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Enhanced Squalene Production by Wild-Type *Saccharomyces cerevisiae* Strains Using Safe Chemical Means

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Supporting Information

ABSTRACT: Interest is increasing in establishing renewable sources for squalene, a functional lipid, as the conventional ones are limited. In the present study, squalene production was achieved in a wild-type laboratory *Saccharomyces cerevisiae* strain by two safe chemical means using terbinafine (0.05−0.55 mM) and methyl jasmonate (MJ) (0−1.00 mM). Bioprocess kinetics optimized by response surface methodology and monitored by high-performance liquid chromatography revealed a clear dependence of growth and squalene content (SQC) and yield (SQY) on the above regulators. Maximum SQC (10.02 ± 0.53 mg/g dry biomass) and SQY (20.70 ± 1.00 mg/L) were achieved using 0.442 mM terbinafine plus 0.044 mM MJ after 28 h and 0.300 mM terbinafine after 30 h, respectively. A 10-fold increase in SQY was achieved in comparison to that in the absence of regulator. The ruggedness of optimum conditions for SQY was verified for five industrial strains. The cellular lipid fraction (∼12% of dry biomass) was rich in squalene (12−13%). Results are encouraging toward bioprocess scale up.

KEYWORDS: squalene, wild-type *Saccharomyces cerevisiae* strains, terbinafine, methyl jasmonate, squalene epoxidase

INTRODUCTION

The incorporation of functional lipids into food products has expanded in the last few decades because each of these lipid classes presents specific health effects.[1] For example, the use of phytosterols, phytostanols, and their esters has become a widely accepted, successful innovation among functional foods.[2] Squalene, a precursor in the biosynthesis of sterols and steroids, is a functional lipid associated with various beneficial properties, despite some uncertainty. Squalene intake becomes effective through the everyday diet or after intravenous injection.[3] This terpenoid hydrocarbon ($C_{30}H_{50}$, 6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) has been associated with a chemopreventive effect on colon cancer upon consumption of virgin olive oil, a decrease in chemotherapy-induced side effects, scavenging properties for toxic metabolites, protection against bacterial and fungal infections, and effective inhibition of photoperoxidation.[3,4] Among known foods, virgin olive oil prevails in dietary squalene content (regularly 7 mg/g),[5] whereas other fruit or seed oils contain much lower quantities (e.g., pumpkin seed oil, <0.5 mg/g).[6] A source of squalene for daily intake that is less-appreciated by consumers is various types of nuts, the maximum level of which is observed in the Brazil nut (∼1.4 mg/g).[7]

With regard to squalene occurrence and biosources, marine sources are currently exhausted, so other sources of animal, plant, or microbe origin have to be evaluated. Well-investigated inedible sources are the distillates of the olive oil industry (10−30%, w/w)[8] and *Amaranthus* seed oil (20−80 mg/g).[9] It is accepted that very few plant materials can provide enough squalene to satisfy foreseen applications, and no animal sources have been mentioned so far for this functional lipid. It seems that microorganisms are currently an unexplored potential source for squalene production, [10−14] having the advantages of renewability and low cost. Food-grade status of microorganisms is a prerequisite when industrial production is intended for human consumption. The yeast *Saccharomyces cerevisiae* is an attractive microorganism for the production of squalene because of the sufficient pool of precursors and its generally recognized as safe (GRAS) status.[13,14] Mantzouridou and collaborators (2009)[13] succeeded in accumulating squalene in yeast cells grown under semianaerobic conditions (1.6 mg/g dry biomass), which was 40 times higher than that reported for the same source under strictly anaerobic conditions (0.04 mg/g dry biomass). Although the literature is rather confined, squalene accumulation using other microorganisms is reported to be of similar size (*Aurantiochytrium mangrovei*, 0.53 mg/g dry biomass;[11] *Schizochytrium mangrovei* cells, 1.17 mg/g dry biomass[12]) under microaerobic conditions. A many fold higher level of squalene production (70.32 mg/g dry biomass) was observed only using an oleagineous yeast strain, *Pseudozyma* sp. JCC 207, grown under microaerobic conditions.[10] Because *Pseudozyma* species is not included in the updated qualified presumption of safety (QPS) list published by the European Food Safety Authority,[15] the optimization of squalene production through the involvement of safe strains of *S. cerevisiae*, which is a well-established microorganism in the history of human food technology (e.g., bread, wine, and beer), becomes a challenging objective.

In the present investigation, squalene accumulation in wild-type *S. cerevisiae* cells was achieved using the safe chemical means terbinafine and methyl jasmonate (MJ) to disrupt the tide regulation of ergosterol synthesis.[14] Specifically, the allylamine terbinafine, a known antifungal agent, was selected because it interferes with ergosterol biosynthesis through the specific inhibition of a key enzyme's activity (squalene epoxidase, Erg1p).[16,17] So

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far, research on terbinafine activity involving *S. cerevisiae* strains is limited to the use of certain yeast mutants as model systems for the elucidation of the mechanism of their resistance to this agent.[18]

The implication of methyl jasmonate—a signaling molecule in plants[19,20] that induces activation of the transcripts of the genes encoding three key enzymes, namely, 3-hydroxy-3-methylglutaryl coenzyme A reductase isoenzyme (Hmg1p),[21] squalene synthase (Erg9p), and squalene epoxidase (Erg1p)[22]—was considered promising for our objectives, too. To our knowledge, this is the first attempt to expose *S. cerevisiae* EGY48 cells to MJ. The effects of terbinafine and MJ on yeast growth and on squalene and ergosterol accumulation were examined in a series of experiments using the laboratory wild-type strain *S. cerevisiae* EGY48. Then, the bioprocess was undertaken, combining the action of the two chemical regulators according to a central composite statistical design (response surface methodology, RSM). Chromatographic methods were applied to monitor the squalene content and the selectivity of the bioprocess in yeast cells and to highlight the composition of cellular lipids. To our knowledge, this is the first study of the combined effect of terbinafine and methyl jasmonate on the kinetics of squalene formation. Optimized conditions for squalene yield were also applied to five commercial *S. cerevisiae* strains as a first step for bioprocess commercialization.

MATERIALS AND METHODS

Microorganisms. The laboratory yeast strain was the wild-type *Saccharomyces cerevisiae* EGY48 (*ura3 trp1 his3 6LexA*-operator-LEU2),[23] generously provided by Dr. Antonios Makris, Mediterranean Agronomical Institute of Maich, Crete, Greece. The above yeast strain was maintained at 4 °C on glucose yeast peptone agar (GYPA) slants. The commercial active dry wine yeast strains Vitilevure LB Rouge MV 95034 (*S. cerevisiae*), Zymaflore F15 (*S. cerevisiae*), Maurivin BP 725 (*S. cerevisiae*), and Fermol Bayanus *Lipari* (*S. cerevisiae* var. *bayanus*) were provided by a Greek wine industry (Tsantalis S.A., Chalkidiki, Greece). Commercially available active dry baker's yeast (*S. cerevisiae*) (Jotis S.A, Athens, Greece) was also used in this study. Commercial dry yeasts were maintained at 4 °C until use.

Standards, Reagents, and Solvents. Squalene (for biochemistry, purity 98%), GYPA, and L-α-phosphatidylcholine (Type IV—S, ~40) was from Sigma Chemical Co. (St. Louis, MO, U.S.), ergosterol (purity >98%) was from BDH Chemicals Ltd. (Poole, U.K.), and DL-α-monoolein (38—40% diacyloglycerols, 16—18% triacyloglycerols, and 4—6% glycerol) was from Fluka AG (Buchs, Switzerland). The culture media contained D-glucose monohydrate (Panreac Quimica S.A., Barcelona, Spain), yeast extract (Merck, Darmstadt, Germany), malt extract (Merck, Darmstadt, Germany), and soy peptone (Lab M Limited, Lancashire, U.K.). Ethanol (95%, v/v), terbinafine hydrochloride (purity >98%), and methyl jasmonate (purity 95%) was from Riedel-de Haën AG (Seelze, Germany), TCI Europe (Brussels, Belgium), and Sigma Chemical Co., respectively. High-performance liquid chromatography (HPLC)-grade methanol, dichloromethane, and acetonitrile were from Merck. Ultra high-purity water was delivered using a Millipore-Milli-Q-system (Barnstead International, Dubuque, Iowa, U.S.). All of the other common reagents and solvents were of the appropriate purity from various suppliers.

Apparatus. Absorbance values were recorded by a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Reversed-phase HPLC (RP-HPLC) of squalene and sterols was performed isocratically using a solvent delivery system consisting of two Marathon IV Series HPLC pumps (Rigas Laboratories, Thessaloniki, Greece) and a Rheodyne 7125 injection valve with a 20 μL fixed loop (Rheodyne 7125 LP, Cotati, CA, U.S.). The chromatogram was coupled to a Linear UVVIS-206 diode array multiple-wavelength detector (Linear Instruments, Fremont, CA, U.S.). Chromatographic data were processed with the software EZChrom 6.6 (Sci Software, Inc., San Ramon, CA, U.S.). Inoculated flasks were placed in a shaking incubator (model MkX, Stoke Poges, U.K.) or in an incubator without agitation (Incucell, MMM Medcenter-Einrichtungen, Gräfelfing, Germany). Yeast cells were lyophilized before further analysis.

Preparation of Inocula. Inocula of EGY48 were prepared as described earlier.[13] Briefly, EGY48 cells stored on a GYPA slant were activated in the same medium by maintaining consecutive transfers. Inocula were prepared by transferring a loopful of yeast cells from the agar slants to a liquid medium of the following composition (g/L): glucose (20), soy peptone (10), yeast extract (5), and malt extract.[5] The initial pH value of the medium was 5.5. The commercial active dry yeast strains were first activated as follows: the selected yeast strains were suspended in water (1:10, w/v; 35—37 °C) and then kept in a water bath for 15—20 min by periodical stirring. Inocula were prepared by transferring 2 mL of the cell suspensions to the above-mentioned liquid medium. To avoid heat shock, the temperature difference between the cell suspensions and the liquid medium was ≤10 °C. Hydrophobic cotton-stopped Erlenmeyer flasks (500 mL) containing 100 mL of the above medium were placed for aerobic growth in a shaking incubator at 30 °C and 200 rpm for two days and then used for the inoculation of the culture medium.

Medium Composition and Aeration Conditions. Medium composition and aeration conditions were as described by Mantzouridou et al.[13] The basal medium (pH 5.5) had the following composition (g/L): glucose (40), soy peptone (20), and yeast extract (10). Cultivation of yeast cells was performed under microaerobic conditions in hydrophobic cotton-stopped Erlenmeyer flasks (500 mL) containing 100 mL of the medium under vigorous shaking (250 rpm) or under semianaerobic conditions by allowing 300 mL of the medium to stand in a similar type of flask. For EGY48, inoculation was accomplished with 8 mL aliquots of the preculture per 100 mL of fresh medium (OD_{600} value of 0.700). For commercial strains, 4 mL aliquots were used to achieve the same OD_{600} value as that of EGY48. In all cases incubation was at 30 °C. Fermentation experiments were carried out in triplicate.

Experimental Design. Examination of the effect of either terbinafine or MJ on squalene accumulation in yeast cells was conducted as follows: yeast cells were exposed to 0.15 mM terbinafine[11,24] in the absence/presence of ergosterol (12 mg/L of culture medium) or 0.1 mM MJ[12,25] for 12 h under microaerobic and semianaerobic conditions. For time course experiments, EGY48 cells were treated with various concentrations of terbinafine (0.15, 0.3, 0.6, and 0.9 mM)[11] or MJ (0.1 and 0.4 mM)[12] under microaerobic conditions as described above. Both terbinafine and MJ were dissolved in ethanol (0.5%, v/v). Ergosterol was dissolved in Tween 80/ethanol (1:1, v/v), giving a final Tween 80 concentration of 0.5% (v/v) in the medium.[25] The control was supplemented with ethanol (0.5%, v/v) for comparison reasons. All of the above solutions were sterilized by passing through 0.45 μm membrane filter (Schleicher Schnell, Dassel, Germany) before inoculation. In all cases, the inoculation process was the same as described above. Fermentation experiments took place in triplicate.

Next, 20 experiments were set according to an unblocked full factorial central composite statistical design[13] for the study of three factors, namely, terbinafine (mM) (X_1), MJ (mM) (X_2), and fermentation time (h) (X_3), each at five experimental levels. The levels of the X_j factor are coded as follows: $-a$, -1, 0, $+1$, and $+a$, where $a = 2^{n/4}$, n = number of variables, and -1, $+1$, and 0 correspond to the low-, high-, and mid-level of X_j. The actual levels of each factor were calculated according to the forumula:

$$\text{coded value} = \frac{\text{actual level} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2}$$

where -1, $+1$ are 0.15, 0.45 mM for X_1; 0.20, 0.80 mM for X_2; and 14.0, 42.0 h for X_3.

Table 1. Experimental Design for Five-Level-Three-Factor Central Composite Design and the Comparison Between Observed and Predicted Responses for Squalene Content (SQC), Squalene Yield (SQY), and Ergosterol Content (ERGC)

	factors			actual/predicted		
	X_1	X_2	X_3			
run	terbinafine (mM)	MJ (mM)	fermentation time (h)	SQC (mg/g of dry biomass)	SQY (mg/L of culture medium)	ERGC (mg/g of dry biomass)
1	0.30	0.50	28.00	6.50/6.24	18.65/16.85	2.12/2.02
2	0.30	1.00	28.00	6.80/6.90	10.34/9.86	1.90/1.93
3	0.30	0.00	28.00	9.40/9.57	24.40/22.34	1.70/1.67
4	0.15	0.20	42.00	4.29/4.30	15.66/18.16	3.32/3.35
5	0.30	0.50	28.00	6.00/6.24	15.00/16.85	1.95/2.02
6	0.30	0.50	51.5	5.00/4.84	18.50/16.22	2.80/2.88
7	0.30	0.50	28.00	6.48/6.24	18.85/16.85	2.10/2.02
8	0.05	0.50	28.00	3.00/2.84	11.04/9.06	2.99/2.91
9	0.45	0.20	42.00	7.80/7.83	20.28/21.09	1.67/1.60
10	0.15	0.20	14.00	5.23/5.26	8.11/8.66	2.15/2.21
11	0.30	0.50	4.45	3.20/3.62	0.61/0.34	2.30/2.22
12	0.30	0.50	28.00	6.05/6.24	14.80/16.85	1.85/2.02
13	0.30	0.50	28.00	6.44/6.24	18.35/16.85	2.15/2.02
14	0.15	0.80	14.00	4.50/4.29	3.06/4.05	2.00/2.07
15	0.45	0.80	14.00	3.40/3.21	2.18/1.48	2.45/2.42
16	0.55	0.50	28.00	4.47/4.90	9.92/9.35	1.65/1.73
17	0.45	0.20	14.00	7.86/7.34	9.27/10.34	1.44/1.47
18	0.15	0.80	42.00	4.98/5.28	11.45/12.19	2.76/2.73
19	0.30	0.50	28.00	6.00/6.24	15.00/16.85	1.95/2.02
20	0.45	0.80	42.00	5.83/5.62	9.61/10.86	2.10/2.04

The 20 runs were set using the software Minitab Release 13.20 (Minitab, Inc., State College, PA, U.S.) (Table 1). The design had six of the factorial points at the center of the design replicated for the estimation of error.

Polynomial response surfaces were fitted to three response variables, namely, squalene content (mg/g of dry biomass) (SQC), squalene yield (mg/L of culture medium) (SQY), and ergosterol content (ERGC) (mg/g of dry biomass). Statistical analysis of the experimental data was performed by RSM using the same software. Initially, the second-order polynomial model was fitted to each response giving an equation of the form: $Y = \beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$ (eq 1), where Y is the predicted SQC, SQY, and ERGC response, X_1, X_2, and X_3 represent the levels of the coded factors, and β_0, β_1, ..., β_{23} represent the estimated coefficients, β_0 being a scaling constant. The quality of the fit of the model was evaluated by the coefficients of determination (R^2), the significance of each parameter through an F-test, and the lack of fit of the model. Coefficients with a p-value lower than 0.05 were considered significant. Where possible, the model was simplified by omission of statistically nonsignificant terms. Optimization of the fitted polynomials for SQC and SQY was performed using the same software. The combination of factor optimal values resulting in optimal responses was verified by conducting a simulation experiment in triplicate. Results were compared with model prediction outcomes.

Analytical Techniques. At specific time intervals, aliquots of cell suspensions were removed from the flasks and subjected to the following determinations.

Determination of Biomass Dry Weight. Biomass obtained by centrifugation (4000*g* for 10 min) of aliquots of cell suspensions was dried in preweighed centrifuge tubes at 120 °C for 2 h. Measurements were obtained in triplicate.

Preparation of the Unsaponified Matter (UM). Yeast cells were collected by centrifugation, washed twice with distilled water, and freeze−dried. Portions of dry biomass (96 mg) were suspended in 5 mL of 60% KOH solution in water, 7.5 mL of methanol, and 7.5 mL of methanolic pyrogallol solution (0.5%, w/v) and incubated in a shaking incubator at 45 °C overnight. The UM was extracted with hexane (3 × 10 mL). The phases were separated by centrifugation at 4000*g* for 10 min. The hexane layers were transferred to the same tube. Emulsions, if present, were dispersed by the addition of 0.5 mL of methanol. The combined hexane fractions were dried over anhydrous Na_2SO_4, and finally the solvent was removed under a vacuum (40 °C). Repeatability of extractions was satisfactory (coefficient of variation (CV)% = 3.6 and 3.4, respectively, for squalene and ergosterol, n = 5). Crude extracts were kept at −18 °C until further analysis.

RP-HPLC of Squalene and Ergosterol. The UM was dissolved in methanol/dichloromethane (9:1, v/v). All of the samples were filtered through a 0.45 μm membrane filter just before HPLC analysis. Separation of squalene and sterols was carried out on a reversed-phase Nucleosil C_{18} column (250 × 4.6 mm i.d.; 5 μm) maintained at 40 °C. Elution with methanol/water (98:2, v/v)[13] was assisted by maintaining column temperature at 40 °C.[26] For the UM derived from cells exposed to terbinafine, the elution system was slightly modified (methanol/water 97:3, v/v) to achieve the separation of peaks corresponding to ergosterol and terbinafine. The flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. Detection and quantification of squalene, ergosterol, and terbinafine was at 208, 285, and 230 nm, respectively. Peak identification was achieved by comparing the retention time with that of authentic standards and confirmed by spiking and comparison of spectral data. Quantification was accomplished with the aid of standard curves calculated by linear regression analysis. Analysis of samples was carried out in duplicate (CV% = 2.3, 2.5, and 2.7, respectively, for a 50 mg/L squalene, a 500 mg/L ergosterol, and a 500 mg/L terbinafine standard solution, n = 5).

Other Methods. In selected cases, the lipid content of the biomass was extracted using acid-assisted disruption of cells and determined according to Fornairon-Bonnefond and Salmon (2003)[27] after slight modifications as follows: 0.5 g of dry biomass was added to a sealed tube with 2.5 mL of hydrochloric acid solution (7.7 M) and 0.75 mL of water. The mixture was boiled for 10 min, and after cooling, carbon tetrachloride (2.5 mL) was added. The mixture was boiled again for 10 min and then cooled, and diethyl ether/ethyl petroleum (1:1, v/v) (18.75 mL) was added. After vigorous shaking (5 min), an aliquot of the upper phase (15 mL) was obtained. After the solvent was dried under a vacuum (40 °C), the obtained lipids were weighed and the lipid concentration in 100 g of yeast (dry weight) was determined according to the formula:

$$\text{lipid concentration } \% = \frac{a(18.75 + 2.5)}{15b} \times 100$$

where a is the recovered lipid weight and b is the initial dry biomass weight. Repeatability of extractions was satisfactory (CV% = 4.6, n = 5). Lipids were kept at −18 °C until further analysis.

The presence of squalene and other lipid classes was tested using thin layer chromatography (TLC) on 20 × 20 cm silica gel (0.2 mm) plates (Merck, Darmstadt, Germany). The separation of neutral lipid classes was accomplished in two steps by two solvent mixtures of different polarities. The first developing mixture of solvents was petroleum ether/diethyl ether/acetic acid (40:40:1.6, v/v/v), and it was developed up to one-third of the plate. The second developing mixture consisted of petroleum ether/diethyl ether (78.4:1.6, *v*/*v*), and it was developed up to the remaining two-thirds of the plate. Spots were visualized using H_2SO_4 (50%, v/v) followed by heating for 10 min in an oven (130 °C). Phospholipid classes were separated in two steps on another TLC plate. The first solvent was acetone, and it was developed to 18 cm above the origin. The second mixture of solvents was chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v), and it was developed up to a height of 17 cm. Visualization of spots was as mentioned above. Squalene content in the lipid matrix was determined on a reversed phase LiChroCART column (125 × 4.0 mm i.d.; 4 μm) maintained at 26 °C. The elution solvent was 100% acetonitrile, the flow rate was 1.2 mL/min, and the injection volume was 10 μL. Detection and quantification of squalene were at 208 nm. Analysis of samples was carried out in duplicate (CV% = 3.5 for a 100 mg/L squalene, n = 5). The column was carefully washed out using acetone after squalene elution.

RESULTS AND DISCUSSION

Squalene Production Assisted by Terbinafine. Aiming at optimizing squalene production using food-grade *S. cerevisiae* strains, we carried out experiments with the EGY48 wild-type strain, which was treated with 0.15 mM terbinafine (middle exposure allylamine level to microbial cells)[11,24] for 12 h under microaerobic and semianaerobic conditions. A 32 and 23% reduction in dry biomass, respectively, was observed with regard to the values for the untreated cells (2.20 vs 3.23 and 1.58 vs 3.08 g/L under microaerobic and semianaerobic conditions, respectively). Only after supplementing the culture medium with ergosterol (12 mg/L) and Tween 80 (0.5%, v/v)[25] could yeast cells exposed to the same level of terbinafine produce a biomass equal to the control culture (3.08 vs 3.23 and 2.15 vs 2.05 g/L under microaerobic and semianaerobic conditions, respectively). The above findings indicate that the growth of treated cells is partially inhibited by becoming deficient in ergosterol. This is supported by the obvious decrease in the ergosterol content of treated cells with regard to the respective values of the control culture (1.74 vs 4.33 and 0.45 vs 2.25 mg/g dry biomass under microaerobic and semianaerobic conditions, respectively). Ergosterol feeding restored the very low intracellular ergosterol level under semianaerobic conditions (2.46 mg/g dry biomass). Because of the oxygen-dependent synthesis of unsaturated fatty acids, which are important structural components of yeast mitochondrial membranes, supplementation with Tween 80 rich in oleic acid is expected to support anaerobic growth of yeast cells.[28] In comparison with control samples, squalene content in treated yeast cells grown under microaerobic conditions was found to be more than 30-fold higher (0.23 vs 7.95 mg/g dry biomass, respectively), whereas semianaerobic conditions did not favor accumulation (1.50 vs 2.67 mg/g dry biomass). All of the above data point to the manipulation of squalene epoxidase as a target enzyme to regulate the ergosterol pathway in favor of squalene formation. This finding, in conjunction with the knowledge of *S. cerevisiae*'s inherent resistance to survive under conditions of low ergosterol and high squalene contents,[14,28] led to conducting a kinetic study on the effect of higher levels of terbinafine (0.3, 0.6, and 0.9 mM) on *S. cerevisiae* cells under microaerobic conditions.

Table 2. Effect of Different Terbinafine Concentrations on the Kinetics of Biomass, Ergosterol, and Squalene Content in Wild-Type *S. cerevisiae* Strain EGY48 Cells Cultivated under Microaerobic Conditions

			ergosterol content[a]	squalene content[a]
fermentation time (h)	terbinafine (mM)	biomass[a] (g/L culture medium)	(mg/g dry biomass)	
12	0.00	3.08 ± 0.14	4.46 ± 0.18	0.30 ± 0.02
	0.15	2.13 ± 0.09	1.63 ± 0.08	8.18 ± 0.38
	0.30	1.72 ± 0.07	1.83 ± 0.08	5.88 ± 0.31
	0.60	1.69 ± 0.09	1.44 ± 0.06	5.51 ± 0.26
	0.90	1.63 ± 0.08	1.62 ± 0.09	4.66 ± 0.21
24	0.00	4.72 ± 0.22	4.71 ± 0.24	0.15 ± 0.01
	0.15	2.15 ± 0.25	2.66 ± 0.11	6.86 ± 0.33
	0.30	2.19 ± 0.23	2.52 ± 0.13	9.27 ± 0.45
	0.60	2.09 ± 0.24	2.22 ± 0.10	9.01 ± 0.42
	0.90	2.12 ± 0.22	1.97 ± 0.08	8.39 ± 0.38
48	0.00	4.91 ± 0.25	2.70 ± 0.12	0.10 ± 0.004
	0.15	4.71 ± 0.22	2.62 ± 0.12	4.93 ± 0.25
	0.30	4.58 ± 0.21	2.12 ± 0.10	7.39 ± 0.35
	0.60	4.36 ± 0.23	2.14 ± 0.10	7.51 ± 0.33
	0.90	4.38 ± 0.21	2.58 ± 0.14	6.04 ± 0.29
72	0.00	3.60 ± 0.17	4.90 ± 0.26	0.31 ± 0.02
	0.15	3.30 ± 0.15	4.28 ± 0.22	2.94 ± 0.15
	0.30	3.60 ± 0.17	4.24 ± 0.21	5.04 ± 0.24
	0.60	3.50 ± 0.18	3.73 ± 0.17	5.10 ± 0.25
	0.90	3.20 ± 0.14	3.03 ± 0.16	4.29 ± 0.20

[a] Mean value of three independent experiments ± standard deviation.

The evolution of biomass formation and squalene transformation to ergosterol, followed by RP-HPLC of the UM of cellular lipids from yeast cells harvested in the middle of the exponential growth phase (12 h), at the beginning of the stationary phase (24 h), at the late growth phase (48 h), and at the end of cultivation (72 h),[13] are presented in Table 2. In the first 24 h of cultivation, a retardation of biomass formation was noticed for the terbinafine-treated cells. Nevertheless, similar values of

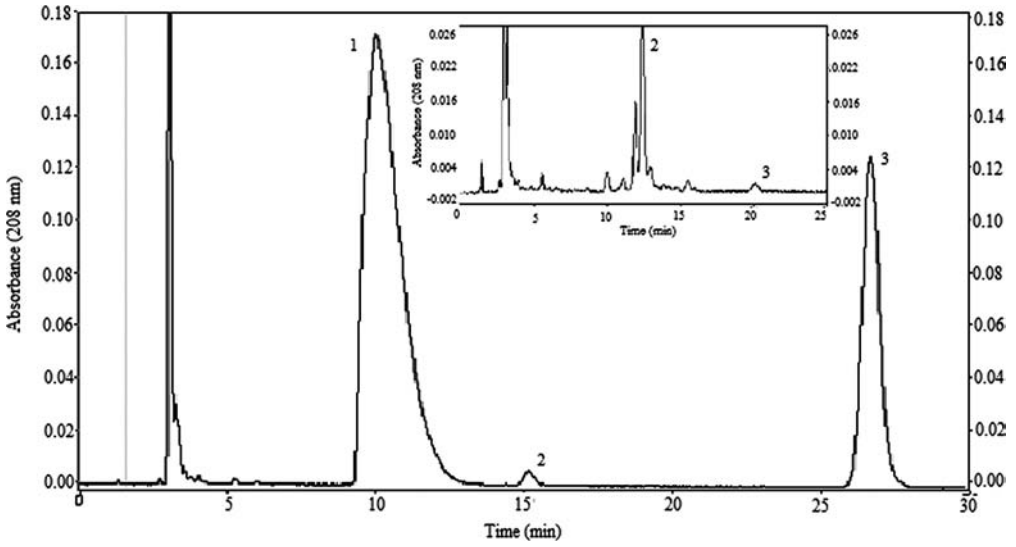

Figure 1. Chromatographic profile of the total unsaponifiable fraction of cellular lipids in the presence of 0.3 mM terbinafine (peaks: 1, terbinafine; 2, ergosterol; 3, squalene) extracted from wild-type *S. cerevisiae* strain EGY48 cells after 24 h of cultivation (inset: in the absence of the regulator).

biomass yield after 48 h of yeast cultivation in control medium and in media containing 0.15, 0.3, 0.6, and 0.9 mM terbinafine (4.91 vs 4.71, 4.58, 4.36, and 4.38 g/L of culture medium, respectively) indicate that there was no impairment of the microorganism's ability to grow under the above terbinafine levels. An explanation is related to the success of terbinafine-treated cells to produce adequate amounts of ergosterol, above the minimum amount required to overcome growth inhibition (Table 2).

HPLC of the UM of cellular lipids demonstrated that squalene accumulation increased up to 9.27 mg/g dry biomass in *S. cerevisiae* EGY48 cells exposed to 0.3 mM terbinafine for 24 h (Table 2). In the ongoing cultivation, a decrease in squalene content with a concomitant increase of ergosterol was observed, which is due to the reversible inhibition effect of this agent on squalene epoxidase activity.[16,29] Specifically, terbinafine reversibly inhibits squalene epoxidase by interacting via its naphthalene ring moiety with a squalene binding site of the enzyme and through its lipophilic side chain with a second lipophilic binding site, resulting in a change of the conformation of the enzyme that renders it inactive.[29] These loose interactions with the binding domains of the enzyme might be easily removed over time. Higher terbinafine concentrations (0.6 and 0.9 mM) led to a reduction in squalene content. This finding is in agreement with previous reported studies[30] and indicates that the magnitude of squalene accumulation depends on the level of terbinafine in the culture medium. In yeast cells treated with high levels of terbinafine, despite the decrease in squalene accumulation, ergosterol content was not increased, but it was lower than that produced by untreated cells. This finding suggests limitation in the pre-squalene part of the pathway because of the accumulation of toxic levels of squalene precursors that inhibit transformation through the later steps of the mevalonate pathway, in line with previous evidence.[13,14]

Chromatographic analysis also revealed that in the presence of terbinafine, the bioprocess is highly selective for squalene formation (Figure 1). This is supported by the fact that in the absence of terbinafine, different intermediates along with ergosterol are formed in EGY48 cells (Figure 1, inset), whereas in its presence, ergosterol was the only sterol formed. It seems that terbinafine treatment reduces the amount of surplus sterols because of the decreased Erg1p activity. It is worth mentioning that the accumulation of squalene achieved in terbinafine-treated cells is clearly higher than that observed by Fan et al. (2010)[11] in *Aurantiochytrium mangrovei* treated cells (9.27 vs 0.53 mg/g dry biomass, respectively) for the same level of terbinafine addition and for time periods of similar length.

Table 3. Effect of Different Methyl Jasmonate Concentrations on the Kinetics of Biomass Formation, Ergosterol, and Squalene Content in Wild-Type *S. cerevisiae* Strain EGY48 Cells Cultivated under Microaerobic Conditions

			ergosterol content[a]	squalene content[a]
fermentation time (h)	MJ (mM)	biomass[a] (g/L culture medium)	(mg/g dry biomass)	
12	0.0	2.93 ± 0.14	4.19 ± 0.22	0.22 ± 0.01
	0.1	2.75 ± 0.14	5.22 ± 0.24	0.19 ± 0.01
	0.4	2.44 ± 0.11	4.35 ± 0.21	0.40 ± 0.02
24	0.0	4.80 ± 0.25	4.16 ± 0.20	0.17 ± 0.01
	0.1	2.93 ± 0.15	5.04 ± 0.23	0.20 ± 0.01
	0.4	2.73 ± 0.13	4.94 ± 0.25	0.06 ± 0.002
48	0.0	4.86 ± 0.23	2.56 ± 0.12	0.15 ± 0.01
	0.1	2.72 ± 0.12	2.78 ± 0.13	0.05 ± 0.002
	0.4	2.54 ± 0.11	2.25 ± 0.10	0.10 ± 0.004

[a] Mean value of three independent experiments ± standard deviation.

Squalene Production Assisted by Methyl Jasmonate. Effects of MJ on growth and squalene and sterol formation in yeast cells were investigated during 48 h of cultivation by adding MJ at two characteristic concentrations (0.1 and 0.4 mM)[12] (Table 3). The yeast was grown under microaerobic conditions because Hmg1p, which has been reported to be induced by MJ,[21] is the predominant isoform under conditions of sufficient oxygen availability.[31]

Table 4. Model Equations for Prediction of the Optimum Response Values of Squalene Content (SQC), Squalene Yield (SQY), and Ergosterol Content (ERGC)

		polynomial equation[a]	
model	response	coded values of factors	actual values of factors
1	SQC (mg/g of dry biomass)	$SQC = 6.2375 + 0.6123X_1 - 0.7939X_2 + 0.3615X_3 - 0.8386X_1^2 + 0.7046X_2^2 - 0.7096X_3^2 - 0.7987X_1X_2 + 0.3537X_1X_3 + 0.4887X_2X_3$ (eq 2)	$SQC = 1.76 + 30.60X_1 - 8.41X_2 + 0.12X_3 - 37.27X_1^2 + 7.83X_2^2 - 0.0036X_3^2 - 17.75X_1X_2 + 0.17X_1X_3 + 0.12X_2X_3$ (eq 3)
2	SQY (mg/L of culture medium)	$SQY = 16.848 + 0.086X_1 - 3.710X_2 + 4.721X_3 - 2.701X_1^2 - 3.028X_3^2$ (eq 4)	$SQY = -13.7 + 80.3X_1 - 0.1X_2 + 1.2X_3 - 120.1X_1^2 - 0.015X_3^2$ (eq 5)
3	ERGC (mg/g of dry biomass)	$ERGC = 2.02 - 0.3532X_1 + 0.0781X_2 + 0.1941X_3 + 0.1062X_1^2 - 0.0777X_2^2 + 0.1875\ X_3^2 + 0.2688X_1X_2 - 0.2563X_1X_3 - 0.1237X_2X_3$ (eq 6)	$ERGC = 2.625 - 4.756X_1 + 0.156X_2 + 0.012X_3 + 4.720X_1^2 - 0.863X_2^2 + 0.001X_3^2 + 5.972X_1X_2 - 0.122X_1X_3 - 0.029X_2X_3$ (eq 7)

[a] X_1, X_2 and X_3 are the coded (eqs 2, 4, 6) or actual (eqs 3, 5, 7) values of factors presented in Experimental Design.

The growth of the cells was negatively influenced in all tested concentrations of MJ after 12 h of cultivation. RP-HPLC profiles of the UM of cellular lipids from the treated cells with low and high MJ levels were qualitatively similar to that illustrated in Figure 1 (inset) for the untreated cells. Differences were mainly quantitative. Within 12 h of cultivation, induction of ergosterol was revealed at the lower addition level of MJ (0.1 mM), whereas accumulation of squalene was slightly lower, compared to what was evidenced in untreated cells. Further increasing MJ concentration to 0.4 mM led to a nearly doubled squalene content and a similar ergosterol content with regard to that in untreated cells (0.40 and 4.35 vs 0.22 and 4.19 mg/g dry biomass, respectively). In the latter case, stimulation of squalene accumulation may be related to the higher activity of key enzymes of the pre-squalene part of the ergosterol pathway, such as Hmg1p and Erg9p.[21,22] Our results are consistent with previous work by Yue and Jiang (2009),[12] who found a 60% increase in squalene content in *Schizochydrium mangrovei* cells exposed to 0.1 mM MJ. Nevertheless, the enhancement of squalene by high MJ concentration could only be maintained for a short time, as squalene decreased to a lower value than that in untreated cells within the next 12 h of cultivation, with a concomitant increase in ergosterol content (0.06 and 4.94 vs 0.17 and 4.16 mg/g dry biomass) as shown in the same table. By comparing the patterns of squalene and ergosterol content in low- and high-MJ treated cells, it seems that the elevated transfer of squalene into the post-squalene part of the ergosterol pathway was enhanced from the beginning and between the 12th and 24th hour of cultivation, respectively. This may be attributed to the activation of the downstream gene *ERG1* involved in the oxidation of squalene to 2,3-oxidosqualene.[22]

From the results obtained so far, it can be stated that the selective accumulation of squalene against sterols by terbinafine is the factor that differentiates its activity from that exhibited by MJ. This is explained by the fact that terbinafine specifically inhibits Erg1p,[16] resulting in a negative control of squalene flow into sterols, whereas MJ is thought to induce the activity of the key enzymes Hmg1p and Erg9p involved in the pre-squalene pathway, stimulating squalene synthesis and Erg1p.[21,22] Nevertheless, the above effects were found to be dose- and time-dependent. On such grounds, it was interesting to evaluate the combined effect of MJ and terbinafine on squalene content and yield of the bioprocess over time. This was achieved using the RSM.

Evaluation of the Combined Effects of Terbinafine and Methyl Jasmonate on Squalene Content and Yield of the Bioprocess Using the RSM. A central composite design was used to select the experimental conditions under which the study of the effects of terbinafine (X_1), MJ (X_2), and the fermentation time (X_3) on squalene content (SQC) (mg/g dry biomass), squalene yield (SQY) (mg/L of culture medium), and ergosterol content (ERGC) (mg/g dry biomass) takes place for the wild-type *S. cerevisiae* EGY48 strain. The statistical models fitted to the data for each response allowed assessment of the interactions among the factors using a reduced number of experiments. The models, in terms of coded (−1, +1) and actual factor levels (see Experimental Design) fitted for each of the response variables, are shown in Table 4 (eqs 2−7). Statistical details and a description of the model fitting are provided in the Supporting Information (text and data in Tables S-I and S-II).

The fitted polynomial equations (eqs 3, 5, and 7 in Table 4) were expressed as response surface plots to visualize relationships between SQC, SQY, and ERGC and the factors (X_1, X_2, and X_3) and to deduce the optimum conditions (Figure 2a−i). The surface plots were made by combining the values of two test variables at a time and keeping the value of the remaining variable constant at the low level.

At the fixed MJ level (0.2 mM), the data in Figure 2a,b show increasing SQC and SQY with regard to terbinafine content and duration of the bioprocess. The optimum response was obtained near the middle level of both factors. In the same figure, the characteristic curvature of the response surfaces illustrates the negative quadratic effects of terbinafine content and time course on SQC and SQY. This finding suggests that the dose- and time-dependent inhibitory activity of terbinafine on squalene epoxidase is related to the specific mechanism of action of this regulator.[29] The response surface of ERGC depicted in Figure 2c shows that ERGC varied inversely with SQC and SQY. This relationship supports the down-regulation of *ERG1* by terbinafine.[16] Nevertheless, increasing terbinafine content resulted in a less marked impact on ERGC. Because of *ERG1* deregulation, treated cells may accumulate toxic levels of squalene precursors so that transformation to squalene and sterols is not favored, in line with recently published findings.[13,14]

Figure 2d−f shows the surfaces of the corresponding responses as a function of MJ content and time course at the fixed terbinafine level (0.15 mM). As it can be observed, at low MJ level SQC and SQY were higher, and the effect was more pronounced at the beginning of the stationary phase (24 h). The positive quadratic

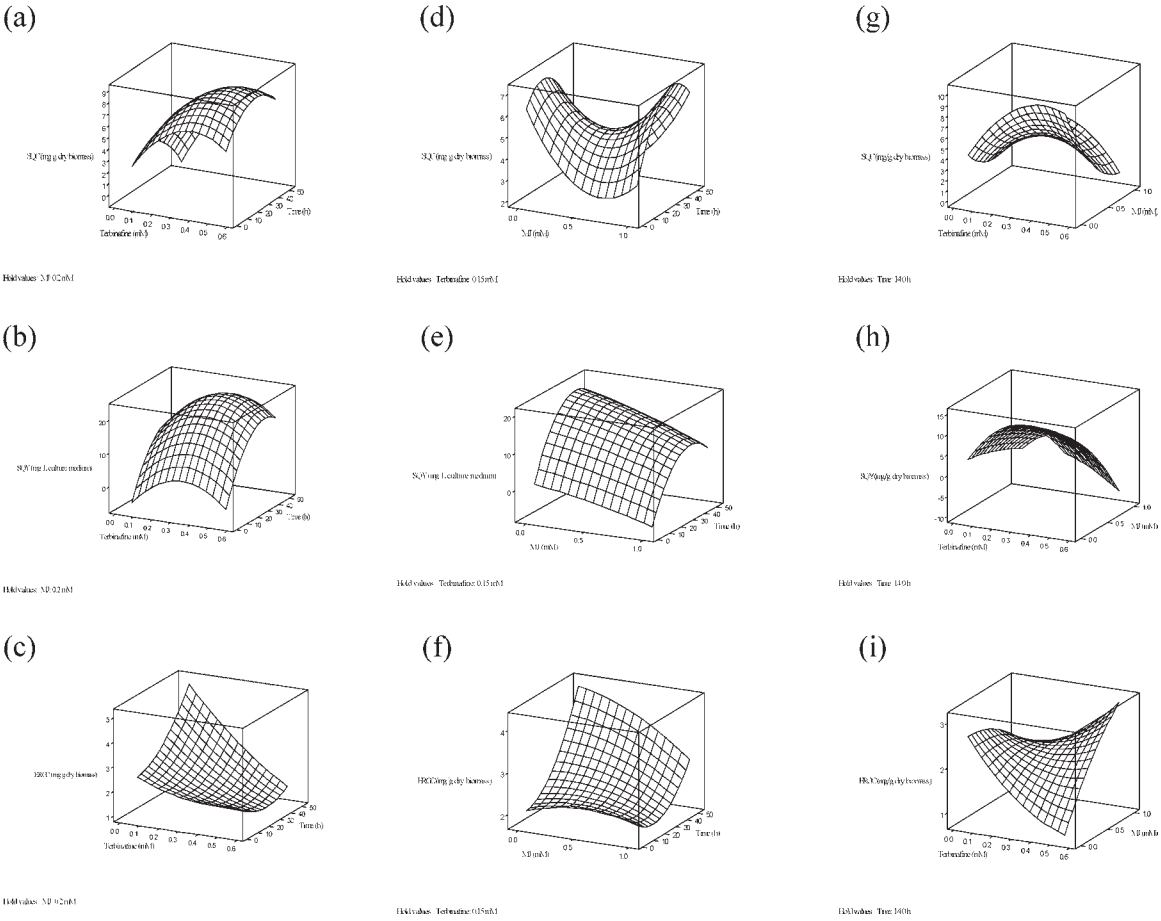

Figure 2. Surface plot for squalene content (SQC) (a, d, g), squalene yield (SQY) (b, e, h), and ergosterol content (ERGC) (c, f, i) at varying levels of terbinafine (mM), methyl jasmonate (mM), and fermentation time (h) for wild-type *S. cerevisiae* strain EGY48.

effect of MJ content on SQC indicates that its negative impact on this response is deccelerated above 0.5 mM, especially after about 30 h that terbinafine effect was weak. On the other hand, the quadratic effect of MJ content on SQY was positive but negligible, indicating dependence of the latter on cell growth that is inversely affected by MJ addition level (data not shown). By comparing data in Figure 2, panels d and f, it is also evident that despite the significant decrease in SQC at MJ content approaching its middle level, ERGC was not proportionally increased. This finding was attributed to the induction of the activation of the transcripts of *ERG1* by MJ in parallel with the inhibition of Erg9p activity by terbinafine at the fixed level. The combined effect of the two factors results in a nonquantitative conversion of squalene to ergosterol.

Keeping fermentation time constant (14 h), data in Figure 2g, h indicated that the combined effect of MJ and terbinafine on SQC and SQY was negative. On the other hand, ERGC was positively affected by the interaction of the two regulators (Figure 2i).

Predicted and Verified Optimum Conditions for SQC and SQY. As the fitted models for eqs 3 and 5 (in Table 4) provide a good approximation of the experimental conditions, these were employed to predict optimum values of the process variables for maximum SQC and SQY. The predicted values for optimum SQC were 0.442 mM terbinafine, 0.044 mM MJ, and 28 h of fermentation. For SQY the respective values were 0.30 mM, 0.00 mM, and 30 h. Then, the above sets of conditions were substituted to eqs 3 and 5 (in Table 4), respectively, to obtain maximum SQC and SQY responses, which were 10.09 mg/g dry biomass and 22.09 mg/L (Table 5). In the same table the verified values of responses are also given. These experimental values fit well with the respective predicted ones. In particular, maximum squalene content and yield obtained were 10.02 ± 0.53 mg/g dry biomass and 20.70 ± 1.00 mg/L of culture medium, respectively. Squalene production was highly selective, and the only sterol formed was the end product, ergosterol (2.23 mg/g dry biomass) (Figure 3, peak 2). Residual terbinafine that did not exceed 10% of the initial terbinafine addition level was observed in all of the samples (e.g., Figure 3, peak 1) due to coextraction with the unsaponifiable fractions.

All of the above findings revealed that terbinafine via its specific activity is the most effective at controlling squalene formation and has the highest contribution to the maximum response value. From a practical point of view, MJ addition cannot be suggested as an inducer along with terbinafine. This finding is also interesting from the mechanistic point of view and may help other researchers, who made a hypothesis similar to the one predicted above for *Aurantiochytrium mangrovei*.[11]

Application of Optimized Conditions for SQY Using Industrial Yeast Strains and Potential for Commercialization. In order to test the ruggedness of the optimum conditions for squalene yield found above, industrial yeast strains were also employed. Considering that *S. cerevisiae* produces the largest

amounts of ergosterol among various *Saccharomyces* and non-*Saccharomyces* yeasts, commercial strains of the former that are used in different manufacturing procedures were selected. Among them, the four wine yeasts, Maurivin BP 725, Vitilevure LB Rouge MV 95034, Zymaflore F15, and Fermol Bayanus *Lipari*, which have different suggested uses (as indicated by the producers), ensure a rapid start and a high reliability of the fermentation process in wine making (e.g., refs 32−34). In addition, the selected industrial *S. cerevisiae* baking yeast was claimed to be appropriate in croissant and bread production.

With reference to EGY48 performance under the current optimized conditions, those of the five different commercial yeast strains subjected to treatment with 0.30 mM terbinafine exhibited marked differences with regard to biomass formation, SQC, ERGC, and SQY after 30 h of bioprocessing (Table 6). Specifically, Maurivin BP 725, Vitilevure LB Rouge MV 95034, and Zymaflore F15 produced 2-fold higher biomass than that of EGY48. On the other hand, Fermol Bayanus *Lipari* and baker's yeast were inferior with regard to the formation of biomass, partially due to the different adaptation abilities of these strains to withstand terbinafine treatment. Nevertheless, Fermol Bayanus *Lipari* was proven to be a better ergosterol producer than the other commercial yeasts and EGY48, resulting in higher final SQC in terbinafine-treated cells (12.68 mg/g dry biomass). Using the rest of the strains, SQC and ERGC ranged from 5.37 to 6.73 and 1.65 to 2.56 mg/g dry biomass, respectively. These values were 3.5−4.5-fold higher than the respective value of EGY48 grown under semianaerobic conditions,[13] although they were lower than that obtained in terbinafine-treated EGY48 cells. RP-HPLC profiles (data not shown) of the UM of cellular lipids extracted from all commercial yeasts indicated high selectivity similar to that shown in Figure 1 for the EGY48 terbinafine-treated cells. In the cases of Maurivin BP 725, Vitilevure LB Rouge MV 95034, and Zymaflore F15, SQY was better (>1.5 fold) than that of EGY48 as a result of the high biomass formation (Table 6).

Table 5. Optimum Values of Terbinafine, Methyl Jasmonate, and Fermentation Time and Maximum Predicted and Experimental Values of Squalene Content (SQC) and Squalene Yield (SQY)

		squalene content (mg/g dry biomass)	
factor	optimum actual values	predicted response value	mean experimental response value[a]
terbinafine (mM)	0.442		
methyl jasmonate (mM)	0.044	10.09	10.02 ± 0.53
fermentation time (h)	28.0		
		squalene yield (mg/L of culture medium)	
		predicted response value	mean experimental response value[a]
terbinafine (mM)	0.300		
methyl jasmonate (mM)	0.000	22.09	20.70 ± 1.00
fermentation time (h)	30.0		

[a] Mean value of three independent experiments ± standard deviation.

Moreover, to highlight the content and composition of cellular lipids, the fat was quantitatively extracted using an appropriate procedure.[27] The lipid content (15.5% for EGY48 and 11.6% for Vitilevure LB Rouge MV 95034) was found to be satisfactory for commercialization in case this fraction has to be recovered from the biomass. The above values are toward the upper end of the range for total yeast lipids indicated in the literature under different growth conditions (3.5−14.5% of dry biomass).[35] Squalene accounted for 12−13% of total lipid content. Previously reported studies point out that the upper limit of squalene content in the total lipids of yeast as a byproduct of brewery is 12.4% (3.53 mg/g dry biomass).[36,37] This content is limited to 0.3% of total lipids (0.2 mg/g dry biomass) in baker's yeast grown aerobically.[36] Under the current optimized conditions, it is pointed out that the fat is enriched with squalene. Extrapolation of the squalene content per gram of dry biomass led to higher values (23.10 ± 0.67 for EGY48 and 11.31 ± 0.15 for Vitilevure LB Rouge MV 95034) than those reported in Table 6. This can be attributed to the acid hydrolysis step that breaks down the cell wall, leading to more efficient extractability of cellular yeast lipids in contrast to the direct extraction of the unsaponifiable lipids from rigid cell walls.

The lipid composition of both EGY48 and Vitilevure LB Rouge MV 95034 was found to contain mainly squalene and free sterols

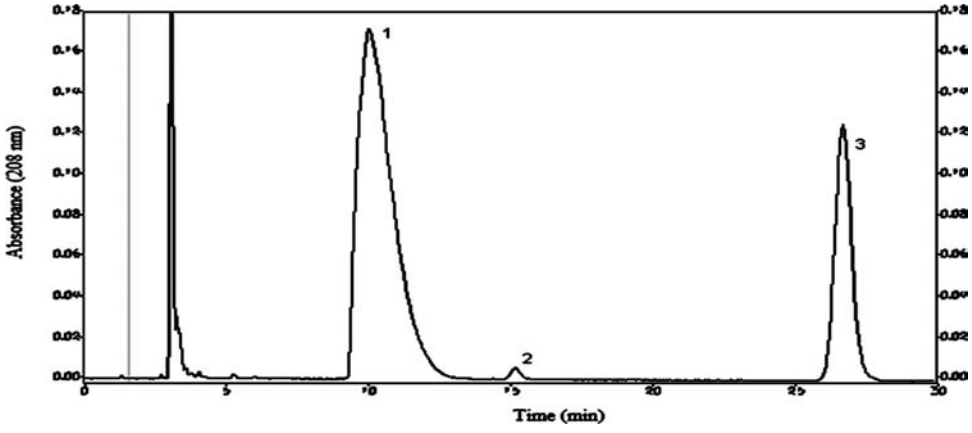

Figure 3. Chromatographic profile of the total unsaponifiable fraction of cellular lipids (peaks: 1, terbinafine; 2, ergosterol; 3, squalene) extracted from wild-type *S. cerevisiae* strain EGY48 cells after 28 h of cultivation in the presence of 0.442 mM terbinafine and 0.044 mM methyl jasmonate.

Table 6. Biomass Formation, Ergosterol Content, and Squalene Content/Yield Using Commercial *Saccharomyces cerevisiae* Strains Treated with Terbinafine (0.3 mM) under Microaerobic Conditions (30 h)

		ergosterol content[a]	squalene content[a]	
yeast strain	biomass[a] (g/L medium)	(mg/g dry biomass)		squalene yield[a] (mg/L medium)
	5.09 ± 0.07	1.96 ± 0.07	6.64 ± 0.33 (3.4)	33.74 ± 1.38
EGY48	2.40 ± 0.09	1.90 ± 0.08	9.10 ± 0.45	21.84 ± 1.10
Maurivin BP 725	6.32 ± 0.01	2.56 ± 0.06	5.37 ± 0.09	33.94 ± 0.51
Vitilevure LB Rouge MV 95034	5.67 ± 0.19	2.20 ± 0.08	6.39 ± 0.34	36.16 ± 1.38
Zymaflore F15	5.09 ± 0.07	1.96 ± 0.07	6.64 ± 0.33	33.74 ± 1.38
Fermol Bayanus *Lipari*	1.11 ± 0.06	3.61 ± 0.21	12.68 ± 0.59	14.09 ± 0.09
baker's yeast	2.31 ± 0.05	1.65 ± 0.06	6.73 ± 0.27	15.55 ± 0.27

[a] Mean value of three independent experiments ± standard deviation.

as well as triacylglycerols and phospholipids (data not shown). No steryl esters were detected on the TLC plates because of the deficiency in ergosterol formation. Free fatty acids were not detected, though they are expected to be present in the total lipid fraction even at low levels.[35,36]

As a conclusion, the data of the present study, along with the wide availability of *S. cerevisiae* commercial strains and their ability to adapt well to a controlled fermentation environment and to grow quickly, suggest the favorability of using this yeast compared to other microorganisms that have been proposed as potent squalene producers, for example, the thraustochytrids microalgae, which are found in tropical and subtropical tidal areas with a high degree of salinity and are available only in private collections. The merits of our approach to investigate ways for a safe alternative production of squalene, considering the progressive increase in its demand, are steadily built up. The total yield of squalene per volume of medium, an important issue, was improved more than 10-fold in comparison to our previous results in the absence of the regulators. These findings seem encouraging toward the direction of scale up in bioreactors that should be carried out together with respective provision of process economics.

■ ASSOCIATED CONTENT

Supporting Information. Table S-I: Analysis of variance of SQC, SQY, and ERGC obtained using the RSM model. Table S-II: Estimated regression coefficients and significance (p-values) for SQC, SQY, and ERGC after analysis using coded values of factors. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Gunstone, F. D., Ed. *Lipids for Functional Foods and Neutraceuticals*; The Oily Press: Bridgwater, England, 2003.

(2) Chen, Z. Y.; Jiao, R.; Ma, K. Y. Cholesterol-lowering nutraceuticals and functional foods. *J. Agric. Food Chem.* **2008**, *56*, 8761–8773.

(3) Reddy, L. H.; Couvreur, P. Squalene: A natural triterpene for use in disease management and therapy. *Adv. Drug Delivery Rev.* **2009**, *61*, 1412–1426.

(4) Rao, C. V.; Newmark, H. L.; Reddy, B. S. Chemopreventive effect of squalene on colon cancer. *Carcinogenesis* **1998**, *19*, 287–290.

(5) Psomiadou, E.; Tsimidou, M. On the role of squalene in olive oil stability. *J. Agric. Food Chem.* **1999**, *47*, 4025–4032.

(6) Stevenson, D. G.; Eller, F. J.; Wang, L.; Jane, J. L.; Wang, T.; Inglett, G. E. Oil and tocopherol content and composition of pumpkin seed oil in 12 cultivars. *J. Agric. Food Chem.* **2007**, *55*, 4005–4013.

(7) Ryan, E.; Galvin, K.; O'Connor, T. P.; Maguire, A. R; O'Brien, N. M. Fatty acid profile, tocopherol, squalene and phytosterol content of brasil, pecan, pine, pistachio, and cashew nuts. *Int. J. Food Sci. Technol.* **2006**, *57*, 219–228.

(8) Bondioli, P.; Mariani, C.; Lanzani, A.; Dedeli, E.; Muller, A. Squalene recovery from olive oil deodorizer distillates. *J. Am. Oil Chem. Soc.* **1993**, *70*, 763.

(9) He, H. P.; Corke, H. Oil and Squalene in *Amaranthus* grain and leaf. *J. Agric. Food Chem.* **2003**, *51*, 7913–7920.

(10) Chang, M. H.; Kim, H. J.; Jahng, K. Y.; Hong, S. C. The isolation and characterization of *Pseudozyma* sp. JCC 207, a novel producer of squalene. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 963–972.

(11) Fan, K. W.; Aki, T.; Chen, F.; Jiang, Y. Enhanced production of squalene in the thraustochytrid *Aurantiochytrium mangrovei* by medium optimization and treatment with terbinafine. *World J. Microbiol. Biotechnol.* **2010**, *26*, 1303–1309.

(12) Yue, C. Y.; Jiang, Y. Impact of methyl jasmonate on squalene biosynthesis in microalga *Schizochytrium mangrovei*. *Process Biochem.* **2009**, *44*, 923–927.

(13) Mantzouridou, F.; Naziri, E.; Tsimidou, M. Z. Squalene versus ergosterol formation using *Saccharomyces cerevisiae*: Combined effect of oxygen supply, inoculum size, and fermentation time on yield and selectivity of the bioprocess. *J. Agric. Food Chem.* **2009**, *57*, 6189–6198.

(14) Mantzouridou, F.; Tsimidou, M. Observations on squalene accumulation in *Saccharomyces cerevisiae* due to the manipulation of HMG2 and ERG6. *FEMS Yeast Res.* **2010**, *10*, 699–707.

(15) EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food or feed (2010 update). *EFSA J.* **2010**, *8*, 1944.

(16) Ryder, N. S. Terbinafine: Mode of action and properties of the squalene epoxidase inhibition. *Br. J. Dermatol.* **1992**, *126*, 2–7.

(17) Veen, M.; Stahl, U.; Lang, C. Combined overexpression of genes of the ergosterol biosynthetic pathway leads to accumulation of sterols in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **2003**, *4*, 87–95.

(18) Leber, R.; Fuchsbichler, S.; Klobučníkov, V.; Schweighofer, N.; Pitters, E.; Wohlfarter, K.; Lederer, M.; Landl, K.; Ruckenstuhl, K.; Hapala, I.; Turnowsky, F. Molecular mechanism of terbinafine resistance

in *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **2003**, *47*, 3890–3900.

(19) De La Pena Moreno, F.; Blanch, G. P.; Ruiz Del Castillo, M. (+)-Methyl jasmonate-induced bioformation of myricetin, quercetin and kaempferol in red raspberries. *J. Agric. Food Chem.* **2010**, *58*, 11639–11644.

(20) Ketchum, R. E. B.; Gibson, D. M.; Croteau, R. B.; Shuler, M. L. The kinetics of taxoid accumulation in cell suspension cultures of *Taxus* following elicitation with methyl jasmonate. *Biotechnol. Bioeng.* **1999**, *62*, 97–105.

(21) Choi, D.; Bostock, R. M.; Avdiushko, S.; Hildebrand, D. F. Lipid-derived signals that discriminate wound- and pathogen responsive isoprenoid pathways in plants-Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 2329–2333.

(22) Lee, M. H.; Jeong, J. H.; Seo, J. W.; Shin, C. G.; Kim, Y. S.; In, J. G.; Yang, D. C.; Yi, J. S.; Choi, Y.-E. Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. *Plant Cell Physiol.* **2004**, *45*, 976–984.

(23) Kampranis, S. C.; Damianova, R.; Atallah, M.; Toby, G.; Kondi, G.; Tsichlisi, P. N.; Makris, A. M. A novel plant glutathione *S*-transferase/peroxidase suppresses Bax lethality in yeast. *J. Biol. Chem.* **2000**, *275*, 29207–29216.

(24) Ryder, N. S. Specific inhibition of fungal sterol biosynthesis by SF 86-327, a new allylamine antimycotic agent. *Antimicrob. Agents Chemother.* **1985**, *27*, 252–256.

(25) Landl, K. M.; Klosch, B.; Turnowsky, F. ERG1, Encoding squalene epoxidase, is located on the right arm of chromosome VII of *Saccharomyces cerevisiae*. *Yeast* **1996**, *12*, 609–613.

(26) Xu, S; Norton, R. A.; Crumley, F. G.; Nes, W. D. Comparison of the chromatographic properties of sterols, select additional steroids and triterpenoids: gravity flow column liquid chromatography, thin-layer chromatography, gas-liquid chromatography and high-performance liquid chromatography. *J. Chromatogr.* **1988**, *452*, 377–398.

(27) Fornairon-Bonnefond, C.; Salmon, J. M. Impact of oxygen consumption by yeast lees on the autolysis phenomenon during simulation of wine aging on lees. *J. Agric. Food Chem.* **2003**, *51*, 2584–2590.

(28) Jollow, D.; Kellerman, G. M.; Linnane, A. W. The biogenesis of mitochondria III. The lipid composition of aerobically and anaerobically grown *Saccharomyces cerevisiae* as related to the membrane systems of the cells. *J. Cell Biol.* **1968**, *37*, 221–230.

(29) Mercer, E. I. Inhibitors of sterol biosynthesis and their applications. *Prog. Lipid Res.* **1993**, *32*, 357–416.

(30) Yates, P. J.; Haughan, P. A.; Lenton, J. R.; Goad, L. J. Effects of terbinafine on qrowth, squalene, and steryl ester content of a celery cell suspension culture. *Pestic. Biochem. Physiol.* **1991**, *40*, 221–226.

(31) Hampton, R.; Dimster-Denk, D.; Rine, J. The biology of HMG-CoA reductase: the pros of contra-regulation. *Trends Biochem. Sci.* **1996**, *21*, 140–145.

(32) Valero, E.; Schuller, D.; Cambon, B.; Casal, M.; Dequin, S. Dissemination and survival of commercial wine yeast in the vineyard: A large-scale, three-years study. *FEMS Yeast Res.* **2005**, *5*, 959–969.

(33) Major, M., Ed. *Technical Review*, No. 182; The Australian Wine Research Institute: Urrbrae, Australia, 2009; http://www.awri.com.au/information_services/technical_review/latest_issue/Technical_Review_Issues/Technical_Review_Issue_182.pdf

(34) Rodríguez, M. E.; Infante, J. J.; Molina, M.; Rebordinos, L.; Cantoral, J. M. Using RFLP-mtDNA for the rapid monitoring of the dominant inoculated yeast strain in industrial wine fermentations. *Int. J. Food Microbiol.* **2010**No. 10.1016/j.ijfoodmicro.2010.11.035.

(35) Slaughter, J. C.; Minabe, M. Fatty acid-containing lipids of the yeast *Saccharomyces cerevisiae* during post-fermentation decline in viability. *J. Sci. Food Agric.* **1994**, *65*, 497–501.

(36) Nurminen, T.; Konttinen, K.; Suomalainen, H. Neutral lipids in the cells and cell envelope fractions of aerobic baker's yeast and anaerobic brewer's yeast Time. *Chem. Phys. Lipids* **1975**, *14*, 15–32.

(37) Rupcic, J.; Juresic, C. G. Influence of stressful fermentation conditions on neutral lipids of a *Saccharomyces cerevisiae* brewing strain. *World J. Microbiol. Biotechnol.* **2010**, *26*, 1331–1336.