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Response Surface Methodological Study of Glucose Laurate Synthesis Catalyzed by Immobilized Lipase from *Candida cylindracea*

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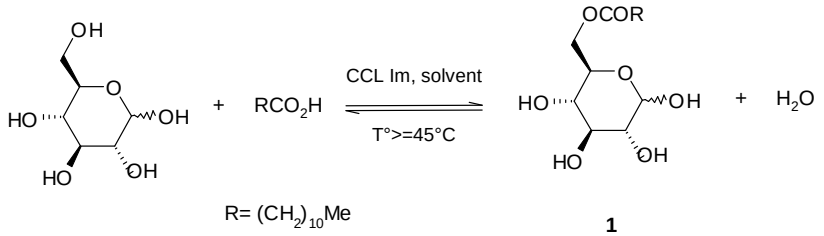
ABSTRACT: The purpose of the present work is to study the reaction conditions affecting the lipase-catalyzed synthesis of fatty acid glucose ester with immobilized *Candida cylindracea* lipase, using response surface methodology. According to the Box-Behnken design principles, response surface methodology with three factors such as temperature, solvent polarity and reaction time at three levels was adopted. The factors influencing the parameters were determined by means of regression analysis. The optimum values for the highest conversion yield of C=76% was obtained at a temperature of 80°C in ethyl methyl ketone as a solvent and at a reaction time of 72h. It was shown that the obtained model can predict conversion of the starting material and the obtained results were in good agreement to measured data.

Keywords: Lipase from *Candida cylindracea*, sugar, optimization, response surface methodology.

INTRODUCTION

Fatty acid sugar esters are nonionic surfactants with high emulsifying, stabilizing, detergency and other useful effects (Favrelle *et al*., 2010; Van Kempen *et al*., 2013). These sugar-based compounds are widely used as emulsifiers in food, detergents, cosmetics and pharmaceutical industries (Piccicuto *et al*., 2001). In recent years, lipase-catalyzed synthesis of sugar fatty acid esters has emerged as an interesting synthetic route (Uswatun *et al*., 2012; Sarney *et al*. 1995), due to the general properties and advantages of the biotechnological over the chemical synthesis (Villeneuve, 2007; Loughlin, 2000). The use of enzymes is a green alternative to organic synthesis and has been widely investigated (Boland *et al*., 1991; Faber 1995; Persson *et al*. 2002). Bio-inspired processes or enzymatic reactions have a low environmental impact, reduce the amount of waste material and can minimize costs, thus serving the requirements to integrate environmental sustainability with economic growth and welfare. The main advantage of the enzymatic synthesis is that its high regioselectivity mainly leads to the production of monoesters (Khaled *et al*.,1992). An important way to improve the performance of enzymes in non-natural environments (Klibanov, 1989) is to immobilize them by adsorption on a solid support. This offers advantages over free enzymes, because of the possibility of rapid termination reactions and enhancement of the catalytic activity of the lipase. In addition, a higher thermostability of the enzyme would be required for long-term process in many applications.

In this paper, optimization of lipase-catalyzed esterification of D-Glucose is reported. The obtained 1-*O*-Dodecanoyl-D-Glucopyranose 1 is a nonionic surfactant with good emulsifying properties (Klai *et al*., 2015; Serradj *et al*. 2008). Conversions obtained for this synthesis with the free lipase are not high enough. So, find a more convenient method to synthesize is considerable. We obtained **1** from D-glucose by treatment with lauric acid and immobilized *Candida cylindracea* lipase (CCL Im) synthesized at the laboratory but the conversion just reach to 57 % (Scheme-I). The goal of the present work is to optimize the conversion of the starting glucose to obtain **1** via a Box-Behnken design (BBD) and response surface methodology (RSM).



Scheme-I.

Response surface methodological studies were conducted in order to optimize and understand the relationship between the important reaction parameters. In our previous work, a screening design was used to identify which factors had significant effects on ester production (Bouzaouit and Bidjou-Haiour, 2015). Subsequently, a Box-Benkhen design (Box *et al.*, 1960; Box *et al*., 1951) is employed to optimize factors, which have significant influence on sugar ester production. The results are analyzed by response surface methodology. RSM has been reported to be an effective tool for optimizing a process. It involves statistical design of experiments in which all factors are varied together over a set of experimental runs (Myers *et al*., 2009, Montgomery *et al*., 2002). The main objective of RSM is to settle the optimum operational conditions of the system or to determine a region that satisfies the operating specifications (Bas *et al*., 2007). It has been employed for optimization of lipase-catalyzed synthesis of various enzymatic reactions (Guvenc *et al*., 2007 , Martins *et al*., 2011). This method is particularly suitable for the lipase-assisted synthesis of sugar esters (Zhang *et al*., 2015 , Galonde *et al*., 2013).

MATERIALS AND METHODS

Candida cylindracea lipase (Type II) was purchased from Sigma Co. (USA). D-glucose from Sigma-Aldrich and lauric acid from Merck were tested as starting materials. All solvents (Merck) were of analytical grade and were distilled once before use. Molecular sieves 3Å (4-8 mesh) was used as a water removal adsorbent (Aldrich, USA). All other chemicals used in this work were of analytical grade and used without further purification.

A. Lipase immobilization

Celite (60 mg) was added to 10 mL of 0, 1 M phosphate buffer (pH = 8) containing the *Candida cylindracea* lipase (100 U/mL). The reaction was then stirred with a magnetic bar at 4 ºC and 100 rpm for 30 mn. 20 mL of cold acetone were then added. After 2 h, the suspension was filtered. The immobilized enzyme (CCL Im) was washed with acetone, dried in a vacuum desiccator and then stored at -18°C.

B. Synthesis of glucose ester by esterification

D-glucose (180 mg, 1mmol) was first dissolved in the solvent for one night. After that, lauric acid (200 mg, 1mmol) was added, the mixture equilibrated for 15 min, the biocatalyst (CCL Im) and molecular sieves finally incorporated. Aliquots were removed at intervals, filtered and analyzed qualitatively by thin layer chromatography and quantitatively by volumetric titration.

C. Analysis

Samples were withdrawn at definite time intervals and the extent of esterification monitored by a titration procedure which estimated the decrease in total acid content of the reaction mixture (Leitgeb *et al*., 1990).

D. Experimental design and statistics

The parameters selected for the experiment were reaction temperature (X_1), reaction time (X_2) and solvent polarity (X_3). The sugar conversion (Y) was used as the response. Before applying the response surface methodology (RSM) to determine the conditions which will give the best conversion for the synthesis of the compound 1, Box-Behnken experimental design was conceived and applied. In the present study a 3-factor, 3-level (high, medium and low) design involving 15 experiments from three important variables (X_1, X_2, X_3) is suitable for exploring quadratic response surfaces and constructing second order polynomial equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_1X_2 + a_5X_1X_3 + a_6X_2X_3 + a_7X_1^2 + a_8X_2^2 + a_9X_3^2$$

where Y = sugar conversion in %, X_1= reaction temperature, in °C, X_2= time, in hours, X_3=solvent polarity, a_o= constant, a_1, a_2, a_3= linear coefficients, a_4, a_5, a_6= quadratic coefficients, and a_7, a_8, a_9= cross product coefficients.

This design was considered to analyze the linear, cross-product and quadratic effects to arrive at a predictive equation for the conversion of the starting sugar. The variables and their respective levels are presented in Table 1.

Minitab 14 software was used to analyze coefficients and their statistical significance and to arrive at the final predictive equation.

RESULTS AND DISCUSSION

Response data for all 15 experimental runs of Box-Behnken experimental design were performed. All trials were performed in triplicate. The actual variables employed and their corresponding coded levels are given in Table 2.

Table 1: Actual and coded values employed for the analysis.

Coded level	Low (-1)	Medium (0)	High (+1)
Solvent (X_1, logP)	THF	*t*-BuOH	EMK
Temperature (X_2, °C)	40	60	80
Time (X_3, h)	24	48	72

Table 2 : Results of Box-Behnken experimental design. Comparison of predicted and experimental conversion in glucose ester synthesis.

Coded values			Actual values			Response	
			X_1	X_2	X_3	Ester conversion, Experimental (%)	Ester conversion, Predicted (%)
1	0	-1	EMK	60	24	10,75	12,37
-1	0	1	THF	60	72	50,00	48,37
0	1	1	*t*-BuOH	80	72	57,74	58,73
0	0	0	*t*-BuOH	60	48	10,00	09,12
-1	1	0	THF	80	48	37,14	37,77
0	1	-1	*t*-BuOH	80	24	01,40	01,47
0	0	0	*t*-BuOH	60	48	08,69	09,12
1	0	1	EMK	60	72	45,16	45,87
-1	-1	0	THF	40	48	23,47	25,17
0	0	0	*t*-BuOH	60	48	08,69	09,12
0	-1	1	*t*-BuOH	40	72	19,13	19,05
1	1	0	EMK	80	48	43,66	41,95
-1	0	-1	THF	60	24	12,22	11,50
1	-1	0	EMK	40	48	20,00	19,36
0	-1	-1	*t*-BuOH	40	24	06,95	05,95

A statistical analysis of data in the table above has allowed us to estimate the coefficients of the mathematical model. From these results, a regression procedure from Minitab 14 was employed to fit the second order polynomial equation. The significance of the coefficients of the full model equation (1) was tested using regression analysis. The linear term corresponding to X_1 and the cross-product term corresponding to X_1 with X_3 were found to be insignificant and were eliminated according to their *p*-value in order to refine the model ($\alpha = 0.05$). The final response equation obtained is as follows:

$$C(\%) = 9{,}126 + 8{,}798X_2 + 17{,}588X_3 + 15{,}084X_1^2 + 6{,}856X_2^2 + 5{,}321X_3^2 + 2{,}497X_1 \bullet X_2 + 11{,}040X_2 \bullet X_3 \quad (1)$$

The corresponding analysis of variance (ANOVA) is given in Table 3.

Table 3: Statistical significance of the response equation.

Regression statistics			
Term	**Coefficient**	***t*-statistic**	***p*-value**
Constant	9,1267	8,907	0,000
X_1	-0,4075	-0,649	0,540
X_2	8,7988	14,023	0,000
X_3	17,5888	28,032	0,000
$X_1 \bullet X_1$	15,0842	16,332	0,000
$X_2 \bullet X_2$	6,8567	7,424	0,000
$X_3 \bullet X_3$	5,3217	5,762	0,001
$X_1 \bullet X_2$	2,4975	2,815	0,031
$X_2 \bullet X_3$	11,0400	12,442	0,000
R square	0,9960		
Adjusted R square	0,9900		
Standard error			

Analysis of variance (ANOVA)

Source	**Degrees of freedom**	**Sum of squares**	**Mean square**	**F-value**	***p*-value**
Regression	8	4621,33	577,67	183,41	0,000
Linear	3	3095,59	1031,86	327,62	0,000
Square	3	1013,27	337,76	107,24	0,000
Interaction	2	512,48	256,24	81,36	0,000
Residual error	6	18,90	3,15	-	-
Lack-of-fit	4	17,75	4,44	7,76	0,117
Pure error	2	1,14	0,57	-	-
Total	14	4640,23	-	-	-

The F value is a measure of the variation of the data about the mean. Generally, the calculated F value should be several times greater than the tabulated F value if the model is a good prediction of the experimental results and the estimated factor effects are real. In this case, the ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the calculated F value (183,41) and a very low probability value p (0,0000) which indicates that the second-order polynomial is highly significant and therefore the significance of the model at 95% probability level. The R^2 of 0.996 is concordant and confirms that 99.6% of variation of the sugar conversion can be explained by the fitted model. The model can be considered as a good model for the prediction of the conversion with the factors used and within the ranges tested.

Fig. 1, showing the plot of experimental values compared to predicted values, demonstrates a good concordance between these values. In addition, Fig. 2 representing the normal probability of residuals, shows a good correlation between the experimental data distribution and the linear regression model. Consequently analysis of variance can be validate from Fig. 1 and 2 prove that the model represents well and accurately the influence of the selected factors on glucose conversion.

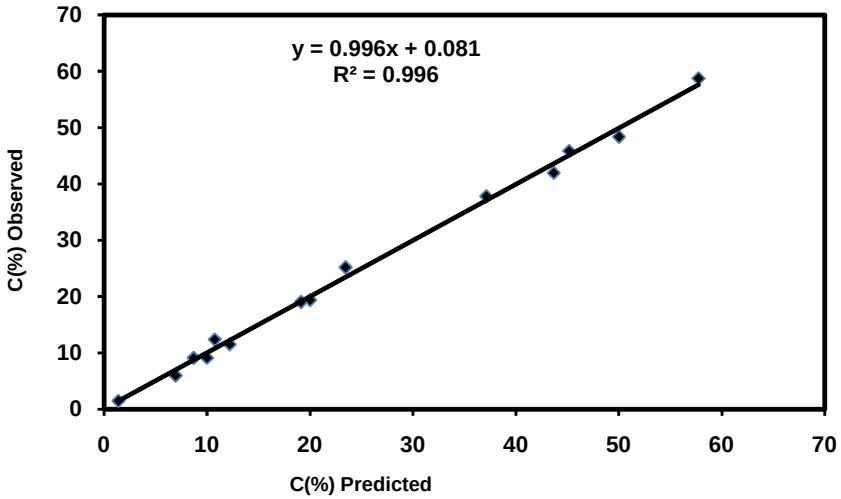


Fig. 1. Plot of experimental values versus predicted values of glucosyl laurate synthesis.

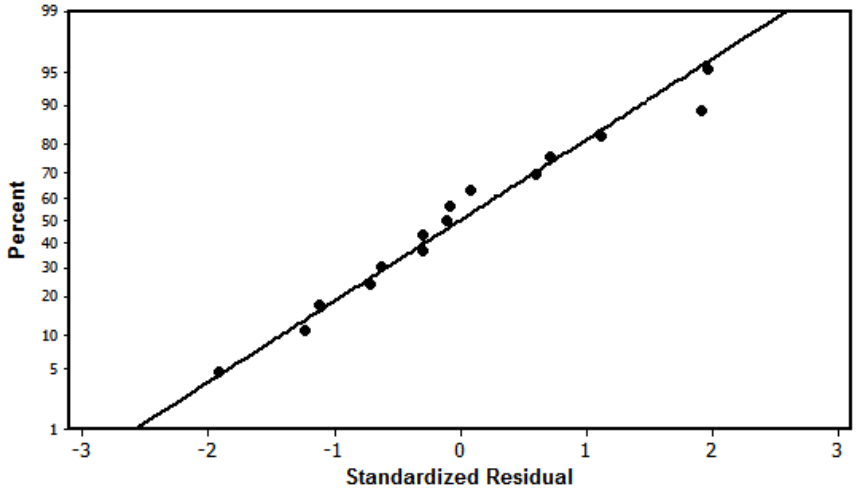


Fig. 2. Normal probability plot of the residuals.

These results show a satisfactory representation of the relationship between the experimental and predicted values. Those calculated from the model equation, indicate a good fit, as observed in Fig 1. The high value of R^2 (R^2 =0,99) indicate a good correlation between the measured and theoretical values predicted by the model.

The mutual effect of the solvent polarity (X_1), the temperature (X_2) and reaction time (X_3) on the conversion was studied by the contour plots generated from the predicted model using MINITAB software 14 by the combination of the three considered factors. The response surface plots are shown in Fig. 1, 2 and 3, which illustrate the relationship between response and the experimental data. According to the interpretation of the contour diagrams, the highest values of the conversion (>70%) are obtained when the three factors are fixed at high levels.

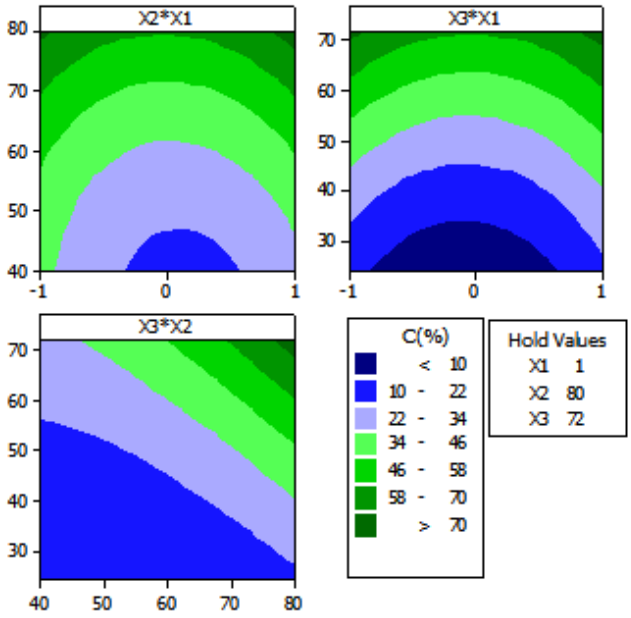


Fig. 3. Contour plots showing the crossed effect of parameters on the predicted conversion (%) of the glucose laurate synthesis (X_1, X_2, X_3 are constant at high levels).

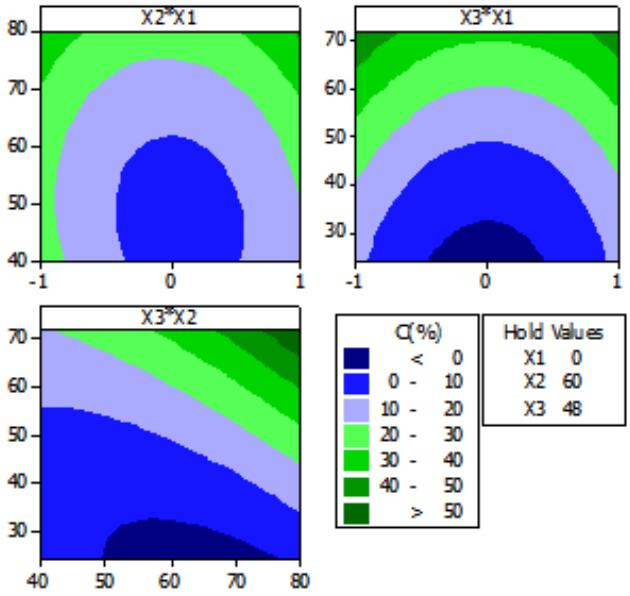


Fig. 4. Contour plots showing the crossed effect of parameters on the predicted conversion (%) of the glucose laurate synthesis (X_1, X_2, X_3 are constant at medium levels).

Consideration of the set of graphs allowed the selection of the optimal point : X_1 = 80°C, X_2 = 72 h, X_3 =EMC; X_1, X_2 and X_3 are the coded levels of hydrophobic solvents, temperature, and reaction time respectively. The conversion value at this point is C = 76 %. As can be seen from the various curves, maximum conversion is obtained when the three factors are maintained at their highest levels. The increase of the conversion with temperature can be explained by an improvement of the enzymatic activity due to a better diffusion process and by a higher sugar solubility in EMK at this temperature (Yan *et al*., 2001). Similar positive effect of the temperature was also observed for the immobilized *Candida antartica* lipase B (Yoshida *et al*., 2006). It has been shown that the lipase from *Candida cylindracea* has a surface with a very high hydrophobicity (Sugimura *et al*., 2000) and hydrophobic interactions are likely to have an important role in the adsorption process. Lipases exist in two structural forms, one closed, where a lid consisting of a polypeptide chain isolates the active site of the reaction medium, and the open form, where the lid moves and the active center is exposed (Derewenda *et al*., 1992). This equilibrium is shifted to the open form in the presence of hydrophobic surfaces like porous celite support (Accurel EP-100), wherein the lipase is absorbed on the hydrophobic material. This permits us to hypothesize that lipase immobilized following this kind of mechanism might be quite specific towards sugars.

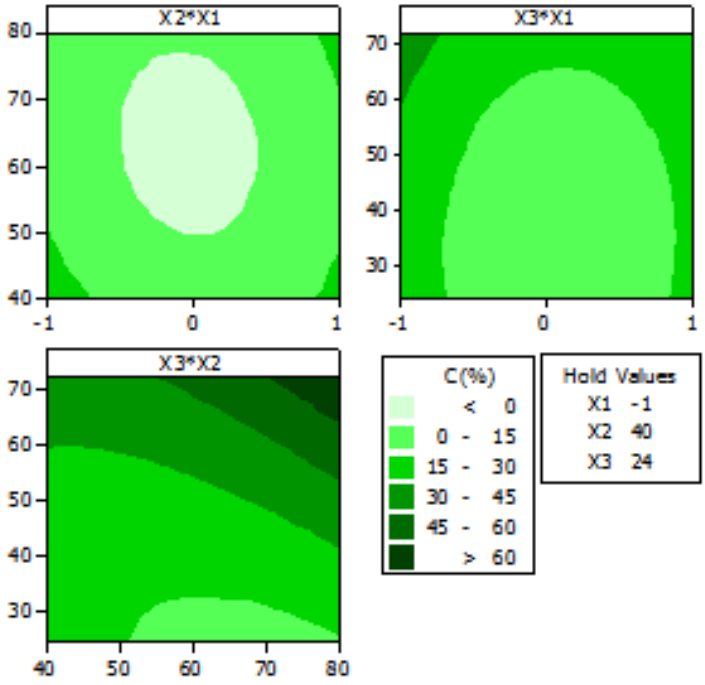


Fig. 5. Contour plots showing the crossed effect of parameters on the predicted conversion (%) of the glucose laurate synthesis (X_1, X_2, X_3 are constant at low levels).

Verification of the predicted values was conducted by using optimal conditions in experiments. The practical corresponding response was 75,35%. This result corroborated the validity and the effectiveness of this model.

CONCLUSION

The modeling and optimization of immobilized *Candida cylindracea* lipase catalyzed synthesis of glucose ester was successfully performed using a response surface methodology based on a Box-Behnken design. Predictability of the results for the above-mentioned reaction was found to be very good. Under the optimal conditions, the conversion was 76% in EMK at 80°C and 72h. The determination coefficient (R^2) was 0.923, which ensure an adequate credibility of the model. The effectiveness of the bio-catalytic esterification of D-Glucose with immobilized CCL was found to depend strongly on the reaction temperature and the solubility of the substrate.

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