ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Production of Miltiradiene by Metabolically Engineered *Saccharomyces cerevisiae*

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ABSTRACT: Metabolic engineering of microorganisms is an alternative and attractive route for production of valuable terpenoids that are usually extracted from plant sources. Tanshinones are the bioactive components of *Salvia miltiorrhizha* Bunge, which is a well-known traditional Chinese medicine widely used for treatment of many cardiovascular diseases. As a step toward microbial production of tanshinones, copalyl diphosphate (CPP) synthase, and normal CPP kaurene synthase-like genes, which convert the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP) to miltiradiene (an important intermediate of the tanshinones synthetic pathway), was introduced into *Saccharomyces cerevisiae*, resulting in production of 4.2 mg/L miltiradiene. Improving supplies of isoprenoid precursors was then investigated for increasing miltiradiene production. Although over-expression of a truncated 3-hydroxyl-3-methylglutaryl-CoA reductase (*tHMGR*) and a mutated global regulatory factor (*upc2.1*) gene did improve supply of farnesyl diphosphate (FPP), production of miltiradiene was not increased while large amounts of squalene (78 mg/L) were accumulated. In contrast, miltiradiene production increased to 8.8 mg/L by improving supply of GGPP through over-expression of a fusion gene of FPP synthase (*ERG20*) and endogenous GGPP synthase (*BTS1*) together with a heterologous GGPP synthase from *Sulfolobus acidocaldarius* (*SaGGPS*). Auxotrophic markers in the episomal plasmids were then replaced by antibiotic markers, so that engineered yeast strains could use rich medium to obtain better cell growth while keeping plasmid stabilities. Over-expressing *ERG20-BTS1* and *SaGGPS* genes increased miltiradiene production from 5.4 to 28.2 mg/L. Combinatorial over-expression of *tHMGR-upc2.1* and *ERG20-BTS1-SaGGPS* genes had a synergetic effects on miltiradiene production, increasing titer to 61.8 mg/L. Finally, fed-batch fermentation was performed, and 488 mg/L miltiradiene was produced. The yeast strains engineered in this work provide a basis for creating an alternative way for production of tanshinones in place of extraction from plant sources.

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KEYWORDS: tanshinones; miltiradiene; terpenoids; isoprenoid precursors; fermentation; *Saccharomyces cerevisiae*

Introduction

Terpenoids represent a diverse class of molecules with important medicinal and industrial properties, most of which are synthesized by plants (Ajikumar et al., 2008; Chang and Keasling, 2006; Chemler et al., 2006). However, terpenoids often accumulate in low content in their native hosts. Because of their complex structures, chemical synthesis of these compounds is inherently difficult and of low yield (Ajikumar et al., 2008; Chang and Keasling, 2006; Chemler et al., 2006). Metabolic engineering of microorganisms is an alternative and attractive route for production of these valuable compounds (Ajikumar et al., 2008; Ajikumar et al., 2010; Chang and Keasling, 2006;

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Leonard et al., 2010; Ro et al., 2006). This strategy has been used to engineer model microorganisms (*Escherichia coli* and *Saccharomyces cerevisiae*) for successful production of many terpenoids and important precursors in their biosynthesis pathways, such as artemisinin (Ro et al., 2006), taxol (Ajikumar et al., 2010; Engels et al., 2008), ginkgolides (Leonard et al., 2010), patchoulol (Albertsen et al., 2011), and so on.

Tanshinones are a group of active diterpenoids found in Chinese medicinal herb *Salvia miltiorrhizha* Bunge, which exhibit diverse pharmacological activities, including antibacterial, antioxidant, neuroprotective, cardioprotective, and antitumor effects (Kai et al., 2011; Zhou et al., 2005). Tanshinones are a mixture of chemicals, mainly including dihydrotanshinone I, tanshinone I, tanshinone IIA, and cryptotanshinone (Dai et al., 2011; Gao et al., 2009). Miltiradiene is the common precursor of the tanshinones biosynthetic pathway (Gao et al., 2009). In *S. miltiorrhizha*, the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP) is catalyzed into miltiradiene by the copalyl diphosphate synthase (SmCPS) and the normal CPP kaurene synthase-like (SmKSL) (Fig. 1) (Gao et al., 2009; Hillwig et al., 2011; Sugai et al., 2011).

Terpenoids are derived from two common building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized through mevalonate (MEV) pathway in yeast (Ajikumar et al., 2008; Ro et al., 2006). Most of the IPP and DMAPP precursors enter the ergosterol synthetic pathway in *S. cerevisiae* (Arnezeder and Hampel, 1990). Similar metabolic engineering strategies have been used in *S. cerevisiae* for improving terpenoids production, which have focused on (1) increasing flux through the MEV pathway to IPP by over-expressing a truncated 3-hydroxyl-3-methylglutaryl-CoA reductase gene (*tHMGR*) (Albertsen et al., 2011; Asadollahi et al., 2010; Engels et al., 2008; Rico et al., 2010; Ro et al., 2006; Tokuhiro et al., 2009; Verwaal et al., 2007), (2) reducing flux toward ergosterol by either deleting the squalene synthase gene (*ERG9*) or down-regulating *ERG9* gene expression by replacing the native *ERG9* promoter with a *MET3* promoter (Albertsen et al., 2011; Asadollahi et al., 2010; Ro et al., 2006), (3) over-expressing *upc2.1* allele (a semi-dominant mutant allele that enhances the activity of sterol uptake control protein 2, UPC2) as a global regulation (Engels et al., 2008; Ro et al., 2006), and (4) increasing flux to farnesyl diphosphate (FPP) or GGPP by over-expressing endogenous FPP synthase (*ERG20*), GGPP synthase (*BTS1*), and/or heterologous bi-functional GGPP synthase genes (Engels et al., 2008; Ohnuma et al., 1994; Tokuhiro et al., 2009).

The *SmCPS* and *SmKSL* genes were previously co-expressed with a GGPP synthase in an *E. coli* strain to enable production of sufficient amounts of miltiradiene for its structural characterization by NMR (Gao et al., 2009). Although miltiradiene production was detected, the titer (2.5 mg/L) was far away from industrial production (Gao et al., 2009). Recently, modular pathway engineering of diterpenoid synthases and MEV pathway was applied in *S. cerevisiae*, leading to production of 365 mg/L miltiradiene (Zhou et al., 2012). In this work, microbial production of miltiradiene was obtained by integrating *SmCPS* and *SmKSL* genes into chromosome of *S. cerevisiae*. Production of miltiradiene was then enhanced by increasing carbon flux to FPP and GGPP through plasmid over-expression. Finally, auxotrophic selection markers in the episomal plasmids were replaced by antibiotic selection markers, and rich medium was used for further improvement of miltiradiene production.

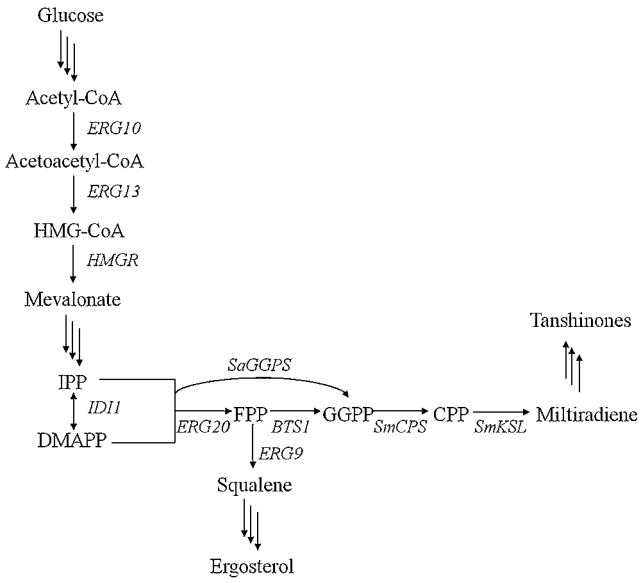

Figure 1. Biosynthetic pathway for miltiradiene in *S. cerevisiae*. The single arrow represents the one-step conversions in the biosynthesis, and the triple arrows represent multiple steps. Intermediates: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; CPP, copalyl diphosphate.

Materials and Methods

Strains and Medium

S. cerevisiae BY4742 (Table I), a derivative of S288C (Ro et al., 2006), was obtained from EUROSCARF and used as the parent strain for all yeast strains. Engineered yeast strains were grown either in SD medium (Burke et al., 2000) lacking leucine, uracil and histidine where appropriate, or in YPD medium (Burke et al., 2000) with geneticin and Hygromycin B where appropriate.

Escherichia coli Trans-10 (TransGen Biotech, Beijing, China) was used for transformation and plasmid DNA extraction. Strains were cultivated at 37°C in LB medium with 100 mg/L ampicillin.

Table I. Strains used in this study.

Name	Description	Source
Strains		
BY4742	*MATα*, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*	Ro et al. (2006)
ZD-T-010	*SmCPS* and *SmKSL* genes integrated into δ site of BY4742	This study
Group 1		
ZD-T-011	ZD-T-010 harboring pLLeu-tHMGR-UPC2.1	This study
ZD-T-012	ZD-T-010 harboring pHLeu-tHMGR-UPC2.1	This study
ZD-T-013	ZD-T-010 harboring pLHis-ERG20/BTS1-SaGGPS	This study
ZD-T-014	ZD-T-010 harboring pHHis-ERG20/BTS1-SaGGPS	This study
Group 2		
ZD-T-021	ZD-T-010 harboring pLKanMX-tHMGR-UPC2.1	This study
ZD-T-022	ZD-T-010 harboring pHKanMX-tHMGR-UPC2.1	This study
ZD-T-023	ZD-T-010 harboring pLhphNT1-ERG20/BTS1-SaGGPS	This study
ZD-T-024	ZD-T-010 harboring pHhphNT1-ERG20/BTS1-SaGGPS	This study
ZD-T-025	ZD-T-010 harboring pLKanMX-tHMGR-UPC2.1 and pLhphNT1-ERG20/BTS1-SaGGPS	This study
ZD-T-026	ZD-T-010 harboring pLKanMX-tHMGR-UPC2.1 and pHhphNT1-ERG20/BTS1-SaGGPS	This study
ZD-T-027	ZD-T-010 harboring pHKanMX-tHMGR-UPC2.1 and pLhphNT1-ERG20/BTS1-SaGGPS	This study
ZD-T-028	ZD-T-010 harboring pHKanMX-tHMGR-UPC2.1 and pHhphNT1-ERG20/BTS1-SaGGPS	This study

Plasmids Construction

The yeast expression plasmids pRS41K and pRS42H (Taxis and Knop, 2006) was obtained from EUROSCARF. Plasmids pYES2.0/CT, pRS406, pRS313, pRS316, pRS426, and pRS425 (Sikorski and Hieter, 1989) were kindly provided by Dr. Qinhong Wang at Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences. Primers used during plasmids construction were summarized in Table S1. Detailed information for plasmids construction was described in supplementary data.

Yeast Transformation and Strain Construction

Transformation of *Saccharomyces* strains was performed by standard electroporation method (Burke et al., 2000). Three to 10 colonies from each transformation were screened for selection of the highest miltiradiene producing transformant. For integration of *SmCPS* and *SmKSL* into δ sites of strain BY4742, strain ZD-T-010 was constructed by transforming strain BY4742 with a DNA fragment amplified from pHUra-SmCPS-SmKSL using primer pair 46/47, followed by selection on SD-URA plates. Integration was verified by PCR analysis. Strains ZD-T-011 to ZD-T-014 (Table I, Group 1) were constructed by transforming strain ZD-T-010 with plasmids containing auxotrophic selection markers, and selection on corresponding auxotrophic SD plates. Strains ZD-T-021 to ZD-T-028 (Table I, Group 2) were constructed by transforming strain ZD-T-010 with plasmids containing antibiotic selection markers, and selection on corresponding antibiotic YPD plates.

Yeast Cultivation

All optical densities at 600 nm (OD_{600}) measurements were taken using a Shimadzu UV-2550 spectrophotometer. SD medium with 2% glucose was used for strains ZD-T-010 and ZD-T-011 to ZD-T-014 (Table I, Group 1). YPD medium with 200 mg/L geneticin and/or 300 mg/L Hygromycin B was used for strains ZD-T-010 and ZD-T-021 to ZD-T-028 (Table I, Group 2). All strains were firstly inoculated into 15 mL culture tubes containing 2 mL medium, and grown at 30°C and 250 rpm to OD_{600} about 1. Unbaffled culture flasks (100 mL) containing 10 mL medium were then inoculated to an OD_{600} 0.05 with these seed cultures. Strains were grown at 30°C and 250 rpm for 4 days.

Fed-Batch Fermentation of Strain ZD-T-026

Strain ZD-T-026 was used for production of miltiradiene through fed-batch fermentation. YPD medium with 200 mg/L geneticin and 300 mg/L Hygromycin B was used for both seed preparation and fermentation. Seed culture was prepared by inoculating several colonies into a 250 mL flask containing 50 mL culture medium, and incubating at 30°C and 250 rpm for 24 h. The seed was then transferred to a 5 L fermentor (Biotech-5BG; Shanghai Baoxin Bioengineering Equipment, Shanghai, China) containing 2 L medium with an initial OD_{600} 0.05. Fermentation was carried out at 30°C with an agitation speed of 600 rpm and air flow rate of 5 L/min. The pH was controlled at 5.5 by automatic addition of 5 M ammonia hydroxide. A fed solution, containing 20 g/L Yeast Extract, 40 g/L peptone, 400 g/L glucose, 200 mg/L geneticin, and 300 mg/L Hygromycin B, was fed to the fermentor within the first 4 days at the feed rate of 5 mL/h.

Miltiradiene Extraction and Analysis

Biomass from fermentation cultures was separated from the culture medium via centrifugation, followed by direct extraction with acetonitrile for analysis of miltiradiene content. Acetonitrile extracts (1 μL) were analyzed by

GC/MS using a Agilent technologies 5975C insert ×1 MSD with triple-Axis Detector equipped with a HP-5 ms (30 m × 0.25 × 0.5 μm) GC column. Compound separation was achieved with an injector temperature of 300°C, and a 20.0 min temperature gradient program for GC-separation starting at 50°C for 2 min followed by heating the column at 20°C/min to 250°C, and a final constant hold at 250°C for 8 min. Mass detection was achieved with electric ionization using a SIM scan mode with diagnostic ion monitored: m/z 272 and m/z 148. A crystallized miltiradiene sample was used as the standard for quantification. Squalene contents were calculated from the ratios of the peak areas of the relevant compounds to those of the standard (purchased from Sigma–Aldrich, St. Louis, MO).

Results and Discussion

Production of Miltiradiene by Integrating *SmCPS* and *SmKSL* Genes into Chromosome of *S. cerevisiae*

In order to produce miltiradiene in *S. cerevisiae*, the *SmCPS*, and *SmKSL* genes were firstly cloned into an integration vector, resulting in plasmid pHUra-δDNA-SmCPS-SmKSL (Fig. 2A). *SmCPS* gene was controlled by the TEF1 promoter, while *SmKSL* gene was controlled by the PGK1 promoter. Both promoters are strong and constitutive, which is compatible with yeast fermentation using glucose as the carbon source (Engels et al., 2008). Integrating *SmCPS* and *SmKSL* genes into chromosome of *S. cerevisiae* BY4742 resulted in strain ZD-T-010. This strain was cultivated in SD medium with 2% glucose for 4 days. GC-MS analysis of cell extraction confirmed that integration of *SmCPS* and *SmKSL* genes did result in miltiradiene synthesis (Fig. 3). This strain produced 4.2 mg/L miltiradiene with a content of 4.4 mg/g dry cell weight.

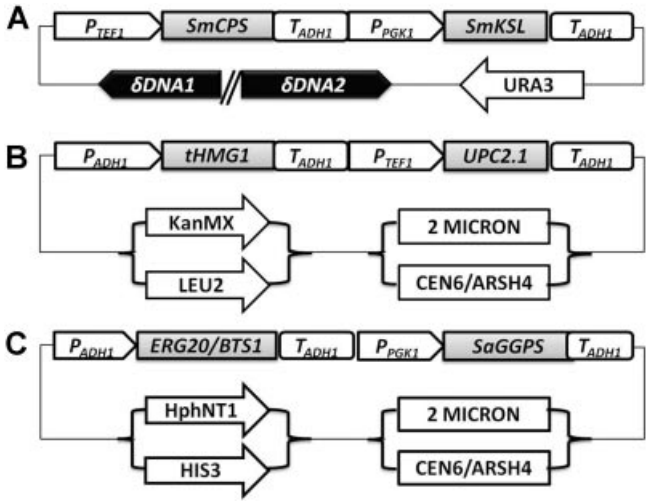

Figure 2. Structures of the integration vector and episomal plasmids. **A**: Structure of the integration vector used for integration of *SmCPS* and *SmKSL* genes into chromosome of *S. cerevisiae*. **B**: Structures of episomal plasmids used for over-expression of *tHMGR* and *upc2.1* genes. **C**: Structures of episomal plasmids used for over-expression of a fusion of *ERG20* and *BTS1* genes together with a codon-optimized *S. acidocaldarius* GGPP synthase (*SaGGPS*) gene. KanMX and HphNT1 represent antibiotic markers, while URA3, LEU2, and HIS3 represent auxotrophic markers. 2 MICRON represents a high copy number plasmid, while CEN6/ARSH4 represents a low copy number plasmid.

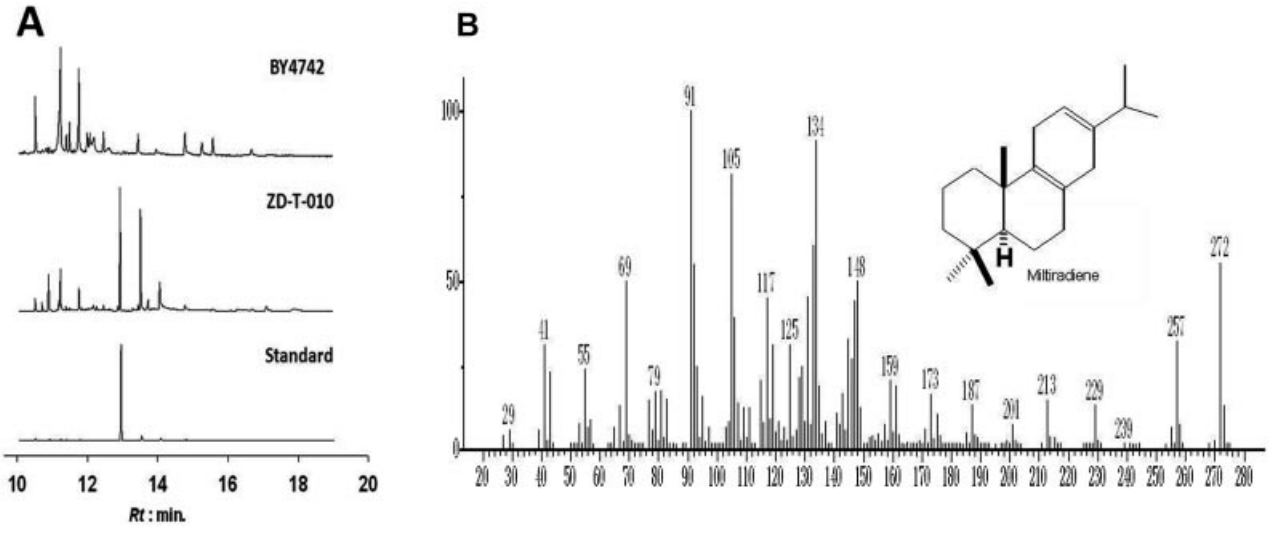

Figure 3. Production of miltiradiene by engineered *S. cerevisiae* strain ZD-T-010. This strain was cultivated in SD medium with 2% glucose for 4 days. **A**: GC-MS analysis of cell extractions of the parent strain BY4742 and ZD-T-010 which had *SmCPS* and *SmKSL* genes integrated in the chromosome. Miltiradiene was used as a standard for comparison. **B**: Mass spectra of miltiradiene.

Improving FPP Supply to Increase Miltiradiene Production

The low quantity of miltiradiene produced by strain ZD-T-010 was assumed to be caused by deficient supply of the isoprenoid precursors. In order to improve supplies of IPP and FPP for increasing miltiradiene production, over-expression of both *tHMGR* and *upc2.1* in episomal plasmids was used. Although high copy number plasmid was usually used for gene over-expression, it might have big metabolic burden on the host cell and be detrimental for maximum productivity in certain metabolic engineering applications (Jones et al., 2000). Thus, we over-expressed the *tHMGR-upc2.1* cassette in ZD-T-010 using both low and high copy number plasmid (Fig. 2B, Table II). The resulting strains, ZD-T-011 and ZD-T012, produced similar amounts of miltiradiene compared to the parent strain ZD-T-010 (Fig. 4). In contrast, large amounts of squalene (71 and 78 mg/L) were produced in these two strains. It was suggested that over-expression of *tHMGR* and *upc2.1* did increase carbon flux toward the MEV pathway and did increase FPP supply. However, conversion of FPP to GGPP seems to be rate-limiting, and the carbon flux was pushed from FPP toward squalene synthesis. The yeast squalene synthase has a K_m value of 2.5 μM for FPP and a turnover number of $0.53\,s^{-1}$ (LoGrasso et al., 1993), whereas the *S. cerevisiae* BTS1 has a K_m value of 3.2 μM for FPP and a turnover number of $0.025\,s^{-1}$ (Chang et al., 2006). The higher K_m value of BTS1 for FPP and lower turnover number suggests that it has lower capacity than squalene synthase, thus leading to accumulation of squalene if carbon flux toward FPP exceeds a certain limit.

Improving GGPP Supply to Increase Miltiradiene Production

Since conversion of FPP to GGPP was rate-limiting for miltiradiene synthesis, supply of GGPP needs to be improved. Co-expression of a fusion of the *BTS1* and *ERG20* genes was utilized since close proximity of ERG20 and BTS1 could speed up FPP turnover in the pathway by reducing the transit time required for FPP to reach BTS1 (Albertsen et al., 2011; Tokuhiro et al., 2009). Over-expressing heterologous GGPP synthase from *S. acidocaldarius* was also utilized since this enzyme can synthesize GGPP through sequential addition of DMAPP to IPP, thus avoiding competition for FPP (Engels et al., 2008; Ohnuma et al., 1994).

Table II. Plasmids used in this study.

Name	Description	Source
Plasmids		
pRS42H	2MICRON, hphNT1	Taxis and Knop (2006)
pRS41K	CEN6/ARSH209, KanMX	Taxis and Knop (2006)
pRS425	2MICRON, LEU2	Sikorski and Hieter (1989)
pRS313	CEN6/ARSH4, HIS3	Sikorski and Hieter (1989)
pRS316	CEN6/ARSH4, URA3	Sikorski and Hieter (1989)
pRS426	2MICRON, URA3	Sikorski and Hieter (1989)
pHUra-δDNA	Containing δ site, URA3	This study
pHUra-δDNA-tHMGR-A	Containing P_{ADH1}-*tHMGR*-T_{ADH1} cassette and δ site, URA3	This study
pHUra-δDNA-tHMGR-T	Containing P_{TEF1}-*tHMGR*-T_{ADH1} cassette and δ site, URA3	This study
pHUra-δDNA-tHMGR-P	Containing P_{PGK1}-*tHMGR*-T_{ADH1} cassette and δ site, URA3	This study
pHUra-δDNA-tHMGR-UPC2.1	Containing P_{ADH1}-*tHMGR*-T_{ADH1} and P_{TEF1}-*UPC2.1*-T_{ADH1} cassettes and δ site, URA3	This study
pHUra-δDNA-SmCPS-SmKSL	Containing P_{TEF1}-SmCPS-T_{ADH1} and P_{PGK1}-SmKSL-T_{ADH1} cassettes and δ site, URA3	This study
pLLeu-tHMGR-UPC2.1	Containing P_{ADH1}-*tHMGR*-T_{ADH1} and P_{TEF1}-*UPC2.1*-T_{ADH1} cassettes, CEN6/ARSH4, LEU2	This study
pHLeu-tHMGR-UPC2.1	Containing P_{ADH1}-*tHMGR*-T_{ADH1} and P_{TEF1}-*UPC2.1*-T_{ADH1} cassettes, 2MICRON, LEU2	This study
pLHis-ERG20/BTS1-SaGGPS	Containing P_{ADH1}-*ERG20/BTS1*-T_{ADH1} and P_{PGK1}-*SaGGPS*-T_{ADH1} cassettes, CEN6/ARSH4, HIS3	This study
pHHis-ERG20/BTS1-SaGGPS	Containing P_{ADH1}-*ERG20/BTS1*-T_{ADH1} and P_{PGK1}-*SaGGPS*-T_{ADH1} cassettes, 2MICRON, HIS3	This study
pLKanMX-tHMGR-UPC2.1	Containing P_{ADH1}-*tHMGR*-T_{ADH1} and P_{TEF1}-*UPC2.1*-T_{ADH1} cassettes, CEN6/ARSH4, KanMX	This study
pHKanMX-tHMGR-UPC2.1	Containing P_{ADH1}-*tHMGR*-T_{ADH1} and P_{TEF1}-*UPC2.1*-T_{ADH1} cassettes, 2MICRON, KanMX	This study
pLhphNT1-ERG20/BTS1-SaGGPS	Containing P_{ADH1}-*ERG20/BTS1*-T_{ADH1} and P_{PGK1}-*SaGGPS*-T_{ADH1} cassettes, CEN6/ARSH4, hphNT1	This study
pHhphNT1-ERG20/BTS1-SaGGPS	Containing P_{ADH1}-*ERG20/BTS1*-T_{ADH1} and P_{PGK1}-*SaGGPS*-T_{ADH1} cassettes, 2MICRON, hphNT1	This study

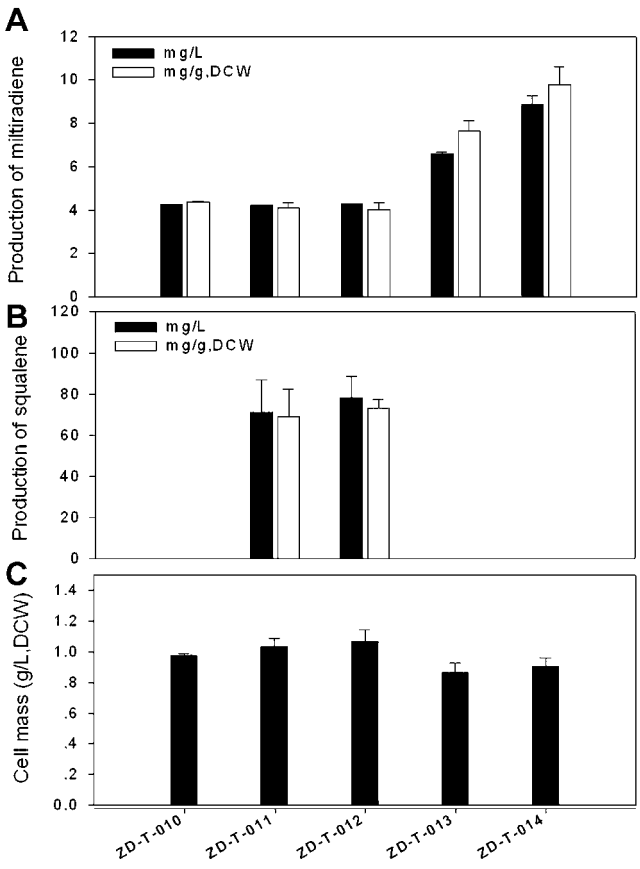

Figure 4. Production of miltiradiene by engineered *S. cerevisiae* strains with *tHMGR-upc2.1* and *ERG20-BTS1-SaGGPS* cassettes over-expressed using episomal plasmids with auxotrophic markers. All strains were cultivated in SD medium lacking leucine and/or histidine with 2% glucose for 4 days. **A**: Production of miltiradiene by strains ZD-T-011 to ZD-T-014; (**B**) accumulation of squalene by strains ZD-T-011 to ZD-T-014; (**C**) cell mass of strains ZD-T-011 to ZD-T-014. Three repeats were performed for each strain, and the error bars represented standard deviation.

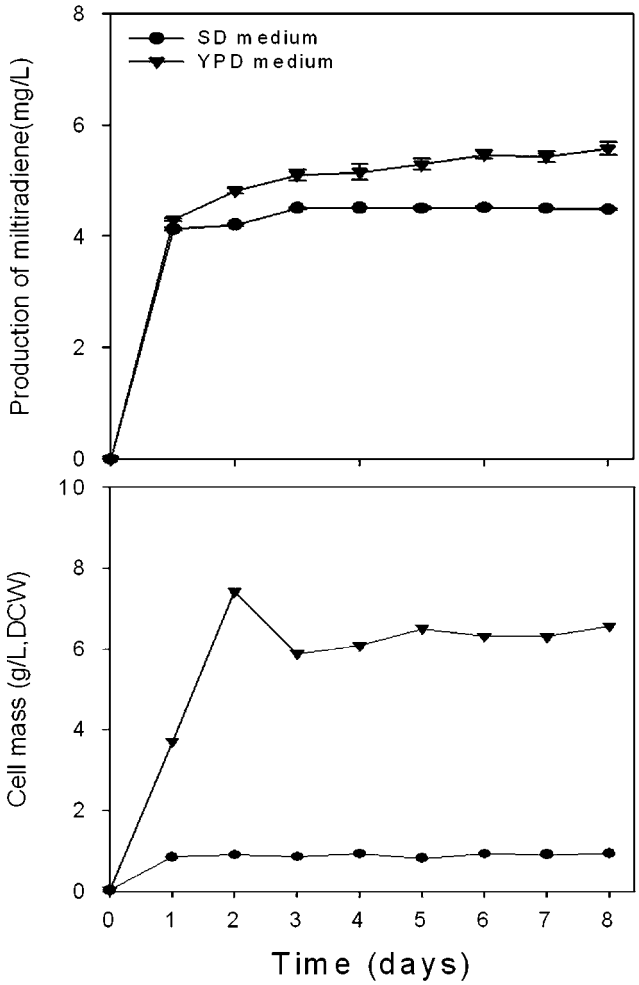

Figure 5. Production of miltiradiene by engineered *S. cerevisiae* strain ZD-T-010. This strain was cultivated in both SD medium with 2% glucose (circle) and YPD medium (triangles) for 8 days. Three repeats were performed for each strain, and the error bars represented standard deviation.

The *ERG20-BTS1-SaGGPS* cassette was over-expressed in ZD-T-010 using either low or high copy number plasmid (Fig. 2C, Table II), resulting in strain ZD-T-013 and ZD-T-014, respectively. ZD-T-013 produced 6.6 mg/L and ZD-T-014 produced 8.8 mg/L miltiradiene, which were 57 and 110% higher than the parent strain ZD-T-010 (Fig. 4). Improving GGPP supply did significantly increase miltiradiene production, and higher miltiradiene concentration using a high copy number plasmid was assumed to be due to higher activity of GGPP synthase.

Replacing Auxotrophic Marker With Antibiotic Marker to Increase Miltiradiene Production

In order to keep stabilities of episomal plasmids containing auxotrophic markers, synthetic medium lacking leucine or histidine was used for cultivation of the engineered yeast strains. However, cell mass was much lower compared to cultivation in rich medium. When cultivating ZD-T-010 in SD medium, cell growth reached the stationary stage within 24 h, and the final cell mass was only 0.94 g/L (Fig. 5). Production of miltiradiene continued to increase until the third day, and the maximum titer was 4.5 mg/L (Fig. 5). In contrast, when cultivating ZD-T-010 in YPD medium, cell growth reached the stationary stage after 2 days, and the final cell mass was 7.4 g/L, which was 7.4-fold higher than in SD medium (Fig. 5). Production of miltiradiene continued to increase, and the maximum titer was 5.6 mg/L (Fig. 5). Although miltiradiene titer increased 24%, the content decreased from 4.4 to 0.7 mg/g dry cell weight.

In order to use rich medium to obtain higher cell mass while keeping stabilities of episomal plasmids, auxotrophic markers (LEU2 and HIS3) in the episomal plasmids over-expressing the *tHMGR-upc2.1* and *ERG20-BTS1-SaGGPS* cassettes were replaced with antibiotic markers

(KanMX and HphNT1) (Table II, Fig. 2). The resulting plasmids were then transformed into strain ZD-T-010 and cultivated in YPD medium for increasing miltiradiene production.

The *tHMGR-upc2.1* cassette was over-expressed in ZD-T-010 using both low and high copy number plasmid (Fig. 2B, Table II), resulting in strain ZD-T-021 and ZD-T-022, respectively. ZD-T-021 produced 6.3 mg/L and ZD-T-022 produced 7.2 mg/L miltiradiene, which were 17 and 33% higher than the parent strain ZD-T-010 (Fig. 6). Large amounts of squalene (446 and 852 mg/L) were also accumulated in these two strains.

The *ERG20-BTS1-SaGGPS* cassette was over-expressed in ZD-T-010 using both low and high copy number plasmid (Fig. 2C, Table II), resulting in strain ZD-T-023 and ZD-T-024, respectively. ZD-T-023 produced 28.2 mg/L and ZD-T-024 produced 25.7 mg/L miltiradiene, which were 5.2-fold and 4.8-fold higher than the parent strain ZD-T-010 (Fig. 6). Over-expressing *tHMGR* and *upc2.1* had little effect on miltiradiene production while over-expressing *ERG20-BTS1* and *SaGGPS* significantly increased miltiradiene production, which was consistent with cultivation in SD medium.

Combinatorial Effects of Improving FPP and GGPP Supply for Increasing Miltiradiene Production

In order to further increase miltiradiene production, the *tHMGR-upc2.1* cassette was over-expressed in strain ZD-T-023 and ZD-T-024. Strains ZD-T-025 and ZD-T-027 were obtained after transforming ZD-T-023 with a low and high copy number plasmid containing the *tHMGR-upc2.1* cassette, respectively. Similar amounts of miltiradiene (41.6 and 43.6 mg/L) were produced in ZD-T-025 and ZD-T-027, which were 48 and 55% higher than the parent strain ZD-T-023 (Fig. 6).

Strains ZD-T-026 and ZD-T-028 were obtained after transforming ZD-T-024 with a low and high copy number plasmid containing the *tHMGR-upc2.1* cassette, respectively. ZD-T-026 produced 61.8 mg/L and ZD-T-028 produced 43.2 mg/L miltiradiene, which were 140 and 68% higher than the parent strain ZD-T-024 (Fig. 6). Miltiradiene production in strain ZD-T026 was 11.4-fold higher than the starting strain ZD-T-010.

It was interesting that squalene was not accumulated in all strains over-expressing both the *tHMGR-upc2.1* and the *ERG20-BTS1-SaGGPS* cassettes (ZD-T-025 to ZD-T-028) (Fig. 6). The amounts of squalene accumulated (446 and 852 mg/L) when over-expressing the *tHMGR-upc2.1* cassette alone were much higher than the increased amounts of miltiradiene produced (35–55 mg/L) after further over-expressing the *ERG20-BTS1-SaGGPS* cassette. It was suggested that carbon flux was pushed away from FPP to GGPP when increasing GGPP synthase activities. However, the catalyzing capacities of the downstream enzymes (CPS and KSL) might not be high enough to convert all the GGPP

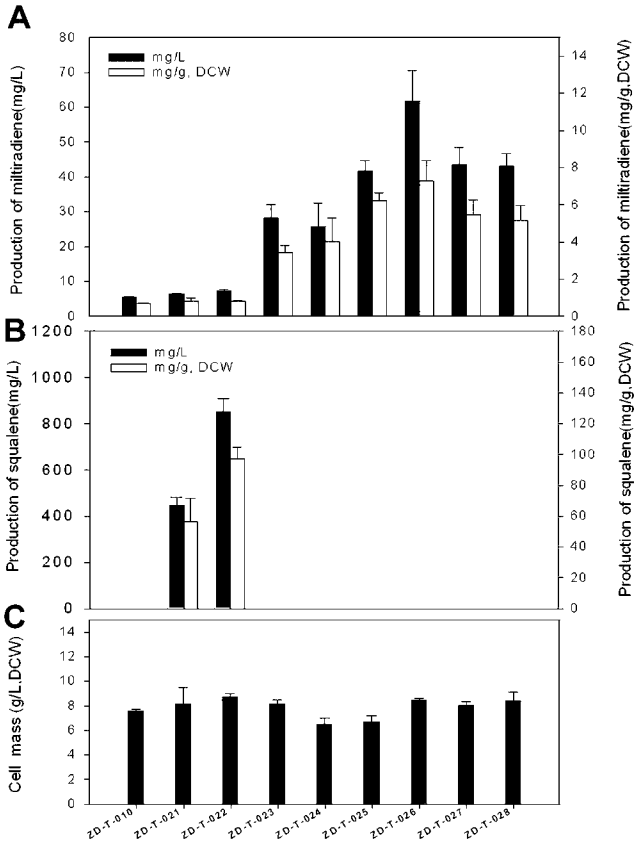

Figure 6. Production of miltiradiene by engineered *S. cerevisiae* strains with *tHMGR-upc2.1* and/or *ERG20-BTS1-SaGGPS* cassettes over-expressed using episomal plasmids with antibiotic markers. All strains were cultivated in YPD medium with geneticin and/or hygromycin for 4 days. **A**: Production of miltiradiene by strains ZD-T-021 to ZD-T-028; (**B**) accumulation of squalene by strains ZD-T-021 to ZD-T-028; (**C**) cell mass of strains ZD-T-021 to ZD-T-028. Three repeats were performed for each strain, and the error bars represented standard deviation.

to miltiradiene. Most of the GGPP was assumed to be converted to geranylgeraniol through endogenous phosphotase (Tokuhiro et al., 2009).

The highest miltiradiene production was obtained in strain ZD-T-026, which over-expressed *tHMGR* and *upc2.1* genes in a low copy number plasmid and over-expressed *ERG20-BTS1* and *SaGGPS* genes in a high copy number plasmid. This was reasonable since conversion of FPP to GGPP and conversion of GGPP to miltiradiene were the most important rate-limiting steps for miltiradiene production. High expression level of *ERG20-BTS1* and *SaGGPS* genes was necessary for providing enough GGPP supply, whereas enough FPP could be supplied for miltiradiene synthesis with a relatively low expression level of *tHMGR* and *upc2.1* genes. In addition, utilization of two high copy number plasmids would have a bigger metabolic burden to the host cell, thus decreasing miltiradiene production (ZD-T-028).

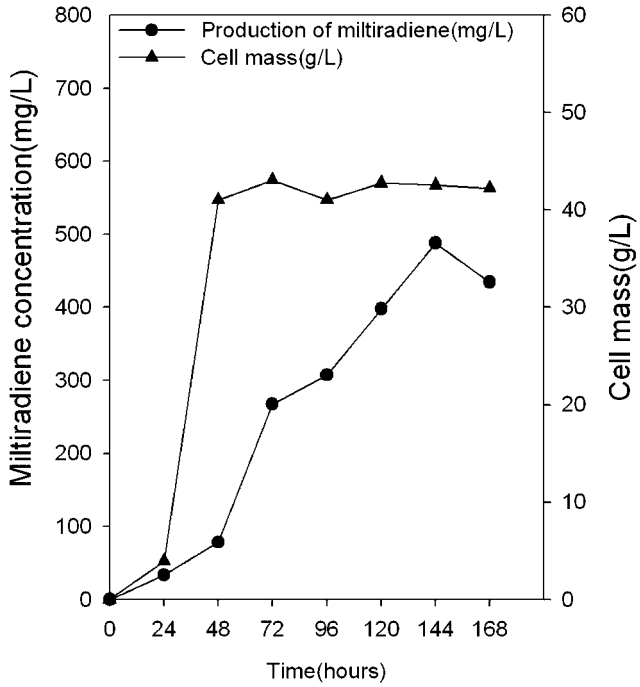

Figure 7. Production of miltiradiene by strain ZD-T-026 through fed-batch fermentation. This strain was cultivated in YPD medium with geneticin and hygromycin for 7 days.

Production of Miltiradiene Through Fed-Batch Fermentation

In order to obtain higher miltiradiene production, fed-batch fermentation of strain ZD-T-026 was performed in a 5 L fermentor with pH controlled at 5.5 (Fig. 7). Cell growth reached stationary stage after 2 days, and the final cell mass was 43 g/L. Production of miltiradiene continued to increase until the sixth day, and the maximum titer was 488 mg/L (Fig. 7). The miltiradiene content was 11.3 mg/g dry cell weight.

In conclusion, we have engineered yeast for efficient synthesis of miltiradiene through integrating *SmCPS* and *SmKSL* genes and providing sufficient supplies of FPP and GGPP precursors. Miltiradiene is the first committed intermediate in the tanshinones synthetic pathway. The yeast strains engineered in this work provided a basis for creating an alternative way for production of tanshinones in place of extraction from plant sources. Although large amount of miltiradiene was produced, antibiotics were added to keep stabilities of the episomal plasmids, which was not feasible for large-scale industrial production. Integration strategies will be used further to over-express essential genes to improve supplies of isoprenoid precursors to obtain genetically stable yeast strains for efficient miltiradiene production.

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