World Journal of Microbiology and Biotechnology (2018) 34:55
https://doi.org/10.1007/s11274-018-2440-9

ORIGINAL PAPER

Rate-limiting steps in the *Saccharomyces cerevisiae* ergosterol pathway: towards improved ergosta-5,7-dien-3β-ol accumulation by metabolic engineering

Bin-Xiang Ma[1,2] · Xia Ke[1,2] · Xiao-Ling Tang[1,2] · Ren-Chao Zheng[1,2] · Yu-Guo Zheng[1,2]

Received: 7 February 2018 / Accepted: 23 March 2018

Abstract
Ergosterol is the predominant nature sterol constituent of plasma membrane in *Saccharomyces cerevisiae*. Herein, the biosynthetic pathway of ergosterol was proposed to be metabolically engineered for the efficient production of ergosta-5,7-dien-3β-ol, which is the precursor of vitamin D4. By target disruption of *erg5*, involved in the end-steps of post-squalene formation, predominantly accumulated ergosta-5,7-dien-3β-ol (4.12 mg/g dry cell weight). Moreover, the rate-limiting enzymes of ergosta-5,7-dien-3β-ol biosynthesis were characterized. Overexpression of Hmg1p led to a significant accumulation of squalene, and induction of Erg1p/Erg11p expression raised the yield of both total sterols and ergosta-5,7-dien-3β-ol with no obvious changes in growth behavior. Furthermore, the transcription factor allele *upc2-1* was overexpressed to explore the effect of combined induction of rate-limiting enzymes. Compared with an obviously enhanced yield of ergosterol in the wild-type strain, decreases of both the ergosta-5,7-dienol levels and the total sterol yield were found in Δ*erg5-upc2-1*, probably due to the unbalanced NADH/NAD^+ ratio observed in the *erg5* knockouts, suggesting the whole-cell redox homeostasis was also vital for end-product biosynthesis. The data obtained in this study can be used as reference values for the production of sterol-related intermediates involved in the post-squalene biosynthetic pathway in food-grade *S. cerevisiae* strains.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11274-018-2440-9) contains supplementary material, which is available to authorized users.

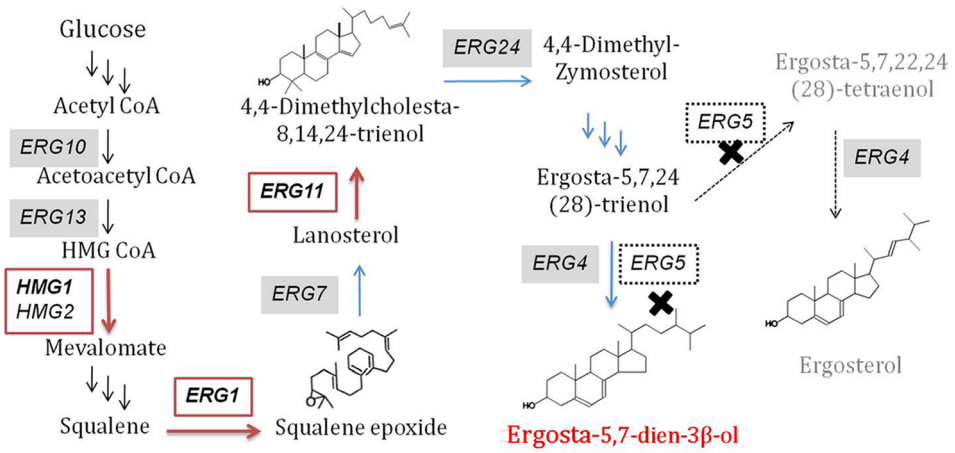
✉ Yu-Guo Zheng
zhengyg@zjut.edu.cn

[1] Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

[2] Engineering Research Center of Bioconversion and Biopurification of Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

Published online: 28 March 2018

Graphical Abstract



✖ disruption of *erg5*: engineering the biosynthetic pathway of ergosterol for the production of ergosta-5,7-dien-3β-ol;

⟶ *tHMG1*, *ERG1*, *ERG11* overexpression: rate-limiting enzymes of the ergosta-5,7-dien-3β-ol biosynthesis pathway;

⟶ *upc2-1* overexpression: induction of the post-squalene pathway.

Keywords Ergosta-5,7-dien-3β-ol · Ergosterol synthesis pathway · Rate-limiting steps · *Saccharomyces cerevisiae* · *Upc2-1*

Introduction

Sterols, also known as steroid alcohols, are key constituents of the plasma membrane of eukaryotic cells, serving as signaling, protective or attractant/repellent molecules (Bloch 1981; Parks et al. 1995). The endogenous ergosterol synthesis pathway in yeast is now well-characterized, with all involved genes having been identified more than 20 years ago (Skaggs et al. 1996). As such, *Saccharomyces cerevisiae* provides an ideal microbial platform for the production of varied sterol-derivatives for high-value pharmaceuticals.

Biosynthesis of sterols in yeast is a complex process with multiple control steps. The early intermediates, including acetyl-CoA and mevalonate from glucose, are the fundamental building blocks for the synthesis of terpenoids in the classic mevalonate pathway (Phillips et al. 2012), which provides the common intermediate for the branch point of a variety of terpenoid synthesis pathways in yeast. Multiple enzymatic steps are involved in the post-squalene pathway, with lanosterol, zymosterol and fecosterol as intermediates (Pierson et al. 2004). In addition to the conserved reactions in the sterol biosynthesis pathway, an additional branch that adds a methylidene/methyl group at position C-24 in a reaction catalyzed by *ERG6*, the C-24 sterol methyl transferase that converts cholesta-8,24-dienol into 24-methylene-cholest-8-enol, fecosterol (Tiedje et al. 2007), and desaturates position C-22 in a reaction catalyzed by *ERG5*, the C-22 sterol desaturase that converts ergosta-5,7,24(28)-trienol into ergosta-5,7,22,24(28)-tetraenol to form ergosterol (MacPherson et al. 2005), was identified in *S. cerevisiae*. This additional pathway differentiates yeast sterols from their counterparts from animal cells, which possess double bonds at C-7 and C-24, as in cholesterol.

Previous reports have demonstrated that sterol intermediates, such as ergosta-5,7-dien-3β-ol, are precursors of vitamin D4 (Phillips et al. 2012). Ergosta-5,7-diene compounds are useful intermediates in the synthesis of the 1α,25-dihydroxyvitamin D_4 compounds. The D_4 compounds are expected to possess an interesting pharmacological activity in association with the active-type vitamins D_3 and D_2 (Tsuji et al. 1992), in addition to its well-defined role in bone calcium homeostasis (Holick 2002). Vitamin D4 is often used for the treatment of postoperative tetany in its chronic, latent, and acute forms (Leidig-Bruckner et al. 2016). Vitamin D4 metabolites had a tissue distribution similar to vitamin D3 but were excreted more quickly and also appear to have lower toxicity in high doses compared to D3 (Phillips et al. 2012). Given its presence in many commonly consumed mushrooms (Krings and Berger 2014), further study of the biological activity and production of vitamin D4 is

warranted. Thus, with the aim to modify the sterol composition and accumulate specific ergosterol precursors in yeast, inhibition of the catalytic activity of non-essential enzymes involved in the final steps of ergosterol synthesis, such as sterol C-24 methyl transferase, which can be achieved by targeted deletion of *erg5*, was proposed to refine the ergosterol biosynthesis pathway for the production of ergosta-5,7-dien-3β-ol in engineered yeast.

Moreover, rate-limiting steps involved in yeast ergosterol biosynthesis have been extensively studied in wild-type *S. cerevisiae* (Polakowski et al. 1998; Veen et al. 2003). HMG-CoA reductase (*HMG*) is reported to be a key rate-limiting enzyme involved in the early stages of sterol synthesis (Kodedova and Sychrova 2015), and its overexpression leads to an increased conversion of HMG-CoA to squalene (Scallen 1965). Overexpression of a truncated HMG-CoA reductase gene (*tHMG*) was commonly used to remove its intrinsic feedback regulation, thus increasing carbon flux through the MVA pathway (Dai et al. 2013). In addition, squalene epoxidase (Erglp) and sterol-14α-demethylase (Erg11p) were also demonstrated to be key regulators involved in the post-squalene synthetic pathway (Korn et al.1969; Veen et al.2003.) In order to maximize the production capacity of the engineered strain, the growth behavior and sterol yields were further investigated after overexpression of each of these enzymes.

The efficiency of biosynthesis is not only determined by the activity of a single rate-limiting enzyme, but more importantly also by the balanced expression of all enzymes in a pathway (Ghodasara and Voigt 2017). Transcription factor Upc2p is reported to upregulate target genes involved in sterol biosynthesis via the trans-activation of sterol-responsive elements in their respective promoter regions. Nine responsive *ERG* genes from *Candida albicans* (*ERG1, ERG2, ERG5, ERG6, ERG10, ERG11, ERG24, ERG26* and *ERG27*) have previously been reported to contain the core motif of sterol-responsive elements (Gaber et al. 1989; Gachotte et al. 1999), and most of these are involved in the post-squalene biosynthetic pathway, providing an alternative approach to elevate the sterol biosynthesis capacity of *S. cerevisiae*.

In the present study, the ergosterol biosynthesis pathway was metabolically reprogrammed to allow the accumulation of ergosta-5,7-dien-3β-ol instead of the natural end-product ergosterol, and the influence of the designed genetic modifications on cell growth and sterol accumulation was investigated in detail (Fig. 1). Our observations further the knowledge surrounding the regulation of the ergosterol pathway in the production of sterol intermediates. At least three rate-limiting enzymes appear to be involved in the biosynthesis of ergosta-5,7-dien-3β-ol in the *erg5* knockout strain: HMG-CoA reductase, the squalene epoxidase Erg1p, and the sterol-14α-demethylase Erg11p. In addition to the combined upregulation of the post-squalene pathway by *upc2-1*, the NADH/NAD$^+$ ratio was also confirmed to be a vitally important determinant of the accumulation of ergosta-5,7-dien-3β-ol in the engineered strains.

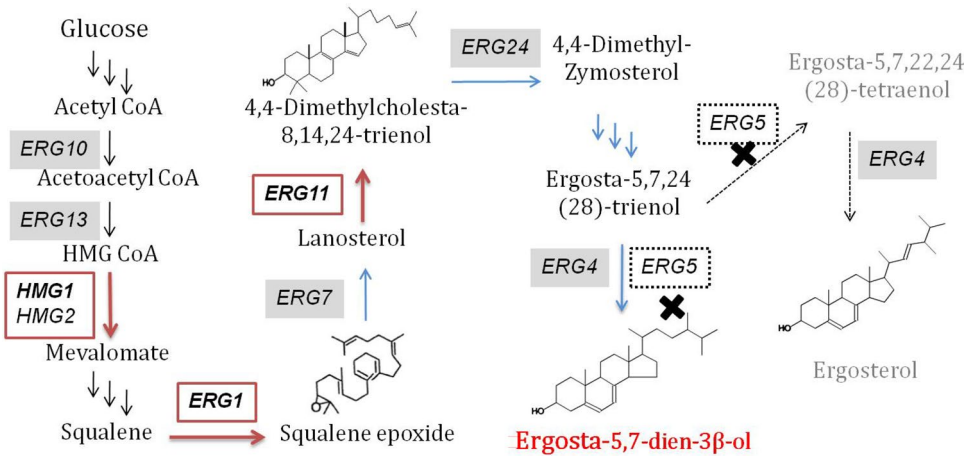


Fig. 1 The workflow of metabolic engineering of *S. cerevisiae* to convert accumulated ergosterol to ergosta-5,7-dien-3β-ol, encompassing the deletion of *erg5*, overexpression of the bona fide rate-limiting enzymes Hmg1p, Erg1p, and Erg11p, and the transcription factor *upc2-1* for the combined induction of the post-squalene pathway, with the aim to increase the synthesis of ergosta-5,7-dien-3β-ol. (Times symbol) disruption of *erg5*: engineering the biosynthetic pathway of ergosterol for the production of ergosta-5,7-dien-3β-ol; (red colored arrow) *tHMG1, ERG1, ERG11* overexpression: rate-limiting enzymes of the ergosta-5,7-dien-3β-ol biosynthesis pathway; (blue colored arrow) *upc2-1* overexpression: induction of the post-squalene pathway

Materials and methods

Media, culture conditions and chemicals

The parent strain *S. cerevisiae* BY4741 was obtained from Euroscarf (http://www.euroscarf.de/search.php?name=Order), and was grown at 30 °C and 250 rpm in YPD medium containing 1% yeast extract (Oxoid Ltd., England), 2% peptone (Becton, Dickinson and Company, France) and 2% dextrose. Mutant selection was conducted using YNBD-URA synthetic minimal medium (0.67% yeast nitrogen base) (BBI Life Sciences Corporation, China), and 2% glucose, supplemented with URA dropout amino acid mixture (Sigma-Aldrich Corporation, USA). The induction medium was YPG (1% yeast extract, 2% peptone and 2% galactose), galactose was obtained from BBI Life Sciences Corporation (China). Oligonucleotide primers were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). The restriction enzymes (RE), ExPfu DNA polymerase and T4 ligase were obtained from Thermo-Fisher Scientific (USA). Authentic reference standards of sterol intermediates including squalene, ergosterol and lanosterol, as well as the antibiotic G418 were purchased from Sigma-Aldrich Corporation (USA). All other chemicals used in this study were from commercial sources and of reagent grade.

Targeted gene disruption of erg5

The PCR-mediated one step gene disruption strategy was applied to obtain the *erg5* deletion strain. The pUG6 plasmid containing the *kanMX* marker was used as template to generate the disruption cassette. The knockout cassette contained 45 nucleotide stretches homologous to sequences upstream of the start codon and downstream of the stop codon of *erg5* as shown in Table 1. The BY4741 strain was transformed with the *erg5::kanMX* cassette by electroshock, and transformants were selected on YPD agar plates containing 200 mg/L of G418. The positive transformants were confirmed by yeast colony PCR.

Molecular cloning of tHMG1, ERG1, ERG11 and upc2-1

The *ERG1, tHMG1* and *ERG11* genes were amplified from chromosomal DNA of *S. cerevisiae* BY4741 using the primers listed in Table 1. The PCR procedure was performed using Expfu DNA polymerase with a total of 30 amplification cycles. Each cycle included three steps: 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min. The mutant allele of *UPC20, upc2-1* (containing a glycine to aspartic acid mutation at position 888, G888D), was amplified from the chromosomal DNA of the *upc2-1* mutant strain (Crowley et al. 1998), which was a kind gift by Dr. Leo W. Parks, North Carolina State University. The PCR procedure was performed using Expfu DNA polymerase with a total of 30 cycles. Each cycle included three steps: 98 °C for 10 s,

Table 1 Primers, plasmids and strains used in this study

Name	Description	Reference
Primers for gene disruption and confirmation		
erg5-F	ATCACATTTTGCTATTCCAATAGACAATAAATACCTTTTAACAAATTCTGTTTTTCCCCA	This study
erg5-R	AAGTAAATATGATTTATTGTCTGGACAAAGTTCTGTTTTTCCCCAGTTTTGTTTATTGTG	This study
Primers for gene overexpression		
tHMG1-F	GGGGATCCATGGACCAATTGGTGA	This study
tHMG1-R	CGGCGGCCGCTTAGGATTTAATGCAGG	This study
ERG1-F	CTGAAGCTTATCATGTCTGCTGTTAACGTTGC	This study
ERG1-R	GGTCTAGATTAACCAATCAACTCACCAAAC	This study
ERG11-F	CCCAAGCTTAACACAATGTCTGCTACCAAG	This study
ERG11-R	CCGCTCGAGCTAGATCTTTTGTTCTGG	This study
UPC2-1-F	CCGGAGCTCATGAGCGAAGTCGGTATACAG	This study
UPC2-1-R	CCGCTCGAGTCATAACGAAAAATCAGAGAAATTTG	This study
Plasmids		
pSH69	Cre-expressing (pGAL1-cre) CEN/ARS plasmid, hygromycin B marker	Euroscarf
pUG6	*loxP-kanMX-loxP* disruption module plasmid	Euroscarf
pYES2	Expression vector with GAL1 promoter and CYC terminator, URA3 marker	Dr.Sun Jie
Strains		
BY4741	*ΔMAT, his3Δ, leu2Δ, met15Δ, ura3Δ*	Euroscarf

56 °C for 30 s, and 72 °C for 105 s. All PCR products were gel-purified and digested using the appropriate REs. The digested products were ligated into the pYES2 plasmid backbone and used to transform *E. coli* BL21 competent cells (TransGen Biotech, China). The positive colonies were confirmed by bacterial colony-PCR and restriction-enzyme double digestion. The confirmed recombinant plasmids were extracted from *E. coli* and used to transform both the wild-type BY4741 and the Δ*erg5* strain using the PEG-LiAc transformation method (Gietz et al. 1995), yielding BY4741-*tHMG1*, Δ*erg5*-*tHMG1*, BY4741-*ERG1*,Δ*erg5*-*ERG1*, BY4741-*ERG11*, Δ*erg5*-*ERG11*, BY4741-*upc2-1*, and Δ*erg5*-*upc2-1*, respectively. Positive transformants were selected on YNBD–URA plates. An empty pYES2 plasmid was used to transform the wild type to generate the BY4741-*empty* control strain. To stabilize the *erg5* disruption strains with recombinant vectors, all of which were confirmed by yeast colony PCR, 200 μg/mL of G418 was added to the medium.

Determination of biomass

The optical density of the cultures was measured at 600 nm on a U-2000 spectrophotometer (Hitachi, Tokyo, Japan), using a control sample comprising sterile medium as the blank. The dry cell weight was obtained after incubating the cells at 80 °C for 6 h.

Extraction of sterols

Sterols were extracted after alkaline saponification in the presence of pyrogallol (Adams and Parks 1968). The yeast cells were collected by centrifugation at 3000×*g* and 4 °C for 10 min, washed twice with distilled water and dried at 80 °C to a constant weight. Samples comprising 300 mg of the dried biomass were resuspended in raeaction mixtures comprising 5 mL of a 60% KOH solution, 7.5 mL of methanol and 7.5 mL of methanolic pyrogallol (0.5% w/v), and incubated at 80 °C for 4 h under constant shaking at 180 rpm. The unsaponified matter was extracted with 10 mL of hexane. Different phases were formed after centrifugation at 3000×*g* for 10 min. The top *n*-hexane layer was removed to a clean tube and the residue was extracted twice with hexane again. The n-hexane phase containing the extracted sterols was recovered, dried under nitrogen, and the final obtained sterols were resuspended in 500 μL of chloroform/methanol (4:1) for GC/MS analysis.

GC/MS analysis of sterols

The sterols were separated and analyzed by GC-MS on an Agilent 3400 system (Agilent, USA) equipped with a fused silica column (DB-5, 15 m × 0.32 mm × 0.25 μm film thickness; J&W Scientific, USA), with helium at a flow rate of 1 mL/min as the carrier gas. The temperature program comprised a ramp from 40 to 300 °C at 10°C/min, and the injection volume was 1 μL in split mode (split ratio 1:20). The injection temperature was kept at 300 °C for 4 min. Mass spectra were generated in electron impact mode at 70 eV, at an ion-source temperature of 150 °C. The mass spectra were recorded from 40 to 700 μ at 1 s intervals. Sterols were identified by searching against the metabolite mass spectra database, comparing their spectra to commercially available authentic reference standards, and on the basis of relative retention times as previously reported (Hughes et al. 2000; Yun et al. 2014). Relative quantification of sterols between the wild type and the *erg5* deletion strain was conducted by comparing the integrated peak areas of each sterol intermediate, and the specific sterol yields were calculated per gram of dried cell weight (DCW).

Measurement of the NADH/NAD$^+$ ratio

The lysis of the yeast cell wall was induced by adding 0.15 U/μL lyticase (TIANGEN Biotech Co. Ltd., China) and incubating at 30 °C for 30 min before measurement. The NADH/NAD$^+$ ratio was measured using the Coenzyme I NAD(H) content test kit from Comin Biotechnology Co. Ltd. (Suzhou, China). All strains were sampled after 24 h of cultivation at 28 °C and 150 rpm in YPG medium.

Statistical analysis

For each calculation, three biological replicates were performed. The mean value and standard curve calculated from three different batches of data were used to represent the production levels of the components. Student's *t* test was applied in the analysis of statistical significance and a $p < 0.05$ (*) threshold was used to identify significant differences in this study.

Results

Deletion of erg5 leads to the accumulation of ergosta-5,7-dien-3β-ol

Targeted deletion of *erg5*, which is involved in the final steps of the ergosterol synthesis pathway, is a workable strategy for the directed synthesis of ergosta-5,7-dien-3β-ol. In YPD medium, only slight differences in growth behavior were observed between the wild type and the Δ*erg5* strain (Fig. 2a). However, an investigation of the strains' sterol profiles revealed significant differences in sterol composition (Fig. 2b, c). Ergosterol was found to be the main component of total sterols in the wild-type strain, accounting for 76.0%

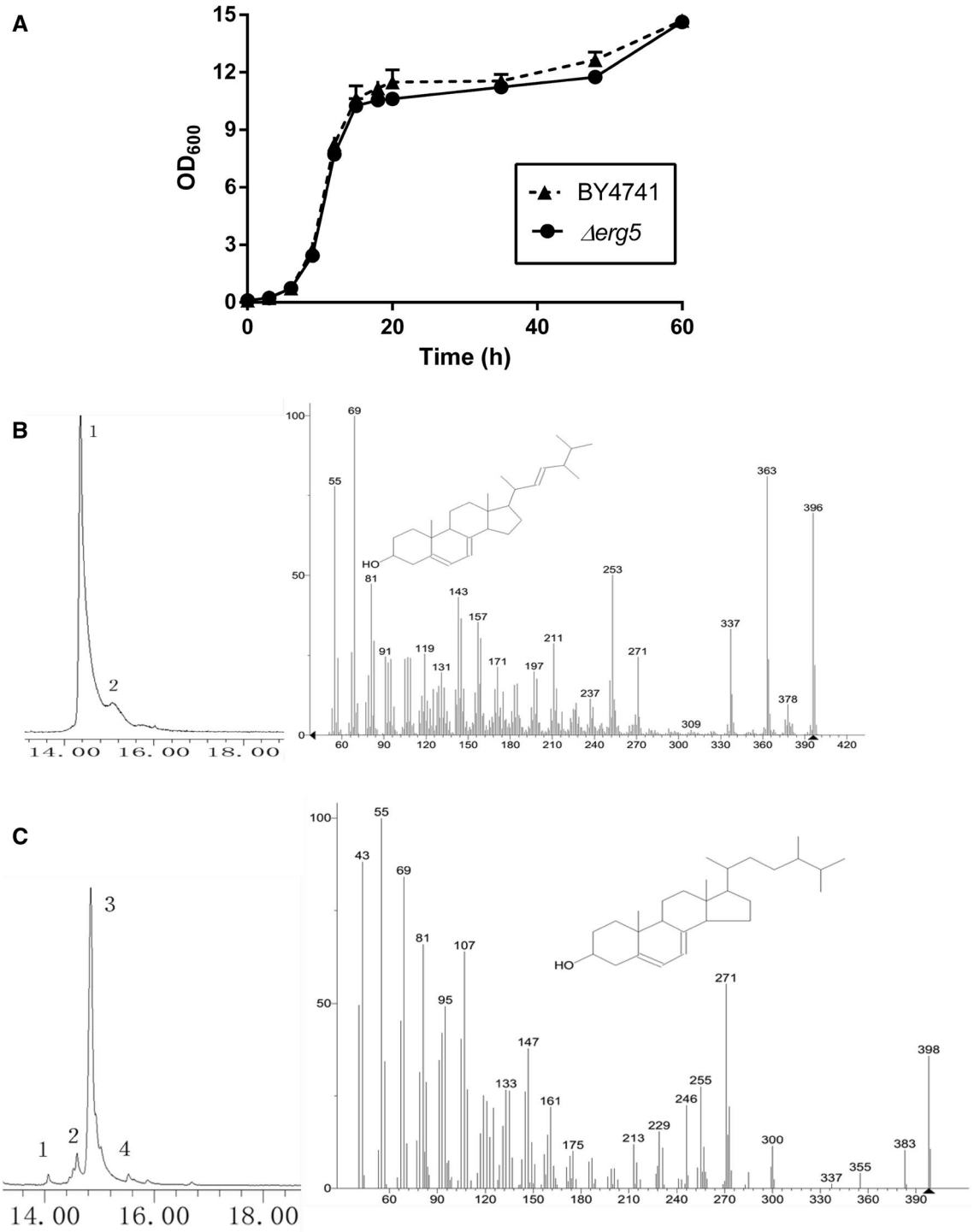


Fig. 2 Growth curves and free sterol profiles of the *S. cerevisiae* wild type strain and *erg5* gene-disrupted mutant (Δ*erg5*). **a** Growth curves of strains cultivated at 30 °C and 250 rpm in YPD medium. The cell concentration was measured via the OD_{600}. GC/MS profiles of free sterols extracted from wild type BY4741 (**b**) and Δ*erg5* (**c**), numbers in the top of the corresponding peaks denote different products. **b** Left: 1, ergosterol; 2. lanosterol; right: the fragmentation profile of ergosterol as the major sterol obtained from BY4741. **c** Left: 1, zymosterol; 2, ergosta-7,24(28)-dien-3β-ol; 3, ergosta-5,7-dien-3β-ol; 4, lanosterol. Right: the fragmentation profile of ergosta-5,7-dien-3β-ol as the major sterol obtained from Δ*erg5*

of total sterols. By contrast, the Δ*erg5* strain accumulated ergosta-5,7-dien-3β-ol as the dominant free sterol (86.6%), in addition to a small quantity of lanosterol (4.4%), and an unidentified sterol intermediate (9.0%) (Online Resource 1: Supplementary Table 1). The productivity of ergosta-5,7-dien-3β-ol in the Δ*erg5* strain was further investigated, and the yield reached 4.12 ± 0.06 mg/g DCW after cultivation in YPD medium for 48 h, after which it decreased to 3.66 ± 0.09 mg/g at 60 h (Fig. 3). The intracellular redox potential is primarily determined by the ratio of NADH/NAD^+ (Vemuri et al. 2007). The total NADH/NAD^+ ratio of BY4741 was 0.431 ± 0.038, while the ratio of Δ*erg5* was 0.708 ± 0.016 as illustrated in Table 2.

Overexpression of the gene encoding tHMG-CoA reductase to improve the squalene supply

As a first step towards a sterol-overproducing yeast strain, a truncated form of the HMG-CoA reductase Hmg1p was overexpressed in both BY4741-*empty* and Δ*erg5-empty* (-*empty*: the strain was transformed with an empty pYES2 plasmid). The results indicated that the growth curve of the BY4741-*tHMG1* strain remained almost synchronous with that of the BY4741-*empty* strain during 20 h of cultivation. At the later stages, a palpable inhibition was noticed, and the cell density of BY4741 decreased to 53.08% of that of the empty-vector control after 60 h of cultivation (Fig. 4a). Overexpression of Hmg1p in BY4741-*tHMG1* resulted in accumulation of squalene (3.38 ± 0.16 mg/g) and a slight decrease of ergosterol production (8.30 ± 0.21 vs. 9.23 ± 0.39 mg/g), as illustrated in Table 3. Growth inhibition was also found in the Δ*erg5-tHMG1* strain, whereby its growth lag phase extended for about 30 h, and its cell density was significantly decreased to 44.51% of that of the empty-vector control after 60 h of cultivation (Fig. 4c). Overexpression of Hmg1p in Δ*erg5-tHMG1* resulted in accumulation of squalene (1.87 ± 0.35 mg/g) and other post-ergosterol intermediates (1.69 ± 0.05 vs. 0.43 ± 0.04 mg/g). However, the yield of ergosta-5,7-dien-3β-ol was significantly reduced (1.79 ± 0.10 vs. 4.12 ± 0.06 mg/g) (Table 3). The production level of ergosta-5,7-dien-3β-ol in Δ*erg5-tHMG1* was obviously decreased, accounting for only 45.13% of the level in the empty-vector control strain. All these data suggested that the overexpression of Hmg1p was directly responsible for the enhanced supply of squalene, while an reduction of ergosta-5,7-dien-3β-ol indicated that further regulatory mechanisms may be present in the synthesis of intermediates of end-product accumulation.

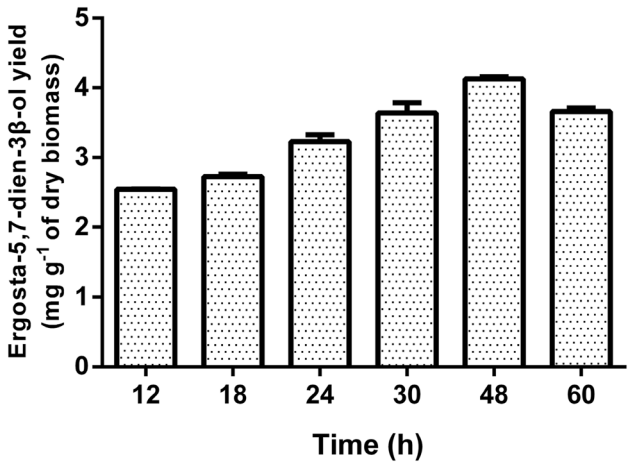


Fig. 3 The yield profile of ergosta-5,7-dien-3β-ol in the engineered strain Δ*erg5* cultivated at 30 °C and 250 rpm in YPD medium. The yields were measured in three separate experiments and expressed as means ± standard deviations

Upregulation of ERG1/ERG11 expression enhanced ergosta-5,7-dien-3β-ol production

The proposed key limiting steps in the post-squalene pathway catalyzed by Erg1p and Erg11p were further investigated in both the wild type and the Δ*erg5* knockout strain. The growth curves of BY4741-*ERG1* and BY4741-*ERG11* remained almost synchronous with that of the BY4741-empty strain, even if their cell density was somewhat lower (Fig. 4a). The production level of ergosterol was increased by 10.29 and 8.34% in BY4741-*ERG1* and BY4741-*ERG11*, respectively (Fig. 4b). A similar phenomenon was also observed when comparing the growth curve of the Δ*erg5*-empty strain to those of Δ*erg5-ERG1* and Δ*erg5-ERG11* (Fig. 4c). The production level of ergosta-5,7-dien-3β-ol was increased by 12.38% in Δ*erg5-ERG1* and 3.40% in Δ*erg5-ERG11*, respectively (Fig. 4d). In addition, total sterol yields in both Δ*erg5-ERG1* and Δ*erg5-ERG11* were increased by 16.8 and 12.6%, respectively, and the differences were statistically significant (Table 3), which further confirmed their vital role in the post-squalene biosynthesis pathway of ergosta-5,7-dien-3β-ol. However, in addition to the clearly increased accumulation of lanosterol in BY4741-*ERG1* (1.72 ± 0.14 vs. 0.19 ± 0.03 mg/g), a slight enhancement of lanosterol production was also found in Δ*erg5-ERG1* (0.30 ± 0.02 vs. 0.21 ± 0.09 mg/g) (Table 3). Moreover, in both BY4741-*ERG11* and Δ*erg5-ERG11*, the

Table 2 Total NADH/NAD^+ ratios of engineered strains derived from BY4741

Strain	BY4741-*empty*	Δ*erg5-empty*	BY4741-*upc2-1*	Δ*erg5-upc2-1*
NADH/NAD^+ ratio	0.431 ± 0.038	0.708 ± 0.016	0.471 ± 0.007	0.836 ± 0.042

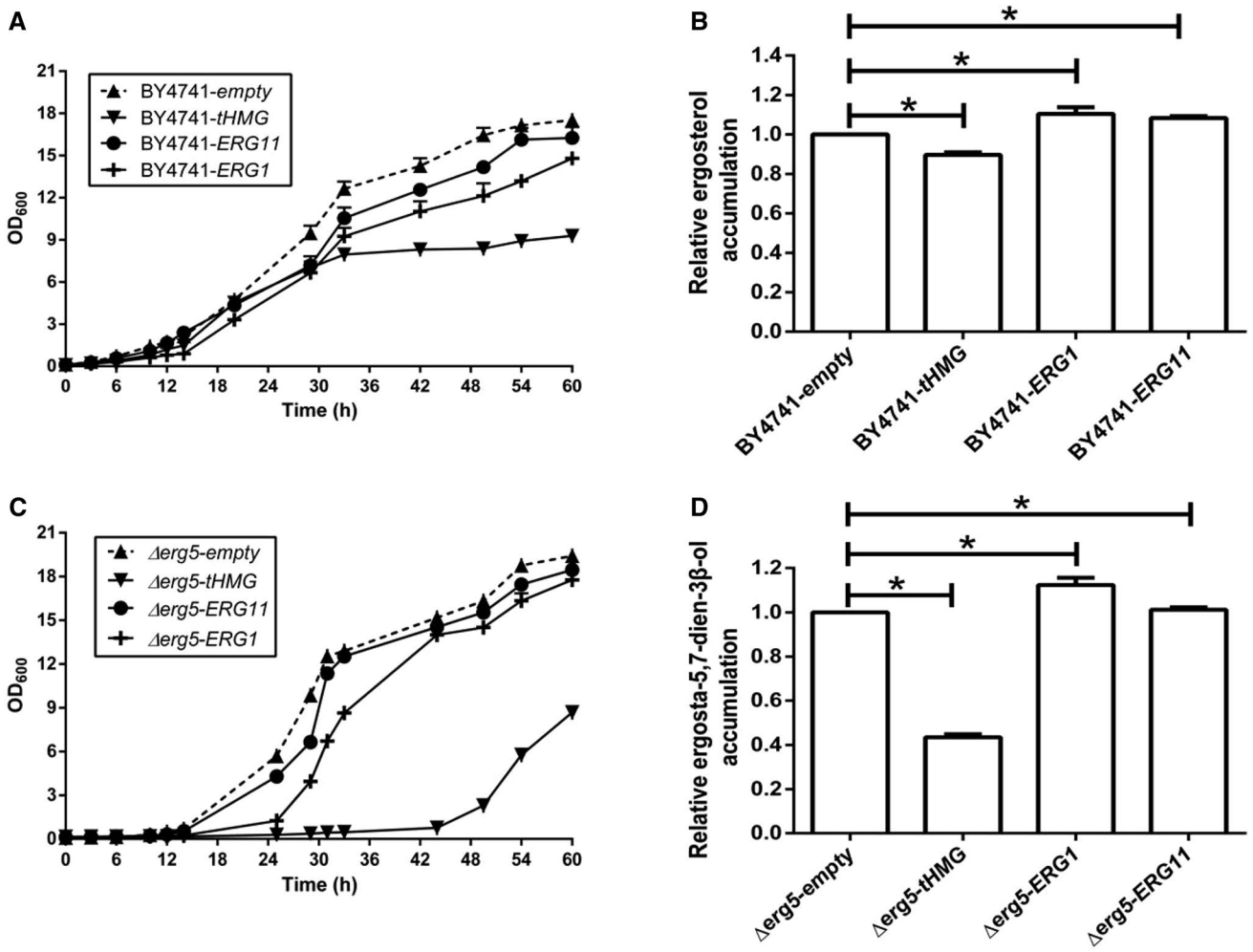


Fig. 4 Growth curves and ergosta-5,7-dien-3β-ol accumulation of Δ*erg5* strains overexpressing *tHMG1, ERG1* and *ERG11*, respectively, compared with the empty-vector control. **a** The OD_{600} value was measured to characterize the growth behavior of the wild-type strains overexpressing *tHMG1, ERG1* or *ERG11*. **b** Fold-change of ergosterol accumulation in BY4741 overexpressing *tHMG1, ERG1* and *ERG11*, respectively. **c** The OD_{600} value was measured to characterize the growth behavior of the Δ*erg5* strains overexpressing *tHMG1, ERG1* or *ERG11*. **d** Fold change of ergosta-5,7-dien-3β-ol accumulation in the Δ*erg5* strains overexpressing *tHMG1, ERG1* or *ERG11*. The relative amounts were measured in three separate experiments and expressed as means ± standard deviations

Table 3 The yields of major free sterols (mg/g DCW) in *S. cerevisiae* overexpressing *tHMG1, ERG1* or *ERG11* and controls

Sterol intermediates	Yield (mg/g DCW)							
	BY4741-*empty*	BY4741-*tHMG1*	BY4741-*ERG1*	BY4741-*ERG11*	*Δerg5-empty*	*Δerg5-tHMG1*	*Δerg5-ERG1*	*Δerg5-ERG11*
Squalene	n.d.	3.38 ± 0.16	0.18 ± 0.12	0.43 ± 0.14	n.d.	1.87 ± 0.35	0.05 ± 0.03	0.24 ± 0.08
Lanosterol	0.19 ± 0.03	0.22 ± 0.02	1.72 ± 0.14	n.d.	0.21 ± 0.09	0.25 ± 0.06	0.30 ± 0.02	n.d.
Post-ergosterol intermediates	2.72 ± 0.41	1.74 ± 0.41	1.61 ± 0.63	2.23 ± 0.33	0.43 ± 0.05	1.69 ± 0.04	0.58 ± 0.04	0.86 ± 0.04
Ergosterol	9.23 ± 0.39	8.30 ± 0.21	10.18 ± 0.56	10.00 ± 0.15	n.d.	n.d.	n.d.	n.d.
Ergosta-5,7-dien-3β-ol	n.d.	n.d.	n.d.	n.d.	4.12 ± 0.06	1.79 ± 0.10	4.63 ± 0.25	4.26 ± 0.15
Total sterols and squalene	12.14 ± 0.83	13.64 ± 0.79	13.69 ± 1.21	12.66 ± 0.61	4.76 ± 0.24	5.60 ± 0.52	5.15 ± 0.34	5.26 ± 0.28

n.d. not detected

content of lanosterol was almost undetectable, indicating that the catalytic activity of lanosterol 14-α-demethylation was increased after overexpression of Erg11p. Moreover, a typical peak (M/S = 441.3) in significant quantity (1.75% of the total peak area) suggested that 4-carboxyl-sterols (4-carboxyl-fecosterol or 4-carboxyl-episterol), which are correlated with components involved in sterol C4-demethylation, were present in both BY4741-*ERG11* and Δ*erg5-ERG11*.

Effect of the combined induction of rate-limiting enzymes using the transcriptional factor allele upc2-1 on ergosta-5,7-dien-3β-ol production

Pleiotropic effects of the allele *upc2-1*, which contains a single guanine-to-adenine transition (with a predicted amino acid change of G888D), of the gene responsible for control of sterol uptake (Tokuhiro et al. 2009), were further investigated. Thus, the mutant allele *upc2-1* was overexpressed in both Δ*erg5* and its parental strain. The growth curve of the BY4741-*upc2-1* strain remained almost synchronous with that of BY4741-*empty* (Fig. 5a). Additionally, only slight growth inhibition was observed in Δ*erg5-upc2-1* and its cell density decreased to 76.4% of that of the empty-vector control after 48 h of cultivation (Fig. 5c). There was a significant increase in the yield of ergosterol accumulated in BY4741-*upc2-1* (11.91 ± 0.40 vs. 9.23 ± 0.39 mg/g, corresponding to an increase of 24.5%) (Fig. 5b). However, to our surprise, the overexpression of *upc2-1* in the Δ*erg5* strain resulted in an obvious decrease in both the ergosta-5,7-dienol level (by 24.0%; Fig. 5d) and the total sterol yield (by 16.8%; Online Resource 1: Supplementary Table 2). The NADH/NAD^+ ratio of BY4741-*upc2-1* was 0.471 ± 0.007, while the ratio of the Δ*erg5-upc2-1* strain reached 0.836 ± 0.042 as shown in Table 2.

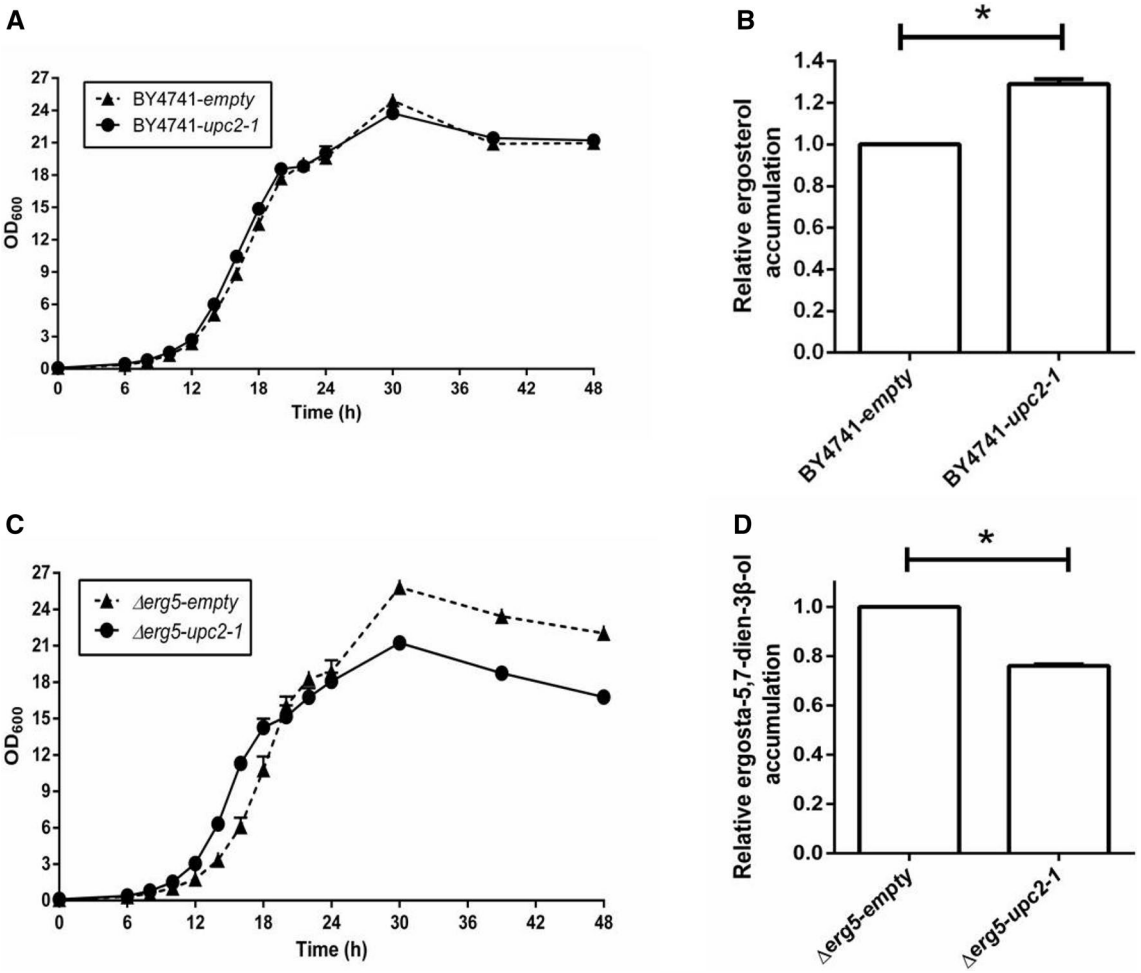


Fig. 5 Growth curve and ergosta-5,7-dien-3β-ol accumulation of the Δ*erg5* strain overexpressing *upc2-1*, compared with the empty vector control. **a** The OD_{600} value was measured to characterize the growth behavior of the wild type strain overexpressing *upc2-1*. **b** Fold-change of ergosterol accumulated by BY4741 overexpressing *upc2-1*. **c** The OD_{600} value was measured to characterize the growth behavior of the Δ*erg5* strain overexpressing *upc2-1*. **d** Fold-change of ergosta-5,7-dien-3β-ol accumulation in the Δ*erg5* strain overexpressing *upc2-1*. The relative amounts were measured in three separate experiments and expressed as means ± standard deviations

Discussion

Sterols are essential lipid components of eukaryotic membranes, controlling their physical state by modulating the fluidity and permeability of their bilayer structure (Caspeta et al. 2014). Interestingly, it is reported that the lipid composition of the cells of yeasts such as *Mucor hiemalis* correlates with their growth rate (Mysiakina et al. 2012). Earlier reports demonstrate that an *erg6* knockout strain exhibited a pleiotropic phenotype encompassing defective conjugation, hypersensitivity to cycloheximide, resistance to nystatin, and a severely diminished capacity for genetic transformation (Bard et al. 1996; Ignea et al. 2014; Veen et al. 2003). Similarly, *erg5* knockouts also show defective conjugation, cross-resistance to azoles and amphotericin B, and a slightly diminished capacity for genetic transformation (Sharma 2006; Sun et al. 2013). Consistently, our study also confirmed that different genetic manipulations of BY4741 that affected the mutants' sterol profiles also changed their growth behavior. For example, in Hmg1p-overexpressing strains, abundant accumulation of squalene tends to induce growth retardation. A deletion of *erg5* preserved the normal growth rate, albeit leading to a small decrease of genetic transformation capacity. It is reasonable that sterols not only contribute to the fluidity of lipid membranes, but also mediate many other vital processes including vesicle formation, protein sorting, cytoskeleton organization, endocytosis and mating (Aguilar et al. 2010; Caspeta et al. 2014; Tiedje et al. 2007).

Key enzymatic steps involved in ergosterol biosynthesis were further investigated to elevate the production capacity of ergosta-5,7-dien-3β-ol. Thus, three potential rate-limiting enzymes were overexpressed both in Δ*erg5* and its parental strain. The measurements of sterol metabolism suggest that (i) Hmg1p is responsible for the enhanced supply of squalene, while the obvious reduction of ergosta-5,7-dienol accumulation indicates that further regulation mechanisms are present in the steps leading from sterol intermediates towards the end-product; (ii) Overproduction of Erg1p or Erg11p facilitates bioconversion of the corresponding intermediates, implying that they play important roles in post-ergosterol biosynthesis; (iii) The presence of 4-carboxylic acid sterols after overexpression of Erg11p implied a partial block of the C-4 demethylation of lanosterol, implying that stepwise oxidation in the sterol C-4 demethylation process, catalyzed by the C-4 demethylation enzyme complex composed of Erg25p (Bard et al. 1996), Erg26p (Gachotte et al. 1998) and Erg27p (Gachotte et al. 1999) and the scaffold protein Erg28p (Mo and Bard 2005), seems to be a rate-limiting step.

The combined overexpression of the three key enzymes is a feasible strategy to enhance the yield of both ergosta-5,7-dien-3β-ol and ergosterol in both Δ*erg5* and its parental strain. However, tandem expression of multiple gene clusters imposes a heavy metabolic burden on the host cells (Borkowski et al. 2017). Moreover, excessive overexpression of specific enzymes can result in unbalanced accumulation of sterol intermediates, leading to an obvious reduction of total cell biomass as well as end-product repression as observed in the *tHMG1*-overexpressing strain. Therefore, there is an implicit requirement to avoid an excessive cellular burden caused by the overexpression of specific enzymes and accumulation of cytotoxic intermediates (Zhou et al. 2017). Moreover, it is necessary to achieve a balance between increased precursor supply and induction of the catalytic activities of enzymes involved in the post-squalene biosynthetic pathway, to maximize the production of the end-product ergosta-5,7-dien-3β-ol.

Induction of specific transcription factors responsive to stress conditions is another method to alter the expression levels of selected target genes (Shimada et al. 1998). Upc2p is a global transcription regulator that specifically recognizes ergosterol (Yang et al. 2015), regulating sterol uptake and biosynthesis (Crowley et al. 1998). Consistently, our results demonstrated that the yield of ergosterol in the BY4741-*upc2-1* strain was increased 1.3-fold, which indicates a potential to use this strategy to further enhance the production of ergosta-5,7-dien-3β-ol in the future. However, overexpression of *upc2-1* resulted in biomass reduction and a decreased yield of ergosta-5,7-dien-3β-ol.

The C-22 sterol desaturase encoded by *ERG5* is an NADH-dependent cytochrome P450 which takes part in the oxidative metabolism of steroids (Skaggs et al. 1996). Since nicotinamides act as electron donors (NADH) or acceptors (NAD^+) in a myriad redox reactions, any change in the NADH/NAD^+ ratio leads to widespread changes of cellular metabolism (Nielsen 2007), including unbalanced oxidation–reduction potential, cell damage, carbon or energy waste and even metabolic arrest (Chen et al. 2014). Elimination of some by-products will break original intracellular redox balance (Cheng et al. 2013). Moreover, defects of ergosterol metabolism may activate certain transcription factors, such as Upc2p, to enhance the expression of genes in the ergosta-5,7-dien-3β-ol synthetic pathway, which normally serves as a feedback loop to reestablish cellular metabolic balance in ergosterol precursor production, but can also result in a further exacerbation of the intrinsic redox imbalance in Δ*erg5* strains (Su et al. 2015). As observed in our study, the NADH/NAD^+ ratio of Δ*erg5-empty* was increased by 1.64-fold compared to that of BY4741-*empty*, while the total sterol content was decreased by 60.8%. Overexpression of *upc2-1* in the Δ*erg5* strain exacerbated this imbalance, so that the NADH/NAD^+ ratio increased 1.94-fold compared with the wild type, albeit representing only a 1.09-fold increase over BY4741-*upc2-1*. The reduction of

biomass brought about by the upregulation of oxidases can mainly be attributed to increased energy dissipation (Vemuri et al. 2007). Therefore, in order to further increase the yield of the target product ergosta-5,7-dien-3β-ol, strategies to alleviate the redox imbalance need to be developed, such as employing a water-forming NADH oxidase (NOX) and an alternative oxidase (AOX1) (Su et al. 2015).

Conclusions

Our study explored an efficient approach for the production of ergosta-5,7-dien-3β-ol, and three key enzymes - Hmg1p, Erg1p and Erg11p, were identified as catalyzing the rate-limiting steps involved in the ergosterol pathway. Other factors such as redox balance also appear to be vital for the yield of the end-product. Taken together, this study offers important guidance for further improvement of the synthesis of sterol-related intermediates involved in the post-squalene biosynthetic pathway in food-grade *S. cerevisiae* strains.

Acknowledgements This work was supported by the National Natural Science Foundation of China (No. 31400978) and the Natural Science Foundation of Zhejiang Province (No. LY18B020019).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Adams BG, Parks LW (1968) Isolation from yeast of a metabolically active water-soluble form of ergosterol. J Lipid Res 9:8–11

Aguilar PS et al (2010) Structure of sterol aliphatic chains affects yeast cell shape and cell fusion during mating. Proc Natl Acad Sci USA 107:4170–4175. https://doi.org/10.1073/pnas.0914094107

Bard M et al (1996) Cloning and characterization of ERG25, the *Saccharomyces cerevisiae* gene encoding C-4 sterol methyl oxidase. Proc Natl Acad Sci USA 93:186–190

Bloch K (1981) Sterol structure and membrane. Curr Top Cell Regul 18:289–299

Borkowski O, Bricio Garberi C, Murgiano M, Stan G-B, Ellis T (2017) Cell-free prediction of protein expression costs for growing cells. bioRxiv. https://doi.org/10.1101/172627

Caspeta L et al (2014) Biofuels. Altered sterol composition renders yeast thermotolerant. Science 346:75–78. https://doi.org/10.1126/science.1258137

Chen X, Li S, Liu L (2014) Engineering redox balance through cofactor systems. Trends Biotechnol 32:337–343. https://doi.org/10.1016/j.tibtech.2014.04.003

Cheng KK, Wang GY, Zeng J, Zhang JA (2013) Improved succinate production by metabolic engineering. BioMed Res Int 2013:538790. https://doi.org/10.1155/2013/538790

Crowley JH, Leak FW Jr, Shianna KV, Tove S, Parks LW (1998) A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. J Bacteriol 180:4177–4183

Dai Z, Liu Y, Zhang X, Shi M, Wang B, Wang D, Huang L (2013) Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides. Metab Eng 20:146–156. https://doi.org/10.1016/j.ymben.2013.10.004

Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M (1989) The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol Cell Biol 9:3447–3456

Gachotte D, Barbuch R, Gaylor J, Nickel E, Bard M (1998) Characterization of the *Saccharomyces cerevisiae* ERG26 gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis. Proc Natl Acad Sci USA 95:13794–13799

Gachotte D, Sen SE, Eckstein J, Barbuch R, Krieger M, Ray BD, Bard M (1999) Characterization of the *Saccharomyces cerevisiae ERG27* gene encoding the 3-keto reductase involved in C-4 sterol demethylation. Proc Natl Acad Sci USA 96:12655–12660

Ghodasara A, Voigt CA (2017) Balancing gene expression without library construction via a reusable sRNA pool. Nucleic Acids Res 45:8116–8127. https://doi.org/10.1093/nar/gkx530

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11:355–360. https://doi.org/10.1002/yea.320110408

Holick MF (2002) Vitamin D. Clin Rev Bone Miner Metab 1(3–4):181–207

Hughes TR et al (2000) Functional discovery via a compendium of expression profiles. Cell 102:109–126

Ignea C, Pontini M, Maffei ME, Makris AM, Kampranis SC (2014) Engineering monoterpene production in *Yeast* using a synthetic dominant negative geranyl diphosphate synthase. ACS Synth Biol 3:298–306. https://doi.org/10.1021/sb400115e

Kodedova M, Sychrova H (2015) Changes in the sterol composition of the plasma membrane affect membrane potential, salt tolerance and the activity of multidrug resistance pumps in *Saccharomyces cerevisiae*. PLoS ONE 10:e0139306. https://doi.org/10.1371/journal.pone.0139306

Korn ED, Von BT, Tobie EJ (1969) The sterols of *Trypanosoma cruzi* and *Crithidia fasciculata*. Comp Biochem Physiol 30:601

Krings U, Berger RG (2014) Dynamics of sterols and fatty acids during UV-B treatment of oyster mushroom. Food Chem 149:10–14. https://doi.org/10.1016/j.foodchem.2013.10.064

Leidig-Bruckner G, Bruckner T, Raue F, Frank-Raue K (2016) Long-term follow-up and treatment of postoperative permanent hypoparathyroidism in patients with medullary thyroid carcinoma: differences in complete and partial disease. Horm Metab Res 48:806–813. https://doi.org/10.1055/s-0042-118181

MacPherson S, Akache B, Weber S, De Deken X, Raymond M, Turcotte B (2005) *Candida albicans* zinc cluster protein *Upc2p*. confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother 49:1745–1752. https://doi.org/10.1128/Aac.49.5.1745-1752

Mo CQ, Bard M (2005) Erg28p is a key protein in the yeast sterol biosynthetic enzyme complex. J Lipid Res 46:1991–1998. https://doi.org/10.1194/jlr.M500153-JLR200

Mysiakina IS, Sergeeva YE, Ivashechkin AA, Feofilova EP (2012) Impact of morphogenetic effectors on the growth pattern and the lipid composition of the mycelium and the yeastlike cells of the fungus *Mucor hiemalis*. Microbiology 81:676–683. https://doi.org/10.1134/S0026261712060100

Nielsen JJMC (2007) The role of metabolomics in systems biology. Metabolomics 18:1–10

Parks LW, Smith SJ, Crowley JH (1995) Biochemical and physiological effects of sterol alterations in yeast: a review. Lipids 30:227–230

Phillips KM, Horst RL, Koszewski NJ, Simon. RR (2012) Vitamin D4 in mushrooms. PLoS ONE 7:e40702. https://doi.org/10.1371/journal.pone.0040702.g001

Pierson CA, Eckstein J, Barbuch R, Bard M (2004) Ergosterol gene expression in wild-type and ergosterol-deficient mutants of *Candida albicans*. Med Mycol 42:385–389

Polakowski T, Stahl U, Lang C (1998) Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Appl Microbiol Biotechnol 49:66–71

Scallen TJ (1965) Chemical synthesis of cholesta-5,7,24-trien-3-beta-ol and demonstration of its conversion to cholesterol in the rat. Biochem Biophys Res Commun 21:149–155

Sharma SC (2006) Implications of sterol structure for membrane lipid composition, fluidity and phospholipid asymmetry in *Saccharomyces cerevisiae*. FEMS Yeast Res 6:1047–1051. https://doi.org/10.1111/j.1567-1364.2006.00149.x

Shimada H, Kondo K, Fraser PD, Miura Y, Saito T, Misawa N (1998) Increased carotenoid production by the food yeast *Candida* utilis through metabolic engineering of the isoprenoid pathway. Appl Environ Microbiol 64:2676–2680

Skaggs BA et al (1996) Cloning and characterization of the *Saccharomyces cerevisiae* C-22 sterol desaturase gene, encoding a second cytochrome P-450 involved in ergosterol biosynthesis. Gene 169:105–109

Su W, Xiao WH, Wang Y, Liu D, Zhou X, Yuan YJ (2015) Alleviating redox imbalance enhances 7-dehydrocholesterol production in engineered *Saccharomyces cerevisiae*. PLoS ONE 10:e0130840. https://doi.org/10.1371/journal.pone.0130840

Sun X et al (2013) Sterol C-22 desaturase *ERG5* mediates the sensitivity to antifungal azoles in *Neurospora crassa* and *Fusarium verticillioides*. Front Microbiol 4:127. https://doi.org/10.3389/fmicb.2013.00127

Tiedje C, Holland DG, Just U, Hofken T (2007) Proteins involved in sterol synthesis interact with Ste20 and regulate cell polarity. J Cell Sci 120:3613–3624. https://doi.org/10.1242/jcs.009860

Tokuhiro K et al (2009) Overproduction of geranylgeraniol by metabolically engineered *Saccharomyces cerevisiae*. Appl Environ Microbiol 75:5536–5543. https://doi.org/10.1128/AEM.00277-09

Tsuji M, Tachibana Y, Yokoyama S, Ikekawa N (1992) 1α,25-dihydroxyvitamin D4 compounds, ergosta-5,7-diene compounds and processes for the preparation thereof. US5157135

Veen M, Stahl U, Lang C (2003) Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. FEMS Yeast Res 4:87–95. https://doi.org/10.1016/s1567-1356(03)00126-0

Vemuri GN, Eiteman MA, McEwen JE, Olsson L, Nielsen J (2007) Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 104:2402–2407. https://doi.org/10.1073/pnas.0607469104

Yang H, Tong J, Lee CW, Ha S, Eom SH, Im YJ (2015) Structural mechanism of ergosterol regulation by fungal sterol transcription factor *Upc2*. Nat Commun 6:6129. https://doi.org/10.1038/ncomms7129

Yun Y, Yin D, Dawood DH, Liu X, Chen Y, Ma Z (2014) Functional characterization of *FgERG3* and *FgERG5* associated with ergosterol biosynthesis, vegetative differentiation and virulence of *Fusarium graminearum*. Fungal Genet Biol 68:60–70. https://doi.org/10.1016/j.fgb.2014.04.010

Zhou P et al (2017) Alleviation of metabolic bottleneck by combinatorial engineering enhanced astaxanthin synthesis in *Saccharomyces cerevisiae*. Enzyme Microb Technol 100:28–36. https://doi.org/10.1016/j.enzmictec.2017.02.006