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Metabolic engineering of *Saccharomyces cerevisiae* for enhanced taxadiene production

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Abstract

Background Metabolic engineering enables the sustainable and cost-efficient production of complex chemicals. Efficient production of terpenes in *Saccharomyces cerevisiae* can be achieved by recruiting an intermediate of the mevalonate pathway. The present study aimed to evaluate the engineering strategies of *S. cerevisiae* for the production of taxadiene, a precursor of taxol, an antineoplastic drug.

Result SCIGS22a, a previously engineered strain with modifications in the mevalonate pathway (MVA), was used as a background strain. This strain was engineered to enable a high flux towards farnesyl diphosphate (FPP) and the availability of NADPH. The strain MVA was generated from SCIGS22a by overexpressing all mevalonate pathway genes. Combining the background strains with 16 different episomal plasmids, which included the combination of 4 genes: *tHMGR* (3-hydroxy-3-methylglutaryl-CoA reductase), *ERG20* (farnesyl pyrophosphate synthase), *GGPPS* (geranyl diphosphate synthase) and *TS* (taxadiene synthase) resulted in the highest taxadiene production in *S. cerevisiae* of 528 mg/L.

Conclusion Our study highlights the critical role of pathway balance in metabolic engineering, mainly when dealing with toxic molecules like taxadiene. We achieved significant improvements in taxadiene production by employing a combinatorial approach and focusing on balancing the downstream and upstream pathways. These findings emphasize the importance of minor gene expression modification levels to achieve a well-balanced pathway, ultimately leading to enhanced taxadiene accumulation.

Keywords Terpenes, Taxol, *S. cerevisiae*, Mevalonate pathway, Metabolic engineering

Background

Terpenes are natural products which are extracted from their original plant sources and can be used in a wide range of applications such as the production of pharmaceuticals, nutraceuticals, cosmetics and biofuels. Among these, the diterpenoid taxol is a potent anti-cancer drug. Since the discovery of taxol in its original source *Taxus brevifolia*, it has been used alone and in combination with other antineoplastic agents in the treatment of many cancers such as metastatic ovarian cancer, metastatic breast cancer, non-small cell lung cancer and AIDS-related Kaposi´s sarcoma [13, 20]. Following its clinical success, meeting the growing demand for taxol from its original natural sources has been challenging due to the

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complex purification process, slow growth, limited distribution and low yield from *Taxus sp.* [4, 25]. On the other hand, the chemical synthesis of taxol is infeasible, as it requires at least 40 steps and presents many obstacles because of its complex structure, with a very hydrophobic nature and 11 stereocentres [2, 15]. Even though recently developed approaches such as using renewable yew parts for semisynthetic production and plant cell cultures are promising, they still have disadvantages due to high cost and low yield [5, 9, 13]. As an alternative production strategy, cell factories metabolically engineered to produce taxol can provide several advantages, such as faster, sustainable and more economical production with the rapid growth rates of microbial hosts. Redirecting cellular metabolism with metabolic engineering enables the production of several desired products in desired amounts using microorganisms, including various terpenes, alkaloids and polyketides [1, 10, 17]. The biosynthetic pathway of taxol, a terpene of particular interest, could be reconstructed in microorganisms to achieve the production of its precursors. Taxa-4(5)-11(12)-diene (taxadiene), the first committed intermediate in the biosynthetic pathway of taxol, can be synthesized through the cyclization of geranylgeranyl diphosphate (GGPP) from the mevalonate (MVA) pathway in *S. cerevisiae* and the methylerythritol-phosphate (MEP) pathway in *Escherichia coli* via the actions of Taxadiene Synthase (TS) [4, 23]. To date, the highest titer of 1 g /L of taxadiene in bioreactor cultivation has been achieved through the adoption of a novel multivariate modular approach by engineering *E. coli* as a host [2]. Researchers showed that expressing a geranyl geranyl diphosphate synthase (*GGPPS*) from *Taxus canadensis* and *TS* from *Taxus brevifolia* in an operon system with a re-designed background strain led to significantly improved taxadiene production. However, despite promising results in *E. coli*, the difficulty of expressing cytochrome P450 proteins in this organism makes it a less than ideal host for taxol production, as several subsequent steps after taxadiene are cytochrome P450 dependent [8, 12, 30]. On the other hand, the eukaryotic host *S. cerevisiae* is a promising background strain for metabolic engineering studies and is more advantageous in the expression of such enzymes. By using different metabolic engineering strategies, Engels et al. [8] produced taxadiene in *S. cerevisiae* for the first time and reported production of up to 8.7 mg/L, and most recently, a 251 mg/L taxadiene titer was achieved in *S. cerevisae* by Malcı et al. (Table 1) [8, 12].

Many studies showed that while examining the metabolic engineering of toxic molecules like taxadiene, pathway balance should be prioritized. Taxadiene is a difficult molecule to work since there is no high-throughput screen for it. Therefore, while engineering our strains, we focused on a combinatorial approach and tried to balance between the downstream and upstream pathways [2, 5].

Table 1 Comparison of taxadiene producing strains

Host	Taxadiene concentration (mg/L)	References
S. cerevisiae	0.7	DeJong et al. [7]
S. cerevisiae	8.7	Engels et al. [8]
E. coli	1020	Ajikumar et al. [2]
Bacillus subtilis	17.8	Abdallah et al. [1]
S. cerevisiae	129	Nowrouzi et al. [16]
S. cerevisiae	215	Malcı et al. [12]
S. cerevisiae	184.2	Zhang et al. [29]
S. cerevisiae	528	This study

In order to engineer the upstream pathway, we utilized an existing background strain (SCIGS22a) specifically designed for sesquiterpene production. To create SCIGS22 (background of SCIGS22a), Scalcinati et al. [18, 19] embarked on the engineering of *S. cerevisiae* by enhancing the flux through FPP and blocking the branch point of the pathway through sterol biosynthesis [18, 19]. This involved overexpressing *ERG20* and *tHMGR*, downregulating *ERG9,* and deleting *LPP* & *DPP1*, thereby achieving elevated sesquiterpene production (Fig. 1). SCIGS22a was created by overexpressing the *tHMG1* in SCIGS22 [11]. Since FPP is also a precursor for taxadiene, we used this strain as the basis for further optimization aimed at enhancing taxadiene production.

A second background strain, MVA, was created by overexpressing six mevalonate pathway genes; *ERG10, ERG13*, *ERG12, ERG8, ERG19, IDI* in SCIGS22a. Hence, we comparatively examined the two engineered strains.

As a downstream taxadiene pathway strategy, we created 16 different episomal plasmids which included combinations of 4 genes; heterologous codon optimized *GGPPS* synthase from *Taxus canadensis*, *TS* from *Taxus brevifolia, and* key flux controlling steps of the mevalonate pathway, *tHMG1* and *ERG20*. Plasmids contained one or both or neither of *tHMG1* and *ERG20*, while *GGPPS* and *TS* were present in all. In addition, fusion proteins of *GGPPS-ERG20* and *GGPPS-TS* and the reverse orientation were also created among the plasmid varieties. By combining the upstream pathway with one of the episomal plasmids representing the downstream pathway, we constructed a total of 32 strains. The 32-strains were analysed to identify the most balanced pathway capable of producing high taxadiene in *S. cerevisiae*. Based on our engineering strategies, we managed to enhance the taxadiene production to 528 mg/L, the highest titer reported until now in shake shake flask cultures.

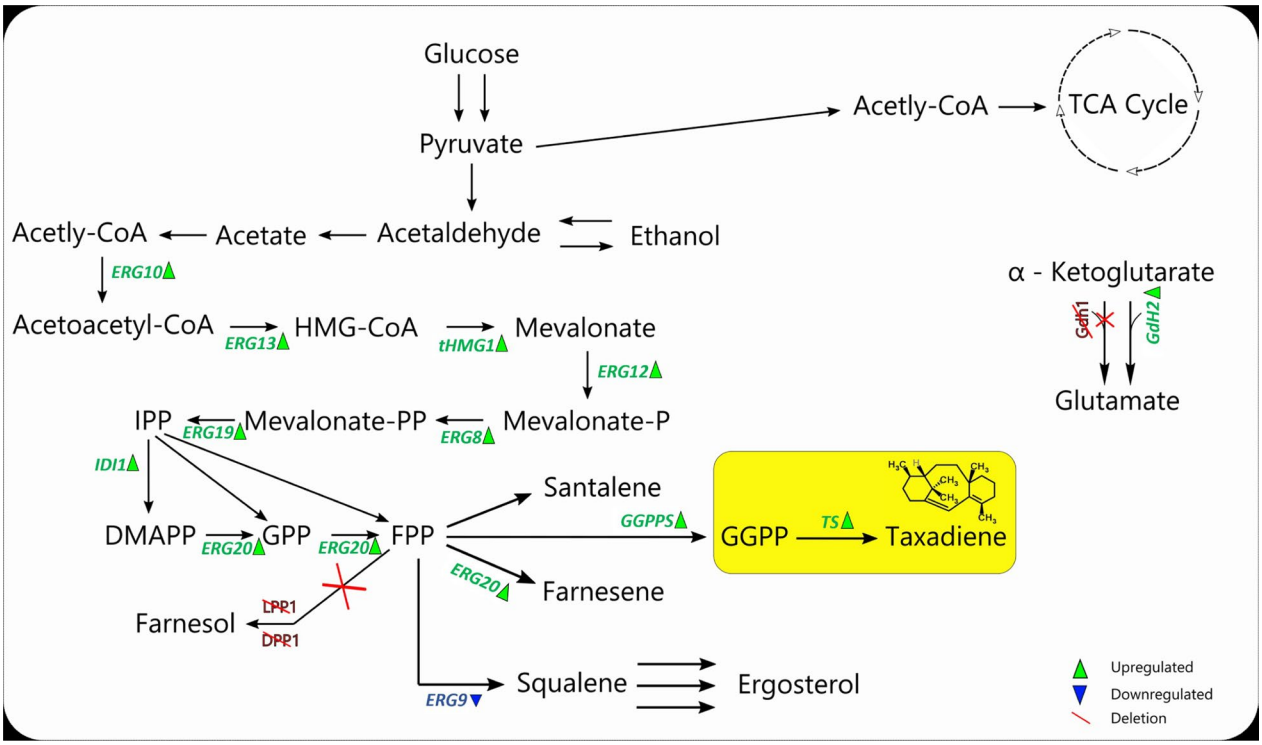

Fig. 1 The engineering strategy of SCIGS22a to produce high amounts of isoprenoids. The downregulated gene ERG9 (squalene synthase) is shown in blue. Deleted genes—LPP1 and DPP1, lipid phosphate phosphatases, and GDH1, NADP-dependent glutamate dehydrogenase are shown in red. Overexpressed genes tHMG1 (HMG-CoA reductase), ERG20 (FPP synthase), GDH2 (NAD dependent glutamate dehydrogenase) are shown in green. On the mevalonate pathway; GGPP is produced right after FPP and taxadiene can be produced using heterologous terpene synthase from GGPP in *S. cerevisiae*

Methods

Strains and cultivation conditions

CEN.PK113-5D (MATa MAL2-8c SUC2 ura3-52), provided by P. Kötter (University of Frankfurt, Germany), was used as the parent strain for all strain engineering experiments. *tHMGR* and *ERG20* were amplified from CEN.PK113-5D. Strain SCIGS22a (*MAT* a *MAL2-8* ^{c} *SUC2 ura3-52 lpp1* Δ::*loxP dpp1* Δ::*loxP* P $_{ERG9}$Δ::*loxP*-P $_{HXT1}$ *gdh1* Δ::*loxP* P $_{TEF1}$ *-ERG20* P $_{PGK1}$ *–GDH2* P $_{TEF1}$ *-tHMG1*) created by Scalcinati et al. [18, 19] and Lopez et al. [11] was used as a backbone strain [11, 18]. Background yeast strains used in this study are presented in detail in Table 2. *Escherichia coli DH5α* was used for general cloning procedures in this study.

Yeast strains for the preparation of competent cells were grown in YPD medium consisting of 10 g/L yeast extract (Merck Milipore), 20 g/L peptone (Difco), and 20 g/L glucose (Merck Millipore). Strains carrying URA3 marker-based plasmids were selected and cultivated on synthetic complete media without uracil, consisting of 6.7 g/L yeast nitrogen base without amino acids (Formedium), 0.77 g/L complete supplement mixture without uracil (Formedium), 20 g/L glucose and 20 g/L agar (Merck Milipore). For shake flask cultivation of yeast strains carrying plasmids for production of taxadiene, defined minimal medium was used. The medium included (per liter): (7.5 g $(NH_4)_2SO_4$, 14.4 g KH_2PO_4, 0.5 g $MgSO_4$ $7H_2O$, 2 mL trace metal solution (per liter,

Table 2 Background yeast strains used in this study and relevant genotype

Strains	Genotypes /Sources	Sources
SCIGS22a	*MAT* **a** *MAL2-8* ^{c} *SUC2 ura3-52 lpp1* Δ::*loxP dpp1* Δ::*loxP* P $_{ERG9}$Δ::*loxP*-P $_{HXT1}$ *gdh1* Δ::*loxP* P $_{TEF1}$ *-ERG20* P $_{PGK1}$ *–GDH2* P $_{TEF1}$ *-tHMG1*	Lopez et al. [11]
MVA	(MATa MAL2-8^{c} *SUC2 ura3-52 lpp1*Δ::loxP *dpp1*Δ::*loxP* P_{ERG9}Δ::loxP-P_{HXT1}gdh1Δ::loxP P_{TEF1}-*ERG20* P_{PGK1}-*GDH2* P_{TEF1}-*tHMG1* P_{PGK1}-*ERG13* P_{TEF1}-*ERG10* P_{PGK1}-*ERG8* P_{TEF}*ERG12* P_{PGK1}-*IDI* P_{TEF1}-*ERG19*)	This study

pH 4.0: 15 g EDTA (sodium salt), 4.5 g $ZnSO_4$ $7H_2O$, 0.84 g $MnCl_2$ $2H_2O$, 0.3 g $CoCl_2$ $6H_2O$, 0.3 g $CuSO_4$ $5H_2O$, 0.4 g; Na_2MoO_4 $2H_2O$, 4.5 g; $CaCl_2$ $2H_2O$, 3 g; $FeSO_4$ $7H_2O$, 1 g H_3BO_3, and 0.1 g KI), 20 g glucose and pH adjusted to 6.5 with 2 M KOH [17]. Overnight cultures were prepared in 5 mL medium at 30 °C and 200 rpm. Cultures prepared from overnight culture at initial OD_{600} of 0.05 in 20 mL medium (100 mL unbaffed shake flask) at 30 °C and 200 rpm.

E. coli was cultivated on Lysogeny Agar (LB- Agar) plates containing antibiotic ampicillin at a final concentration 100 µg/ml at 37 °C and were used for culturing as required [11].

Construction of background strain MVA

In order to increase flux towards FPP in *S. cerevisiae,* three different integration cassettes were created. Each cassette consisted of 2 genes under control of the dual promoters (pTEF1- pPGK1), terminators, and previously characterized chromosomal integration sites upstream and down-stream for homologous recombination [14]. To construct integrative cassettes, a dual promoter was amplified from plasmid pBS01. The genes *ERG10*, *ERG13*, *ERG12*, *ERG8*, *ERG19* and *IDI* were amplified from the genomic DNA of CEN-PK113-5D by PCR with primers designed for overlap extension and listed in Supp Table 1. The first integrative cassette's upstream site of XI-3, harboring tADH1, and the downstream site of XI-3 harboring the marker URA and terminator tCYC1, were amplified from plasmid pXI-3 (Fig. 2A). Gel purified subunits; XI-3-up, ERG10, promoters, ERG13 and XI-3-dw were fused using one step PCR with a molar ratio of 1:3:3:1 [31]. The first PCR product was used as a template for second round PCR, which was performed with primers Fus1F and Fus1R and PrimeSTAR HS DNA polymerase. For transforming the first and second integrative cassettes together, a new recyclable dominant marker cassette, *amdSYM,* flanked by directed repeats was used for the second cassette [21] (Fig. 2B).

The upstream site of the selected integration site XI-2, harboring terminator tADH1, and the downstream site of XI-2, harboring tCYC1, were amplified from plasmid XI-2. Genes *ERG12* and *ERG8*, promoters, cassette *amdSYM*, directed repeats(DR), tCYC1, and integration sites were fused using the same method mentioned above. PCR products of the first and second cassettes were purified from agarose gel and transformed into SCIGS22a, 2000 ng in total with electroporation at

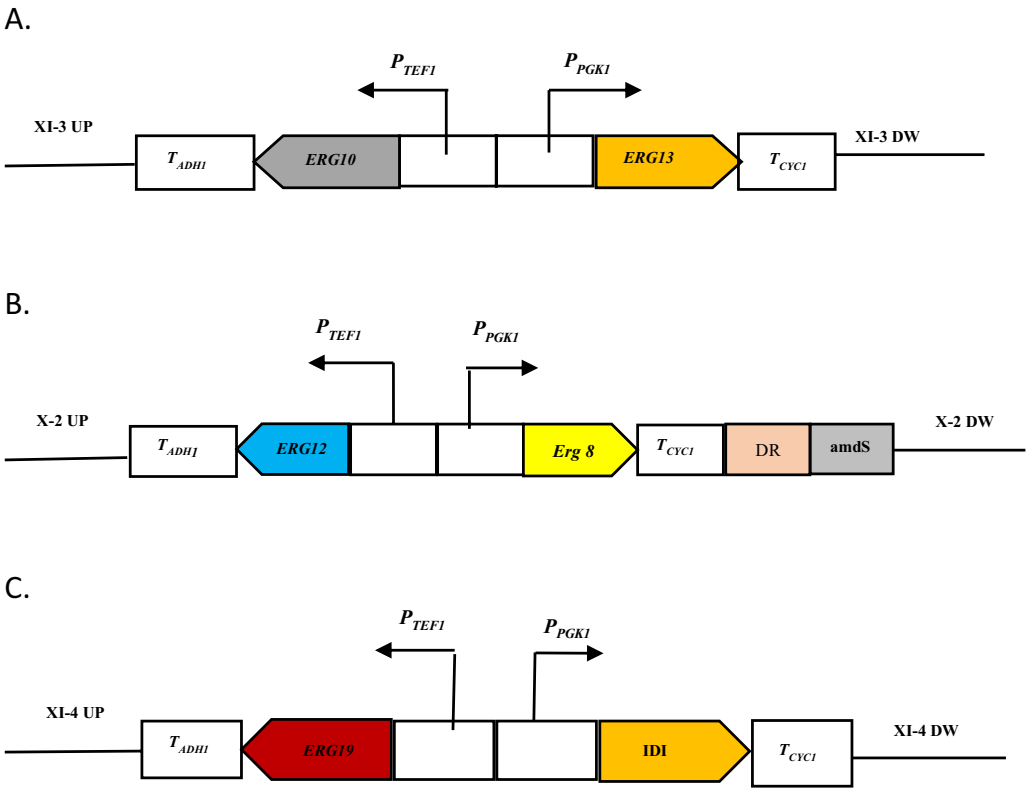

Fig. 2 **A**–**C** Chromosomally integrated constructs. **A** The first construct was integrated in to XI-3 site. **B** The second construct integrated in to XI-2 site. **C** The third construct integrated in to XI-4

1.5 kV, 10μF and 200 Ω in a 0.2 cm gap electroporation cuvette using the Eppendorf Eporator (Eppendorf AG, Hamburg, Germany) [21] with selection on SD-Ac medium (3 g/L KH2PO4, 0.5 g/L MgSO47H2O, 6.6 g/L K2SO4 g/L, 1 ml/L trace element solution as previously described by [18, 19]), 1 ml/L vitamin solution [18, 19, 21], 0.6 g/L acetamide, and 20 g/L glucose [21], and new colonies verified by colony PCR. To excise the markers via mitotic recombination, verified colonies were initially cultivated on 5-fluoroorotic acid (5-FOA) plates to excise the URA3 marker, and selected markerless cell colonies from 5-FOA medium were inoculated on 5-fluoroacetamide (SM-Fac) plates to excise the amdS marker. Growing colonies were selected and verified with colony PCR and then with sequencing.

The last integrative cassette was created with genes *ERG19* and *IDI* of the pathway (Fig. 2C). The upstream part of the integration site harboring tADH1, and the downstream part, harboring tCYC1 and the URA3 marker, were amplified from plasmid XI-4. Purified subunits were fused by first and second round PCR.

Plasmid construction

For episomal plasmid construction, pSP-GM1 was used as a backbone plasmid. All the primers used in this study to create plasmids are listed in Supp. Table 1. Truncated and codon optimized genes (Supp. Table 2); taxadiene synthase (*TS*) from *Taxus brevifolia* and geranyl–geranyl diphosphate synthase (*GGPPS*) from *Taxus canadensis* were flanked by SpeI/SacI and BamHI/NheI, respectively, and were synthesized synthetically and cloned in pUC57 by GeneScript. A Kozak sequence (AAAACA) was introduced immediately upstream of the start codon of GGPPS and TS to promote translation [24]. All restriction enzymes used in this study were from Thermo Fisher Scientific (Waltham, MA, USA). P_{HXT}, *ERG20,* and truncated *tHMG1* were amplified from CENPK-113-5D by Phusion DNA Polymerase (ThermoFisher). Purification of DNA fragments from gel or PCR was conducted with Gene Jet PCR/Gel purification kits (ThermoScientific). All fusion proteins were constructed by OE-PCR [21]. CEN.PK-113-5D was used as a template to prepare fusion pHXT-tHMG1 using primers tHMG1-F1/tHMG1-MreI-R1 and pTHXT7FW-Kpn2I/ptHXT7-R. To prepare the first expression plasmid, pB1**;** *TS* was cloned in to the pSP-GM1(SpeI/SacI) under the control of a P_{TEF} promoter; *GGPPS* was cloned into the same plasmid (BamHI/NheI) under the control of P_{GK1} promoter yielding pB1 (Supp. Figure 1). Plasmids pB2, pB3, and pB4 originated from pB1, including pHXT-*tHMG1* at the Kpn2I/MreI site in pB1, resulting in pB2, including pPGK1-*ERG20* at the AvrII/AscI site in pB1, resulting in pB3, and including pPGK1-*ERG20* at the AvrII/AscI site and pHXT-*tHMG1* at the Kpn2I/ MreI site in pB1, resulting in pB4, respectively.

For constructing pD1 (Supp. Figure 1); *TS* was amplified from pUC57-*TS* using the primers TS-Spe-F1/TS-R1, and *GGPPS* was amplified from pUC57-*GGPPS* using the primers GGPPS-F1/GGPPS-SacI-R1. Fusion enzymes of TS–GGPPS were constructed by inserting a short and flexible GSG linker, (GGT TCT GGT) [Solis-Escalante et al., 20] and fusion genes were cloned into pSP-GM1 (SpeI/SacI). Likewise, pD2, pD3 and pD4 were derived from pD1 (Supp. Figure 1).

Plasmid pE1 (Supp. Figure 1) containing the different order of genes *GGPPS-TS* was created as described above using GGPPS-SpeI-F2/GGPPS-R2 and TS-F2/TS-SacI-R2 primers. After fusion PCR of fragments, the product was cloned in to the pSP-GM1 (SpeI/SacI). Another three plasmids (pE2, pE3 and pE4) were created based on pE1 (Supp. Figure 1).

ERG20, amplified with Erg20-BamHI-F1/Erg20-R1 primers from CEN.PK-113-5D and *GGPP* amplified from PUC57-GGPPS using primers GGPPS-F3/GGPPS-NheI-R3, were fused by OE-PCR in the *ERG20-GGPPS* direction. A reverse order expression cassette, *GGPPS-ERG20* was created through the amplification of *ERG20* with primer Erg20-F2 and Erg20-NheI-R2 and amplification of *GGPP* with primers GGPPS–BamHI-F and GGPPS-R2. Fusion products of the resulting fragments were cloned into BamHI/NheI site of pSP-GM1, which contains TS in the SpeI/SacI site, yielding pF1 and pG1, respectively**.** Version of pF2 and pG2 included ptHXT-tHMG1 in a Kpn21/MreI site. All plasmids represented in Supp. Figure 1.

Strain construction

Strains carrying plasmids were constructed by transforming SCIGS22a and MVA with 16 plasmids separately, resulting in the formation of 32 strains. While, background of strains HK01-16 was SCIGS22a, MVA was the background of HK17-32.

Yeasts were transformed using electroporation. Plasmids transformed into the background strains were 2000 ng in total, with electroporation at 1.5 kV, 10μF and 200 Ω in a 0.2 cm gap electroporation cuvette using the Eppendorf Eporator (Eppendorf AG, Hamburg, Germany) [31]. Five biological replicates verified by colony PCR were used for further experiments.

Shake flask cultivation for production of taxadiene

5 mL defined synthetic medium (SM) was used to prepare pre-cultures as explained above. These pre-cultures were used to inoculate 20 mL fresh medium in a 100 mL un-baffeled flask at a starting OD_{600} of 0.05. Dodecane was added aseptically to 10% (v/v) of the volume. The

cultivations were grown at 30 °C with agitation in an orbital shaker at 200 rpm [18]. 1 mL of samples were collected from the dodecane layer for GC–MS analysis at 72 h. The strains (SCIGS22a and MVA) without plasmids proceeded the same way and were used as negative controls.

Analytical methods

The dodecane layer was analyzed in a Shimadzu GC–MS-QP2010 ultra system equipped with an AOC-20i auto injector and an Agilent J&W HP-5MS (5% Phenyl)-methylpolysiloxane column (0.25 mm inner diameter, 0.25 µm thickness, 30 m length) used as the stationary phase. The samples (1 µL) were injected splitless onto the GC column, and helium was used as the carrier gas. The injector temperature was 250 °C, and the oven initial temperature was 50 °C with an increase of 10 °C per minute up to 300 °C. The initial and final temperatures were held for 1 min each. The selected ion mode (SIM) (m/z ion 122) was used in the MS instrument for quantification of taxadiene using β-caryophyllene as an internal standard. GC–MS solution (version 4.20) was used for processing the chromatograms. A calibration equation was created using β-caryophyllene, and taxadiene concentrations were quantified from this equation (Supp Fig. 2). Before calculation, the taxadiene peak areas were corrected with the correction factor obtained from the ratio of the peak area of reference β-caryophyllene to the β-caryophyllene peak area of the sample to eliminate errors due to injection or loss during extraction [1].

Results and discussions

Production capacity of independent and fused *TS* and *GGPPS* in background cells

In this study, we engineered the SCIGS22a strain further to create MVA, followed by overexpression of six endogenous genes in the mevalonate pathway under a constitutive promoter to enhance the possible production capability. Our first effort was to transform a basic plasmid containing *TS* and *GGPPS* expressed independently under the control of the P_{TEF1} and P_{PGK1} promoters into SCIGS22a and MVA, creating strains HK1 and HK17, respectively. However, contrary to our expectations, the SCIGS22a-based strain HK1 produced a higher amount of taxadiene (109.7 mg/L) compared to the MVA-based strain HK17 (2.9 mg/L) (Fig. 3). Malcı et al. [12] findings, employing OptForce, indicate that the overexpression of genes in the early steps of the mevalonate pathway does not lead to an increase in flux towards taxadiene [12]. On the other hand, several studies have demonstrated that overexpression of all the mevalonate pathway genes, results in the effective synthesis of the desired final product, including taxadiene [16, 26]. This may indicate the well-established balance in the desired flux between the upstream and downstream pathways. In order to identify the optimal, well-balanced pathway, we used a modular approach, drawing inspiration from

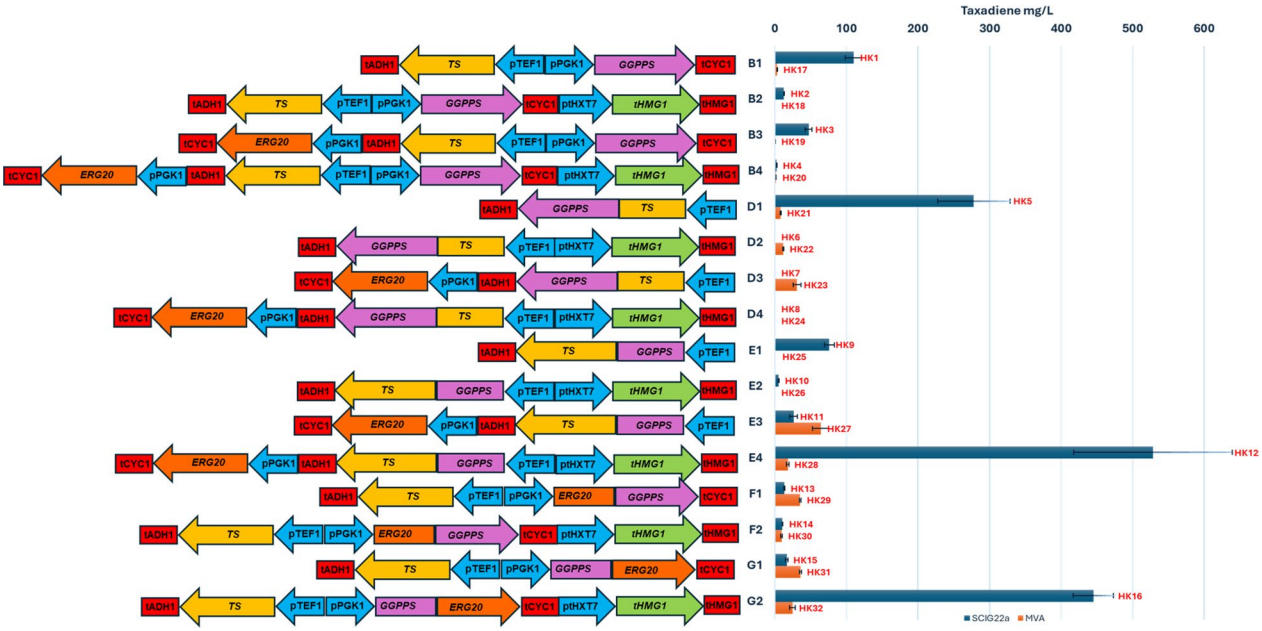

Fig. 3 Taxadiene production by recombinant yeasts harboring constracts and episomal plasmids overproducing various enzymes. Values shown are mean ± standard deviation for 72-h triplicate cultivations

previous successful studies to create a variety of combinations for the downstream pathway [2]. Molecular interactions between sequential enzymes have been shown in previous studies to be beneficial for the efficient conversion of *GGPP* to diterpenoids through substrate channelling in vivo. Therefore, we fused *TS* and *GGPPS* in both *TS-GGPPS* and *GGPPS-TS* orientations under the control of the P_{TEF1} promoter. The titres obtained for the *TS-GGPP* orientation in HK5 was 277.8 mg/L and 7.9 mg/L in HK21 (Fig. 3). Hence, a significant increase in taxadiene production was observed in the SCIGS22a and MVA background strains with the fusion combination, resulting in almost 2.5-fold increase comparred with the separate expression of *TS* and *GGPPS.* On the other hand, cultivation of strain HK9 including *GGPPS-TS* orientation yielded approximately 76.0 mg/L of taxadiene, whereas we could not observe any taxadiene titre in HK25 (Fig. 3). When aligning the gene sequences in the *TS-GGPPS* direction, we observed enhanced results for MVA, whereas the reverse orientation had an advantage in SCIGS22a over MVA. Consistently, Ajikumar et al. [2] showed that the *TS-GGPPS* orientation resulted in an increase in taxadiene titre in an upstream engineered *E. coli* strain compared to the reverse direction. Based on these results, it can be asserted that minor modifications can yield significant differences in achieving balance between the downstream and upstream pathways. The differences in protein expression levels observed among different gene orientations in fusion proteins can be attributed to variations in mRNA secondary structure and processing. These factors can influence mRNA stability and translation efficiency. One possible explanation for these differences is the formation of distinct stem-loop structures within the mRNA, particularly in the reverse orientation. This structural variability can impact the ability of the mRNA to be translated into protein effectively [3].

The impact of *tHMG1* on taxadiene production

The tightly regulated MVA pathway in yeast relies on feedback mechanisms, with HMG-CoA reductase (HMGR) acting as the primary regulatory target. HMGR catalyzes the conversion of HMG-CoA to mevalonate and is subject to both transcriptional and posttranscriptional control. Eliminating the N-terminal regulatory domain of yeast HMGR disrupts the negative feedback exerted by steroids, leading to an increased availability of isopentenyl diphosphate for alternative metabolic pathways. Additionally, HMG-CoA reductase, a bottleneck enzyme within the MVA pathway, can be modified in terms of its structure and activity to enhance the flux of the pathway. These advances in manipulating activity provide valuable means for augmenting metabolite production in yeast and hold promise for the development of more efficient microbial cell factories [8, 22, 27].

In order to investigate the impact of expressing a truncated form of integrative *HMG1*, which encodes an HMGR, in strain design, we aimed to assess its effect on taxadiene production by also expressing it through the plasmid. We were unable to identify any high production associated with cells of either HK2 (12.6 mg/L) or HK18 (no production), which included *tHMG1*, *GGPPS* and *TS* expressed seperately (Fig. 3). Similarly, no taxadiene titre was detected in the HK6 expressing the fusion of *TS-GGPPS* with *tHMG1*, as well as in HK26 expressing the fusion of *GGPPS-TS* with *tHMG1*. Expressing the fusion of *TS-GGPPS* and *tHMG1* seperately in HK22 resulted in a yield of 11.4 mg/L taxadiene (Fig. 3). Cultivation of HK10, expressing the fusion of *GGPPS-TS* and *tHMGR* seperately, resulted in a taxadiene titre of 4.9 mg/L. In our study, we demonstrated the compatibility of the *GGPPS-TS* fusion within the SCIGS22a background strain, whereas the *TS-GGPPS* fusion exhibited compatibility within the MVA background strain. The co-expression of *tHMG1* with each successfully fused construct resulted in a 1.4-fold increase in taxadiene titer within the MVA background strain, while a pronounced 15-fold decrease was observed in the SCIGS22a background strain. Early studies showed that an increased amount of the catalytic domain of HMGR induces the production of isoprenoids; however, it is essential to note that in these studies, the tHMG1 cassette was integrated into the genome but in this study, besides integration, it was expressed through an episomai plasmid [6, 8, 11, 16].

The impact of *ERG20* on taxadiene production

Farnesyl diphosphate synthase, encoded by *ERG20* catalyzes the formation of the geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) metabolites, two key enzymatic steps in the mevalonate pathway. In this study, the expression of *ERG20* in the episomal plasmid was regulated under the P_{PGK1} promoter. The MVA background strain HK19 exhibited a markedly diminished yield of 0.08 mg/L taxadiene, while the titer in strain HK3 was 47 mg/L (Fig. 3). Concerning the plasmid containing the *TS-GGPPS* fusion and *ERG20* expression, no discernible production was observed for SCIGS22a. In contrast, the MVA background strain HK23 yielded 30.9 mg/L of taxadiene. The highest yield of taxadiene for MVA-background strain, amounting to 64.0 mg/L, was obtained for the HK27 strain when *GGPPS-TS* fusion and *ERG20* were expressed separately. Cultivation of HK11 resulted in 25.9 mg/L of taxadiene (Fig. 3). The expected outcome was that all MVA background cells containing the *TS-GGPPS* fusion had an advantage over SCIGS22a background cells in taxadiene production. However, with

the incorporation of *ERG20*, the expression of the plasmid containing the *GGPPS-TS* fusion in the MVA background strain yielded the highest taxadiene result among the MVA background strains. These results indicate that a well-established balance can be achieved with minor changes in the flow.

The GGPPS and TS fusion results motivated us to construct a fusion between ERG20 and GGPPS, enzymes catalyzing the adjacent steps in the mevalonate pathway. Plasmids expressing the *ERG20-GGPPS* and *GGPPS-ERG20* fusions were designed to include the *TS* separately under the control of P_{TEF1} promoter. Our results showed that the *ERG20-GGPPS* fusion yielded a 2.6-fold increase in taxadiene titer in the HK29 strain compared to the HK13 strain. Likewise, the reverse fusion of *ERG20* and *GGPPS* maintained the same ratio, with the HK31 strain still achieving a roughly 2.2-fold higher titer than HK15. Each combination involving the expression of *ERG20* exhibited higher taxadiene production in MVA background cells compared to SCIGS22a background cells. However, the maximum production achieved in previous MVA strains was not attained in these fusion combinations, and an almost twofold decrease was observed. A previous experimental study reported that *ERG20* significantly improves isoprenoid concentration when expressed together with terpene synthases. Protein complex formation is biologically significant as it brings active sites closer together, facilitating efficient substrate channeling and preventing intermediates from diffusing and degrading by other enzymes. Recent proteomic studies affirm the commonality of protein complexes, underscoring their role in enhancing specific pathways through effective substrate channeling. As previous reports indicated, the increased availability of precursors and protein solubility might be the main reason for the enhanced production of the desired product [28, 31]. According to our findings, any fusion involving *ERG20*, *GGPPS*, and *TS* in strains with an MVA background yielded results that exceeded those in cells with a SCIGS22a background. However, in the SCIGS22a background cells, where only *TS* and *GGPPS* are expressed, and *tHMG1* and *ERG20* are not, a remarkable taxadiene production of 109 mg/L was achieved, representing the most favorable outcome among the combinations analyzed thus far. These results underscore the importance of investigating options that effectively balance both pathways rather than exclusively modifying the upstream or downstream pathway.

Taxadiene production in plasmid combinations co-expressing *TS*, *GGPPS*, *tHMGR*, and *ERG20*

In plasmid combinations where these four genes were individually expressed under separate promoters, a taxadiene titre of 2.1 mg/L and 1.3 mg/L was achieved for the HK4 and HK20 strains, respectively (Fig. 3). Our most efficient strain, HK12 containing the fusion of *TS* and *GGPPS* with *tHMG1* and *ERG20* expressed separately, and having the *GGPPS-TS* orientation, reached the highest taxadiene titer 528.5 mg/L noted to date in shake flask culture (Fig. 3). The co-expression of the *GGPPS-ERG20* fusion, along with separately expressed *tHMG1* and *TS* in the HK16 strain, resulted in a notably high taxadiene titer of 445 mg/L, whereas strain HK32 showed a limited production of 24.4 mg/L of taxadiene (Fig. 3). It is important to accommodate the downstream pathway with the capacity of the upstream pathway to reach a high product capacity. In this study, a well balanced pathway was achieved in HK12 and HK16 strains.

In this study, while we have identified promising taxadiene-producing strains, it is important to acknowledge the limitations in our current approach. Specifically, we did not conduct follow-up studies on gene expression or intermediate metabolite pools in these strains. Understanding both weaker and stronger strain responses through such detailed analysis could provide valuable insights into the underlying mechanisms of taxadiene biosynthesis. Future work should include RNA sequencing or qPCR for gene expression analysis, proteomics for protein expression analysis, and metabolite profiling to quantify intermediates in the biosynthetic pathway. These additional studies would not only validate our findings but also optimize strains for industrial applications. Therefore, we recommend incorporating these analyses in future research to provide a more comprehensive evaluation of taxadiene production and address the limitations of the current study.

Conclusion

In previous studies, the highest levels of taxadiene production have been achieved in *E. coli*, yet ongoing endeavors in *S. cerevisi*ae are focused on attaining elevated titers of this molecule. These efforts encompass diverse approaches, including the utilization of different promoters, culture optimization studies, enzyme truncation experiments, and strategies to address challenges associated with the expression of membrane-bound enzymes in *S. cerevisiae*. The rationale behind these efforts lies in the pronounced advantages that *S. cerevisiae* offers over *E. coli* in terms of its genetic and biosynthetic capabilities. Despite the inherent complexities associated with membrane-bound enzyme expression, *S. cerevisiae* emerges as a promising platform for taxadiene production. In this study combinatorial design has been used to determine the optimal combination for the production of the early-step Taxol precursor, taxadiene. Combinatorial design is widely

used in synthetic biology and metabolic engineering areas for microbial production of diverse chemicals. The creation of the background strain MVA was realized through the integrative expression approach. Simultaneously, the expression of crucial pathway genes and enzymes involved in isoprenoid production was pursued using episomal plasmids.

We observed substantial changes in strains derived from SCIGS22a within the created combinations. The best result was achieved with the fusion of *GGPPS-TS*, where four different genes are expressed in the specified orientations. A typical inference that can be drawn from this is that fusions positively influence substrate accessibility. This study surpasses the 300 mg/L achieved for *E. coli* in shake flask experiments. These findings illustrate that significant alterations in taxadiene accumulation can be achieved by modulating expression levels within a limited range for both the upstream and downstream pathways.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Author contributions

HK designed and supervised the study, designed the experiments, performed the experiments, analyzed the results, and drafted the manuscript. MK contributed experimentally to the construction of plasmids and strains. HAK contributed to the design of the experiments and reviewing of the manuscript SL conducted the GC analyses YÖ provided guidance and supervision for the GC analyses SDO provided support for the collection of samples for GC. JN supervised and contributed revieving of the manuscript. AK designed and supervised the study. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this article with supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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