JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 6189–6198 6189
DOI:10.1021/jf900673n

Squalene versus Ergosterol Formation Using *Saccharomyces cerevisiae*: Combined Effect of Oxygen Supply, Inoculum Size, and Fermentation Time on Yield and Selectivity of the Bioprocess

Fani Mantzouridou, Eleni Naziri, and Maria Z. Tsimidou*

Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

The dynamics of two wild type strains of *Saccharomyces cerevisiae* (BY4741 and EGY48) that vary in the ability to produce sterols were compared in batch cultures under different aeration conditions. Poor supply of oxygen enhanced selectivity of the bioprocess in favor of squalene formation. Optimization of inoculum size and fermentation time arranged according to a central composite statistical design revealed significant differences between the strains in terms of yield and productivity. Experimental verification showed that an optimized bioprocess under semianaerobic conditions is competitive with regard to those reported in the literature. Maximum squalene yield and productivity were, respectively, 2967.6 ± 118.7 μg/L of culture medium and 104 ± 4.2 μg/Lh for BY4741 and 3129 ± 109.5 μg/L of culture medium and 155.9 ± 5.5 μg/Lh for EGY48. The prospect of developing high-purity squalene preparations that meet food safety regulation demands is expected to attract the interest of the food industry.

KEYWORDS: Squalene; ergosterol; *Saccharomyces cerevisiae*; aeration conditions; inoculum size

INTRODUCTION

Squalene ($C_{30}H_{50}$, 6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an important member of the isoprenoid polyenes that plays a dominant role in the biosynthesis of sterols and steroids. The established importance of squalene in cosmetics is due to its effectiveness as a $^1O_2$ quencher, which is currently extended to the food sector. Health effects through squalene consumption have not been extensively investigated, despite the recent evidence for protection against certain types of cancer (see, e.g., ref (*1*)). Antioxidant properties of importance in edible oil stability attribute its presence with a delay in lipid autoxidation or photo-oxidation or during heating of the matrix (see, e.g., refs (*2*−*4*)).

The availability of squalene in the animal and plant kingdoms is rather confined to specific species. As a result of international concern for the protection of marine animals, the use of certain sharks having livers rich in squalene is now under scrutiny. Among possible alternatives are distillates of the oil industry (*5*) as well as *Amaranthus* seed oil (*6*) and other plant origin materials. Because microbial production of high-value products is an emerging area, the use of microorganisms for squalene production presents also a potential industrial interest. In most of the limited number of publications, mainly patents, on the production of squalene by yeasts (*Saccharomyces*, *Candida*, *Torulaspora*) (*7*−*10*), genetically modified strains of the bacterium *Pseudomonas* (*11*), the algae *Euglena* (*12*), and the marine microorganisms *Pseudozyma* sp. JCC207 and *Aurantiochytrium* sp. BR-MP4-A1 (*13*, *14*) were used.

In the present study, taking into account food safety requirements for products of biotechnology as well as the need for sufficient pools of isoprenoid precursors for high-yield production of squalene by microorganisms, our interest was focused on *Saccharomyces cerevisiae*. In this yeast, the mevalonate biosynthetic route to isoprenoid precursors leads mainly to the formation of ergosterol, the level of which has been found to range from 0.03 to 4.6% of dry biomass in relation with the type of yeast strains, the composition of the culture medium, the stage of growth, and the oxygen availability (*15*, *16*). It is now well-known that squalene is the first specific precursor of ergosterol. The whole process of biosynthesis is separated into pre- and post-squalene formation steps. The former start from acetyl-CoA, provide common precursors of the routes leading to essential cellular constituents such as heme, quinones, and dolichols, and end with squalene. In the latter, the enzymatic transformation of squalene to ergosterol takes place.

S. cerevisiae is able to accumulate squalene under anaerobic conditions, which prevent ergosterol biosynthesis (*16*). This is due to the fact that there are several reaction steps in the ergosterol biosynthetic pathway that require molecular oxygen (*17*). Nevertheless, ergosterol or molecular oxygen is essential for the viability of yeast cells (*18*) so that, under anaerobic conditions, cell growth slows with a concomitant reduction in biomass yield and fermentation rate.

Optimizing the industrial performance of *S. cerevisiae* in terms of squalene production aims at establishing conditions that favor

*Corresponding author (telephone ++302310997796; fax ++302310997779; e-mail tsimidou@chem.auth.gr).

Published on Web 06/19/2009

both active growth and a relatively high squalene content in yeast cells. Our study was undertaken to explore the effects of oxygen supply, inoculum size, and fermentation time on the intensification of the bioprocess for squalene yield and selectivity. The dynamics of two commonly used *S. cerevisiae* laboratory wild type strains (BY4741 and EGY48) that vary in their ability to produce sterols were compared. Optimization of the bioprocess conditions was based on a central composite statistical design. RP-HPLC was employed to monitor changes in the squalene yield and sterol composition of the unsaponified lipid fraction. Biochemical and kinetic elucidation regarding biomass formation, extracellular pH value, and dissolved oxygen evolution, as well as substrate assimilation, were also considered and discussed. To our knowledge, this is the first attempt to optimize the bioprocess for squalene production using *S. cerevisiae* wild type strains. Taking into account the safety status of *S. cerevisiae*, our results are expected to attract the interest of the food industry.

MATERIALS AND METHODS

Microorganisms. The two wild type strains of *S. cerevisiae* used in this study, BY4741 and EGY48, were provided generously by Dr. Antonios Makris (Mediterranean Agronomical Institute of Maich, Crete, Greece). Both strains were maintained at 4 °C on glucose yeast peptone agar (GYPA) slants.

Standards, Reagents, and Solvents. Squalene (for biochemistry, purity = 98%) was from Sigma Chemical Co. (St. Louis, MO), ergosterol (purity > 98%) was from BDH Chemicals Ltd. (Poole, U.K.), and lanosterol (purity > 98%) was from Carl Roth OHG (Karlsruhe, Germany). GYPA was from Sigma Chemical Co Culture media contained D-glucose monohydrate (Panreac Quimica S.A., Barcelona, Spain), yeast extract (Merck), malt extract (Merck), and soy peptone (Lab M Limited, U.K.). HPLC-grade methanol and dichloromethane were from Merck). Ultrahigh-purity water was delivered using a Millipore Milli-Q system (Barnstead International, Dubuque, IA). All 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). 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, Cotati, CA). The chromatograph was coupled to a Linear UVVIS-206 diode array multiple-wavelength detector (Linear Instruments, Fremont, CA). The data from the detector were processed with EZChrom 6.6 software (Sci Software, Inc., San Ramon, CA). The pH value of the culture media was measured with the aid of a Consort C532 pH probe (Turnhout, Belgium). Dissolved oxygen (DO) concentration in the culture medium was determined with a microprocessor oxymeter (OXI 96, WTW, Weinheim, Germany). 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. Yeast cells stored on GYPA slants 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) (*10*). The initial pH value of the medium was 5.5. 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 to a final OD_{600} value of approximately 2.

Medium Composition and Aeration Conditions for Squalene Production. The medium used for all experiments had the following composition (g/L): glucose (40), soy peptone (20), and yeast extract (10); the pH value was 5.5 (*10*). Experiments were conducted under different oxygen supplies, namely, microaerobic and semianaerobic ones. In microaerobic experiments, defined as non-oxygen-limited cultures, cultivation of yeast cells was performed in hydrophobic cotton-stopped Erlenmeyer flasks (500 mL) containing 100 mL of the medium under vigorous shaking (250 rpm). Semianaerobic experiments, defined as oxygen-limited cultures, were carried out by allowing 300 mL of the medium to stand in a similar type of flask. Inoculation was accomplished with 5 mL aliquots of the precultures per 100 mL of fresh medium unless otherwise stated. In all cases incubation was at 30 °C. Fermentation experiments were carried out in triplicate.

Table 1. Levels of Factors in Actual and Coded Values Used in the Experimental Design

factor	name					
		Coded Values				
		$-a$	-1	0	$+1$	$+a$
		Actual Values				
X_1	inoculum size (%, v/v)	0.75	2.00	5.00	8.00	9.24
X_2	fermentation time (h)	5.0	12.0	28.5	45.0	52.0

Experimental Design. Thirteen experiments were set according to an unblocked full factorial central composite statistical design (*19*) for the study of two factors, namely, inoculum size (X_1) and fermentation time (X_2) (h), each at five experimental levels (see Supporting Information). The levels of the X_j factor are coded as follows: $-a$, -1, 0, $+1$, $+a$, where $a = 2^{n/4}$, n = number of variables, and -1, $+1$, and 0 correspond to the low, high, and midleveld of X_j (**Table 1**). The actual levels of each factor were calculated using the following equation:

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

The 13 runs were set using the software Minitab release 13.20 (Minitab, Inc., State College, PA) (**Table 2**). The design shown in **Table 2** was arranged to allow for fitting an appropriate regression model. It combines the vertices of the hypercubes for which the coordinates are given by the 2^n factorial design to provide for the estimation of the curvature of the model. Five replicates at the center of the design were used to allow for the estimation of experimental error and to check the adequacy of the model (lack of fit). Experiments were randomized to maximize the effect of unexplained variability in the observed responses due to extraneous factors.

Polynomial response surfaces were fitted to three response variables, namely, squalene yield (SQY) (mg/L of culture medium), productivity (P) (μg/Lh), and biomass (B) (g/L). Statistical analysis of the experimental data was performed by response surface methodology (RSM) using the same software. Details of the methodology can be found elsewhere (*19*). Initially, the second-order polynomial model was fitted to each response, giving an equation of the form,

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \qquad (1)$$

where Y is the predicted SQY, P, and B response, X_1 and X_2 represent the levels of the coded factors according to **Table 1**, and $\beta_0, \beta_1, ..., \beta_{12}$ 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 of < 0.05 were considered to be significant. When possible, the model was simplified by the omission of nonstatistically significant terms. Optimization of the fitted polynomials for SQY and P 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 OD and Biomass Dry Weight. The OD value of the inoculated medium was measured at 600 nm against a control sample. 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. All measurements were obtained in triplicate.

Determination of DO Concentration. DO concentration was measured off-line as described in a recent paper (*20*). To avoid infection, a

Table 2. Experimental Design for Five-Level−Two-Factor Central Composite Design and Comparison between Observed and Predicted Responses for Squalene Yield (SQY), Productivity (*P*), and Biomass (*B*) of BY4741 and EGY48

			responses					
	factors		SQY (μg/L of culture medium)		P (μg/Lh)		B (g/L of culture medium)	
run	inoculum size (%)	fermentation time (h)	actual	predicted	actual	predicted	actual	predicted
			I: BY4741					
1	5.00	28.5	3150.000	3114.000	111.000	109.400	2.900	2.860
2	5.00	28.5	3060.000	3114.000	107.000	109.400	2.800	2.860
3	5.00	28.5	3150.000	3114.000	111.000	109.400	2.900	2.860
4	5.00	28.5	3060.000	3114.000	107.000	109.400	2.800	2.860
5	8.00	12.0	864.000	972.848	72.000	67.994	1.610	1.590
6	5.00	28.5	3150.000	3114.000	111.000	109.400	2.900	2.860
7	5.00	52.0	1680.000	1752.105	32.000	29.695	2.530	2.480
8	2.00	45.0	1980.000	1880.519	44.000	45.510	2.430	2.445
9	8.00	45.0	1950.000	1950.254	43.000	44.019	2.790	2.844
10	9.24	28.5	1990.000	1914.800	70.000	71.594	2.810	2.785
11	5.00	5.0	201.000	119.529	39.000	43.801	0.440	0.495
12	0.75	28.5	1500.000	1565.835	53.000	53.901	1.980	2.010
13	2.00	12.0	540.000	549.13	45.000	41.484	0.950	0.891
			II: EGY48					
1	5.00	28.5	2008.000	1848.807	71.000	65.400	2.425	2.475
2	5.00	28.5	1610.000	1848.807	57.000	65.400	2.550	2.475
3	5.00	28.5	2008.000	1848.807	71.000	65.400	2.550	2.475
4	8.00	45.0	3004.000	2905.266	67.000	65.521	2.495	2.486
5	5.00	28.5	1610.000	1848.807	57.000	65.400	2.425	2.475
6	2.00	12.0	1510.000	1393.201	126.000	120.478	1.330	1.285
7	5.00	28.5	2008.000	1848.807	71.000	65.400	2.425	2.475
8	8.00	12.0	1420.000	1287.448	118.000	117.377	1.630	1.537
9	9.24	28.5	2410.000	2528.886	85.000	85.036	2.470	2.531
10	0.75	28.5	1630.000	1726.610	57.000	63.964	2.080	2.073
11	5.00	52.0	2082.000	2165.854	40.000	44.105	2.050	2.017
12	2.00	45.0	1748.000	1665.019	39.000	32.622	2.050	2.090
13	5.00	5.0	698.000	829.679	140.000	142.896	0.690	0.777

number of microaerobic and semianaerobic experiments were started simultaneously. Each flask was used for a single measurement only. All measurements were obtained in triplicate.

Extraction of Squalene and Sterols. Squalene and sterols were extracted after methanolic pyrogallol saponification (*21*) as follows: After collection by centrifugation, yeast cells were washed twice with distilled water and freeze-dried. Portions of dry biomass (96 mg) were resuspended 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 unsaponified matter (UM) was extracted by the addition of 10 mL of hexane. The phases were separated by centrifugation at 4000*g* for 10 min. The top hexane layer was removed to a clean tube, and the residue was extracted two more times with hexane. Emulsions, if present, can be dispersed by the addition of 0.5 mL of methanol. The hexane fractions were combined and dried over anhydrous Na_2SO_4, and finally the solvent was removed under vacuum (40 °C). Repeatability of extraction was satisfactory (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 Analysis of Squalene and Sterols. The UM was dissolved in methanol/dichloromethane (9:1, v/v). All samples were filtered through a 0.45 μm membrane filter (Schleicher Schnell, Dassel Germany) just before HPLC. Separation of squalene and sterols was achieved on a reversed phase Nucleosil C_{18} column (particle size = 5 μm, 250 × 4.6 mm i.d.) maintained at 40 °C. The elution solvent was methanol/water (98:2, v/v), the flow rate was set at 1.0 mL/min, and the injection volume was 10 μL. Detection and quantification of squalene and ergosterol were at 208 and 285 nm, respectively. Peaks were identified by comparison of retention time with that of authentic standards and confirmed by spiking and comparison of spectral data. Owing to the lack of availability of standard zymosterol and ergosta-5,7,22,24(28)-tetraen-3β-ol, identification was solely based on elution order and spectral data in the UV region with regard to published information (*22*, *23*). 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 and 3.5, respectively, for 50 ppm squalene and 500 ppm ergosterol standard solutions, $n = 5$).

Determination of Residual Sugar and Nitrogen Content. Residual sugars and nitrogen were determined in the filtered aliquots using standard colorimetric procedures (*20*). All measurements were obtained in triplicate.

RESULTS AND DISCUSSION

Effect of Aeration on Growth of *S. cerevisiae* with Regard to Squalene Production and Selectivity. In the first part of the investigation, the effect of aeration upon cell growth and squalene production by two *S. cerevisiae* wild type strains, namely, BY4741 and EGY48, which differ in their ability to produce sterols, was studied. In this series of experiments, yeast cells were allowed to grow under microaerobic, that is, in shake-flask cultures, or semianaerobic conditions, that is, in standing cultures. In our experiments, we used a high initial level of glucose (40 g/L of culture medium), in line with previous works (*10*, *24*−*26*), which suggested an inhibitory effect on the synthesis of ergosterol under low oxygen supply and, consequently, a rather selective accumulation of squalene.

The evolution of the biochemical responses (biomass, substrate consumption, extracellular pH, and DO) of BY4741 and EGY48 under the two different aeration conditions is presented in **Figure 1**. Squalene transformation to ergosterol was followed by RP-HPLC of the UM of cellular lipids from yeast cells harvested at the beginning (5 h) and in the middle of the exponential growth phase (12 h), at the beginning of the stationary

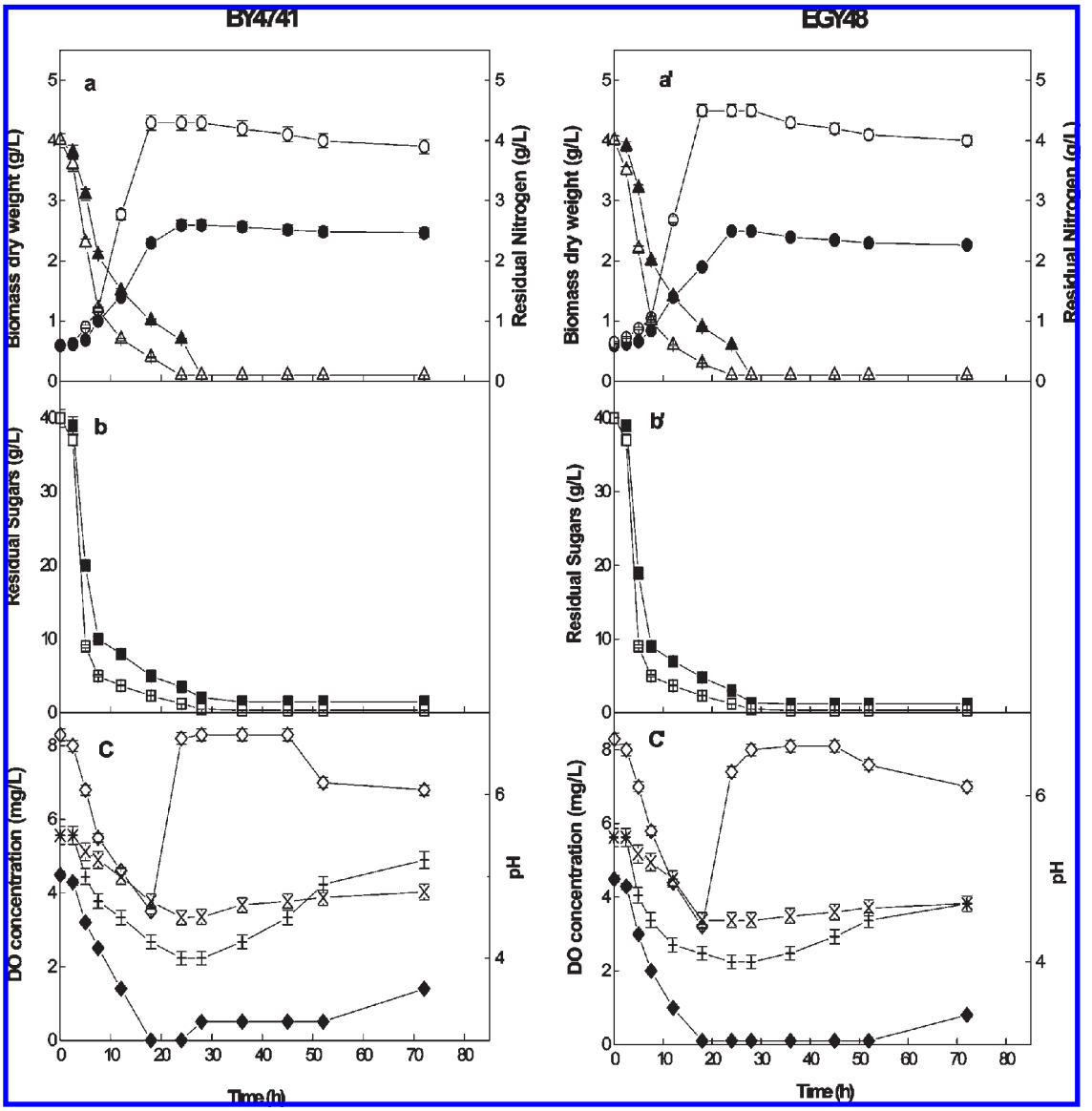

Figure 1. Kinetics of total biomass dry weight (○, ●), residual sugars (□, ■), residual nitrogen (△, ▲), medium pH (+, ×), and DO concentration (◇, ◆) in BY4741 and EGY48 cultivation under microaerobic (open symbols) and semianaerobic (solid symbols) conditions (see Materials and Methods for descriptions of cultivation conditions). Error bars represent the SD of the mean of three independent experiments.

phase (28 h), at the late growth phase (45 h and 52 h), and at the end of cultivation (72 h) (**Figures 2**−**4**). The results are critically discussed below in terms of effectiveness of experimental conditions.

Effect of Aeration Conditions on Growth Characteristics of Yeast Strains. As is obvious in **Figure 1**, the growth patterns for BY4741 and EGY48 depended strongly on the aeration conditions. However, for the same oxygen supply, no significant differences were observed between the two strains. Decreases of glucose by 4- and 2-fold were observed during the early phase of the bioprocess (0−5 h) under microaerobic and semianaerobic conditions, respectively, and the amount of biomass per unit of substrate consumed in both cases was very low (**Figure 1a**,**a′**,**b**,**b′**). As expected, semianaerobic conditions resulted in a 2-fold lower initial DO level in the culture medium, in comparison with the respective one under microaerobic conditions (4.5 versus 8.3 mg/L of culture medium) (**Figure 1c**,**c′**). DO values near zero indicated oxygen limitation after 12 h of cultivation. It seems that in both aeration conditions fermentative metabolism dominated due to glucose overfeeding (Crabtree effect), which could not be prevented even at higher DO level. The decreased respiratory activity of yeast cells was reflected in the low biomass yield and the slow DO consumption (**Figure 1a**,**a′**,**c**,**c′**). In the exponential phase (5−18 h), for lower glucose concentration, oxidative respiration advanced progressively, causing an increase in DO consumption and a rapid growth rate. Growth was accompanied by nitrogen consumption and a decrease in the extracellular pH value due to the production of organic acids (**Figure 1a**,**a′**,**c**,**c′**). However, semianaerobic conditions led to a 50% reduction in the maximum biomass values for both strains in comparison with those obtained under microaerobic conditions (2.5 and 2.6 g/L of culture medium after 24 h vs 4.3 and 4.5 g/L of culture medium after 28 h for BY4741 and EGY48, respectively) (**Figure 1a**,**a′**). When most of the available glucose has been consumed, yeast cells ceased growing, and, consequently, DO levels increased. After 28 h, the cells aged gradually with some degree of autolysis. This resulted in cell dry weight loss due to release of intracellular material into the medium with a parallel increase in pH value. Noticeably, under microaerobic conditions, near the end of cultivation (after 50 h) the DO level was greatly reduced, whereas,

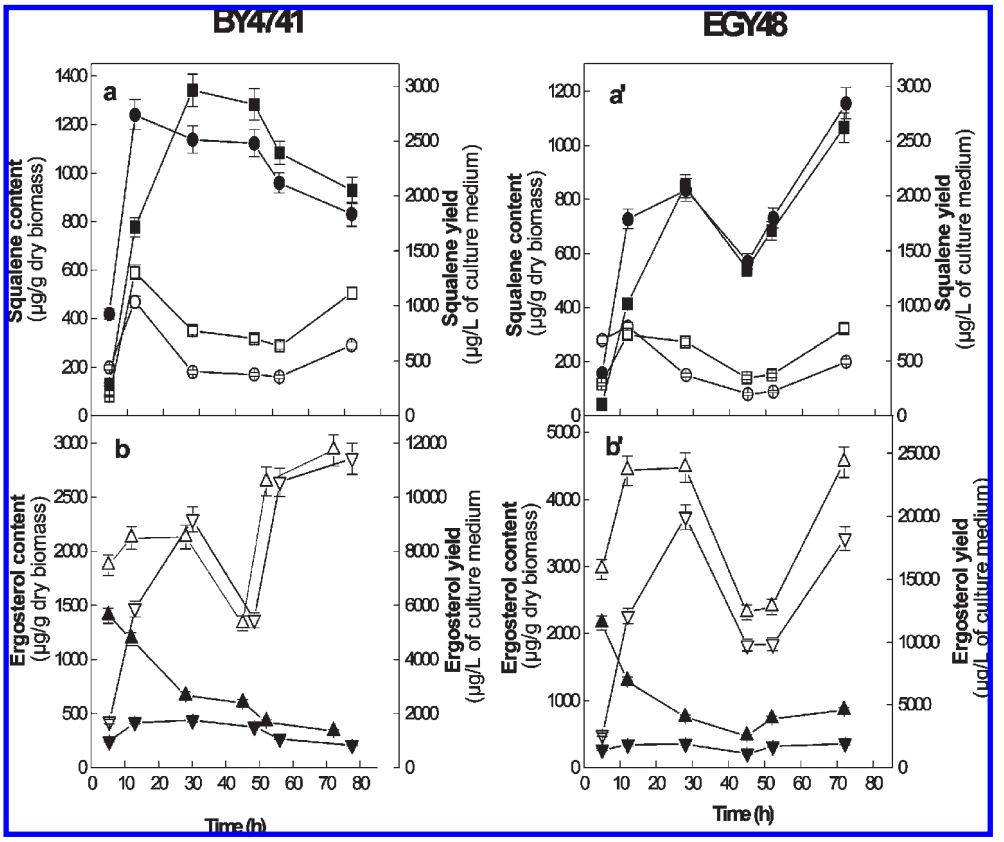

Figure 2. Kinetics of squalene content (○, ●) and yield (□, ■) and ergosterol content (△, ▲) and yield (▽, ▼) in BY4741 and EGY48 cultivation under microaerobic (open symbols) and semianaerobic (solid symbols) conditions (see Materials and Methods for descriptions of cultivation conditions). Error bars represent the SD of the mean of three independent experiments.

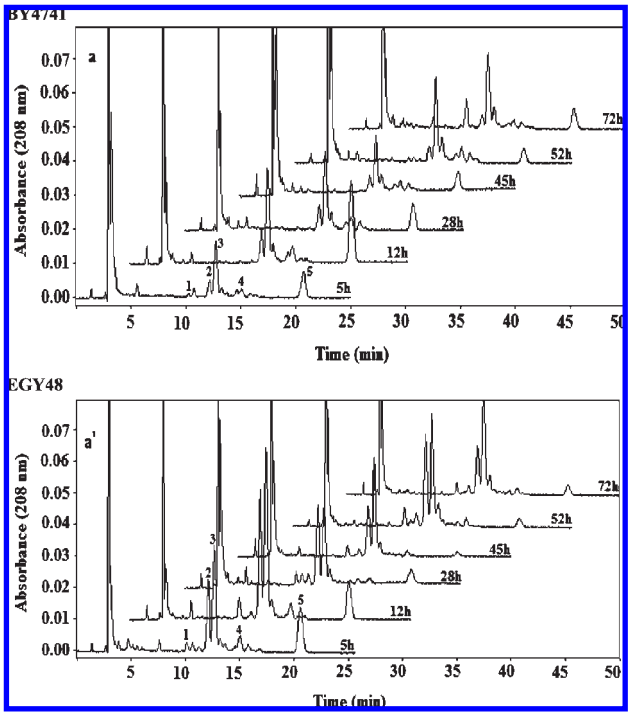

Figure 3. Chromatographic profile of the UM of cellular lipids extracted from BY4741 (**a**) and EGY48 (**a**′) cultivated under microaerobic conditions. Peaks: 1, ergosta-5,7,22,24(28)-tetraen-3β-ol; 2, zymosterol; 3, ergosterol; 4, lanosterol; 5, squalene) (chromatographic conditions as in Materials and Methods).

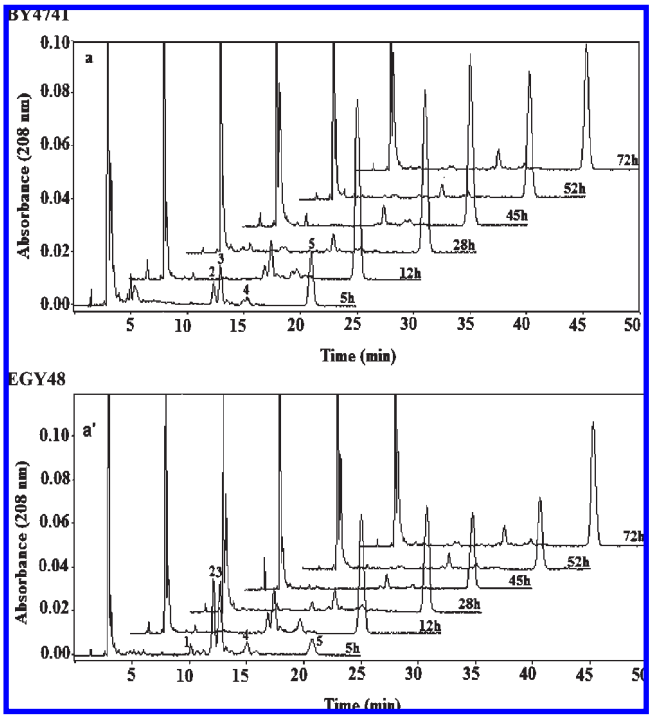

Figure 4. Chromatographic profile of the UM of cellular lipids extracted from BY4741 (**a**) and EGY48 (**a**′) cultivated under semianaerobic conditions. Peaks: 1, ergosta-5,7,22,24(28)-tetraen-3β-ol; 2, zymosterol; 3, ergosterol; 4, lanosterol; 5, squalene (chromatographic conditions as in Materials and Methods).

under semianaerobic conditions, an increase of DO level was observed after 44 h of growth ceasing (**Figure 1a**,**a′**,**c**,**c′**). These findings may be due to a metabolic activity of yeast cells during the late growth phase that contributes to the utilization of molecular oxygen. However, under semianaerobic conditions, the low rate of atmospheric oxygen transfer into the interior of the liquid in standing flasks should not be neglected.

Our results clearly indicate that microaerobic conditions are more efficient than semianaerobic ones in terms of cell growth and biomass accumulation. This can be attributed either to the favored synthesis of sterols and unsaturated fatty acids, which are essential for important membrane physical characteristics such as intensity and permeability (*16*, *18*), or to the effect of alternative oxygen-consuming pathways on yeast metabolism (*17*).

Effect of Aeration Conditions on Squalene Content of Yeast Cells and Yield of the Bioprocess. HPLC of the UM of cellular lipids demonstrated that, under semianaerobic conditions, significantly higher amounts of squalene were accumulated in BY4741 and EGY48 when compared with those determined under microaerobic conditions (1241 μg/g dry of biomass at 12 h and 1155 μg/g of dry biomass at 72 h vs 470 and 330 μg/g dry of biomass at 12 h) (**Figure 2a**,**a′**). This finding is the consequence of the inhibition of squalene epoxidase formation and activity under low levels of oxygen (*26*). As a result of the strong increase in squalene content in yeast cells grown under semianaerobic conditions, yield per volume medium was at least 2-fold greater (i.e., 2959 and 2622 vs 1300 and 740 μg/L of culture medium for BY4741 and EGY48, respectively) (**Figure 2a**,**a′**). Another important observation under the semianaerobic conditions was the different time patterns of squalene formation in the two strains. Specifically, in BY4741, squalene content reached its maximum (1241 μg/g of dry biomass) in the exponential growth phase (at 12 h of cultivation) and then declined to a final value of 830 μg/g of dry biomass (**Figure 2a**). In EGY48, squalene content showed a strong fluctuation throughout the cultivation. Thus, although accumulation progressed for the first 24 h (up to 835 μg/g of dry biomass), it dropped within the next 17 h and then an upward trend was evidenced up to a final value of 1155 μg/g of dry weight (**Figure 2a′**). Squalene content showed a similar, although not so sharp, type of fluctuation in both strains cultivated under microaerobic conditions (**Figure 2a**,**a′**). According to the latter pattern of squalene accumulation, it seems that yeast cells continue to synthesize it during the late growth phase, coinciding with glucose depletion, after a period of adaptation.

It is also important to note that the patterns for ergosterol accumulation in the two yeast strains cultivated under semianaerobic conditions were analogous to the ones observed for squalene content in the respective cases (**Figure 2b**,**b′**). For example, in BY4741, ergosterol content showed a constant decrease throughout the cultivation to a final value of 334 μg/g of dry biomass and a similar trend in EGY48 during the first 45 h. Inversion of the pattern in the second strain after 45 h of cultivation led to a 2.5-fold increase of the end product (850 μg of ergosterol/g of dry biomass). Our observation can be explained by the fact that, after 45 h, yeast cells may continue to synthesize squalene and ergosterol using organic acids and ethanol produced in former steps (*27*). Such an activity may contribute to a higher ethanol tolerance of EGY48 under semianaerobic conditions (*28*).

Effect of Aeration Conditions on the Selectivity of the Bioprocess for Squalene Production. Because various intermediates are formed in the post-squalene pathway, enhancement of its synthesis at the expense of ergosterol and related intermediates may be achieved only under controlled conditions. In our study, the overall sterol formation pattern in the different aeration conditions was monitored by RP-HPLC of the UM at 208 nm and ergosterol content determination at 285 nm.

Analysis revealed the significant differences between the sterol profile of the two strains and the effect of aeration strategy on the selectivity of the bioprocess. Under microaerobic conditions, ergosterol (**Figure 3a**, peak 3) was the main sterol in BY4741 throughout the monitoring period, whereas zymosterol (**Figure 3a**, peak 2) and lanosterol (**Figure 3a**, peak 4) were detected in traces. In contrast, in EGY48, zymosterol and ergosterol dominated at all stages of the growth cycle, whereas only traces of the direct precursor of ergosterol, ergosta-5,7,22,24-(28)-tetraen-3β-ol (**Figure 3a′**, peak 1) and lanosterol (**Figure 3a′**, peak 4) were present. According to previous results by Makzec et al. (*29*), our observations can be explained by an insufficient conversion of zymosterol to fecosterol at elevated content of the former intermediate in EGY48 cells. The results under semianaerobic conditions showed a general sterol deficiency in favor of squalene formation, especially after 28 h of cultivation (**Figure 4a**,**a′**). Ergosterol was produced in significantly lower amounts by both yeast strains grown in comparison with those obtained under microaerobic contitions (1407 and 2154 vs 2930 and 4560 μg/g of dry biomass for BY4741 and EGY48, respectively). Restricted oxygen supply enhanced the synthesis of squalene at the expense of ergosterol in both yeasts; thus, as was previously shown in **Figure 2a**,**a′**, additional squalene reserves were formed. However, ergosterol levels under semianaerobic conditions indicate that both yeast cells were not completely defective for its biosynthesis. Despite the significant decrease in ergosterol formation in EGY48 cells under these conditions, squalene content was not proportionally increased. This strain formed squalene to a level lower than that produced by BY4741, which could be due to the accumulation of toxic levels of squalene precursors that inhibit transformation through the later steps of the mevalonate pathway, in line with the work of Polakowski et al. (*30*). With regard to the presence of only traces of zymosterol in both strains, it seems that the biochemical transformation of zymosterol to fecosterol is complete or that other factors, for example, ethanol, may also be involved. For example, it is known that the presence of ethanol leads to a decrease of the total sterol content, although not at the expense of ergosterol content (*28*). In this regard, ethanol, which is produced under semianaerobic conditions, might lower zymosterol synthesis in both yeast cells.

Optimization Process for Squalene Production Using the Response Surface Methodology. From the results obtained so far, semianaerobic conditions were found to be a suitable aeration strategy for squalene production by *S. cerevisiae* and the selectivity of the bioprocess. On such grounds it was challenging to direct, under the above aeration conditions, other cultural stimulants to achieve higher squalene yields. The importance of inoculum size and its effects on the parameters accounting for growth (i.e., duration of the lag phase, specific growth rate, biomass yield) and ethanol inhibitory effect on growth and production rate during ethanol fermentation by *S. cerevisiae* are known (see, e.g., ref (*31*)). Therefore, in the next part of our study, a central composite statistical design was used to select the experimental conditions under which the effects of inoculum size (X_1) and fermentation time (X_2) on squalene yield (SQY) (μg/L of culture medium), productivity (P) (μg/Lh), and biomass (B) (g/L of culture medium) were investigated for BY4741 and EGY48. The model allowed assessment of factor interactions so that the response surface models fitted enabled optimization of the bioprocess conditions. Verification of the models was then tested experimentally.

Model Fitting for Squalene Yield, Productivity, and Biomass. For BY4741 and EGY48, experimental results for SQY, P,

Table 3. Model Equations for Prediction of the Optimum Response Values of BY4741 and EGY48

model	response	polynomial equation[a] coded values of factors	polynomial equation[a] actual values of factors
		I: BY4741	
1	squalene yield (SQY, μg/L of culture medium)	$SQY = 1347+184X_1+711X_2 -5X_1^2 -1089X_2^2$ (2)	$SQY = -3500.21+855.105X_1+271.941X_2 -76.3028X_1^2 -4.00034X_2^2$ (3)
2	productivity (P) (mg/L/h)	$P = 46.27+6.42X_1+5.63X_2 -0.16X_1^2 -36.33X_2^2 -0.58X_1X_2$ (4)	$P = -85.7230+32.0288X_1+8.01025X_2 -2.59135X_1^2 -0.133429X_2^2 -0.141414X_1X_2$ (5)
3	biomass (B) (g/L)	$B = 1.9122+0.0813X_1+0.8157X_2 -0.0016X_1^2 -0.6863X_2^2$ (6)	$B = -1.71513+0.391499X_1+0.193799X_2 -0.0256912X_1^2 -0.00252070X_2^2$ (7)
		II: EGY48	
1	squalene yield (SQY, μg/L of culture medium)	$SQY = 1741.8 -9.6X_1 -43.6X_2+22.4X_1X_2$ (8)	$SQY = 1392.53 -254.142X_1 +31.3890X_2 -0.644703X_1X_2$ (9)
2	productivity (P) (mg/L/h)	$P = 64.67 -0.43X_1 -48.73X_2 +14.05X_2^2+0.60X_1X_2$ (10)	$P = 193.778 -7.75317X_1 -5.96755X_2 +0.0516075X_2^2+0.181818X_1X_2$ (11)
3	biomass (B) (g/L)	$B = 2.0230+0.0285X_1+0.3830X_2 -0.0004X_1^2 -0.5391X_2^2$ (12)	$B = -0.296940+0.129320X_1+0.135779X_2 -0.00961712X_1^2 -0.00198004X_2^2$ (13)

[a] X_1 and X_2 are the coded (eqs 2, 4, 6, 8, 10, and 12) or actual (eqs 3, 5, 7, 9, 11, and 13) values of factors presented in **Table 1**.

and B were analyzed by ANOVA (data in Tables I and II provided as Supporting Information) to test the validity of each model on the basis of F-test values (see **Table 2**, Experimental Design). If the F test for the model is significant at the 5% level ($p < 0.05$), then the model can adequately account for the variation observed. If the F test for lack of fit is significant, then a more complicated model is needed. R^2 ranged from 0.920 to 0.997, and the F test for the regression was significant at a level of 5% ($p < 0.05$), indicating that >92% of the variability in the responses could be explained by the models (data in Table I provided as Supporting Information). The models, in terms of coded and actual factor values (**Table 1**), fitted for each of the response variables are shown in **Table 3** (eqs 2−13). When needed, the full second-order polynomial model was reduced by omitting the insignificant terms ($p > 0.05$) (data in Tables I and II provided as Supporting Information). In the case of SQY, the full second-order model was reduced by omitting the insignificant terms ($p \gg 0.05$) X_1X_2 for BY4741 and X_{11}^2 and X_{22}^2 for EGY48. X_1 and X_2 appear to have no significant linear effect on SQY for EGY48; however, these factors were included in eqs 8 and 9 as their interaction effect on SQY was significant at the 5% level. In the case of P, the full second-order model was reduced by omitting the insignificant term X_{11}^2 for EGY48. X_1 and X_2 appear to have no significant linear effect on P for EGY48 and BY4741, respectively; however X_1 was included in eqs 10 and 11 and X_2 was included in eqs 4 and 5 as the former gave interaction with X_2 that had a significant effect on P for EGY48 and the latter had a strong quadratic effect ($p \ll 0.05$) as well as interaction effect with X_1 ($p \ll 0.05$) on P for BY4741. In the case of B, the full second-order model was reduced by omitting the insignificant term X_1X_2 for both strains (eqs 6, 7, 12, and 13). Consequently, the above equations can give an adequate description of the experimental data and are suitable for use in the optimization of the SQY, P, and B responses.

Main Effects of Factors and Interactions on SQY, P, and B Responses. The fitted polynomial equations (eqs 3, 5, 7, 9, 11, and 13) were expressed as response surface plots (**Figure 5a**,**a′**) to visualize the relationship between the responsesof SQY, P,and B and experimental levels of each factor (X_1, X_2) and to deduce the optimum conditions. Our results suggest that the inoculum size is an important variable when one is attempting to determine the kinetics of squalene production and productivity. Moreover, comparison of panels **a** and **a′** of **Figure 5** indicates that the two strains were differently affected by inoculum size and fermentation time. For BY4741, panels **a(i)** and **a(ii)** of **Figure 5** show increasing SQY and P as a function of inoculum size and fermentation time, with a maximum near the middle level of both factors (**Table 1**). In the same figures, the negative quadratic effect and interaction effect of the factors can be seen by the characteristic curvature of the response surfaces, especially at values of factors higher than their respective middle level. In contrast, in the SQY response surface plot for EGY48 (**Figure 5a′(i)**), the strong positive interaction effect of the above factors on SQY is clear, indicating that this response reaches a maximum when both X_1 and X_2 approach their high levels. These effects suggest that it might be possible to get a slight growth of yeast cells along with low cell viability under semianaerobic conditions by selecting an inoculum size below 3% (v/v). It can be also assumed that the higher the inoculum volume, the greater the amounts of factors that stimulate growth, in line with observations made by Thomas et al. (*32*) for the positive effect of cell-free culture filtrate on anaerobic cell growth of the yeast. Moreover, increasing the inoculum level may decrease the ethanol inhibition effect (*31*). The above assumption is strengthened by the trend observed for B in BY4741 and EGY48 (**Figure 5a(iii)**,**a′(iii)**). High inoculum size values lead more quickly to nutrient and oxygen consumption and increase in oxygen demand. Because semianaerobic conditions are not expected to provide the increased oxygen

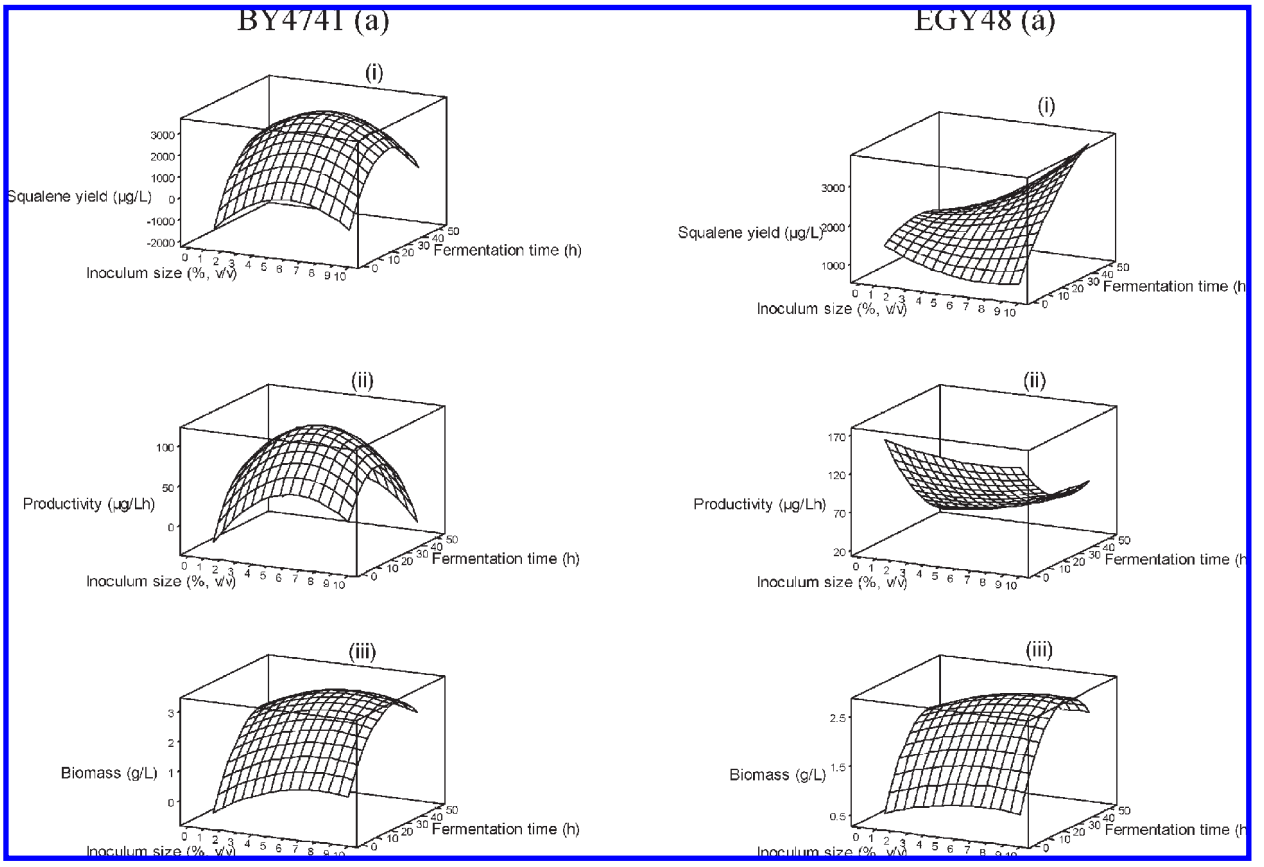

Figure 5. Surface plot for squalene yield (SQY) (**i**), productivity (*P*) (**ii**), and biomass (*B*) (**iii**), at various levels of inoculum size (%, v/v) and fermentation time (h) for BY4741 (**a**) and EGY48 (**a′**).

Table 4. Predicted and Experimental Values of Squalene Yield (SQY), Productivity (*P*), and Biomass (*B*) for BY4741 and EGY48 at Optimum Values of Inoculum Size and Fermentation Time

		SQY (µg/L of culture medium)		productivity (µg/Lh)	
factor	optimum actual value	predicted response value	mean exptl response value[a]	predicted response value	mean exptl response value[a]
			BY4741		
inoculum size (%)	5	3114	2967.6 ± 118.7		
fermentation time (h)	28.5				
inoculum size (%)	5.2			109	104 ± 4.2
fermentation time (h)	27.3				
			EGY48		
inoculum size (%)	8.1	3000	3129 ± 109.5		
fermentation time (h)	47.7				
inoculum size (%)	2.6			150	155.9 ± 5.5
fermentation time (h)	5.2				

[a] Mean value of three independent experiments.

requirement, anaerobic metabolism can be triggered along with more ethanol production. Again, with regard to the work of Walker-Caprioglio et al. (*28*), excess ethanol might be the reason for the decrease in SQY and *P* at high inoculum size values for BY4741. In contrast, it seems likely that EGY48, which exhibits higher levels of ergosterol than the respective one from BY4741 (**Figure 2b**,**b′**), might also have a higher ethanol tolerance. With this in mind, we propose that at high inoculum size values of EGY48, ethanol that is produced during the early stages of cultivation could be used as a substrate for squalene production under glucose depletion at the late stage of fermentation (*27*). As shown in **Figure 5a′(ii)**, in all inoculum size values a trend toward higher *P* values is noted by decreasing fermentation time to low level for EGY48. As the influences from the aerobically grown preculture were negligible (data not shown), this can be explained by the fact that the steepest slope of squalene synthesis is during the early growth phase. This is in line with our initial experiments and previous investigations (*15*, *28*).

Optimum Conditions for SQY and P. X_1 and X_2 values determined to be optimum for SQY and *P* responses for BY4741 and EGY48 by RSM optimization approach are presented in **Table 4**. For BY4741, inoculum size and fermentation time combination of (5.0%, 28.5 h) and (5.2%, 27.3 h) can be recommended as optimum for SQY and *P*, respectively. The above sets of conditions were substituted into the eqs 3 and 5, respectively, to predict maximum SQY and *P* values; the optimum values calculated were 3114 µg/L and 109 µg/Lh for SQY and *P*, respectively (**Table 4**). For EGY48, inoculum size and fermenta-

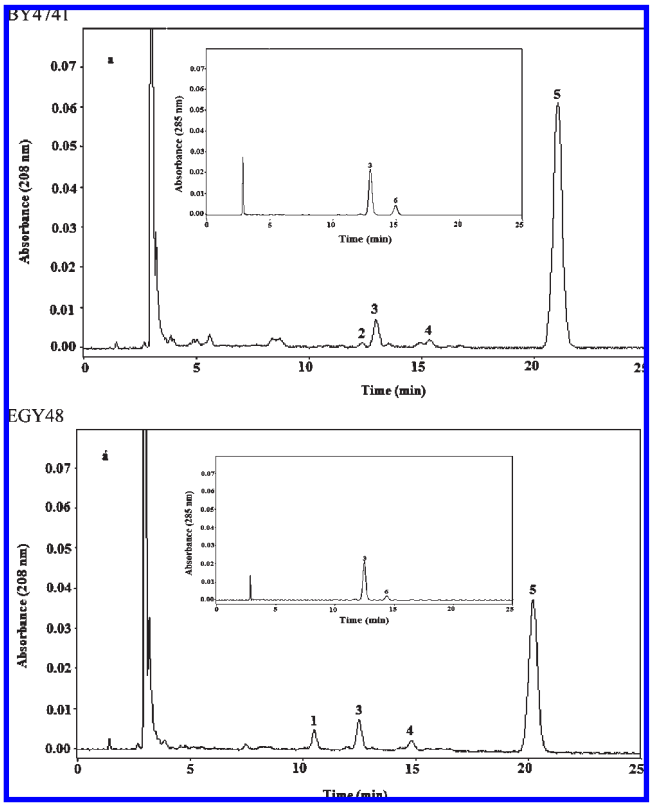

Figure 6. Chromatographic profile of the total unsaponifiable fraction of cellular lipids extracted from BY4741 (**a**) and EGY48 (**a′**) cultivated under the predicted optimum conditions for squalene yield. Peaks: 1, ergosta-5,7,22,24(28)-tetraen-3β-ol; 2, zymosterol; 3, ergosterol; 4, lanosterol; 5, squalene; 6, 22,23-dihydroergosterol) (optimum conditions as in **Table 4**).

tion time combinations of (8.1%, 47.7 h) and (2.6%, 5.2 h) can be recommended as optimum for SQY and *P*, respectively. The above sets of conditions were substituted into eqs 9 and 11, respectively, to predict maximum SQY and *P* values; the optimum values calculated were 3000 μg/L and 150 μg/Lh for SQY and *P*, respectively (**Table 4**).

Verification of Results. The suitability of the model equations for predicting the optimum response values were tested experimentally using the predicted optimum conditions. As shown in **Table 4**, the experimental values fit well with the respective predicted ones. Maximum squalene yield and productivity obtained experimentally using the optimized conditions were, respectively, 2967.6 $\pm$ 118.7 μg/L of culture medium after 28.5 h (1000 μg/g of biomass dry weight) and 104 $\pm$ 4.2 μg/Lh for BY4741 and 3129$\pm$109.5 μg/L of culture medium after 48 h (1565 μg/g of biomass dry weight) and 155.9 $\pm$ 5.5 μg/Lh for EGY48. Under the predicted optimum conditions, squalene production was highly selective for both strains (**Figure 6a**,**a′**). Nevertheless, a slightly higher proportion of the direct precursor of ergosterol, ergosta-5,7,22,24(28)-tetraen-3β-ol (**Figure 6a′**, peak 1) and lanosterol (**Figure 6a′**, peak 4) was observed for EGY48. The sterol fraction extracted from yeast strains contained also a compound with the same absorption maxima as ergosterol and a slightly longer retention time than that for ergosterol (**Figure 6a**,**a′**, peak 6). According to literature data (*33*), this peak can be assigned to an oxidation product of ergosterol, that is, 22,23-dihydroergosterol.

We showed that the type of yeast strain, aeration strategy, inoculum size, and stage of growth cycle are critical factors in achieving high squalene yield and selectivity of the bioprocess. Our optimized bioprocess under semianaerobic conditions is a competitive process compared to performances reported in the literature, that is, 41 μg/g of biomass dry weight after 48 h with commercially available compressed baker's yeast under anaerobic cultivations (*10*) nd 3000 μg/g in 168 h with two *S. cerevisiae* leaky mutants of ergosterol biosynthesis in YPD medium (*8*). Thus, our results are of practical importance in the development of a commercial bioprocess for squalene production by *S. cerevisiae*. From an industrial point of view, the economic feasibility of the bioprocess depends strongly on the magnitude of costs involved for the production and commercialization of the final product and its intended utilization. The prospect of developing high-purity squalene preparations that meet food safety regulation demands would offer indisputable profit to the food industry. Such an effort is in process in our laboratory.

ABBREVIATIONS USED

Acetyl-coA, acetyl-coenzyme A; ATP, adenosine triphosphate; RP-HPLC, reversed-phase high-performance liquid chromatography; YPD, yeast peptone dextrose.

Supporting Information Available: Analysis of variance of squalene yield (SQY), productivity (*P*), and biomass (*B*) for response surface quadratic model for BY4741 and EGY48; estimated regression coefficients and significance (*P*) values for squalene yield (SQY), productivity (*P*), and biomass (*B*) after analysis using coded values of factors for BY4741) and EGY48; response surface methodology description. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Rao, C. V.; Newmark, H. L.; Reddy, B. S. Chemopreventive effect of squalene on colon cancer. *Carcinogenesis* **1998**, *19*, 287–290.
(2) Psomiadou, E.; Tsimidou, M. Stability of virgin olive oil. I. Autoxidation studies. *J. Agric. Food Chem.* **2002**, *50*, 716–721.
(3) Psomiadou, E.; Tsimidou, M. Stability of virgin olive oil. II. Photooxidation studies. *J. Agric. Food Chem.* **2002**, *50*, 722–727.
(4) Kalogeropoulos, N.; Andrikopoulos, N. K. Squalene in oils and fats from domestic and commercial fryings of potatoes. *Int. J. Food Sci. Nutr.* **2004**, *55*, 125–129.
(5) Bondioli, P.; Mariani, C.; Lanzani, A.; Dedeli, E.; Muller, A. Squalene recovery from olive oil deodorizer distillates. *J. Am. Oil Chem. Soc.* **1993**, *70*, 763–766.
(6) Jahaniaval, F.; Kakuda, Y.; Marcone, M. F. Fatty acid and triacylglycerol compositions of seed oils of five *Amaranthus* accessions and their comparison to other oils. *J. Am. Oil Chem. Soc.* **2000**, *77*, 847–852.
(7) Kawai, H. Manufacture of squalenes with yeast mutant strains. Japan Kokai Tokkyo Koho JP 04,311,393. **1992**; *Chem. Abstr. 118*, 123141.
(8) Kamimura, N.; Hidaka, M.; Masaki, H.; Uozumi, T. Construction of squalene-accumulating *Saccharomyces cerevisiae* mutants by gene disruption through homologous recombination. *Appl. Microbiol. Biotechnol.* **1994**, *42*, 353–357.
(9) Tsujiwaki, G.; Yamamoto, H.; Minami, K. Manufacture of squalene with *Candida famata*. Japan Kokai Tokkyo KohoJP 07,289,272, **1995**; *Chem. Abstr. 124*, 230184.
(10) Bhattacharjee, P.; Shukla, V. B.; Singhal, R. S.; Kulkarni, P. R. Studies on fermentive production of squalene. *World J. Microbiol.* **2001**, *17*, 811–816.
(11) Uragami, S.; Koga, H. Bacterial production of squalene. Japan Kokai Tokkyo Koho JP 61,212,290, **1986**; *Chem. Abstr. 106*, 65872.
(12) Kawaura, S.; Matsuda, N.; Kobayashi, N. Squalene manufacture with Euglena. Japan Kokai Tokkyo Koho JP 07,115,981, **1995**; *Chem. Abstr. 125*, 8714.
(13) 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.
(14) Li, Q.; Chen, G. Q.; Fan, K. W.; Lu, F. P.; Aki, T.; Jiang, Y. Screening and characterization of squalene-producing thraustochytrids from Hong Kong mangroves. *J. Agric. Food Chem.* **2009**, *57*, 4267−4272

(15) Arnezeder, C.; Hampel, W. A. Influence of growth rate on the accumulation of ergosterol in yeast-cells. *Biotechnol. Lett*. **1990**, *12*, 277–282.
(16) 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.
(17) Rosenfeld, E.; Beauvoit, B. Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast* **2003**, *20*, 1115–1144.
(18) Daum, G.; Lees, N. D.; Bard, M.; Dickson, R. Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. *Yeast* **1998**, *14*, 1471–1510.
(19) Neter, J.; Kutner, M. H.; Nachtsheim, C. J.; Waaerman, W. *Applied Linear Statistical Models*; McGraw-Hill: London, U.K., 1996.
(20) Mantzouridou, F.; Naziri, E.; Tsimidou, M. Z. Industrial glycerol as a supplementary carbon source in the production of β-carotene by *Blakeslea trispora*. *J. Agric. Food Chem*. **2008**, *56*, 2668–2675.
(21) Adams, B. G.; Parks, L. W. Isolation from yeast of a metabolically active water-soluble form of ergosterol. *J. Lipid Res*. **1968**, *9*, 8–11.
(22) Thompson, E. D.; Starr, P. R.; Parks, L. W. Sterol accumulation in a mutant of *Saccharomyces cerevisiae* defective in ergosterol production. *Biochem. Biophys. Res. Commun*. **1971**, *43*, 1304–1309.
(23) Mukhopadhyay, K.; Kohli, A.; Prasad, R. Drug susceptibilities of yeast cells are affected by membrane lipid composition. *Antimicrob. Agents Chemother*. **2002**, *46*, 3695–3705.
(24) Johnson, B.; Nelson, S. J.; Brown, C. M. Influence of glucose concentration on the physiology and lipid composition of some yeast. *Antonie van Leeuwenhoek* **1972**, *38*, 129–136.
(25) Rogers, P. J.; Yue, S. B.; Stewart, P. R. Effects of oxygen tension and glucose repression on mitochondrial protein synthesis in continuous cultures of *Saccharomyces cerevisiae*. *J. Bacteriol*. **1974**, *118*, 523–533.
(26) Jahnke, L.; Klein, H. P. Oxygen requirement for formation and activity of the squalene epoxidase in *Saccharomyces cerevisiae*. *J. Bacteriol*. **1983**, *155*, 488–492.
(27) Tan, T.; Zhang, M.; Gao, H. Ergosterol production by fed-batch fermentation of *Saccharomyces cerevisiae*. *Enzyme Microb. Technol*. **2003**, *33*, 366–370.
(28) Walker-Caprioglio, H. M.; Casey, W. M.; Parks, L. W. *Saccharomyces cerevisiae* membrane sterol modifications in response to growth in the presence of ethanol. *Appl. Environ. Microbiol*. **1990**, *56*, 2853–2857.
(29) Maczek, J.; Junne, S.; Nowak, P.; Goetz, P. Metabolic flux analysis of the sterol pathway in the yeast *Saccharomyces cerevisiae*. *Bioprocess Biosyst. Eng*. **2006**, *29*, 241–252.
(30) Polakowski, T.; Stahl, U.; Lang, C. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. *Appl. Microbiol. Biotechnol*. **1998**, *49*, 66–71.
(31) Vega, J. L.; Navarro, A. R.; Clausen, E. C.; Gaddy, J. L. Effect of inoculum size on ethanol inhibition modelling and other fermentation parameters. *Biotechnol. Bioeng*. **1987**, *29*, 633–638.
(32) Thomas, K. C.; Hynes, S. H.; Ingledew, W. M. Initiation of aerobic growth of *Saccharomyces cerevisiae* by amino acids or nucleic acid bases: ergosterol and unsaturated fatty acids cannot replace oxygen in minimal media. *J. Ind. Microbiol. Biotechnol*. **1998**, *21*, 247–253.
(33) Böcking, T.; Barrow, K. D.; Netting, A. G.; Chilcott, T. C.; Coster, H. G. L.; Höfer, M. Effect of singlet oxygen on membrane sterols in the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem*. **2000**, *26*, 1607–1618.

Received February 27, 2009. Revised manuscript received May 26, 2009. Accepted May 27, 2009.