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DoE oriented reaction optimization on the lipase-catalyzed monostearin synthesis

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ABSTRACT

Recognition that *trans* and saturated fats have negative health effects drive researchers to develop alternative systems that can structure liquid oils into semi-solid plastic pastes for food applications. Monoacylglicerols (MAG) can be used as a promising molecule to achieve this structuring so we have optimized a biocatalytic batch process to the esterification reaction between 1,2-O-isopropylidene glycerol and stearic acid, catalyzed by Lipozyme RM IM, using response surface methodology (RSM) in a laboratory setting with 95% of conversion after 4 h.

1. Introduction

A better understanding of the role of dietary lipids in the coronary heart disease (CHD) continues to give us insights into metabolic effects of individual fatty acids and their impact on surrogate markers of risk [1]. The recognition that *trans* and saturated fats have negative health effects drive researchers to develop alternative systems that can structure liquid oils into semi-solid plastic pastes for food applications because the traditional processes for manufacturing spreads, margarines, and shortenings uses hydrogenation and/or saturated fats to achieve the desired structuring [2,3].

In this way, monoacylglicerols (MAG) can be used as a promising molecule to achieve this structuring by utilizing the properties of gel phases (alpha gel and coagel) [4,5]. These MAG are commonly produced based on alkaline-catalyzed chemical glycerolysis of natural oil and fats at high temperatures (220–250 °C) and elevated pressure under nitrogen atmosphere leading to products with low yield, poor quality, dark-colored and burned-tasting characteristics [6,7]. To overcame this issues the use of enzymatic process can lead to an environmentally friendly approach, employing enzyme-catalyzed synthesis of MAG by selective hydrolysis or alcoholysis using 1,3-regiospecific lipases [8–10], esterification of glycerol with fatty acids [11,12], and glycerolysis of fats or oils [13].

In this work we have optimized a biocatalytic batch process to the esterification reaction between solketal and stearic acid using response surface methodology (RSM) in a laboratory setting [14]. The lipase-catalyzed esterification [15] has been investigated as a potential substitute to the traditional chemical glycerolysis, since lipases as biocatalysts demand milder reaction conditions which minimize energy costs, allow a better reaction control and consequently provide higher-quality products [16]. RSM is a statistical tool for developing and optimizing processes with one or more responses influenced by several variables. The RSM advantage is that it allows the user to gather large amounts of information from a small number of experiments. The RSM use also enables to observe the effects of individual variables and their combination of interactions on the response.

2. Experimental

Heptane was purchased from Tedia Co. (R,S)-1,2-isopropylidene glycerol from Sigma–Aldrich as well as all chromatofigureic

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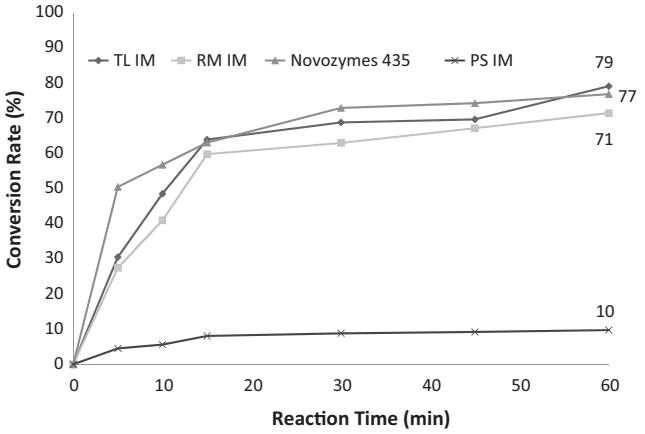

Fig. 1. Initial screening for immobilized lipases. Experimental conditions: 60 °C, 250 rpm and 1% (w/v) of enzyme (Lipozyme TL IM, Lipozyme RM IM, PS Amano IM and Novozym 435).

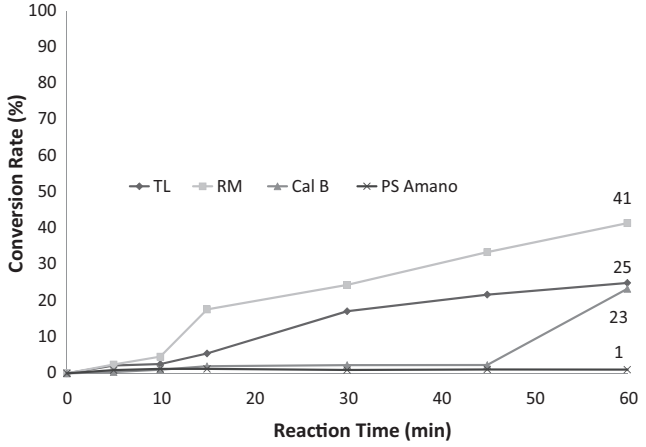

Fig. 2. Initial screening for free and lipases. Experimental conditions: 60 °C, 250 rpm and 1% (v/v or w/v) of enzyme (*Thermomyces lanuginosus, Rhizomucor miehei*, *Candida antarctica B* and PS Amano).

standards. Stearic acid (>98%) was purchased from Vetec Ltd. Lipases were purchased from Novozymes and Amano.

2.1. GC–MS analysis

The GC–MS analysis was performed by using modified method from EN 14105. Free fatty acids and solketal were transformed into more volatile silylated derivatives in presence of pyridine and N-methyl-N-trimethysilyltrifluoroacetamide (MSTFA). All GC–MS measurements were carried out in duplicate (Dizge & Keskinler; 2008) using a DB 5–HT (Agilent, *J & W. Scientific*®, USA) capillary column (10 m × 0.32 mm × 0.1 μm). The quantifying was done based on calibration curves with internal standards. The GC–MS samples were prepared by dissolving 0.1 g of the final product on 1 ml of n-heptane. 100 μl of this solution and pyridine solutions of butanetriol (1 mg/ml) and tricaprine (8 mg/ml), used as internal standards, were added on a flask forward by an addition of 100 μl of MSTFA. After 15 min, these reactants were dissolved on 8 ml de n-heptane. 1 μl of this sample was then injected into a Shimadzu CG2010 equipment.

2.2. Lowry–Tinsley analysis

The esterification rate was also measured using a modification of the Lowry and Tinsley assay [17]. The depletion of fatty acid was monitored as follows: 0.30 ml of the reaction solution, including the buffer solutions was added to a tube containing 0.6 ml of n-heptane and 1 ml of cupric acetate-pyridine (5%, w/v; pH 6.0). The final solutions were vigorously mixed for 30 s in vortex, and the upper organic phase was measured by a UV/visible spectrophotometer at 715 nm. Each reaction was analyzed in triplicate, and content conversion, calculated according to the percentage difference for the absorbance shown by the stock solution.

2.3. Batch procedure

A stock solution containing 1,2-O-isopropylidene glycerol and stearic acid in n-heptane in proportions of 2:1 (150 mM and 75 mM respectively) was prepared. In 2 ml cryotubes, were poured 1 ml of reaction medium, followed by the addition of appropriate enzyme (1%, w/w or w/v). The cryotubes were then incubated in shaker at 250 rpm and 60 °C. The esterification rate was measured by a modification of the Lowry and Tinsley assay, at time intervals between 0 and 60 min. The depletion of fatty acid was monitored as follows: 0.30 ml of the reaction solution was added to a cryotube containing 0.6 ml of n-heptane and 1 ml of cupric acetate–pyridine (5%, w/v; pH 6.0). The final solutions were vigorously mixed for 30 s in vortex, and the upper organic phase was measured by a UV/visible spectrophotometer at 715 nm. Each reaction was analyzed in triplicate, and content conversion, calculated according to the percentage difference for the absorbance shown by the stock solution. The results were confirmed by GC–MS analysis.

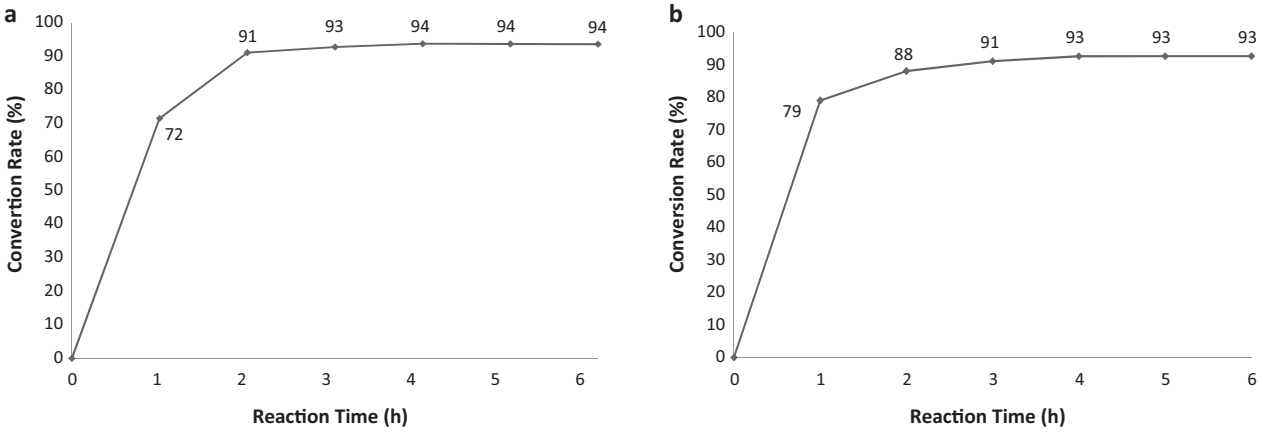

Fig. 3. Conversion rates of 1,2-O-isopropylidene glycerol stearate (2:1, 60 °C, 250 rpm and 1%, w/v) by using Lipozyme RM IM (a) and Lipozyme TL IM (b) as biocatalysts.

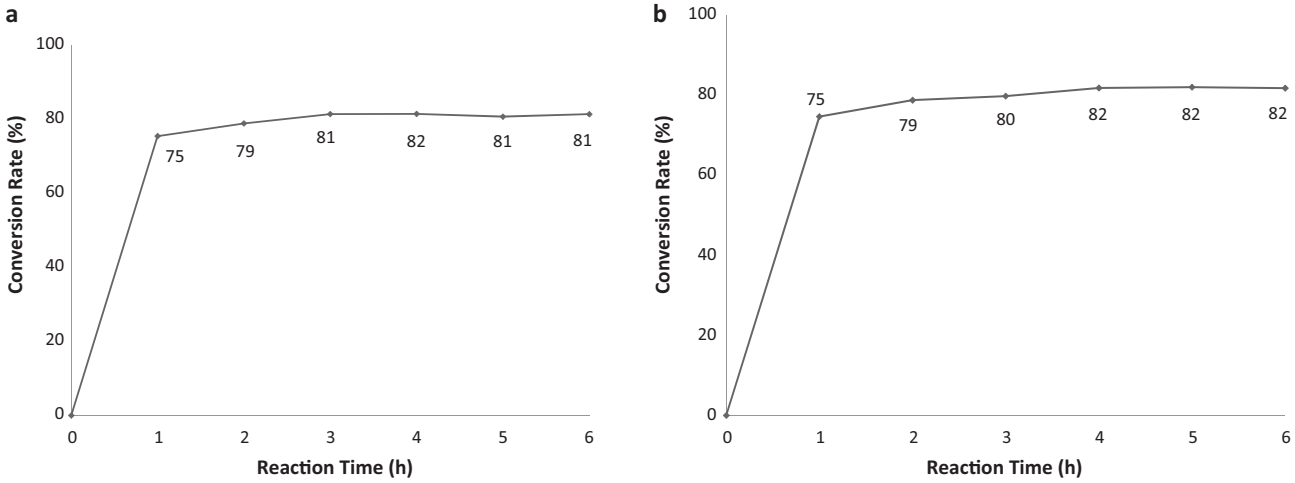

Fig. 4. Conversion rates of 1,2-O-isopropylidene glycerol stearate (1:1, 60 °C, 250 rpm and 1%, w/v) by using Lipozyme TL IM (a) and Lipozyme RM IM (b) as biocatalysts.

Table 1
Real and coded values (+ higher level, 0 intermediate, − lower level) for the independent variables, 2^{4-1}.

Variables	−1	0	+1
Temperature (°C)	40	50	60
Amount of enzyme (%)[a]	0.1	0.55	1
Substrate Concentration (mM)	50	75	100
Stirring (rpm)	50	150	250

[a] By weight of total system.

2.4. Statistical analysis

The experimental designs and results analysis were carried out using the *software* Statistica 6.0 (Statsoft, Inc., USA), according with the significance level established to obtain the mathematical model. The significance of the regression coefficients and the associated probabilities, $p(t)$, were determined by Student's t test; the model equation significance was determined by Fisher's F test. The variance explained by the model is given by the multiple determination coefficients, R^2.

3. Results and discussion

3.1. Initial screening of commercial available free and immobilized lipases

In order to evaluate the best biocatalyst for the synthesis of 1,2-O-isopropylidene glycerol stearate, we perform an initial screening with 4 commercial immobilized lipases (Lipozyme TL IM, Lipozyme RM IM, PS Amano IM and Novozym 435) and 4 commercial free lipases (*Thermomyces lanuginosus*, *Rhizomucor miehei*, *Candida antarctica B* and PS Amano). A stock solution containing 1,2-O-isopropylidene glycerol and stearic acid (in a molar ratio 1:2) in n-heptane was prepared in order to avoid pipetting errors. In 2 ml ependorfs, were added 1 ml of stock solution, followed by the addition of appropriate enzyme (1%, w/w or w/v) and the reaction analyzed each 5 min until 15 min. After that, the reaction was analyzed at 30, 45 and 60 min. The results are summarized in Figs. 1 and 2 for the immobilized and free enzymes, respectively.

As Lipozyme TL IM and Lipozyme RM IM are less studied in the synthesis of MAG and lead to good results in the initial screening, we decide to go further with these two enzymes evaluating the reaction time needed for full conversion of substrates. As shown in Fig. 3a and b, after 4 h of reaction it is not possible to detect any considerable improvement on conversion rate for both Lipozyme RM IM and Lipozyme TL IM immobilized enzymes. As from 1 to 4 h a significant raise on reaction conversion is still clearly observed, we decided to use 4 h as a standard for total reaction time in our work.

Considering MAG as a food ingredient in industry process, minor costs with starting materials such as the reagent 1,2-O-isopropylidene glycerol represent a relevant and crucial step. Thus, we also evaluate the ability of Lipozyme TL IM lipase and Lipozyme RM IM lipase to catalyze the esterification reaction of stearic acid and 1,2-O-isopropylidene glycerol in a molar ratio of 1:1 under the same experimental conditions mentioned in Fig. 3, saving 1,2-

Table 2
Experimental factorial design and results of FFD for enzymes studied.

Entry	Variable levels				Conversion (%)[a]	
	T (°C)	E (%)[a]	S (mM)	St (rpm)	RMIM	TLIM
1	−1 (40)	−1 (0.1)	−1 (50)	−1 (50)	70	70
2	+1 (60)	−1 (0.1)	−1 (50)	+1 (250)	85	78
3	−1 (40)	+1 (1)	−1 (50)	+1 (250)	90	91
4	+1 (60)	+1 (1)	−1 (50)	−1 (50)	88	84
5	−1 (40)	−1 (0.1)	+1 (100)	+1 (250)	43	48
6	+1 (60)	−1 (0.1)	+1 (100)	−1 (50)	64	65
7	−1 (40)	+1 (1)	+1 (100)	−1 (50)	80	86
8	+1 (60)	+1 (1)	+1 (100)	+1 (250)	79	80
9	0 (50)	0 (0.55)	0 (75)	0 (150)	72	72
10	0 (50)	0 (0.55)	0 (75)	0 (150)	72	73
11	0 (50)	0 (0.55)	0 (75)	0 (150)	73	72

[a] Measured by Lowrey–Tinsley and confirmed by GC–MS.

Table 3
Estimated effect for variables studied for of 1,2-O-isopropylidene glycerol stearate production.

Variable	Effect		*p*-Value	
	RMIM	TLIM	RMIM	TLIM
Mean	80.2625	78.6625	<0.0001*	<0.0001*
Curvature check	−12.6917	−11.8983	0.0197*	0.0050*
Temperature (*T*)	7.6450	1.6150	0.0149*	0.0676
Amount of enzyme (*E*)	17.8600	20.3150	0.0027*	<0.0001*
Substrate concentration (*S*)	−17.3850	−10.4650	0.0029*	0.0017*
Stirring (St)	−1.8400	−0.6350	0.1912	0.2880

* Statistically significant at 95% confidence level.

Table 4
Real and coded values (+ level, 0 intermediate, − lower level) for the independent variables, 2^2.

Variables	−1, 41	−1	0	+1	+1, 41
Temperature (°C)	50	51.4	55	58.5	60
Substrate concentration (mM)	50	53.6	62.5	71.3	75

O-isopropylidene glycerol, which is the limiting reagent in the reaction.

Again, a stock solution containing 1,2-O-isopropylidene glycerol and stearic acid in n-heptane (1:1 proportion) was prepared in order to avoid pipetting errors. In 2 ml cryotubes, were added 1 ml of reaction medium, followed by the addition of appropriate enzyme (1%, w/w) and the reaction analyzed every hour, until 6 h. The results are summarized in Fig. 4a and b.

The results presented in Fig. 4 shows that with 1:1 proportion of substrates, we still obtained good conversion rates with Lipozyme TL IM and Lipozyme RM IM, although they were slightly lower than the conversions presented in a 2:1 ratio (Fig. 3). However, it is still advantageous in terms of industrial and economic development, working with equimolar proportions of reagents, avoiding waste and decreasing costs in the final product.

3.2. Initial evaluation of reaction parameters using fractional factorial design (FFD)

In order to optimize the reaction conditions we have proposed fractional factorial design (FFD) carried out in order to compare the Lipozyme TL IM and Lipozyme RM IM as biocatalysts for the synthesis of 1,2-O-isopropylidene glycerol stearate.

A two level factorial design was adopted for a preliminary study designed to identify the lipase with the best performance in synthesizing 1,2-O-isopropylidene glycerol stearate. First, we carried out a fractional factorial designs 2^{4-1} for each biocatalyst, to determine the variables of most influence for the esterification reaction catalyzed by Lipozyme TL IM and Lipozyme RM IM. This first part of the study required 22 experiments, eleven trials for each lipase.

The variables studied in the fractional factorial design (FFD) were temperature (*T*), amount of lipase (*E*), substrate concentration (*S*) and stirring (St). These parameters were chosen based on earlier studies of our group. The reaction time was not considered as a variable in the present experimental design, since that a reaction kinetic study was previously held to establish the best time in which good conversion rates were achieved. The variables with the respective levels used of fractional factorial design (FFD) for Lipozyme TL IM and Lipozyme RM IM lipase are presented in Table 1 and the experimental design with corresponding results are shown in Table 2.

As shown in Table 2, in most cases, Lipozyme RM IM and Lipozyme TL IM present similar results independently of the variables studied. Only in two experiments is possible to observe a considerable difference in conversion in between these two enzymes (entries 2 and 7, Table 2). The best result was achieved when using 1% (w/w) of enzyme at 40 °C (50 mM) and 250 rpm which leads to 90% of conversion (entry 3, Table 2). But, it is important to highlight that working on lower catalyst concentration (0.1%, w/w) good conversions (85%) could also be obtained as can be seen in Table 2, entry 2. The Lipozyme RM IM enzyme showed high efficiency at low concentrations since the use of 0.1% (w/w) of this enzyme has lead to good conversions.

The estimated effects and the p values are shown in Table 3 where is possible to see that temperature had a positive effect (7.64) within the range studied. An increase in temperature causes a significant increase in product conversion, as can be seen in Table 2, entries 1 and 2. When the temperature goes from 40 °C (Table 2, entry 1) to 60 °C (Table 2, entry 2), under the same concentration of substrate (50 mM), the conversion increase from 70% to 85% for

Table 5
Experimental factorial design and results of CCRD for Lipozyme RM IM.

Entry	Variable levels[a]		Conversion (%)[b]
	T (°C)	*S* (mM)	
1	−1 (51.4)	−1 (53.6)	75
2	−1 (51.4)	+1 (71.3)	69
3	+1 (58.5)	−1 (53.6)	95
4	+1 (58.5)	+1 (71.3)	68
5	−1.41 (50)	0 (62.5)	70
6	+1.41 (60)	0 (62.5)	86
7	0 (55)	−1.41 (50)	81
8	0 (55)	+1.41 (75)	69
9	0 (55)	0 (62.5)	74
10	0 (55)	0 (62.5)	74
11	0 (55)	0 (62.5)	74

[a] Coded variables and real values.
[b] Measured by Lowrey–Tinsley and confirmed by GC–MS.

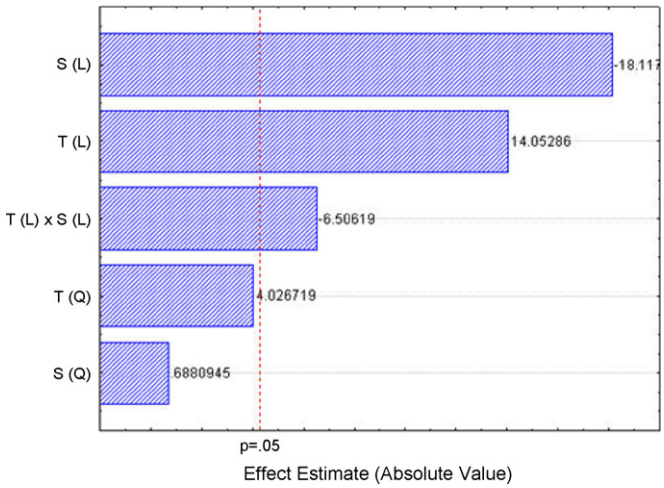

Fig. 5. Pareto chart from CCRD showing the significance of experimental variables: *T*, temperature; *S*, substrate concentration; *L*, linear effect; and *Q*, quadratic effect.

Table 6
Variance analysis for validation of mathematical models (ANOVA).[a]

Factor	Sum of squares	Degrees of freedom	Mean square	*F* calculated	*F* tabulated	*p*-Value
Regression	812.27	3	270.75	17.80	4.34	0.0011
Residuals	106.42	7	15.20			
Lack of fit	103.55	5				
Pure error	2.85	2				
TOTAL	918.69	10				

[a] Confidence level 95%.

Lipozyme RM IM and from 70% to 78% for Lipozyme TL IM since stirring was not significant in the process, presenting a $p > 0.05$ (Table 3). In Table 3 we can also observe that the amount of enzyme presented a high estimate effect (17.86).

Thus, the enzyme Lipozyme RM IM was chosen to continue the optimization of reaction variables in the CCRD because of higher average conversion results presented in Table 2. Besides the fact that amount of enzyme presented a high estimate effect, it remained constant by 0.1% (by weight of total system) on CCRD to enable the industrial applicability. As for the enzyme Lipozyme RM IM was possible to obtain 85% of conversion using only 0.1% of this biocatalyst, the process becomes cheaper than using 1% of this enzyme to obtain 90% of the final product. The temperature and concentration of substrate also had significance in the process and the CCRD was further applied in order to determine their optimal values maximizing the conversion rates. It was also observed in Table 2 the need for curvature in the model by including axial points for presenting a $p < 0.05$.

3.3. Central composite rotatable design (CCRD) for the production of 1,2-O-isopropylidene glycerol stearate

Following the first factorial design mentioned previously, a central composite rotatable design (CCRD) was employed to obtain the optimum conditions for esterification synthesis. The study was carried out using Lipozyme RM IM, which showed the best performance in the previous experiments. The reaction parameters involved were the temperature (T) and substrate concentration (S). The stirring was fixed at 250 rpm according with previous design and also the amount of enzyme was fixed at 0.1% aiming an industrial applicability, since good results were obtained with this amount of biocatalyst. Variables along with their coded and uncoded values are given in Table 4.

In the CCRD, both selected variables were varied at five levels resulting in 11 experiments, including four factorial points and three central points allowing to check the curvature (Table 5). To fit a second order model, four extra points with the same distance from the central point were added at the matrix for this design. The results obtained for the production of 1,2-O-isopropylidene glycerol stearate are presented in Table 5.

The results presented in Table 5, shows that excellent conversion can be obtained by the optimization of reaction conditions affording the desired product in 95% of conversion (entry 3, Table 5).

We have also obtained the quadratic effect which shows that variables temperature and substrate concentration and the interaction between each other were significant in the process. These results are shown in Fig. 5.

Fig. 5 shows the effects for CCRD estimatives were similar to FFD. The variables temperature, substrate concentration and the interaction between each other were significant in the process. Analyzing the figure a negative effect of substrate concentration in the range studied is observed, perhaps due to the low homogeneity of the medium generated by increasing the concentration of the substrate, which in turn undermines the contact of the substrate with the active site of the enzyme. Another plausible explanation may be that there is a small enzyme inhibition by high concentrations of substrate. In experiments 1, 2 and 3, 4 (Table 5) there is a clearly negative effect of this variable, since when increasing the concentration of substrates, the conversion of product also decreases.

The experimental data have been adjusted to the proposed model and adequacy was performed by the analysis of variance and parameter R^2 and statistical testing of the model was done by

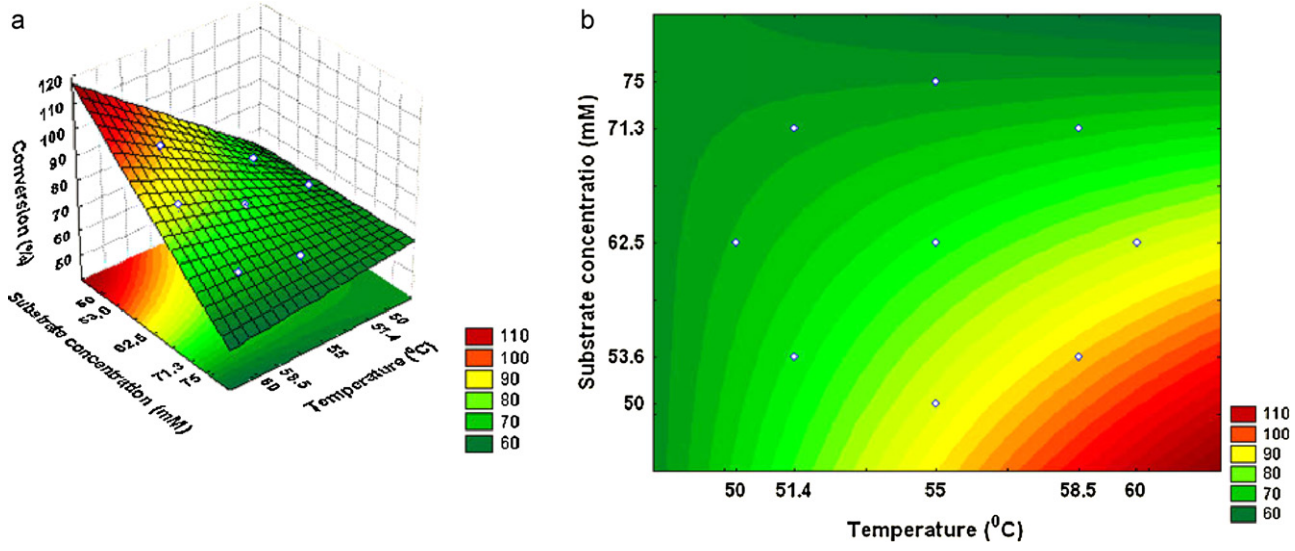

Fig. 6. Response surface (a) and contour curves (b) for the esterification reaction of the enzyme RMIM as a function of temperature and concentration of the substrate.

the Fisher's statistical test for ANOVA. Eq. (1) represents the mathematical model of the conversion of 1,2-O-isopropylidene glycerol monoester in function of the variables.

$$Y = 74.77 + 5.94T - 7.65S - 3.89T \cdot S \quad (1)$$

where Y is the percentage yield conversion, and T and S are the uncoded values of temperature and substrate concentration, respectively. Statistical testing of the model was done by the Fisher's statistical test for ANOVA (Table 6).

Table 6 represents the analysis of variance (ANOVA) which shows the validity of the model by F test and residue that shows the magnitude of experimental error. The calculated F (17.80) was higher than the tabulated F ($F_{3,7}$ = 4.34), showing the validity of the experimental model. The goodness of the model can be checked by the determination (R^2). The determination coefficient (R^2 = 0.90) implies that the sample variation of 90% for ester production is attributed to the independent variables and can be accurately explained by the model.

Fig. 6 shows that the decrease in substrate concentration and temperature increase results in an optimal response. But there is a limit to the increase in temperature which avoids denaturation of the enzyme source.

The monostearin synthesis was accomplished by 1,2-O-isopropylidene cleavage using boric acid as standard procedure described over literature [18].

4. Conclusion

In conclusion we have used DoE as an important tool for reaction optimization of esterification reaction between 1,2-O-isopropylidene glycerol and stearic acid with both Lipozyme RM IM and Lipozyme TL IM. After initial evaluation, Lipozyme RM IM was chosen for further development leading to an increase on reaction conversion from initial 71% to final 95%. The mathematic model proposed suggested a satisfactorily representation of the process and good correlation among the experimental results and the theoretical values predicted by the model equation were achieved.

Acknowledgements

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