Romanian Biotechnological Letters *Vol. 16, No. 1, 2011*

ORIGINAL PAPER

Response surface methodology-based optimization of lipase-catalyzed triolein hydrolysis in hexane

Received for publication, September 10, 2010
Accepted, January 24, 2011

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Abstract

Response surface methodology (RSM) is an effective statistical technique for the investigation of complex processes. The main advantage of this technique is the reduced number of experimental runs required to acquire sufficient information for statistically acceptable result. Lipase research is often time and resource consuming because of its complex dependence on various reaction parameters (factors), such as time, temperature, pH, molar ratio of reactants etc. RSM based experimental design enables to analyze all parameters at once and model the response. In our case the effectiveness of triolein hydrolysis reaction catalyzed by commercial lipases Palatase ® 20000L, Lecitase® Ultra, Lipopan™ F BG and Lipopan™ 50 BG was studied. Central composite design of RSM was employed to evaluate the effects of reaction parameters on the catalytic activity of lipase. These include reaction time (20–180 min), reaction temperature (30.0-60.0°C), and type of the enzyme. Statistical analysis and optimization were conducted in order to reach the maximum hydrolysis of triolein and the maximum oleic acid yield with preference to minimize reaction temperature and time. The optimal conditions were 139 minutes at 50.5ºC using Palatase, to yield 40.4% residual triolein and 38.9% oleic acid concentrations.

Keywords: Triolein hydrolysis; Palatase® 20000L; Lecitase® Ultra; Lipopan™ F BG; Lipopan™ 50 BG; Process optimization; Response Surface Methodology.

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are among the most useful and most studied enzymes in fats and oils industry [1, 2]. Large quantities of lipases are being produced on an industrial scale for medical and industrial uses. These enzymes have been studied for use as food additives for flavor modification, in medicines as a digestive aid, for industrial reagent production and as detergent additives for fat (triacylglycerols, TAG) hydrolysis, as enzymes catalyzing esterification reactions and in commercial processes for ingredients used in personal care products [3].

The hydrolysis of TAG to yield free fatty acids and glycerol represents an important group of chemical reactions relevant to the industrial processing of natural oils and fats. Hydrolysis is the primary reaction for production of free fatty acids that may then be interesterified, transesterified, or converted into high-value fatty alcohols. The mainstream current technologies for hydrolysis are based on high-temperature, high-pressure contacting processes with steam or superheated liquid water, involving high temperatures and high-pressure equipment requirements [4]. Enzymatic fat splitting (hydrolysis) has been studied extensively using enzymes immobilized on hydrophobic polymeric supports [5, 6] and also using enzymes freely attached at a liquid–liquid interface [7].

New effective and cheap lipases are being searched for various purposes. Lipases used for food industry can be a good choice because of their low cost and sufficient supply. Consequently for our study we have selected four in food industry commonly used enzymes

Palatase® 20000L; Lecitase® Ultra; Lipopan™ F BG; Lipopan™ 50 B (in subsequent text Palatase, Lecitase, Lipopan F BG and Lipopan 50 BG), kindly provided by Biopolis Ltd, distributor of Novozymes A/S in Lithuania.

Palatase is a purified 1,3-specific lipase *from Rhizomucor miehei* produced by submerged fermentation of a genetically modified *Aspergilus oryzae* microorganism. It is a purified lipase developed for the production of cheese flavors or enzyme-modified cheese. Its preference for hydrolyzing small fatty acid esters results in optimal flavor formation.
Palatase has a high activity in the pH range 6-8.5, the optimum being pH 7.5. The temperature optimum is 40°C.

Lecitase is a phospholipase A_1, obtained from the fusion of the genes of the lipase from *Thermomyces lanuginosus* and the phospholipase from *Fusarium oxysporum.* Production organism is *Aspergilus oryzae.* Lecitase Ultra is a liquid phospholipase preparation intended for use in vegetable oil degumming and in hydrolysis of lecithin. Lecitase Ultra is a phospholipase, which converts non-hydratable phosphatides/gums (NHP) into hydratable phosphatides (HP) in a gentle and natural way [8, 9].

Lipopan F BG is a purified lipolytic enzyme obtained from *Fusarium oxysporum* produced through the submerged fermentation of the genetically modified microorganism *Aspergillus oryzae*. Lipopan FBG has an inherent activity on phospholipids, glycolipids and triglycerides. Lipopan F BG is a dough-strengthening lipase that acts by modifying both polar and non-polar flour lipids.

Lipopan 50 BG is a purified 1,3- specific lipase from *Thermomyces lanuginosus* produced by submerged fermentation of a genetically modified *Aspergilus oryzae*. Lipopan 50 BG is a dough-conditioning lipase solution yielding improved crumb structure and enhanced dough stability [8].

Traditionally, selected lipases are used in aqueous solutions, though there is data on use of Palatase in organic media, for example surfactant production in various solvents [10] and potential food flavor fatty ester synthesis in hexane [11]. The use of lipases in organic media is of growing importance because of enhanced solubilities of reaction composites or products, better yields and possibly higher stabilities of enzymes. Our goal was to explore the possibility and compare the enzymatic efficiency of triolein hydrolysis in hexane, catalyzed by Palatase, Lecitase, Lipopan F BG and Lipopan 50 BG.

If the classical method of optimization is used to find the best conditions for the particular reaction, it involves varying one parameter at a time and ignores the combined interactions between physicochemical parameters. Alternatively, response surface methodology (RSM) is one of the most popular techniques used for optimization of chemical and biochemical processes, varying several parameters at a time [12]. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable result. It is a faster and less expensive method for gathering research results than the classical method [13]. RSM is a combination of statistical experimental design fundamentals, regression modelling techniques, and optimization methods [14]. We have employed central composite design (CCD) to evaluate the temperature and time effects on triolein hydrolysis in hexane by four different enzymes.

Materials and methods

Materials

Lipases in this study were purchased from Novozymes, Denmark. The reagents of analytical grade were purchased from Sigma (triolein) and Chempur (Oleic acid, diethyl ether, acetic acid and hexane). Pre-coated thin-layer chromatographic (TLC) plates DC-Fertigplatten SIL G-25 UV_{254} were purchased from Macherey-Nagel, Germany.

Experimental design

A five-level-two numerical and one-categorical-factor CCD were employed in this study. The fractional factorial design consisted of 4 factorial points, 4 axial points and 5 center points (13 runs), multiplied by the number of categorical factors (4 different enzyme types), resulting in 52 experimental runs. The variables and their levels selected for the triolein hydrolysis were 2 numerical factors: time (20–180 min); temperature (20–60°C); 1 categorical factor: type of enzyme (Palatase, Lecitase, Lipopan F BG and Lipopan 50 BG). In order to have a possibility to investigate the reaction at lower reaction times, we selected the value of Alpha (to define axial points) as "practical" = 1.18921, so that there would be no reaction times with negative values. The experiment design in actual and coded values is shown in Table 1.

The experimental design, data analysis and regression model building were performed using Design Expert software (version 8.0.1.0, Stat-Ease Inc., Minneapolis, MN).

Hydrolysis and analysis

Hydrolysis of triolein (TO). The reaction was performed in 2ml Eppendorf plastic test tubes, the mixture contained 40µl of TO, 360µl of hexane and 50µl or 0.01KLU (kilo-lipase units) of lipase solution in buffer (final volume 450µl). The mixture of this composition was thoroughly selected from a number of special experiments (modified method of Yadav et al. was used [15]). The mixture was incubated at required reaction temperature under continuous stirring by thermo-shaker at 1400 rpm. For reaction termination 450µl of diethyl ether was added and samples were frozen. The samples were analyzed by thin layer chromatography method as described below.

Thin layer chromatography (TLC). A modified method of Yadav et al. was used [15]. Analysis of the reaction products was carried out on TLC plates (5x10cm and 10x10cm) pre-coated with 0.25 mm Silica Gel 60 (Macherey-Nagel). The samples were applied to the marked start edge of the TLC plate (1,0cm height from lower edge of the plate) using the specified TLC – Hamilton syringe. The sample volume for all experiments was 2µl. The plate was then allowed to be air-dried for 10-15 min before its transferring to the TLC tank for the development. Chromatograms were developed in solvent system (mobile system) of light petroleum (b.p. 40-60^{o}C) : diethyl ether : acetic acid (80:20:2), v/v) [16,17]. The tank with the poured mobile system was covered with a lid and pre-saturated with mobile system vapor for at least 20–30 min at room temperature before use. The sample – loaded TLC plate was transferred to the TLC tank and was then developed for not less than 4cm (for 5x5cm TLC plate) and 8cm (for 5x10cm TLC plate) migration distance of the solvent from the start line. The developed TLC plates were air–dried for about 10-15 min. Visualization of spots was developed by using a saturated iodine vapor chamber and spots were identified with reference to standards. Pure TO, diacylglycerols (DAG), monoacylglycerols (MAG) and oleic acid (OA) solutions in diethyl ether were used as standards.

Quantitative analysis (%) of reaction products separated by TLC was performed by Uvitec Cambridge Fire-reader imaging system and Uvitec Fire-reader software considering the spot area and color intensity.

The standard spectrophotometric assay [18, 19]. Hydrolytic activity of lipase upon p-nitrophenylbutyrate (p-NPB) solution in 2-propanol was investigated measuring the change of optical density at 410 nm during 3-6 min at 30ºC and pH 7.0-10.0, 100 mM universal buffer (ub) (Britton – Robinson buffer, composed of acetate, ortho-borate and ortho-phosphate at a ratio of 1:1:1), providing buffering capacity over a wide range of pH [20, 21]. One unit of lipase hydrolytic activity corresponds to the amount of the enzyme releasing 1µmol of p-nitrophenol per minute under standard conditions.

Data analysis

The experimental data were analyzed using Design Expert 8.01 and then interpreted. Analysis of variance (ANOVA), a regression analysis and the plotting of response surface were performed to establish optimum conditions for the hydrolysis. ANOVA was used to test adequacy and fitness of the responses for linear, 2 function interaction (2fi) and quadratic functions of the variables. A model with *P*-values ($P > F$) less than 0.05 was regarded as significant. The lack-of-fit test was used to compare the residual and pure errors at the replicated design points. If the model fits the data well, lack of fit is not significant. Quadratic model was chosen as a highest-order significant polynomial having a non-significant lack of fit. The optimal conditions were predicted to obtain the highest oleic acid yield and maximize triolein hydrolysis. The experiment in given condition was repeated in four replicates to check the reliability of the predicted values and experimental data.

Table 1. Experimental design table and effect of factors on triolein and oleic acid concentrations (actual and predicted values).

Standard order	Run order	Factor A: time, minutes	Factor B:temp, °C	Factor C: enzyme	Response 1, Triolein, %		Response 2, Oleic acid, %	
					AV	PV	AV	PV
1	13	20 (-1)	30.0 (-1)	P (1 0 0)	65.00	71.41	19.00	21.14
2	6	180 (1)	30.0 (-1)	P (1 0 0)	61.00	62.62	41.00	45.37
3	42	20 (-1)	60.0 (1)	P (1 0 0)	53.00	55.50	18.00	21.09
4	41	180 (1)	60.0 (1)	P (1 0 0)	43.00	52.60	43.00	44.62
5	33	5 (-1.188)	45.0 (0)	P (1 0 0)	80.00	64.79	12.00	16.32
6	4	195 (1.188)	45.0 (0)	P (1 0 0)	75.00	57.85	41.00	44.68
7	12	100 (0)	27.2 (-1.189)	P (1 0 0)	57.00	62.03	42.00	36.74
8	31	100 (0)	62.8 (1.189)	P (1 0 0)	45.00	46.61	40.00	36.26
9	52	100 (0)	45.0 (0)	P (1 0 0)	33.00	51.32	27.00	34.56
10	37	100 (0)	45.0 (0)	P (1 0 0)	48.00	51.32	43.00	34.56
11	34	100 (0)	45.0 (0)	P (1 0 0)	57.00	51.32	39.00	34.56
12	22	100 (0)	45.0 (0)	P (1 0 0)	62.00	51.32	41.00	34.56
13	51	100 (0)	45.0 (0)	P (1 0 0)	51.00	51.32	33.00	34.56
14	15	20 (-1)	30.0 (-1)	L (0 1 0)	88.00	79.81	16.00	14.66
15	25	180 (1)	30.0 (-1)	L (0 1 0)	69.00	75.07	26.00	28.17
16	8	20 (-1)	60.0 (1)	L (0 1 0)	83.00	83.97	0.00	-1.70
17	*27*	*180 (1)*	*60.0 (1)*	*L* (0 1 0)	*84.00*	*85.12*	~~*0.00*~~	11.13
18	46	5 (-1.188)	45.0 (0)	L (0 1 0)	76.00	82.84	1.00	2.70
19	14	195 (1.188)	45.0 (0)	L (0 1 0)	86.00	80.71	21.00	18.32
20	5	100 (0)	27.2 (-1.189)	L (0 1 0)	79.00	70.56	22.00	26.44
21	48	100 (0)	62.8 (1.189)	L (0 1 0)	91.00	79.01	0.00	6.57
22	11	100 (0)	45.0 (0)	L (0 1 0)	75.00	71.78	16.00	14.57
23	47	100 (0)	45.0 (0)	L (0 1 0)	68.00	71.78	14.00	14.57
24	44	100 (0)	45.0 (0)	L (0 1 0)	68.00	71.78	17.00	14.57
25	1	100 (0)	45.0 (0)	L (0 1 0)	79.00	71.78	19.00	14.57
26	38	100 (0)	45.0 (0)	L (0 1 0)	50.00	71.78	16.00	14.57
27	20	20 (-1)	30.0 (-1)	LF (0 0 1)	96.00	92.66	0.00	-0.069
28	3	180 (1)	30.0 (-1)	LF (0 0 1)	90.00	87.60	7.00	2.63
29	18	20 (-1)	60.0 (1)	LF (0 0 1)	95.00	94.36	0.00	-2.17
30	2	180 (1)	60.0 (1)	LF (0 0 1)	95.00	95.20	1.00	-0.18
31	49	5 (-1.188)	45.0 (0)	LF (0 0 1)	90.00	94.49	0.00	-3.89

32	10	195 (1.188)	45.0 (0)	LF	89.00	91.99	0.00	-1.11
33	28	100 (0)	27.2 (-1.189)	LF	79.00	83.48	2.00	4.96
34	26	100 (0)	62.8 (1.189)	LF	89.00	89.01	0.00	2.04
35	9	100 (0)	45.0 (0)	LF	98.00	83.24	0.00	1.56
36	35	100 (0)	45.0 (0)	LF	77.00	83.24	0.00	1.56
37	7	100 (0)	45.0 (0)	LF	96.00	83.24	0.00	1.56
38	50	100 (0)	45.0 (0)	LF	90.00	83.24	0.00	1.56
39	39	100 (0)	45.0 (0)	LF	61.00	83.24	0.00	1.56
40	19	20 (-1.0)	30.0 (-1)	L50	70.00	75.99	17.00	11.22
41	24	180 (1.0)	30.0 (-1)	L50	60.00	61.61	28.00	23.67
42		*20 (-1.0)*	*60.0 (1)*	*L50*	~~*79.00*~~	82.37	~~*21.00*~~	4.94
43	16	180 (1.0)	60.0 (1)	L50	76.00	73.93	15.00	16.67
44	21	5 (-1.188)	45.0 (0)	L50	79.00	81.06	2.00	4.39
45	43	195 (1.188)	45.0 (0)	L50	60.00	67.49	19.00	18.76
46	17	100 (0)	27.2 (-1.189)	L50	68.00	61.71	18.00	21.52
47	32	100 (0)	62.8 (1.189)	L50	71.00	72.84	20.00	13.62
48	29	100 (0)	45.0 (0)	L50	64.00	64.27	14.00	15.63
49	*40*	*100 (0)*	*45.0 (0)*	*L50*	*58.00*	*64.27*	*16.00*	*15.63*
50	23	100 (0)	45.0 (0)	L50	75.00	64.27	14.00	15.63
51	45	100 (0)	45.0 (0)	L50	68.00	64.27	13.00	15.63
52	30	100 (0)	45.0 (0)	L50	67.00	64.27	12.00	15.63

P – Palatase, L – Lecitase, LF – Lipopan F BG, L50 – Lipopan 50 BG; AV – actual value, PV – predicted value, coded values of factors shown in brackets.

Results and discussions

The hydrolysis of TO in hexane was investigated in the range of 20-180 min reaction time and 30–60°C reaction temperature, using 4 different enzymes (Palatase, Lecitase, Lipopan F BG, Lipopan 50 BG). These parameters were considered as reaction factors. Concentrations of reaction products - triolein and oleic acid - were analyzed as response variables. To optimize the response and make the process robust we have chosen response surface modeling as a mathematical and statistical technique.

Model selection

The diagnostics of residuals and influence (externally studentized residuals - outlier t) helped to detect outliers in the data that were not fit well by the model and which were later disregarded in the analysis (values crossed out in Table 1.). Fitting the data to various models (linear, two factor interaction and quadratic) and their subsequent ANOVA indicated that the reactions were best described by second-order polynomial models both for triolein and oleic acid concentrations.

The quadratic polynomial regression model for predicting responses Y_1 and Y_2 (triolein and oleic acid concentrations respectively) is as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad \text{(Equation 1.)}$$

where Y is the response variable, Xi and Xj are independent variables, β0, βi and βj, βii, βij are the constant coefficients of intercepts (linear, quadratic and interaction terms).

The models were statistically significant and adequate to represent the relationship between the response and the significant variables, with a very small model p values

(<0.0001). The residual lack of fit p value was 0.9032 for triolein concentration and 0.1073 for oleic acid, meaning non-significant lack of fit. The ANOVA for triolein and oleic acid response models is represented in Tables 2 and 3 respectively.

Table 2. ANOVA for response surface quadratic model, response triolein

Source	Sum of squares	Degrees of freedom	Mean square	F value	P-value, Prob > F	
Model	8800.07	14	628.58	6.47	<0.0001	significant
A-Time	151.25	1	151.25	1.56	0.2203	
B-Temperature	19.42	1	19.42	0.2	0.6575	
C-Enzyme	7043.22	3	2347.74	24.16	<0.0001	
AB	31.2	1	31.2	0.32	0.5745	
AC	88.46	3	29.49	0.3	0.8227	
BC	511.05	3	170.35	1.75	0.1736	
A^2	859.49	1	859.49	8.84	0.0052	
B^2	77.3	1	77.3	0.8	0.3784	
Residual	3498.51	36	97.18			
Lack of fit	1411.31	20	70.57	0.54	0.9032	not significant
Pure error	2087.2	16	130.45			
Cor total	12298.59	50				

Cor total – total sum of squares corrected for the mean.

Table 3. ANOVA for response surface quadratic model, response oleic acid

Source	Sum of squares	Degrees of freedom	Mean square	F Value	P-value, Prob > F	
Model	9310.4	14	665.03	37.54	<0.0001	significant
A-Time	1032.94	1	1032.94	58.3	<0.0001	
B-Temperature	231.38	1	231.38	13.06	0.0009	
C-Enzyme	7173.13	3	2391.04	134.96	<0.0001	
AB	0.4	1	0.4	0.02	0.8816	
AC	396.48	3	132.16	7.46	0.0005	
BC	229.97	3	76.66	4.33	0.0107	
A^2	139.3	1	139.3	7.86	0.0082	
B^2	31.8	1	31.8	1.79	0.1890	
Residual	620.1	35	17.72			
Lack of fit	426.9	19	22.47	1.86	0.1073	not significant
Pure error	193.2	16	12.07			
Cor total	9930.5	49				

Cor total – total sum of squares corrected for the mean.

The models' F-values of 6.47 and 37.54 imply the models are significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise (p values <0.0001).

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case C and A^2 are significant model terms. It is considered that reducing the model and leaving only the significant model terms could improve the model. However, term B was not excluded because it is significant in oleic acid model (Table 3) and the amount of oleic acid depends directly on triolein hydrolysis.

In this case A, B, C, AC, BC, A^2 are significant model terms.

The coefficients of determination R^2 (adjusted for the number of parameters in the model relative to the number of points in the design) were 0.6049 and 0.9126 for the triolein and oleic acid models respectively. This is an estimate of the fraction of overall variation in the data accounted by the model, and thus the models are capable of explaining 60.49% and 91.26% of the variation in response. The values were high enough (especially for the oleic acid model) and, what is more important for design of experiment, predicted R^2 values (0.4969 and 0.8526 for triolein and oleic acid models respectively) were in good agreement with the adjusted R^2 values (the values should differ by no more than 0.2).

The adequate precision value is an index of the signal-to-noise ratio, value of more than 4 is desirable. For both models the adequate precision values were greater than 9.

According to Eq. 1, the response value in final equations is the sum of intercept and products of coefficients and independent variables. The lists of coefficients for both responses in terms of coded and actual factors are shown in Tables 4 and 5.

Table 4. Model coefficients for equations in terms of coded factors

Term/ response	Intercept	A	B	C_1	C_2	C_3	AB	AC_1	AC_2	AC_3	BC_1	BC_2	BC_3	A^2	B^2
Y_1	67.65	-2.65	1.02	-16.34	4.13	15.59	1.47	-0.28	1.75	1.59	-7.5	2.53	1.31	7.09	2.12
Y_2	16.58	6.44	-3.28	17.98	-2.01	-15.02	-0.18	5.51	0.14	-5.26	3.08	-5.08	2.05	-2.88	1.37

Y_1 and Y_2 are response values (triolein and oleic acid concentrations respectively), A is time, B temperature and C is enzyme.

Table 5. Model coefficients for equations in terms of actual factors

Term/ response	Intercept	A	B	$AB \cdot 10^{-3}$	$A^2 10^{-3}$	$B^2 10^{-3}$
Y_{1Pal}	110.13	-0.31	-1.40	1.23	1.11	9.43
Y_{1Lec}	97.95	-0.29	-0.74	1.23	1.11	9.43
Y_{1LipF}	113.29	-0.29	-0.81	1.23	1.11	9.43
Y_{1Lip50}	93.08	-0.35	-0.66	1.23	1.11	9.43
Y_{2Pal}	27.43	0.25	-0.55	-0.15	-0.45	6.10
Y_{2Lec}	38.61	0.18	-1.09	-0.15	-0.45	6.10
Y_{2LipF}	10.97	0.11	-0.62	-0.15	-0.45	6.10
Y_{2Lip50}	25.23	0.17	-0.76	-0.15	-0.45	6.10

where Y_{1Pal}, Y_{1Lec}, Y_{1LipF}, Y_{1Lip50} correspond to triolein concentrations after reaction with Palatase, Lecitase, Lipopan F BG, Lipopan 50 BG respectively. Y_{2Pal}, Y_{2Lec}, Y_{2LipF}, Y_{2Lip50} correspond to oleic acid concentrations after reaction with Palatase, Lecitase, Lipopan F BG, Lipopan 50 BG respectively.

Mutual effect of reaction parameters

The relationships between reaction factors and responses can be better understood by examining the series of contour plots.

Figures 1-4 show response surface contour plots of triolein hydrolysis model. The triolein concentration is plotted by contours as a function of time and temperature (two independent variables in X and Y-axes) for 4 different enzymes: Palatase (Fig. 1), Lecitase (Fig. 2), Lipopan F BG (Fig. 3), Lipopan 50 BG (Fig. 4).

The optimal reaction time for all the enzymes varied between 100 and 140 min. Generally, the best enzyme to hydrolyze triolein was Palatase (about 52% of triolein hydrolyzed), it tended to hydrolyze triolein more efficiently within higher temperature range (54-60ºC).

Meanwhile using Lecitase, Lipopan F BG and Lipopan 50 BG triolein was better hydrolyzed in lower temperatures (up to 42°C, 48°C and 48°C respectively). It is expected that in appropriate temperature and time conditions, about 28%, 16% and 40% of triolein could be hydrolyzed by Lecitase, Lipopan F BG and Lipopan 50 BG respectively.

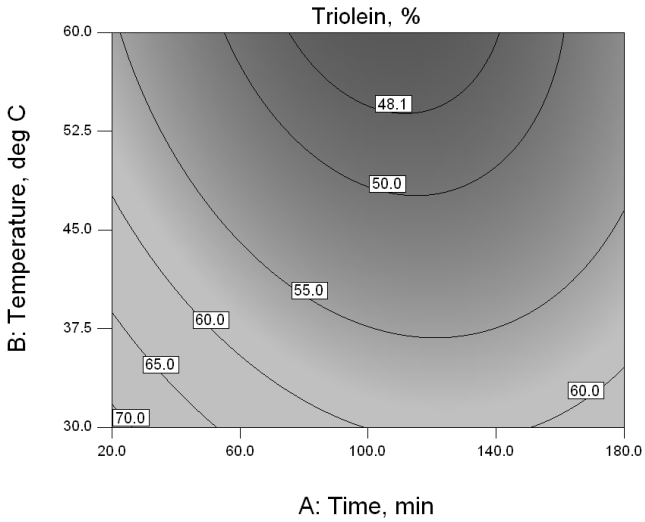

Figure 1. Response surface plot showing the mutual effect of time and temperature on Palatase - catalyzed triolein hydrolysis (residual amount, %).

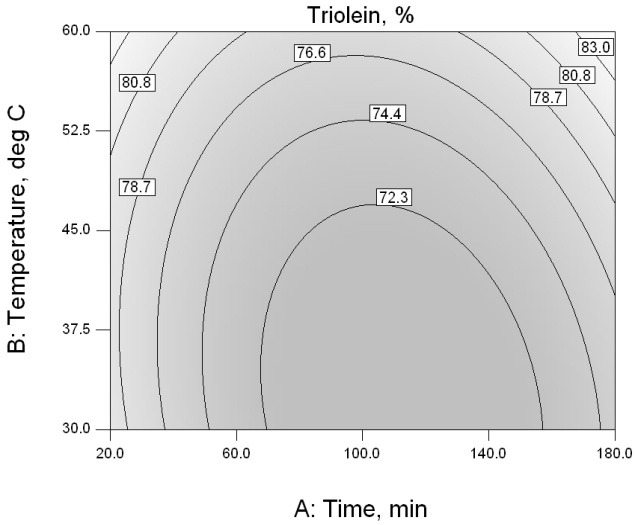

Figure 2. Response surface plot showing the mutual effect of time and temperature on Lecitase - catalyzed triolein hydrolysis (residual amount, %)

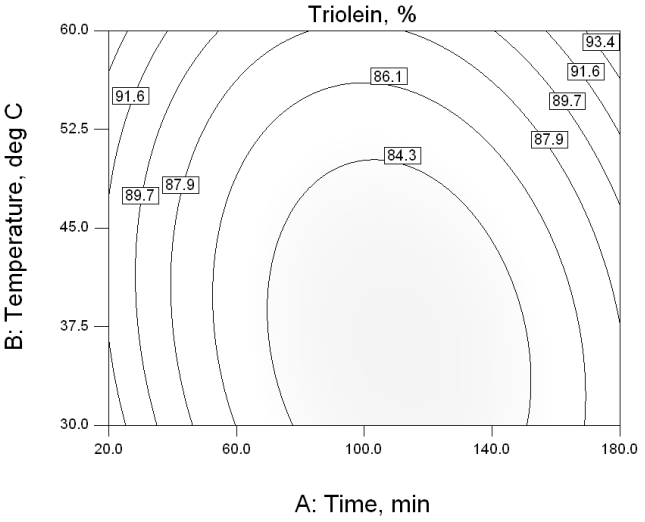

Figure 3. Response surface plot showing the mutual effect of time and temperature on Lipopan F BG - catalyzed triolein hydrolysis (residual amount, %).

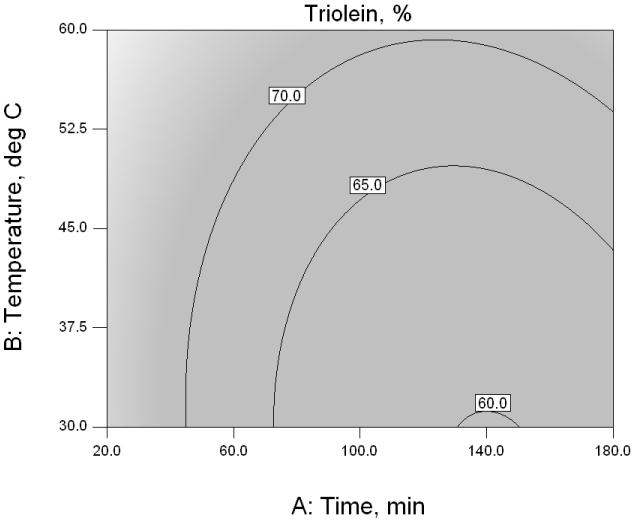

Figure 4. Response surface plot showing the mutual effect of time and temperature on Lipopan 50 BG - catalyzed triolein hydrolysis (residual amount, %).

Another parameter to analyze was oleic acid formation (concentration, %). Contour plots are shown in Figures 5-8 for Palatase, Lecitase, Lipopan F BG and Lipopan 50 BG enzymes respectively. A similar pattern is visible in all cases: the increase of oleic acid concentration corresponds with the longer reaction time. The highest amount of oleic acid (over 40%) was produced by Palatase (as shown above it was also the best enzyme for triolein hydrolysis, see Fig. 1). In the case of oleic acid formation catalyzed by Palatase, the effect of reaction time was much bigger than the effect of reaction temperature (the optimal temperature being around 45ºC), what is different from triolein hydrolysis pattern, where the highest hydrolysis yield was reached in the highest temperatures shown in Fig. 2. This might be due to diacylglycerol formation and subsequent hydrolysis, because Palatase is 1,3-specific. In the cases of Lecitase and Lipopan 50 BG, oleic acid yields increased when the

reactions were carried in lower reaction temperatures for longer times (Figs. 6 and 8), what correlates well with triolein hydrolysis model, only that the oleic acid yield tended to increase all the time. In the case of Lipopan F BG, the concentration of oleic acid was too low to predict effective reaction conditions, though some oleic acid was produced in low temperatures (around 30ºC) at about 120 min (Fig. 7).

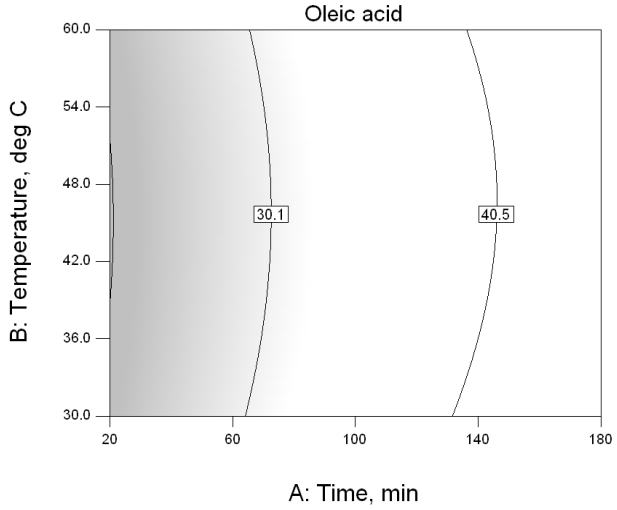

Figure 5. Response surface plot showing the mutual effect of time and temperature on Palatase – catalyzed oleic acid formation (amount, %).

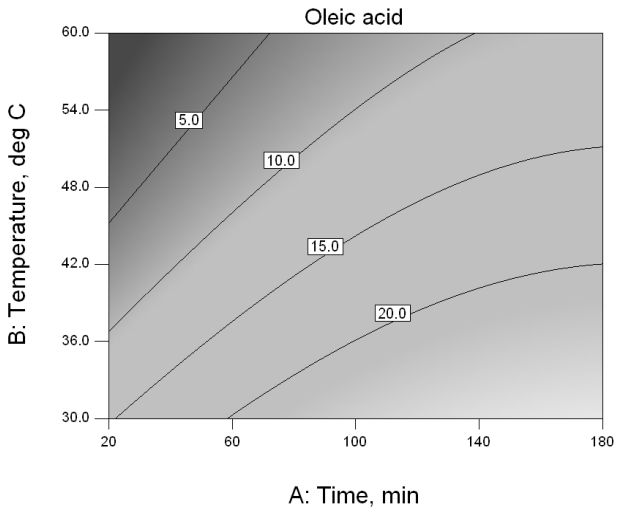

Figure 6. Response surface plot showing the mutual effect of time and temperature on Lecitase – catalyzed oleic acid formation (amount, %).

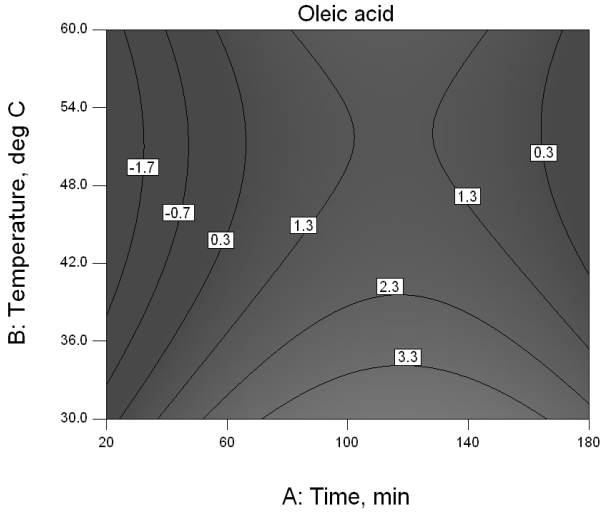

Figure 7. Response surface plot showing the mutual effect of time and temperature on Lipopan F BG – catalyzed oleic acid formation (amount, %).

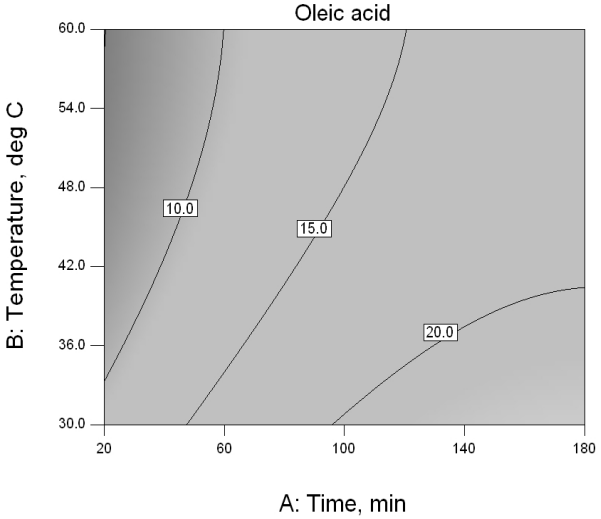

Figure 8. Response surface plot showing the mutual effect of time and temperature on Lipopan 50 BG – catalyzed oleic acid formation (amount, %).

Reaction optimization

Optimization tool in the Design Expert software was used to determine the optimal factor settings to reach the defined goals (the goals are combined into an overall desirability function; the program seeks to maximize this function). The optimization was meant to satisfy given criteria: minimize triolein concentration, maximize oleic acid concentration (medium importance) with preference to shorter reaction times and lower temperatures (lower importance). Not only the lower importance of time and temperature parameters was selected, but also the upper weights were set to be 0.1, what meant the broader selection of parameter solutions, not only the strict objective of the desired values. No preference for enzyme was

defined (Table 6.). Optimization options were 30 random starting points, using up to 50 design points, default epsilon = 0 for duplicate solution filter, and simplex fraction of 0.1, maximum number of solutions 100.

Table 6. Reaction optimization criteria for enzymatic triolein hydrolysis

Constraints Name	**Goal**	**Lower limit**	**Upper limit**	Lower weight	Upper weight	Importance (range 1-5)
A:Time, min	minimize	20	180	1	0.1	2
B:temperature, °C	minimize	30	60	1	0.1	2
C:Enzyme	in range	Palatase	Lipopan 50	1	1	3
Triolein	minimize	33	98	1	1	3
Oleic acid	maximize	0	43	1	1	3

The first four (out of 17) given solutions are shown in Table 7. The first solution with the highest mach to our preferences was chosen and reaction in 4 replicates was performed at 50.5°C for 139 minutes using the Palatase enzyme as a catalyst. The obtained results were 40.4% residual triolein concentration and 38.9% oleic acid concentration that reasonably matched and even exceeded (for triolein concentration) the predicted values (49.8% triolein, 39.8% oleic acid).

Table 7. The first four solutions for reaction optimization

Solution Number	Time, min	Temperature, °C	Enzyme	Triolein, %	Oleic acid, %	Desirability
1	139.4	50.5	Palatase	49.7747	39.8192	0.850
2	139.579	50.4	Palatase	49.82	39.834	0.85
3	141.54	30.0	Lipopan 50 BG	59.89	22.7821	0.684
4	142.672	30.0	Lipopan 50 BG	59.9	22.83	0.68

Conclusions

Response surface methodology (central composite design) was chosen as a time and material-saving option to detect the optimal enzyme and time-temperature related conditions for enzymatic triolein hydrolysis. Out of four enzymes selected for the study, Palatase was shown to be the most effective enzyme to hydrolyze triolein in hexane. With a desire to minimize reaction time and temperature, but maximize triolein hydrolysis to oleic acid, the numerical optimization was carried out. In predicted optimal conditions (enzyme Palatase at 50.5ºC for 139 min), 59.6% of triolein was hydrolyzed and 38.9% of oleic acid formed, what matched and even exceeded the predicted values, therefore, the response surface methodology was an appropriate and convenient tool to find the active factors, analyze and optimize the process. In our opinions, even better yields could be achieved by optimizing the physical conditions of the process. The use of immobilized enzyme could solve the problem of equal distribution of aqueous enzyme solution phase in hexane, still a minimal amount of water is necessary for ester-linkage hydrolysis. Therefore it is possible to proceed the work further also in defining other variables such as substrate and enzyme concentrations in the reaction medium, solvent choice, etc. and further increase triolein hydrolysis and oleic acid formation.

Acknowledgments

Research Council of Lithuania (Contract No. PBT-07/2010-2) is gratefully acknowledged for financial support and Biopolis Ltd the distributor of Novozymes A/S in Lithuania for the kind gift of the enzymes.

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