Concentration of ω-3 Polyunsaturated Fatty Acids of Marine Oils Using *Candida cylindracea* Lipase: Optimization of Reaction Conditions

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ABSTRACT: Production of ω-3 fatty acid concentrates from seal blubber oil (SBO) and menhaden oil (MHO) upon enzymatic hydrolysis by *Candida cylindracea* lipase was optimized. In this process, the content of total ω-3 fatty acids, Y_1; eicosapentaenoic acid, Y_2; and docosahexaenoic acid, Y_3, in the final product was maximized. A three-factor central composite rotatable design was used to study the effect of enzyme concentration (X_1), reaction time (X_2), and reaction temperature (X_3). Second-order polynomial regression models for Y_1, Y_2, and Y_3 were employed to generate response surfaces. After hydrolysis, a maximum of 54.3% total ω-3 fatty acids was obtained from SBO at an enzyme concentration of 308 U/g oil, a reaction time of 40 h, and a reaction temperature of 37°C. Similarly, a maximum of 54.5% total ω-3 fatty acids was obtained from MHO at an enzyme concentration of 340 U/g oil, a reaction time of 45 h, and a reaction temperature of 38°C.

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KEY WORDS: *Candida cylindeacea* lipase, docosahexaenoic acid, docosapentaenoic acid, eicosapentaenoic acid, enzyme hydrolysis, ω-3 fatty acids, menhaden oil, process optimization, seal blubber oil.

Efficiency of eight microbial lipases from *Aspergillus niger, Candida cylindracea, Chromobacterium viscosum, Geotrichum candidum, Mucor miehei, Pseudomonas* spp., *Rhizopus niveus,* and *R. oryzae* in preparation of ω-3 polyunsaturated fatty acid (PUFA) concentrates from seal blubber oil (SBO) and menhaden oil (MHO) in the form of acylglycerols was reported in a previous paper (1). Microbial lipases selectively hydrolyzed fatty acids other than ω-3 PUFA from the triacylglycerol molecules, thus producing concentrates of ω-3 PUFA in the remaining acylglycerols of both SBO and MHO. Among lipases screened for ω-3 PUFA concentration, *C. cylindracea* lipase exhibited the highest efficiency at a concentration of 200 U lipase/g oil and a reaction temperature of 35°C. However, many other factors affect the yield (concentration of ω-3 fatty acids) of lipase-catalyzed hydrolysis of triacylglycerols. These include pH and temperature of the reaction medium, reaction time, substrate and enzyme concentrations, among others (2). Therefore, it is necessary to study these factors collectively to find the optimal reaction conditions to obtain the maximal possible ω-3 PUFA content from SBO and MHO by *C. cylindracea* lipase-assisted hydrolysis.

The results of one-factor-at-a-time experiments do not reflect actual changes in the environment as they ignore interactions between factors which are present simultaneously. When many factors and interactions affect desired responses, response surface methodology (RSM) (3,4) is an effective tool for optimizing the process (5). This method was successfully adapted in many optimization studies (6–10). The central composite rotatable design (CCRD) is the preferred experimental design for fitting polynomial models to analyze response surfaces of multifactor combinations. The design is considered rotatable because the variance of the predicted response, Y, at the point X is a function only of the distance of the point from the design center irrespective of the direction. This implies that the variance contours of predicted responses are concentric circles. Also rotatable design has the property that the variance of predicted response does not change when the design is rotated around the center point (11). Thus, CCRD with RSM is a very effective tool for reducing the number of combinations required without compromising the validity of the results in studies where several independent variables are included. If the proposed model is adequate, as revealed by the diagnostic checking provided by analysis of variance (ANOVA) and residual plots, contour plots can be usefully employed to study response surface and locate the optimum. The method of process optimization by RSM is a faster and more economical method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation (6,7).

In this study, reaction parameters such as enzyme concentration (X_1), reaction time (X_2), and reaction temperature (X_3) were selected for optimization of hydrolysis of SBO and MHO by *C. cylindracea* lipase. Enzyme concentration and reaction time are major factors that affect the economy of preparation of ω-3 fatty acids concentrates *via* lipase-assisted hydrolysis. The *C. cylindracea* lipase possesses a wide range

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of optimal pH activity (5.0–8.0); however, pH 7.0 was found to be most suitable (12). Furthermore, the temperature of the reaction medium and also the reaction time may be considered important because they could influence the oxidative state of the prepared ω-3 concentrates.

MATERIALS AND METHODS

Hydrolysis of SBO and MHO by C. cylindracea *lipase.* Hydrolysis of SBO and MHO by *C. cylindracea* lipase and separation of the ω-3 enriched fraction were carried out according to the method described in a previous paper (1). Oil (4.0 g) and phosphate buffer (6.0 mL of a 0.1 M solution; pH 7.0) containing *C. cylindracea* lipase (amounts are given in Table 1) were placed in a glass container (4-cm diameter and 7-cm height). The container was flushed with nitrogen and sealed with a rubber cap and parafilm. Containers were then placed in a Gyratory water bath shaker, and the shaking speed was set to 200 rpm. Hydrolyzed samples were removed periodically (separate sample container for each time). Unhydrolyzed fraction of oils (acylglycerols) was extracted with hexane after addition of 0.5 N ethanolic KOH to the reaction mixture. The fatty acid composition of unhydrolyzed fraction of oils was determined according to the method of Shahidi *et al.* (13). Optimization of hydrolysis parameters such as enzyme concentration, reaction time, and reaction temperature to acquire a maximal concentration yield of total ω-3 fatty acid from oils was performed as described below.

Experimental design. A CCRD (14,15) was employed to study the responses (Y variables), namely, concentrations of total ω-3 fatty acids Y_1, eicosapentaenoic acid (EPA) Y_2, and docosahexaenoic acid (DHA) Y_3 by *C. cylindracea* lipase-assisted hydrolysis of SBO and MHO. The enzyme concentration (X_1), reaction time (X_2), and reaction temperature (X_3) were independent variables studied. Each variable to be optimized was coded at five levels: –1.68, –1, 0, +1, and +1.68. Table 1 summarizes the variables, their symbols, and levels used in the experiments. The selection of variable levels was based on the results of preliminary studies. The CCRD combined the vertices of a hypercube whose coordinates are given by the 2^n factorial design (runs 1–8; Table 2) with the "star" points (runs 9–14). The star points were added to the factorial design to provide for estimation of curvature of the model (16). Five replicates (runs 15–19) at the center (0,0,0) of the design were performed to allow the estimation of the "pure error." All experiments were carried out in a randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors.

Statistical analysis. A quadratic polynomial regression model was assumed for predicting individual Y variables. The model proposed for each response of Y was:

$$Y = \beta_o + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i<j=1}^{3}\sum\beta_{ij} x_i x_j \qquad [1]$$

where β_o, β_i, β_{ii}, and β_{ij} are intercept, linear, quadratic, and interaction regression coefficient terms, respectively, and x_i and x_j are independent variables. The Statistical Analytical System (17) was used for multiple regression analysis, ANOVA, canonical analysis, and analysis of ridge maximum of data using response surface regression (RSREG) procedure. Response surfaces and contour plots were developed using the fitted quadratic polynomial equations obtained from RSREG analysis and holding the independent variables with the least effect on the response at a constant value and changing the levels of the other two variables.

RESULTS AND DISCUSSION

Table 2 summarizes the contents of total ω-3 fatty acids, EPA, docosapentaenoic acid (DPA), and DHA as well as percentage recovery (yield) of the nonhydrolyzed fractions of SBO and MHO in the acylglycerol form. For all responses (total ω-3 fatty acids, EPA, DHA, and yield), higher values were obtained for MHO as compared to SBO, except for DPA which was higher in SBO than MHO. The contents of total ω-3 fatty acids, EPA, and DHA were higher in the original MHO (total ω-3 fatty acids, 30.1%; EPA, 13.3%; and DHA, 10.1%) than SBO (total ω-3 fatty acids, 20.1%; EPA, 6.4%; and DHA, 7.6%), while the content of DPA in the original SBO (4.7%) was higher than that of MHO (2.4%). Therefore, during enzymatic hydrolysis, a similar pattern of abundance of these fatty acids was observed in the nonhydrolyzed fraction of SBO and MHO, because these fatty acids are resistant to lipase hydrolysis and are retained in the intact acylglycerol forms.

TABLE 1
Independent Variables and Their Levels for Central Composite Rotatable Design in Optimization of ω-3 Fatty Acid Concentrate Production by *Candida cylindracea* Lipase-Assisted Hydrolysis of Seal Blubber Oil and Menhaden Oil

		Coded variable levels[a]				
Independent variables	Symbol	–1.68 (–α)	–1	0	1	1.68 (α)
Enzyme concentration (U/g oil)	X_1	45	140	280	420	515
Reaction time (h)	X_2	3	12	25	38	47
Reaction temperature (°C)	X_3	26.6	30	356	40	43.4

[a]Transformation of coded variable (X_i) levels to uncoded variable (x_i) levels could be obtained from: $x_1 = 140X_1 + 280, x_2 = 13X_2 + 25$ and $x_3 = 5X_3 + 35$.

TABLE 2
Central Composite Rotatable Design Arrangement and Responses for *Candida cylindracea* Lipase-Assisted Hydrolysis Experiment of Seal Blubber Oil (SBO) and Menhaden oil (MHO)

	Coded variables			Responses (%, *Y*)									
				Yield[a]		EPA		DPA		DHA		Total (ω-3)	
Run	X_1	X_2	X_3	SBO	MHO	SBO	MHO	SBO	MHO	SBO	MHO	SBO	MHO
1	−1	−1	−1	35.8	47.5	13.9	17.2	7.62	3.89	17.6	17.5	41.1	45.3
2	1	−1	−1	29.2	44.0	13.2	17.9	7.80	3.79	20.5	18.7	43.3	44.7
3	−1	1	−1	25.4	52.9	13.4	18.5	8.10	4.30	20.5	19.2	43.9	46.7
4	1	1	−1	22.2	36.2	12.8	19.8	8.01	4.37	24.2	21.5	46.8	50.4
5	−1	−1	1	40.8	54.2	14.1	18.3	7.92	4.03	17.4	17.4	41.4	46.1
6	1	−1	1	29.1	43.6	14.9	19.6	8.12	3.95	22.1	19.6	47.0	48.5
7	−1	1	1	21.8	52.7	13.9	18.5	8.30	3.96	25.3	21.9	49.3	50.7
8	1	1	1	23.6	40.1	12.2	19.9	8.36	4.46	26.6	22.9	48.9	52.7
9	−1.68	0	0	41.1	65.7	13.9	17.0	7.80	3.59	15.4	14.6	39.0	38.6
10	1.68	0	0	25.6	49.3	15.0	20.2	6.88	3.92	25.0	21.2	48.5	49.5
11	0	−1.68	0	35.0	64.4	14.0	16.9	7.63	3.36	19.4	19.8	42.9	45.7
12	0	1.68	0	23.5	45.7	15.5	20.2	8.20	4.61	26.3	24.4	53.9	55.8
13	0	0	−1.68	26.1	37.8	12.0	16.7	8.30	4.58	19.9	20.3	42.1	47.5
14	0	0	1.68	23.1	47.0	14.1	20.2	8.31	4.19	21.3	22.0	45.5	51.8
15	0	0	0	25.6	50.9	17.1	20.3	7.13	3.86	24.3	24.8	50.9	53.7
16	0	0	0	25.9	49.2	16.3	20.0	7.43	3.78	24.3	25.7	50.8	52.9
17	0	0	0	27.1	46.8	17.1	20.9	7.74	3.81	24.8	24.6	51.5	52.4
18	0	0	0	29.4	48.0	16.3	21.3	7.38	3.94	26.2	25.8	52.7	54.4
19	0	0	0	25.9	49.1	16.5	20.2	7.47	3.96	26.4	25.1	52.2	54.5

[a]Percentage recovery of nonhydrolyzed fraction of oil. X_1 = Enzyme concentration (U/g oil); X_2 = reaction time (h); X_3 = reaction temperature (°C); EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Diagnostic checking of the fitted models. The data obtained for total ω-3 fatty acids, EPA, and DHA from the 19 experimental points in both SBO and MHO were used for statistical analysis to optimize process variables: enzyme concentration, reaction time, and reaction temperature. Multiple regression coefficients obtained using the least squares method to predict quadratic polynomial models for total ω-3 fatty acids (Y_1), EPA (Y_2), and DHA (Y_3) for both SBO and MHO are summarized in Table 3. Examination of these coefficients with the *t*-test indicated that in SBO, linear and quadratic terms for test variables, enzyme concentration, and reaction temperature were significant ($P < 0.01$ or 0.05) for total ω-3 PUFA content. The linear term for reaction time for total ω-3 content was not significant ($P > 0.05$), but quadratic term was significant ($P < 0.05$). For the content of EPA in SBO, only the linear term of reaction temperature and the quadratic

TABLE 3
Regression Coefficients of Predicted Quadratic Polynomial Model for Response Variables (total ω-3, EPA, and DHA contents) in *Candida cylindracea* Lipase-Assisted Hydrolysis Experiment of SBO and MHO[a]

	Coefficients (β)					
	Total ω-3 (%, Y_1)		EPA (%, Y_2)		DHA (%, Y_3)	
Variables	SBO	MHO	SBO	MHO	SBO	MHO
Intercept	−110.5355***	−37.56674	−48.60494***	−28.04923**	−61.24276**	−67.05821***
Linear						
X_1	0.101038**	0.091352**	0.006971	0.018149	0.067948**	0.086240***
X_2	0.375423	0.257621	0.185442	0.415054**	0.031836	0.242539
X_3	7.691455***	3.779578**	3.410418***	2.127598***	3.970611***	4.135811***
Quadratic						
X_{11}	−0.000139***	−0.000168***	−0.000037***	−0.000032***	−0.000082***	−0.000134***
X_{22}	−0.006311**	−0.005393	−0.003578	−0.003720***	−0.003950	−0.006709***
X_{33}	−0.108737***	−0.052472**	−0.048938***	−0.027114***	−0.058109***	−0.059370***
Interaction						
X_{12}	−0.000371	0.000277	−0.000018	0.000039	−0.000179	0.000014
X_{13}	0.000000	0.000275	0.000436	0.000114	−0.000079	−0.000044
X_{23}	0.006808	0.003462	0.000038	−0.005389	0.011077	0.005981
X_{123}	—	—	—	—	—	—
R^2	0.94	0.92	0.91	0.89	0.93	0.97

[a]See Table 1 for description of variables. See Tables 1 and 2 for abbreviations. **$P < 0.05$; ***$P < 0.01$.

terms of enzyme concentration and reaction temperature were highly significant ($P < 0.01$). The coefficient obtained for DHA content in SBO showed that linear and quadratic terms of enzyme concentration and reaction temperature were significant ($P < 0.01$), whereas reaction time was not. In MHO, linear terms of enzyme concentration and reaction temperature were significant for both total ω-3 fatty acids ($P < 0.05$) and DHA ($P < 0.01$) contents, but for EPA content, reaction time ($P < 0.05$) and reaction temperature ($P < 0.01$) were significant. All quadratic terms were highly significant ($P < 0.01$) for EPA and DHA contents of MHO, whereas for total ω-3 fatty acids content only enzyme concentration and reaction temperature were significant (at $P < 0.01$ and $P < 0.05$, respectively). No statistically significant ($P > 0.05$) interactions existed between any two of the three factors observed, suggesting that linear and/or quadratic effects of enzyme concentration, reaction time, and reaction temperature are the primary determining factors for the amounts of total ω-3 fatty acids, EPA, and DHA in the prepared concentrates of both SBO and MHO by *C. cylindracea* lipase-assisted hydrolysis. The contribution of linear and quadratic terms to the models of SBO concentrate was 0.44 and 0.49 for total ω-3 fatty acids, 0.12 and 0.77 for EPA, and 0.63 and 0.26 for DHA, respectively. The contributions of these two terms to the models of MHO concentrate were 0.45 and 0.46 for total ω-3 fatty acids, 0.51 and 0.35 for EPA, and 0.32 and 0.64 for DHA, respectively. The coefficients of independent variables, enzyme concentration (X_1), reaction time (X_2), and reaction temperature (X_3) determined for quadratic polynomial models for total ω-3 fatty acids (Y_1), EPA (Y_2), and DHA (Y_3) of prepared concentrate of SBO were:

$$Y_1 = -110.536 + 0.101X_1 + 0.375X_2 + 7.691X_3 - 0.00014X_1^2 - 0.00631X_2^2 - 0.10873X_3^2 - 0.00037X_1X_2 + 0.00681X_2X_3 \quad [2]$$

$$Y_2 = -48.605 + 0.007X_1 + 0.185X_2 + 3.410X_3 - 0.00004X_1^2 - 0.00358X_2^2 - 0.04894X_3^2 - 0.00002X_1X_2 + 0.00044X_1X_3 + 0.00004X_2X_3 \quad [3]$$

$$Y_3 = -61.243 + 0.068X_1 + 0.032X_2 + 3.971X_3 - 0.00082X_1^2 - 0.00395X_2^2 - 0.05811X_3^2 - 0.00018X_1X_2 - 0.00008X_1X_3 + 0.01108X_2X_3 \quad [4]$$

The quadratic polynomial models for Y_1, Y_2, and Y_3 for the prepared concentrate of MHO were:

$$Y_1 = -37.567 + 0.091X_1 + 0.258X_2 + 3.779X_3 - 0.00017X_1^2 - 0.00539X_2^2 - 0.05247X_3^2 + 0.00028X_1X_2 + 0.00028X_1X_3 + 0.00346X_2X_3 \quad [5]$$

$$Y_2 = -28.049 + 0.018X_1 + 0.415X_2 + 2.128X_3 - 0.00003X_1^2 - 0.00372X_2^2 - 0.02711X_3^2 + 0.00004X_1X_2 + 0.00011X_1X_3 - 0.00539X_2X_3 \quad [6]$$

$$Y_3 = -67.058 + 0.086X_1 + 0.243X_2 + 4.136X_3 - 0.00013X_1^2 - 0.00671X_2^2 - 0.05937X_3^2 - 0.00001X_1X_2 - 0.00004X_1X_3 + 0.00598X_2X_3 \quad [7]$$

These models predicted Y_1, Y_2, and Y_3 for both oils adequately, as indicated by error analysis that showed nonsignificant ($P > 0.05$) lack-of-fit. The regression models for total ω-3 fatty acids, EPA, and DHA were highly significant ($P < 0.01$) with satisfactory coefficients of determination (R^2) of 0.94, 0.91, and 0.93 for SBO and 0.92, 0.89, and 0.97 for MHO, respectively.

Response surface plotting and optimization based on canonical analysis. The relationship between independent and dependent variables is shown in the three-dimensional representation of the response surfaces generated for models developed for the contents of total ω-3 fatty acids, EPA, and DHA in SBO concentrate (Figs. 1–3). Canonical analysis was performed on predicted quadratic polynomial models to examine the overall shape of response surface curves and was used to characterize the nature of stationary points. This showed that all three responses, total ω-3 fatty acids, EPA and DHA contents in SBO, had maximal stationary points (Table 4). A maximal total ω-3 fatty acids content of 53.5% was predicted at enzyme concentration, reaction time, and reaction temperature of 308 U/g oil, 40 h, and 37°C, respectively. The EPA content of the SBO concentrate was predicted to increase from 6.4% in the original oil to 16.5% in the concentrate at an enzyme concentration of 297 U/g oil, reaction time of 26 h, and reaction temperature of 36°C. The DHA content was maximized to 28.1% at an enzyme concentration of 342 U/g oil, reaction time of 51 h, and reaction temperature of 39°C. The maximal points for total ω-3 fatty acids, EPA, and DHA contents are clearly indicated graphically in the contour plots (Figs. 1–3), and these were also located in the experimental region.

Response surfaces for total ω-3 fatty acids, EPA, and DHA contents of MHO concentrate are given in Figures 1–3. Canonical analysis of MHO data on these responses also showed maximal stationary points similar to SBO (Table 4). A maximum of 56.3% total ω-3 fatty acids was predicted in MHO at an enzyme concentration of 340 U/g oil, reaction time of 45 h, and reaction temperature of 38°C (Table 5). Maximal concentrations of 21.1 and 25.9% EPA and DHA in the MHO concentrate were predicted at enzyme concentrations of 370 and 314 U/g oil, reaction times of 31 and 34 h, and reaction temperatures of 37 and 36°C, respectively. Graphical representations of data on contour plots (Figs. 1–3) showed that these maxima were located in the experimental region.

Results of independent experiments carried out to examine the adequacy of the predicted values by the models for both oils showed very close correspondence of values for all three responses (Tables 4 and 5). These verification results revealed that the predicted values from models were reasonable and reproducible. Therefore, hydrolysis of SBO and MHO by *C. cylindracea* lipase can increase the content of total ω-3 fatty acids up to 54.3 ± 3.2 and 54.5 ± 2.3%, with yields of 30 and 43% from the original oils, respectively.

Finally, it is important to note that even though the initial ω-3 fatty acid contents in both SBO and MHO were different,

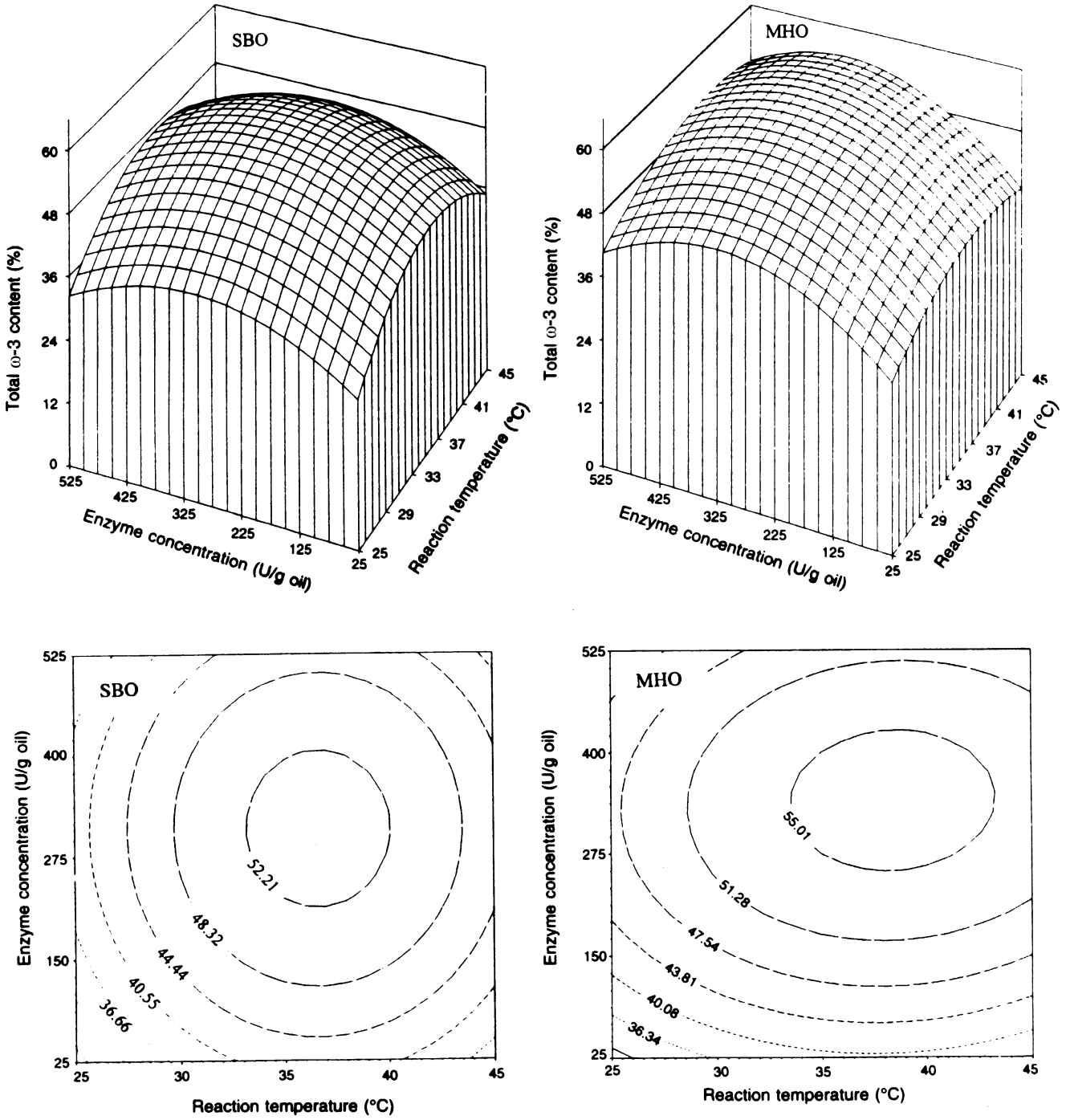

FIG. 1. Response surface and contour plots for the effect of enzyme concentration and reaction temperature on total ω-3 fatty acid content of the prepared concentrate of seal blubber oil (SBO) and menhaden oil (MHO) using *Candida cylindracea* lipase.

the levels of ω-3 fatty acids in the final products were the same. The positional distributions of ω-3 fatty acids in the triacylglycerol (TAG) molecules of SBO and MHO are different (18). EPA, DPA, and DHA are located mainly in the *sn*-1 and *sn*-3 positions of the TAG of SBO. In MHO, DPA and DHA are mainly in the *sn*-2 position of TAG; however, EPA is equally distributed in the *sn*-2 and *sn*-3 positions and is present only in small amounts in the *sn*-1 position. Therefore, if the lipase is *sn*-1,3-specific, it may easily hydrolyze fatty acids other than those of the ω-3 type present in the *sn*-1 and *sn*-3 positions of MHO (in MHO, ω-3 fatty acids are mainly present in *sn*-2 position) as compared to SBO (in SBO, ω-3 fatty acids are mainly present in *sn*-1 and *sn*-3 positions). However, the *C. cylindracea* lipase used in this study is nonspecific and hydrolyzes fatty acids (mainly saturates and monounsaturates) randomly from the TAG molecules of the oils, leaving long-

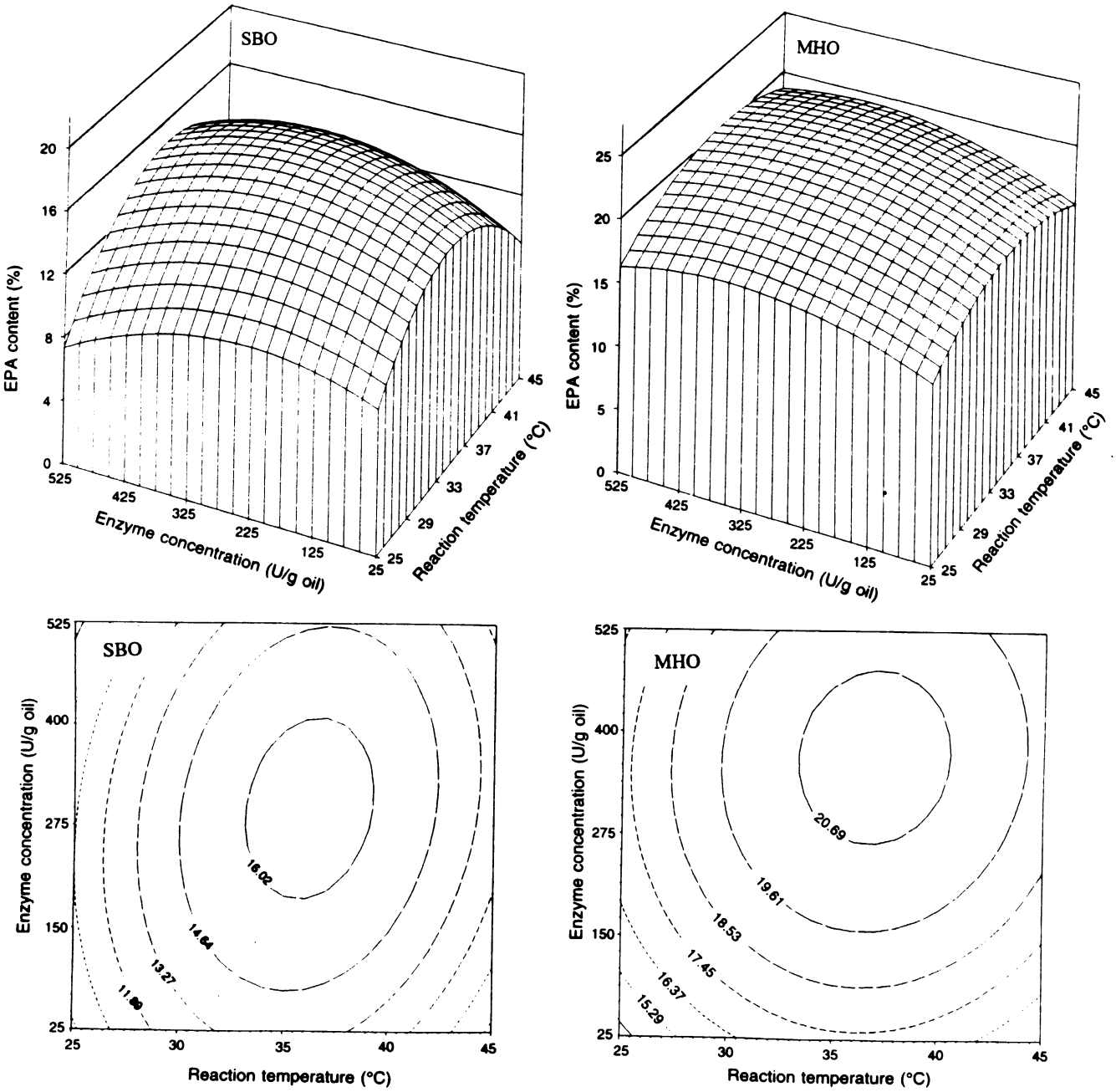

FIG. 2. Response surface and contour plots for the effect of enzyme concentration and reaction temperature on eicosapentaenoic acid (EPA) content of the prepared concentrate of SBO and MHO using *C. cylindracea* lipase. See Figure 1 for other abbreviations.

chain ω-3 fatty acids in acylglycerols until hydrolytic activity of the enzyme ceases. Therefore, these results justify the assumption that hydrolytic activity of *C. cylindracea* lipase is controlled by the content of ω-3 fatty acids in the acylglycerol molecules of the oils under investigation.

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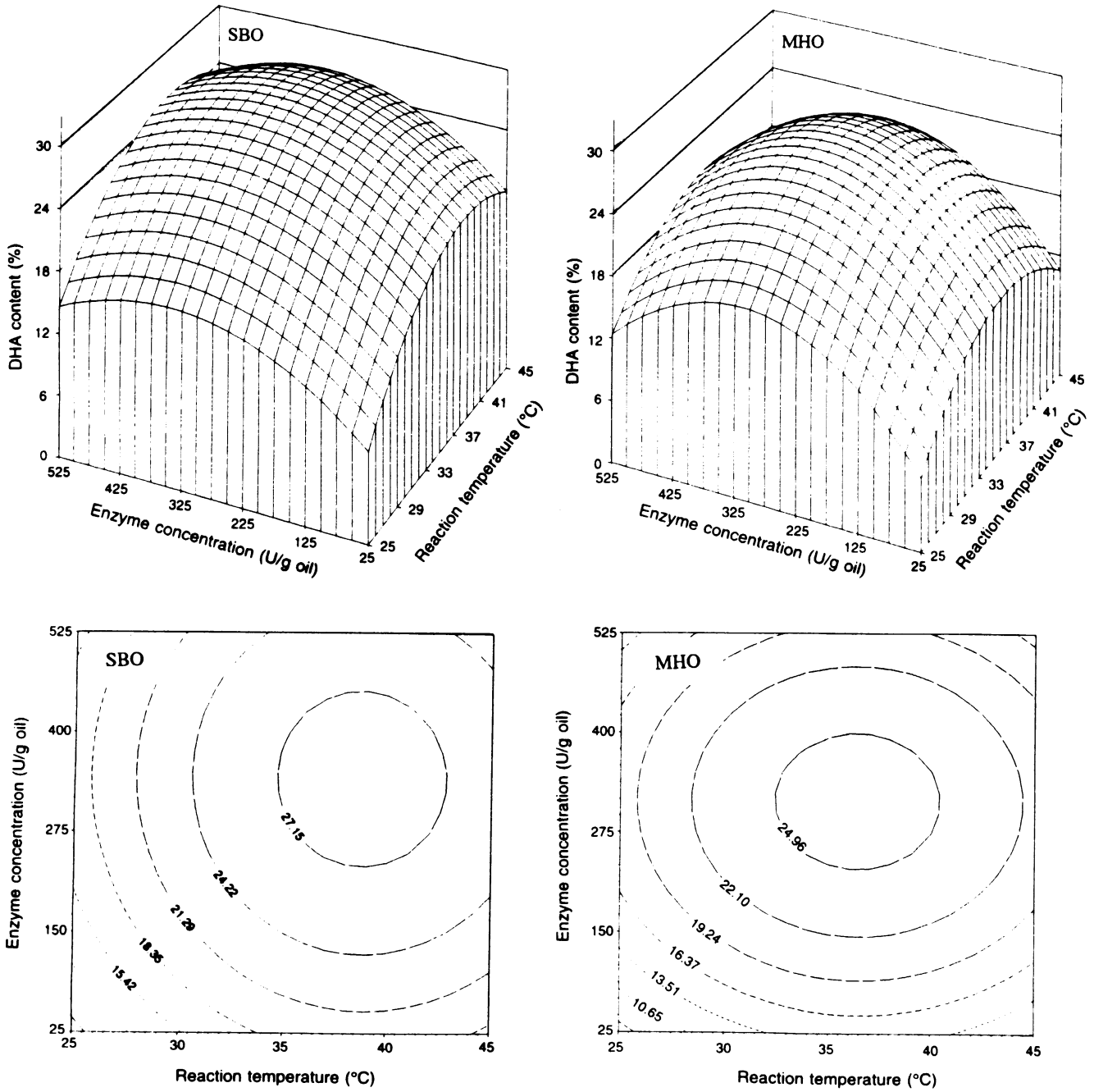

FIG. 3. Response surface and contour plots for the effect of enzyme concentration and reaction temperature on docosahexaenoic acid (DHA) content of the prepared concentrate of SBO and MHO using *C. cylindracea* lipase. See Figure 1 for other abbreviations.

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TABLE 4
Predicted and Observed Values for Response Variables (total ω-3, EPA, and DHA contents) in *Candida cylindracea* Lipase-Assisted Hydrolysis of SBO

Response variables	Critical values of independent variables			Stationary point	Predicted value (%)	Observed value (%)[a]
	Enzyme concentration (U/g oil)	Reaction time (h)	Reaction temperature (°C)			
Total ω-3 fatty acids (%)	308	40	37	Maximum	53.5	54.3 ± 3.2
EPA (%)	297	26	36	Maximum	16.5	19.4 ± 2.5
DHA (%)	342	51	39	Maximum	28.1	27.6 ± 2.1

[a]Means ± SD (n = 3). See Tables 1 and 2 for abbreviations.

TABLE 5
Predicted and Observed Values for Response Variables (total ω-3, EPA, and DHA contents) in *Candida cylindracea* Lipase-Assisted Hydrolysis of MHO

Response variables	Critical values of independent variables			Stationary point	Predicted value (%)	Observed value (%)[a]
	Enzyme concentration (U/g oil)	Reaction time (h)	Reaction temperature (°C)			
Total ω-3 fatty acids (%)	340	45	38	Maximum	56.3	54.5 ± 2.3
EPA (%)	370	31	37	Maximum	21.1	18.1 ± 2.8
DHA (%)	314	34	36	Maximum	25.9	26.1 ± 3.4

[a]Means ± SD (n = 3). See Tables 1 and 2 for abbreviations.

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