UDC 66.095.13:547.266:577.152.311
ISSN 1330-9862

scientific note

(FTB-1209)

Optimization of Enzymatically Prepared Hexyl Butyrate by Lipozyme IM-77

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Received: December 12, 2002
Accepted: April 24, 2003

Summary

Hexyl butyrate, a green note flavour compound, is widely used in the food industry. The ability of immobilised lipase (Lipozyme IM-77) from *Rhizomucor miehei* to catalyse the transesterification of hexanol and tributyrin was investigated in this study. Response surface methodology (RSM) and five-level-five-factor central composite rotatable design (CCRD) were employed to evaluate the effects of synthesis parameters, such as reaction time (2 to 10 h), temperature (25 to 65 °C), enzyme amount (10 to 50 %), substrate amount (in mol) ratio of tributyrin to hexanol (1:1 to 3:1), and added water content (0 to 20 %), on percentage amount (in mol) conversion of hexyl butyrate by transesterification. Reaction time and enzyme amount were the most important variables and substrate amount (in mol) ratio had less effect on the percentage of amount (in mol) conversion. Based on canonical analysis, the optimum synthesis conditions were: reaction time 8.3 h, temperature 50 °C, enzyme amount 42.7 %, substrate amount (in mol) ratio 1.8:1, and added water 12.6 %. The predicted value was 96.2 % and actual experimental value 95.3 % of the amount (in mol) conversion.

Key words: biosynthesis, hexanol, lipase, response surface methodology, transesterification

Introduction

Hexyl esters, green note flavour compounds, are widely used as flavour and fragrance in food, beverage and pharmaceutical industries. There is a growing demand for natural flavours containing »green note« represented by hexanol (C_6 alcohols) derivatives (*1*). Hexyl butyrate is of special interest as it represents a model of flavour ester. Traditionally, it has been isolated from natural sources or produced by chemical synthesis. Consumers are more interested in the source and compositions of flavourings in foods and prefer the word »natural« instead of »artificial« or »synthetic«. Therefore, the biosynthesis of such esters by lipase-catalysed chemical reactions under mild conditions attains much current commercial interest. An optimised enzymatic synthesis of hexyl butyrate would benefit the manufacturers and might be more appealing to the consumers.

Bourg-Garros *et al.* synthesised (Z)-3-hexen-1-yl butyrate by direct esterification using lipases *Rhizomucor miehei* (Lipozyme IM) and *Candida antarctica* (Novozym 435) in *n*-hexane with high yield (>90 %) (2). Carvalho *et al.* reported that hexyl acetate was synthesised by the cutinase-catalysed transesterification reaction of butyl

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acetate with hexanol in reversed micelles system and the optimisation of the transesterification was described using response surface methodology (RSM) (*3*).

The present work focuses on the reaction parameters that affect lipase from *R. miehei* (Lipozyme IM-77)-catalysed transesterification of hexyl butyrate using tributyrin as acyl donor in *n*-hexane. Our purposes were to better understand the relationships between the factors (reaction time, temperature, enzyme amount, substrate amount (in mol) ratio, and added water content) and the response (percent amount (in mol) conversion); and to determine the optimal conditions and procedure for hexyl butyrate synthesis using central composite rotatable design (CCRD) and RSM analysis.

Materials and Methods

Materials

Immobilised lipase (triacylglycerol hydrolase, EC 3.1.1.3; Lipozyme IM-77, 7.7 BAUN/g) from *R. miehei* supported on macroporous weak anionic resin beads was purchased from Novozyme, Inc. (Bagsvaerd, Denmark). Hexanol (98 % pure), triacetin (99 % pure) and tributyrin (99 % pure) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Molecular sieve 4Å was purchased from Davison Chemical Co. (Baltimore, MD, USA) and *n*-hexane was obtained from Merck Chemical Co. (Darmstadt, Germany). All other chemicals were of analytical reagent grade.

Experimental design

A five-level-five-factor CCRD with replicate in each point was employed in this study, requiring 32 experiments in total (*4,5*). The fractional factorial design consisted of 16 factorial points, 10 axial points (two axial points on the axis of each design variable at a distance of two from the design center), and 6 center points. The variables and their levels selected for the study of hexyl butyrate synthesis were: reaction time (2–10 h); synthesis temperature (25–65 °C); enzyme fraction (10–50 %, by mass of hexanol); substrate amount (in mol) ratio (3:1–1:1; tributyrin:hexanol); and added water content (0–20 %, by weight of hexanol). Table 1 presents the in-

Table 1. Central composite rotatable second-order design in terms of coded, uncoded and experimental data for five-level-five-factor response surface analysis

Treatment No.	*t* / h	*t* / °C	*w* (enzyme by *wt* of hexanol) / %	*R* (tributyrin/ hexanol)	*w* (H_2O by *wt* of hexanol)/ %	η (in amount (in mol) conversion) / %
	x_1	x_2	x_3	x_4	x_5	Y
1	–1(4)[a]	–1(35)	–1(20)	–1(1.5:1)	1(15)	40.86
2	1(8)	–1(35)	–1(20)	–1(1.5:1)	–1(5)	75.99
3	–1(4)	1(55)	–1(20)	–1(1.5:1)	–1(5)	61.89
4	1(8)	1(55)	–1(20)	–1(1.5:1)	1(15)	74.52
5	–1(4)	–1(35)	1(40)	–1(1.5:1)	–1(5)	72.84
6	1(8)	–1(35)	1(40)	–1(1.5:1)	1(15)	87.13
7	–1(4)	1(55)	1(40)	–1(1.5:1)	1(15)	71.79
8	1(8)	1(55)	1(40)	–1(1.5:1)	–1(5)	94.53
9	–1(4)	–1(35)	–1(20)	1(2.5:1)	–1(5)	48.08
10	1(8)	–1(35)	–1(20)	1(2.5:1)	1(15)	35.20
11	–1(4)	1(55)	–1(20)	1(2.5:1)	1(15)	28.99
12	1(8)	1(55)	–1(20)	1(2.5:1)	–1(5)	89.40
13	–1(4)	–1(35)	1(40)	1(2.5:1)	1(15)	48.50
14	1(8)	–1(35)	1(40)	1(2.5:1)	–1(5)	90.89
15	–1(4)	1(55)	1(40)	1(2.5:1)	–1(5)	81.99
16	1(8)	1(55)	1(40)	1(2.5:1)	1(15)	89.51
17	–2(2)	0(45)	0(30)	0(2:1)	0(10)	22.59
18	2(10)	0(45)	0(30)	0(2:1)	0(10)	89.81
19	0(6)	–2(25)	0(30)	0(2:1)	0(10)	46.20
20	0(6)	2(65)	0(30)	0(2:1)	0(10)	69.22
21	0(6)	0(45)	–2(10)	0(2:1)	0(10)	55.95
22	0(6)	0(45)	2(50)	0(2:1)	0(10)	75.04
23	0(6)	0(45)	0(30)	–2(1:1)	0(10)	78.95
24	0(6)	0(45)	0(30)	2(3:1)	0(10)	73.28
25	0(6)	0(45)	0(30)	0(2:1)	–2(0)	86.90
26	0(6)	0(45)	0(30)	0(2:1)	2(20)	72.30
27	0(6)	0(45)	0(30)	0(2:1)	0(10)	80.89
28	0(6)	0(45)	0(30)	0(2:1)	0(10)	80.30
29	0(6)	0(45)	0(30)	0(2:1)	0(10)	83.24
30	0(6)	0(45)	0(30)	0(2:1)	0(10)	84.62
31	0(6)	0(45)	0(30)	0(2:1)	0(10)	86.60
32	0(6)	0(45)	0(30)	0(2:1)	0(10)	85.11

[a]Numbers in parentheses represent actual experimental amounts

dependent factors (x_i), levels and experimental design in terms of coded and uncoded values.

Esterification method

All substrates and *n*-hexane were dehydrated with molecular sieve 4Å for 24 h before reaction. Hexanol (100 mM, 30.66 mg) and different amount (in mol) ratios of tributyrin were added to 3 mL of *n*-hexane, followed by different amounts of added water and enzyme. The control of water content was important through the reaction, in which the added water was observed to dissolve in the organic phase not forming a separate phase. The reaction mixtures were incubated in an orbital shaking water bath (200 rpm) at different reaction temperatures and reaction times (Table 1).

Extraction and analysis

The immobilised enzyme and any residual water were removed by passing reaction media through an anhydrous sodium sulfate column. Before sample analysis, triacetin (50 mM) was added to each sample as an internal standard. The analyses of samples were performed by injecting a 1 μL aliquot in a splitless mode into a Hewlett Packard 4890 gas chromatograph equipped with a flame-ionization detector (Avondale, PA, USA) and a DB-5 fused-silica capillary column (30 m × 0.32 mm i.d.; film thickness 1 μm; J&W Scientific, Folsom, CA, USA). Injector and detector temperatures were set at 280 and 300 °C, respectively. Oven temperature was held at 50 °C for 2 min, elevated to 200 °C at 50 °C/min, held for 4 min, and then increased up to 300 °C at 70 °C/min. Nitrogen was used as a carrier gas. The calculated yield (percent amount (in mol) conversion) was defined as n(hexyl butyrate)/n(initial hexanol)/(mmol/mmol) · 100 % and was estimated using peak area integrated by on-line software Hewlett Packard 3365 Series II ChemStation (Avondale, PA, USA).

Statistical analysis

The actual experimental data (Table 1) were analysed by a response surface regression (RSREG) procedure with ridge max option to fit the following second-order polynomial equation *(6)*:

$$Y = \beta_{k0} + \sum_{i=1}^{5}\beta_{ki}x_i + \sum_{i=1}^{5}\beta_{kii}x_i^2 + \sum_{i=1}^{4}\sum_{j=i+1}^{5}\beta_{kji}x_ix_j \qquad /1/$$

where Y is response (amount (in mol %) conversion); β_{k0}, β_{ki}, β_{kii} and β_{kij} are constant coefficients and x_i the uncoded independent variables. Canonical analysis is one part of the RSREG output and the method of ridge analysis computes the estimated ridge of maximum response for increasing radii from the centre of original design.

Results and Discussion

Model fitting

The five-level-five-factor CCRD in terms of coded, uncoded and actual experimental data are presented in Table 1. Among the various treatments, the greatest molar conversion (94.53 %) was treatment No. 8 (8 h, 55 °C, 40 % enzyme, substrate amount (in mol) ratio 1.5:1, and added water content 5 %), and the smallest conversion (only 22.59 %) was treatment No. 17 (2 h, 45 °C, 30 % enzyme, substrate amount (in mol) ratio 2:1, and added water content 10 %). The RSREG procedure with lack-of-fit test was employed to fit the second-order polynomial Eq. /1/ to the experimental data – percent amount (in mol) conversions (Table 1).

Table 2. Analysis of variance (ANOVA) for synthesis variables pertaining to the response percent amount (in mol) conversion

Source	Degrees of freedom	Sum of squares	F-ratio	p-value
Model	20	11241.000	6.601	0.001
Linear	5	8445.406	19.837	0.000
Quadratic	5	1977.221	4.644	0.016
Cross product	10	818.180	0.961	0.521*
Lack of fit	6	691.026	2.345	0.184*
Pure error	5	245.580		
Total error	11	936.606		

*Not significant at p = 0.05; R^2 (coefficient of determination) = 0.923

Table 2 shows the analysis of variance (ANOVA), indicating that the model with very small p-value (0.001) is highly significant. The lack-of-fit test (p=0.184) was not significant and the squared correlation coefficient (R^2) was 0.923, both indicating that the model is adequate to represent the actual relationship between response percent amount (in mol) ratio and the variables. Therefore, the second-order polynomial Eq. /1/ is given below /2/:

$$Y = -114.864 + 19.265x_1 + 4.150x_2 + 2.081x_3 - 14.934x_4 - 0.636x_5 - 1.461x_1^2 + 0.076x_2x_1 - 0.055x_2^2 - 0.026x_3x_1 - 0.010x_3x_2 - 0.035x_3^2 + 0.791x_4x_1 + 0.517x_4x_2 + 0.452x_4x_3 - 3.463x_4^2 + 0.064x_5x_1 + 0.016x_5x_2 + 0.066x_5x_3 - 1.930x_5x_4 \qquad /2/$$

Furthermore, the overall effect of the five synthesis variables on the percent amount (in mol) conversion of hexyl butyrate was further analysed by a joint test (Table 3). The results revealed that the time (x_1), temperature (x_2), enzyme amount (x_3), and added water content (x_5) were the important factors, exerting a statistically significant overall effect on the response ($p < 0.05$) on the amount (in mol) conversion of hexyl butyrate. Sub-

Table 3. Analysis of variance for joint test

Factor	Degrees of freedom	Sum of squares	F-ratio	p-value
Time (x_1)	6	5237.892	10.253	0.0006
Temperature (x_2)	6	1854.631	3.630	0.0309
Enzyme amount (x_3)	6	2662.052	5.211	0.0090
Substrate amount (in mol) ratio (x_4)	6	848.525	1.661	0.2204*
Added water content (x_5)	6	1742.434	3.411	0.0375

*Not significant at p = 0.05

strate amount (in mol) ratio (x_4) had a less significant effect ($p > 0.05$), indicating that the synthesis with low substrate amount (in mol) ratio (2:1) is possible. It was concluded that reducing the amounts of co-substrate (tributyrin) would reduce the production cost to synthesise hexyl butyrate.

Effects of synthesis parameters

Fig. 1 (A) shows the effect of reaction time, enzyme amount, and their mutual interaction on hexyl butyrate synthesis at 45 °C, substrate amount (in mol) ratio 2:1, and added water amount 10 %. At the lowest reaction time (2 h) with the lowest enzyme amount (10 %), amount (in mol) conversion was indistinguishable from zero. As reaction time increased, the amount (in mol) conversion increased at each enzyme amount. Similarly, increase in the enzyme amount resulted in increased amount (in mol) conversion at each reaction time. The reaction with low enzyme amount (10 %) and high reaction time (10 h) attained the amount (in mol) conversion (~55 %), indicating that lower enzyme dosage can be compensated for by a longer reaction time. The reaction with the lowest reaction time (2 h) and highest enzyme amount (50 %) reached 40 % of the amount (in mol) conversion. In general, inverse proportionality between reaction time and enzyme dosage was found for many industrial enzyme processes.

A reaction with high reaction time (10 h) with highest enzyme amount (50 %) favoured maximal esterification.

The effect of varying reaction temperature and enzyme amount at constant reaction time (6 h), substrate amount (in mol) ratio (2:1), and added water content (10 %) is shown in Fig. 1 (B). At each temperature from 25 to 65 °C, an increase in enzyme amount led to higher yields-up. A moderate reaction temperature (55 °C) with highest enzyme amount favoured maximal yield, and the increase in temperature up to 65 °C resulted in less esterification at any given enzyme amount probably due to the inhibition of enzyme by temperature over 55 °C, indicating that the optimal temperature for lipase IM-77 was around 55 °C.

Fig. 1 (C), depicting the variation of enzyme amount and added water content, shows clearly that the added water content affected esterification negatively at low enzyme amount. With an increase in water content from 0 to 20 % at enzyme amount 10 %, esterification decreased at reaction time of 6 h, temperature 45 °C, and substrate amount (in mol) ratio = 2:1. However, the water content did not affect the yields at high enzyme amount (50 %).

Overall effects

The entire relationships between reaction factors and response can be better understood by examining the planned series of contour plots (Fig. 2) generated from the predicted model (Eq. 2) by holding the enzyme amount (10, 20 and 30 %) and added water content (0, 10 and 20 %) constant. Figs. 2 A, B and C represent the same added water content (0 %); and A, D and G represent the same enzyme amount (10 %).

Substrate amount (in mol) ratio was constant (2:1) with less significant effect on response in the optimisation studies. Such an application could be employed to study the synthesis variables simultaneously in a five-dimensional space and easily observe the overall effects of synthesis variables on amount (in mol) conversions.

Reaction time (x_1) and temperature (x_2) were the most important variables for hexyl butyrate synthesis with the small p-values (see Table 2) and considered as indicators of effectiveness and economical performance. In general, all nine contour plots in Fig. 2 exhibited similar behaviour in that the predicted amount (in mol) conversion increased by the reaction time. Therefore, a 10-h synthesis gave the highest percent of amount (in mol) conversion compared to the others in the experimental region. An increase in reaction temperature resulted in higher esterification in the range of 25 to 55 °C. However, a reaction with temperature over 55 °C resulted in lower esterification, which revealed that high temperature may lead to evaporation of the product or denaturation of lipase IM-77. A reaction with higher enzyme amount (in mol) gave higher amount (in mol) conversion compared to the one with fewer enzymes.

Attaining optimum conditions

The optimum points of synthesis of hexyl butyrate were obtained by graphic method and canonical analysis. A near-optimum condition (Fig. 2), which allowed

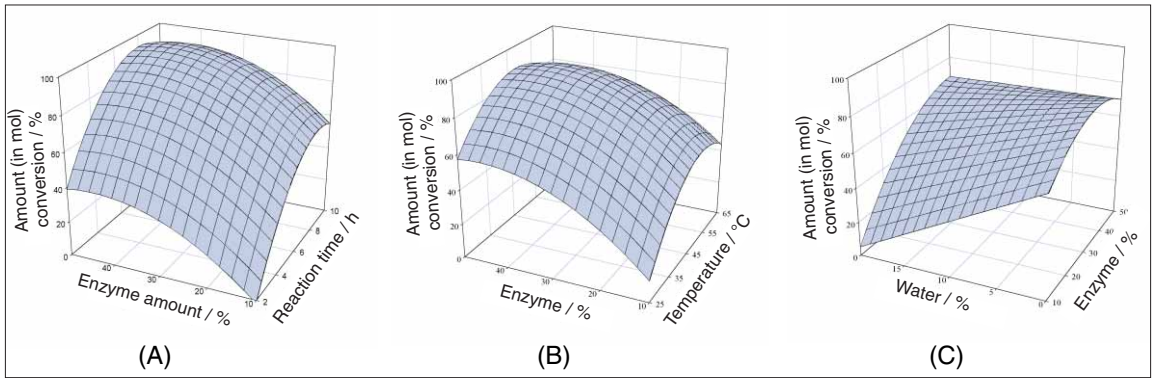

Fig. 1. Response surface plot showing (A) the effect of reaction time, enzyme amount; (B) the effect of reaction temperature, enzyme amount; (C) the effect of enzyme amount, added water content; and their mutual interaction on hexyl butyrate synthesis. Other synthesis parameters are constant at 0 level

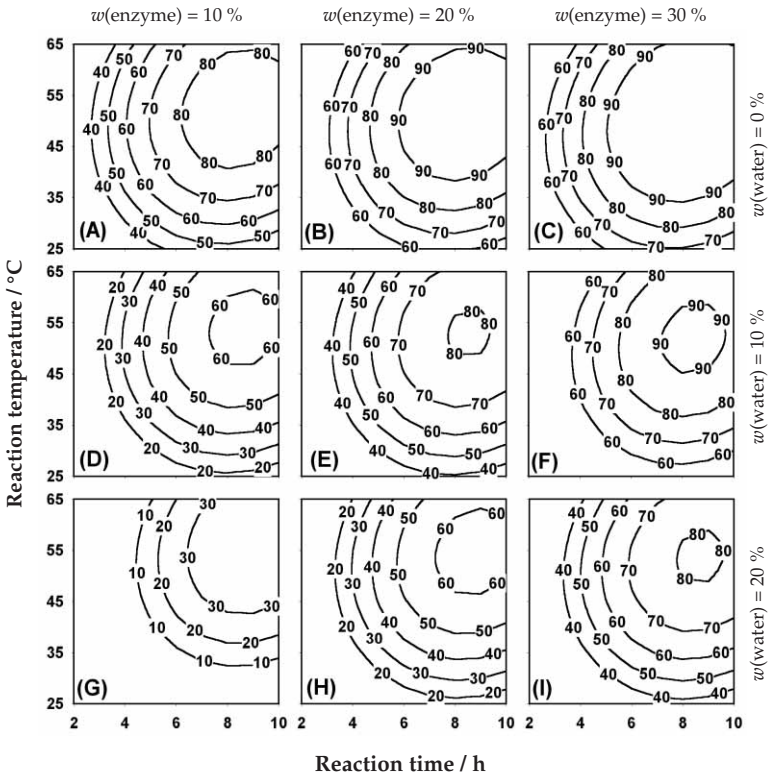

Fig. 2. Contour behaviour of percent amount (in mol) conversion for hexyl butyrate synthesis. Substrate amount (in mol) ratio (R) was constant (tributyrin/hexanol) = 2:1. Enzyme amount and added water amount were by weight of hexanol. The numbers inside contour plots indicate amount (in mol) conversions at given reaction conditions

us to compare all of the factors simultaneously, could be suggested for the industry. The most efficient condition of this reaction would use the lowest amount of enzymes to achieve the acceptable conversion (say ~80 % for the practical range) of substrate in minimal time at the lowest temperature. Fig. 2 (A) suggests what seems to be a reasonable range of acceptable temperatures (50–55 °C) and reaction time (8–10 h) should be employed for the practical near-optimum conditions because it required only 10 % enzyme, 0 % added water and substrate amount (in mol) ratio (1:1) to achieve 80 % compared to the others, because enzyme is more expensive than substrate in this case.

In addition, the optimum point (reaction time 8.3 h, synthesis temperature 50.3 °C, enzyme amount 42.7 %, substrate amount (in mol) ratio 1.8:1, and added water content 12.6 %) was determined by canonical analysis. The stationary point, values of variables at which the first derivative of response was zero, was located exactly in the experimental region with the predicted value of 96.2 %. The canonical analysis, based on the stationary point, resulted in the following equation:

$$Y = 96.184 + 8.468\ W_1^2 - 4.562\ W_2^2 - 15.506\ W_3^2 - 24.522\ W_4^2 - 26.238\ W_5^2 \qquad /3/$$

where, W_1, W_2, W_3, W_4 and W_5 are eigenvalues based on coded data and Y is the amount (in mol) conversion of hexyl butyrate (%). The mixed signs of eigenvalues indicated that the predicted response surface of the stationary point is shaped like a saddle. The response behavior of reaction time and synthesis temperature (Fig. 3) was followed while holding the other reaction parameters constant at the suggested optimum point with the maximum value (96 %) at combination of 8.3 h and 50.3 °C. Therefore, the reaction conditions (reaction time 8.3

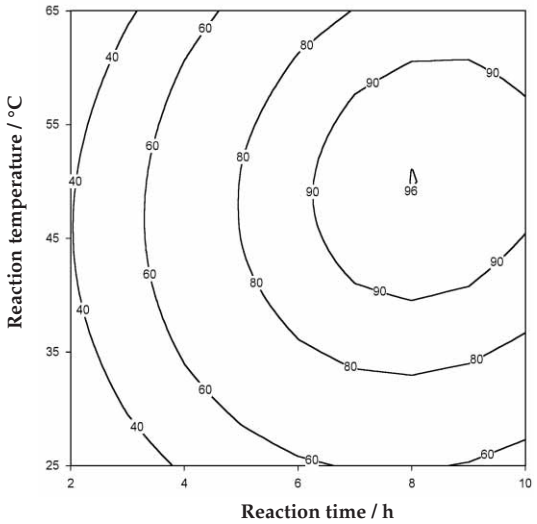

Fig. 3. Contour plots showing response behaviour of reaction time and temperature for the optimum synthesis condition at the stationary point suggested by canonical analysis. Other parameters were constant (enzyme amount 42.7 %; substrate amount (in mol) ratio 1.8:1; added water content 12.6 %)

h, synthesis temperature 50 °C, enzyme amount 42.7 %, substrate amount (in mol) ratio 1.8:1, and added water content 12.6 %) were recommended as the optimisation for hexyl butyrate synthesis with 96.2 % of amount (in mol) conversion in this study.

Model verification

The adequacy of the predicted model here was examined by additional independent experiments at the suggested optimal synthesis conditions. The predicted value was 96.2 % obtained by canonical analysis and the actual value was (95.3 ± 0.4) %. Chi-square test (p-value = 0.99, degrees of freedom = 3) indicated that the observed values were significantly the same as the predicted values and the generated model adequately predicted the percent amount (in mol) conversion (7). Thus, the optimised enzymatic synthesis for hexyl butyrate by lipase IM-77 was successfully developed by CCRD and RSM.

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Optimiranje priprave heksilbutirata katalizirane lipozimom IM-77

Sažetak

Heksilbutirat je miris koji se često koristi u prehrambenoj industriji. U radu je ispitana sposobnost imobilizirane lipaze (Lipozyme IM-77) iz *Rhizomucor miehei* da katalizira transesterifikaciju heksanola i tributirina. Da bi se procijenio utjecaj vremena reakcije (2–10 h), temperature (25–65 °C), količine enzima (10–50 %), množinskog omjera tributirina prema heksanolu (od 1:1 do 3:1), te dodane vode (0–20 %) na množinsku konverziju heksilbutirata tijekom transesterifikacije, primijenjena je metodologija odzivnih površina (response surface methodology – RSM) i pet-razinskog-pet-faktorskog složenog središnjeg rotacijskog dizajna (five-level-five-factor central composite rotatable design – CCRD). Vrijeme reakcije i količina enzima bile su najvažnije varijable, dok je množinski omjer supstrata manje utjecao na postotak konverzije. Na osnovi kanoničke analize utvrđeni su optimalni uvjeti sinteze: vrijeme reakcije 8,3 h, temperatura 50 °C, količina enzima 42,7 %, množinski omjer supstrata 1,8:1 i dodana voda 12,6 %. Predviđena vrijednost množinske konverzije iznosila je 96,2 %, dok je eksperimentalno utvrđena vrijednost 95,3 %.