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Maximization of fructose esters synthesis by response surface methodology

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Enzymatic synthesis of fructose fatty acid ester was performed in organic solvent media, using a purified lipase from *Candida antartica* B immobilized in acrylic resin. Response surface methodology with a central composite rotatable design based on five levels was implemented to optimize three experimental operating conditions (temperature, agitation and reaction time). A statistical significant cubic model was established. Temperature and reaction time were found to be the most significant parameters. The optimum operational conditions for maximizing the synthesis of fructose esters were 57.1°C, 100 rpm and 37.8 h. The model was validated in the identified optimal conditions to check its adequacy and accuracy, and an experimental esterification percentage of 88.4% (±0.3%) was obtained. These results showed that an improvement of the enzymatic synthesis of fructose esters was obtained under the optimized conditions.

Introduction

Sugar esters are non-ionic biosurfactants that consist of a carbohydrate moiety as hydrophilic group and one or more fatty acids as lipophilic component(s). By controlling the esterification degree and the nature of fatty acid and sugar, it is possible to synthesize sugar esters within a wide range of properties.

An increasing interest in the production of sugar esters has been reported, because they can be used as surface-active components in many industrial fields, as cosmetics, health-care, pharmaceuticals and food industries [1,2]. Furthermore, these compounds have certain advantages over synthetic surfactants, such as being prepared from renewable sources; tasteless, odorless, stable over a broad pH range and non-irritant. In food industry, fructose esters can be used in the production of aromas and maturation of cheeses, bakery products, cakes and biscuits, mayonnaise and sauces, instant products and sausages, among others [3]. In addition, sugar ester properties as antibiotics [4], anti-tumor agents [5] and insecticides [6] are well reported and might open new markets. For the past few years, several researchers have investigated the lipase-catalyzed synthesis of sugar containing acrylic esters for their biomedical applicability [7–9]. Moreover, these compounds are biodegradable, biocompatible and essentially non-toxic [10,11].

Sugar esters can be synthesized either by chemical or enzymatic processes. Chemical production of sucrose esters is usually base-catalyzed at high temperatures, has a low selectivity, forming colored derivatives as side-products [1]. Enzymes have been successfully applied to the regioselective transformations of mono- and oligosaccharides, including acylation, deacylation and oxidation reactions. The enzyme-catalyzed synthesis of sugar esters provides regio- and stereoselective products [12–14]. Previously, sugar esters were synthesized mostly by esterification in aqueous media causing hydrolytic side reactions. To prevent these side reactions, solvents such as pyridine and dimethylformamide were used as reaction media [15]. However, the solubility of sugars and the activity of enzyme were decreased due to the increased hydrophobicity introduced by these organic solvents in the reaction system. In addition, the use of sugar esters as food additives and pharmaceuticals was incompatible with the use of these toxic solvents. Because of the high regiospecificity of enzymes, enzy-

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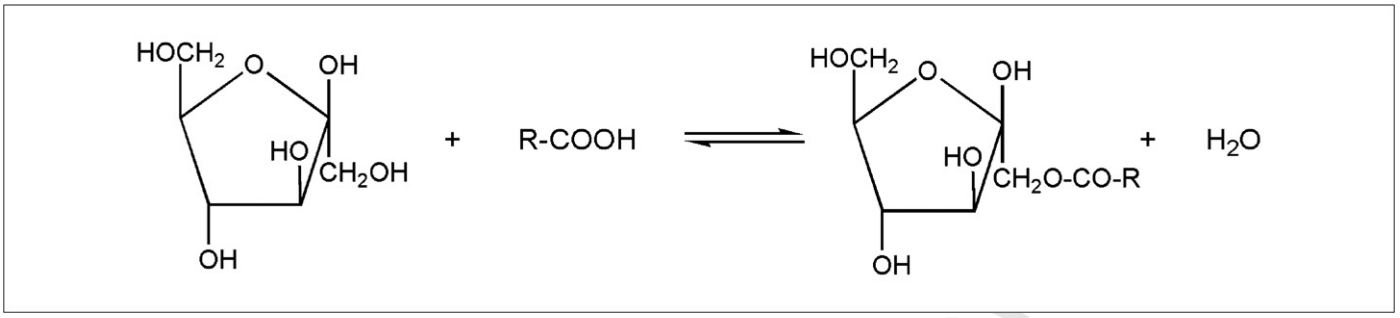

FIGURE 1

Lipase-catalyzed synthesis of fructose fatty acid esters [18].

matic synthesis is characterized by the production of a more defined product sugar mono ester, whereas chemical process usually leads to a mixture of sugar polyesters [16]. Therefore, various sugar esters (e.g. fructose or sucrose esters) can now be prepared by a single reaction step employing enzymes – lipase – as a biocatalyst [3,17,18]. Enzymatic synthesis in organic medium is based on the ability of lipases to catalyze reverse hydrolysis, that is, the formation of ester bonds. These reactions take place in a medium presenting a low water activity and allow much higher conversions in a shorter time. In these conditions, the thermodynamic equilibrium of the reaction is shifted towards synthesis reaction instead of hydrolysis. The enzymatic process yields up to 80% conversion within eight hours of incubation, and synthesis can be performed in a batch reactor at a temperature as low as 64°C in the presence of microbial lipase like *Candida antartica*. Enzymatic synthesis of fructose fatty acid esters is shown in Fig. 1.

The enzymatic esterification of sugar esters is gaining importance due to mild reaction conditions and excellent selectivity associated with lipase-catalyzed reactions. Therefore, the optimization of sugar ester synthesis is very important for its economical manufacturing [19]. Nevertheless, several factors can affect both the conversion yield and the rate of esterification. These factors include the reaction solvent, reaction temperature, reaction time, the type and concentration of the acyl donor, enzyme content and initial substrate concentration. Thus, it is difficult to search for the major factors and to optimize them because several parameters are involved [20]. The classical method of optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time consuming method often does not guarantee determination of optimal conditions, neither takes into account possible interactions among various operational factors. Experimental design and optimization are tools that enable building models and evaluating the significance of the different factors considered, as well as their interactions. Furthermore, with these models, a small number of experimental trials are used to search the optimal factor levels that conduct to the desired response [19,21].

The aim of this work is to optimize, using a response surface method (RSM), the operational conditions (temperature, agitation and reaction time) that maximize the synthesis of fructose esters. Therefore, a 2^k full-factorial central composite design, based on a preliminary design that used five factors (temperature, agitation, reaction time, fructose concentration and enzyme concentration) (*data not shown*) was conducted. Fructose esters are synthesized by esterification of oleic acid with fructose using a lipase from *C. antarctica* type B (CALB) immobilized in acrylic resin.

Materials and methods

Chemicals

All chemicals used were of analytical grade. The commercial triacylglycerol lipase from *C. antartica* B immobilized in acrylic resin (CALB) (Novozym 435) was purchased from Sigma–Aldrich (Sigma–Aldrich Co., St. Louis, MO).

Synthesis of fructose esters

The fructose esters synthesis experiments (esterification reactions) were conducted in flasks by adding oleic acid (0.5 mmol), fructose (0.6 mmol), CALB (12.5 mg), sodium sulfate anhydrous (0.1 g) and ethanol 99% (0.6 mL). The flasks were incubated for different reaction times under controlled agitation and temperature. The synthesis procedure has been previously described by Sabeder *et al.* [18].

Experimental design and data analysis

The optimal temperature, agitation and reaction time levels for maximizing the synthesis of fructose esters were studied using a 2^k full-factorial design with 3 factors and 3 replicates of the central point. On the basis of previous experiments (*data not shown*) and studies, the amounts of the reactants showed no significant effect on the esterification percentage.

The initial experimental design was augmented using an additional central composite design allowing the optimization of those experimental conditions of operation by means of a response surface methodology. Because the new runs were made after a period of 15-days from the initial ones, to control day-to-day variation, blocking technique was considered. Therefore, a new block of experimental data was included, consisting in nine other experimental points, being three of them replicates of the initial central point. The other six new points, also called star points, were introduced to make the central composite design rotatable, setting the distance from the central design equal to ±1.682. The parameter range was chosen according to previous experiments and reported knowledge on the optimum conditions for enzymatic synthesis of fructose esters [18,22–24]. In total, the 3 independent factors (temperature, agitation and reaction time corresponding to the actual factors x_1, x_2 and x_3, respectively) were studied at 5 levels (±1.682; ±1 and 0) and 20 experiments was carried out randomly (Table 1).

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TABLE 1

Experimental range and levels of the factors tested in the 2^3 full factorial central composite rotatable design

Variable	Symbol	Coded (X_i) variable level				
		−1.682	**−1**	**0**	**+1**	**+1.682**
Temperature (°C)	x_1	46.6	50	55	60	63.4
Agitation (rpm)	x_2	58	75	100	125	142
Reaction time (h)	x_3	3.8	12	24	36	44.2

For the statistical treatment, the actual factors were coded according to the following equation:

$$X_i = \frac{x_i - x_0}{\Delta x_i}, \quad i = 1, 2, 3 \tag{1}$$

where X_i is the coded value of the independent factor, x_i is the real value of the independent factor, x_0 is the real value of the independent factor at the central point and Δx_i is the step change value.

It is expected that the behavior of the system could be explained by a quadratic or cubic equation, which is used for predicting the optimal esterification percentage point (Y), based on the coded values of the independent factors (X_i):

$$Y = \beta_0 + \sum_{i=1}^{3}(\beta_i X_i + \beta_{ii} X_i^2 + \beta_{iii} X_i^3) + \sum_{i<j}^{3}(\beta_{ij} X_i X_j + \beta_{iij} X_i^2 X_j + \beta_{ijj} X_i X_j^2) + \beta_{123} X_1 X_2 X_3 + \alpha_{block} + \varepsilon \tag{2}$$

where Y is the predicted response, which takes into account the block correction (α_{block} is equal to the value of the block 1 or block 2 correction for the experimental data obtained at the first or second block of essays, respectively); the βs are the first, second and third order parameters whose values are to be determined using multiple linear regression model (MRLM) and the statistically significant ones selected using a stepwise method. The first order parameters are related with the screening process, the second order with the model curvature and the third order parameters due to asymmetry issues. Furthermore, to ensure that the model is hierarchical parameters could be included in the final model regardless of their statistical significance. X_1, X_2 and X_3 are the coded independent factors and ε is a random error term of the regression model.

Design-Expert 6.0.6., Trial version and Statistical Package for Social Sciences (SPSS), version 14, were used for the experimental design and regression analysis of the experimental data. The significance of the regression model was evaluated using analysis of variance (ANOVA). The quality of the fit obtained using the regression model equation was statistically checked by means of two diagnostic residuals: the multiple or adjusted coefficient of determination (R^2 or R^2_{adj}, respectively) and the predicted coefficient of determination (Q^2). The R^2-values describe the goodness of fit, giving an idea of how well current runs can be reproduced by the mathematical model. The Q^2-value describes the goodness of prediction, showing how well new experiments can be predicted using the mathematical model. R^2 and Q^2 values higher than 0.75 and 0.60 indicate that the model is good, and Q^2 values lower than 0.25 indicate that the model is useless [25].

The discrimination ability of the model was also inferred by calculating the adequate precision value, which compares the range of the predicted values at the design points to the average prediction error. A value greater than 4 is envisaged to assure adequate model discrimination. The significance of the regression coefficients was tested using a *t*-test. Also, the required non multicollinearity condition between the independent variables was evaluated using the variance inflation factor (VIF). Values lower than 10 should be obtained to ensure that the independent variables are not collinear. Finally, the contour plots obtained from the fitted quadratic or cubic model were also used to infer about the optimal experimental conditions keeping the independent factors within the experimental range studied. To validate the optimal operation condition levels that maximize the synthesis of fructose esters, 3 additional experimental trials were carried out at the optimal operating conditions to confirm the predicted esterification value obtained by the analysis of the response surface.

Quantification of fructose esters

The ester content was calculated taking into account the residual fatty acid amount in the reaction mixture, which was determined by the volumetric method. Briefly, 0.1 g of a sample from the reaction mixture was diluted in 20 mL of 0.1 wt% phenolphthalein solution in absolute ethanol, and then titrated with a sodium hydroxide solution (0.1 mol/L) [26].

Fructose ester purification and characterization

At the end of the esterification reaction, the lipase, together with the sodium sulfate anhydrous (non reactive species), was removed by filtration using filter paper with a pore-size of 60-μm (Macherey-Nagel Inc.). Afterwards, the ethanol was evaporated from the reaction media using a rotoevaporator. The remaining product (fructose ester) was then analyzed and identified by thin layer chromatography (TLC), using a chloroform/hexane (1:1, v/v) mixture for elution. Subsequently, the fructose ester spot was identified with iodine according to Ducret and collaborators' work [27].

Additionally, the purified reaction product was characterized by infrared spectroscopy (IR). The solid product was crushed with a mulling agent, Nujol. Subsequently, a thin film of the mull was applied on the surface of a NaCl cell and measured. Infrared absorption spectra were recorded on a Bio-Rad model FTS 165 spectrophotometer with a spectral band between 450 and 4000 cm^{-1}.

Results and discussion

Sugar esters have been attracting a considerable interest in several fields, such as food industry, mainly due to their advantages as compared to synthetic surfactants [1–3]. Sugar esters can be synthesized either by chemical or enzymatic processes, although enzymatic esterification of sugar esters is gaining importance due to mild reaction conditions and excellent selectivity associated with lipase-catalyzed reactions [18]. The rate of esterification, as well as the conversion yield, is affected by several factors such as the solvent, temperature, time, type and concentration of the acyl

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donor, enzyme content and initial substrate concentration [17,22,23,28,29]. Consequently, as several parameters are involved, the optimization of the esterification rate and conversion yield can be very laborious if no alternative approaches, such as experimental design and optimization tools, are used [20]. Even using an experimental design to build models and study interactions among different factors, when several factors are used the optimization may be difficult to assess. On the basis of this discussion, a preliminary design was conducted with five factors (temperature, agitation, reaction time, fructose concentration and enzyme concentration) (*data not shown*) and, although the ratio substrate/enzyme has been reported as an important factor [18], the significance of substrate and enzyme concentrations were found to be not significant, as well as their interactions. Also, it is well known that water concentration is a crucial factor in ester synthesis reactions catalyzed by lipases, because an excess of water would favor the reverse reaction, that is, the hydrolysis of ester bonds. Nevertheless, in the current work this factor was excluded from the design because a desiccant (sodium sulfate anhydrous) was added to the reaction medium to prevent the reverse reaction. Therefore, a response surface methodology (RSM) with a three-factor-five-level central composite rotatable design (CCRD) was employed for modeling and optimization of the enzymatic esterification of fructose esters. The influence of three operating variables, namely temperature, agitation and reaction time, on the esterification process was evaluated by means of a 2^3-full factorial rotatable central composite design. In total 20 runs were carried out, being six of them at the central point. The ranges of values of the variables used in the augmented experimental design were 46.6–63.4°C, 58–142 rpm and 3.8–44.2 h, respectively (Table 1).

A statistically significant cubic polynomial model ($P < 0.0001$) was fitted to the experimental data (Table 2) with an R^2-value and an R^2adj-value of 0.9995 and 0.9981, respectively. Two data blocks were considered for establishing the model allowing controlling day-to-day variation between the first 11 experimental runs and the last 9, corresponding to the initial design and to the augmented design, respectively. The model had no lack of fit ($P = 0.0227$), an adequate model discrimination (adequate precision value of 96.4) and a Q^2-value of 0.7056, showing a very satisfactory predictive performance of the model. The main effects, quadratic and cubic effects and interactions of the three operating variables on the esterification percentage were evaluated. Globally, all effects evaluated were statistically significant ($P \leq 0.0020$) except agitation ($P = 0.8625$). However, because second and third order interaction parameters involving agitation were statistically significant, agitation was included in the final form of the cubic model to ensure a hierarchical model. The parameters of the final cubic model estimated using the response surface methodology and their standard errors are shown in Table 3. Considering the results obtained Eq. (2) takes the simpler form of:

$$Y = \beta_0 + \sum_{i=1}^{3}(\beta_i X_i + \beta_{ii} X_i^2) + +\beta_{111} X_1^3 + \beta_{333} X_3^3 + \sum_{i<j}^{3}(\beta_{ij} X_i X_j) + \beta_{112} X_1^2 X_2 + \beta_{123} X_1 X_2 X_3 + \alpha_{block} + \varepsilon \quad (3)$$

TABLE 2

Experimental design and results (experimental and model prediction) obtained using the 2^3 full factorial central composite rotatable design used for the optimization of the synthesis of fructose esters

Run	Block	Temperature (°C)	Agitation (rpm)	Time (h)	Esterification (%)	
					Experimental	Model Prediction*
1	1	60	75	12	67.24	67.15
2	1	50	125	12	64.79	64.70
3	1	50	75	36	79.62	79.53
4	1	55	100	24	84.84	84.92
5	1	60	125	12	75.77	75.68
6	1	50	75	12	85.91	85.82
7	1	55	100	24	84.35	84.92
8	1	60	75	36	87.45	87.36
9	1	60	125	36	86.16	86.07
10	1	55	100	24	84.81	84.92
11	1	50	125	36	79.77	79.68
12	2	55	100	24	83.48	83.10
13	2	55	100	24	83.25	83.10
14	2	55	100	24	83.37	83.10
15	2	55	100	44.2	84.34	84.47
16	2	55	142	24	85.42	85.55
17	2	55	100	3.8	78.19	78.32
18	2	55	58	24	85.32	85.45
19	2	63.4	100	24	54.84	54.97
20	2	46.6	100	24	71.96	72.09

*Model predicted values include block corrections.

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TABLE 3

Regression parameters of the optimal cubic model selected using a stepwise method, for the 2^3 full factorial central composite design

Factor	β's coefficient (coded factors)	Standard errors	*P*-values
Intercept	84.0	0.2	<0.0001
Block 1 (α_{block})	0.9	–	–
Block 2 (α_{block})	−0.9	–	–
X_1	4.1	0.2	<0.0001
X_2	0.03	0.2	0.8625*
X_3	6.6	0.2	<0.0001
X_1^2	-6.9	0.1	<0.0001
X_2^2	0.9	0.1	0.0004
X_3^2	−0.6	0.1	0.0020
$X_1 X_2$	3.5	0.1	<0.0001
$X_1 X_3$	2.7	0.1	0.0001
$X_2 X_3$	1.4	0.1	<0.0001
X_1^3	−3.2	0.1	<0.0001
X_3^3	−1.7	0.1	<0.0001
$X_1^2 X_2$	−1.8	0.2	0.0004
$X_1 X_2 X_3$	−3.9	0.1	<0.0001

* Parameter with no statistical significance included to keep a hierarchical model.

Also, no statistical evidence of multi-collinearity was found because the variance inflation factor (VIF) values calculated for all the terms included in the model (linear, quadratic and cubic terms) were lower than 5. The predicted values were in good agreement with the experimental values (Table 2), showing that the cubic model could be used to predict and optimize the esterification percentage by determining the optimal operating conditions (temperature, agitation and reaction time). The optimization process was carried out based on the contour plots and the 3D response surface (Fig. 2). For optimization purposes agitation was set equal to 100 rpm (equal to the value of the initial design central point), because this effect was not statistically significant. Under this agitation, the fitted surface showed a possible maximum point, based on the contour plots analysis. The predicted maximum esterification percentage was equal to 89.8% (±0.4%) for a temperature of 57.1°C and a reaction time of 37.8 h. Three model validation experiments were carried out at those optimal operating conditions and showed good correspondence between experimental (88.4 ± 0.3%) and predicted maximum esterification percentage. Furthermore, according to Fig. 2 and model predictions, the conversion of fatty acid into ester after 3.5 h, 57.1°C and 100 rpm is about 78%. However, after 37.8 h under the same temperature and agitation conditions, conversion reaches 89.8%. Thus, from a practical point of view it would be beneficial to stop the reaction after 3.5 hours instead of waiting for the optimum reaction time to be reached (37.8 h) because it would cause a pronounced decrease in productivity (moles of ester per hour). It is important to notice that, before the optimization of the operational conditions for the enzymatic synthesis of fructose ester, the maximum esterification percentage obtained was 74.3 ± 0.2%, for 72 h, 40°C and 250 rpm. Also, enzymatic process yields up to 80% conversion have been reported for the synthesis of sugar esters using a lipase from *C. antartica* [3,17,18].

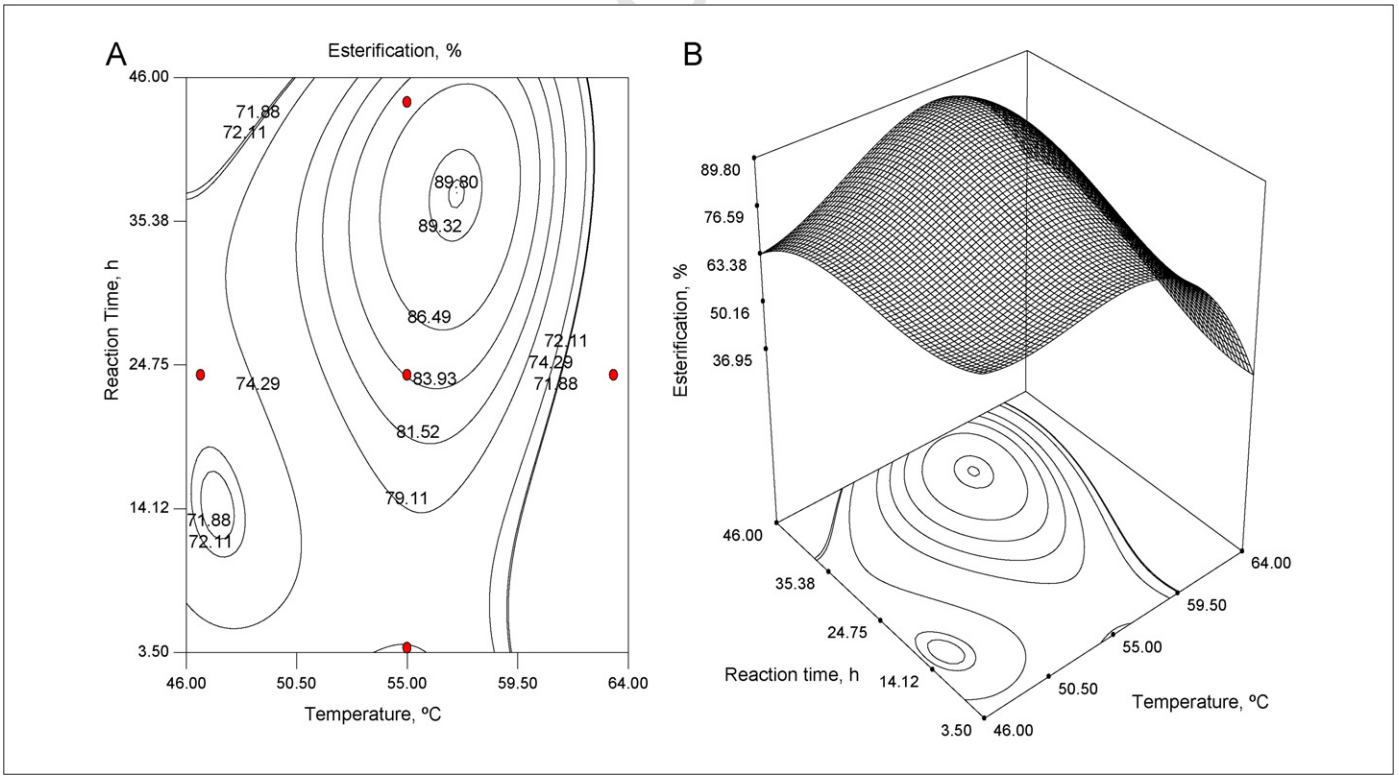

FIGURE 2

Response surface described by the model for an agitation set equal to 100 rpm (central point), in the region explored experimentally: **(a)** contour plots showing the predicted esterification percentage; **(b)** 3D surface.

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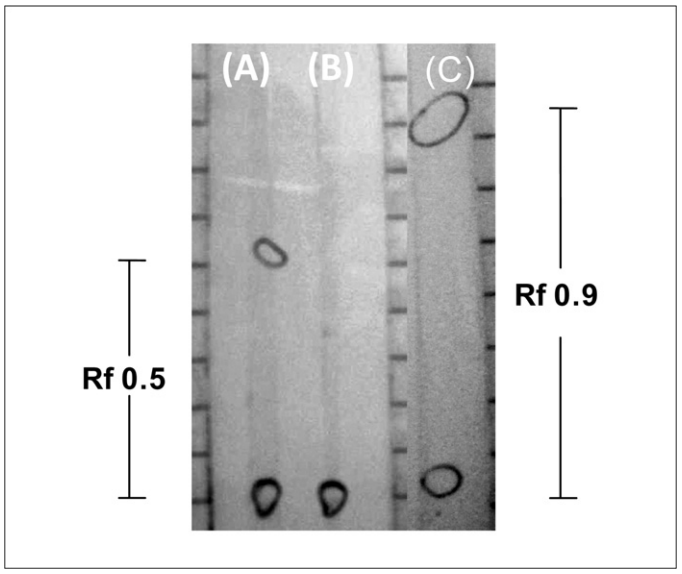

FIGURE 3

Thin-layer chromatography of the fructose ester. Lane **(a)** corresponds to the product synthesized at the validated maximum point of the design, namely the fructose ester. Lane **(b)** corresponds to a control experiment where no enzyme was used, thus no fructose ester was formed. Lane **(c)** corresponds to a standard of ethyl oleate.

Afterwards, the esterification product obtained at the validated maximum point was analyzed by TLC and IR spectroscopy to confirm the synthesis of the fructose ester according to the reaction scheme presented in Fig. 1, because ethyl oleate is a possible by-product of the reaction that occurs in the presence of ethanol and oleic acid [30–34]. The TLC plate is illustrated in Fig. 3, where a spot with a retention factor (*Rf*) (distance traveled by the compound divided by the distance traveled by the solvent) of 0.5 was found to correspond to the fructose ester. The fructose ester *Rf* value was found to be in accordance with previous reports [35,36]. Several authors reported higher *Rf* values ($Rf \sim 0.9$) for ethyl oleate ester [30,33,36,37], as confirmed also by TLC in the current work (Fig. 3 – lane C). Furthermore, the fructose ester was analyzed by infrared spectroscopy (Fig. 4) and the band peaks obtained confirmed the presence of an ester: 1741 cm^{-1} (C═O, ester); 2923 cm^{-1} (CH); 1463 cm^{-1} (CH_2); 1178 cm^{-1} (C═C) (Seino *et al.* [37]). Therefore, it was possible to conclude that the product synthesized at the maximum point of the experimental design corresponds to the expected fructose ester.

Although the enzymatic synthesis of highly biodegradable surfactants from renewable resources (sugar and fatty acids) has been widely investigated [18,28], statistical design of experiments and RSM have been only applied in a few studies [22,23]. Therefore, the current work can be regarded as a useful input for the development of more efficient processes for the enzymatic synthesis of fructose esters. However, it is important to refer that the results of such optimization are more limited in application than those which come from a more mechanistic standpoint, and therefore the optimum conditions found are only valid under the same experimental domain and reaction system used.

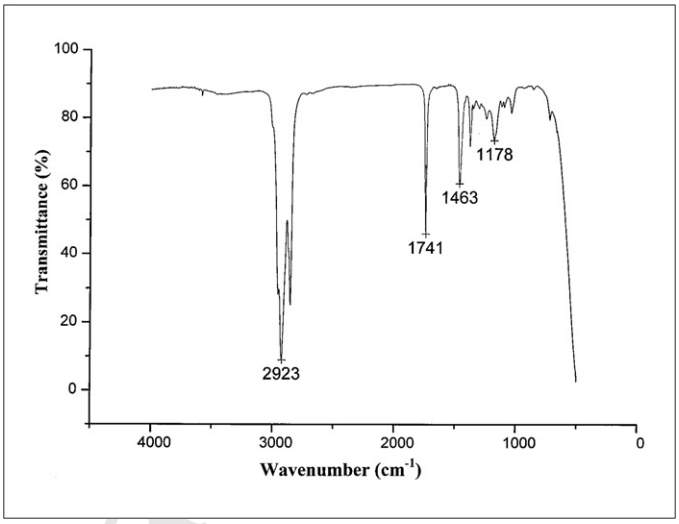

FIGURE 4

Infrared absorption spectra of the fructose ester obtained in the optimized conditions for enzymatic synthesis.

Conclusions

The modeling and optimization of immobilized *C. antartica* B catalyzed esterification reaction to synthesize fructose ester was successfully performed using a response surface methodology based on a central composite rotatable design (R^2 and Q^2 equal to 0.9995 and 0.7056, respectively). Furthermore, fructose ester was confirmed to be the product of the esterification process by TLC and IR spectroscopy. The effects of three main reaction operating parameters (temperature, agitation and reaction time) and of their interactions were evaluated over the given ranges. The results obtained showed that the established cubic model can be used to predict the esterification percentage under any given conditions within the experimental range. Moreover, under the optimized operating conditions an effective enhancement of the synthesis of fructose esters was achieved. An 88.4% (±0.3%) esterification percentage was obtained in a 37.8 h experiment conducted at 57.1°C and 100 rpm, which corresponds to an improvement of about 15% comparing to the values previously reported in the literature. Finally, this study can be seen as an effective contribution to the development of more efficient bioprocesses for industrial synthesis of fructose esters.

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