG2p, respectively (Burg and Espenshade, 2011). Both enzymes are regulated on the transcriptional level, but SchHMGIp is also regulated during translation, while SchHMG2p is post-translationally regulated by ubiquitination and endoplasmic reticulum-associated degradation. The negative feedback regulation of SchHMGIp can be overcome by truncating the membrane-anchored N-terminal (tSCHMGIp), resulting in higher HMGp-activity (Polakowski et al., 1998). This led to the hypothesis that the same principle applies to *Y. lipolytica* HMGp, but several studies show that non-truncated HMGp outperforms tHMGp for terpenoid production in *Y. lipolytica* (Gao et al., 2016; Huang et al., 2018; Jia et al., 2019; Kildegaard et al., 2017; H. Liu et al., 2020; S. C. Liu et al., 2020), while a few other studies do not point to large differences (Li et al., 2020; Li et al., 2019). While some studies utilized heterologous NADH-dependent HMGp enzymes based on a presumed large abundance of NADH in *Y. lipolytica*, they did not compare these enzymes to overexpression of NADPH-dependent HMGp versions (Gao et al., 2021; Y. Liu et al., 2019). Therefore, it remains unclear whether shifting HMGp dependency from NAPDH to NADH is a superior strategy for terpenoid production in *Y. lipolytica*.

Besides HMGp upregulation, some studies demonstrate the benefits of overexpressing single or multiple MVA-genes for terpenoid production in *Y. lipolytica*. However, these modifications are typically co-overexpressed with HMGp, due to the long track record of this strategy for boosting terpenoid production. Two studies found that overexpression of ERG12p increased limonene yield (mass of product/dry biomass) 6-fold and seemingly also α-pinene titers (product concentration in cultivation broth) (Gao et al., 2016; Wei et al., 2021). Conversely, another study showed no significant amorphadiene titers increase when ERG12p was overexpressed (Marsafari and Xu, 2020). Overexpression of ERG19p increased lycopene yield, and α-farnesene titer and yield in different studies (S. C. Liu et al., 2020; Schwartz et al., 2017). Overexpression of ERG13p also improved limonene titers by ~20% in one study and β-carotene yield in two studies (Li et al., 2022; Qiang et al., 2020; X.-K. Zhang et al., 2020). Overexpression of IDIp increased α-farnesene titers in a tHMGp-expressing background, but not without HMGp-expression (Yang et al., 2016). IDIp overexpression also...
| Compound  | Carbon Source | Parental Strain | Modifications related to terpenoid biosynthesis | Titer | Reference |
|-----------|---------------|-----------------|-----------------------------------------------|-------|-----------|
| **Hemiterpenoids:** | | | | | |
| Isoprene: | glucose | Po1g | ↑HMG ↑ERG13 ↑IDI ↑PImIISP5 | 530.4 μg/L (sealed vials) | Shaikh and Odaneth (2021) |
| Monoterpenoids: | | | | | |
| Limonene: | Glucose Pyruvate | Po1f | ↑TαLS ↑SINDPS1 ↑HMG ↑ERG12 | 23.6 mg/L (shakeflask) | Cao et al. (2016) |
| | Glucose | Po1g | ↑HMG (↑CLS or ↑MaLS) | D-limonene: 11.7 mg/L or L-limonene: 11.1 mg/L, respectively (bioreactor) | Pang et al. (2019) |
| Limonene: | Glycerol Citrate | Po1f | ↑TαLS ↑SINDPS1 ↑HMG ↑ERG12 | 165.3 mg/L (bioreactor) | Cheng et al. (2019) |
| | Glucose | ATCC 20460 | ↑HMG ↑ERG12 ↑AACL1 ↑ScAACS444F ↑IDI ↑ERG20PPIV ↑N119W ↑SQS ↑PES | 35.9 mg/L (glass tube) | Arnesen et al. (2020) |
| | Lignocellulosic hydroxylate Citric acid | Po1f | ↑aXr ↑aXyX ↑KKS | ↑TαLS ↑SINDPS1 ↑HMG ↑ERG12 | 20.57 mg/L (shake flasks) | Yang et al. (2020) |
| | Waste cooking oil | Po1f | ↑CLS or ↑MaLS | ↑HMG IDI ↑SINDPS1 | 91.24 (D-limonene) and 83.06 (L-limonene) mg/L (shake flasks) | Li et al. (2022) |
| Linalool: | Citrate Pyruvate | Po1f | ↑aAASL ↑ERG29PPIV ↑N119W ↑HMG ↑IDI | 6.96 mg/L (shake flask) | Cao et al. (2017) |
| | Waste cooking oil, soybean oil, or lignocellulosic hydroxylate medium | Po1f | ↑HMG ↑SINDPS1 ↑PIPS ↑ERG8,12 TMBP-ERG12 ↑AMPD | 33.8, 36.6 or 36.1 mg/L for each carbon source, respectively (shake flasks) | Wei et al. (2021b) |
| Sesquiterpenoids: | | | | | |
| Abscisic acid: | Glucose | ATCC 20460 | ↑HMG ↑ERG12 ↑AACL1 ↑ScAACS444F ↑IDI ↑ERG20 ↑SQS ↑BAABA1-4 ↑BcCPR1 ↑P055 ↑ADTX50 | 263.5 mg/L (deepwell plate) | Arnesen et al. (2022) |
| Amorphadiene: | Glucose | Po1g | ↑aAASL ↑HMG ↑ERG12 | 171.5 mg/L (shakeflask) | Marsafari and Xu (2020) |
| | Waste cooking oil | Po1g | ↑HMG ↑GcABCG1 (↑AqalA, ↑ZcAqA, or ↑HxqA) | 973.1 mg/L | Zhao et al. (2021) |
| | (—)α-bisabolol | Po1f | ↑POT1 ↑MrBBS ↑HMG ↑ERG20 ↑SQS | 364.23 mg/L (shake flasks) | (Yirong Ma et al., 2021) |
| | α-farnesene | Glucose Fructose | Po1h | ↑ScHMG ↑IDI ↑MdFS-L-ERG20 | 260 mg/L (bioreactor) | Yang et al. (2016) |
| | α-farnesene | Glucose | Po1f | ↑bAbo818HMG ↑ERG13 ↑MdFS-L-ERG20 ↑ERG12 ↑IDI ↑ERG8,19 ↑aGPPS | 25.55 g/L (bioreactor) | (Y. Liu et al., 2019) |
| | α-farnesene | Glucose Glycerol | Po1f | ↑FS-L-ERG20 ↑ScHMG1 ↑IDI ↑HMG ↑ERG19 | 2.57 g/L (bioreactor) | (S. C. Liu et al., 2020) |
| | α-farnesene | Oleic acid | Po1f | ↑VHb ↑MdFS-L-ERG20 ↑ERG12 ↑IDI ↑ERG8,19 | 10.2 g/L (bioreactor) | (Y. Liu et al., 2021) |
| | β-farnesene | Glucose | ATCC 20460 | ↑HMG ↑ERG12 ↑AACL1 ↑ScAACS444F ↑IDI ↑ERG20 ↑AabFS | 955 mg/L (glass tube) | Arnesen et al. (2020) |
| | β-farnesene | Glucose | Po1f | ↑DGA1 ↑DGA2 ↑HMG ↑BF5 ↑ERG8,10,12,13,19,20 DΔ2 ↑aXpC3,4,5,6 | 22.8 g/L (bioreactor) | (T. Shi et al., 2021) |
| | α-humulene | Glucose | Po1f | ↑POT1 ↑AACH2PS ↑RbHMG35 ↑DIANTI ↑ERG12,8,20,10,13,19,25 ↑DIET5S | 3.2 g/L (bioreactor) | Guo et al. (2021) |
| | α-santalene | Glucose | ATCC 201249 | ↑CST5 ↑ERG8 ↑HMG | 27.92 mg/L (bioreactor) | Jia et al. (2019) |
| Nootkatone: | Valencene | Glucose | ATCC 201249 | ↑CnVS ↑CnCYP706M1-ADDATRI ↑ScHMG ↑ERG20 | Nootkatone: 978.2 μg/L | Guo et al. (2019) |
| | | | | | Valencene: 22.8 mg/L (shake flasks) |
| Valencene: | Glucose | ATCC 20460 | ↑HMG ↑ERG12 ↑AACL1 ↑ScAACS444F ↑IDI ↑ERG20 ↑SQS ↑CnVS | 113.9 mg/L (bioreactor) | Arnesen et al. (2020) |
| Sterols: | Campesterol: | Sunflower seed oil | ATCC 201249 | Δerg5 ↑XIHDRCR7 | 453 mg/L (bioreactor) | Du et al. (2016) |
| | | | | | Zhang et al. (2017) |
| | Campesterol: | Sunflower seed oil | ATCC 201249 | Δerg5 ↑DrDHCRC7 ↑P0X2 | 942 mg/L (bioreactor) | Sun et al. (2019) |
| | | | | | Arnesen et al. (2019) |
| | | | | | Jin et al. (2019) |
| | | | | | Li et al. (2019) |
| | | | | | Sun et al. (2019) |
| | Triterpenoids: | Betulinic acid | Glycerol | ATCC 201249 | ↑HMG1 ↑SQS ↑AULUP1 ↑McCYP716A12-1AAPTR1 | 26.53 mg/L (shake flask) | Sun et al. (2019) |
| | | | | | ↑RcLUS ↑BCPO3 ↑CPR ↑P055 ↑HMG ↑MEF1 | 204.89 mg/L total triterpenoid (shake flask) | Zhang et al. (2020) |
| | | | | | ↑P055 ↑P085 ↑P085-L-AATR1 ↑P0GUT1 | 161.8 mg/L (bioreactor) | Zhang et al. (2020) |
| | | | | | ↑RcLUS ↑HMG ↑SQS ↑OELI1 ↑aXpC4 ↑aXpC2 | 411.72 (shake flasks) | D. Li et al., (2020) |
| | | | | | ↑ScHMG ↑ERG20 ↑SQS ↑GpBAS ↑McCYP716A12-L-LAAPTR1 | 540.7 mg/L (bioreactor) | Zhang et al. (2020) |
| | | | | | 232.5 mg/L (deepwell plate) | Arnesen et al. (2020) |
| | Oleanolic acid | Glucose | ATCC 201249 | ↑HMG ↑ERG20 ↑SQS ↑GpBAS ↑McCYP716A12-L-LAAPTR1 | 22 mg/L (deepwell plate) | Arnesen et al. (2020) |
(continued on next page)
| Compound | Carbon Source | Parental Strain | Modifications related to terpenoid biosynthesis | Titer | Reference |
|----------|---------------|----------------|-----------------------------------------------|-------|-----------|
| Protopanaxadiol | Xylose | ATCC 201249 | **[ScX][ScXH][ScX][ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 300.65 mg/L (bioreactor) | Wu et al. (2019) |
| Squalene | Glucose Citrate | PoI | **[ScX][ScXH][ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 10 mg/gDCW (shake flask) | Huang et al. (2018) |
| Squalene | Glucose | PoI | **[ScX][ScXH][ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 531.6 mg/L | Gao et al. (2017b) |
| Squalene | Glucose | ATCC 20460 | **[ScX][ScX]** 18**[ScX]** 19**[ScX]** | 402.4 mg/L (deepwell plate) | Arnesen et al. (2020) |
| Squalene | Glucose | PoIg | **[ScX]** 18**[ScX]** 19**[ScX]** | 502.7 mg/L (shake flask) | Wei et al. (2021a) |
| Squalene | Glucose | PoI | **[ScX]** 18**[ScX]** 19**[ScX]** | 240.5 mg/L (shake flask) | Wei et al. (2021a) |
| Diterpenoids: | | | | | |
| Gibberellins | Glucose | GB20 | **[ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 12.81 mg/L GA<sub>4</sub>, 16.41 mg/L GA<sub>20</sub>, 0.79 mg/L GA<sub>30</sub>, 4.70 mg/L GA<sub>8</sub> | Kildegaard et al. (2021) |
| Tetraterpenoids: | | | | | |
| Astaxanthin | Glucose | GB20 | **[ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 54.6 mg/L (microtitreplate) | Kildegaard et al. (2017) |
| Astaxanthin | Glucose | GB20 | **[ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 285 mg/L (bioreactor) | Tramontin et al. (2019) |
| Astaxanthin | Safflower oil | GB20 | **[ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 167 mg/L (bioreactor) | N. Li et al., (2020) |
| Cantaxanthin | Glucose | Not described | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 858 mg/L (shake flask) | Yongshuo Ma et al., (2021) |
| β-carotene | glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 4 g/L (bioreactor) | Gao et al. (2017b) |
| β-carotene | glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 0.41 mg/gDCW | Gao et al. (2017a) |
| β-carotene | Glucose | ATCC 20460 | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 6.5 mg/L (bioreactor) | Larroude et al. (2018) |
| β-carotene | Glucose | PoIg | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 12.1 mg/gDCW | Cui et al. (2019) |
| β-carotene | Glucose | S11073 (in-house strain collection) | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 75 mg/L (shake flask) | Bruder et al. (2020) |
| β-carotene | Glucose | ATCC 20460 | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 164 mg/L (deepwell plate) | Arnesen et al. (2020) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 4.5 mg/gDCW | X.-K. Zhang et al., (2020) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 2.4 g/L (bioreactor) | Qiang et al. (2020) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 2.01 g/L (bioreactor) | Lv et al. (2020) |
| β-carotene | Glucose | IMUFRJ 50682 | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 50.1 mg/L (shake flask) | de Souza et al. (2020) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | −2.6 g/L (bioreactor) | Yang et al. (2021) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 1.7 g/L (bioreactor) | L. Liu et al. (2021) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | −12.5 mg/gDCW (96-well plate) | Liu et al. (2022) |
| β-carotene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 7.6 g/L (bioreactor) | M. Liu et al. (2021) |
| Lycopene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 39.5 g/L β-carotene or 17.6 g/L lycopene, respectively (bioreactor) | Ma et al. (2022) |
| Lycopene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 242 mg/L (bioreactor) | Nambou et al. (2015) |
| Lycopene | Glucose | H222 | **[ScX][ScX][ScX]** 18**[ScX][ScX]** 19**[ScX]** | 16 mg/gDCW (bioreactor) | Matthaus et al. (2014) |
| Lycopene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 213 mg/L (bioreactor) | Schwartz et al. (2017) |
| Lycopene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 745 mg/L (bioreactor) | Zhang et al. (2019) |
| Lycopene | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 4.2 g/L (bioreactor) | Luo et al. (2020) |
| Apocarotenoids: | | | | | |
| α-ionone | Glucose | PoI | Proprietary information | 408 mg/L (bioreactor) | Czajka et al. (2020) |
| β-ionone | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 380 mg/L (bioreactor) | Czajka et al. (2018) |
| β-ionone | Glucose | PoI | **[ScX]** 18**[ScX][ScX]** 19**[ScX]** | 0.98 g/L (bioreactor) | Lu et al. (2020) |
increased linalool titers 2.8-fold in an HMGp-overexpressing back
ground (Cao et al., 2017). Interestingly, three-copy, but not single-,
or double-copy, overexpression of IDI benefitted linalool titers, but
not limonene yield, without HMG-overexpression (Cao et al., 2016, 2017).
This suggests that HMG-upregulation influences the effects of other
MVA-pathway modifications on terpenoid production. Another strategy
was to upregulate the entire MVA-pathway. It was found that over-
expression of all MVA-pathway genes (ERG10, 13, 12, 8, 19, 20, HMG,
and IDI) boosted β-carotene production by 46% and β-ionone titers by
2.8-fold in separate studies, with the caveat that non-neutral genomic
loci like POX genes were targeted for DNA construct integration (Gao
et al., 2017b; Lu et al., 2020).
Furthermore, increasing the conversion of DAMPP/IPP into the
appropriate terpene precursor GPP, FPP, or GGPP can be advantageous.
The expression of a mutated farneyl diphosphate synthase (ERG20
389p,119p) resulted in 0.56 mg/L linalool while the parental strain produced
0.09 mg/L (Cao et al., 2017). This strategy was based on a previous study in S. cerevisiae that demonstrated that the mutation of similar residues in S. cerevisiae ERG20 changed its function into a geranyl diphosphate synthase (Ignea et al., 2014). ERG20 over-
expression resulted in 54.68 mg/L of the sesquiterpenoid
(−)-â-bisabolol, while the parental strain only produced 39.83 mg/L
(Yirong Ma et al., 2021). Likewise, overexpression of ERG20p alongside
S. cerevisiae tHMGP-expression tsCHMGP resulted in 22.8 mg/L (+)-valencene and 978.2 µg/L (+)-nootkatone, while only expressing
tsCHMGP provided 10.9 mg/L (+)-valencene and 551.1 µg/L (+)-nootkatone (Guo et al., 2018). Increasing the copy number of ERG20
from two to three copies increased abscisic acid production with a
strain-dependency (Arnesen et al., 2022).

The overexpression of GGPPS resulted in a 4-fold increase in
β-carotene titer (Kildegaard et al., 2017). In another study, the expres-
sion of the archaeal Archaeoglobus fulgidus GGPPS increased carotenoid
yield 2.6-fold, while the combined overexpression of ERG20p and native
GGPPS only increased carotenoid yield by 1.9-fold (M. Liu et al., 2021).
Likewise, expression of Synecochoccus sp. (cyanobacterium)
(SsGGPSP77) increased β-carotene titers by 272%, while expression of a
second Xanthophyllomyces dendrorhous GGPP synthase (XdCM) copy
increased β-carotene titers by 49% (Tramontin et al., 2019). Interest-
ing, a comparison of GGPP productivity of GGPPS/crtE enzymes from
various organisms put them in the order of Taxus canadensis, Pantoaea
glomerans, Y. lipolytica, Sulfobolus acidocaldarius, and X. den-
drorhous from lowest to highest (Ma et al., 2022). Interestingly, the expression of S. acidocaldarius GGPPS (SsGGPSP) provided the high-
est β-carotene titer and β-carotene to lycopene conversion ratio to the
other GGPPS enzymes. This was explained by the slightly lower flux of SaGGPPSp, avoiding too rapid lycopene build-up and therefore preventing substrate inhibition.

Another option for improving terpenoid production is to limit the flux of intermediates towards undesired side-products. In the case of non-terpenoid or -sterol products, this may be achieved by reducing flux towards squalene. Replacement or truncation of the native SQS promoter was found to increase β-carotene titers by 2-2.5-fold (Kildegaard et al., 2017). Interestingly, some of these modifications also increased squalene titers. Contrarily, SQS-promoter replacement did not positively affect increased squalene titers. Contrarily, SQS-promoter replacement did not non-triterpenoid or -sterol products, this may be achieved by reducing availability. The mannitol dehydrogenase makes NADPH from NADP⁺, which has attempted to improve terpenoid production by increasing NADPH synthesis (Christen and Sauer, 2011; Dahlin et al., 2019). Nevertheless, strategies that increase acetyl-CoA flux and their effect on terpenoid production in Y. lipolytica have been studied (Fig. 2). The ATP-citrate lyase (ACLp) forms acetyl-CoA, oxaloacetate, and ADP + Pi from citrate and CoA in the cytosol (Blazeck et al., 2014; Dulermo et al., 2015). ACLp consists of two subunits, ACL1p and ACL2p, in Y. lipolytica. Overexpression of ACL1p with co-expression of a mutated version of the Salmonella enterica acetyl-CoA synthetase (SeACS), that forms acetyl-CoA, AMP, and diphosphate from acetate, CoA, and ATP, improved squalene yield by 3.2-fold (Huang et al., 2018). Individual expression of either SeACS or ACL1p did not significantly enhance squalene accumulation. Overexpression of SeACS or ACL1p did not significantly enhance squalene accumulation. Overexpression of ACL1p and ACL2p also improved β-farnesene titer and yield (Arnesen et al., 2020). Furthermore, overexpression of ACL1p increased acetyl-CoA production in the PO1f background, while ACL2p overexpression decreased acetyl-CoA accumulation (Huang et al., 2018). Curiously, the overexpression of ACL2p, but not ACL1p, increased the triterpenoid titers in some strain backgrounds, while both ACL1p and ACL2p overexpression increased lycopene production independently (Jin et al., 2019; Zhang et al., 2019). ACL2p overexpression also improved squalene titer, but decreased the yield due to a corresponding increase in biomass (H. Liu et al., 2020). ACLp overexpression increased campesterol yield 1.3-fold (Zhang et al., 2017). Overexpression of the adenine monophosphate deaminase (AMPDp) increased lycopene titer and yield (Zhang et al., 2019). AMPDp inhibit the isocitrate dehydrogenase, which increase citrate and, by extension, acetyl-CoA levels.

Expression of a non-native pathway for acetyl-CoA generation consisting of the Bifidobacterium bifidum phosphoketolase (BbPTp) and Bacillus subtilis phosphotransacetylase (BbPTA) increased β-ionone titer by 2-fold in the PO1f background and lycopene yield in Y. lipolytica, but more research on this topic is warranted.

Y. lipolytica is presumed to have high acetyl-CoA abundance relative to other common microbial chassis, which may have been inferred by the medium-to-high lipid accumulation obtained by some Y. lipolytica strains during particular cultivation conditions (Beopoulos et al., 2008; Kerkhoven et al., 2016). Some studies indicate that Y. lipolytica possesses higher acetyl-CoA flux and abundance than S. cerevisiae under similar cultivation conditions, but more evidence is needed to justify such generalizations (Christen and Sauer, 2011; Dahlin et al., 2019). Nevertheless, strategies that increase acetyl-CoA flux and their effect on terpenoid production in Y. lipolytica have been studied (Fig. 2). The ATP-citrate lyase (ACLp) forms acetyl-CoA, oxaloacetate, and ADP + Pi from citrate and CoA in the cytosol (Blazeck et al., 2014; Dulermo et al., 2015). ACLp consists of two subunits, ACL1p and ACL2p, in Y. lipolytica. Overexpression of ACL1p with co-expression of a mutated version of the Salmonella enterica acetyl-CoA synthetase (SeACS), that forms acetyl-CoA, AMP, and diphosphate from acetate, CoA, and ATP, improved squalene yield by 3.2-fold (Huang et al., 2018). Individual expression of either SeACS or ACL1p did not significantly enhance squalene accumulation. Overexpression of SeACS or ACL1p did not significantly enhance squalene accumulation. Overexpression of ACL1p and ACL2p also improved β-farnesene titer and yield (Arnesen et al., 2020). Furthermore, overexpression of ACL1p increased acetyl-CoA production in the PO1f background, while ACL2p overexpression decreased acetyl-CoA accumulation (Huang et al., 2018). Curiously, the overexpression of ACL2p, but not ACL1p, increased the triterpenoid titers in some strain backgrounds, while both ACL1p and ACL2p overexpression increased lycopene production independently (Jin et al., 2019; Zhang et al., 2019). ACL2p overexpression also improved squalene titer, but decreased the yield due to a corresponding increase in biomass (H. Liu et al., 2020). ACLp overexpression increased campesterol yield 1.3-fold (Zhang et al., 2017). Overexpression of the adenine monophosphate deaminase (AMPDp) increased lycopene titer and yield (Zhang et al., 2019). AMPDp inhibit the isocitrate dehydrogenase, which increase citrate and, by extension, acetyl-CoA levels.

Expression of a non-native pathway for acetyl-CoA generation consisting of the Bifidobacterium bifidum phosphoketolase (BbPTp) and Bacillus subtilis phosphotransacetylase (BbPTA) increased β-ionone titer by

![Fig. 2. Overview of metabolic engineering strategies pertaining to acetyl-CoA, fatty acid metabolisms, and substrate utilization used in Y. lipolytica for terpenoid production in different studies.](image-url)
32% (Lu et al., 2020). The phosphoketolase can convert fructose-6-phosphate or xylose-5-phosphate into erythrose-4-phosphate or glyceraldehyde-3-phosphate, respectively, and acetyl-CoA, while the latter can be converted into acetyl-CoA by the phosphotransacetylase (Bergman et al., 2016).

Alternatively, acetyl-CoA accumulation can be affected by decreasing flux towards lipid biogenesis or increasing rates of fatty acid degradation. Knocking out the diacylglycerol acyltransferase genes Δgag1 and Δgag2 reduced the lipid content from 26.3% to 8.7% and increased β-farnesene titers by 56.2% (T. Shi et al., 2021). Knocking out Δgag1 or Δgag2 separately seemingly provided lesser reductions in lipid content and lesser increases in β-farnesene titers. The peroxisome acetyl-CoA oxidase 2 (POX2p) overexpression increased campesterol yield 1.3-fold (Zhang et al., 2017). The growth media contained sunflower oil with abundant long-chain fatty acids, likely providing a substrate for the enhanced β-oxidation pathway. Interestingly, overexpression of POX1,4, 5,6p under the same conditions did not affect campesterol yield, while overexpression of POX3p, the multifunctional-oxidation protein (MFEp), and the peroxisomal o xoacyl thiolase (POT1p) decreased campesterol yield. MFEp catalyzes hydration and dehydrogenation reactions during β-oxidation, while POT1p catalyzes the thiolytic cleavage of β-ketoacyl-CoA into acetyl-CoA and a shortened acyl-CoA molecule (Boopoulos et al., 2009; Hanko et al., 2018; Smith et al., 2020). Interestingly, overexpression of MFE1p and POT1p increased (−)-α-bisabolol titers (Yirong Ma et al., 2021). Overexpression of POT1p also enhanced α-humulene titer and yield in a strain where the MVA- and α-humulene biosynthetic pathways were targeted to the peroxisomes, while overexpression of MFE1p or the peroxisomal biogenesis factor 10 (PARK10p) did not provide substantial benefits (Guo et al., 2021). MFE1p and POT1p overexpression also increased triterpenoid titers in some strain backgrounds (Jin et al., 2019). Strain-dependent increases in triterpe- noid titers were also found when the long-chain fatty acid transporter (PXM1p) or triacylglycerol lipase (TGL3p) were overexpressed. Alternatively to genetic engineering, adding the lipid biosynthesis blocking compound cerulenin to the growth media enhanced the amorpha diene titer by 231.13% with a strain-specific dependency (Marsafari and Xu, 2020). Lastly, expression of Vitreoscilla hemoglobin (Vhbp) was demonstrated to improve α-farnesene production by 12.7%, likely by enhancing oxygen delivery to the cells (Y. Liu et al., 2021).

5. Modulation of lipid storage

Some hydrophobic terpenoids like β-carotene are stored in the lipid bodies of Y. lipolytica (Larroude et al., 2018). Therefore, it may be beneficial to increase sequestration of such lipophilic products by expanding the lipid pool. Larroude et al. developed an obese Y. lipolytica platform strain by overexpression of DGA2, the glycerolaldehyde-3-phosphate dehydrogenase gene (GPD1), and deletion of Δpox1-6 and Δa2g4 (Larroude et al., 2018). This obese strain accumulated 3.6-fold more lipids and the β-carotene titer and yield were boosted by 1.9- and 2.6-fold compared to the wt. Overexpression of DGA1 increased squalene yield by 2.9-fold and seemingly increased lipid accumulation (Wei et al., 2021a). Likewise, deletion of Δpox10 increased squalene production 9-fold. Furthermore, the co-deletion of Δa2g2, which encodes a protein putatively involved in oxidative stress responses and nitrogen metabolism, and Δpox10 improved squalene production more than the individual deletions. Overexpression of DGA1 and the acetyl-CoA carboxylase gene (ACC1) increased the lipid content by 2.97- or 1.76-fold depending on the strain background, increased the accumulation of GPP, FPP, and GGPP, and seemingly benefitting lycopene production (Luo et al., 2020). Furthermore, overexpression of either GPD1p, DGA1p, or DGA2p increased lycopene titer and yield (Zhang et al., 2019). Presumably, engineering strategies for increased fatty acid accumulation may be advantageous specifically for intracel- lularly accumulated products. Indeed, deletion of Δpox10 decreased the titer and yield by >50% of the extracellularly accumulated α-farnesene, possibly due to reduced acetyl-CoA availability (S. C. Liu et al., 2020).

The overexpression of the Δ9-fatty acid desaturase gene (OLE1) combined with the deletion of the diacylglycerol kinase (Δdkg1) and phosphatidic acid phosphatase (Δap1h1) genes increased the lupeol titer 4.7-fold while skewing the fatty acid profile from dominantly saturated to unsaturated (J.-L. Zhang et al., 2020). These gene edits also increased the ratio of extracellularly to intracellularly accumulated lupeol during two-phase cultivations with an organic overlay and were shown to benefit the production of α- and β-amyrin, and longifolene-type sesqui- terpenes. These results were primarily attributed to increased plasma membrane permeability following lipid unsaturation allowing increased efflux into the extracellular phase.

A β-carotene producing strain was used to construct a mutant library by NHEJ-mediated random mutagenesis (Liu et al., 2022). This approach identified four new gene targets that improved β-carotene when a leucine prototrophy conferring gene-cassette (LEU2) was inte- grated with proximity to or into the gene. The largest increase in β-carotene production was achieved by NHEJ-mediated insertion of LEU2 into the NDH80 locus. Reverse engineering by deletion of NDTS0 in the parental strain increased β-carotene production by 62%. Furthermore, the lipid content increased, while the ergosterol content decreased in the ΔNDTS0-strain compared to the parental strain, which may simultaneously expand the β-carotene storage capabilities and redirect MVA-pathway flux towards carotenoid biosynthesis.

6. Compartmentalization, morphology, and transport engineering

Several studies have applied pathway compartmentalization for terpenoid production in yeast (Dusséaux et al., 2020; G. S. Liu et al., 2020; Y. Shi et al., 2021).

Targeting the peroxisomes for α-humulene biosynthesis was achieved by fusing a peroxisomal targeting signal (PTSp) peptide to the MVA-pathway enzymes and the Agnullaria grassna α-humulene synthase (AchHspsp) (Guo et al., 2021). The report demonstrated that only complete, but not partial, peroxisomal re-construction of the MVA-pathway improved α-humulene titers (50-fold) together with AchHspsp expression. Further increases in α-humulene production were achieved by β-oxidation engineering (see previous chapter) and overexpression of the peroxisomal adenine nucleotide transporter gene (ANT1). This latter strategy increased the α-humulene titer by 11%, likely due to increased transport of cytoplasmic ATP into the peroxi- somes. While the cited work resulted in a highly productive α-humulene strain (3.2 g/L), it is unclear whether the peroxisomal targeting strategy was superior to conventional cytoplasmic expression. However, another report does provide some evidence that compartmentalization can be beneficial for terpenoid production in Y. lipolytica. Increasing the copy number of a non-targeted astaxanthin biosynthetic fusion enzyme gene, consisting of the Paracoccus sp. β-carotene ketolase fused to the N-terminal of the Haematococcus pluvialis β-carotene hydroxylase (PsCrtW-HpCrtZ), did not affect astaxanthin production (Yongshuo Ma et al., 2021). Yet, the astaxanthin titer increased 1.62- to 1.84-fold when PsCrtW-HpCrtZp was targeted to either the lipid bodies, ER, or peroxi- somes by fusion with the appropriate signal peptides. When PsCrtW-HpCrtZp was simultaneously targeted to all three compart- ments, the astaxanthin titer increased 4.8-fold. Expression of either the Escherichia coli resistance-nodulation-division family efflux pump (EcAcraBp) or the Gromania clavigera ATP-binding cassette transporter (GcABC1Gp) increased the titers of various bisabolene isomers during two-phase organic cultivation (Zhao et al., 2021).

Deleting genes involved in the single cells to hyphae transition can improve terpenoid production (M. Liu et al., 2021). Deleting the protein kinase gene (ΔCLA4) involved in the transition from yeast cells to fila- ments increased the β-carotene yield by 81%, although pseudohyphal state could still be observed. Likewise, the deletion of the transcription factor gene (ΔMYH1) abolished hyphae formation and increased the β-carotene production of Y. lipolytica. The overexpression of the Δ9-fatty acid desaturase gene (OLE1) combined with the deletion of the diacylglycerol kinase (Δdkg1) and phosphatidic acid phosphatase (Δap1h1) genes increased the lupeol titer 4.7-fold while skewing the fatty acid profile from dominantly saturated to unsaturated (J.-L. Zhang et al., 2020). These gene edits also increased the ratio of extracellularly to intracellularly accumulated lupeol during two-phase cultivations with an organic overlay and were shown to benefit the production of α- and β-amyrin, and longifolene-type sesqui- terpenes. These results were primarily attributed to increased plasma membrane permeability following lipid unsaturation allowing increased efflux into the extracellular phase. A β-carotene producing strain was used to construct a mutant library by NHEJ-mediated random mutagenesis (Liu et al., 2022). This approach identified four new gene targets that improved β-carotene when a leucine prototrophy conferring gene-cassette (LEU2) was inte- grated with proximity to or into the gene. The largest increase in β-carotene production was achieved by NHEJ-mediated insertion of LEU2 into the NDH80 locus. Reverse engineering by deletion of NDTS0 in the parental strain increased β-carotene production by 62%. Furthermore, the lipid content increased, while the ergosterol content decreased in the ΔNDTS0-strain compared to the parental strain, which may simultaneously expand the β-carotene storage capabilities and redirect MVA-pathway flux towards carotenoid biosynthesis.
yield by 45%. Overexpression of endosomal sorting complex subunit DID2p increased β-carotene yield by 260%. In addition, it increased the expression of specific genes involved in β-carotene biosynthesis, the pentose phosphate pathway, the tricarboxylic acid pathway, and the hexokinase encoding gene (Hxk) (Yang et al., 2021).

While the above examples provide exciting investigations into the topics of compartmentalization, morphology engineering, and cellular transport, these research areas remain relatively unexplored in Y. lipolytica. It would be exciting for future research to expand these areas, which show great promise.

7. Enzyme engineering

Some papers demonstrate the utility of engineered enzymes for terpenoid production in Y. lipolytica. A common pathway improvement strategy is fusing proteins catalyzing consecutive biocatalytic steps or linking enzymes with supporting protein partners.

The pairing of the Paracoccus sp. ketolase (PsCrtWp) and the Haematococcus pluvialis hydroxylase (HpCrtZp) provided the highest astaxanthin titers in a small combinatorial screen (Yongshuo Ma et al., 2021). Fusing PsCrtWp and HpCrtZp via a protein linker increased the astaxanthin titers compared to separate expression, with 2.2- to 2.8-fold increases for the HpCrtZ-linker-PsCrtWp or PsCrtW-linker-HpCrtZp fusion protein, respectively. The fusion of the Malus x domestica α-farnesene synthase (MdFsp) with ERG20p increased α-farnesene titers compared to separate expression (Yang et al., 2016). The MdF8s-linker-ERG20p fusion outperformed the ERG20p-linker-MdF8sp fusion by 30%. Direct fusion of the Callitropsis nootkatensis cytochrome P450 (CnCYP706M1p) with the N-terminally truncated A. thaliana cytochrome P450 (AtATR1p) increased (−)-nootkatone titers ~6-fold compared to separate expression of CnCYP706M1p and non-truncated AtATR1p, although multi-loci integration was used for both constructs (Guo et al., 2018). Interestingly, adding a “GSTSSG” linker between CnCYP706M1p and tAtATR1p seemingly reduced the (−)-nootkatone titers. Fusion of the propanoxadiol synthase (PpDSp), a cytochrome P450, and AtATR1p also increased propanoxadiol titers 2.3-fold compared to co-expression (Wu et al., 2019). Truncation of the AtATR1p N-terminal is likely necessary due to the presence of a transmembrane domain. Indeed, no benefits to terpenoid production were found when non-truncated cytochromes P450 and reductases were fused with varying linker lengths or C-/N-terminal configurations (Jin et al., 2019). Other studies have also used cytochrome P450 and reductases fusions to produce oleanolic acid, betulinic acid, and ginsenoside compound K (D. Li et al., 2020; Li et al., 2019; Sun et al., 2019).

Protein tagging can improve stability and solubility. For example, expression of ERG12p N-terminally tagged with maltose-binding protein (MBP) increased α-pinene 1.84-fold compared to overexpression of the untagged ERG12p (Wei et al., 2021b).

The effects of protein fusion and removal of the plastidial targeting sequences by N-terminal truncation of the A. thaliana copalyol diphoste synthase (AtCPFSp), ent-kaurene synthase (AtKSp), ent-kaurene oxidase (AtKOp) on GA-biosynthesis was investigated (Kildegaard et al., 2021). The highest GA-titers were achieved by expressing the non-fused truncated enzymes.

Structure-guided protein engineering of the R domain/lycopene cyclase from McCarRPp successfully removed substrate inhibition by lycopene (Ma et al., 2022). Structure modeling and a Position-Specific Scoring Matrix (PSSM) based on the lycopene cyclase domain and its homologs provided an evolutionary basis for selecting amino acid residues for substitution. A sampling of the variance space allowed the isolation of three McCarRPp variants, V175W, T31R–F92W, and Y27R, with improved β-carotene to lycopene ratios. The Y27R-variant had a 98% selectivity compared to 18% for the WT for β-carotene.

8. Alternative substrate utilization

The broad substrate utilization of Y. lipolytica makes it an excellent chassis for turning unconventional low-value or waste carbon sources into high-value products. Although glucose is commonly used as a substrate for terpenoid production in Y. lipolytica (Table 1) in the lab, for industrial processes, it can be advantageous to use cheaper or more abundant feedstock. It was demonstrated that oleic acid could be used as a carbon source for α-farnesene production with the final titer of 10.2 g/L achieved during fed-batch cultivation (Y. Liu et al., 2021). Oleic acid seemed to provide slightly better titers compared to glucose. α-farnesene was also produced in the 1–2 g/L range when cultivated in shake flasks with soybean oil, either fresh or from as waste cooking oil (WCO), olive, palm, glycerol trioleate, or rapeseed oil. Likewise, substituting oleic acid for glucose at carbon equivalent concentrations increased α-humulene titers by 18.5% for a strain with the MVA- and α-humulene pathway targeted to the peroxisomes (Guo et al., 2021). Using safflower oil with an oleic acid content of 77.0% as a carbon source during fed-batch cultivation resulted in 167 mg/L astaxanthin with 48% accumulated extracellularly (N. Li et al., 2020). The production of astaxanthin per cmol of carbon when using safflower oil as a carbon source was similar to that of glucose. Using sunflower seed oil at as carbon source resulted in higher campesterol titers than glucose at carbon equivalent concentrations (Du et al., 2016). It was also observed that sunflower oil increased lipid accumulation and lipid body formation compared to glycerol or glucose. Limonene titers were higher with WCO than glycerol or glucose at carbon equivalent concentrations (Li et al., 2022).

Conversely, bisabolene titers were lower when using WCO than glucose at carbon equivalent concentrations, despite higher biomass accumulation when using WCO (Zhao et al., 2021). The addition of Mg2+ to WCO-based rich media increased the bisabolene titers, which was also found for limonene production (Pang et al., 2019). It was demonstrated that WCO, sunflower, rapeseed, or soybean oil could be used for α-pinene production, with the latter noted to increase α-pinene titer 80% compared to glucose (Wei et al., 2021b). The expression of the xyllose metabolism genes encoding the Scheffersomyces stipites xylitol reductase (SsXR), S. stipites xylitol dehydrogenase (SsXDH), and native xylulokinase (XK) enabled α-pinene production and highly enhanced growth on xylene-containing rich media. But the expression of the xylene metabolism genes did not benefit α-pinene production when detoxified lignocellulosic hydrolysate, with major constituents being glucose, xyllose, and acetate, was used as carbon source. Another report demonstrated that the genes encoding SsXR, SsXDH, and S. stipites xylulokinase (SsXK) or XK enabled growth on xylene-based rich media (Yao et al., 2020). The expression of XK yielding better growth than SsXR. A mixture of 8 g/L glucose and 32 g/L xylene provided higher limonene titers compared to 40 g/L of either pure glucose or xylene for the engineered strain. Likewise, it was demonstrated improved growth on xylene based media by expressing SsXRX427W/R272D, SsXDH, and XK (Wu et al., 2019). The ability of the strain to consume xylene was improved by an adaptation period on xylene containing media, after which further engineering enabled protopanaxadiol (PPD) production. Overexpression of the transketolase (TKLP) and transaldolase (TALP), which connect the xylene degradation pathway with the pentose phosphate pathway, improved the PPD titer and biomass accumulation on xylene based media. The final engineered strain exhibited the best PPD titers on pure xylene as a carbon source compared to glucose or mixed sugar compositions.

Using glycerol instead of glucose, soybean, corn oil, or oleic acid at the same concentrations resulted in the highest betulinic acid titer (Sun et al., 2019). Interestingly, the expression of specific MVA-pathway genes and acetyl-CoA accumulation increased during cultivation with glycerol compared to glucose. Another report found that glycerol improved limonene yield compared to glucose, citrate, fructose, maltose, sucrose, mannose, or galactose at similar concentrations (Cheng et al., 2019). Adding auxiliary carbon sources like citrate,
pyruvate, malate, but not acetate increased limonene titers further. Similarly, α-farnesene titer and yield during fed-batch bioreactor cultivation also improved when glycerol was fed instead of glucose, although the batch phase used glucose as a carbon source (S. C. Liu et al., 2020). Contrarily, glycerol decreased limonene yield or α-pinenene titer and yield compared to glucose at similar concentrations (Cao et al., 2016; Wei et al., 2021b).

Using citrate as a carbon source resulted in the highest linalool yield and titer compared to fructose, glucose, or glycerol, with the latter being the second-best carbon source (Cao et al., 2017). The addition of pyruvate further increased linalool titer and yield. Likewise, the addition of pyruvic acid also increased limonene yield (Cao et al., 2016). The addition of citrate or acetate increased squalenone yield for a strain expressing ACL1p and SeACS6Δ4IPp, which respectively can convert these substrates to acetyl-CoA (Huang et al., 2018). The addition of 4 g/L citrate toYPD medium enhanced α-pinenene yield and titer, which was not found for the addition of pyruvate, acetate, or malate in the range of 0–4 g/L, or lower concentrations of citrate (Wei et al., 2021b).

The ability of *Y. lipolytica* to utilize glucose was enhanced by overexpression of the hexokinase (HXKp), which catalyzes the phosphorylation of glucose as the initial step in the glycolysis pathway (Qiang et al., 2020). HXKp overexpression enhanced β-carotene yield by 98% and led to faster glucose consumption. Using palmitic acid as an addition to glucose-based rich media enhanced lycopene titers, lipid accumulation, and the accumulation of MVA-pathway metabolites like FPP and GGPP (Luo et al., 2020). Experiments using C12-labeled glucose showed that >90% of C16:0 and C18:0 lipids were unlabeled and therefore most likely derived from the exogenous lipid source, while ~75% acetyl-CoA was derived from the labeled glucose. These findings are supported by (Ma et al., 2022), that demonstrated abundant unlabeled IPP/DMAPP and GGPP during cultivation with labeled glucose and unlabeled stearic acid. Therefore, the addition of fatty acids could enhance intracellular terpenoid storage and contribute to the acetyl-CoA pool via β-oxidation.

In summary, carbon sources like glycerol or WCO can be used as substrates for terpenoid production in *Y. lipolytica*. Furthermore, the research shows that the substrate acceptance of *Y. lipolytica* can be widened by engineering. Various auxiliary carbon sources also show promise; but their utility may be decided by their price-to-benefit ratio. Therefore, terpenoid production by cultivating *Y. lipolytica* with alternative and cheap carbon sources represents a promising avenue for developing economic and sustainable bioprocesses.

### 9. Outlook

The current research has demonstrated numerous varied strategies for improving terpenoid production in *Y. lipolytica*. However, while some strategies achieve different results across studies, it is important to consider that the contexts vary greatly; factors like strain background, cultivation conditions, properties of the specific terpenoid product and pathway, and previous strain modifications potentially affect the outcomes. Nevertheless, direct MVA-pathway engineering has been shown in multiple studies to improve terpenoid titers several-fold. Cofactor engineering by redirecting flux towards cytosolic acetyl-CoA has also shown some utility. Conversely, increasing lipid accumulation for intracellular storage is highly effective for producing some terpenoids, although likely dependent on the product’s propensity to accumulate intra- or extracellularly. Emerging evidence demonstrates new strategies like morphology or compartmentalization engineering that increase terpenoid production in *Y. lipolytica*. Still, more research is needed to identify in which contexts these strategies are effective. Besides modifying the native metabolism, the careful selection and engineering of heterologous pathway and supportive enzymes by protein fusion, tagging, or modifying select amino residues can lead to several-fold increased terpenoid titers in *Y. lipolytica*. Media optimization is a valuable addition to genetic engineering, and alternative substrates and additives have been used to modestly increase terpenoid production in some reports. In summary, there are ample well-described and nascent strategies for improving terpenoid production in *Y. lipolytica*, and highly productive *Y. lipolytica* cell factories for terpenoids with short heterologous biosynthetic pathways have been developed. Indeed, the implementation of long heterologous biosynthetic pathways in *Y. lipolytica* remains a challenge with a good example being the production of gibberellic plant hormones (Kildegaard et al., 2021). Before the high-level production of complex terpenoids becomes possible, greater knowledge about the expression of individual enzymes and balancing long non-native pathways in *Y. lipolytica* is needed. Computational approaches utilizing genome-scale modelling, omics, and machine learning have yet to substantially impact terpenoid production in *Y. lipolytica*. These tools may become more relevant as they develop and the knowledge of *Y. lipolytica* metabolism expands.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgements

The research was funded by the Novo Nordisk Foundation (Grant Agreement No. NNFP200CC0035580, NNFP200CC006809, and NNFP15CC0016592) and by the European Research Council under the European Union’s Horizon 2020 research and innovation programme (YEAST-TRANS project, Grant Agreement No. 757384) and European Union’s Horizon 2020 research and innovation programme under grant agreement No. 760798 (OLEFINE). Figs 1 and 2 were created with BioRender.com.

### References

Arnesen, J.A., Jacobsen, L.H., Dyekjær, J.D., Rago, D., Kristensen, M., Klitgaard, A.K., Randelovic, M., Martinez, J.L., Borodina, I., 2022. Production of asbacic acid in the oleaginous yeast *Yarrowia lipolytica*. FEMS Yeast Res. 20, 1–34. https://doi.org/10.1093/femsyr/foac015.

Arnesen, J.A., Klitgaard, K.R., Cermuda Pastor, M., Jayachandran, S., Kristensen, M., Borodina, I., 2020. *Yarrowia lipolytica* strains engineered for the production of terpenoids. Front. Bioeng. Biotechnol. 8, 1–14. https://doi.org/10.3389/fbioe.2020.00045.

Ashour, M., Wink, M., Gershenzon, J., 2010. Biochemistry of terpenoids: monoterpens, sesquiterpenes and diterpenes. In: Biochemistry of Plant Secondary Metabolism. Wiley-Blackwell, Oxford, UK, pp. 258–303. https://doi.org/10.1002/9780470622503.ch9.

Beopoulos, A., Cescut, J., Haddouche, R., Uribelearena, J.L., Molina-Jouve, C., Nicaud, J. M., 2009. *Yarrowia lipolytica* as a model for bio-oil production. Prog. Lipid Res. 48, 375–387. https://doi.org/10.1016/j.plipres.2008.09.005.

Beopoulos, A., Mozová, Z., Ževenieka, F., Le Dall, M.T., Hapala, I., Papanikolaou, S., et al., 2008. Control of lipid accumulation in the yeast *Yarrowia lipolytica*. Appl. Environ. Microbiol. 74, 7779–7789. https://doi.org/10.1128/AEM.01412-08.

Bergman, A., Stiewers, V., Nielsen, J., Chen, Y., 2016. Functional expression and evaluation of heterologous phosphoketolases in *Saccharomyces cerevisiae*. Amb. Express 6, 115. https://doi.org/10.1186/s13566-016-0290-0.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., 2000. The protein data bank. Nucleic Acids Res. 28, 235–242. https://doi.org/10.1093/nar/28.1.235.

Blazek, J., Hill, A., Liu, L., Knight, R., Miller, J., Pan, A., Otoupal, P., Alper, H.S., 2014. Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. Nat. Commun. 5. https://doi.org/10.1038/ncomms4131.

Bonanno, J.B., Edo, C., Ewar, N., Pieper, U., Romanzowski, M.J., Ilyin, V., Gerchman, S. E., Kycia, H., Studier, F.W., Sali, A., Burke, S.L., 2001. Structural genomics of enzymes involved in sterol/isoprenoid biosynthesis. Proc. Natl. Acad. Sci. USA 98, 12896–12901. https://doi.org/10.1073/pnas.181466998.

Bruder, S., Melcher, F.A., Zoll, T., Hackenschmidt, S., Kabisch, J., 2020. Evaluation of a *Yarrowia lipolytica* strain collection for its lipid and carotenoid production capabilities. Eur. J. Lipid Sci. Technol. 122, 1900172 https://doi.org/10.1002/ejlt.201900172.

Burg, J.S., Espeneshade, P.J., 2011. Regulation of HMG-CoA reductase in mammals and yeast. Prog. Lipid Res. 50, 403–410. https://doi.org/10.1016/j.plipres.2011.07.002.

Cao, X., Lv, Y.B., Chen, J., Imanaka, T., Wei, L.I., Hua, Q., 2016. Metabolic engineering of oleaginous yeast *Yarrowia lipolytica* for limonene overproduction. Biotechnol. Biofuels 9, 1–11. https://doi.org/10.1186/s13068-016-0526-7.
Cao, X., Wei, L.J., Lin, J.Y., Hua, Q., 2017. Enhancing linalool production by engineering oleaginous yeast Yarrowia lipolytica. Bioreourc. Technol. 245, 1641–1644. https://doi.org/10.1016/j.biortech.2017.05.102.

Cheng, B.-Q., Wei, L.-J., Ly, V.-B., Chen, J., Hua, Q., 2019. Elevating limonene production in oleaginous yeast Yarrowia lipolytica via genetic engineering of limonene biosynthesis pathway and optimization of medium composition. Biotechnol. Bioproc. Eng. 24, 500–506. https://doi.org/10.1007/s12018-019-00497-9.

Christen, S., Sauer, U., 2011. Intracellular characterization of aerobic glucose metabolism in seven yeast species by 13C flux analysis and metabolomics. FEMS Yeast Res. 11, 263–272. https://doi.org/10.1111/j.1567-6004.2010.00713.x.

Costa Perez, J., Morales Rodriguez, A.R., De La Fuente Moreno, J.L., Rudy, J., Jacobo Saiz, M., Diez Garcia, B., Ceopen, E.P., Cabri, W., Barredo Fuente, J.L., 2017. Method of production of β-ionone fermentation in mixed culture using (+)- and (-)-strains of B. braunii. US Patent no. 7,752,965 B2.

Cui, Z., Jiang, X., Zheng, H., Qi, H., Hou, J., 2019. Homology-independent genome integration enables rapid library construction for enzyme expression and pathway optimization in Yarrowia lipolytica. Biotechnol. Bioeng. 116, 354–363. https://doi.org/10.1002/bit.26863.

Cui, Z., Zheng, H., Jiang, J., Zuo, Z., Liu, X., Qi, H., Hou, J., 2021. A CRISPR-Cas9-Mediated, homology-independent tool developed for targeted genome integration in Yarrowia lipolytica. Appl. Environ. Microbiol. 87, 1–16. https://doi.org/10.1128/AEM.00666-20.

Czajka, J.J., Kambhampati, S., Tang, Y.J., Wang, Y., Allen, D.K., 2020. Application of stable isotope tracing to elucidate metabolic dynamics during Yarrowia lipolytica α-ionone fermentation. iScience 23, 100854. https://doi.org/10.1016/j.isci.2020.100854.

Dahlin, J., Holkenbrink, C., Marella, E.R., Wang, G., Liebal, U., Lieven, C., Weber, D., Donnell, T.J., Kurasaki, R., Kajihara, L., Williams, P.G., Tang, Y., Su, W., 2018. iScience 23, 100854. https://doi.org/10.1016/j.isci.2018.10.018.

Holkenbrink, C., Dam, M.I., Klidgeard, K.R., Beder, J., Dahlin, J., Domenech Belda, D., Borodina, I., 2018. EasyCloneYALL: CRISPR/Cas9-based synthetic toolbox for engineering of the yeast Yarrowia lipolytica. Biotechnol. J. 13, 1–8. https://doi.org/10.1002/biot.201700543.

Huang, Y.-Y., Jian, X.-X., Ly, Y.-B., Nian, K.-Q., Gao, Q., Chen, J., Wei, L.-J., Hua, Q., 2018. Enhanced squalene biosynthesis in Yarrowia lipolytica based on metabolically engineered acetyl-CoA metabolism. J. Biotechnol. 281, 106–114. https://doi.org/10.1016/j.jbiotec.2018.07.001.

Idris, F.N., Mohd Nadzir, M., 2021. Comparative studies on different extraction methods of Centella asiatica and extracts bioactive compounds effects on antimicrobial activities. Antibiotics 10, 1–24. https://doi.org/10.3390/antibiotics10010015.

Ignea, C., Poiniti, M., Maftei, M.E., Makris, A.M., Kampranis, S.C., 2014. Engineering monoterpene production in yeast using a synthetic dominant negative geranyl diphasphate synthase. ACS Synth. Biol. 3, 298–306. https://doi.org/10.1021/sb400257w.

Jia, D., Xu, S., Sun, J., Zhang, C., Li, D., Lu, W., 2019. Yarrowia lipolytica construction for heterologous synthesis of α-santalene and fermentation optimization. Appl. Microbiol. Biotechnol. 103, 3511–3520. https://doi.org/10.1007/s00253-019-09322-z.

Jin, C.-C., Zhang, J.-L., Song, H., Cao, Y.-X., 2019. Boosting the biosynthesis of betulinic acid and related triterpenoids in Yarrowia lipolytica via multimodal metabolomic engineering. Microbiol. Cell Factories 18, 1–18. https://doi.org/10.1186/s12934-019-1217-x.

Jumper, J., Evans, R., Pritzel, A., Green, T., Hassabis, D., 2021. Highly accurate protein structure prediction using deep neural networks. Nat. Struct. Mol. Biol. 28, 445–451. https://doi.org/10.1038/s41594-021-00819-2.

Kerkhoven, E.J., Pommraning, K.R., Baker, S.E., Niehren, J., 2016. Regulation of amino acid metabolism controls flux to lipid accumulation in Yarrowia lipolytica. npj Syst. Biol. Appl. 2, 16005. https://doi.org/10.1038/npjsba.2016.5.

Klidgeard, K.R., Adiego-Perez, B., Domenech Belda, D., Khangura, J.K., Holkenbrink, C., Borodina, I., 2017. Engineering of Yarrowia lipolytica for production of astaxanthin. Synth. Biotechnol. 2, 287–294. https://doi.org/10.1016/j.synbio.2017.10.002.

Kleis, K., Arnesen, J., Busse, B., Romer, R., Kragelund, K., Klidgeard, K.K., Hansen, E.H., Hansen, J., Borodina, I., 2021. Tailored biosynthesis of gibberellin via multimodal engineering of the yeast Yarrowia lipolytica. Plant Cell Physiol. 62, 1–11. https://doi.org/10.1093/pcp/pcab010.

Larralde, M., Celinska, E., Back, A., Thomas, S., Nicula, J.M., Ledema-Amaro, R., 2018. A synthetic biology tool to transform Yarrowia lipolytica into a competitive biotechnological producer of β-carotene. Biotechnol. Bioeng. 115, 464–472. https://doi.org/10.1002/biot.201605047.

Li, D., Wu, Y., Peng, Q., Cao, X., Li, M., Zhang, C., Zhou, Z., Lu, W., 2020. Metabolic engineering of Yarrowia lipolytica for heterologous oleic acid production. Chem. Eng. Sci. 218, 1–11. https://doi.org/10.1016/j.ces.2021.117342.

Li, D., Wu, Y., Zhang, C., Sun, Z., Zhou, L., Wu, L., 2019. Production of triterpene ginsenoside compound K in non-conventional yeast Yarrowia lipolytica. Agric. Food Chem. 67, 2581–2587. https://doi.org/10.1021/acs.acsfoodchem.8b00587.

Li, N., Han, Z., O’Donnell, T.J., Kurasaki, R., Kajihara, L., Williams, P.G., Tang, Y., Su, W., 2020. Production and excretion of astaxanthin by engineered Yarrowia lipolytica using yeast oil as host cell source and the biocompatible extractant. Appl. Microbiol. Biotechnol. 104, 6977–6989. https://doi.org/10.1007/s00253-020-10753-2.

Li, S., Rong, L., Wang, S., Liu, S., Sun, Z., Zhao, L., Zhao, B., Zhang, C., Xiao, D., Pu, J., Zhou, K., Wu, M., Cao, Y., 2018. Enhanced limonene production by metabolically engineered Yarrowia lipolytica from cheap carbon sources. Chem. Eng. Sci. 219, 5751–5760. https://doi.org/10.1016/j.ces.2019.11.001.

Liu, H., Marnaslot, M., Deng, L., Xu, P., 2014. Understanding lipogenesis by dynamically profiling transcriptional activities and lipogenic promoters in Yarrowia lipolytica. Appl. Microbiol. Biotechnol. 103, 3176–3179. https://doi.org/10.1007/s00253-019-09664-8.

Lin, H., Wang, F., Deng, L., Xu, P., 2020. Genetic and bioprocess engineering to improve squalene production in Yarrowia lipolytica. Bioreourc. Technol. 317, 1–8. https://doi.org/10.1016/j.biortech.2020.123991.
