Synthetic and Systems Biotechnology 8 (2023) 331–338

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Original Research Article

Improving solubility and copy number of taxadiene synthase to enhance the titer of taxadiene in *Yarrowia lipolytica*

Man Xu, Wenliang Xie, Zhen Luo, Chun-Xiu Li, Qiang Hua, Jianhe Xu *

State Key Laboratory of Bioreactor Engineering, Shanghai Collaborative Innovation Center for Biomanufacturing and School of Biotechnology, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, China

ARTICLE INFO

Keywords:
Taxadiene
Yarrowia lipolytica
"Push–pull" ostrategy
Multi-copy integration
Fed-batch fermentation

ABSTRACT

Taxadiene is an important precursor for the biosynthesis of highly effective anticancer drug paclitaxel, but its microbial biosynthesis yield is very low. In this study, we employed *Yarrowia lipolytica* as a microbial host to produce taxadiene. First, a "push–pull" strategy was adopted to increase taxadiene production by 234%. Then taxadiene synthase was fused with five solubilizing tags respectively, leading a maximum increase of 62.3% in taxadiene production when fused with *SUMO*. Subsequently, a multi-copy iterative integration method was used to further increase taxadiene titer, achieving the maximum titer of 23.7 mg/L in shake flask culture after three rounds of integration. Finally, the taxadiene titer was increased to 101.4 mg/L by optimization of the fed-batch fermentation conditions. This is the first report of taxadiene biosynthesis accomplished in *Y. lipolytica*, serving as a good example for the sustainable production of taxadiene and other terpenoids in this oleaginous yeast.

1. Introduction

Paclitaxel is a derivative of tricyclic diterpenoids derived from Pacific yew trees, consisting of three parts, respectively, a taxane skeleton, skeleton modification groups, and a side chain [1]. Paclitaxel was approved by the Food and Drug Administration (FDA) in 1992 for the treatment of ovarian cancer [2] and was later confirmed to have a significant effect on some noncancer diseases [3]. Paclitaxel was originally isolated from the bark of *Taxus brevifolia* [4], but this species has a relatively long growth cycle, poor regeneration ability, and very low paclitaxel content in the bark [5]. These characteristics seriously hinder the development of paclitaxel as an excellent anticancer drug. Owing to the rapid development of synthetic biology and metabolic engineering, the microbial production of high value-added plant-originating compounds has become a promising alternative approach. Taxadiene is the first key intermediate in biosynthesis of paclitaxel, and high taxadiene production is an important prerequisite for the efficient biosynthesis of paclitaxel.

Taxadiene is produced from the cyclization of geranylgeranyl pyrophosphate (GGPP) by taxadiene synthase (*TASY*) [6]. GGPP is formed by the condensation of one dimethylallyl pyrophosphate (DMAPP) and three isopentenyl pyrophosphates (IPPs). DMAPP and IPP can be produced by the mevalonate (MVA) pathway in eukaryotes (Fig. 1), or by the 2-*C*-methylerythritol 4-phosphate (MEP) pathway in prokaryotes [7]. In recent years, the biosynthesis of taxadiene has been investigated in different microbial organisms as host strains (Table 1). Among them, the highest taxadiene titer of 1020 mg/L was achieved in *Escherichia coli* by strengthening the MEP pathway and using multiple modular metabolic engineering strategies to optimize the balance between taxadiene synthesis and growth metabolism [8]. However, taxadiene must undergo 18 enzymatic steps to finally synthesize paclitaxel, including hydroxylation, acetylation, and rearrangement [9]. The following expression of multiple heterologous P450 hydroxylases in *E. coli* may causes many unexpected difficulties owing to the lack of a complete endomembrane system and organelles [10].

Yarrowia lipolytica is recognized as a "Generally Recognized as Safe" (GRAS) by FDA [11] and with a complete inner membrane system which can express plant-derived cytochrome P450s more efficiently than *E. coli*. Owing to its high lipid production capacity, *Y. lipolytica* can supply sufficient acetyl coenzyme A (acetyl-CoA), adenosine triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADPH) for the endogenous MVA biosynthesis pathways [12,13],

Peer review under responsibility of KeAi Communications Co., Ltd.

* Corresponding author. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, China.

E-mail address: jianhexu@ecust.edu.cn (J. Xu).

https://doi.org/10.1016/j.synbio.2023.04.002
Received 5 January 2023; Received in revised form 10 April 2023; Accepted 11 April 2023
Available online 26 April 2023

which is an effective strain for the production of terpenoids, such as squalene [14,15], farnesene [16], and α-humulene [17]. Furthermore, due to the absence of Crabtree effect, contrary to *Saccharomyces cerevisiae* [18], and the compatibility to many inexpensive media [19], *Y. lipolytica* has become one of the most widely studied unconventional yeasts in recent years.

In this study, multiple metabolic engineering approaches were introduced to achieve the enhanced production of taxadiene in *Y. lipolytica*. Firstly, a taxadiene producing *Y. lipolytica* strain YP11 was obtained by expressing the taxadiene synthase (*TASY*) gene from *Taxus cuspidata*, which produced about 0.02 mg/L of taxadiene. Then, a "push-pull" strategy was applied to increase the supply of precursors and decreased the bypass metabolism. Next, according the accumulation of geranylgeraniol (GGOH), the bottleneck of the taxadiene biosynthesis was supposed to be the step catalyzed by *TASY*, which were subsequently alleviated by increasing the soluble expression and copy number of the *TASY*. Finally, the taxadiene titer reached 101.4 mg/L after fed-batch fermentation for 144 h under the optimized fermentation conditions, which is approximately 5000-fold compared with initial strain YP11. This is the first example for utilizing *Y. lipolytica* for taxadiene biosynthesis, which provides a foundation for the production of taxadiene and other diterpenoids by *Y. lipolytica*.

2. Results

2.1. Construction of a taxadiene basal strain

Previous reports have shown that expression of the genes encoding the taxadiene synthase (*TASY*) from *Taxus cuspidate* in *S. cerevisiae* resulted in taxadiene production [20]. In this study, *TASY* of the same origin controlled by a strong constitutive promoter, php4d, was integrated into Po1f genome, generating a basal strain YP11. The resulting strain produced approx. 0.02 mg/L taxadiene when cultured in YPD medium for 120 h (Fig. 2).

The diterpene backbone contains 20 carbon atoms (Fig. 1). In *Y. lipolytica*, these carbon atoms derive from common terpene building blocks IPP and DMAPP, produced via the MVA pathway [21]. Therefore, increasing the MVA flux would enhance the terpene production by filling the building blocks pool. In the MVA pathway, hydroxymethylglutaryl-CoA reductase (*HMG1*) has been reported to be the rate-limiting enzyme [22]. The truncated form of *HMG1* (*tHMG1*) without the *N*-terminal membrane-targeting signal can avoid the self-degradation mediated by its *N*-terminal domain and is more effective in increasing isoprenoid production in *Y. lipolytica* [23,24]. The precursor of taxadiene, GGPP, is formed by the condensation of FPP (C15) with IPP (C5), which catalyzed by geranylgeranyl diphosphate synthase (*GGSP1*). The high flux of GGPP is very important for the production of downstream products. For example, when *GGSP1* is overexpressed, the production of carotene and miltiradiene was significantly increased [25,26]. To enhance the taxadiene concentration, one copy of endogenous *tHMG1* and *GGSP1* was co-expressed in the chromosome of strain YP11, the resulting strain YP12 produced 0.07 mg/L taxadiene after 120 h of flask fermentation (Fig. 2). These results indicated that the overexpression of rate-limiting enzymes in the MVA pathway could increase the taxadiene titer.

2.2. Enhancing the titer of taxadiene by "push–pull" strategy

To further improve the taxadiene titer, we adopted a "push–pull" strategy [27]. About the 'push' aspect, we overexpressed the key enzyme *tHMG1* in the MVA pathway on the basis of strain YP12, the taxadiene titer of resulting recombinant strain YP21 reached 0.13 mg/L after 120 h of fermentation (Fig. 3a). To push the FPP to the direction of taxadiene synthesis, another one copy of *GGSP1* was also overexpressed on the basis of strain YP12, the taxadiene titer of resulting recombinant strain YP22 reached 0.15 mg/L. To achieve higher accumulation of taxadiene, we simultaneously overexpressed *tHMG1* and *GGSP1*, giving recombinant strain YP23, which resulted in a taxadiene production of 0.27 mg/L.

FPP is a common precursor of squalene and GGPP, while squalene was reported to be a precursor for the biosynthesis of ergosterol which is an important cell wall component and essential for the growth of microorganisms [28]. Kildegaard and Arenesen replaced the native squalene synthase promoter (p*ERG9*) with the relatively weak lanosterol 14-a-demethylase promoter (p*ERG11*) to prevent carbon loss to squalene and increase the titer of mono-, sesqui-, diterpenoid and carotenoid

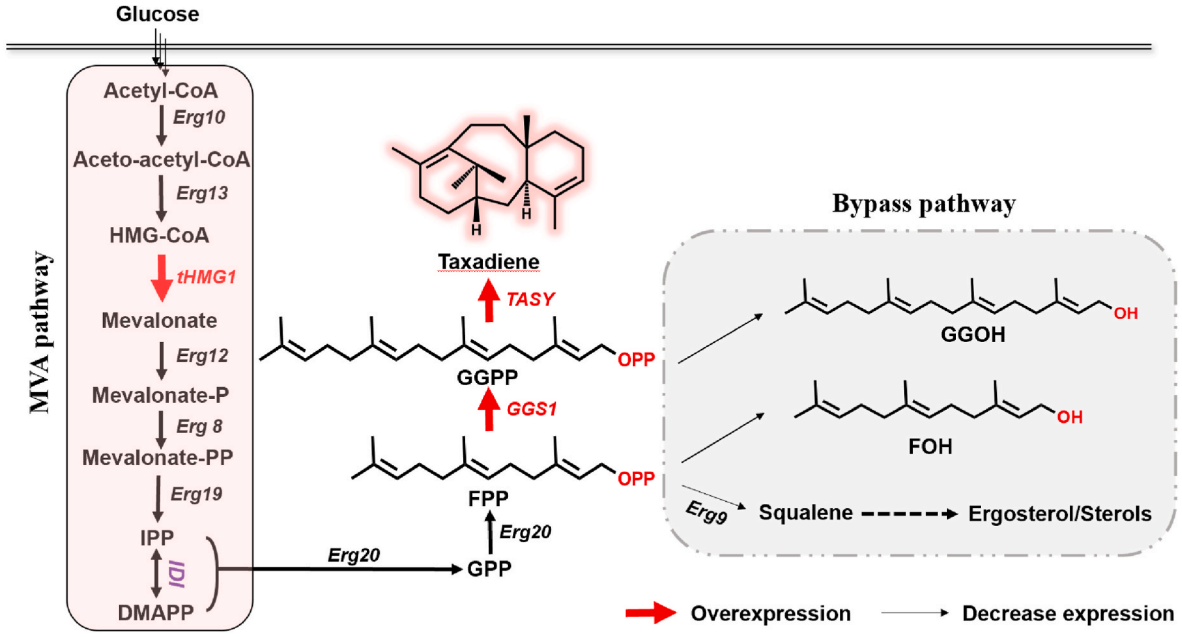


Fig. 1. Production pathways of taxadiene from glucose in *Y. lipolytica*. In *Y. lipolytica*, isoprenoids are synthesized from acetyl-CoA through the MVA pathway. Pink box represents the endogenous MVA pathway. Dashed boxes represent alternative metabolic pathways for the biosynthesis of taxadiene. Red arrows indicate overexpression and thin black arrows indicate reduced expression.

[21,23]. In order to reduce the flow of FPP to ergosterol, the endogenous promoter *pERG9* of key gene *erg9* in ergosterol biosynthesis was also replaced with a relatively weaker promoter *pERG11* in the starting strain YP23, the taxadiene titer of resulting recombinant strain YP24 reached 0.31 mg/L (Fig. 3a). Fusion proteins generated by fusing two genes with a linker region can shorten the distance between the enzyme and the substrate, and enhance the catalytic capacities of enzymes [29]. To further pull the flux of FPP to GGPP, the fusion protein of *ERG20* and *GGSP1* with different linkers (G3S, G4S, $(G4S)_2$) was overexpressed in three recombinant strains (YP25, YP26 and YP27) based on the strain YP21. As shown in Fig. 3a, the titer of taxadiene was further increased after fusion expression of the two proteins, and the maximum titer reached 0.80 mg/L when the linker was $(G4S)_2$. Besides, when the linker was G3S and G4S, the titers of taxadiene was 0.68 mg/L and 0.70 mg/L, respectively.

However, GGOH, a hydrolysis product of GGPP, was observed to accumulate gradually with the increase of taxadiene titer, the titer of GGOH reached 115 mg/L especially in recombinant strain YP27 (Fig. 3b). GGOH is known to form spontaneously through the degradation of GGPP by endogenous phosphatase [30], while the titer of GGOH in the Po1f was relatively low with only 0.40 mg/L (Fig. 3b). The accumulation of GGOH and taxadiene demonstrate the feasibility of the "push-pull" strategy, but the large accumulation of GGOH reveals the metabolic bottleneck on the pathway of taxadiene biosynthesis.

2.3. Solubilizing tags and multiple-rounds of genomic integration to enhance taxadiene production

Fusion tags has been shown to increase the solubility and stability of exogenous proteins, and increase the titer of compounds of interest [31]. According to previous studies, the soluble expression of taxadiene synthase in *S. cerevisiae* is poor, and fusion with soluble tag can increase the taxsdiene titer [20]. To increase the taxadiene levels by increasing the soluble expression of taxadiene synthase, five solubilizing protein tags, including small ubiquitin modifying protein (*SUMO*) [32], glutathione *S*-transferase (*GST*) [33], maltose-binding protein (*MBP*) [20,34], *N*-utilization substance protein A (*NusA*) [35], and thioredoxin A (*TrxA*) [36], was fused to the *N*-terminus of *TASY* respectively, then integrated into genome, obtaining a set of recombinant strains (YP–51S, YP-51G, YP-51 M, YP-51N, and YP-51T). The ORF-*tasy* was also integrated into genome alone, the resulting recombinant strain YP51 served as a negative control (Fig. 4a). Different fusion tags performed differently. After fusion expression with the *SUMO* and *MBP,* the titers of taxadiene reached 3.55 mg/L and 3.45 mg/L respectively. However, overexpression of ORF-*tasy* alone produced only 2.01 mg/L taxadiene (Fig. 4b). However, the taxadiene production was only increased by 12.4% when *TASY* was fused with *NusA*, and even decreased by 15.3% when fused with *TrxA*. This might be related to the varied degree to which the correct folding of taxadiene synthase was affected during the fusion expression.

Exhilaratingly, we also noticed for the first time, that the recombinant strain YP51 with an additional copy of *TASY* showed a 173% increase in taxadiene production (Fig. 4b) and significant decrease in GGOH level (Fig. 4c) when compared with the starting strain YP27. To improve the conversion rate of GGPP to taxadiene, the *tasy* gene should be integrated in multiple copies. In *Y. lipolytica*, plasmid-based gene expression systems are less stable compared with chromosomes, the number of selectable markers in *Y. lipolytica* is limited, and homologous recombination is less efficient. These features limit our ability to perform multiple rounds of efficient gene integration. In *Y. lipolytica*, on the other hand, there are more than two hundred 26s rDNA loci that can be used to randomly integrate heterologous pathways genes [37]. Here we employed the method described by Xu et al. [38] which combines the Cre-loxP system with multicopy site 26s rDNA. In brief, we employed (*SUMO-TASY*)*2 as a unit for modular assembly of *SUMO* and *TASY* to integrated in the position between loxp-ura3-loxp and downstream 26s rDNA (Fig. 5a). The expression of Cre recombinase effectively removed the marker and facilitated the next round of gene integration. After one round of integration, about 20 transformants were randomly selected from the transformed plates and cultivated in 24 deep-well plates for 120 h, the recombinant strain with the highest titer was selected for the next round of integration. After three rounds of integration, taxadiene production reached a new maximum of 23.7 mg/L (Fig. 5b). In *S. cerevisiae*, increasing the copy number of *TASY* also increased the titer of taxadine [20].

The three recombinant strains with the highest titers of each round of integration were selected and designated as M1-1 to M1-3, M2-1 to M2-3, and M3-1 to M3-3, respectively. We analyzed the copy number of *tasy* in these nine recombinant strains and the negative control strain YP-51 (Fig. 5c). The titers of taxadiene in corresponding recombinant strains increased gradually with the increasing number of integrations (Fig. 5b). With the copy number of the *tasy* increasing, the level of GGOH decreased gradually. After three rounds of iterative integration, the production of undesired GGOH decreased significantly from 115 mg/L to 31.4 mg/L (Fig. 5c).

2.4. Medium optimization and fed-batch fermentation

Before fed-batch fermentation, four key parameters that might affect the final titer of taxadiene of recombinant strain M2-3 were optimized, including temperature, pH, Mg^{2+} concentration, and initial glucose concentration in the medium. The taxadien titer was highest at culture temperature of 25 °C, controlled pH at 6.5, and initial glucose concentration of 20 g/L and without Mg^{2+} addition (Fig. S1). As the auxotrophic strain could not perform high-density fermentation [39], strain M4 was constructed by supplementing the M3-3 with Leu marker. To examine the potential of engineered strain M4 for large-scale taxadiene production, we used a 5-L bioreactor for fed-batch fermentation. During the fed-batch cultivation process, the temperature was controlled at 25 °C, and 3 M NaOH solution was used to maintain a pH around 6.5 because a large amount of acid was produced during the fermentation process. The initial glucose concentration was set as 20 g/L. Once the glucose concentration in the medium decreased to ≤5 g/L, it was increased to 20 g/L using a feed solution of 200 g/L glucose. The corresponding fermentation curves are shown in Fig. 6. Strain growth rate was faster during the fermentation for 0–72 h and slowed down after 72

Table 1
Production of taxadiene by various engineered microorganisms.

Host	Taxadiene concentration	Engineering strategy	Cultivation condition	Reference
E. coli	94% of total product		A cell-free assay was conducted	[44]
E. coli	1.3 mg/L	Engineering Isoprenoid pathway	1 L LB medium culture, 37 °C	[45]
S. cerevisiae	1.0 mg/L	Engineering Isoprenoid pathway	700 mL Selective SG-URA medium	[46]
S. cerevisiae	8.70 ± 0.85 mg/L	Engineering Isoprenoid pathway	500 mL baffled shake flask, 28 °C	[47]
E. coli	1.02 ± 0.08 g/L	Optimizing MEP pathway	1 L fed-batch cultivation, 22 °C	[8]
S. cerevisiae	20 mg/L	Engineering Isoprenoid pathway	5 mL YP-galactose medium culture, 30 °C	[31]
N. benthamiana	56.6 ± 3.2 μg g^{-1} FW	Engineering Isoprenoid pathway and chloroplastic compartmentalize		[48]
S. cerevisiae	129.0 ± 5.6 mg/L	Engineering Isoprenoid pathway and diterpenoid synthase	500-mL MiniBio bioreactor, 20 °C	[20]
Y. lipolytica	101.4 mg/L	Engineering Isoprenoid pathway and multi-copy integration	5 L fed-batch cultivation, 25 °C	This study

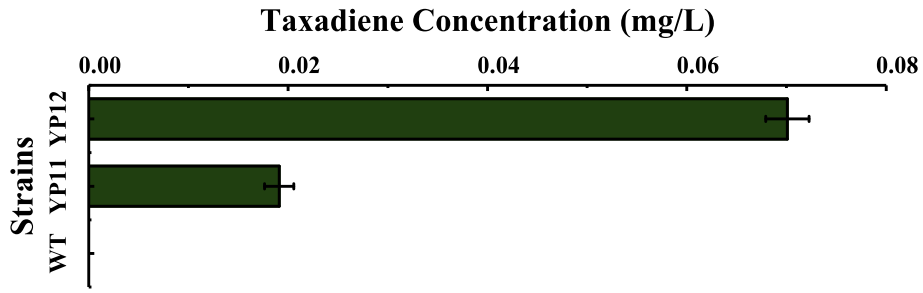
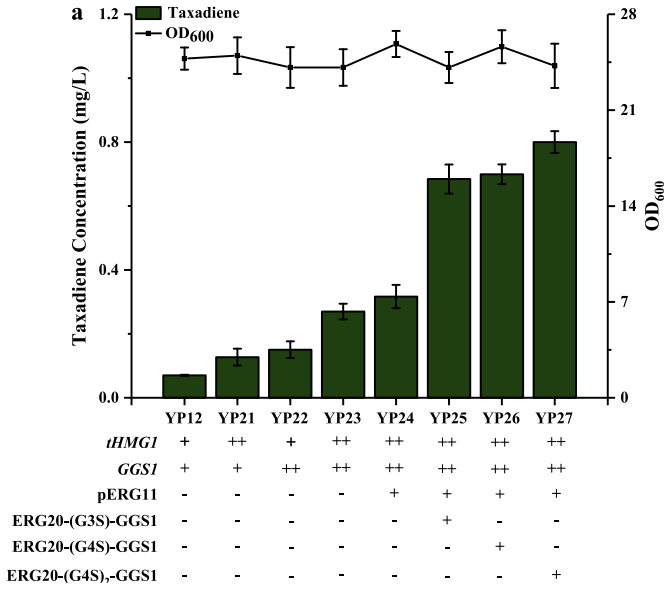


Fig. 2. Engineered taxadiene biosynthesis pathways in *Y. lipolytica*. Taxadiene production in engineered strains overexpressing *TASY* alone and simultaneously overexpressing MVA pathway rate-limiting enzymes *tHMG1*, *GGSP1*, and *TASY*. Experiments were conducted in triplicate, with error bars representing the standard deviation from the mean value.



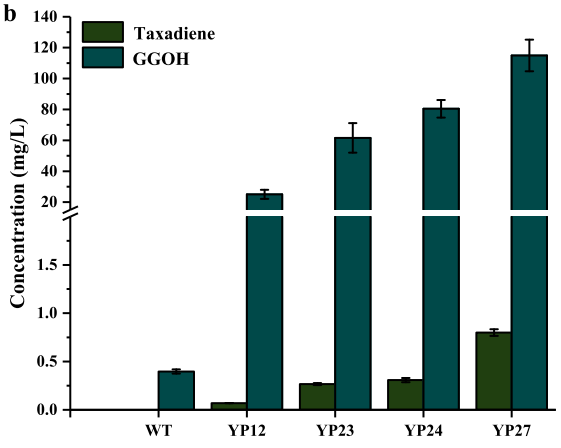


Fig. 3. "Push–pull" strategy analysis of taxadiene biosynthesis. (a) Taxadiene production in engineered strains obtained from increasing the gene copy number, decreasing *SQS* expression by replacing the strong promotor, and fusion by expressing *ERG20* with *GGSP1*. (b) Concentration of GGOH in strains WT, YP12, YP23, YP24, and YP27 after 140 h of cultivation. Experiments were conducted in triplicate, with error bars representing the standard deviation from the mean value.

h, the biomass reached an OD_{600} of 175 at 72 h. With fermentation proceeding, the taxadiene production also increased steadily. At the end of fed-batch fermentation, the OD_{600} was stably maintained at 210, meanwhile the taxadiene production reached 101.4 mg/L.

3. Materials and methods

3.1. Strains and medium

E. coli strain DH5α was used for plasmid construction, propagation and storage, which was cultivated at 37 °C in a Luria-Bertani (LB) medium (agar plate containing 15 g/L Bacto agar (Difco) supplementing with ampicillin (100 mg/L) or kanamycin (50 mg/L) for selection. *Y. lipolytica* strain ATCC MYA2613 (Po1f) were cultivated at 30 °C in a yeast rich medium, which was prepared with 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose, supplemented with 15 g/L Bacto agar (Difco) for solid plates. The selective YNB medium, which was made of 0.67% yeast nitrogen base without amino acids, 20% glucose, and 1.5% agar, was supplemented with an appropriate amount of uracil or leucine. YNB-5-FoA (YNB medium with 1 g/L 5-fluoroorotic acid (5-FoA)), CSM-LEU-5-FoA (YNB medium without leucine but with 1 g/L 5-FoA) and CSM-URA-5-FoA (YNB medium without uracil but with 5-FoA) were used for recycling of the URA3 selection marker. All strains constructed and used in this study are listed in supplementary Information.

3.2. Construction of vectors

Taxadiene synthase (*TASY*) from *Taxus cuspidate* were codon optimized and synthesized from the Generay Biotech Co., Ltd. (Generay, Shanghai, China). Sequences of codon-optimized gene are list in Supplementary Information, Table S3. All primers used in this study are also shown in supplementary Information, Table S2. The plasmids for CRISPR/Cas9 system including homologous donor and single gRNA plasmids, were used for gene knock-in and knock-out. For example, firstly we used the restriction enzyme *Spe* I and *Avr* II to digest the donor plasmid, then the *tasy* sequences were inserted into the *Spe* I and *Avr* II sites of plasmid pHR_F1_hrGFP to generate the homologous donor plasmid pHR_F1_tasy. The F1-n20 containing 20-bp homologous arms to the plasmid backbone in both ends was inserted into the *Asc* I site of blank plasmid pCRISPRyl-F1 to produce the F1 site sgRNA. To integrate genes into the 26s rDNA locus on the *Y. lipolytica* genome, tandem of two *SUMO-TASY* expression cassette was inserted into the *EcoR* I site of plasmid pUC19-rDNA to generate the plasmid pUC19-rDNA-(SUMO-TASY)*2, then the vector was linearized by *Avr* II restriction enzymes prior to *Y. lipolytica* transformation.

3.3. Construction of strains

For iterative integration of genes into the genome we used the CRISPR/Cas9 system [40]. To integrate *TASY* into the multicopy 26s rDNA loci, we used the homologous recombination through linearized plasmids. The exogenous DNAs were transformed to *Y. lipolytica* by the kit Frozen-EZ yeast transformation II. All colonies were screened by PCR and about 10–20 clones were selected for analysis of each strain. All

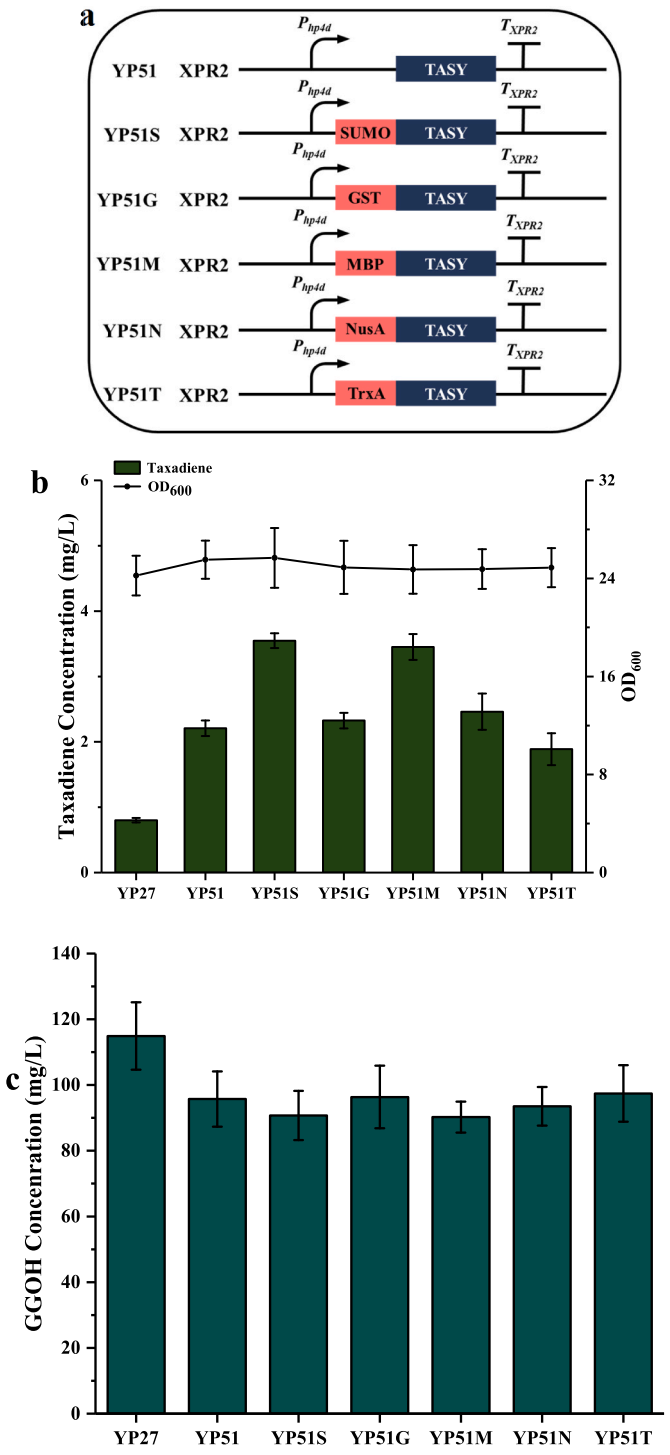


Fig. 4. Test of fusion tag universality. (a) Scheme of chromosomally integrated fusion tags with *tasy* genetic constructs. *SUMO*, *GST*, *MBP*, *NusA*, and *TrxA* were fused with *TASY* before being integrated into the chromosome XPR2 site. (b) Taxadiene production in the engineered strains by overexpressing *TASY* with different fusion tags. (c) Concentration of GGOH in the engineered strains detected at 140 h. Experiments were conducted in triplicate, with error bars representing the standard deviation from the mean value.

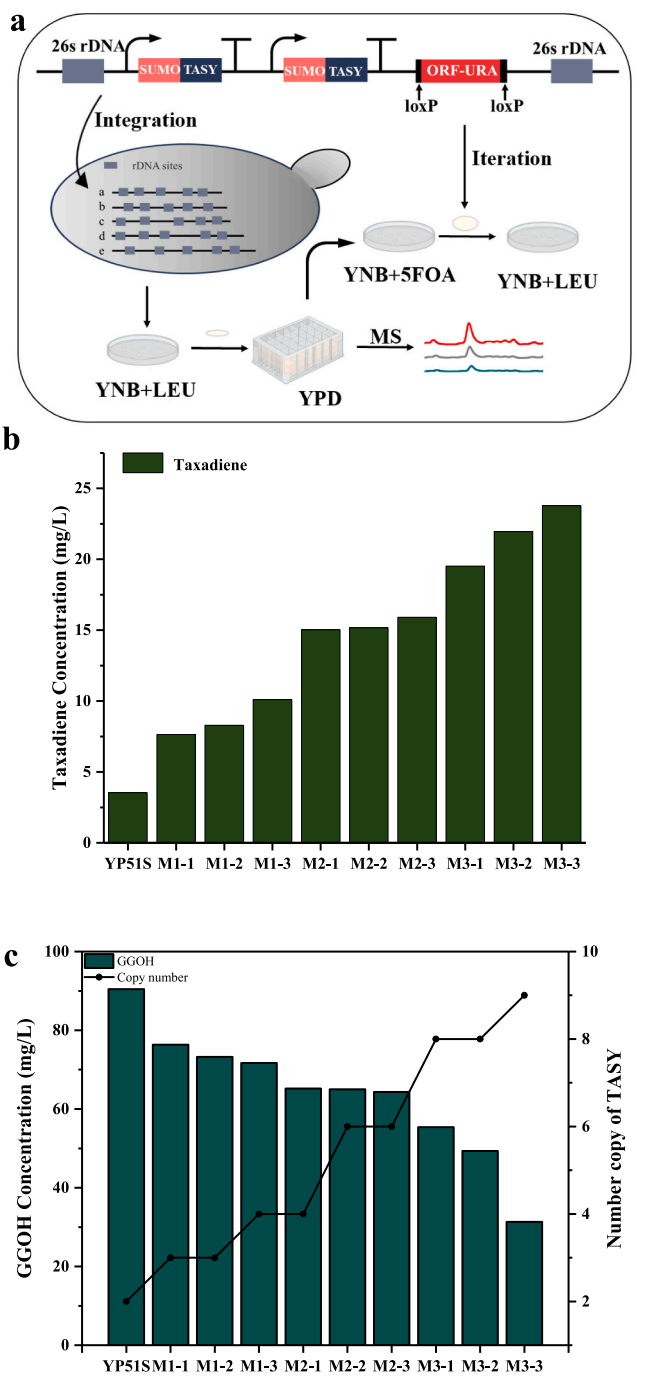


Fig. 5. Multicopy integration of 26s rDNA into genome to improve taxadiene production. (a) Multicopy integration scheme of (*SUMO-TASY*)*2 in *Y. lipolytica*. After two loxP sequences were recognized by *Cre*, the ORF-URA between the loxP sites was excised for the next round of genes integration. (b) Effect of increasing rounds of integration of (*SUMO-TASY*)*2 into the genome on the formation of taxadiene in engineered *Y. lipolytica*. (c) The copy number of *tasy* and GGOH concentration in the highest titer strain per integration. Experiments were conducted in triplicate, with error bars representing the standard deviation from the mean value.

strains are listed in supplementary Information, Table S1.

3.4. Strain cultivation and fed-batch fermentation

The *Y. lipolytica* strains were first pre-cultured in 4 mL YPD medium at 30 °C (24 h, 220 rpm), then 1 mL of the preculture was inoculated into 50 mL YPD medium contained in a 250-mL shake flask (covered with 10% dodecane) with an initial OD_{600} of 0.02 and fermented for 120 h under the same condition.

During the fermentation process, the engineered *Y. lipolytica* was first pre-cultured in 4 mL of YPD medium at 30 °C (220 rpm) for 18 h, then

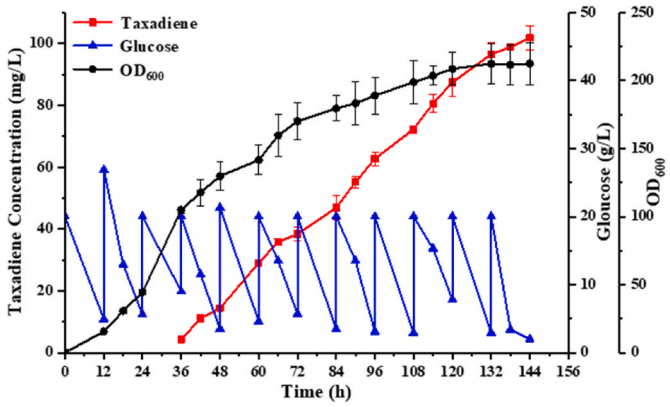


Fig. 6. Fed-batch fermentation of metabolically engineered strain M4. Taxadiene production of recombinant strain M6 in batch fermentation. Fermentation was performed in a 5-L jar fermenter containing 2 L of medium.

transferred to a 250-mL flask containing 50 mL YPD, and cultivated at 30 °C, 220 rpm for 16–18 h. Lastly, the seed culture was inoculated into a 5-L bioreactor (Zhenjiang Grenada, China) loaded with 2 L YPD medium (20 g/L glucose, 20 g/L yeast extract and 40 g/L peptone) and the initial OD_{600} was set to 0.5. During the fermentation, the dissolved oxygen (DO) concentration was controlled to be around 35% by adjusting agitation rate between 200 and 800 rpm, and the pH was controlled around 6.5 through addition of 3 M NaOH or 3 M HCL. A 10× YPD medium, consisting of 200 g/L yeast extract, 400 g/L peptone and 200 g/L glucose, was fed into the bioreactor when the glucose concentration was lower than 5 g/L during fermentation. Dodecane 20% (V/V) was added to the medium, when the fermentation reached 36 h.

3.5. Taxadiene and immediate metabolite quantification

Before cultivation, 10% (V/V) dodecane was add to the medium to capture the metabolites taxadiene and GGOH. The supernatant of the fermentation broth (1 mL) was centrifuged at 8000 rpm for 3 min, and the dodecane layer was taken for taxadiene detection. We used octadecane as an internal standard, so the samples with dodecane overlay were first mixed with octadecane (dissolved in dodecane, 50 mg/L) in an equal volume, and then analyzed by GC-MS (Agilent Technologies 6890 N gas chromatography combined with 5975C mass spectrometry with triple-axis detector).

3.6. Measurement of tasy gene copy number by quantitative PCR

The *tasy* gene transcription was measured by quantitative PCR. Genimic DNA samples were prepared by using the EasyPure® Genomic DNA Kit (Transgene Biotech, Beijing, China). The qPCR was performed using One step SYBR® Green RT-qPCR with MMLV & hot-start Taq DNA Polymerase. A *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene of *Y. lipolytica* was used as reference sequence.

4. Discussion

Due to its broad-spectrum anticancer effects, paclitaxel has become one of the most popular anticancer drugs in the market with a huge market demand. As a key intermediate for the biosynthesis of paclitaxel, taxadiene has attracted extensive attention. In this study, improving the solubility and copy number of the key rate-limiting enzyme taxadiene synthase in *Y. lipolytica* resulted in a significant increase in taxadiene production. This is the first report on the biosynthesis of taxadienes in *Y. lipolytica*, demonstrating the potential of *Y. lipolytica* as a platform for the biosynthesis of taxadiene.

tHMG1 and *GGSP1* are key enzymes for the biosynthesis of taxadiene. Overexpressing *tHMG1* and *GGSP1* on the basis of the strain YP11 increased the titer of taxadiene by 250%, which illustrates the fact that these two enzymes are key enzymes for the biosynthesis of taxadiene. However, the absolute titer was still very low. The successful uses of "push-pull" strategies have improved the production of terpenoids in various microorganisms, such as *E. coli* [41], *S. cerevisiae* [27], and *Y. lipolytica* [42]. In this study, we overexpressed above two enzymes for the second time to push the carbon flux to the synthesis of GGPP, which improved the titer of taxadiene by 286%. About 'pull' aspect, the FPP was pulled to synthesize GGPP by weakening bypass through replacing the naive squalene synthade promoter (p_{ERG9}) of squalene synthetase with a relatively weak promoter (p_{ERG11}). In addition, the fusion expression of *ERG20* and *GGSP1* accompanied with optimization of the linker for fusion protein dramatically increased the production of taxadiene by 296%. Owning to the fusion expression, the reduction of the shuttle distance between the substrate FPP and the enzyme *GGSP1* might cause the pulling effect of FPP toward the direction of GGPP synthesis, which led to the significant increase of taxadiene production. The titer of the recombinant strain YP27 was 11.4-fold higher than that of the strain YP12 after adopting the "push-pull" strategy. Combined with previous experimental results, it is confirmed that the "push-pull" strategy can effectively increase the precursor supply of the interested product and redirect the carbon flow of metabolism, thereby improving the final titer of the target product.

In the recombinant strain YP27, a large amount of GGOH was detected, which revealed that the metabolic bottleneck of taxadiene biosynthesis may be located at the taxadiene synthase. It has also been reported that the low solubility and poor expression of *TASY* in *S. cerevisiae* were identified as critical bottlenecks for taxadiene biosynthesis [20,43]. We hypothesized that the majority of GGPP naturally hydrolyzes to GGOH due to the poor soluble expression of *TASY* in *Y. lipolytica*. In order to solve this problem, we measured the titers of taxadiene after respective fusion expression of five different solubilizing tags fusion with *TASY* at *N*-terminus. When the *TASY* fused with *SUMO* the taxadiene titer was 60.6% higher than that of the control strain (YP51), which showed the best performance. The application of these solubilizing tags also provides a good reference for the selection of solubilizing tags in *Y. lipolytica*. In addition, increasing the copy number of a gene is another approach to solve this problem and thus increase the conversion rate. We therefore integrated the unit of (*SUMO-TASY*)*2 into the rDNA multi-copy loci. After three rounds of iterative integration, the titer of taxadiene reached 23.7 mg/L, while the titer of GGOH was significantly reduced form 115 mg/L to 31.4 mg/L. These results indicated that GGPP hydrolysis is a competing pathway against the taxadiene biosynthesis in *Y. lipolytica*, and confirmed that increasing the soluble expression of the key enzymes and multi-copy integration of their encoding genes into chromosome can effectively improve the conversion efficiency of GGPP to taxadiene.

In a word, the taxadiene biosynthesis was firstly achieved and optimized using *Y. lipolytica* as chassis cells in this study. We confirmed the feasibility of a "push–pull" strategy and multicopy loci integration to enhance the biosynthesis of taxadiene, achieving the maximum titer of 101.4 mg/L taxadiene by optimizing the medium and fed-batch fermentation. Also, the above-mentioned strategies could provide important insights and powerful tools to further improved the production of taxadiene or other diterpene compounds by using *Y. lipolytica*.

5. Associated content

The Supporting Information is available free of charge on the ACS Publications website. *Y. lipolytica* strains used in this study (Table S1), oligonucleotide primers used in this study (Table S2), codon optimized gene used in this study (Table S3), and the optimization of fermentation conditions for the M2-3 strain in shake flasks (Fig. S1).

Key points

- *First report of taxadiene biosynthesis accomplished in Y. lipolytica*
- *The "push-pull" strategy significantly increased the titer of taxanediene*
- *A multi-copy chromosomal integration led to efficient synthesis of taxadiene.*

Notes

The authors declare no competing financial interest.

CRediT authorship contribution statement

Man Xu: designed the research and drafted the manuscript. **Wenliang Xie:** revised articles and analyzed data. **Zhen Luo:** revised articles and analyzed data. **Chun-Xiu Li:** revised the manuscript, All authors read and approved the final version of manuscript. **Qiang Hua:** supervised the project. revised the manuscript. **Jianhe Xu:** supervised the project, revised the manuscript.

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.

Acknowledgments

This work was financially supported by the National Key Research and Development Program of China (2019YFA0905000), the National Natural Science Foundation of China (21871085 and 31971380), and the Fundamental Research Funds for the Central Universities (222201714026).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2023.04.002.

Abbreviations

Y. lipolytica	*Yarrowia lipolytica*
FDA	Food and Drug Administration
DMAPP	dimethylallyl diphosphate
IPP	*isopentenyl* diphosphate
GPP	geranyl diphosphate
FPP	farnesyl diphosphate
MVA	mevalonate
MEP	2-*C*-methylerythritol 4-phosphate
GRAS	Generally Recognized as Safe
ATP	adenosine triphosphate
NADPH	nicotinamide adenine dinucleotide phosphate
5-FoA	5-fluoroorotic acid
DO	dissolved oxygen
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
TASY	taxadiene synthase
HMGR	hydroxymethylglutaryl-CoA
SQS	squalene synthase
yltHMG1	truncated 3-hydroxy-3-methyl-glutaryl-CoA reductase from *Y lipolytica*
GGSP1	geranylgeranyl diphosphate synthase
GGPP	geranylgeranyl diphosphate
GGOH	geranylgeraniol
SUMO	small ubiquitin modifying protein
GST	glutathione *S*-transferase
MBP	maltose-binding protein
NusA	*N*-utilization substance protein A
TrxA	thioredoxin A

References

[1] McElroy C, Jennewein S, Xu M. Taxol® biosynthesis and production: from forests to fermenters. Springer; 2018. p. 145–85.
[2] Abu Samaan TM, Samec M, Liskova A, Kubatka P, Busselberg D. Paclitaxel's mechanistic and clinical effects on breast cancer. Biomolecules 2019;9:789.
[3] Yoshida H, Sumi T, Abe K, Ishiko O. Pharmacokinetics of paclitaxel and carboplatin in a hemodialysis patient with advanced ovarian cancer. Eur J Gynaecol Oncol 2009;30:583–5.
[4] Shi Q W, Kiyota H, Xu M. New natural taxane diterpenoids from Taxus species since 1999. Chem Biodiversity 2005;2:1597–623.
[5] Żwawiak J, Zaprutko L. A brief history of taxol. J Med Sci 2014;83:47–52.
[6] van Rijn JPM, Escorcia AM, Thiel W. QM/MM study of the taxadiene synthase mechanism. J Comput Chem 2019;40:1902–10.
[7] Vranova E, Coman D, Gruissem W. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu Rev Plant Biol 2013;64:665–700.
[8] Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G. Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 2010;330:70–4.
[9] Jennewein S, Croteau R. Taxol: biosynthesis, molecular genetics, and biotechnological applications. Appl Microbiol Biotechnol 2001;57:13–9.
[10] Kitaoka N, Wu Y, Xu M, Peters RJ. Optimization of recombinant expression enables discovery of novel cytochrome P450 activity in rice diterpenoid biosynthesis. Appl Microbiol Biotechnol 2015;99:7549–58.
[11] Groenewald M, Boekhout T, Neuveglise C, Gaillardin C, van Dijck PW, Wyss M. *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential. Crit Rev Microbiol 2014;40:187–206.
[12] Markham KA, Alper HS. Synthetic biology expands the industrial potential of *Yarrowia lipolytica*. Trends Biotechnol 2018;36:1085–95.
[13] Miller KK, Alper HS. *Yarrowia lipolytica*: more than an oleaginous workhorse. Appl Microbiol Biotechnol 2019;103:9251–62.
[14] Liu H, Wang F, Deng L, Xu P. Genetic and bioprocess engineering to improve squalene production in *Yarrowia lipolytica*. Bioresour Technol 2020;317:123991.
[15] Tang WY, Wang DP, Tian Y, Fan X, Wang C, Lu XY, Li PW, Ji XJ, Liu HH. Metabolic engineering of *Yarrowia lipolytica* for improving squalene production. Bioresour Technol 2021;323:124652.
[16] Liu Y, Wang Z, Cui Z, Qi Q, Hou J. Progress and perspectives for microbial production of farnesene. Bioresour Technol 2022;347:126682.
[17] Guo Q, Shi TQ, Peng QQ, Sun XM, Ji XJ, Huang H. Harnessing *Yarrowia lipolytica* peroxisomes as a subcellular factory for α-humulene overproduction. J Agric Food Chem 2021;69:13831–7.
[18] Sun L, Lee JW, Yook S, Lane S, Sun Z, Kim SR, Jin YS. Complete and efficient conversion of plant cell wall hemicellulose into high-value bioproducts by engineered yeast. Nat Commun 2021;12:4975.
[19] Sun T, Yu Y, Wang K, Ledesma-Amaro R, Ji XJ. Engineering *Yarrowia lipolytica* to produce fuels and chemicals from xylose: a review. Bioresour Technol 2021;337:125484.
[20] Nowrouzi B, Li RA, Walls LE, d'Espaux L, Malci K, Liang L, Jonguitud-Borrego N, Lerma-Escalera AI, Morones-Ramirez JR, Keasling JD, Rios-Solis L. Enhanced production of taxadiene in *Saccharomyces cerevisiae*. Microb Cell Factories 2020;19:200.
[21] Arnesen JA, Kildegaard KR, Cernuda Pastor M, Jayachandran S, Kristensen M, Borodina I. *Yarrowia lipolytica* strains engineered for the production of terpenoids. Front Bioeng Biotechnol 2020;8:945.
[22] Li ZJ, Wang YZ, Wang LR, Shi TQ, Sun XM, Huang H. Advanced strategies for the synthesis of terpenoids in *Yarrowia lipolytica*. J Agric Food Chem 2021;69:2367–81.
[23] Kildegaard KR, Adiego-Perez B, Domenech Belda D, Khangura JK, Holkenbrink C, Borodina I. Engineering of *Yarrowia lipolytica* for production of astaxanthin. Synth Syst Biotechnol 2017;2:287–94.
[24] Yang X, Nambou K, Wei L, Hua Q. Heterologous production of α-farnesene in metabolically engineered strains of *Yarrowia lipolytica*. Bioresour Technol 2016;216:1040–8.
[25] Gao S, Tong Y, Zhu L, Ge M, Zhang Y, Chen D, Jiang Y, Yang S. Iterative integration of multiple-copy pathway genes in *Yarrowia lipolytica* for heterologous β-carotene production. Metab Eng 2017;41:192–201.
[26] Hu T, Zhou J, Tong Y, Su P, Li X, Liu Y, Liu N, Wu X, Zhang Y, Wang J, et al. Engineering chimeric diterpene synthases and isoprenoid biosynthetic pathways enables high-level production of miltiradiene in yeast. Metab Eng 2020;60:87–96.
[27] Jiang GZ, Yao MD, Wang Y, Xiao WH, Yuan YJ. A "push-pull-restrain" strategy to improve citronellol production in *Saccharomyces cerevisiae*. Metab Eng 2021;66:51–9.
[28] Gu YA, Jiao X, Ye LD, Yu HW. Metabolic engineering strategies for de novo biosynthesis of sterols and steroids in yeast. Bioresour. Bioprocess 2021;8:110.
[29] Woolston BM, Edgar S, Stephanopoulos G. Metabolic engineering: past and future. Annu Rev Chem Biomol Eng 2013;4:259–88.
[30] Bu X, Lin JY, Duan CQ, Koffas MAG, Yan GL. Dual regulation of lipid droplet-triacylglycerol metabolism and ERG9 expression for improved β-carotene production in *Saccharomyces cerevisiae*. Microb Cell Factories 2022;21:3.
[31] Reider Apel A, d'Espaux L, Wehrs M, Sachs D, Li RA, Tong GJ, Garber M, Nnadi O, Zhuang W, Hillson NJ, et al. A Cas9-based toolkit to program gene expression in *Saccharomyces cerevisiae*. Nucleic Acids Res 2017;45:496–508.

[32] Butt TR, Edavettal SC, Hall JP, Mattern MR. SUMO fusion technology for difficult-to-express proteins. Protein Expr Purif 2005;43:1–9.
[33] Harper S, Speicher DW. Purification of proteins fused to glutathione S-transferase. Methods Mol Biol 2011;681:259–80.
[34] Bernier SC, Cantin L, Salesse C. Systematic analysis of the expression, solubility and purification of a passenger protein in fusion with different tags. Protein Expr Purif 2018;152:92–106.
[35] Costa SJ, Almeida A, Castro A, Domingues L, Besir H. The novel Fh8 and H fusion partners for soluble protein expression in *Escherichia coli*: a comparison with the traditional gene fusion technology. Appl Microbiol Biotechnol 2013;7:6779–91.
[36] Savitsky P, Bray J, Cooper CDO, Marsden BD, Mahajan P, Burgess-Brown NA, Gileadi O. High-throughput production of human proteins for crystallization: the SGC experience. J Struct Biol 2010;172:3–13.
[37] Magnan C, Yu J, Chang I, Jahn E, Kanomata Y, Wu J, Zeller M, Oakes M, Baldi P, Sandmeyer S. Sequence assembly of *Yarrowia lipolytica* atrain W29/CLIB89 shows transposable element diversity. PLoS One 2016;11:e0162363.
[38] Lv Y, Edwards H, Zhou J, Xu P. Combining 26s rDNA and the cre-loxp system for iterative gene integration and efficient marker curation in *Yarrowia lipolytica*. ACS Synth Biol 2019;8:568–76.
[39] Xie W, Ye L, Lv X, Xu H, Yu H. Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in *Saccharomyces cerevisiae*. Metab Eng 2015;28:8–18.
[40] Schwartz C, Shabbir-Hussain M, Frogue K, Blenner M, Wheeldon I. Standardized markerless gene integration for pathway engineering in *Yarrowia lipolytica*. ACS Synth Biol 2017;6:402–9.
[41] Liu D, Wan N, Zhang FZ, Tang YJJ, Wu SG. Enhancing fatty acid production in *Escherichia coli* by Vitreoscilla hemoglobin overexpression. Biotechnol Bioeng 2017;114:463–7.
[42] Czajka JJ, Nathenson JA, Benites VT, Baidoo EEK, Cheng Q, Wang Y, Tang YJ. Engineering the oleaginous yeast *Yarrowia lipolytica* to produce the aroma compound β-ionone. Microb Cell Factories 2018;17:136.
[43] Wei LJ, Zhong YT, Nie MY, Liu SC, Hua Q. Biosynthesis of α-pinene by genetically engineered *Yarrowia lipolytica* from low-cost renewable feedstocks. J Agric Food Chem 2021;69:275–85.
[44] Williams DC, Wildung MR, Jin AQW, Dalal D, Oliver JS, Coates RM, Croteau R. Heterologous expression and characterization of a "pseudomature" form of taxadiene synthase involved in paclitaxel (Taxol) biosynthesis and evaluation of a potential intermediate and inhibitors of the multistep diterpene cyclization reaction. Arch Biochem Biophys 2000;379:137–46.
[45] Huang QL, Roessner CA, Croteau R, Scott AI. Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. Bioorg Med Chem 2001;9:2237–42.
[46] DeJong JM, Liu YL, Bollon AP, Long RM, Jennewein S, Williams D, Croteau RB. Genetic engineering of Taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotechnol Bioeng 2006;93:212–24.
[47] Engels B, Dahm P, Jennewein S. Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metab Eng 2008;10:201–6.
[48] Li J, Mutanda I, Wang K, Yang L, Wang J, Wang Y. Chloroplastic metabolic engineering coupled with isoprenoid pool enhancement for committed taxanes biosynthesis in *Nicotiana benthamiana*. Nat Commun 2019;10:4850.