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Original Research Article

Combinatorial metabolic engineering of *Saccharomyces cerevisiae* for improved production of 7-dehydrocholesterol

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ABSTRACT

7-Dehydrocholesterol (7-DHC), a key pharmaceutical intermediate in the production of vitamin D_3, has a wide range of applications. To explore fermentative synthesis of 7-DHC, a 7-DHC-producing *Saccharomyces cerevisiae* strain was constructed by blocking the competitive pathway, eliminating rate-limiting steps, altering global regulation, and pathway compartmentalization. After blocking the competitive pathway by disrupting *ERG5* and *ERG6* and introducing *DHCR24* from *Gallus gallus, S. cerevisiae* produced 139.72 mg/L (17.04 mg/g dry cell weight, hereafter abbreviated as DCW) 7-DHC. Subsequent alteration of global regulation by deleting *ROX1* and overexpressing *UPC2-1* increased 7-DHC production to 217.68 mg/L (37.56 mg/g DCW). To remove the accumulated squalene, the post-squalene pathway was strengthened by co-overexpression of P_{GAL1}-driven *ERG11* and P_{GAL10}-driven *ERG1*, which improved 7-DHC titer and yield to 281.73 mg/L and 46.78 mg/g DCW, respectively, and reduced squalene content by 90.12%. We surmised that the sterol precursors in the plasma membrane and peroxisomes may not be accessible to the pathway enzymes, thus we re-localized DHCR24p and Erg2p-GGGGS-Erg3p to the plasma membrane and peroxisomes, boosting 7-DHC production to 357.53 mg/L (63.12 mg/g DCW). Iron supplementation further increased 7-DHC production to 370.68 mg/L in shake flasks and 1.56 g/L in fed-batch fermentation. This study demonstrates the power of global regulation and subcellular relocalization of key enzymes to improve 7-DHC synthesis in yeast.

1. Introduction

Vitamin D_3 (VD_3), which regulates calcium-phosphorus homeostasis and bone metabolism, is widely used in the healthcare, food, and feed industries to prevent vitamin D_3 deficiency [1]. Currently, VD_3 is primarily produced using 7-dehydrocholsterol (7-DHC) as a key intermediate. The supply of 7-DHC is dependent on the chemical conversion of cholesterol extracted from lanolin [2], raising concerns about the use of toxic organic solvents and generation of various byproducts [3]. To address these issues, fermentative production of 7-DHC using engineered cell factories has attracted increased attention (Table 1).

De novo biosynthesis of 7-DHC has been achieved in *Saccharomyces cerevisiae* by blocking the competitive pathway in the inherent ergosterol pathway and introducing the heterologous Δ24-sterol reductase (*DHCR24*) [5]. In the sterol synthesis pathway in yeast, *ERG2–6* are non-essential genes because zymosterol and its downstream sterols play substitutive roles for ergosterol in ergosterol-deficient yeast [9]. Owing to the low substrate specificity of these enzymes, diverse sterols can be accumulated using different knockout strategies [10]. For example, the cholesta-5,7,24-trienol content generated from zymosterol though Erg2p- and Erg3p-mediated catalysis was enhanced following *ERG6* disruption, making cholesta-5,7,24-trienol the main sterol component in the plasma membrane instead of ergosterol [11]. The effects of disrupting *ERG5* alone or as a double knockout of *ERG5* and *ERG6* on 7-DHC production have been reported [5]. However, the effects of *ERG6* single knockout, *ERG6/ERG4* double knockout, and *ERG4/ERG5/ERG6* triple knockout on 7-DHC production remain to be investigated.

Common strategies employed to enhance 7-DHC yield include overexpression of mevalonate (MVA) pathway genes (*ERG10, IDI*, and *tHMG1*) to improve squalene supply and promote the expression of key enzymes in the post-squalene pathway (encoded by *ERG1, ERG11*, and *DHCR24*) to accelerate the conversion of squalene into sterols [7,8]. With one or a few genes as the engineering targets, expression levels should be fine-tuned to avoid severe disturbances in the metabolic balance. Manipulation of the transcriptional factors that regulate the sterol synthetic pathway is an alternative strategy to strengthen metabolic flux. For example, inactivation of the repressors Rox1p and Mot3p boosts the transcription of genes involved in the sterol synthesis pathway and enhances 7-DHC production [8,12], whereas overexpression of the sterol regulatory element (SRE)-binding proteins Upc2p and Ecm22p

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Table 1
Engineering strategies for *de novo* biosynthesis of 7-DHC in *Saccharomyces cerevisiae*.

Strain	Strategies	Engineering targets			Cultivation mode	Yield	References
		Genes deleted	Genes introduced	Genes overexpressed			
S. cerevisiae (BY4742)	Alleviating redox imbalance, strengthening the supply of acetyl-CoA	*ERG5*	*DHCR24, ACS, ACL*	*tHMG1, ADH2, ALD6*	5 L Bioreactor	44.490 ± 9.630 mg/L	[4]
S. cerevisiae (CENPK2-1D)	Screening *DHCR24* sources, enhancing the transcriptional level of *DHCR24*, engineering lipid metabolism genes	*ERG5, ERG6, NEM1*	*Gg_DHCR24*	All the MVA pathway genes, *Gg_DHCR24*	5 L Bioreactor	1.070 g/L	[5]
S. cerevisiae (CENPK2-1D)	Compartmentalized reconstitution of post-squalene pathway	*ERG5, ERG6*	*Gg_DHCR24*	All the MVA pathway genes, *DHCR24*, LD-targeted *ERG2-3-DHCR24*, ER-targeted *ERG1-11-24*, LD-targeted *ERG25-26-27*	Shake-flask	360.600 mg/L	[6]
S. cerevisiae (CEN.PK2-1C)	*In silico* prediction and validation of gene knockout targets by GSMM, enhancing the expression level of *DHCR24* through yeast transposon, redistribution of metabolic flux using CRISPRi system	*ROX1, GDH1*	*DHCR24*, P_{GAL}-dcas9-4 20 nt site (*ERG6* promoter)	*tHMG1, IDI1, ERG20, INO2, ERG1, ERG11*	3 L Bioreactor	1.328 g/L	[7]
S. cerevisiae (CEN.PK2-1C)	Upregulation/ downregulation of the expression of key genes using the CRISPR/dCpf1-mediated or CRISPR/ dCas9-mediated transcriptional activation system, harnessing yeast ER for compartmentalization of the 7- DHC pathway	-	*Gg_DHCR24*, P_{TEF1}-*dCpf1-VP*-crRNA$_{GAL1, GAL7}$, P_{TEF1}-*dCas9-TUP1-MIG1-UME6*-sgRNA$_{erg6,mls1,cit2}$	*tHMG1, IDI1, ERG7, INO2, ERG1, ERG11, ADH2, UPC2-1, ERG24, ERG25, ERG26, ERG27, ERG20, MVD1, ERG8, ERG12*	5 L bioreactor	2.870 g/L	[3]
S. cerevisiae (CENPK2-1D)	Engineering ER-related genes, multi-copy gene integration	*ERG5, ERG6, MOT3, NEM1*	*Gg_DHCR24*	*tHMG1, IDI1, ERG2, ERG3, DHCR24, POS5, CTT1*	5 L bioreactor	2.000 g/L	[8]

improves the production of phytosterol and ergosterol, respectively [13,14].

Most enzymes in the post-squalene pathway, including DHCR24p, are located in the endoplasmic reticulum (ER), whereas sterol intermediates are transported to different organelles, such as lipid droplets (LDs) and peroxisomes [3,15], which may hamper substrate accessibility. Therefore, compartmentalized reconstitution of the 7-DHC synthetic pathway is a promising strategy to improve 7-DHC yield. Relocating ER-localized enzymes to the LDs where esterified sterols are stored boosted 7-DHC yield in engineered *S. cerevisiae* by 64.20% [9]. ER-derived peroxisomes play a role similar to that of LDs in maintaining lipid homeostasis [16,17], thus they may be another subcellular compartment to target for rate-limiting enzymes, which may improve 7-DHC yield. In contrast, free sterols, such as zymosterol, cholesta-7,24-dienol, and cholesta-5,7,24-trienol, are mainly transported to the plasma membrane (PM) (up to 90%), whereas only approximately 5% remains in the ER [18]. Therefore, re-localization of post-squalene pathway enzymes to the PM, with its rich stock of sterol intermediates, is a prospective strategy for promoting 7-DHC synthesis.

In this study, combinatorial metabolic engineering of *S. cerevisiae*, integrating competition elimination, transcriptional regulation, and pathway compartmentation, was conducted to obtain a 7-DHC high-producing yeast strain (Fig. 1).

2. Materials and methods

2.1. Strains, plasmids, and genes

Engineered strains were generated from *S. cerevisiae* YXWP113 (BY4741, *MATα*, *HIS3*Δ1, *LEU2*Δ0, *MET15*Δ0, *URA3*Δ0, *GAL80*::*LEU2*) by episomal plasmid expression and genome integration employing pUMRI plasmids or CRISPR/Cas9. The LiAc/SS carrier DNA/PEG method was used for DNA transformation of *S. cerevisiae* [19]. The yeast strains used in this study are listed in Table 2.

The *DHCR24* (Sterol Δ^{24}-dehydrocholesterol reductase) gene from *Gallus gallus* [5] was synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). The *UPC2-1* gene encodes the yeast transcription factor UPC2 with the G888D mutation [21]. Plasmid construction and propagation were conducted using *Escherichia coli* Top10 cells. The fragments were cloned into plasmids using Gibson assembly, restriction digestion, and ligation. Multi-gene editing plasmids required for CRISPR/Cas9 manipulation were constructed using the Golden Gate method with sgRNA designed using Editor in Benchling (https://benchling.com/editor). EGFP was fused to the C-terminus of DHCR24p with a GGGS linker using overlap extension PCR, and the DHCR24-GGGS-EGFP fragment was ligated to the high-copy number plasmid pESC via restriction digestion and ligation. The primers used in this study are listed in Table S1. All plasmids, including the yeast episomal and integration plasmids constructed in this study, are shown in Table S2.

2.2. Media, culture conditions, and reagents

E. coli Top10 cells were cultured in Luria-Bertani (LB) medium containing antibiotics (100 g/mL ampicillin or 50 μg/mL kanamycin) at 37 °C for plasmid amplification. Yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% D-glucose) supplemented with G418 (200 μg/mL), if necessary, was used for routine culture of *S. cerevisiae* strains. Engineered *S. cerevisiae* cells with auxotroph

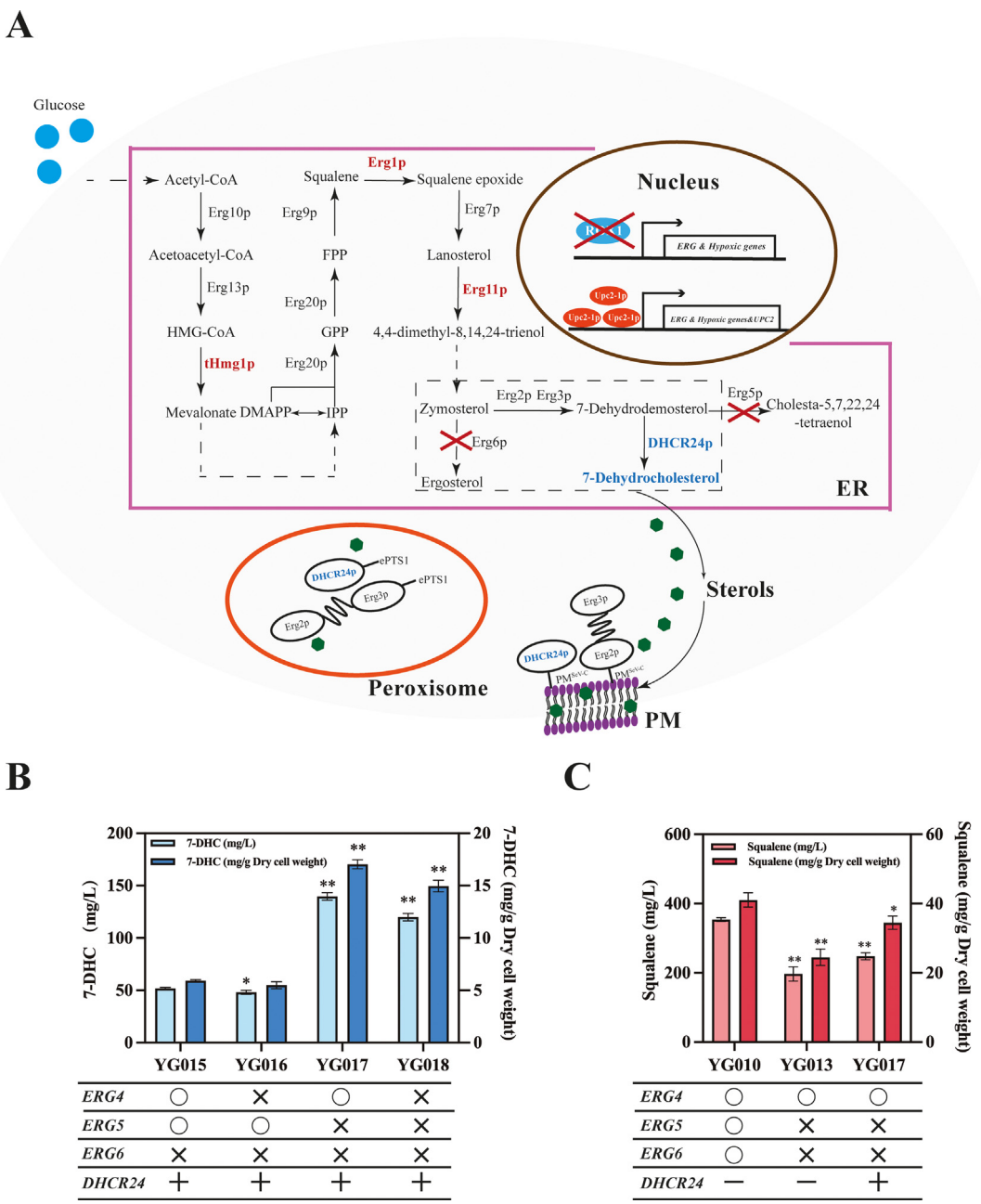

Fig. 1. 7-DHC synthesis in *S. cerevisiae* enabled by pathway assembly and competition elimination. (A) Overview of the 7-DHC synthetic pathway and the metabolic engineering strategies used in this study. Heterologous enzymes and products are labeled in blue, and overexpressed endogenous enzymes are shown in red. Knocked out genes are indicated by red crosses. (B) Effect of blocking different competitive pathways on 7-DHC production. (C) Effect of disrupting the competitive pathways on squalene content. In B and C, cells were cultured in YPD medium for 72 h. ○ represents the control condition of the gene without manipulation, × indicates a gene knockout, and +/- indicates the presence/absence of the heterologous gene, respectively. The data presented are the means of three biological replicates. Error bars represent standard deviations (*, $P < 0.05$ and **, $P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

markers were selected and cultivated in synthetic drop-out (SD) medium lacking corresponding amino acids at 30 °C.

For shake-flask fermentation, single colonies of recombinant yeast strains were picked from a plate and cultivated for 20 h in SD medium or for 16 h in YPD medium at 30 °C and 220 rpm. These precultures were then inoculated into 50 mL SD or YPD medium to an initial OD_{600} of 0.05 and cultured at 30 °C and 220 rpm for 72 h.

Restriction enzymes were purchased from Takara (China). *BsaI*-HFv2 and its paired T4 DNA ligase were purchased from New England BioLabs (China). Primers were synthesized by Tsingke Biotechnology (China). Standard references for 7-DHC and squalene were purchased from Sigma-Aldrich (China).

2.3. Visualization of EGFP-GGGS-DHCR24p fused to anchors

EGFP-GGGS-DHCR24 was fused to anchors using circular polymerase extension cloning. Localization of the fusion proteins DHCR24p-EGFP, $PM^{SeV\text{-}C}$-DHCR24p-EGFP, and DHCR24p-EGFP-ePTS1 in yeast was visualized using confocal laser scanning microscopy. After cells containing episomal plasmids were cultured on SD plates for 48 h, single

Table 2
Strains used in this study.

Strain	Description	Plasmid	Reference
YXWP113	BY4741, Δ*GAL8*::*LEU2*		[20]
YG010	YXWP113, Δ*DPP1*::P_{GAL1}-*tHMG1*-T_{CYC1}		This study
YG011	YG010, Δ*ERG6*		This study
YG012	YG010, Δ*ERG4*, Δ*ERG6*		This study
YG013	YG010, Δ*ERG5*, Δ*ERG6*		This study
YG014	YG010, Δ*ERG4*, Δ*ERG5*, Δ*ERG6*		This study
YG015	YG010, Δ*ERG6*::P_{GAL1}-*DHCR24*-T_{CYC1}		This study
YG016	YG010, Δ*ERG4*, Δ*ERG6*::P_{GAL1}-*DHCR24*-T_{CYC1}		This study
YG017	YG010, Δ*ERG5*, Δ*ERG6*::P_{GAL1}-*DHCR24*-T_{CYC1}		This study
YG018	YG010, Δ*ERG4*, Δ*ERG5*, Δ*ERG6*::P_{GAL1}-*DHCR24*-T_{CYC1}		This study
BY4741-C-04	BY4741, Δ*LPP1*::T_{CYC1}-*ERG10*-P_{GAL1}-P_{GAL10}-*HMGS*-T_{ADH1}; Δ*HO*::T_{TPS1}-*tHMG1*-P_{GAL7}-P_{GAL2}-*ERG12*-T_{PGK1};Δ*DPP1*::T_{CYC1}-*tHMG1*-P_{GAL1}-P_{GAL10}-*PMK*-T_{ADH1}; Δ*GAL80*::T_{TPS1}-*MVD1*-P_{GAL7}-P_{GAL2}-*IDI1*-T_{PGK1}		This study
YG019	BY4741-C-04, Δ*ERG5*, Δ*ERG6*::P_{GAL1}-*DHCR24*-T_{CYC1}		This study
YG021	YG017, Δ*ROX1*		This study
YG022	YG017, Δ*ROX1*::P_{GAL1}-*UPC2-1*-T_{CYC1}		This study
YG023	YG017, Δ*MOT3*		This study
YG024	YG017, Δ*MOT3*::P_{GAL1}-*ECM22*-T_{CYC1}		This study
YG025	YG017, Δ*ROX1*::P_{GAL1}-*UPC2*-T_{CYC1}		This study
YG030	YG022, Δ*LPP1*		This study
YG031	YG022, Δ*LPP1*::P_{GAL1}-*ERG1*-T_{CYC1}		This study
YG032	YG022, Δ*LPP1*::P_{GAL1}-*ERG11*-T_{CYC1}		This study
YG033	YG022, Δ*LPP1*::T_{ADH1}-*ERG11*-P_{GAL10}-P_{GAL1}-*ERG1*-T_{CYC1}		This study
YG034	YG022, Δ*LPP1*::T_{ADH1}-*ERG1*-P_{GAL10}-P_{GAL1}-*ERG11*-T_{CYC1}		This study
YG035	YG022, Δ*LPP1*::P_{GAL1}-*ERG2*-T_{CYC1}		This study
YG036	YG022, Δ*LPP1*::P_{GAL1}-*ERG3*-T_{CYC1}		This study
YG037	YG022, Δ*LPP1*::T_{ADH1}-*ERG3*-P_{GAL10}-P_{GAL1}-*ERG2*-T_{CYC1}		This study
YG038	YG022, Δ*LPP1*::T_{ADH1}-*ERG2*-P_{GAL10}-P_{GAL1}-*ERG3*-T_{CYC1}		This study
YG040	YG022, Δ*HO*		This study
YG041	YG022, Δ*HO*::P_{GAL1}-*DHCR24*-T_{CYC1}		This study
YG042	YG022, Δ*HO*::P_{GAL10}-*ERG2-GGGGS-ERG3*-T_{ADH1}		This study
YG043	YG022, Δ*HO*::P_{GAL1}-$PM^{SeV\text{-}C}$ -*DHCR24*-T_{CYC1}		This study
YG044	YG022, Δ*HO*::P_{GAL10}-$PM^{SeV\text{-}C}$-*ERG2-GGGGS-ERG3*-T_{ADH1}		This study
YG045	YG022, Δ*HO*::T_{ADH1}-$PM^{SeV\text{-}C}$-*ERG2-GGGGS-ERG3*-P_{GAL10}-P_{GAL1}-$PM^{SeV\text{-}C}$-*DHCR24*-T_{CYC1}		This study
YG050	YG022, Δ*Ty4*		This study
YG051	YG022, Δ*Ty4*::P_{GAL1}-*DHCR24*-ePTS1-T_{CYC1}		This study
YG052	YG022, Δ*Ty4*::P_{GAL10}-*ERG2-GGGGS-ERG3*-ePTS1-T_{ADH1}		This study
YG053	YG022, Δ*Ty4*::T_{ADH1}-*ERG2-GGGGS-ERG3*-ePTS1-P_{GAL10}-P_{GAL1}- *DHCR24-ePTS1*-T_{CYC1}		This study
YG060	YG034, Δ*HO*::T_{ADH1}-$PM^{SeV\text{-}C}$-*ERG2-GGGGS-ERG3*-P_{GAL10}-P_{GAL1}-$PM^{SeV\text{-}C}$-*DHCR24*-T_{CYC1}		This study
YG061	YG060, Δ*Ty4*::T_{ADH1}-*ERG2-GGGGS-ERG3*-ePTS1-P_{GAL10}-P_{GAL1}- *DHCR24-ePTS1*-T_{CYC1}		This study
YG022-A	YG022	pESC-URA-EGFP	This study
YG022-B	YG022	pESC-URA-DHCR24-EGFP	This study
YG022-C	YG022	pESC-URA-PM-DHCR24-EGFP	This study
YG022-D	YG022	pESC-URA-DHCR24-EGFP-ePTS1	This study
YG061(+)	YG061, Δ*his3*::*HIS3*, Δ*ura3*::*URA3*, Δ*met15*::*MET15*		This study

colonies were picked and washed twice with phosphate buffer (PBS). The cells were resuspended in PBS and observed under a Leica TCS SP5 inverted confocal microscope (LEICA, Germany).

2.4. Analytical methods

A 1 mL aliquot of culture was harvested by centrifugation at 12,000 rpm for 2 min, and the cell pellets were washed twice and re-suspended in ddH_2O, followed by disruption in an automatic sample grinding machine (Tissuelyser-24; Shanghai, China) at 65 Hz for 5 min. After centrifugation and removal of the supernatant, the pellets were resuspended in 1 mL of 1.5 M KOH-methanol solution and incubated at 80 °C for 90 min. Then, 500 μL n-hexane was added twice to extract sterols from the saponification liquid with vortexing. The n-hexane phase was collected via centrifugation and dried using a vacuum evaporator. The dry residue was solubilized in 300 μL ethanol and filtered for HPLC analysis. The 7-DHC content was determined using an HPLC system (SHIMADZU LC-20 AT) equipped with a Hypersil GOLD C18 column (4.6 × 250 mm, 5 μm) and a UV/VIS detector. The mobile phase was 100% methanol at a flow rate of 1.0 mL/min. The column temperature was 40 °C, and the signals were detected at 280 nm [8].

Squalene was extracted according to the procedure described by W. Xie et al. [20] with some modifications. The cells were resuspended in acetone and disrupted in a Tissuelyser-24 (Shanghai, China) at 65 Hz for 5 min. Squalene was also quantified using an HPLC (LC 20AT; SHIMADZU) equipped with an Amethyst C18-H column (4.6 mm × 150 mm, 5 μm), as previously described [20].

During fermentation, the glucose and ethanol concentrations were measured as previously described [22,23].

2.5. Fed-batch fermentation

A single colony was selected from a YPD plate, inoculated into 5 mL YPD medium, and incubated for 12 h as the seed preculture. This was then inoculated into 100 mL YPD medium and cultured for 24 h. Two seed cultures were inoculated into a 5 L bioreactor (Shanghai Baoxing Bioengineering Equipment, China) containing 1.8 L of YPD medium. During fermentation, the pH was maintained at 5.5 by automatic addition of 60% ammonia hydroxide, dissolved oxygen was maintained at approximately 25% by manually adjusting the air flow rate (1–4.0 vvm), the agitation speed was 300–600 rpm, and the temperature was maintained at 30 °C. The glucose concentration was maintained below 2 g/L

by feeding 500 g/L glucose. After 72 h, glucose feeding was discontinued, and 50% ethanol (v/v) was used as the carbon source. The ethanol concentration was maintained at approximately 5 g/L by regulating the feeding rate.

2.6. Statistical analysis

Data are presented as the arithmetic mean ± standard deviation (SD). Unpaired two-sided t-tests were employed to compare the means of two groups, while one-way analysis of variance (ANOVA) was used for comparing multiple groups with a control group. $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Construction of 7-DHC-producing S. cerevisiae

The 7-DHC biosynthetic pathway was constructed in *S. cerevisiae* by extending the endogenous ergosterol synthesis pathway through the addition of the sterol Δ24-dehydrocholesterol reductase from *Gallus gallus* (encoded by *DHCR24*). To decouple cell growth from 7-DHC synthesis, *GAL* promoters were used to regulate the expression of pathway genes in strain YXWP113 [20] in a *GAL80* deletion background to eliminate the dependence of the *GAL* system on galactose induction. To enhance the supply of MVA pathway-derived squalene, a vital precursor for 7-DHC synthesis, truncated *HMGR* (encoded by *tHMG1*), which is a rate-limiting enzyme, was overexpressed in YXWP113. The resulting strain, YG010, accumulated 354.00 mg/L squalene (Fig. S1). In contrast, no squalene was detected in the parent strain YXWP113. In *S. cerevisiae*, squalene is naturally metabolized to sterols via the ergosterol biosynthesis pathway. Therefore, blocking ergosterol biosynthesis is essential to produce 7-DHC. In the ergosterol biosynthesis pathway, *ERG2–6* are non-essential genes, and their deletion did not significantly impair cell growth (Fig. S2). Integration of the *DHCR24* gene at the *ERG6* locus in the YG010 genome (strain YG015) resulted in production of 51.70 mg/L 7-DHC in shake flasks within 72 h (Fig. 1B). Deletion of *ERG6* blocked the conversion of zymosterol to fecosterol, leading to the accumulation of cholesta-5,7,24-trienol, the direct precursor of 7-DHC. However, because of the broad substrate specificity of Erg5p, cholesta-5,7,24-trienol can also be converted to cholesta-5,7,22,24-tetraenol [10]. Thus, *ERG5* was disrupted in YG015 to construct strain YG017, which improved 7-DHC production by 170.25% to 139.72 mg/L (Fig. 1B). In addition to blocking the competitive pathway, ergosterol deficiency resulting from the deletion of *ERG5* and *ERG6* also relieved the suppressive effect of ergosterol on Upc2p, which might strengthen the target pathway [24], especially the post-squalene pathway, as inferred from decreased squalene accumulation (Fig. 1C). The biomass of YG017 was slightly lower than that of YG010 (8.20 g/L vs. 8.63 g/L) (Fig. S3). Knockout of *ERG4* in strains YG015 and YG017 (generating YG016 and YG018, respectively) did not augment 7-DHC synthesis, indicating that the sterol C24 (28) reductase encoded by *ERG4* was not functional in strains with *ERG6* deletion, as they failed to generate the proper substrate with the C28 methyl group (Fig. 1B). Based on comprehensive considerations of both 7-DHC production and strain biomass, YG017, with the *ERG5/ERG6* double knockout, was used in subsequent experiments.

To boost 7-DHC yield, it is necessary to strengthen metabolic flux of the 7-DHC synthesis pathway. Since squalene often accumulates as a key endogenous intermediate, the 7-DHC biosynthesis pathway can be divided into pre- and post-squalene synthesis pathway modules. In the pre-squalene module, flux relies largely on the MVA pathway, with 3-hydroxy-3-methylglutaryl-CoA reduction as the main rate-limiting step, which can be ameliorated by overexpressing *tHMG1* (encoding truncated HMGR). Overexpression of other genes in the MVA pathway also improves squalene content and promotes 7-DHC production [5]. However, the growth of YG019 cells overexpressing all the MVA pathway genes was severely impaired, possibly because of the metabolic burden caused by excessive protein expression. The biomass of YG019 was 46.64% lower than that of YG017 overexpressing only tHmg1p, and 7-DHC production was also much lower (41.04 mg/L vs. 139.72 mg/L) (Fig. S4).

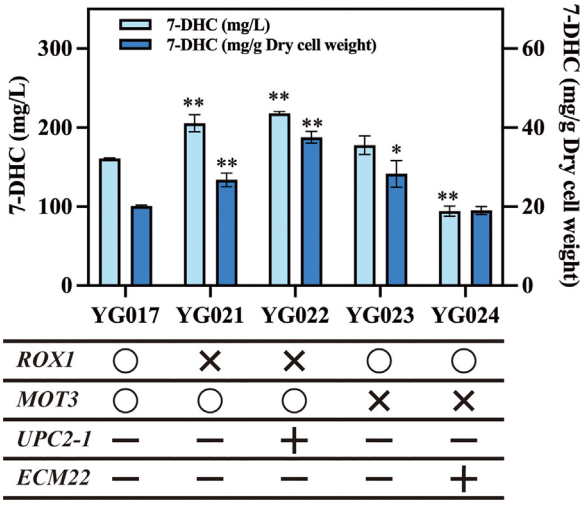

Fig. 2. Effect of manipulating transcriptional regulators on 7-DHC production. ○ indicates that the gene is present, as in the parent strain, × indicates a gene knockout, + indicates introduction of the gene, - indicates that the gene is not present. The data presented are the means of three biological replicates. Error bars represent standard deviations (*, $P < 0.05$ and **, $P < 0.01$).

3.2. Enhancing 7-DHC synthesis by manipulating transcriptional factors

As an alternative to overexpression of the entire MVA pathway, global regulation via manipulation of transcription factors is a promising strategy to enhance metabolic flux of the pre-squalene pathway module to supply sufficient precursors for the synthesis of downstream sterols. Rox1p, Mot3p, Ecm22p, and Upc2p regulate the ergosterol synthesis pathway in *S. cerevisiae* in response to sterol deficiency or low oxygen. Under ergosterol deficiency, Ecm22p-mediated activation of *ERG* genes is suppressed by direct interaction of the repressor Mot3p with Ecm22p, while the amount of Upc2p and its binding to the *ERG* gene promoters are highly increased, which contributes to enhanced metabolic flux of sterol synthesis [24]. For instance [12], increased mevalonate levels in the pre-squalene pathway by deleting the *ROX1* gene. To explore the effect of these transcription factors on 7-DHC biosynthesis, we followed the principle of knocking out repressors and overexpressing activators and disrupted the *ROX1* gene; integrated the *UPC2-1* gene, which encodes the G888D mutant of Upc2p and allows cells to take up sterols aerobically [21], into the *ROX1* site; disrupted the *MOT3* gene; and integrated the *ECM22* gene into the *MOT3* site of strain YG017 to generate YG021, YG022, YG023, and YG024, respectively. In strains YG021, YG022, and YG023, 7-DHC production was improved (Fig. 2). Strain YG022, generated by deletion of *ROX1* and overexpression of Upc2-1p, exhibited the highest 7-DHC titer of 217.68 mg/L (37.56 mg/g DCW), which was 55.80% higher than that of strain YG017. The *ROX1* knockout increased sterol synthesis because Rox1p negatively regulates genes encoding plasma membrane transporters and genes involved in sterol biosynthesis and turnover [25]. A previous study showed that *ROX1* deletion in *S. cerevisiae* increased 7-DHC production by enhancing the expression of *ERG9, ERG1, ERG11, ERG25*, and *ERG3* [26], whereas overexpression of *UPC2-1* increased the expression of *ERG13, ERG12*, and *ERG8* in the pre-squalene pathway [27]. However, overexpression of Ecm22p led to a 46.93% decrease in 7-DHC production (Fig. 2). We speculated that increasing the amount of Ecm22p might interfere with the function of Upc2p in strains unable to synthesize ergosterol because

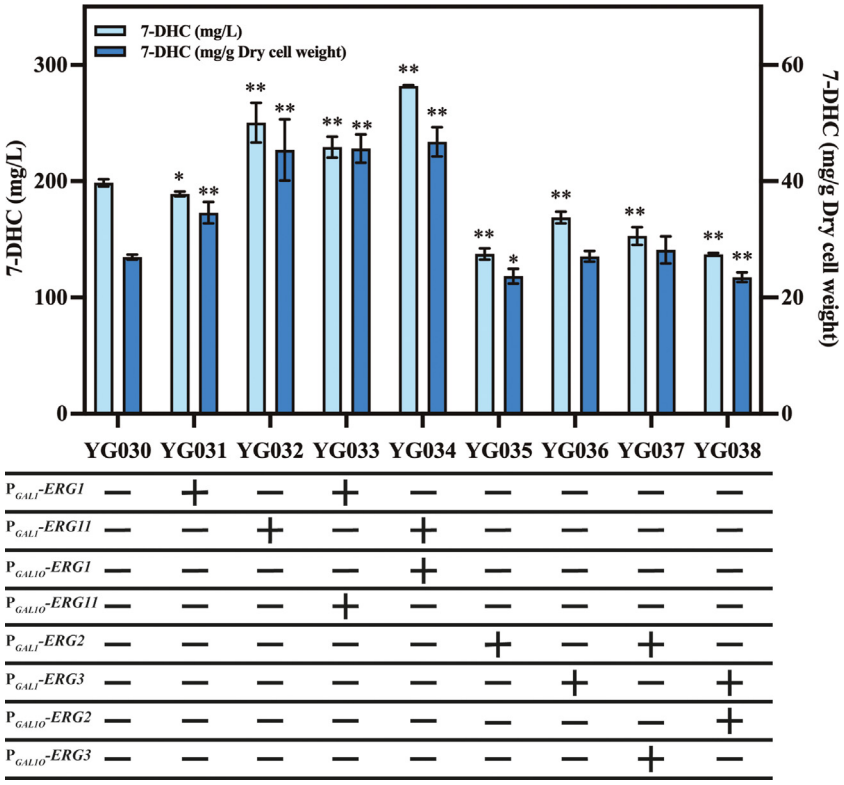

Fig. 3. Effect of regulating the expression of key enzymes in the post-squalene pathway module on 7-DHC production. + indicates that the gene was introduced, - indicates that the gene is not present. The data presented are the means of three biological replicates. Error bars represent standard deviations (*, $P < 0.05$ and **, $P < 0.01$).

Upc2p plays a role in activating *ERG* gene expression in ergosterol-deficient strains, whereas under sterol-rich, aerobic conditions Ecm22p is responsible for maintaining basal expression of *ERG* genes [28–30].

3.3. Overexpression of rate-limiting enzymes in the post-squalene pathway

In strain YG022, which had the highest 7-DHC production, the squalene content increased by 91.04%, reaching 475.94 mg/L (Fig. S5). This implies that enhancing the conversion of squalene to downstream sterols is crucial to further improve 7-DHC production. *ERG1, ERG11, ERG2*, and *ERG3* are key genes in the post-squalene pathway for sterol synthesis [9]. Overexpression of Erg1p promoted the transformation of squalene, leading to a significant increase in lanosterol and a slight elevation in later sterols, from zymosterol to ergosterol, whereas overexpression of Erg11p increased the conversion of lanosterol to downstream sterols [31]. Therefore, *ERG11* was introduced into the *LPP1* locus of YG022, generating strain YG032; the strain produced 250.16 mg/L 7-DHC, which was 26.04% higher than that produced by YG030. In contrast, overexpression of Erg1p alone did not increase 7-DHC production, which may have been caused by the limited substrate supply due to the rate-limiting upstream enzyme. Introduction of both P_{GAL1}-driven *ERG11* and P_{GAL10}-driven *ERG1* into the *LPP1* locus (in strain YG034) led to the highest 7-DHC yield of 281.73 mg/L (Fig. 3). These changes also dramatically decreased the squalene content, from 521.26 mg/L in YG030 to 51.81 mg/L in YG034 (Fig. S6). However, overexpression of Erg2p, Erg3p, or Erg2p and Erg3p had a negative effect on 7-DHC production (Fig. 3).

3.4. Re-localization of key post-squalene pathway enzymes

Our finding that overexpression of ER-localized Erg2p and Erg3p failed to improve 7-DHC production contrasts with a previous observation that overexpression of LD-targeted *ERG2/3* and *DHCR24* improved 7-DHC production [6], suggesting the importance of the subcellular localization of these enzymes. The failure of Erg2p and Erg3p overexpression to enhance 7-DHC synthesis may have been caused by the distance between the enzymes and their substrates. Similar to LDs, peroxisomes are generated in the ER, which is the main site of sterol storage. Therefore, relocalization of the pathway to peroxisomes might have an effect similar to that of LD relocalization. To verify whether free sterols were used for 7-DHC synthesis in peroxisomes, DHCR24p and Erg2p-GGGS-Erg3p were re-localized to peroxisomes using enhanced C-terminal peroxisome targeting signal type 1 (ePTS1). As expected, 7-DHC biosynthesis increased. Co-localization of Erg2p, Erg3p, and DHCR24p to peroxisomes led to the highest 7-DHC production of 275.28 mg/L, which was 17.67% higher than that of the control (Fig. 4B).

In *S. cerevisiae*, excess free sterols are esterified in the ER, and steryl esters (SE) are hydrolyzed in the PM and LDs, where three ester hydrolases (Yeh1p, Yeh2p, and Tgl1p) are located, implying a rich stock of free sterols. Moreover, Yeh2p, which is located in the PM, has a higher hydrolase activity than those of Yeh1p and Tgl1p located in LDs [18]. In Δ*ERG6* strains, zymosterol, cholesta-7,24-dienol, and cholesta-5,7,24-trienol, which are the direct substrates of Erg2p, Erg3p, and DHCR24p, respectively, were the main sterol components in the PM, accounting for 67% of the total sterols in the PM [11]. Therefore, relocalization of Erg2p, Erg3p, and DHCR24p to the PM may enhance 7-DHC production by making better use of PM-stored sterol precursors. Recently, a PM anchor peptide called $PM^{SeV\text{-}C}$ was reported in *S. cerevisiae*, and its application enhanced the production of lutein and canthaxanthin by shortening the distance between the rate-limiting enzyme and its substrate stored in the PM [22,32]. We fused $PM^{SeV\text{-}C}$ to the N-termini of DHCR24p and Erg2p-GGGS-Erg3p. As expected, we observed a positive effect on 7-DHC accumulation (Fig. 4A). The highest 7-DHC production (27.73% higher than that of the control) was achieved when Erg2p, Erg3p, and DHCR24p were all re-localized to the PM. When combined with overexpression of ER-localized Erg1p and Erg11p (in strain YG060), 7-DHC production reached 337.39 mg/L (Fig. 4C). Enhanced green fluorescent protein (EGFP) was used as a reporter to examine the subcellular localization of the anchor-fusion enzymes. As shown in Fig 4D, freely ex-

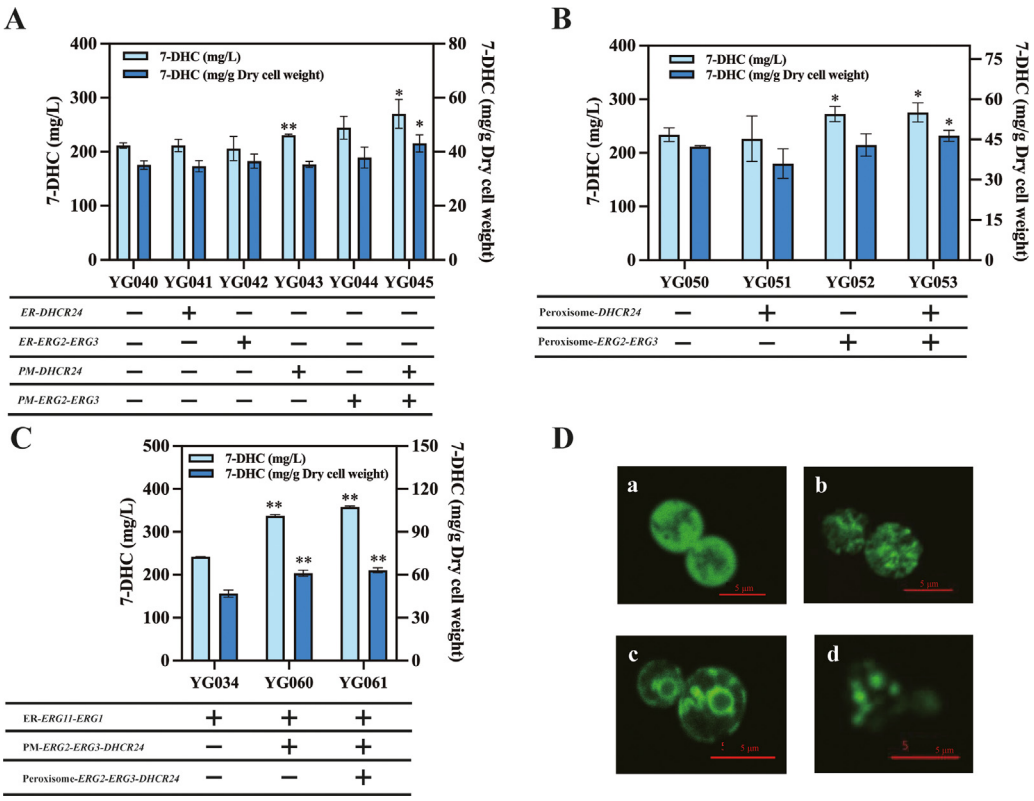

Fig. 4. Effect of re-localization of key enzymes on 7-DHC production. (A) Effect of re-localization of Erg2p, Erg3p, and DHCR24p to the plasma membrane (PM) on 7-DHC production. (B) Effect of re-localization of Erg2p, Erg3p, and DHCR24p to peroxisomes on 7-DHC production. (C) Effect of re-localization of Erg2p, Erg3p, and DHCR24p to the PM and peroxisomes on 7-DHC production. (D) EGFP-reported subcellular localization of enzymes fused to different anchors pESC-URA-EGFP (a), pESC-URA-DHCR24-EGFP (b), pESC-URA-PM-DHCR24-EGFP (c), and pESC-URA-DHCR24-EGFP-ePTS1 (d). + indicates introduction of a gene, - indicates that the gene was not introduced. The data presented are the means of three biological replicates. Error bars represent standard deviations (*, $P < 0.05$ and **, $P < 0.01$).

pressed DHCR24p was located in the cytoplasm, as previously reported [6], whereas fusion to $PM^{SeV\text{-}C}$ or ePTS1 re-localized the enzymes to the PM or peroxisomes, respectively (Fig. 4D).

Finally, re-localization of DHCR24p and Erg2p-GGGS-Erg3p to the PM and peroxisomes was combined with overexpression of ER-localized Erg1p and Erg11p to generate strain YG061, which produced 357.53 mg/L 7-DHC in shake flasks (Fig. 4C). These results show that compartmentalized reconstitution of the post-squalene pathway is an efficient strategy for enhancing 7-DHC production by shortening the distance between the enzymes and free sterols stored in the PM and peroxisomes. Based on a recent study [8], inhibition of *ERG2* transcription was fully relieved in *MOT3* deletion mutants, and the *ERG2* transcript level could be further enhanced by integrating one copy each of *ERG2* and *ERG3* at the *MOT3* site, which improved 7-DHC yield. In contrast, Guo, Yao, Xiao, Wang, Zhao and Yuan [6] reported that transcript levels of modified genes (*ERG2* and *ERG3*) were increased by overexpression of ER- and LD-localized Erg2p/Erg3p in a strain with intact *MOT3*. Taken together, these findings suggest that modifying *ERG2* and *ERG3* by adding targeting sequences might help them escape inhibition by Mot3p, which could be another possible reason for the increase in 7-DHC production when paired with overexpression of PM-localized and peroxisome-localized Erg2p/Erg3p. However, additional evidence and further investigation are required.

3.5. Fed-batch fermentation of the 7-DHC high-producing strain

Iron plays an important role in the activity of several enzymes in the sterol synthesis pathway, including Erg11p, Erg25p, and Erg3p [24]. Iron deficiency is associated with reduced activity of these enzymes. Thus, the effect of Fe^{2+} concentration on 7-DHC accumulation was evaluated by exogenous addition of $FeSO_4{\cdot}7H_2O$. The results show that 7-DHC production peaked at 370.68 mg/L when 0.4 mM ferrous sulfate was added (Fig. 5).

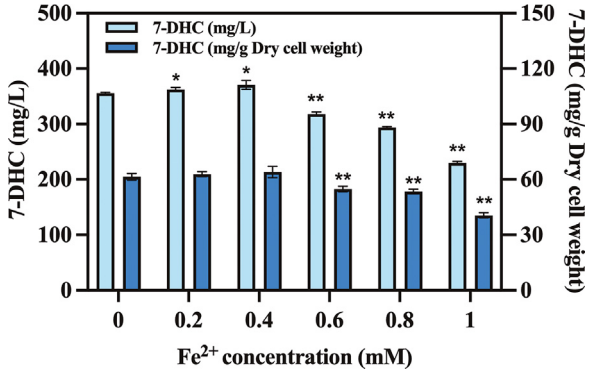

Fig. 5. Effect of Fe^{2+} concentration on 7-DHC production by strain YG061(+) in shake flasks. The data presented are the means of three biological replicates. Error bars represent standard deviations (*, $P < 0.05$ and **, $P < 0.01$).

Fed-batch fermentation was conducted using strain YG061(+), which was generated from YG061 by complementation of the auxotrophic markers, in a 5 L bioreactor containing 2 L YPD medium supplemented with 0.4 mM $FeSO_4{\cdot}7H_2O$. Dissolved oxygen was maintained at approximately 25% because although the enzymatic steps catalyzed by Erg1, Erg11, Erg25, and Erg3 are oxygen-consuming [5,24], excessive oxygen inhibits the production of sterols owing to oxygen-related transcriptional regulation [8]. After 120 h fermentation, 7-DHC production reached 1.56 g/L (Fig. 6).

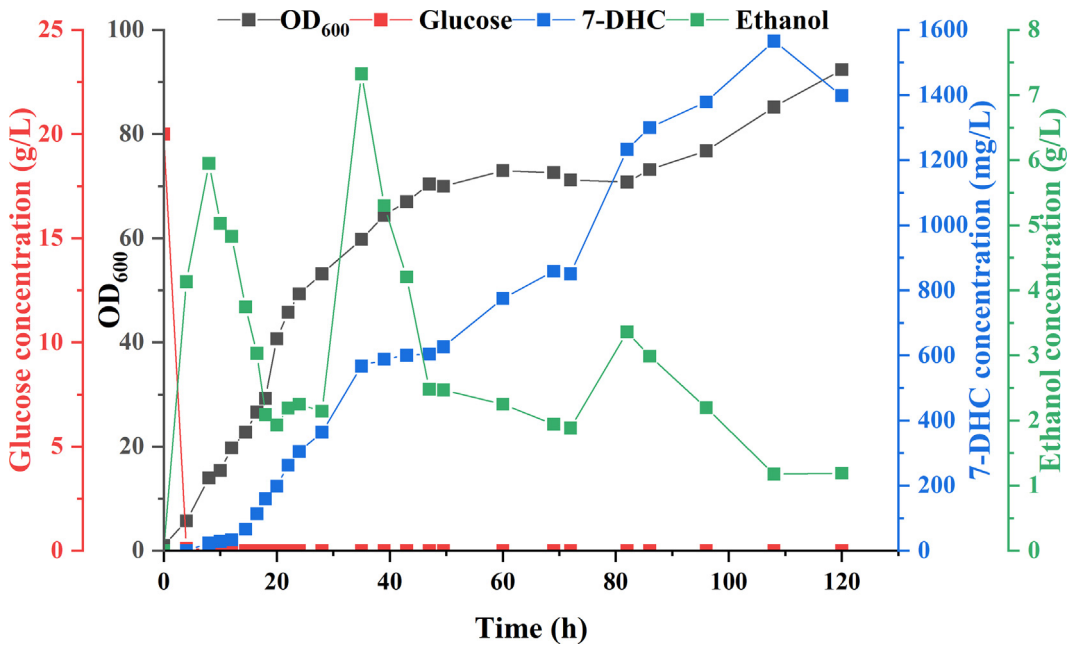

Fig. 6. Time courses of OD_{600}, 7-DHC production, and carbon source consumption of YG061(+) during fed-batch fermentation.

4. Conclusion

In this study, we constructed a 7-DHC-producing yeast strain through combinatorial regulation of endogenous competitive pathways, precursor supply and conversion, and subcellular re-localization of key pathway enzymes. Our best strain produced 370.68 mg/L 7-DHC in shake flasks and 1.56 g/L 7-DHC in a 5 L bioreactor. This work not only generated an engineered yeast strain with high 7-DHC production capability but also demonstrated the effectiveness of global regulation and subcellular re-localization strategies, which may provide hints for engineering yeast to produce other sterols. Nevertheless, the 7-DHC titer was still too low to meet industrial requirements. Based on the results of two recent studies with higher 7-DHC titers (Table 1), multi-copy integration of key genes and maintenance of basal ergosterol content using the CRISPRi system, instead of knocking out related genes, might further improve 7-DHC production. In addition, future endeavors to finely rearrange the distribution of enzymes in the post-squalene pathway module among different subcellular compartments, such as the ER, LDs, peroxisomes, and PM, may also further enhance 7-DHC production.

Take-away

In addition to gene overexpression, manipulation of transcription factors can be an alternative approach to efficiently enhance the pre-squalene pathway. Our study shows that the plasma membrane and peroxisomes are promising subcellular environments for 7-DHC production. These strategies provide promising approaches for engineering yeast to produce other sterols.

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.

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CRediT authorship contribution statement

YHG: Conceptualization, Investigation, Methodology, Writing-original draft; SHC: Investigation; XJ: Methodology, Resources; QB: Resources; LDY: Conceptualization, Supervision, Funding acquisition, Writing-review&editing; HWY: Conceptualization, Supervision, Funding acquisition.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2023.100100.

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