



# High-level recombinant production of squalene using selected *Saccharomyces cerevisiae* strains

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## Abstract

For recombinant production of squalene, which is a triterpenoid compound with increasing industrial applications, in microorganisms generally recognized as safe, we screened *Saccharomyces cerevisiae* strains to determine their suitability. A strong strain dependence was observed in squalene productivity among *Saccharomyces cerevisiae* strains upon overexpression of genes important for isoprenoid biosynthesis. In particular, a high level of squalene production ( $400 \pm 45$  mg/L) was obtained in shake flasks with the Y2805 strain overexpressing genes encoding a bacterial farnesyl diphosphate synthase (*ispA*) and a truncated form of hydroxyl-3-methylglutaryl-CoA reductase (*tHMG1*). Partial inhibition of squalene epoxidase by terbinafine further increased squalene production by up to 1.9-fold ( $756 \pm 36$  mg/L). Furthermore, squalene production of  $2011 \pm 75$  or  $1026 \pm 37$  mg/L was obtained from 5-L fed-batch fermentations in the presence or absence of terbinafine supplementation, respectively. These results suggest that the Y2805 strain has potential as a new alternative source of squalene production.

**Keywords** Squalene · *Saccharomyces cerevisiae* · Metabolic engineering · Farnesyl diphosphate synthase · HMG-CoA reductase

## Introduction

Squalene (2,6,10,15,19,23-hexamethyltetracosane-2,6,10,14,18,22-hexaene; C<sub>30</sub>H<sub>50</sub>) is a linear polyunsaturated triterpene with common uses mainly related to the cosmetics industry as a moisturizing agent and emollient. Recently, squalene attracted attention due to multiple studies showing its therapeutic effects and pharmaceutical applications [32, 35], including significant antitumor activities [22, 28, 33]. In addition, it has also been used as a common method of delivering drugs into cells (squalenoylation) [7] and as an emollient in adjuvants for vaccines [9].

The major commercial sources of squalene are liver oil from deep-sea sharks and certain plant-seed oils. However, continuous supply of the liver oils is uncertain due to

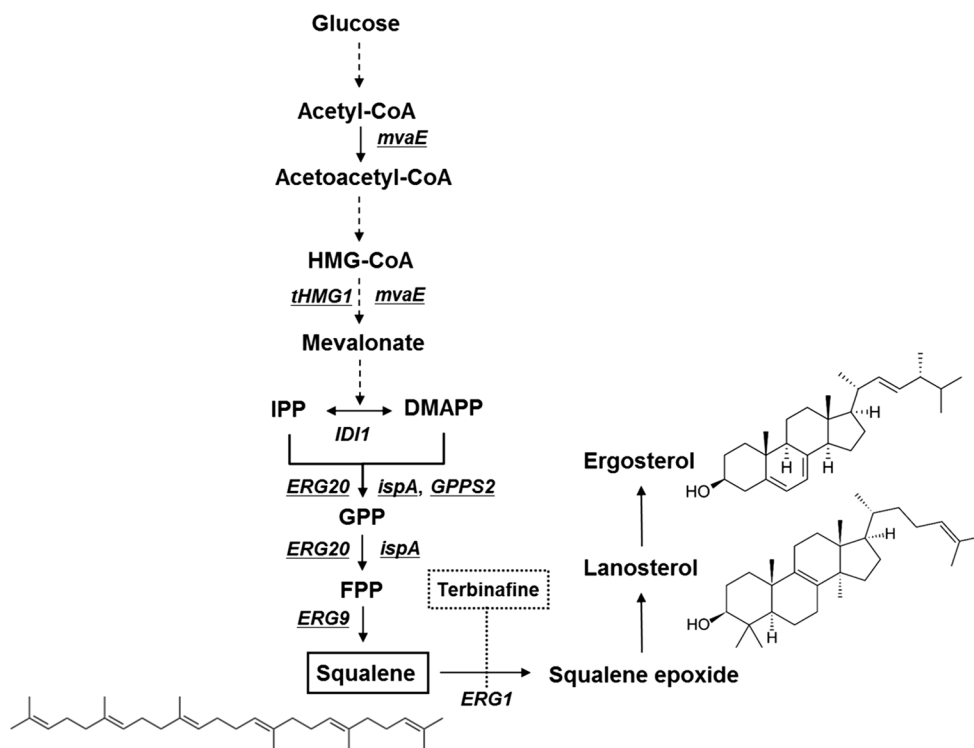
environmental concerns, and the supply of plant-seed oils is also uncertain due to the low squalene yield and unstable production in certain plant species [24, 36, 39]. Recently, microalgae and other microorganisms have emerged as new alternative sources for squalene production. Among these microorganisms, yeast is a good alternative due to the ease of genetic and physiological manipulation necessary for squalene production. In particular, *Saccharomyces cerevisiae* is an attractive microorganism for the production of isoprenoids, such as squalene, due to its intrinsically large pools of precursors [20, 21] and its status as generally recognized as safe (GRAS). Moreover, the food-grade status of microorganisms is an essential prerequisite when industrial production is intended for human consumption. Therefore, *S. cerevisiae* have been successfully employed in metabolic engineering for the production of sterols [42].

In yeast *S. cerevisiae*, squalene is synthesized as the first precursor via the mevalonate pathway to produce sterols, such as ergosterol (Fig. 1) [6]. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and squalene synthase are the major control enzymes for squalene production in the yeast metabolic pathway [6]. Overexpression of a cytosolic truncated form of HMG-CoA reductase (encoded by *tHMG1*) leads to squalene accumulation in *S. cerevisiae*

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**Fig. 1** Description of the ergosterol-biosynthesis pathway in *Saccharomyces cerevisiae*. Abbreviations of the pathway intermediates and genes are as follows: *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate, *mvaE* fused acetoacetyl-CoA thiolase/HMG-CoA reductase gene from *Enterococcus faecalis*, *tHMG1* a truncated form of HMG-CoA reductase, *IDI1* IPP isomerase, *ERG20* farnesyl diphosphate synthase, *ispA* farnesyl diphosphate synthase from *Escherichia coli*, *GPPS2* geranyl diphosphate synthase gene from *Abies grandis*, *ERG9* squalene synthase, *ERG1* squalene epoxidase. Underlined genes represent engineering targets used in this study. Broken arrows represent multiple enzymatic steps and unbroken arrows represent a single enzymatic step. The broken lined box indicates a specific inhibitor

[27]. In a recent study, Mantzouridou and Tsimidou (2010) reported that an extra copy of the *HMG2* gene with a K6R-stabilizing mutation induced a strong increase in the size of the squalene pool [21]. In addition, numerous metabolic engineering studies have been performed to improve the production of squalene in *S. cerevisiae* (summarized in Table 1). Based on these studies, squalene productivity shows a remarkable degree of strain dependence [25], with large variability in squalene production levels appearing to be a consequence of different genetic backgrounds in the strains.

In this study, we screened several *S. cerevisiae* strains with high potential for squalene accumulation to determine a suitable host for recombinant production of squalene. The selected strain, Y2805 overexpressing *tHMG1* and bacterial farnesyl pyrophosphate (FPP) synthase (*ispA*), produced a 1.7-fold higher yield of squalene as compared with strain BY4741 which has been most frequently employed as the host for metabolic engineering related to squalene overproduction. The suitability of Y2805 as a platform strain for large-scale production was confirmed through fed-batch fermentation experiments under optimal conditions. During the

course of this study, we noted that commonly used protocols for the determination of squalene content in *S. cerevisiae* showed variable results. Therefore, we compared the different protocols for squalene determination frequently used in the literature and proposed simple and efficient methods for squalene extraction from *S. cerevisiae* cells.

## Materials and methods

### Strains and plasmids

Strains and plasmids used in this study are listed in Table 2. The expression plasmid pYEG $\alpha$  and the host strain *S. cerevisiae* Y2805 were described previously [2, 34]. *S. cerevisiae* ATCC200589, ATCC201238, ATCC201741, BY4741, and W303 were obtained from the American Type Culture Collection (Manassas, VA, USA), and *S. cerevisiae* YPH499 was purchased from Stratagene (La Jolla, CA, USA). *Escherichia coli* DH5 $\alpha$  was used for plasmid construction and propagation.

**Table 1** Squalene production by metabolically engineered *S. cerevisiae* strains

Strain name	Description	Squalene titer (mg/L)	Squalene content (mg/g)	References
37 ATCC strains	ATCC strains; harboring pRS434GAP-HMG1	0.010 (ATCC200179) ~ 10.0 (ATCC201741) mg/L		[25]
AM63	EGY48; <i>Mata URA3 trp1 his3 P<sub>GAL</sub>-(K6R) HMG2:: HO</i>		18.5 mg/g dry biomass	[21]
BY4742-TRP	BY4742; <i>Trp1Δ</i>	9.6 mg/L		[5]
BY-T1	BY4742; <i>Trp1Δ δDNA::P<sub>PGK1</sub>-tHMG1-T<sub>ADH1</sub>-P<sub>TEF1</sub>-LYS2-T<sub>CYC1</sub></i>	150.9 mg/L		[5]
BY-βA-G	BY4742; <i>Trp1Δ δDNA::P<sub>PGK1</sub>-tHMG1-T<sub>ADH1</sub>-P<sub>TEF1</sub>-LYS2-T<sub>CYC1</sub> rDNA::P<sub>PGK1</sub>-GgbAS-T<sub>ADH1</sub>-P<sub>TDH3</sub>-ERG1-T<sub>TPH1</sub>-P<sub>TEF1</sub>-ERG9-T<sub>CYC1</sub></i>	183.4 mg/L		[5]
EPY213	BY4742; <i>erg9::P<sub>MET3</sub>-ERG9 P<sub>GALI</sub>-Thmgr P<sub>GALI</sub>-upc2-1</i> , harboring pRS424ADS	9 mg/L/OD <sub>600</sub>		[26]
H1246	W303; <i>MATα are1::HIS3 are2::LEU2 dgal1::KanMX4 lra1::TRP1 met ura3</i> , harboring pEG-α-synuclein		23 μg/10 <sup>9</sup> cells	[31]
HMG1/YPH499	YPH499; harboring pRS504-HMG1	191.9 mg/L		[37]
HX4HE	D452-2; <i>leu2::LEU2 pYS10 ura3::URA3 pSR6-X123 his1::HIS1 pSR3-X23 P<sub>CCW12</sub>-TAL1::P<sub>TDH3</sub>-tHMG1-T<sub>CYC1</sub>-P<sub>TDH3</sub>-ERG10-T<sub>CYC1</sub></i>	150 mg/L		[17]
JN10	CEN.PK2-1C; <i>MATα ura3 trp1 leu2::P<sub>TEF1</sub>-tHMG1 his3::coGES1</i>		15 mg/g DCW	[18]
KLN1-ERG1	JS204; <i>MATα leu2 ura3 trp1 ERG1::URA3</i> (pNS1), harboring pRS315-erg1 <sup>L37P</sup>		120 μg/10 <sup>9</sup> cells	[11]
KLN1-erg1 <sup>L37P</sup>	JS204; <i>MATα leu2 ura3 trp1 ERG1::URA3</i> (pAB1), harboring pRS315-erg1 <sup>L37P</sup>		1700 μg/10 <sup>9</sup> cells	[11]
KLN1-erg1 <sup>Q443UAG</sup>	JS204; <i>MATα leu2 ura3 trp1 ERG1::URA3</i> (pCN1), harboring pRS315-ERG1		1100 μg/10 <sup>9</sup> cells	[11]
M1EG	INVScl; <i>δ1-P<sub>HMF2</sub>-HMG1 P<sub>IRA1</sub>-ID11 P<sub>PET9</sub>-ERG20 P<sub>TEF1</sub>-hphNT1 and P<sub>RHO1</sub>-ERG9-δ2</i>	34 mg/L		[29]
Y15922	BY4742; <i>MATα his3Δ1 leuΔ0 lys2Δ0 ura3Δ0 yta7::kanMX4</i>		114 μg/g DCW	[16]
YIP-MC-05	CEN.PK113-5D; <i>MATα MAL2-8<sup>C</sup> SUC2 ura3-52</i> , harboring pGAL1-GFTpsC and pGAL10-tHMG1		9 mg/g DCW	[1]
YIP-MC-10	CEN.PK113-5D; <i>MATα MAL2-8<sup>C</sup> SUC2 ura3::P<sub>TPH1</sub>-tHMG1</i> , harboring pGAL1-GFTpsC and pGAL10-tHMG1		8 mg/g DCW	[1]
YUG37-ERG1	YUG37; <i>erg1Δ</i>	1.76 mg/L		[14]
YXWP-P3-01	BY4741; <i>Δgal80::LEU2 Δho::T<sub>TPS1</sub>-tHMG1-P<sub>GAL7</sub>-P<sub>GAL2</sub>-CrtYB11 M-T<sub>PGK1</sub>-T<sub>CYC1</sub>-CrtI-P<sub>GALI</sub>-P<sub>GALI0</sub>-CrtE-T<sub>ADH1</sub></i>		25.6 mg/g DCW	[43]
ZD-PPD-010	BY4742; <i>rDNA::P<sub>PGK1</sub>-PgDDS-T<sub>ADH1</sub> P<sub>TDH3</sub>-AtCPRI-T<sub>TPH1</sub> and P<sub>TEF</sub>-PgPPDS-T<sub>CYC1</sub> P<sub>PGK1</sub>-tHMG1-T<sub>ADH1</sub></i>		13.8 mg/g DCW	[4]
ZD-PPD-013	BY4742; <i>rDNA::P<sub>PGK1</sub>-PgDDS-T<sub>ADH1</sub> P<sub>TDH3</sub>-AtCPRI-T<sub>TPH1</sub> and P<sub>TEF</sub>-PgPPDS-T<sub>CYC1</sub> P<sub>PGK1</sub>-tHMG1-T<sub>ADH1</sub> P<sub>PGK1</sub>-ERG20-T<sub>ADH1</sub> P<sub>TEF1</sub>-ERG20-T<sub>CYC1</sub></i>		16.5 mg/g DCW	[4]
ZD-T-012	BY4742; <i>rDNA::SmCPS and SmKSL</i> , harboring pHLeu-tHMGR-UPC2.1	78.0 mg/L		[3]
ZD-T-022	BY4742; <i>rDNA::SmCPS and SmKSL</i> , harboring pHKanMX-tHMGR-UPC2.1	852.0 mg/L		[3]
ZXA	BY4741; <i>SUE erg9Δ::HPH erg1Δ::KanMX4</i>	87.2 mg/L		[44]
ZXB	BY4741; <i>SUE erg9Δ::HPH erg1Δ::tHMGR-KanMX4</i>	250 mg/L		[44]
βamy4	BY4742; <i>rDNA::P<sub>GAL</sub>-tHMG1 P<sub>MET3</sub>-ERG7</i> , harboring pESC-AaBAS	10 μg/mL		[15]

**Table 2** Strains and plasmids used in this study

Name	Description	References
<b>Strains</b>		
<i>E. coli</i> DH5 $\alpha$	<i>F</i> <sup>-</sup> $\Phi$ 80 <i>lacZ</i> $\Delta$ <i>M15</i> $\Delta$ <i>lacU169</i> <i>thi-1</i> <i>endA1</i> <i>hsdR17</i> (r <sup>-</sup> m <sup>-</sup> ) <i>supE44</i> ) <i>recA1</i> <i>gyrA96</i> <i>relA1</i>	ATCC98040
<i>S. cerevisiae</i> ATCC200589	<i>MAT</i> $\alpha$ <i>can1</i> <i>leu2</i> <i>trp1</i> <i>ura3</i> <i>aro7</i>	ATCC200589
<i>S. cerevisiae</i> ATCC201238	<i>MAT</i> $\alpha$ <i>leu2-3</i> <i>leu2-112</i> <i>trp1-1</i> <i>ura3-1</i> <i>his3-11</i> <i>his3-15</i> <i>ade2-1</i> <i>can1-100</i>	ATCC201238
<i>S. cerevisiae</i> ATCC201741	<i>MAT</i> $\alpha$ <i>ade2-1</i> <i>trp1-1</i> <i>can1-100</i> <i>leu2-3</i> <i>leu2-112</i> <i>his3-11</i> <i>his3-15</i> <i>ura3-53</i> <i>GAL</i> <sup>+</sup>	ATCC201741
<i>S. cerevisiae</i> BY4741	<i>MAT</i> $\alpha$ <i>his3</i> $\Delta$ 1 <i>leu2</i> $\Delta$ 0 <i>met15</i> $\Delta$ 0 <i>ura3</i> $\Delta$ 0	ATCC201388
<i>S. cerevisiae</i> W303	<i>MAT</i> $\alpha$ <i>can1-100</i> <i>ade2-1</i> <i>his3-11,15</i> <i>leu2-3</i> <i>trp1-1</i> <i>ura3-1</i>	ATCC200060
<i>S. cerevisiae</i> Y2805	<i>MAT</i> $\alpha$ <i>pep4::HIS3</i> <i>prb1-<math>\Delta</math>1.6R</i> <i>can1</i> <i>his3-20</i> <i>ura3-52</i>	[2, 34]
<i>S. cerevisiae</i> YPH499	<i>MAT</i> $\alpha$ <i>ura3-52</i> <i>lys2-801</i> <i>ade2-101</i> <i>trp1-<math>\Delta</math>63</i> <i>his3-<math>\Delta</math>200</i> <i>leu2-<math>\Delta</math>1</i>	Stratagene
<b>Plasmids</b>		
pYEG $\alpha$	pBR322 origin, yeast 2 $\mu$ origin, <i>GAL10</i> promoter, <i>URA3</i> , Amp <sup>r</sup>	[34]
pG-tHMG1	pYEG $\alpha$ carrying <i>tHMG1</i> gene	This study
pG-ERG20	pYEG $\alpha$ carrying <i>ERG20</i> gene	This study
pG-ERG9	pYEG $\alpha$ carrying <i>ERG9</i> gene	This study
pG-mvaE	pYEG $\alpha$ carrying <i>mvaE</i> gene	This study
pG-ispA	pYEG $\alpha$ carrying <i>ispA</i> gene	This study
pG-GPPS2	pYEG $\alpha$ carrying <i>GPPS2</i> gene	This study
pG-H-I	pYEG $\alpha$ carrying <i>tHMG1</i> and <i>ispA</i>	This study
pG-H-E20	pYEG $\alpha$ carrying <i>tHMG1</i> and <i>ERG20</i>	This study
pG-H-G	pYEG $\alpha$ carrying <i>tHMG1</i> and <i>GPPS2</i>	This study
pG-M-I	pYEG $\alpha$ carrying <i>mvaE</i> and <i>ispA</i>	This study
pG-M-G	pYEG $\alpha$ carrying <i>mvaE</i> and <i>GPPS2</i>	This study
pG-H-E9	pYEG $\alpha$ carrying <i>tHMG1</i> and <i>ERG9</i>	This study

ATCC American Type Culture Collection

**Table 3** Primers used in this study

Name	Oligonucleotides (5'–3')	Description
tHMG1-F	ATGGAATTCAAAAATGGACCAATTGGTGA	pG-tHMG1
tHMG1-R	GAGGTCGACTTAGGATTTAATGCAGGT	pG-tHMG1
ERG20-F	ATGGAATTCAAAAATGGCTTCAGAAAAAGAAATTAGGAGAGAG	pG-ERG20
ERG20-R	GAGCTCGAGTTATTTGCTTCTCTTGTAACCTTTGTTCAAG	pG-ERG20
ERG9-F	ATGGAATTCAAAAATGGGAAAGCTATTACAATTGGC	pG-ERG9
ERG9-R	GAGGTCGACTCACGCTCTGTGTAAGTGATATATA	pG-ERG9
mvaE-F	ATGGAATTCAAAAATGAAGACTGTCGTTATTATTGATGC	pG-mvaE
mvaE-R	GAGGTCGACCTATAGTCCTTGATATCTGCCTTCTTT	pG-mvaE
ispA-F	ATGGAATTCAAAAATGGACTTTCCGCAGCAACTCGAA	pG-ispA
ispA-R	GAGGTCGACTTATTTATTACGCTGGATGATGTAGTCCG	pG-ispA
GPPS2-F	ATGGAATTCAAAAATGTACATGGATTCCAAGGCAATG	pG-GPPS2
GPPS2-R	GAGGTCGACTTAATTTTGGTCTGAATGCCACGTAATC	pG-GPPS2
Infusion smaI-F	TCGAGCTCGGTACCCGGGATCCATCGCTTCGCTGATTAA	Various combinations
Infusion smaI-R	AAGCGATGGATCCCCACAATGAGCCTTGCTGCAACATCC	Various combinations

Restriction sites are bolded

## Plasmid construction

The primers designed for plasmid construction are listed in Table 3. The reagents for molecular manipulations were obtained from Takara (Shiga, Japan) and Qiagen (Valencia, CA, USA). DNA fragments were amplified by polymerase chain reaction (PCR) using a Phusion Hot Start II high-fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The *tHMG1* (a truncated form of HMG-CoA reductase lacking the regulatory membrane-spanning domain), yeast farnesyl diphosphate synthase (*ERG20*), and squalene synthase (*ERG9*) genes were amplified using corresponding primers, digested with *EcoRI* and *SalI* (*XhoI* for *ERG20*), and placed downstream of the *GAL10* promoter by cloning into the *EcoRI* and *SalI* sites of pYEG $\alpha$  to obtain plasmids pG-tHMG1, pG-ERG20, and pG-ERG9, respectively. The DNA sequences of acetyl-CoA acetyltransferase/HMG-CoA reductase (*mvaE*) from *Enterococcus faecalis*, *ispA* from *E. coli*, and geranyl diphosphate synthase (*GPPS2*) from *Abies grandis* were codon optimized for expression in yeast and synthesized (GenScript Corp., Piscataway, NJ, USA). The synthesized *mvaE*, *ispA*, and *GPPS2* genes were digested with *EcoRI/SalI* and ligated into the corresponding sites in pYEG $\alpha$  to obtain plasmids pG-mvaE, pG-ispA, and pG-GPPS2, respectively. To construct plasmids pG-H-I and pG-H-E20, cloning was performed using the InFusion HD cloning kit (Takara). First, the *tHMG1* fragment, including promoter (521 bp) and terminator (427 bp) regions, was amplified from plasmid pG-tHMG1 using the primer pair Infusion smaI-F and Infusion smaI-R. The PCR product was inserted into the *SmaI*-digested pG-ispA and pG-ERG20 plasmids by homologous recombination, generating plasmids pG-H-I and pG-H-E20, respectively. Combinations of other genes were constructed using the same method.

## Chemicals, media, and culture conditions

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and BD Bioscience (Franklin Lakes, NJ, USA). UD medium (20-g/L glucose, 6.7-g/L yeast nitrogen base without amino acids, and 0.77-g/L Ura dropout supplement mixture) was used for transformant selection and pre-cultures of recombinant yeasts harboring episomal plasmids. YPD medium (10-g/L yeast extract, 20-g/L peptone, and 20-g/L glucose) and YPDG medium (10-g/L yeast extract, 20-g/L peptone, 10-g/L glucose, and 10-g/L galactose) were used for fermentation experiments in baffled flasks, with galactose used for induction of genes under the control of galactose-inducible promoters. The plasmids were transformed into the *S. cerevisiae* strains using the LiAc/TE method [13], and cells were plated on UD agar medium for selection of transformants. The colonies formed on the

plates were checked by PCR for the presence of the correct plasmids. Test-tube cultures containing 3 mL of UD medium were used as seed cultures and were grown overnight at 30 °C before use to inoculate cultures in 250-mL baffled flasks containing 25 mL of medium at an OD<sub>600</sub> of 0.1. Flask cultures were performed in triplicate at 30 °C and 200 rpm in a shaking incubator (HB-201MS-2R; Hanbaek Scientific Co., Bucheon, Korea) for 72 h.

## Fed-batch fermentation

Fed-batch fermentations were performed in a 5-L fermenter (CNS, Daejeon, Korea), with an initial medium comprising 10-g/L yeast extract, 20-g/L peptone, and 20-g/L glucose. A pre-culture was prepared by inoculating a single colony into 25 mL of UD medium for 24 h, followed by transfer of the culture into a 1-L baffled flask containing 200 mL of YPD medium and growth for 16 h. The seed culture was then inoculated into a 5-L fermenter containing 2-L medium. Fed-batch fermentations were performed at 30 °C with aeration at 1 vvm and agitation at 600 rpm was maintained using two six-bladed impellers. Culture pH was maintained at 6.0 by automatic feeding of a 10-N NaOH solution. Dissolved oxygen was monitored using OxyProbe (Broadley-James, Irvine, CA, USA) and maintained at > 30% saturation with an agitation cascade. The temperature, air-flow rate, agitation, pH, and dissolved oxygen were monitored and controlled using CNS monitoring and control systems (CNS). A feeding media comprising 600-g/L glucose and 40-g/L yeast extract was fed to the 5-L fermenter at specific rates of between 0-g/L/h and 5-g/L/h following depletion of the initially added glucose. Galactose was added as an inducer every 24 h at 5 g/L, and antifoam 204 (Sigma-Aldrich) was added manually when foam was generated during fermentation. The culture was sampled every 12 h to measure cell growth, glucose concentration, and squalene production.

## Squalene analysis

Four different protocols for squalene extraction in *S. cerevisiae* were tested: the hot alcoholic KOH–dodecane method [30], the hot HCl–acetone method [10], the bead-methanolic KOH–hexane method [41], and the bead–pentane method (this study). The procedure for the hot alcoholic KOH–dodecane method is as follows. Cells from 2 mL of the yeast culture were collected by centrifugation at 15,000g and resuspended in 0.4 mL of the alcoholic KOH solution. Resuspended cells were placed into boiling water for 5 min and allowed to cool on ice. Dodecane (0.4 mL) was added to the resuspended cells and vortexed vigorously for 5 min. After centrifugation at 3,000g for 5 min, the dodecane layer was analyzed by gas chromatography (GC). For the hot HCl–acetone method, cells were pelleted and treated with 3-M



HCl at 95 °C for 5 min, chilled on ice for 10 min, and then washed with distilled water before extracting squalene with 1 mL of acetone. The extracts were centrifuged at 13,000g for 5 min, and the supernatants were analyzed by high-performance liquid chromatography (HPLC) using an Agilent 1200 instrument equipped with a C18 column and a UV detector (Agilent Technologies, Santa Clara, CA, USA). For the bead-methanolic KOH-hexane method, cells were harvested by centrifugation for 5 min, washed with deionized water, and disrupted with glass beads. The cell homogenate was incubated in three volumes of methanolic KOH at 70 °C for 2 h. The saponification mixture was extracted twice with three volumes of hexane, and the combined extracts were dried under nitrogen. The lipid residue containing squalene was dissolved in a desired volume of hexane, and the sample was analyzed by HPLC. The detailed procedures of these protocols were described previously [10, 30, 41] and the bead-pentane method used in this study is subsequently described here in detail.

For the bead-pentane protocol, cells from 1 mL of yeast culture were pelleted and resuspended in 600 µL of 50-mM Tris-HCl (pH 7.5). Glass beads (425–600 µm; Sigma-Aldrich) were added to the resuspended cells, disrupted by vortexing vigorously for 10 min, and interspersed with cooling on ice for 5 min. Pentane (400 µL) containing 0.1% (v/v) methyl laurate as an internal standard was added to the resuspended cells, mixed by vortexing for 10 min, and centrifuged at 10,000g for 10 min. When necessary, a saponification step (boiling with 0.4 mL of alcoholic KOH) described in the first protocol was included following the bead-beating step. The top pentane layer was analyzed by GC using an Agilent 7890A (Agilent Technologies) equipped with a flame-ionization detector and an HP-5 capillary column (30 m × 0.25 µm; Agilent Technologies). The sample (2 µL) from the pentane layer was analyzed in a capillary column with a split ratio of 10 using helium as a carrier gas and at a flow rate of 1 mL/min. The GC oven temperature was initially held at 40 °C for 3 min, followed by an increase to 240 °C at a rate of 10 °C/min where it was maintained for 5 min until increasing to 300 °C at a rate of 10 °C/min and maintained for 1 min. The injector temperature was 300 °C and the detector temperature was 320 °C. Squalene quantification was performed using standard curves. All analyses were performed in duplicate.

## Results and discussion

### Basal levels of squalene in non-engineered *S. cerevisiae* strains

In *S. cerevisiae*, squalene biosynthesis is tightly regulated and accumulates to a negligible level during normal growth.

Under normal conditions, yeast cells rapidly metabolize squalene to sterols, such as ergosterol, which is an essential component for maintaining yeast-cell viability and is synthesized from squalene as the first precursor. Many enzymes are involved in the squalene conversion to ergosterol, but as the first step of the ergosterol-biosynthesis pathway, squalene epoxidase encoded by *ERG1* is the key enzyme necessary for increased squalene production. Partial inhibition of squalene epoxidase activity with the addition of its inhibitor, terbinafine, resulted in squalene accumulation [8, 11, 23]. The basal levels of squalene reported for different strains in the absence or presence of terbinafine are summarized in Table 4 and ranged from 0.056- to 0.8-mg/g dry cell weight (DCW) and from 0.5- to 34-mg/L in the absence of terbinafine. In the presence of terbinafine, squalene accumulation increased from 0.31 to 10.02 mg/g DCW or from 34 to 119.8 mg/L. In this study, we compared strains of *S. cerevisiae* (BY4741, W303, and YPH499) frequently used for squalene accumulation (Tables 1 and 4) and ATCC strains (ATCC200589, 201238, and 201741) reportedly exhibiting comparatively enhanced levels of squalene accumulation among the 37 strains tested upon *HMG1* overexpression (Table 1) [25]. Our laboratory strain, Y2805, which has been previously used for isoprenoid production (unpublished data), was also included for comparison. The level of squalene accumulation among the seven strains in the presence or absence of terbinafine was determined in shake-flask experiments. The squalene content of the seven strains ranged from  $0.3 \pm 0.01$  to  $1.4 \pm 0.3$  mg/L in the absence of terbinafine and from  $11.4 \pm 0.3$  (BY4741) to  $40.5 \pm 4.5$  mg/L (W303) in the presence of terbinafine when assayed using the bead-pentane method (a comparison of the squalene-determination protocols is provided later) (Fig. 2). We noted that despite the more frequent use of BY4741 and 4742 as hosts for metabolic engineering to improve squalene production (Table 1), the non-engineered BY4741 strain showed the lowest level of squalene accumulation, even in the presence of terbinafine (Fig. 2). By contrast, the W303 strain showed the highest level of squalene accumulation in the presence of terbinafine; however, following metabolic engineering, the level of squalene accumulation was negligible (more details are provided later). These contradictory results suggest that squalene accumulation in yeast was highly dependent upon strains with different genetic backgrounds, and that other yeast strains might also represent potential as hosts for squalene production.

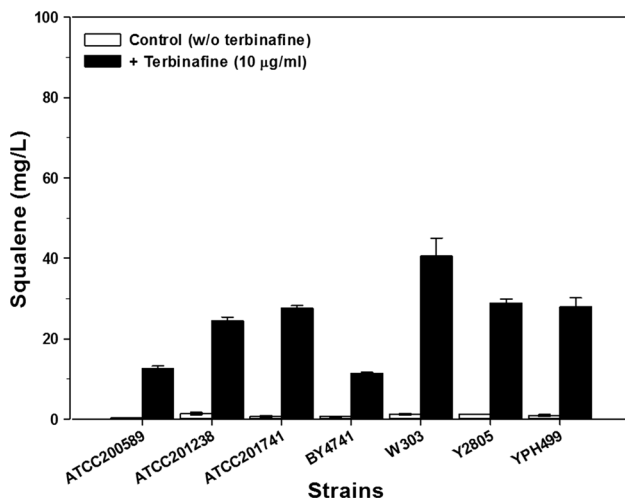
### Overexpression of genes involved in the early steps of ergosterol biosynthesis in *S. cerevisiae*

Although *S. cerevisiae* is an attractive host for the production of isoprenoids, such as squalene, due to the intrinsically large pools of isoprenoid precursors, these intermediates do

**Table 4** Squalene production by non-engineered *S. cerevisiae* strains

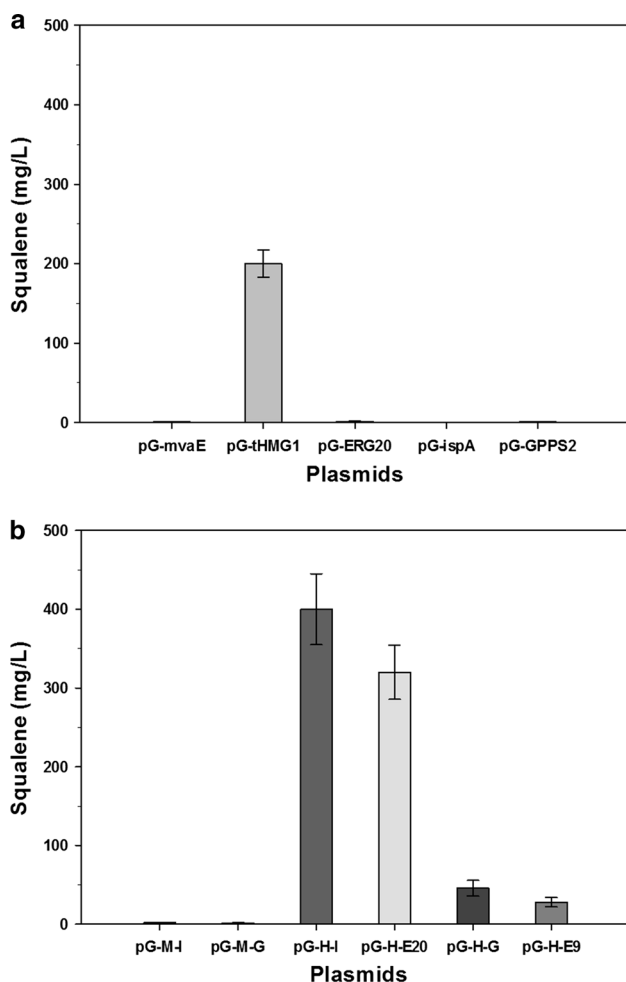
Strain name	Description	Squalene titer (mg/L)	Squalene content (mg/g)	References
BY4742	<i>MATα his3Δ1 leuΔ0 lys2Δ0 ura3Δ0</i>		56 μg/g DCW	[16]
BY4742	<i>MATα his3Δ1 leuΔ0 lys2Δ0 MET15 ura3Δ0</i>		0.8 mg/g DCW	[4]
CEN.PK113-7D	<i>MATα MAL2-8<sup>C</sup> SUC2 ura3-52</i>		0.2 mg/g DCW	[1]
EGY48	<i>MATα ura3Δ trp1Δ his3Δ 6LexA-operator-LEU2</i>		0.31 mg/g dry biomass	[23]
EGY48	EGY48; + 0.442 mM terbinafine and 0.044 mM methyl jamonate		10.02 mg/g dry biomass	[23]
G175	<i>MATα his3Δ leu2Δ trp1Δ ura3Δ</i>		23 μg/10 <sup>9</sup> cells	[31]
INVScl	<i>MATα his3Δ1 leu2 trp1-289 ura3-52/MATα his3Δ1 leu2 trp1-289 ura3-52</i>	3.1 mg/mL		[29]
M1EG	INVScl; $\delta 1$ - <i>P<sub>HMF2</sub>-HMG1</i> , <i>P<sub>IRA1</sub>-IDI1</i> , <i>P<sub>PET9</sub>-ERG20</i> , <i>P<sub>TEF1</sub>-hphNT1</i> and <i>P<sub>RHO1</sub>-ERG9-<math>\delta 2</math></i>	34 mg/L		[29]
M1EG	INVScl; $\delta 1$ - <i>P<sub>HMF2</sub>-HMG1</i> , <i>P<sub>IRA1</sub>-IDI1</i> , <i>P<sub>PET9</sub>-ERG20</i> , <i>P<sub>TEF1</sub>-hphNT1</i> and <i>P<sub>RHO1</sub>-ERG9-<math>\delta 2</math></i> + 30 ng/mL terbinafine	119.08 mg/L		[29]
W303-1B	<i>MATα ade2-1 ura3-52 trp1-1 leu2-3,112 his3-11,15 can1-100</i>		–	[8]
W303-1B	W303-1B; + 7.5 μg/mL terbinafine		300 μg/10 <sup>9</sup> cells	[8]
W303-1B	W303-1B; + 10 μg/mL terbinafine		400 μg/10 <sup>9</sup> cells	[40]
W303-1B LD-less mutant	W303-1B; <i>dga1Δ lro1Δ are1Δ are2Δ</i> + 2.5 μg/mL terbinafine		220 μg/10 <sup>9</sup> cells	[40]
YPH499	<i>MATα lys2-801 ade2-101 trp1-<math>\Delta 63</math> his3-<math>\Delta 200</math> leu2-<math>\Delta 1</math> ura3-52</i>	–		[37]
YUG37	<i>MATα ura3-52 trp1-63 leu-<math>\Delta 1</math>::ITA-LEU2</i>	0.50 mg/L		[14]
YUG37- <i>ERG1</i>	YUG37; <i>erg1Δ</i>	1.76 mg/L		[14]
YUG37- <i>ERG1</i>	YUG37; <i>erg1Δ</i> + 0.025 μg/mL doxycycline	18.0 mg/L		[14]

– Not detected



**Fig. 2** Squalene accumulation in non-engineered *Saccharomyces cerevisiae* strains in the absence and presence of terbinafine. *S. cerevisiae* strains were grown for 72 h in YPD medium containing terbinafine in shake flasks. White bars indicate squalene production by *S. cerevisiae* strains grown in medium without terbinafine (control). Black bars indicate squalene production by *S. cerevisiae* strains grown in medium containing 10 μg/mL terbinafine. Error bars represent the standard deviation from three independent cultivations

not usually accumulate in the cell. To overproduce squalene, we overexpressed enzymes required for the biosynthesis of important intermediates (mevalonate and prenyl diphosphate) during the early steps of isoprenoid biosynthesis. Therefore, the genes *mvaE*, *tHMG1*, *ERG20*, *ispA*, and *GPPS2* were overexpressed in *S. cerevisiae* Y2805 and evaluated as potential key enzyme for squalene production (Fig. 1). In addition, *ERG9*, which is necessary for squalene synthesis in *S. cerevisiae*, was also tested. The genes encoding these enzymes were cloned in a yeast-expression plasmid (pYEG $\alpha$ ) in various combinations, and *S. cerevisiae* Y2805 strains harboring these plasmids were cultivated in a shake flask containing YPDG medium for 72 h. As shown in Fig. 3a, *S. cerevisiae* Y2805 strains overexpressing *ERG20*, *mvaE*, *ispA*, or *GPPS2* alone did not produce any detectable amount of squalene, whereas Y2805 strain overexpressing *tHMG1*, the gene involved in the first committed step of ergosterol biosynthesis, produced high levels of squalene (200 ± 17 mg/L). The effect of *tHMG1* overexpression on squalene accumulation was also tested in other commonly used *S. cerevisiae* strains, including W303 and YPH499. In contrast to the high degree of squalene accumulation in Y2805, levels in W303 and YPH499 were negligible (2.4 ± 0.4 and 9.2 ± 1.5 mg/L, respectively). Numerous



**Fig. 3** Comparison of squalene production in *Saccharomyces cerevisiae* Y2805 overexpressing various genes. Squalene production by *S. cerevisiae* Y2805 overexpressing individual genes (a) and various combinations (b) after a 72-h cultivation. Strains were grown at 30 °C and 200 rpm in YPDG medium for 72 h. Abbreviations of plasmids are as follows: pG-M-I overexpressing *mvaE* and *ispA*, pG-M-G overexpressing *mvaE* and *GPPS2*, pG-H-I overexpressing *tHMG1* and *ispA*; pG-H-E20 overexpressing *tHMG1* and *ERG20*, pG-H-G overexpressing *tHMG1* and *GPPS2*, pG-H-E9 overexpressing *tHMG1* and *ERG9*. Error bars represent the standard deviation from three independent cultivations

reports previously showed that *tHMG1* overexpression results in improved squalene production in yeast (Table 1). Moreover, compared with the wild-type EGY48 strain, squalene production increased by 20-fold in *tHMG1*-overexpressing variants [21]. Polakowski et al. (1998) also reported that squalene accumulation was increased by overexpressing the truncated *HMG1* gene [27].

Co-expression of the *ispA* or *ERG20* gene with the *tHMG1* gene further increased squalene production (Fig. 3b). In particular, the Y2805 strain harboring pG-H-I (co-overexpression of the *tHMG1* and *ispA* genes) resulted in a significant increase in squalene production by

up to twofold ( $400 \pm 45$  mg/L) as compared with the same strain overexpressing *tHMG1* alone. Wang et al. (2010) also reported that overexpression of the *ispA* gene significantly increased farnesol production in an *E. coli* incorporating the exogenous mevalonate pathway. However, overexpression of *GPPS2* or *ERG9* along with *tHMG1* was ineffective for squalene accumulation, resulting in squalene production levels lower than those observed in strains overexpressing *tHMG1* alone. These results suggested that truncated HMG-CoA reductase encoded by *tHMG1* and FPP synthase encoded by *ispA* were the two key enzymes involved in the isoprenoid-biosynthesis pathway for production of isoprenoids, such as squalene.

### Comparative evaluation of protocols for squalene determination in *S. cerevisiae*

Several protocols for squalene determination in *S. cerevisiae* are available in the literature [10, 30, 41], and comprise steps for cell breakage, saponification, and solvent extraction, although, in some cases, the saponification step is omitted. Cell breakage is commonly performed by bead-beating with glass beads, whereas some protocols describe boiling in the presence of strong acid or alkali to omit the bead-beating step. Here, we compared protocols commonly used for squalene determination in *S. cerevisiae* and also compared these with our new protocol. Four different protocols were evaluated using *S. cerevisiae* strain Y2805 overexpressing *tHMG1* and *ispA*. Two protocols, the hot alcoholic KOH-dodecane [30] and hot HCl-acetone [10] methods, omit the bead-beating step for cell breakage and instead employ treatment with boiling alcoholic KOH or hot HCl, followed by solvent extraction with dodecane or acetone. The boiling alcoholic KOH treatment in the former protocol appears to serve also as the saponification step. The other two protocols include bead-beating with glass beads for cell breakage in the presence or absence of the saponification step. The third protocol (bead-methanolic KOH-hexane) [41] consists of separate steps of cell disruption with bead-beating, saponification with hot methanolic KOH, and extraction with hexane. The final protocol (bead-pentane) developed in this study included cell breakage with bead-beating and extraction with pentane.

As shown in Table 5, the hot alcoholic KOH-dodecane protocol yielded considerably lower efficiency of squalene extraction ( $27.7 \pm 2.8$  mg/L) as compared with other protocols, likely due to the omission of physical disruption of the cell. By contrast, the hot HCl-acetone protocol resulted in a good extraction efficiency ( $317 \pm 27$  mg/L), but also not as good as that observed from protocols involving bead-beating. The addition of a cell breakage step (bead-beating with glass beads) replacing the boiling treatment in the hot alcoholic KOH-dodecane protocol greatly improved



**Table 5** Comparison of different protocols for squalene extraction in *S. cerevisiae*

Protocol	Description	Squalene production	References
Hot alcoholic KOH–dodecane	Boiling with alcoholic KOH, followed by dodecane extraction	27.7 ± 2.8 mg/L	[30]
Bead-alcoholic KOH–dodecane	Same as above, but with bead-beating instead of boiling	384 ± 13 mg/L	This study
Bead-alcoholic KOH–pentane	Same as above, but pentane replacing dodecane	404 ± 41 mg/L	This study
Hot HCl–acetone	Hot HCl treatment followed by acetone extraction	317 ± 27 mg/L	[10]
Bead-methanolic KOH–hexane	Bead-beating, followed by saponification and hexane extraction	256 ± 31 mg/L	[41]
Bead–pentane	Bead-beating followed by pentane extraction	400 ± 35 mg/L	This study
Bead–dodecane	Same as above, but dodecane replacing pentane	379 ± 24 mg/L	This study
Bead–pentane-alcoholic KOH	Bead-beating, followed by saponification and pentane extraction	395 ± 10 mg/L	This study

squalene extraction, resulting in levels ( $384 \pm 13$  mg/L) comparable with those obtained in other protocols. Therefore, these findings suggested that the bead-beating process frequently used for cell breakage in yeast-cell biology is a prerequisite for efficient extraction of squalene from yeast cells, and that this process cannot be adequately replaced with the use of strong acid or alkali treatment with heating. The bead–pentane protocol appeared to be the most efficient method, yielding  $400 \pm 35$  mg/L. Notably, the omission of the saponification step did not influence the efficiency of squalene extraction ( $395 \pm 10$  vs.  $400 \pm 35$  mg/L); however, the choice of extraction solvent did influence efficiency, with pentane being slightly superior to dodecane ( $404 \pm 41$  vs.  $384 \pm 13$  mg/L and  $400 \pm 35$  vs.  $379 \pm 24$  mg/L).

These data suggested that the bead–pentane protocol proposed in this study might serve as a simple and efficient method for squalene extraction from *S. cerevisiae* cells. Spiking with a known amount of squalene in the bead–pentane protocol showed an excellent recovery percentage (97%), which confirmed its potential use as a simple and efficient method for squalene extraction from yeast. Spiking experiments in the other protocols suggested that the additional squalene appeared to be lost (20–30%) during this process, possibly explaining the poor extraction yields from these two methods (hot alcoholic KOH–dodecane and bead-methanolic KOH–hexane).

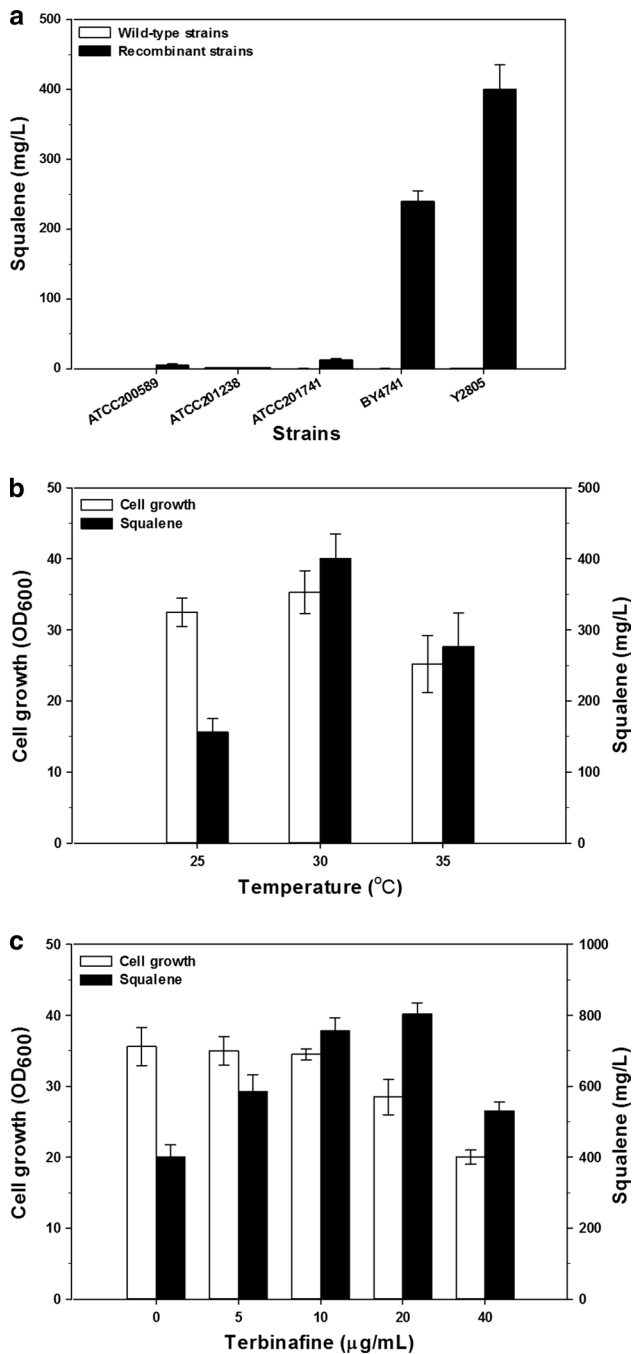
### Strain dependence for squalene accumulation from engineered *S. cerevisiae* strains overexpressing *tHMG1* and *ispA*

Using the new protocol described here, the strains employed for testing basal squalene levels were evaluated for their potential use as hosts for squalene accumulation following metabolic engineering. The ATCC strains 200589, 201238, and 201741, which reportedly accumulated high levels of squalene when engineered to overexpress *HMG1* [25], were selected for this test, and the BY4741 strain, the most frequently used host for engineering the squalene-biosynthesis pathway, was also included. The YPH499 and W303 strains

were not tested, because squalene accumulation was negligible in these strains following engineering for *tHMG1* overexpression.

The ATCC strains and BY4741 were transformed with the plasmid pG-H-I, which resulted in the highest squalene production, and their squalene production levels were determined after cultivation in a shake flask for 72 h and compared with those from the Y2805 strain containing the same plasmid. As shown in Fig. 4a, recombinant strains harboring pG-H-I exhibited higher squalene production as compared with wild-type strains without recombinant plasmids. The Y2805 strain produced the highest squalene levels ( $400 \pm 35$  mg/L), followed by BY4741, which produced  $240 \pm 15$  mg/L squalene. The three ATCC strains (ATCC200589, ATCC201238, and ATCC201741) harboring pG-H-I produced a considerably lower amounts of squalene ( $5.2 \pm 1.6$ ,  $1.6 \pm 0.5$ , and  $12.8 \pm 2.0$  mg/L, respectively) as compared with the other two strains (BY4741 and Y2805) harboring the same plasmid. Despite the non-engineered BY4741 strain showing the lowest squalene accumulation in the presence of terbinafine, the metabolically engineered BY4741 strain showed a considerably higher squalene level as compared with the ATCC strains. Therefore, determining the level of squalene accumulation in non-engineered strains in the presence of terbinafine did not represent a criterion for the selection of host strains for engineering yeast metabolic pathways for squalene biosynthesis.

Our results showed that Y2805 was the optimal strain among the seven yeast strains tested for squalene production. To investigate variations in genetic background possibly responsible for this pronounced difference in squalene accumulation among strains, the nucleotide sequences of the relevant genes important for squalene biosynthesis were determined for Y2805 and ATCC200589. The genes (*ERG9*, *ERG1*, and *MGA2*) did not show any variations in nucleotide sequences in the coding and promoter region between each other or as compared with the standard strain (288C). Therefore, it remains unclear why Y2805 accumulated considerably higher amounts of squalene as compared with the other tested strains. Further studies at the levels of



**Fig. 4** Optimization of squalene production in *Saccharomyces cerevisiae* strains. **a** Comparison of squalene production by different *S. cerevisiae* strains. White bars indicate squalene production in wild-type yeast strains, and black bars indicate squalene production in yeast recombinant strains harboring pG-H-I. **b** Effect of cultivation temperature on squalene production in *S. cerevisiae* Y2805 harboring pG-H-I. **c** Inhibition effect of terbinafine in *S. cerevisiae* harboring pG-H-I. White bars indicate cell growth of Y2805 and black bars indicate squalene production in Y2805. Strains were grown in YPDG medium for 72 h in all cases. Error bars represent the standard deviation from three independent cultivations

transcription and translation along with other characterizations are required.

### Optimization of fermentation conditions for squalene production

Prior to fermentation studies, fermentation conditions, especially with respect to the temperature, were optimized in flask experiments. According to the previous studies, squalene levels in yeast cells are altered by changing culture conditions, such as media composition and cultivation temperature [12, 19, 38]. Among these, temperature optimization is a facile method used to improve the production of desired compounds and affect cell growth. Recently, Henderson et al. (2013) reported that ethanol fermentation using *S. cerevisiae* strains (Prise de Mousse and Sake A18) increased squalene production by up to twofold at 35 °C [12], whereas Loertscher et al. (2006) and Tronconi et al. (2012) reported that cultivation of *S. cerevisiae* (JRY527 and T73, respectively) at low temperatures accumulated high levels of squalene [19, 38]. Therefore, we tested the effect of high (35 °C) and low (25 °C) temperatures on squalene production and cell growth in Y2805 strains harboring pG-H-I. However, in contrast to the previous reports, cultivation of Y2805 overexpressing the *tHMG1* and *ispA* genes resulted in significantly decreased squalene levels ( $156 \pm 19$  mg/L) at 25 °C and also reduced squalene accumulation ( $277 \pm 47$  mg/L) at 35 °C as compared with cultivation at 30 °C as a control (Fig. 4b). Similar to these results, *S. cerevisiae* KLN1-ERG1 harboring a chromosomal copy of the *ERG1* gene disrupted and transformed with a plasmid containing *ERG1*, also showed significantly reduced squalene levels at low (22 °C) and high (35 °C) temperatures [11]. These results indicated that optimization of culture conditions, such as cultivation temperature, for squalene production in *S. cerevisiae* appeared specific to strains harboring different genetic backgrounds.

As described in a previous section, partial inhibition of squalene epoxidase by terbinafine in non-engineered *S. cerevisiae* strains, including Y2805, results in a considerable increase in squalene accumulation within cells. Based on this result, we tested the possibility for further improvements in squalene productivity by inhibiting squalene epoxidase with terbinafine in the engineered Y2805 strain (transformed with pG-H-I). Y2805 harboring pG-H-I was cultivated in YPDG medium containing different concentrations of terbinafine (5–40 µg/mL) for 72 h. As shown in Fig. 4c, squalene levels in cells gradually increased along with the concentration of terbinafine. In particular, when terbinafine was added at a concentration of 20 µg/mL, the highest level of squalene ( $804 \pm 31$  mg/L) was obtained, although the growth of terbinafine-treated cells was slightly effected. However, treatment with 10 µg/mL of terbinafine did not affect the growth

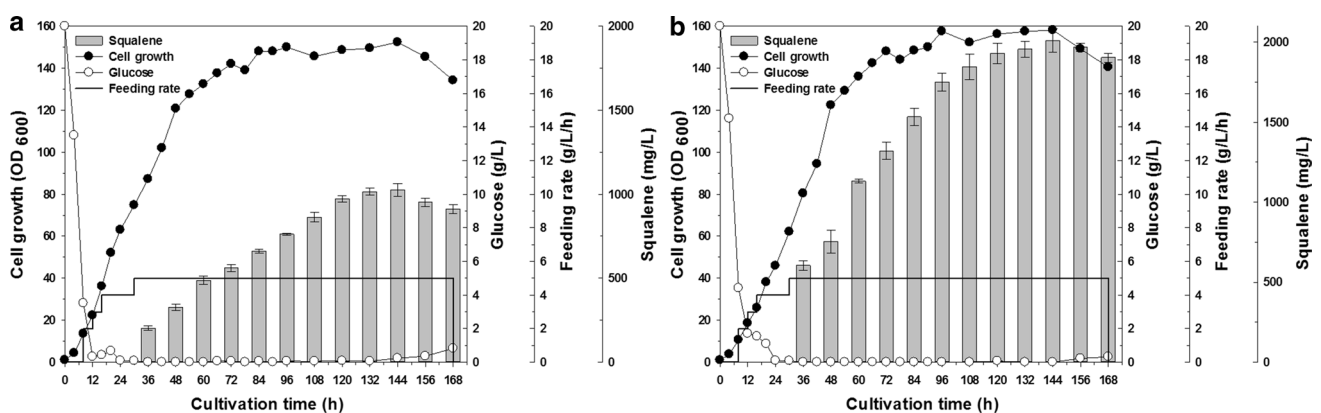
of yeast cells and showed a similar level of squalene accumulation ( $756 \pm 36$  mg/L). Increasing the concentration of terbinafine to  $40 \mu\text{g/mL}$  was expected to result in a higher squalene accumulation; however, the growth of terbinafine-treated cells was significantly inhibited, and squalene levels were, therefore, considerably lower ( $531 \pm 24$  mg/L). The terbinafine concentration ( $10 \mu\text{g/mL}$ ) that did not affect cell growth was selected as the optimal concentration and subsequently used for squalene production during fermentation. Terbinafine treatment during fermentation with Y2805 harboring pG-H-I increased squalene accumulation by up to twofold, indicating that squalene epoxide activity in Y2805 dissipated half the amount of accumulated squalene further downstream to ergosterol. These results also suggested that strain development to contain diminished squalene epoxidase activity might partially replace the need for terbinafine treatment.

### Squalene production in 5-L fed-batch fermentation

Based on the results obtained from flask cultivation under various conditions, we chose the best squalene-producing strain (Y2805 transformed with pG-H-I) and performed fed-batch fermentation in a 5-L fermenter with a working volume of 2 L. To evaluate the performance of *S. cerevisiae* Y2805 transformed with pG-H-I for squalene production, 20 g/L of glucose was used as the initial carbon source, and glucose feeding was initiated after the initial glucose was consumed. Galactose was also added at regular intervals every 24 h to induce genes under the control of a galactose-inducible promoter. The glucose-feeding rate was specifically controlled to prevent carbon catabolite repression caused by the presence of high concentrations of glucose in the medium. As

shown in Fig. 5, squalene production began within 24 h and gradually increased as along with cell growth. Consequently, during 7 days of fermentation, we obtained a maximum squalene production of  $1026 \pm 37$  mg/L at a cell density of  $\text{OD}_{600} = 152$  at 144 h (Fig. 5a). This production level corresponded to a 2.5-fold increase relative to the highest level obtained from shake-flask cultivation. Similar to fed-batch fermentation performed to evaluate squalene production in the absence of terbinafine, fed-batch fermentation was conducted to measure squalene accumulation using the same recombinant strain and in the presence of  $10 \mu\text{g/mL}$  terbinafine. As shown in Fig. 5b, squalene production was initiated after galactose induction and continued steadily, reaching the highest level of  $2011 \pm 75$  mg/L after 144 h of fermentation, with no significant inhibition of the growth of terbinafine-treated yeast cells. Compared with levels obtained from fermentation of the same recombinant strain in the absence of terbinafine, squalene production increased by  $\sim$  twofold. Treatment with  $10\text{-}\mu\text{g/mL}$  terbinafine was sufficient to reduce of the flux of accumulated squalene to post-squalene in the ergosterol-biosynthesis pathway, thereby allowing a maximal increase in squalene production ( $\sim$  twofold higher) relative to that observed in shake-flask experiments. This verified that squalene epoxidase was an important target for improving squalene productivity. Strain improvement involving further diminishing of squalene epoxidase activity by genetic engineering is under way to further improve the development of Y2805 as a suitable host for large-scale squalene production.

In summary, this study reported considerable strain dependence in squalene accumulation among different strains of *S. cerevisiae*, and the metabolically engineered *S. cerevisiae* Y2805 was selected as a new alternative source



**Fig. 5** Squalene production in fed-batch fermentation using *Saccharomyces cerevisiae* Y2805 harboring pG-H-I. **a** Time profile of fed-batch fermentation of *S. cerevisiae* Y2805 transformed with pG-H-I. **b** Time profile of fed-batch fermentation of *S. cerevisiae* Y2805 transformed with pG-H-I in medium containing  $10 \mu\text{g/mL}$  terbinafine. The strain was grown under glucose-limited conditions and

galactose was added at regular intervals every 24 h. Typical profiles observed for cell growth ( $\text{OD}_{600}$ , black circles), glucose concentration (g/L, white circles), feeding rate (g/L/h, lines), and squalene concentration (mg/L, gray bars) are represented. Data were obtained during fed-batch fermentation and represent the average values from two independent analyses

exhibiting improved squalene production. This study also provided a simple and efficient protocol for the extraction of squalene from *S. cerevisiae* cells.

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## Compliance with ethical standards

**Conflict of interest** There are no conflicts of interest to declare.

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