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Lipase-Catalyzed Monostearin Synthesis under Continuous Flow Conditions

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ABSTRACT: Biodegradable, biocompatible and nontoxic nonionic surfactants are widely used in food, pharmaceutical and industrial applications being commonly produced based on alkaline-catalyzed chemical glycerolysis of natural oil and fats at high temperatures and elevated pressure under nitrogen atmosphere. In this work we have optimized a biocatalytic continuous flow process with packed bed reactor for the esterification reaction between (*R*,*S*)-1,2-isopropylidene glycerol and stearic acid using response surface methodology (RSM) leading to the desired product in excellent conversion (95%) and short reaction time (40 s of residence time).

1. INTRODUCTION

Partial acylglycerols, mono- and diacylglycerols (MAG and DAG) are well-known biodegradable, biocompatible and nontoxic nonionic surfactants[1,2] widely used in food, pharmaceutical and industrial applications.[3,4] The hydrophobic part consists of fatty acid (i.e., lauric, myristic, palmitic, oleic and stearic acid), whereas the hydrophilic part can be formed by of glycerol or one of its ester derivatives from organic acids such as lactic, citric, acetic or tartaric acid.

They are commonly produced on the basis of a batch alkaline-catalyzed chemical glycerolysis of natural oil and fats at high temperatures (220−250 °C) and elevated pressure under nitrogen atmosphere. Besides the high-energy consumption, high temperatures are the responsible for the low yield (<50%) and poor product quality leading to dark-colored and burned-tasting products formation, which requires extensive and costly purification steps.[5,6] In this way, the use of enzymatic process can overcome these issues and lead to an environmentally friendly approach, employing enzyme-catalyzed synthesis of MAG by selective hydrolysis or alcoholysis using 1,3-regiospecific lipases,[7] esterification of glycerol with fatty acids[8] and glycerolysis of fats or oils.[9]

Among the monoacylglycerols, monostearin stands out due to its wide application usually as additive in candy, ice cream, cake and bread, which has functions of emulsifying, dispersing, antifroth, bulge, antistarch, improving preservation, protecting freshness and controlling lipid to agglutinate in the food. It is also used in chocolate, candy and ice cream to avoid crystallization and prevent separations between oil and water. It can increase shine and exquisite feeling.[10]

For industrial purposes, the continuous flow system is preferred to batch reactors due to its greater process control, high productivity and improvement of quality/purity and yield.[11,12] Several types of reactor can be used in continuous operation, among these reactors, packed bed reactors (PBR) are the most popular due to high efficiency, low cost and ease of construction, operation and maintenance.[13−15]

In this work we have optimized a biocatalytic continuous flow process with a packed bed reactor for the esterification reaction between (*R*,*S*)-1,2-isopropylidene glycerol[16] and stearic acid using response surface methodology (RSM)[17] in a laboratory setting. To the best of our knowledge this is the first report for this reaction under continuous flow conditions. The lipase-catalyzed esterification 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.[4] 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. Using RSM also enables observation of the effects of individual variables and their combination of interactions on the response.

2. RESULTS AND DISCUSSION

We began our study evaluating the esterification reaction between (R,S)-1,2-isopropylidene glycerol and stearic acid catalyzed by immobilized lipase from *Rhizomucor miehei* (RM IM) under continuous-flow conditions. The reaction proceeded at 60 °C and different flow rates (0.2, 0.6, and 1.0 mL/min). To this purpose we have used a 35 mM stock solution containing stearic acid and (*R*,*S*)-1,2-isopropylidene glycerol (equimolar proportion)

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Scheme 1. Esterification reaction between (*R*,*S*)-1,2-isopropylidene glycerol and stearic acid catalyzed by immobilized lipase from *Rhizomucor miehei* (RM IM) under continuous-flow conditions

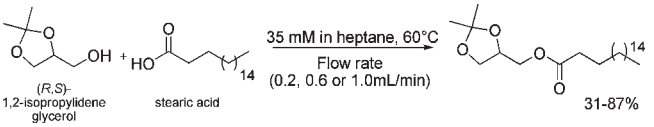

Table 1. Initial screening of RM IM lipase on the esterification of (*R*,*S*)-1,2-isopropylidene glycerol with stearic acid under continuous flow conditions

Q (mL/min)	residence time (min)	conversion (%)[a]
0.2	3.0	65
0.6	1.0	87
1.0	0.6	31

[a] Measured by Lowry and Tinsley assay and confirmed by GC−MS. Reaction conditions: 60 °C, stearic acid and (*R*,*S*)-1,2-isopropylidene glycerol 1:1 (35 mM).

Table 2. Real and coded values (+ higher level, 0 intermediate, − lower level) for the independent variables, 2^{3-1}

variables	−1	0	+1
T (°C)	40	50	60
S (mM)	35	67.5	100
Q (mL/min)	0.2	1.6	3

in *n*-heptane (Scheme 1). The results obtained are summarized in Table 1.

Preliminary results presented in Table 1 show moderate to good conversions when 0.2−0.6 mL/min flow rates are used. In order to have a better understanding on the flow rate/residence time role in the esterification reaction studied we decided to use the RSM tool to find the best reaction conditions.

To identify variables with important effects in our reaction system we performed the synthesis of solketoylestearate according to the initial screening, applying a fractional factorial design 2^{3-1}. Independent variables were temperature (*T*), flow (*Q*) and substrate concentration (stearic acid) (*S*), varying in two levels with three replications of the central point. The fractional factorial design (FFD) is presented in Table 2 that shows the variables with the respective levels used.

In a FFD 2^{3-1}, the main effects can be calculated and used to indicate which variables must be included in the following design as well as to define the new levels for variables. The results obtained by using the FFD matrix for the esterification reaction in continuous flow conditions are presented in Table 3.

As observed in Table 3, moderate results could be achieved for the esterification reaction between (*R*,*S*)-isopropylidene glycerol and stearic acid catalyzed by immobilized lipase from *Rhizomucor miehei* (RM IM) under continuous-flow conditions. The estimated effects for each parameter evaluated is presented in Table 4.

Variables *Q* and *S* were the ones showing significance in the process due to *p* value <0.05 and therefore selected to be optimized in the central composite rotatable design (CCRD).

Table 3. Matrix of the fractional factorial experimental design 2^{3-1} with coded values

entry	*T* (°C)	*Q* (mL/min)	*S* (mM)	conversion (%)[a]
1	−1 (40)	−1 (0.2)	+1 (100)	68
2	+1 (60)	−1 (0.2)	−1 (35)	61
3	−1 (40)	+1 (3)	−1 (35)	45
4	+1 (60)	+1 (3)	+1 (100)	51
5	0 (50)	0 (1.6)	0 (67.5)	66
6	0 (50)	0 (1.6)	0 (67.5)	64
7	0 (50)	0 (1.6)	0 (67.5)	65

[a] Measured by Lowry and Tinsley assay and confirmed by GC−MS.

Table 4. Estimated effect of parameters of FFD 2^{3-1} for studied enzyme

variables	effect	*p* value
mean	56.42	<0.0001[a]
curvature[b]	17.21	0.0038[a]
temperature (*T*)	−0.65	0.4524
amount of substrate *S*	6.85	0.0103[a]
flow (*Q*)	−16.25	0.0018[a]

[a] Statistically significant at 95%. [b] Obtained with all experiments from FFD.

Table 5. Complete factorial experimental design 2^2 with coded values

entry	*S* (mM)	*Q* (mL/min)	conversion (%)[a]
1	−1 (71.87)	−1 (0.4)	95 (92)[b]
2	−1 (71.87)	+1 (0.8)	57
3	+1 (95.27)	−1 (0.4)	52
4	+1 (95.27)	+1 (0.8)	61
5	−1.41 (67.5)	0 (0.6)	76
6	+1.41 (100)	0 (0.6)	59
7	0 (83.75)	−1.41 (0.3)	60
8	0 (83.75)	+1.41 (0.8)	61
9	0 (83.75)	0 (0.6)	80
10	0 (83.75)	0 (0.6)	83
11	0 (83.75)	0 (0.6)	82

[a] Measured by Lowry and Tinsley assay and confirmed by GC−MS. [b] Isolated yield.

The *T* variable did not show any significance and was kept constant (60 °C) in the next design of experiments.

The results obtained in entries 3 and 4 (45% and 51%, respectively, Table 3), indicate the negative influence of flow rate on the reaction conversion, which is confirmed by the negative value obtained in Table 4, where estimated main effects and their *p* values are shown.

The negative effect observed to variable *Q* indicates that higher conversion can be achieved as the flow rate decreases. Such results can be explained by the fact that in shorter flow rates there is increased residence time leading to higher conversion to products, since the contact time between enzyme and substrate is also increased.

The *S* variable had a 6.85 positive effect. This probably occurs since the presence of greater amounts of substrate increases

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Table 6. Effect of estimated parameters for CCRD 2^2 studied

variables	effect	p value[a]
mean	81.63	0.0001
flow	−15.78	0.0044
flow^2	−12.77	0.0096
concentration substrate	−6.8318	0.0232
concentration substrate^2	−19.78	0.0040
flow × concentration substrate	23.30	0.0041

[a] Statistically significant at 95% of confidence level.

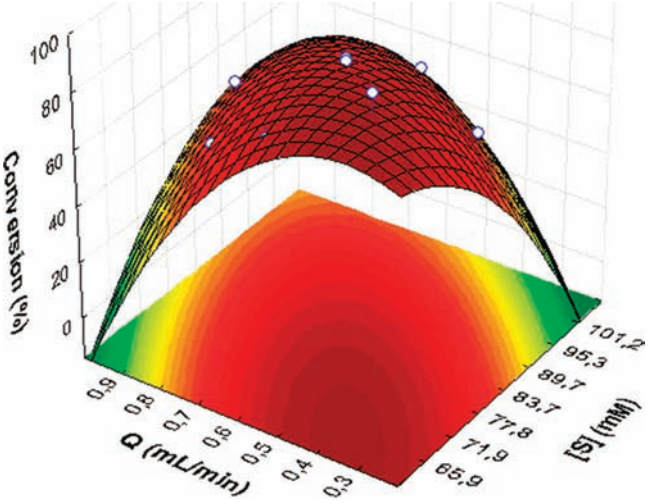

Figure 1. Response surface for the esterification reaction catalyzed by RM IM in continuous flow in the function of flow rate and substrate concentration.

Table 7. Variance analysis for validation of mathematical models (ANOVA)*

factor	sum of squares	degrees of freedom	mean square	F calculated	F tabulated	p-value
regression	1761.51	5	352.30	11.03	5.05	9.8×10^{-3}
residuals	159.66	5	31.93			
lack of fit	155.15	3				
pure error	4.50	2				
TOTAL	1921.16	10				

the substrate−enzyme contact, leading to increased yield conversion.

Table 5 shows 11 treatments of two selected variables and the conversion of each experiment. The first eight treatments are sufficient for determining the mathematical model and referred as complete factorial experimental design. The experiments from 9 to 11 are triplicates of central points for estimating the experimental error.

The highest conversion was obtained in experiment 1, indicating a negative flow rate effect in the process. It is important to note that a combination of factors must be performed to obtain higher conversions; in this case, low flow rates and low concentrations. Table 6 shows the estimated effects for CCRD. All variables and their quadratic and interaction effects were significant in the process. In the table we observe once again the negative flow effect on the studied range, justified by the increased substrate residence time. Substrate concentration, unlike the fractional design, showed a negative effect within the studied range.

Table 8. Effect of enzyme source on the esterification of (*R*,*S*)-1,2-isopropylidene glycerol with stearic acid under continuous flow conditions

entry	lipase	conversion (%)[a]
1	Cal. A	81
2	Cal. C	91

[a] Measured by Lowry and Tinsley assay and confirmed by GC−MS.

Negative effects of variables studied are easily observed in the surface response (Figure 1). When the flow decreases, ester conversion increases. This also happens with the substrate concentration in the studied range. There is an optimal working range between 0.3 to 0.4 mL/min for the flow and from 65.9 to 71.9 mM for the substrate concentration reaching up to 95.2% yields as observed in entry 1 in Table 5.

Equation 1 represents the conversion mathematical model to solketoilmonoestearate, depending on variables obtained from experimental data.

$$Y = 81.63 - 7.89Q - 6.38Q^2 - 3.41S - 9.89S^2 + 11.65Q \times S \quad (1)$$

where Y is the conversion percentage and, Q and S are the uncoded values of flow rate and substrate concentration, respectively. Statistical testing of models was carried out by the Fisher's statistical test for ANOVA (Table 7).

Regarding the analysis of variance (ANOVA), Table 7 shows the model validity by the F test and the residue showing the experimental error magnitude. The calculated F (11.03) was higher than the tabulated one, showing the experimental model's validity. The model can be checked by the determination coefficient (R^2). It implies that the 83% sample variation for ester production (R^2 = 0.83) is attributed to the independent variables and can be explained by the model accurately.

We have also explored the scope of enzyme source on the esterification reaction between (*R*,*S*)-1,2-isopropylidene glycerol and stearic acid. To this purpose, we have used Lipase Cal. A and Cal. C. under the best conditions obtained (60 °C/0.4 mL/min/71.87 mM). Results are shown in Table 8.

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

3. CONCLUSION

In conclusion we have developed a continuous flow process to the (*R*,*S*)-1,2-isopropylidene glycerol esterification with the assistance of the surface response methodology (RSM) and stearic acid, leading to the desired product in excellent conversions (95%) and short reaction time (40 s).

4. EXPERIMENTAL SECTION

4.1. Materials. Heptane was purchased from Tedia Co., (*R*,*S*)-1,2-isopropylidene glycerol from Sigma-Aldrich as well as all chromatographic standards. Stearic acid (>98%) was purchased from Vetec Ltd.a. Immobilized lipase (triacylglycerol hydrolase,

EC 3.1.1.3; Lipozyme IM-20, 25 BIU/g) from *Rhizomucor miehei*, supported on a macroporous weak anionic-exchange resin beads, was purchased from Novozymes.

4.2. GC−MS Analysis. The GC-MS analysis was performed by using modified method from EN 14105. Free fatty acids and (*R*,*S*)-1,2-isopropylidene glycerol were transformed into more volatile silylated derivatives in the presence of pyridine and *N*-methyl-*N*-trimethysilyltrifluoroacetamide (MSTFA). All GC−MS measurements were carried out in duplicate using a DB 5-HT (Agilent, J & W. Scientific, U.S.A.) capillary column (10 m × 0.32 mm × 0.1 μm). The quantifying was done on the basis of calibration curves with internal standards. The GC−MS samples were prepared by dissolving 0.1 g of the final product in 1 mL of *n*-heptane. One hundred microliters of this solution and pyridine solutions of butanetriol (1 mg/mL) and tricaprine (8 mg/mL), used as internal standards, were added into a flask that held 100 μL of MSTFA. After 15 min, these reactants were dissolved in 8 mL *n*-heptane. One microliter of this sample was then injected into a Shimadzu CG2010 equipment.

4.3. Lowry−Tinsley Analysis. The esterification rate was also measured using a modification of the Lowry and Tinsley[19] assay. 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 was calculated according to the percentage difference for the absorbance shown by the stock solution.

4.4. Continuous Flow Reaction Procedure. A 1-L HPLC bottle was equipped with desired reaction mixture in heptane and a stir bar. The starting mixture was stirred for 5 min, while the X-Cube (ThalesNano) instrument was equipped with the packed bed reactor containing immobilized lipase from *Rhizomucor miehei* (0.6 mL volume, 70 mm × 4 mm). The reaction parameters/temperature (40−60 °C), 0.1−3.0 mL/min flow rate and pressure (10 bar) were selected on the flow reactor, and processing was started, whereby only pure solvent (heptane) was pumped through the system until the instrument had achieved the desired reaction parameters and stable processing was assured. At that point the inlet tube was switched from the solvent flask to the 1-L HPLC bottle containing the freshly prepared reaction mixture. After processing through the flow reactor, the inlet tube was dipped back into the flask containing pure heptane and processed for 10 min further, thus washing from the system any remaining reaction mixture. The excess of heptane was removed under vacuum, and the product was obtained and analyzed by GC.

4.5. Statistical Analysis. The experimental designs and results analysis were carried out using the software *Statistica* 6.0 (Statsoft, Inc., U.S.A.), according to 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 is given by the multiple determination coefficients, R^2.

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