Available online at www.derpharmachemica.com

Scholars Research Library

Der Pharma Chemica, 2015, 7(1):261-269
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

Optimization of lipase catalyzed synthesis of fatty acid xylose ester using statistical experimental designs

Nadia Bouzaouit and Chahra Bidjou-Haiour

LOMOP. Department of Chemistry, Faculty of Sciences, Badji-Mokhtar University, El-Hadjar, Annaba, Algeria

---

ABSTRACT

The present work is devoted to study the effect of various factors affecting the enzymatic synthesis of fatty acid xylose ester using the methodology of experimental designs. To optimize the different parameters that have a direct influence on the production of lauric xylose ester, experiments were conducted using first a design of Plackett-Burman to initially screen five of which the three factors were found to have significant effect on xylose ester production. Response surface methodology under Box-Behnken design was then employed to analyze the effects of important parameters on the preparation of fatty acid xylose ester. Experimental ester conversions were found to be in good agreement with predictions. The search for optimal conditions, verified by analysis of contour plots, helped to locate the optimal value of the conversion, C = 85 %. This corresponds to the following values of the factors : temperature 60°C, time 72 hours, and 30 mg of molecular sieves.

Keywords: xylose, lipase, Plackett-Burman design, Box-Behnken design, Response surface methodology

---

INTRODUCTION

Naturally occurring sugar esters [1] are of particular interest in the field of biocompatible surfactants. Because of their properties of amphiphilic nature, nontoxic, bio-degradability, among others, they are often used in the pharmaceutical, cosmetic and food industry [2,3]. Sugar esters can be synthesized using either chemical or biological catalysts. As a result of regioselectivity and mild reaction condition of enzymatic process [4], there is growing interest in the application of lipases as biocatalyst for carbohydrate fatty acid esters production [5, 6]. Numerous reports have shown that lipases are good biocatalyst in non aqueous media [7, 8]. In our previous study, the enzymatic synthesis of 1-*O*-dodecanoyl-D-xylopyranose by esterification of D-xylose with lauric acid using immobilized lipases was carried out in organic solvents [9]. In the present study, the esterification of D-xylose was carried out using immobilized *Candida cylindracea* lipase as a catalyst and optimized with response surface methodology. The study is aimed at a better understanding of the relationships between the important reaction parameters (sugar/fatty acid ratio, amount of enzyme, amount of molecular sieves, temperature and reaction time) and sugar conversion.

The study of enzymatic sugar ester synthesis is so complicated because it depends on several factors [10] and important aspects of this reaction remain to be clarified. The use of experimental design has become more common in several sciences such as bioprocess, environmental chemistry etc...[11]. A well defined statistical experimental design is considered to be necessary for optimization of such process, since it would be possible to get more information through conducting fewer measurements during the process.

The Plackett-Burman design (PBD) has been frequently used for screening process variables that make the greatest impact on a process [12]. It is a set of small and efficient experimental design, which is very powerful, widely

applicable and especially well suited for biotechnology research and development [13]. Recent reports on the use of PBD include its application toward improving lipase catalyzed esterification [14].

To carry out this study, a first screening design of Plackett-Burmann was used to identify which factors have significant effects on xylose ester production. Such factors are then studied by Box-Benkhen design [15]. The results were analyzed by response surface methodology (RSM). RSM has been reported to be an effective tool for optimizing a process [16]. It is defined as the statistical tool that uses quantitative data from appropriate experimental design to determine and simultaneously solve multivariate equations. The graphical representation of these equations are called as response surfaces, could be used to describe the individual and cumulative effect of the test variables on the response and to determine the mutual interaction between the test variables and their subsequent effect on the response [17]. Recently, optimization of lipase-catalyzed production of various esters by RSM has been investigated like synthesis of emulsifier in food industry such as lauroyl lactic acid [18] and synthesis of antioxidant such as octyl hydroxyphenyl propionate [19]. Although the enzymatic synthesis of highly biodegradable surfactants from renewable resources (sugar and fatty acids) has been widely investigated [20,21], statistical design of experiments and RSM have been only applied in a few studies [22,23].

The aim of this investigation is to optimize the synthesis of xylose laurate production by immobilized *Candida cylindracea* lipase (CCL Im) as well as to study the application of RSM to assess the relative importance of process variables.

MATERIALS AND METHODS

Candida cylindracea lipase (Type II) was purchased from Sigma Co. (USA). D-xylose from Sigma-Aldrich and lauric acid from Merck were tested as starting materials. Ethanol (EtOH), tetrahydrofurane (THF) were from Merck. 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.

General procedure for xylose ester synthesis

D-xylose (200 mg, 1,33 mmol) was first dissolved in THF for one night. After that, lauric acid (from 1,33 to 1,99 mmol) was added, the mixture equilibrated for 15 min and the biocatalyst (CCL Im) finally incorporated. Aliquots were removed at intervals, filtered and analyzed qualitatively by thin layer chromatography and quantitatively by volumetric titration.

Analysis

The sugar content was quantified by calculating the residual fatty acid amount in the reaction mixture. Samples were analyzed by volumetric method : 0.1 g of sample of reaction mixture was diluted in 20 mL of 0.1 wt % phenolphthalein solution in absolute ethanol and then titrated with standardized sodium hydroxide solution of 0.1 M in water. Samples were withdrawn at definite time intervals and the extent of esterification monitored by a back titration procedure which estimated the decrease in total acid content of the reaction mixture.

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 was washed with acetone, dried in a vacuum desiccator and then stored at -18 ºC.

Experimental designs and data analysis

Before applying the response surface methodology (RSM) to determine the conditions which will give the best conversion for xylose laurate synthesis, two experimental designs were conceived and applied : a preliminary experiment based on Plackett-Burman Design (PBD) followed by a Box-Behnken Design (BBD). The first design's objective was to highlight the most influential parameters, which were studied in more depth with the second experimental design.

The Plackett-Burman statistical experimental design is a reliable method to short-list the most significant parameters from a wide range or understand the extent of esterification possible under a given set of conditions in a very small number of experiments. PBD considers the statistical interactions between variables to obtain maximum interferences for a minimum number of tests, thus reducing process variability, time of development and overall costs. The parameters selected for the experiment were xylose/acid lauric ratio (LA), immobilized *Candida cylindracea* lipase concentration (CCL Im), amount of molecular sieves (MS), temperature (T°) and reaction time (t). All trials were performed in triplicate and the sugar conversion (C) was used as the response. In the present study

the five independent variables were organized according to the PBD matrix. For each variable, high (+1) and low (-1) levels were tested (Table 1).

Table 1. Process variables and levels in Plackett-Burman design

Parameter	*Symbol*	*Unit*	*Levels*	
			Low(-)	*High(+)*
Xylose/Lauric acid ratio	LA		0,5	1
Candida cylindracea lipase	CCL Im	mg	10	30
Molecular sieves	MS	mg	10	30
Temperature	T°	°C	40	60
Time	t	h	24	72

As per the design, various combinations of the five parameters used, along with the results obtained, are summarized in Table 2.

Table 2. Plackett-Burman design matrix with coded and actual values for 5 independent variables

Run. N°	Coded values					Actual values				C (%)
				LA	CCL Im (mg)	MS (mg)	T (°C)	t (h)		
1	-1	1	1	1	-1	0,5	30	30	60	24	35
2	-1	-1	-1	-1	-1	0,5	10	10	40	24	20
3	1	1	-1	1	-1	1,0	30	10	60	24	40
4	-1	1	-1	-1	-1	0,5	30	10	40	24	20
5	-1	-1	1	1	1	0,5	10	30	60	72	50
6	1	-1	1	-1	-1	1,0	10	30	40	24	10
7	1	1	1	-1	1	1,0	30	30	40	72	20
8	1	-1	1	1	-1	1,0	10	30	60	24	45
9	1	-1	-1	-1	1	1,0	10	10	40	72	30
10	-1	1	1	-1	1	0,5	30	30	40	72	32
11	1	1	-1	1	1	1,0	30	10	60	72	70
12	-1	-1	-1	1	1	0,5	10	10	60	72	60

To obtain a proper model for the optimization of xylose ester synthesis, the Box-Benhnken design, for three variables was then applied. This design was preferred because relatively a few experimental combinations of the variables are adequate to estimate the response function. A3 -factor, 3-level design used is suitable for exploring quadratic response surfaces and constructing second order polynomial model.

Statistical analysis

The experimental data were analyzed by the statistical software package Minitab 14. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variances.

RESULTS AND DISCUSSION

Plackett-Burman Design

A preliminary screening was carried out based on PBD with 5 variables (xylose/acid lauric ratio, immobilized *Candida cylindracea* lipase concentration, amount of molecular sieves, temperature and reaction time) and 2 levels (Table 2).

According to their *p*-value (> 0.05), the less significant factors were discriminated, whilst the parameters that significantly influenced the sugar conversion were selected later for the BBD.

From the statistical analysis, it was found that ester production was affected by the amount of molecular sieves, the temperature and the reaction time [24] as evident from their *p*-values as shown in Table 3 (a *p*-value less than 0.05 is considered significant).

Moreover the model coefficients allow to assess the influence of factors on the response (Table 3). It was found that coefficients with high values are the most important factors.

The final response equation obtained with the coded factors values is as follows:

$$C\ (\%) = 36 + 14T° + 7{,}667t - 4MS + 0{,}167CCL\ Im - 0{,}167LA \quad (1)$$

Table 3. Estimated effects and coefficients for conversion

Term	Effect	Coefficient	*p*-value
Constant	-	36,000	0,000
LA	-0,333	-0,167	0,934
CCL Im	0,333	0,167	0,934
MS	-8,00	-4,0	0,085
T°	28,000	14,000	0,000
t	15,333	7,667	0,008
R^2 = 92,28%	R^2adj = 91.5%.		

Model fitting and analysis of variance (ANOVA)

It is always necessary to examine the fitted model to ensure that it provides an adequate approximation of the true system. The ANOVA results of the developed model is calculated using the statistical software Minitab 14 and are shown in Table 4. The significance of the model has been analyzed by the F-test (Table 4). The F-value and the *p*-value of the model are 14,34 and 0,003 respectively, showing a statistical relation between the response and the selected factors and therefore the significance of the model at 95% probability level. The fit of the model has been tested by the analysis of the regression equation and the R^2. The R^2 of 0.9228 is concordant and confirms that 92,28 % of variation in the ester formation can be explained by the fitted model.

Table 4. Analysis of variance for the experimental PBD.

Source	DF*	SS**	MS***	F-value	*p*-value
Model	5	3250	650	14,34	0,003
Residual Error	6	272	45,33		
Total	11	3522			

****** Degrees of freedom ****** Sum of square ******* Mean of square*

Figure 1, representing the plot of experimental values compared to predicted values, shows a good concordance between them.

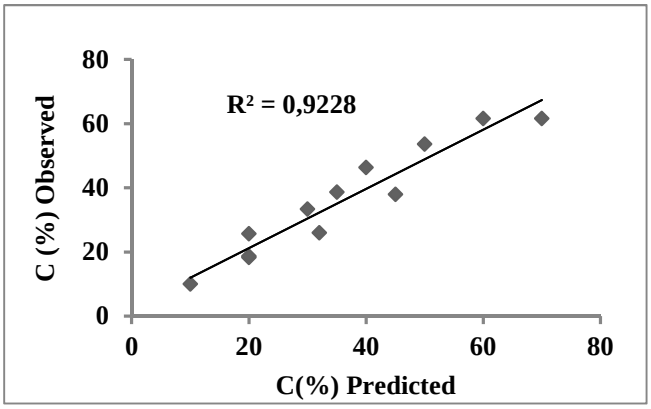

Figure 1. Plot of experimental versus predicted values of conversion

Therefore, ANOVA can be validated since Figure 1 prove that the model accurately represents the influence of the chosen factors on the sugar conversion. This means that there is a good correlation between the theoretical and measured responses. This correlation is confirmed by the value of adjusted R squared (R^2adj= 91.5%).

Main effect plots

The main effect is calculated as the difference between the average of measurements made at the high level setting (+1) and the average of measurements observed at low level setting (-1) of each factor (figure 2).

Diagram of main effects informs us about the simultaneous influence of all factors on conversion. According to this diagram (Figure 2) we found that the temperature, time and amount of the molecular sieves, are the most influential factors on the progress of the enzymatic esterification of D-xylose. Increasing the temperature and reaction time increases the conversion. Increasing the amount of molecular sieves causes a decrease of the response. For factors, xylose/lauric acid ratio and lipase concentration, no significant effect on the conversion is observed.

The Plackett-Burman design was proved to be a powerful tool to determine the effects of various parameters on xylose conversion. However the optimal conditions of each factor that significantly affect xylose ester production could not be obtained. Further work needed to be done to find out this information.

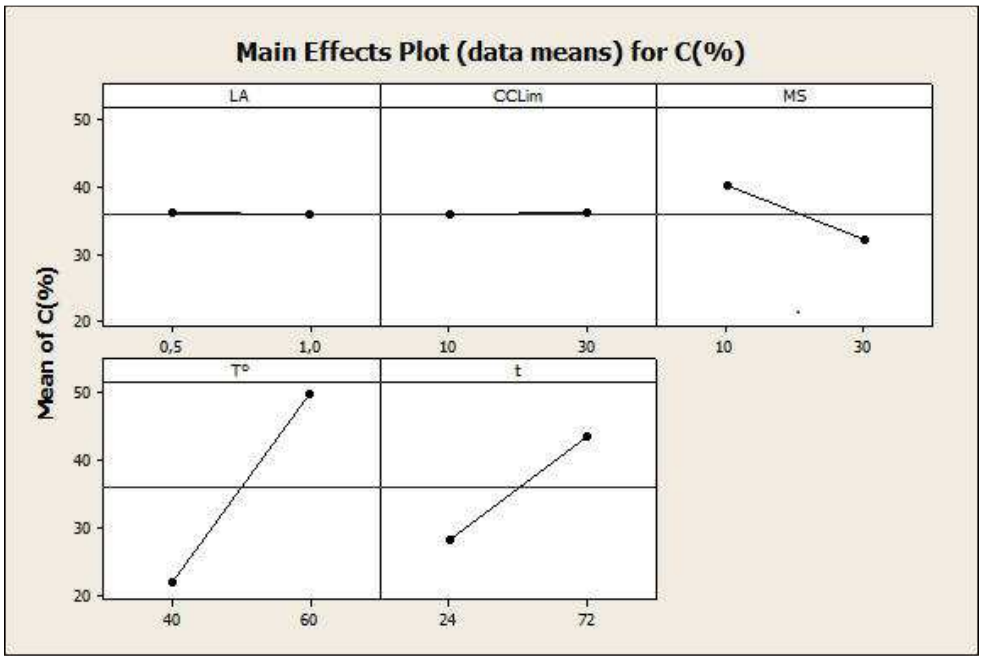

Figure 2. Main effects plot for the conversion C (%)

Optimization

The enzymatic production of xylose laurate was thus optimized by RSM under Box-Benhken design [25,27] and the influence of parameters as well as their interactions on the response was studied. The experimental conditions for BBD were selected according to PBD results for each variable based on prior studies. Experiments were carried out according to the design points with independent variable such as temperature (T°), reaction time (t) and molecular sieves concentration (MS). Three levels, such as low, medium and high, denoted as -1, 0, +1, were employed to fit a full quadratic response surface model and later approximated to obtain the optimal response (Table 5).

Table 5. Process variables and levels in Box-Benhken design

Parameters	*Levels*		
	Low (-1)	*Medium (0)*	*High (+1)*
T°	40	50	60
t	24	48	72
MS	10	20	30

Table 6. Box-Behnken three variables experimental design

Run N°	Coded values			Actual values			C (%)	
				T°	t	MS	Experimental values	Predicted values
1	1	0	-1	60	48	10	10	08,37
2	-1	0	1	40	48	30	22	23,62
3	0	1	1	50	72	30	48	54,87
4	0	0	0	50	48	20	25	31,66
5	-1	1	0	40	72	20	41	32,50
6	0	1	-1	50	72	10	29	31,37
7	0	0	0	50	48	20	35	31,66
8	1	0	1	60	48	30	80	73,87
9	-1	-1	0	40	24	20	25	25,75
10	0	0	0	50	48	20	35	31,66
11	0	-1	1	50	24	30	55	52,62
12	1	1	0	60	72	20	61	60,25
13	-1	0	-1	40	48	10	12	18,12
14	1	-1	0	60	24	20	30	38,50
15	0	-1	-1	50	24	10	12	05,12

The design variables selected in this study with actual and coded levels along with response variables with 3 replicates are shown in Table 6.

The aim of the study is to find the best reaction conditions to obtain the highest conversion [28]. All of the 15 designed experiments were performed and the results were multi-regression analyzed. Coefficients were evaluated by regression analysis and tested for their significance (Table 7). Finally, the best-fitting model was determined by regression.

Table 7. Estimated regression coefficients and R-squared values of the models

Term	Coefficient	*p*-value
Constant	31,667	0,002
T°	10,125	0,024
t	7,125	0,074
MS	17,750	0,002
$T°^2$	1,292	0,792
T^2	6,292	0,234
MS^2	-1,958	0,691
T.t	3,750	0,439
T°.MS	15,000	0,020
T.MS	-6,000	0,237
R^2 = 92,65%	R^2 adj =79,41	

The resulting fitted second-order polynomial equation with the coded factors values is given below :

$C(\%) = 31,667 + 10,125T° + 7,125t + 17,750MS + 1,292T°^2 + 6,292t^2 – 1,958MS^2 + 3,750T°.t + 15T°.MS – 6t.MS$ (2)

Model fitting and analysis of variance (ANOVA)

For estimation of significance of the model, the analysis of variance (ANOVA) was applied. Results are given in Table 8.

Table 8. Analysis of variance for the conversion

Source	DF*	SS**	MS***	F-value	*p*-value
Regression	9	5018,92	557,66	7,00	0,023
Linear	3	3746,75	1248,92	15,67	0,006
Square	3	171,92	57,31	0,72	0,582
Interaction	3	1100,25	366,75	4,60	0,067
Residual error	5	398,42	79,68	-	-
Lack-of-fit	3	331,75	110,58	3,32	0,240
Pure error	2	66,67	33,33	-	-
Total	14	5417,33	-	-	-

****** Degrees of freedom ****** Sum of square ******* Mean of square*

The irrelevant coefficients including quadratic coefficients (T^2, MS^2, t^2) and the cross-product coefficients (T.t, t.MS) were eliminated according to their *p*-value (Table 8) in order to refine the model (= 0.05). Thus, the non significant terms were eliminated (Table 10).

Therefore, the simplified polynomial expression for equation (3) in terms of coded factors values was expressed as follows :

$C(\%) = 34,667+10,125T°+7,125t+17,750MS+15T°.TM$ (3)

Table 9: Estimated regression coefficients and R-squared values of the models (after elimination of no-significant terms)

Terme	Coefficient	T	*p*-values
Constant	34,667	15,295	0,000
T°	10,125	3,262	0,009
t	7,125	2,296	0,045
MS	17,750	5,719	0,000
T°.MS	15,000	3,418	0,007
R^2 = 85,78%	R^2(adj)=80,09%		

Table 10: ANOVA after elimination of no-significant terms

Source	Degrees of freedom	Sum of squares		F-values	*p*-values
Model	4	4646,75	1161,69	15,08	0,000
Linear	3	3746,75	1248,92	16,21	0,000
Interaction	1	900,00	900,00	11,68	0,007
Residual error	10	770,58	77,06	-	-
Lack- of - fit	8	703,92	87,99	2,64	0,304
Pure error	2	66,67	33,33	-	-
Total	14	5417,33	-	-	-

The goodness of fit of the regression equation was evaluated by the determination coefficient (R^2). The value of determination R^2 (0.8578) indicates that the response model can explain 85.78% of the total variations. The value of adjusted determination coefficient R^2_{Adj} (80.09%) was also high enough to indicate the significance of the model. The corresponding analysis of variance (ANOVA) is given in Table 10. In addition, the calculated F-value (15.08) obtained in Table 10 is greater than the previously obtained F-value (7.00) before removing non-significant terms (Table 8). In this case, the ANOVA of the regression model demonstrates that the model is highly significant and is capable of representing the system under the given experimental domain. Thus the improved model is statistically better. It should be noted that polynomial models are reasonable approximations of the true functional relationship over relatively small regions of the entire space of the independent variables.

Furthermore, the analysis of variance indicated that the variance due to the lack-of-fit is not significant. The lack-of-fit value increases from 0.240 (Table 8) to 0.304 (Table 10), which means a better fitting model.

The clustering of the points around the diagonal line in Figure 3 indicates a satisfactory correlation between the experimental and predicted values, thereby confirming the soundness of the model.

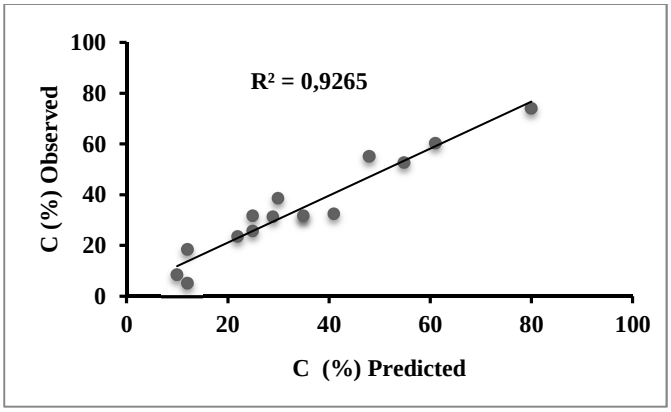

Figure 3. Plot of experimental versus predicted values of conversion C (%)

The predicted values were in good agreement with the experimental values showing that the cubic model could be used to predict and optimize the esterification percentage by determining the optimal operating conditions (temperature, molecular sieves and reaction time). The optimization process was carried out based on the contour plots.

Contour plots

The final step consists in finding the values of factors that give the optimal response [29]. From the validated mathematical model, using the software, we performed graphically 2D contours. The boundary curves are generated using MINITAB software 14 by the combination of three factors induced. To visualize the combined effects of the three factors on xylose ester synthesis, contour plots were generated for the fitted model that displays the effects of the three variables. Figure 4 shows the response surface plots as function of temperature (T°), reaction time (t) and molecular sieves concentration (MS).

According to the interpretation of the contour diagrams, the highest values of the conversion (C> 80%) are obtained when the three factors are fixed at high levels. Consideration of the set of graphs allowed the selection of the optimal point : T° = 60 °C, t = 72 h, MS = 30 mg and the conversion value at this point is equal to C = 85.041 % .

Model verification

The validity of the predicted model was verified by performing the lipase-assisted synthesis of xylosyl laurate under the predicted optimum conditions, corresponding to a temperature, molecular sieves quantity and time of 60 °C, 30 mg, 72 h respectively. Therefore, the model represented by the equation (3) accurately predicts the optimal conversion of the lipase-catalyzed synthesis of 1-*O*-dodecanoyl-D-xylopyranose. Hence, this statistical model showed that the chosen parameters and their adjustments have a significant influence on the substrate conversion.

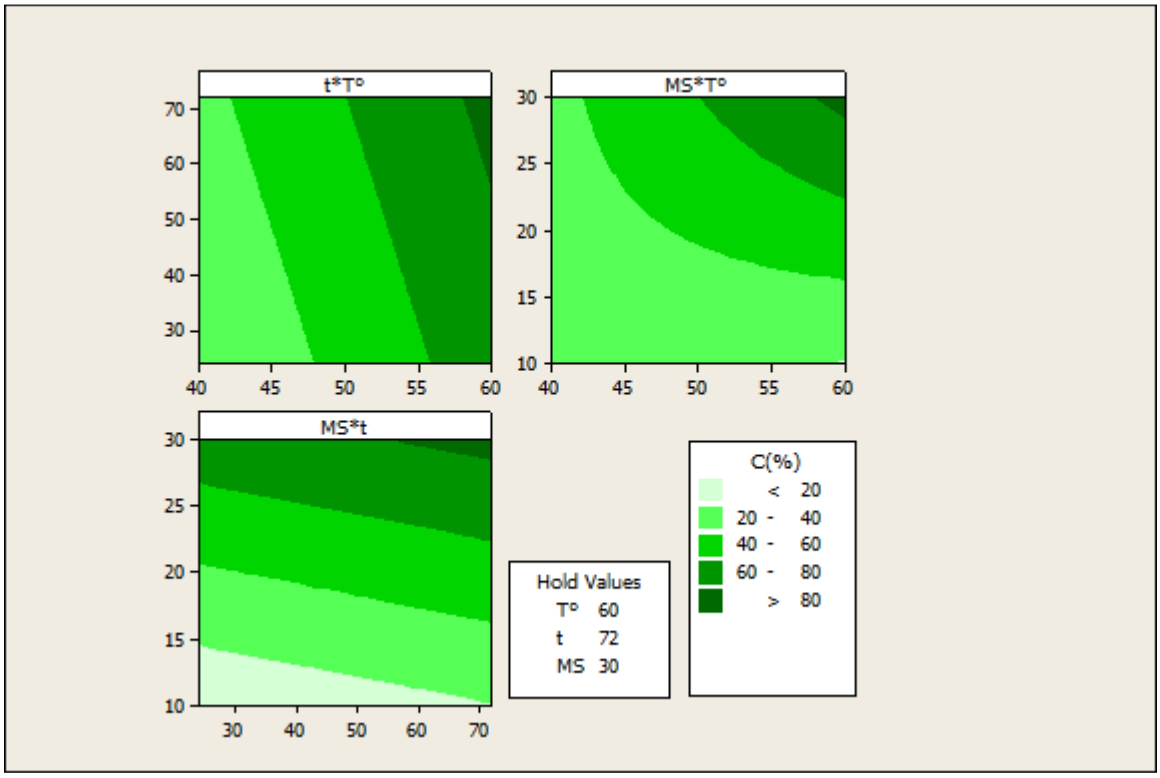

Figure 4. Contour plots of C (%) versus (T°, t, MS) constant at high levels

CONCLUSION

Statistical experimental designs combined with RSM for optimization of enzymatic synthesis of 1-*O*-dodecanoyl-D-xylopyranose were developed. First, Plackett-Burman design was implemented to screen the variables that significantly influence the conversion. This preliminary design displayed a model with a *p*-value of 0.003 and a coefficient of determination R^2 of 0.9228, thus indicating that the model is highly significant and the relationship between the conversion and the factors is adequately represented. It made it possible to highlight, among the five tested parameters, the three most significant ones : the temperature, the reaction time and molecular sieves concentration.

In the second step, the optimum values were determined by Box-Benhken design under response surface methodology. The determination coefficient R^2 was 0.8578, which ensure an adequate credibility of the model. The statistical analysis showed that the optimum reaction conditions led to the maximum conversion (85%). Comparison of predicted and experimental values revealed good matching between them, implying that empirical models derived from RSM can be applied to adequately describe the relationship between the factors and response in xylose laurate synthesis. This model can then be used to predict the conversion under any given conditions within the experimental range.

Acknowledgements

We are grateful to the Algerian Ministry of Scientific Research for providing financial assistance to carry out this project and to the UBMA University for the facilities provided to conduct this research work in LOMOP Laboratory.

REFERENCES

[1] T. Ivankovic, J. Hrenovic, *Arch. Ind. Hyg. Toxicol*., **2010**, 61, 95.
[2] H. Maag, *J. Am. Oil Chem. Soc.*, **1984**, 61, 259.
[3] S. Nakamura, *Oleochemicals*, **1997**, 8, 866.
[4] P. Villeneuve, *Biotechnol. Adv.*, **2007,** 25, 515.
[5] A.M. Gumel, M.S.M. Annuara, T. Heidelberg, Y. Chisti, *Process Biochemistry*, **2011**, 46, 2079.
[6] S.W. Chang, J.F. Shaw, *New Biotechnology*, **2009**, 26, 109.
[7] M. Therisod, A. M. Klibanov, *J. Am. Chem. Soc*., **1986**, 108, 5638.
[8] A. M. Klibanov, *Nature*, **2001**, 409, 241.
[9] C. Bidjou-haiour, N.Klai, *Asian J. Chem*., **2013**, 25 (8), 4347.
[10] D. Coulon, M. Ghoul, *Agro. Food Industry Hi-Tech.*, **1998**, 9, 22.
[11] M, Kılıc¸ E. Bayraktar, S. Ates ¸ Û. Mehmetgolu, *Process Biochem.*, **2002**, 37, 759.
[12] R. L. Plackett, and J. P. Burman, *Biometrika*, **1946** ,33, 305.
[13] N. B. Ghanem, H. H. Yusef, H. K. Mahrouse, *Bioresour. Biotechnol*., **2000**, 73, 113.
[14] L. Rao, S. Divakar, *Process Biochem*., **2001**, 36, 1125.
[15] G.E.P. Box, D.W. Behnken, *Technometrics*, **1960**, 2, 455.
[16] T.U. Nwabueze, *International Journal of Food Science & Technology*, **2010**, 45, 1768.
[17] R.H. Meyer, D.C. Montgomery, Response Surface Methodology: Design and Analysis of Experiments, (John Wiley and sons, New York, **2002**), 445.
[18] K. R. Kiran, B. Manohar, S. Divakar, *Enzyme Micro. Tech.*, **2001**, 29 (2-3), 122.
[19] Y.K. Twu, I. L. Shih, Y. H. Yen, C.J. Shieh, *J. Agric. Food Chem.*, **2005**, 53(4), 1012.
[20] S. Sabeder,M. Habulin, Z. Knez, *J. Food Eng*., **2006**, 77, 880.
[21] T. Polat, and R. J. Linhardt, *J. Surf. Det*., **2001**, 4 (4), 415.
[22**]** C.J. Shieh, P.E. Koehler, C.C. Akoh, *J. Food Sci*., **1996**, 6 (1), 97.
[23] N. S. Neta, A.M. Peres, J.A. Teixeira, L.R. Rodrigues, *New Biotechnology*, **2011**, 28, 349.
[24] Y. Yan, U.T. Bornscheuer, G. Stadler, S. Lutz-Wahl, R. T. Otto, M. Reuss, R. D. Shmidt, *Europ. J. Lipid Sci. Technol*., **2001**, 103 (9), 583-587.
[25] G. E. P. Box, *Biomertika Trust*, **1952**, 39 (1), 49.
[26] G. E. P. Box, K.B. Wilson, *Journal of the Royal Statistical Society. Series B (Methodological)*,**1951**, 13 (1), 1.
[27] R.F. Teofilo, M.M.C. Ferreira, *Quim. Nova*, **2006,** 29, 338.
[28] T. Lundstedt, E. Seifert, L. Abramo, B. Thelin, A. Nyström, J. Pertensen, R. Bergman, *Chemom. Intell. Lab. Sys*., **1998**, 42, 3.
[29] M. A. Bezerra, R. E. Santelli, E. P. Oliveira, L. S. Villar, L. A. Escaleira, *Talanta*, **2008,** 76, 965.