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Ganapati D. Yadav* Somnath Dattatray Shinde†

*Institute of Chemical Technology, Mumbai, gdyadav@gmail.com

†Institute of Chemical Technology, Mumbai, somnath_uict@yahoo.co.in

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Abstract

Response surface methodology (RSM) was used to model and optimize the immobilized Candida antarctica lipase B catalysed synthesis of butyl-4-methyl-3-oxopentanoate. To determine optimum conditions of the transesterification, a four-factor and five-level central composite rotatable design (CCRD) was used. The factors studied were enzyme load (A), reaction temperature (B), methyl-4-methyl-3-oxopentanoate concentration (C) and n-butanol concentration (D). A quadratic polynomial regression model was used to analyze the experimental data at a 95% confidence level ($p < 0.05$). The results indicated that the RSM approach gave reasonable results for the optimization of the reaction parameters in the range of tested parameters. The optimal conditions for the enzymatic reaction were obtained at 0.01 mol of methyl-4-methyl-3-oxopentanoate and 0.03 mol of n-butanol using 104 mg of Novozym 435 at 55 °C and 300 rpm for 6 h. Under these conditions, the transesterification percentage was 87 %. Further, kinetic modelling of the enzymatic synthesis was illustrated. Initial rate data and progress curve data were used to arrive at a suitable model. The kinetics was found to obey the ternary complex ordered bi-bi model with inhibition by the substrate methyl-4-methyl-3-oxopentanoate. The values of kinetic parameters obtained from non-linear regression analysis were found to be Vmax of 0.04 mol/L.min; Km(A) 0.11 mol/L; Km(B) 2 mol/L and Ki(A) 2.2 mol/L.

KEYWORDS: isobutyrylacetate esters, response surface methodology (RSM), Candida antarctica lipase, enzyme kinetics, non-aqueous enzymology

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INTRODUCTION

Isobutyryl acetate esters are commercially attractive because of their applications as intermediates in the synthesis of heterocycles such as furan, pyrazolone, quinolone (Antonioletti *et al.,* 2002; Jung *et al*., 2002; Shetty and Moffett, 2006; Wang and Hollingsworth, 1999). There is a dearth of information on the synthesis of isobutyryl acetate esters. Base catalysed condensation of ethyl isobutyrate to ethyl isobutyryl isobutyrate has been reported in earlier literature (Hauser and Renfrow, 1937). Synthesis of isobutyryl acetate esters has been achieved by homogeneous acid catalysis which requires expensive materials of construction, neutralisation of acidic waste leading to pollution and results in to presence of impurities in the final ester. Therefore, a process that could avoid homogeneous liquid acids and be environmentally friendly and also inexpensive is the most desirable for organic transformations (Krishna et al., 2001; Yadav, 2005; Yadav and Bokade, 1996; Yadav and Devi, 2002, 2004a,b; Yadav and Kirthivasan, 1995,1997; Yadav and Lathi, 2003, 2004a,b, 2006; Yadav et al., 2005,2007, 2008; Yadav and Sivakumar, 2004). Several general routes of ester preparation have been listed amongst which heterogeneous solid acids as catalysts could be used using high temperature (Yadav and Mehta, 1993; Yadav and Nair, 1999). In contrast with solid acids, biocatalysts allow synthesis of esters to be performed at moderate temperatures (Yadav and Borkar, 2006, 2008, 2009a,b, 2010; Yadav and Devendran, 2012; Yadav and Dhoot, 2009; Yadav and Jadhav, 2005; Yadav and Pawar, 2012). Production drugs and active pharmaceutical intermediates by lipase catalyzed reactions has also been performed under mild conditions (Yadav et al. 2007). Optimization of process parameters by using statistical methods has been studied these days in a number of cases.

Many models of lipase-catalyzed esterification and transesterification in non-aqueous media or solvent-free systems have already been reported and shown to kinetically proceed via ping–pong bi–bi mechanism or ternary complex bi–bi mechanism (Kraai et al., 2008 Perez et al., 2007; Romero et al., 2007; Segel, 1975; Vazquez Lima et al., 1996; Yadav and Trivedi, 2003; Yadav and Devi, 2002, 2004a,b; Yadav and Lathi, 2003, 2004a,b, 2006; Yadav et al., 2005,2007, 2008). Mechanisms in some cases involve inhibition by either substrate or product or both. For instance, a ternary complex bi–bi mechanism with inhibition by *n*-octanol substrate was used to model the transesterification of *n*-octanol with vinyl acetate in *n*-heptane (Yadav and Trivedi, 2003) and a ping-pong bi-bi model with dead-end inhibition caused by lauric acid was proposed for the esterification of lauric acid with geraniol in isooctane (Vazquez Lima et al., 1996). A ping–pong bi–bi mechanism with inhibition by acetic anhydride was also reported for isoamyl acetate synthesis in acylation of isoamyl alcohol with acetic anhydride using Novozym 435 in *n*-hexane (Romero et al., 2007).

Response surface methodology (RSM) is an effective statistical technique used to study simultaneously independent parameter optimization and effect of parameter interaction on response variable (Montgomery, 1984; Vicente et al., 1998; Rodriguez-Nogales et al., 2005; Hamsaveni et al., 2001). The reduction in number of experiments needed to provide sufficient information for statistically acceptable results has made RSM a widely acceptable tool for process parameter optimization and empirical modelling. RSM is faster and less expensive than the conventional method (Montgomery, 1984). The conventional method for optimization of process parameters involves changing one variable at a time, keeping others at fixed levels (Moreno *et al*., 1995; Yadav and Borkar, 2008). It is single-dimensional, laborious and time-consuming and often does not guarantee determination of optimal conditions. Thus it was thought worthwhile to apply RSM to study and optimize the enzymatic transesterification of methyl-4-methyl-3-oxopentanoate with *n*-butanol. Various authors have used RSM for optimization of process parameters in enzymatic reactions (Boulifi et al., 2010; Kim and Akoh, 2007; Macedo et al., 2004; Mahapatra et al., 2009 and Sontakke and Yadav, 2011a,b; Yadav and Sontakke, 2011). However, no information is available in open literature on enzymatic synthesis of butyl-4-methyl-3-oxopentanoate using RSM. The current work addresses the optimization of process parameters and development of a kinetic model for transesterification of *n*-butanol with methyl-4-methyl-3-oxopentanoate catalyzed by immobilized *Candida antarctica* lipase B (Novozym 435), using a four-factor and five-level central composite rotatable (CCRD) design. The four factors studied were enzyme loading (A), reaction temperature (B), methyl-4-methyl-3-oxopentanoate concentration (C) and *n*-butanol concentration (D). Effect of these independent variables was studied on conversion of substrate. This work delineates the results here.

MATERIALS AND METHODS

Enzymes and Chemicals

Novozym 435 (lipase B from *Candida antarctica*; immobilized on macro-porous polyacrylic resin beads, bead size 0.3 - 0.9 mm, bulk density 0.430 g/cm^3, water content approximately 0.66% (w/w), activity 7000 PLU/g, where PLU is the ester synthesis activity expressed in "propyl laurate units") was received from Novo Nordisk, Denmark. Lipozyme RM IM (*Rhizomucor meihei* lipase immobilized on an anionic resin) and Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on silica) were procured as gift samples from Novo Nordisk, Denmark. *Thermomyces lanuginosus* is produced from genetically modified *Aspergillus oryzae*. Methyl-4-methyl-3-oxopentanoate (98 % pure) was obtained as a gift sample from Litmus Industries, Mumbai, India. *n*-Butanol was obtained

from E-Merck, Germany. Toluene was obtained from S. D. Fine Chemicals, Mumbai. All other chemicals were standard analytical grade reagents obtained from reputed firms and used as such.

Experimental set-up and procedure

The experimental set-up consisted of a 4 cm i.d. fully baffled mechanically agitated reactor of 50 ml capacity, equipped with four baffles and a six-bladed turbine impeller. The entire reactor assembly was immersed in a thermostatic water bath, which was maintained at a desired temperature within ±1 ◦C. A typical reaction mixture consisted of specified quantities of *n*-butanol and methyl-4-methyl-3-oxopentanoate diluted up to 15 mL with toluene as solvent. The reaction mixture was agitated at the specified temperature for 15 min at a speed of 300 rpm and then the enzyme was added to initiate the reaction. Samples were withdrawn periodically and analysed by using gas chromatography (GC). All experiments were carried out with fresh immobilized enzyme. Control experiments, without the enzyme, were also carried out. All experimental data are average of triplicate values within an error of ±2 %.

Analytical method

The analysis was performed by GC (Chemito model) equipped with flame ionisation detector. BPX-50 capillary column (Make: SGE, USA, 50% phenyl polysilphenylene-siloxane; 30m×0.32mm; 0.25µm film thickness) was used for analysis. The temperature of the oven was maintained at 100 °C for 1 min; then increased to 210 °C at a ramp rate of 15 °C/min and kept for 1 min. Further it was increased to 270 °C at a ramp rate of 15 °C/min and kept for 1 min. Nitrogen was used as the carrier gas at a flow rate of 1 ml/min. Both injector and detector port temperatures were set at 280 °C. *n*-Decane (1.3 % v/v) was used as an internal standard to quantify the collected data for conversions and rates of reactions. Butyl-4-methyl-3-oxopentanoate was also confirmed by GC-MS (Make: PerkinElmer, USA, Model: Clarus 500).

Statistical design and analysis

To examine the combined effect of four different independent variables on transesterification of methyl-4-methyl-3-oxopentanoate, central composite rotatable experimental design (CCRD) of 2^4 =16 plus 6 centre points and (2×4 = 8) star points leading to a total of 30 experiments was performed. The factors studied were enzyme loading (A); reaction temperature (B); methyl-4-methyl-3-oxopentanoate concentration (C) and *n*-butanol concentration (D) (Table 1). The

design was extended up to ±α (axial point) of 2. The center values for variables were based on previously reported studies and carried out at least thrice for estimation of error. The design of experiments employed is presented in Table 2. The experiments were produced in a random order and triplicate measurements of transesterification percentage were run on each experiment.

Coding of the variables was done according to Equation 1:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i} \qquad i = 1, 2, 3, \ldots .k \tag{1}$$

Where: x_i, is the dimensionless value of an independent variable; X_i, real value of an independent variable; X_{cp}, real value of an independent variable at the center point; and ΔX_i, step change of real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i.

The relationship of the independent variables and the response was calculated by the second order polynomial (Equation 2).

$$Y = \beta_O + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i X_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j \tag{2}$$

Where: Y is the predicted response; β_0 a constant; β_i the linear coefficient; β_{ii} the squared coefficient; and β_{ij} the cross-product coefficient, k is number of factors.

The second order polynomial coefficients were calculated using the Design Expert Version 6.0.10 (Stat-Ease, Minneapolis, MN, USA) to estimate the responses of the dependent variable. The RSM statistical model was validated using numerical optimization for butyl-4-methyl-3-oxopentanoate production under the conditions predicted by the model. The R^2 statistic indicates the percentage of the variability of the optimization parameter that is explained by the model. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones.

Enzyme kinetics

An intricate kinetic analysis was carried out in order to elucidate the mechanism of this particular bi-substrate reaction. The effect of concentration of both the substrates on the rate of reaction was studied systematically over a wide range. For determination of initial rates, different sets of experiments were conducted using 3.89 g/L Novozym 435. Concentration of methyl-4-methyl-3-oxopentanoate (C) was varied from 0.0005 to 0.03 mol at different fixed quantities of *n*-butanol

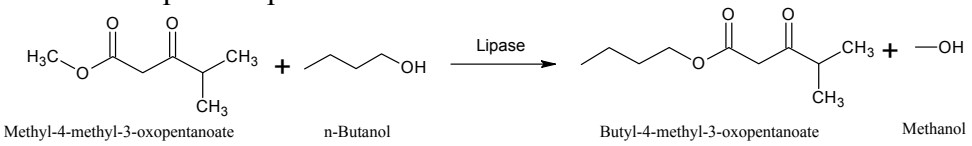
(0.005-0.04 mol). In another set, concentration of *n*-butanol (D) was varied from 0.005 to 0.04 mol at different fixed quantities of methyl-4-methyl-3-oxopentanoate (0.0005-0.03 mol). The initial rates were determined from the quantified data.

Table 1: Experimental domain and level distribution of the variables used for optimization

Independent variable	**Levels**				
	-2	**-1**	**0**	**+1**	**+2**
Enzyme loading, A (mg)	29.2	58.4	87.6	116.8	146
Temperature, B (°C)	40	45	50	55	60
Methyl-4-methyl-3-oxopentanoate, C (mol)	0.005	0.01	0.015	0.02	0.025
n-butanol, D (mol)	0.01	0.02	0.03	0.04	0.05

RESULTS AND DISCUSSIONS

Lipase catalyzed transesterification of methyl-4-methyl-3-oxopentanoate in toluene as solvent is represented by Scheme 1. RSM with CCRD was used for optimization of process parameters.



Scheme 1: Reaction scheme for synthesis of butyl-4-methyl-3-oxopentanoate.

RSM experiments and model fitting

The objective of the current study was to optimize the process parameters and study the kinetics of the transesterification of methyl-4-methyl-3-oxopentanoate to butyl-4-methyl-3-oxopentanoate with Novozym 435 as the enzymatic catalyst. RSM enabled to obtain sufficient information for statistically acceptable results using reduced number of experiments, and was found to be an efficient method to evaluate the effects of multiple parameters, alone or in combination, on response variables. Table 2 lists the actual and predicted conversions at each of the 30 experimental sets generated by the principles of RSM and the response ranged from as low as 33 to as high as 85 %. Second order polynomial equation was used to correlate the independent process variables with conversion. The second order

polynomial coefficient for each term of the equation was determined through multiple regression analysis using the Design Expert. The design of experiments and respective experimental yields are given in Table 2.

The data were analyzed by using analysis of variance (ANOVA) (Table 3). The Model F-value of 20.71 implies that the model is significant. There is only a 0.01% chance that a "Model F-Value" as large as this value could occur due to noise. Model F-value is calculated as the ratio of mean square regression and mean square residual. Model P-value (Prob > F) is very low (< 0.0001). This reconfirms the significance of the model.

The P values were used as a tool to verify the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.05 indicate model terms are significant. The coefficient estimates and the corresponding *P* values suggest that among the test variables used in the study, A, B, C, A^2, B^2, AB, BD and CD are significant model terms. Other interactions were found to be insignificant.

The corresponding second-order response model in terms of coded variables (Equation 3) was found after regression analysis as follows:

$$\text{Conversion (\%)} = 75 + 8.79A + 9.04B - 6.38C - 0.29D - 5.05A^2 - 4.18B^2 - 0.80C^2 - 1.30D^2 - 3.69AB + 0.56AC + 1.94AD + 0.063BC + 3.19BD + 4.94CD \quad (3)$$

Where: A= Enzyme loading, B= Temperature, C= Methyl-4-methyl-3-oxopentanoate, D= *n*-butanol in appropriate units as given earlier.

The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.95, indicating that 95 % of the variability in the response could be explained by the model. The "Pred R-Squared" of 0.7166 is in reasonable agreement with the "Adj R-Squared" of 0.9649. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 14.706 indicates an adequate signal. This model can be used to navigate the design space. Accordingly, three-dimensional plots were generated for the pair-wise combination of the four factors, while keeping the other two at their center point levels. The relevant plots are given here to highlight the roles played by various factors (Fig 2). Thus, the optimal conditions for the enzymatic reaction were obtained at 0.01 mol methyl-4-methyl-3-oxopentanoate and 0.03 mol n-butanol using 104 mg of Novozym 435 at 55 ^{o}C for 6 h. Under these conditions the transesterification conversion was 87 %. The plot of experimental conversion values vs. predicted values also showed almost linear distribution, which is

indicative of a good model (Figure 1). Thus, a response surface quadratic model was well fitted.

Table 2: Four variable composite experimental design and the corresponding response.

Expt #	Enzyme loading (mg)	Temperature (°C)	Methyl-4-methyl-3-oxopentanoate (mol)	*n*-butanol (mol)	Conversion (%)	
					Actual	Predicted
1	58.4	55	0.02	0.02	49 ± 1.2	55
2	116.8	45	0.01	0.02	77 ± 1.9	79
3	58.4	45	0.02	0.04	33 ± 0.9	35
4	146	50	0.015	0.03	75 ± 1.1	72
5	87.6	60	0.015	0.03	84 ± 1.4	76
6	87.6	50	0.015	0.03	75 ± 1.1	75
7	58.4	45	0.01	0.02	62 ± 1.2	60
8	58.4	55	0.02	0.04	64 ± 1.4	67
9	116.8	55	0.02	0.04	79 ± 1.2	82
10	29.2	50	0.015	0.03	40 ± 1.6	37
11	87.6	50	0.025	0.03	66 ± 0.8	59
12	116.8	45	0.01	0.04	72 ± 1.3	67
13	116.8	45	0.02	0.04	59 ± 1.4	65
14	87.6	50	0.015	0.05	75 ± 1.3	69
15	87.6	50	0.015	0.03	75 ± 1.1	75
16	58.4	45	0.02	0.02	36 ± 0.9	36
17	116.8	55	0.01	0.02	85 ± 1.5	84
18	116.8	55	0.01	0.04	78 ± 1.6	83
19	116.8	55	0.02	0.02	59 ± 1.0	62
20	58.4	55	0.01	0.04	68 ± 0.7	71
21	87.6	50	0.015	0.03	75 ± 1.1	75
22	58.4	45	0.01	0.04	37 ± 1.5	39
23	58.4	55	0.01	0.02	79 ± 1.2	78
24	87.6	50	0.015	0.01	70 ± 1.1	70
25	87.6	50	0.005	0.03	83 ± 1.3	85
26	87.6	50	0.015	0.03	75 ± 1.1	75
27	87.6	50	0.015	0.03	75 ± 1.1	75
28	87.6	40	0.015	0.03	38 ± 1.2	40
29	87.6	50	0.015	0.03	75 ± 1.1	75
30	116.8	45	0.02	0.02	60 ± 1.8	58

Validation of the model

The good quality of the fitted model was confirmed by ANOVA (Table 3). The validity of the model was established by comparing the results obtained at the two

check points with the predicted values (Table 4). These results seem to confirm the validity of the model and give optimized conversion response for the transesterification reaction using Novozym 435.

Table 3: ANOVA for conversion response surface model (quadratic)

Factor[a]	Sum of squares	Mean squares	DF[b]	F value	P[c]
Model	6668.783	14	476.3417	20.70551	< 0.0001*
A	1855.042	1	1855.042	80.63451	< 0.0001*
B	1962.042	1	1962.042	85.28556	< 0.0001*
C	975.375	1	975.375	42.39737	< 0.0001*
D	2.041667	1	2.041667	0.088747	0.7699†
A2	700.0744	1	700.0744	30.43067	< 0.0001*
B2	478.5744	1	478.5744	20.80256	0.0004*
C2	17.64583	1	17.64583	0.767025	0.3949†
D2	46.50298	1	46.50298	2.02138	0.1756†
AB	217.5625	1	217.5625	9.456955	0.0077†
AC	5.0625	1	5.0625	0.220056	0.6457†
AD	60.0625	1	60.0625	2.610782	0.1270†
BC	0.0625	1	0.0625	0.002717	0.9591†
BD	162.5625	1	162.5625	7.066228	0.0179†
CD	390.0625	1	390.0625	16.95514	0.0009*

[a] A = Enzyme loading, B= Temperature, C= Methyl-4-methyl-3-oxopentanoate, D= n-butanol
[b] Degree of freedom [c] † - not significant; * - significant $p < 0.05$, $R^2 = 0.9508$

Table 4: Validation of the model

A	B	C	D	Actual	Predicted
103.98	55.28	0.01	0.03	87 ± 1.5	88.3883
87.07	56.96	0.01	0.02	85 ± 1.3	85.612

Analysis of response surfaces

Response surfaces of the dependent variables were estimated for the response on the basis of the samples in the central composite. From these response surfaces, it is possible to study the sensitivity of different parameters of transesterification such as enzyme loading, temperature, methyl-4-methyl-3-oxopentanoate concentration, and *n*-butanol concentration. Prediction plots of effects of various parameters on conversion of the substrate were obtained (data not shown). The conversion was found to increase with an increase in enzyme loading. Within the given range of temperature of 45–55 °C, conversion increased almost linearly with increase in temperature. There was an exponential increase in the conversion when the temperature was raised from 50 to 55 °C. Prediction of the effect of methyl-4-methyl-3-oxopentanoate concentration on conversion showed that there was a decrease in the conversion with an increase in quantity of methyl-4-methyl-3-oxopentanoate from 0.01 to 0.02 mol. It could be attributed to the formation of a dead-end complex between enzyme and excesses of methyl-4-methyl-3-oxopentanoate (data not shown). These results were further confirmed by the three dimensional response surface plots. There was a small increase in the conversion when *n*-butanol concentration was increased from 0.02 to 0.04 mol.

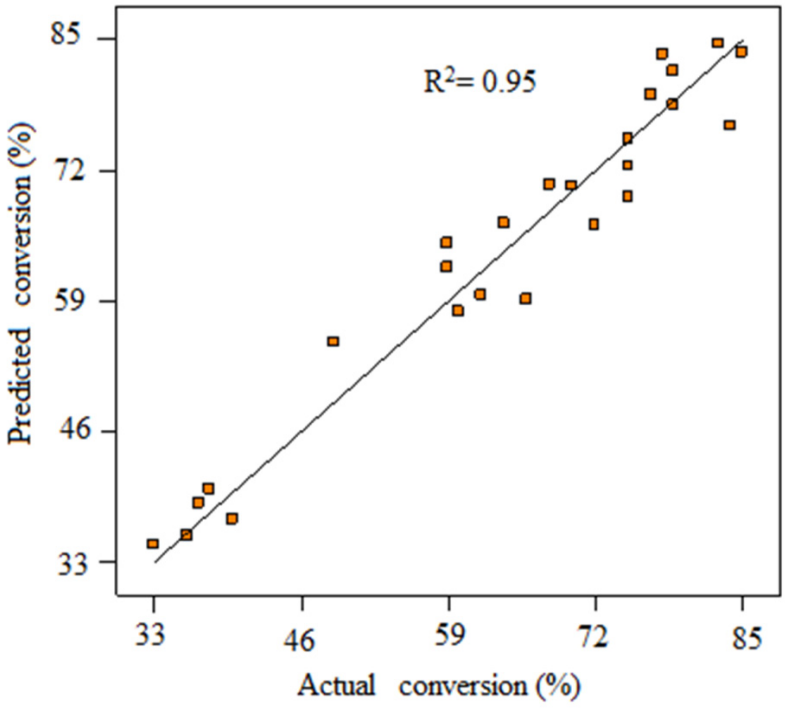


Figure 1. Parity plot

Figure 2 shows the 3-D response surface plots of conversion against other two variables. Figure 2a shows the response surface plot of conversion against concentration of methyl-4-methyl-3-oxopentanoate and temperature. The conversion was found to increase with increase in temperature from 40 °C to 55 °C for all concentrations of methyl-4-methyl-3-oxopentanoate. Decrease in conversion was observed at 60 °C which could be attributed to thermal inactivation of enzyme at this temperature. This was also observed in 3-D three-dimensional response plot between *n*-butanol concentration and temperature for conversion (Figure 2b). Many researchers have reported stability of Novozym 435 in the range of 30 to 55 °C while there was a decrease in conversion beyond 60 °C due to the thermal degradation of enzyme (Duan et al., 2010 and Yadav and Lathi, 2006; Yadav and Borkar, 2006; Yadav et al., 2007). The conversion decreased with an increase in quantity of methyl-4-methyl-3-oxopentanoate from 0.005 to 0.025 mol. It could be attributed to the formation of a dead-end complex between the enzyme and excess of methyl-4-methyl-3-oxopentanoate. The best results were obtained in the range of 50 to 55 °C and at lower methyl-4-methyl-3-oxopentanoate concentration (Figure 2a). In general, the increase of methyl-4-methyl-3-oxopentanoate concentration lowered the transesterification capacity of the enzyme. Increase in concentration of *n*-butanol from 0.01 to 0.05 mol increased the conversion (Figure 2b). Enzyme loading is known to be an important variable for esterification reactions for the synthesis of various esters. Figure 2c shows the interactive plot between temperature and enzyme loading. A positive effect of the enzyme concentration on the rate of reaction of butyl-4-methyl-3-oxopentanoate was observed. The conversion was found to increase when enzyme loading was increased from 29.2 to 142 mg. However, at higher enzyme loading from 85.6 to 142 mg, the conversion increased only marginally which might be due to the fact that more active sites were available for reaction thereby bringing into onset of diffusion resistance.

Enzyme kinetics

Figure 3 shows the effect of the concentration of methyl-4-methyl-3-oxopentanoate on the initial reaction rate when the initial *n*-butanol concentration was fixed. It shows that for a given *n*-butanol concentration, the initial rate increases when the methyl-4-methyl-3-oxopentanoate concentration is increased until it reaches a maximum. A subsequent increase in methyl-4-methyl-3-oxopentanoate concentration leads to a decrease in the initial rate. The influence of the concentration of *n*-butanol on the initial rate at fixed initial methyl-4-methyl-3-oxopentanoate concentration is shown in Figure 4. It indicates that for a given methyl-4-methyl-3-oxopentanoate concentration, the initial rate increases as the *n*-butanol concentration increases. Figure 3 suggests that the Novozyme 435

catalyzed transesterification is inhibited by methyl-4-methyl-3-oxopentanoate. There was no evidence of inhibition by *n*-butanol at all the concentrations studied. The Lineweaver–Burk double inversion plots (1/*V* *versus* 1/[D]) show that the lines are not parallel, ruling out the possibility of a ping-pong bi-bi mechanism. In fact, the lines intersected at a point suggesting a ternary complex mechanism

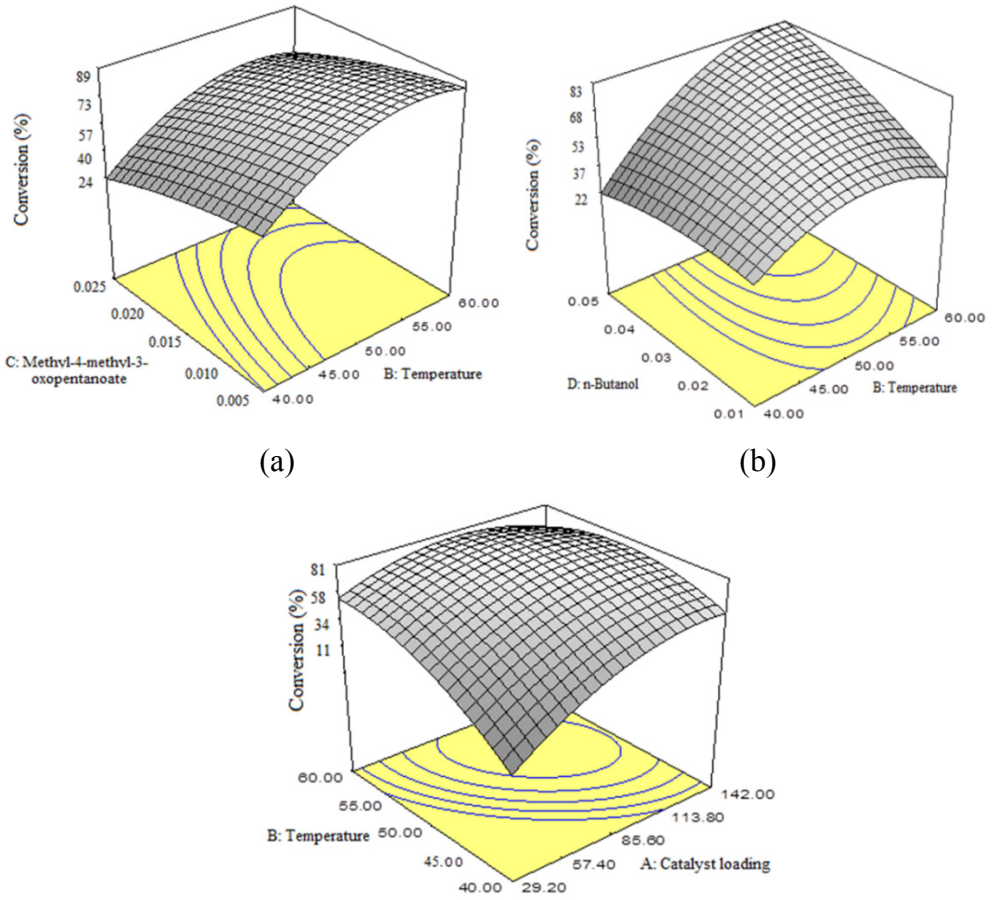
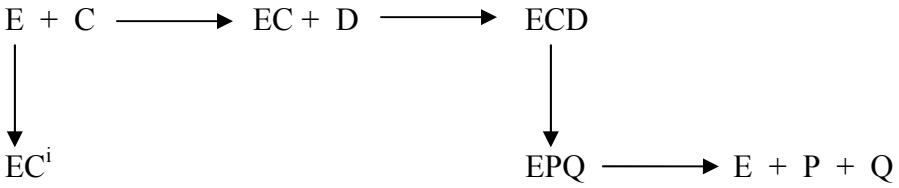


Figure 2. Three-dimensional plot between any two parameters for the conversion of reaction. Conditions: A 87.6 mg, D 0.03 mol for (a); A 87.6 mg, C 0.015 mol for (b); C 0.015 mol, D 0.03 mol for (c).

(Figures 3 and 4). The lipase-catalyzed reaction mechanism involves the formation of an acyl enzyme complex with the acyl donor, which is methyl-4-methyl-3-oxopentanoate. This explanation rules out a random mechanism and it can only be an ordered bi-bi mechanism. According to this, the lipase (E) will react with acyl donor (C) to form a complex (EC). The second substrate (D) then

reacts to form a ternary complex (ECD). At higher concentrations of methyl-4-methyl-3-oxopentanoate (C^i) a dead-end complex (EC^i) is formed. A typical reaction sequence is shown in Scheme 2.



Scheme 2: A typical reaction sequence for ternary complex ordered bi-bi mechanism

The appropriate equation obtained for this mechanism (Segel, 1975) is:

$$\frac{V}{V_{\max}} = \frac{[C][D]}{K_{i(C)}K_{m(D)} + K_{m(C)}[D] + K_{m(D)}[C] + [C][D]} \tag{4}$$

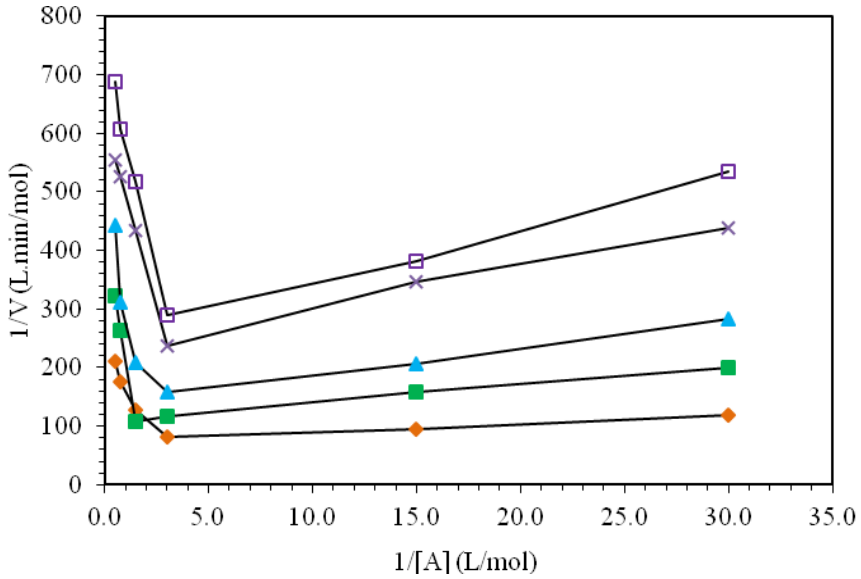


Figure 3. Lineweaver-Burk plot of 1/initial rate (L.min/mol) *versus* 1/[methyl-4-methyl-3-oxopentanoate] (L/mol) at constant *n*-butanol concentration; (◆ 0.04 mol; ■ 0.03 mol; ▲ 0.02 mol; × 0.01 mol; □ 0.005mol).

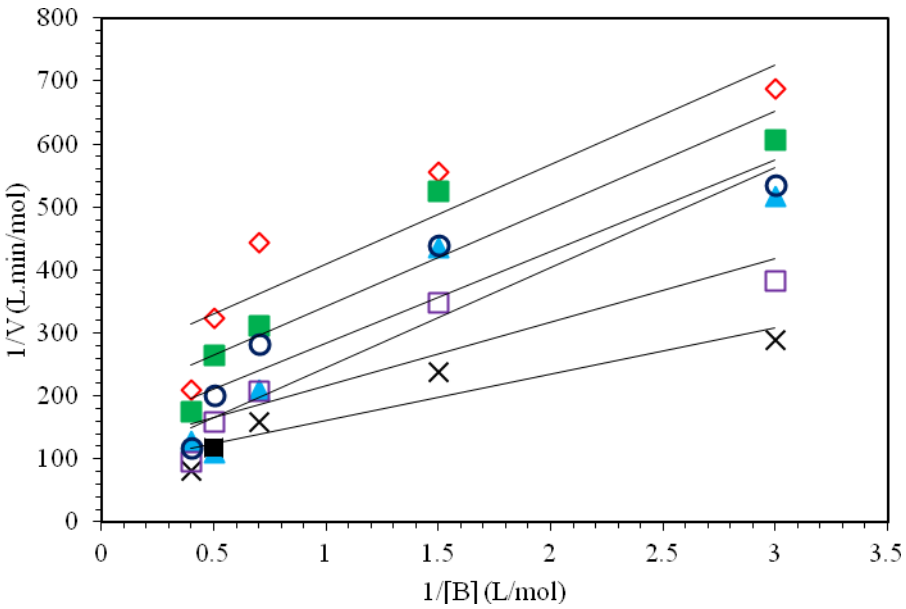


Figure 4. Lineweaver-Burk plot of 1/initial rate (L.min/mol) *versus* 1/[*n*-butanol] (L/mol) at constant methyl-4-methyl-3-oxopentanoate concentration; (◇ 0.03 mol; ■ 0.02 mol; ▲ 0.01 mol; × 0.005 mol; □ 0.001mol; ○ 0.0005 mol).

where V = initial rate of reaction (mol/L.min), V_{max} = maximum rate of reaction (mol/L.min), [C] = initial concentration of methyl-4-methyl-3-oxopentanoate (mol/L), [D] = initial concentration of *n*-butanol (mol/L), $K_{m(C)}$ = Michaelis constant for methyl-4-methyl-3-oxopentanoate (mol/L), $K_{m(D)}$ = Michaelis constant for *n*-butanol (mol/L), and $K_{i(C)}$ = inhibition constant of methyl-4-methyl-3-oxopentanoate (mol/L).

Table 5: Kinetic parameters for ternary complex ordered bi-bi model obtained for transesterification of methyl-4-methyl-3-oxopentanoate with *n*-butanol

Sr. No.	Kinetic Constants	Ternary Complex Ordered Bi-Bi Model Values
1	V_{max} (mol/lit.min)	0.04
2	$K_{m(A)}$ (mol/lit)	0.11
3	$K_{m(B)}$ (mol/lit)	2
4	$K_{i(A)}$ (mol/lit)	2.2

The data from initial rate measurements were used for the optimization of parameters by the least square error estimation using the software Polymath

(Willimantic, CT, USA). A plot of simulated *versus* experimental rate showed that the experimental model fit the data very well (Figure 5). The values for kinetic parameters obtained from nonlinear regression analysis are given in Table 5.

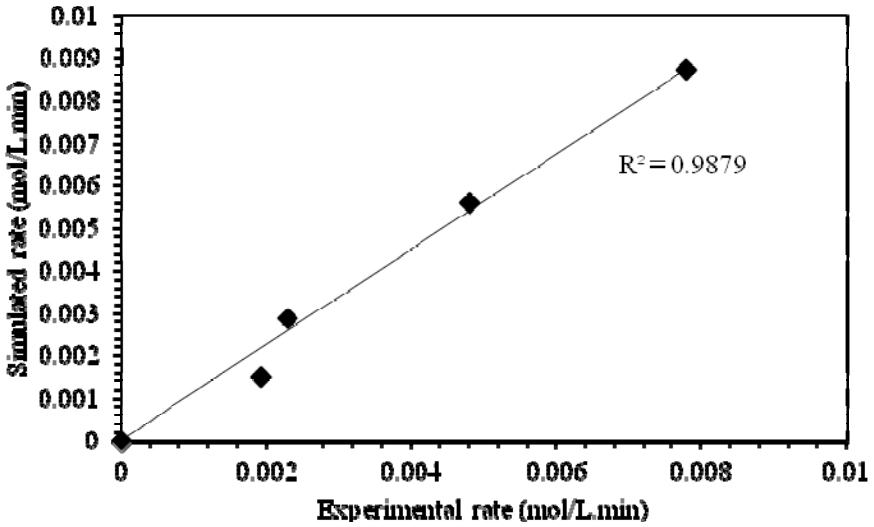


Figure 5. Experimental rate vs. simulated rate

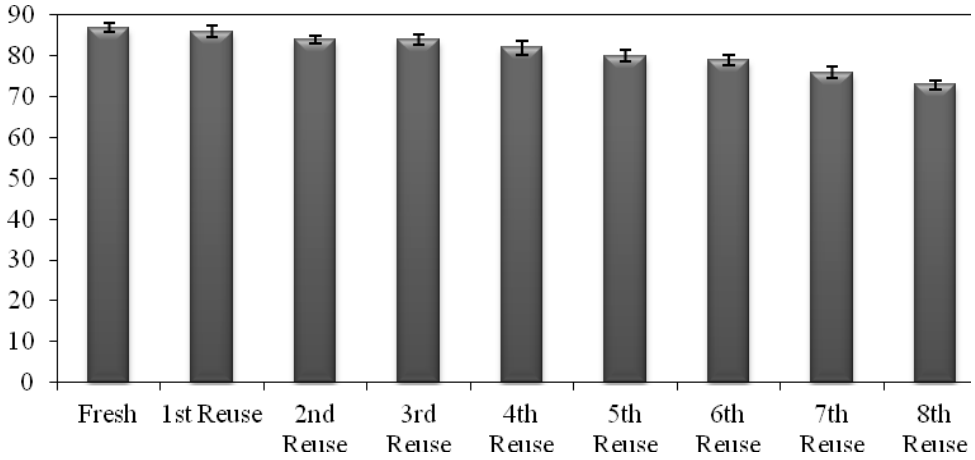


Figure 6. Biocatalyst reusability study Reaction conditions: Speed of agitation - 300 rpm, Temperature - 55 °C, Enzyme load – 104 mg , Methyl-4-methyl-3-oxopentanoate- 0.01 mol, n-Butanol- 0.03 mol, Reaction time- 6 h, Toluene to make volume up to 15 mL.

Operational stability of enzyme

Operational stability of enzyme was conducted under the optimum reaction conditions obtained from the RSM. Hence, experiments for operational stability of enzyme were conducted at 0.01 mol of methyl-4-methyl-3-oxopentanoate and 0.03 mol of *n*-butanol using 104 mg of Novozym 435 at 55 ◦C and 300 rpm for 6 h. After each run, the biocatalyst was allowed to settle and the supernatant solution was removed. Then, toluene was added to the solid particles, and the mixture was shaken to wash away the remaining substrate and product species. The washing was carried out three times. Then enzyme was filtered, air dried and weight loss during process was adjusted by making up the weight to the original enzyme loading and then enzyme used during the next run. To investigate the effect of the substrate on the stability of the enzyme, the enzyme reuse study was carried out under otherwise similar conditions. It was found that there was a marginal decrease in conversion from 87 % to 73 % after eighth reuse (Figure 6).

CONCLUSION

In the present study, the process for synthesis of butyl-4-methyl-3-oxopentanoate using immobilized lipase Novozym 435 has been optimized applying the response surface methodology (RSM). Second order polynomial equation has been obtained for the conversion of the substrate ester. From this equation, it is possible to predict the operational conditions required to obtain optimum amount of enzyme loading, temperature, methyl-4-methyl-3-oxopentanoate concentration and *n*-butanol concentration for transesterification reaction with minimal number of experiments. Moreover, RSM was fairly accurate in predictive modelling and process parameter optimization, which suggests that the relation between the process parameters and conversion can be reasonably approximated by a quadratic non-linearity. Further, initial rate data and progress curve data were used to arrive at a suitable model. The apparent fit of the kinetic data to the assumed ternary complex ordered bi-bi mechanism with methyl-4-methyl-3-oxopentanoate substrate inhibition provided support for the mechanism. This model was used to simulate the rate data, which were in excellent agreement with the experimental values. The enzyme is reusable.

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