Biotecnia
Journal of biological and health sciences
http://biotecnia.unison.mx
Universidad de Sonora
ISSN: 1665-1456

Original Article

Synthesis of acylglycerols structured with n-3 PUFA in a packed bed reactor with recirculation: Op-timization by response surface

Síntesis de acilgliceroles estructurados con AGPI n-3 en un reactor de lecho empacado con recirculación: Optimización por superficie de respuesta

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ABSTRACT
The enzymatic esterification of n-3 PUFA and glycerol (G), catalyzed by a *Candida antarctica* lipase, was studied in a recirculating packed-bed reactor for the synthesis of acylglycerols. An n-3 PUFA concentrate was prepared by chemical hydrolysis of Menhaden's oil followed by urea treatment. A rotatable central composite design was used to evaluate the effect of molar ratio (0.47 – 5.52 mol n-3 PUFA / mol G), temperature (28.14 – 71.86°C) and time (0.24 – 2.76 h), on the production structured acylglicerols. The analysis of variance shows that all principal factors have a significant effect ($p < 0.05$). It was determined through response surface methodology that around 80 % of global esterification can be reached, by operating with a molar ratio of 0.5 mol PUFA/mol G, 70°C and 2.75 h. In general, models had a good adjust to experimental data ($R^2 > 0.92$), and the optimal operating conditions for the formation of the different acylglycerols produced during the esterification (monocylglycerols, diacylglycerols and triacyglicerols) can be established.
Keywords: Esterification, n-3 polyunsaturated fatty acids, immobilized lipase, HPTLC, regression models.

RESUMEN
Se estudió la esterificación enzimática de AGPI n-3 y glicerol (G) catalizada por una lipasa de *Candida antarctica* en un reactor de lecho empacado con recirculación para la síntesis de acilgliceroles estructurados. Se preparó un concentrado de AGPI n-3 por hidrólisis química de aceite de Menhaden seguido de un tratamiento con urea. Se utilizó un diseño central compuesto rotable para evaluar el efecto de la relación molar (0.47 – 5.52 mol AGPI n-3 / mol G), la temperatura (28.14 – 71.86 °C) y el tiempo (0.24 – 2.76 h) en la producción de acilgliceroles. El análisis de varianza mostró que todos los factores principales de los modelos presentaron un efecto significativo en las diferentes respuestas ($p < 0.05$). Mediante metodología de superficie de respuesta se determinó que se puede alcanzar una esterificación global cercana a 80 % al operar con una relación molar de 0.5 mol AGPI/mol G, 70 °C y 2.75 h de reacción. En general, los modelos tuvieron un buen ajuste a los datos experimentales ($R^2 > 0.92$) y permiten establecer las condiciones de operación óptimas para la formación de los diferentes acilgliceroles producidos durante la esterificación (monocilgliceroles, diacilgliceroles y triacilgliceroles).

Palabras clave: Esterificación, ácidos grasos poliinsaturados n-3, lipasa inmobilizada, HPTLC, modelos de regresión.

INTRODUCTION
N-3 polyunsaturated fatty acids (PUFA), usually present in fish oil, play several important health-related functions and are considered essential because the human body cannot synthesize them (Khan *et al*., 2023, Hernández *et al*., 2016; Baeza *et al*., 2014). Different scientific studies indicate that the consumption of n-3 PUFA reduces the risk for coronary heart disease, neuromuscular diseases, allergies, diabetes, cancer and depression (Kołodziej *et al*., 2023; Shahidi and Ambigaipalan, 2018; Rimm *et al*., 2018; Damasco *et al*., 2018; Hallahan *et al*., 2016; Wang and Huang, 2015; Castellanos and Rodríguez, 2015). The great benefits of n-3 PUFA consumption have motivated the study of the formation of structured acylglycerols (AG) enriched with these fatty acids for nutraceutical and pharmaceutical purposes. Enzymatic processes have multiple advantages over conventional chemical processes, such as operation under mild temperature and pH conditions, reduced energy cost, high catalytic efficiency, and a wide range of fatty acid selectivity of lipases leading to specific and pure products. One method for the production of FAs enriched with n-3 PUFAs is through the direct enzymatic esterification reaction of PUFAs and glycerol (Correa *et al*., 2017). During the stages of the esterification reaction, different products with high added value are produced such as monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG). MAGs are recognized for being excellent emulsifiers used for food, cosmetic and pharmaceutical applications. The consumption of oils consisting mainly of DAG contributes to weight loss, reduction of body fat, lower liver fat content, improvement of lipid metabolism by favoring oxidation and decreased storage of TAG in the adipose tissue (Nurul Nadiath *et al*., 2020).

The operation in packed bed reactors (PBR) presents multiple advantages compared to batch reactors, for example, it facilitates the contact and separation of the enzyme with the substrates (Zhang *et al*., 2022), allows the reuse of the enzyme, can be operated in continuous mode, and is usually more economical (Lee *et al*., 2016). Furthermore, immobilizing the enzyme provides it with greater catalytic and thermal stability, allowing a longer useful activity time. The immobilized

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Received: August 12,2024
Accepted: November 21, 2024
Published: December 17, 2024

DOI: 10.18633/biotecnia.v26.2414

lipase from *Candida antarctica* has shown good stability at high temperatures and long operating times (Monteiro *et al*., 2021). The development of a continuous process in an PBR is the most appropriate to carry out reactions catalyzed by lipases involving fats and oils for different industrial applications (Hong, 2014). However, when the substrates injected into the PBR are immiscible, such as n-3 PUFA and glycerol (G), the efficiency of the continuous process decreases considerably. When there is little miscibility in the substrates, an operation mode with recirculation can be chosen for the effective execution of the process (Zhao *et al*., 2012).

Regression analysis using an experimental design, is the construction of predictive models that adequately describe the relationship between the dependent and independent variables of a study process. When the variables of a bioprocess are measurable, continuous and controllable during the experimental runs, a response surface methodology (RSM) study can be carried out considering a rotatable central composite design (RCCD) to determine the effect of the variables and optimize bioprocess responses (Filli *et al*., 2010). RSM is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. The most extensive applications are particularly in situations where several input variables influence performance measures or quantity or quality characteristics of a product or a process (Montgomery, 2019).

In the present work, the effect of temperature, molar ratio and time on the direct enzymatic esterification reaction of n-3 PUFA and G, catalyzed by the immobilized lipase of *Candida antarctica* (NV-435) in a PBR with recirculation, was studied by means of a series of experiments based on a RCCD and analyzed using response surface methodology to optimize the production of structured acyglycerols.

MATERIAL AND METHODS

Reagents

Menhaden fish oil (Sigma-Aldrich) and food grade glycerol (J. T. Baker) were used for the reaction medium; tertbutylhydroxyquinone (TBHQ, Química Dresen) as an antioxidant, and dimelsulfoxide (DMSO) as an emulsifier. The NV-435 lipase from *Candida antarctica* (Novo Nordisk Denmark) immobilized in macroporous acrylic resin was used as catalyst. For high-performance thin layer chromatography (HPTLC) analysis, 10x10 cm plates (Uniplate) were used with analytical grade silica gel, hexane and ethyl ether (Sigma-Aldrich), glacial acetic acid (Fermont) and reagent grade iodine (Fermont).

Isolation and concentration of PUFA

To isolate and concentrate PUFAs from Menhaden oil, the procedure described by Correa *et al*. (2017) was used. One hundred g of oil were weighed and TBHQ (0.02 % w/w) was added as an antioxidant. The mixture was saponified with 200 mL of 7 M KOH in 70 % ethanol by heating at reflux for one hour. A liquid extraction was carried out with distilled water (240 mL) and hexane (200 mL), the aqueous phase was then acidified to pH 1.0 with HCl to subsequently extract the free fatty acids (FFA) with hexane (200 mL). The hexane was removed by evaporation in a rotovapor (40 rpm, 40°C, vacuum 25 mmHg), and the remaining moisture was removed with anhydrous sodium sulfate. In a flask, each 15 g of FFA were mixed with 25 g of urea in 100 mL of 95 % ethanol and heated until the mixture turned a homogeneous yellow color. The solution was transferred to centrifuge tubes and quickly cooled in an ice water bath for subsequent cooling (4°C, 8 h). The crystals formed were separated by centrifugation at 6000x*g* (15 min, 0°C). The supernatant was acidified to pH=4 and transferred to a separatory funnel to extract the PUFA with a 1:1 hexane:water mixture, stirring for 30 min. Finally, the organic phase was evaporated in a rotary evaporator (40 rpm, 40°C, vacuum 25 mmHg) to obtain the PUFA concentrate.

Enzymatic esterification in PBR with recirculation

The experimental runs to study the enzymatic esterification reaction were carried out based on a RCCD. Mixtures of 5 g with different molar proportions of n-3 PUFA and G (0.48, 1.5, 3, 4.5, 5.52 mol/mol), TBHQ (0.02 % w/ p) as antioxidant, and DMSO (1 % w/w) as emulsifier, were prepared in glass vials (20 mL) with magnetic stirrer. Each vial was placed inside a double arm cooling jacket reactor (Wheaton Celstir®) placed on a magnetic stirring plate at 800 rpm. After stirring the mixture for 30 minutes, a peristaltic pump was used to recirculate the medium at a flow rate of 1.65 mL/min through an PBR (length = 5.104 cm and diameter = 0.717 cm) with 0.9115 g of immobilized lipase NV- 435 of *Candida antarctica*. A water recirculator bath was used to maintain an isothermal process, experimental runs were carried out at different operating temperatures (28.14, 37, 50, 63, 71.86 °C) and 35 µL samples were taken at different reaction times (t = 0.24 , 0.75, 1.5, 2.25, 2.76 h). A representative diagram of the process is shown in Figure 1.

Analysis of acylglycerols (AG) and global esterification

The analysis was carried out in a HPTLC equipment (CAMAG). Aliquots (5 µL) of the sample were diluted in a chloroform:methanol solution (2:1 v/v) to obtain a final concentration of 20 µg AG/ µL sample. One microliter of each diluted sample was applied on a silica gel plate 1 cm from the edge and with a separation of 1 cm between each sample. The plate was saturated with the mobile phase of hexane:ethyl ether (38:62 v/v) with 1 % glacial acetic acid in a vertical double-channel glass chamber for thin layer chromatography by 40 minutes. The plate was placed into the mobile phase for chromatographic development until a path of 9 cm was reached. The plate was then removed from the chamber and the solvent was evaporated into the air. The plate was developed by atomizing a solution of iodine in ethanol (0.5 % w/v), until achieving a light yellow color on the surface of the plate and the solvent was cold evaporated for 30 min. The developed plate was scanned, and shadow area analysis of the spot bands was performed using ImageJ 1.45 software

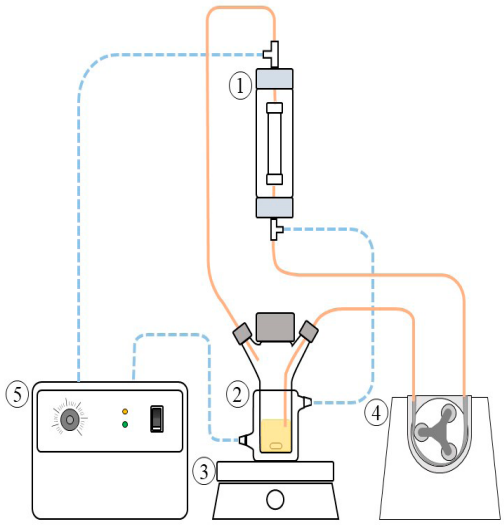

Figure 1. Operation diagram of the enzymatic esterification reaction in the packed bead reactor (PBR) with recirculation. 1: jacketed PBR, 2: jacketed double sidearm Celstir®, 3: magnetic stirrer, 4: peristaltic pump and 5: recirculator water bath with temperature control.
Figura 1. Diagrama de operación de la reacción de esterificación enzimática en el reactor de lecho empacado (RLE) con recirculación. 1: RLE enchaquetado, 2 Celstir ® de doble brazo enchaquetado, 3: agitador magnético, 4: bomba peristáltica y 5: baño recirculador de agua con control de temperatura.

(NIH; Rasband, 2018). For the quantification of MAG, DAG and TAG, a standard curve was generated using lipid standards (Sigma-Aldrich) containing a mixture of *cis*-9-monoolein, *cis*-9-1,2-diolein, *cis*-9-1,3-diolein and *cis*-9-triolein.

The G concentration was calculated using a stoichiometric mass balance considering the concentrations of MAG, DAG and TAG, determined by HPTLC analysis using the following equation:

$$C_G = Co_G - (C_{MAG} + C_{DAG} + C_{TAG}) \quad (1)$$

To determine the global esterification (GE), 30 µL aliquots were taken and diluted in ethanol. The samples were titrated with a 0.05 N NaOH solution using phenolphthalein as an indicator.

Experimental design and statistic analysis

A RCCD was selected to evaluate the effect of the molar ratio between PUFA and G (x_1=*M*), temperature (x_2=*T*) and time (x_3=*t*), on GE (y_1), MAG (y_2); DAG (y_3) and TAG (y_4) production.

To predict the dependent variables (y_i) a third order polynomial regression model was considered:

$$y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \beta_{iii} x_i^3 + \sum_{i=1}^{k-1} \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

Where β_0, β_i, β_{ii}, β_{iii}, and β_{ij} represent the interaction coefficients in the regression, the linear effect, quadratic effect, cubic effect and the combined effects, respectively.

A simplified model was considered for each response according to the significative variables (α = 0.05) resulted from the analysis of variance. Statistical analysis was performed in a JMPpro v17.0 ® platform.

RESULTS AND DISCUSSION

Table 1 records the experimental results of the enzymatic esterification of n-3 PUFA and G from the different experimental runs established for the RCCD. The coefficient of determination (R^2) is a value that varies from 0 to 1 that expresses the prediction of a model to the experimental data, with a value close to 1 indicating a better prediction, however, in these models the value of R_{adj}^2 is usually considered, which is a corrected measure of goodness of fit to determine the precision of the model that identifies the percentage of variance in the target field that is explained by the model inputs. Gutierrez and De la Vara (2012) suggest that an R^2 greater than 0.75 is adequate to determine a good fit of the model to the experimental data. In this study, R^2 greater than 0.92 and R_{adj}^2 values greater than 0.74 were obtained. Different bioprocess investigations have obtained R^2 values similar to those reported in this study (Pereira *et al*., 2018). The statistical analysis showed that the models had a significant effect with respect to the different responses ($p < 0.05$).

The F statistic was performed in the ANOVA, high F values were obtained, higher than the critical values, which indicate that the models are statistically significant (Nahemiah *et al*., 2015). The results of the statistical analysis of the models are recorded in Table 2. To determine the fit model for each of the responses, the least significant parameters ($p > 0.2$) were eliminated to optimize the value of R_{adj}^2.

Global esterification (GE)

The parameters with the greatest effect on the GE are the linear and quadratic terms of the molar ratio and reaction time ($p < 0.005$). In the analysis of variance, the effect of temperature did not have a representative effect on GE ($p = 0.3954$), but it was included in the equation to have a hierarchical model because the interaction term between temperature and time had a significant effect ($p < 0.1$). The model to describe the GE obtained through second-order polynomial regression is the following:

$$GE = 3.019\,M^2 - 6.157\,t^2 - 2.647\,M\,t + 0.223\,T\,t - 15.369\,M + 21.593\,t - 0.180\,T + 48.124 \quad (3)$$

The RSM graphs show that a GE close to 80 % can be achieved by operating with a molar ratio of 0.5 mol PUFA/mol G and a temperature of 70°C for 3 h (Fig. 2a). It is important to clarify that by decreasing the molar ratio (M), n-3 PUFAs have more binding sites in the G structure due to the surplus of this substrate considering that the stoichiometric ratio is 3:1 mol PUFA/mol G, therefore, it is normal to obtain higher degrees of esterification. Linder *et al*. (2005) studied the enzymatic esterification reaction of PUFA and G in a batch reactor, and obtained a higher GE at a molar ratio of 0.83 mol PUFA/mol G at 46°C. It is important to highlight the high reaction rate that occurs in the PBR, since a high GE was

Table 1. Experimental and predicted results of the enzymatic esterification of n-3 PUFAs and G in the PBR with recirculation.
Tabla 1. Resultados experimentales y predichos de la esterificación enzimática de los AGPI n-3 y G en el RLE con recirculación.

Run	Factors			GE%		MAG%*		DAG%*		TAG%*	
	M	***T***	***t***	exp	pred	exp	Pred	exp	pred	exp	pred
1	1.5	37	0.75	43.76	40.48	56.87	56.61	28.13	28.28	15.00	14.22
2	1.5	37	2.25	50.16	52.79	59.86	61.39	22.00	24.00	18.14	17.08
3	1.5	63	0.75	59.64	42.86	59.42	59.76	26.91	26.03	13.67	13.86
4	1.5	63	2.25	61.44	59.93	53.41	52.88	26.88	26.68	19.71	20.18
5	4.5	37	0.75	42.17	41.91	40.68	43.40	28.89	28.45	30.43	29.47
6	4.5	37	2.25	42.17	43.33	48.04	48.18	27.53	27.77	24.43	23.56
7	4.5	63	0.75	46.49	44.28	53.14	53.80	26.64	26.20	20.22	20.59
8	4.5	63	2.25	49.70	50.46	46.16	46.91	29.04	30.45	24.80	25.08
9	0.47	50	1.5	65.29	66.25	50.99	65.62	26.61	26.19	22.40	22.81
10	5.52	50	1.5	33.12	59.49	51.38	49.49	29.72	29.50	18.89	19.31
11	3	28.14	1.5	42.75	39.61	55.20	53.39	28.62	27.69	16.18	18.07
12	3	71.86	1.5	47.10	47.61	55.05	54.97	27.77	28.05	17.18	16.12
13	3	50	0.24	23.09	26.18	47.45	46.50	26.30	27.48	26.25	26.67
14	3	50	2.76	43.86	41.73	50.79	44.73	29.28	27.45	19.93	20.34
15	3	50	1.5	44.44	43.61	52.72	45.62	22.82	35.89	24.46	21.37
16	3	50	1.5	38.88	43.61	43.55	45.62	36.00	35.89	20.45	21.37
17	3	50	1.5	44.19	43.61	45.00	45.62	35.68	35.89	19.33	21.37
18	3	50	1.5	43.50	43.61	48.93	45.62	25.26	35.89	25.81	21.37

M= molar ratio (mol PUFA / mol G), T= Temperature (°C), t= time (h), GE = Global esterification (%), MAG % = Monoacylglycerols (% mol), DAG % = Diacylglycerols (% mol), TAG % = Triacylglycerols (% mol).
* The percentage molar fraction was calculated based on the total fatty acids.

Table 2. Statistical terms obtained in the statistical analysis of the GE, MAG %, DAG % and TAG % models.
Tabla 2. Términos estadísticos obtenidos en el análisis estadístico de los modelos de GE, % MAG, % DAG y % TAG.

Statistic	GE	MAG%*	DAG%*	TAG%*
R^2	0.946	0.927	0.922	0.921
R^2_{adj}	0.899	0.854	0.834	0.747
F value	23.913	12.740	10.467	5.301
P value	<0.0001	0.0017	0.0028	0.0392

GE = Global esterification (%), MAG % = Monoacylglycerols (% mol), DAG % = Diacylglycerols (% mol), TAG % = Triacylglycerols (% mol).
* The percentage molar fraction was calculated based on the total fatty acids.

obtained at relatively short operating times, unlike different studies in intermittent reactors where long reaction times are required to achieve a significant GE (Noriega *et al*., 2013; Wang *et al*., 2011). The RSM of temperature versus molar ratio provides very interesting information (Fig. 2b). Firstly, the model suggests that at higher molar ratios (M > 4) the GE can increase a little, however, it is not advisable to operate under these conditions because PUFA are the limiting reagent in esterification, and these are the most expensive substrate of the process due to its laborious obtaining process (Torres *et al*., 2014). Secondly, it can be seen that temperature is not as significant a variable in the GE as time and molar ratio, obtaining similar GE at different operating temperatures in relation to the proportions of the substrates in the medium. Correa *et al*. (in press) studied the kinetics of enzymatic esterification of n-3 PUFA and G at different temperatures in a batch reactor, and it was observed that an increase in temperature raised the GE reached during the reaction relatively little.

Synthesis of monoacylglycerols

MAGs are the first product formed in the enzymatic esterification reaction, and as the reaction proceeds, the formation of DAG consumes the MAGs present in the reaction medium. The parameters with the greatest effect on the percentage molar fraction of MAG (% MAG) are the linear term of the molar ratio, and the quadratic terms of the molar ratio and temperature ($p < 0.005$). The statistical model that describes the MAG % is:

$$MAG\,\% = 1.876\,M^2 + 0.018\,T^2 + 0.093\,M\,T - 0.299\,T\,t - 19.099\,M - 1.585\,T + 14.251\,t + 107.631 \quad (4)$$

Figure 3 shows the RSM graphs of the MAG %. The model suggests that the MAG % can be optimized, reaching 80 %, by operating with a molar ratio of 0.5 mol PUFA/mol G and 30°C for 2.75 h. Byun *et al*. (2007) studied the formation of MAG in the enzymatic esterification of fish oil, and determined that, at low operating temperatures, greater MAG formation occurs. Similarly, Zhao *et al*. (2011) sought to optimize the formation of MAG in an enzymatic esterification reaction without solvents, and obtained conversions of 49.6 % at a molar ratio of 0.16 mol AGL/mol G and a temperature of 50°C. Figure 3b shows the response surface of the molar ratio against temperature (t = 0.5 h), observing that operating with higher molar ratios can slightly increase the MAG %, this result is similar to that obtained by Noriega *et al*. (2013), in which the enzymatic esterification reaction of n-3 PUFA and G was studied in an intermittent reactor.

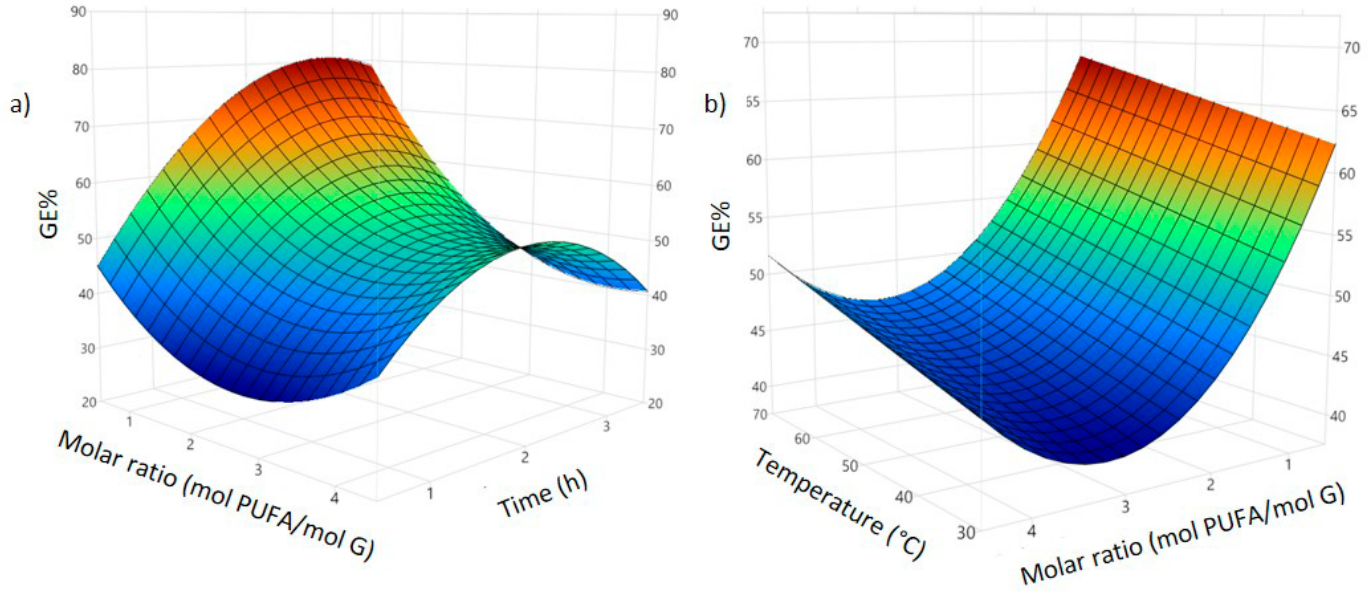

Figure 2. Response surface for global esterification (GE) in the PBR with recirculation. a) versus molar ratio and time at T=70°C, b) versus temperature and molar ratio at t = 1.5 h.
Figura 2. Superficie de respuesta para la esterificación global (EG) en el RLE con recirculación. a) contra la relación molar y el tiempo a T=70°C, b) contra la temperatura y la relación molar a t = 1.5 h.

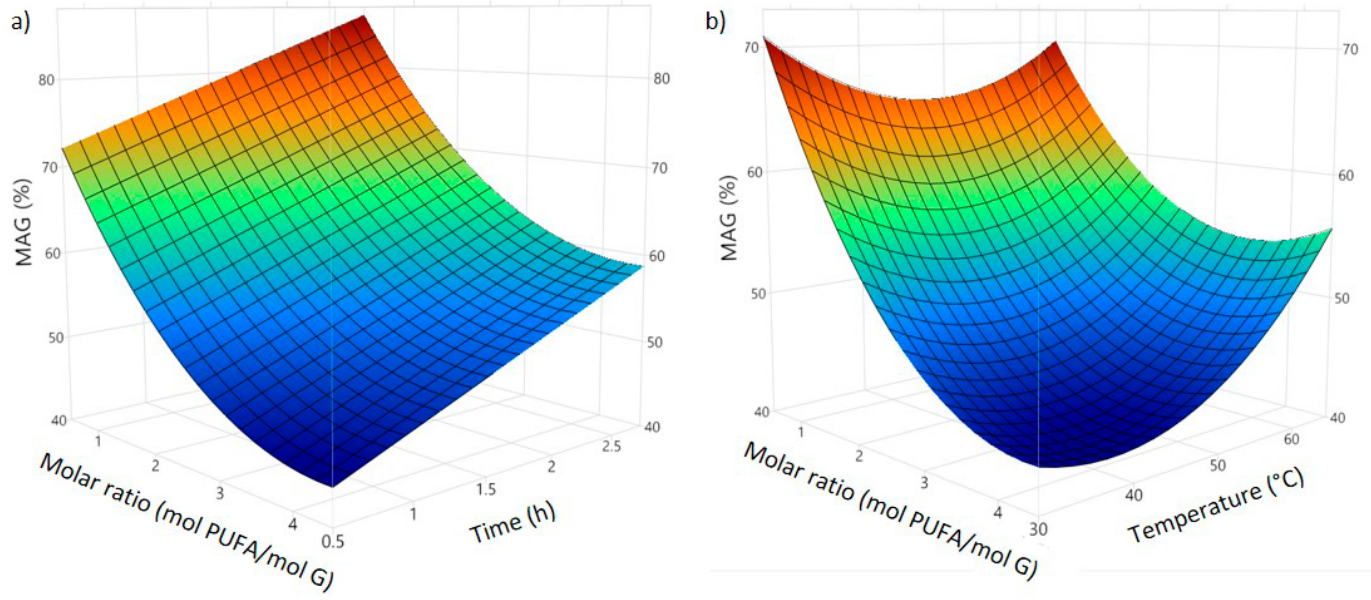

Figure 3. Response surface for monoacylglyerols (MAG) in the PBR with recirculation. a) versus molar ratio and time at T = 28°C, b) versus temperature and molar ratio at t = 0.5 h.
Figura 3. Superficie de respuesta para los monoacilgliceroles (MAG) en el RLE con recirculación. a) contra la relación molar y el tiempo a T = 28°C, b) contra la temperatura y la relación molar a t = 0.5 h.

Synthesis of diacylglycerols

DAGs are an intermediate product in the enzymatic esterification reaction, however, these are lipids with high added value due to their multiple benefits. The parameters with the greatest effect in the model are the quadratic effects of time, molar ratio and temperature, presenting values $p < 0.001$. The model that determines the percentage mole fraction of DAG (% DAG) is the following:

$$DAG\,\% = -1.265\,M^2 - 0.016\,T^2 - 5.297\,t^2 + 0.799\,M\,t + 0.126\,T\,t + 7.045\,M + 1.497\,T + 7.166\,t - 18.672 \quad (5)$$

The RSM analysis performed on temperature versus time (Fig. 4a) shows that at a temperature of 50.33°C, molar ratio of 3.26 mol PUFA/mol G and reaction time of 1.52 h, the production of DAG can be maximized in the enzymatic esterification reaction (DAG %=36). This behavior is due to the fact that, in the reaction mechanism, at one of the first stages

of esterification, the MAG react with PUFA to form DAG, but in the next stage these are consumed for the formation of TAG. Different investigations have published kinetic profiles of DAG formation similar to those obtained in this investigation (Correa *et al*., 2017; Bornadel *et al*., 2013). Duan *et al*. (2010) sought to optimize the formation of 1,3-DAG from an enzymatic esterification reaction in a batch reactor. They obtained optimal formation conditions at 60°C and a molar ratio of 2.5 mol FFA/ mol G, reaching a concentration of 40 %. Lo *et al*. (2007) studied the enzymatic esterification of palmitic and oleic acid catalyzed by the lipozyme lipase RM IM, obtaining optimal conditions for the formation of DAG at 66.29°C, operating with a molar ratio of 2.14 mol FFA/mol G. Although the results obtained differ slightly from those reported by other researchers, the enzymatic esterification reaction presents many factors that can influence the performance and behavior of the process, such as the composition of the substrate (Rivero *et al*., 2020; Kahveci and Xu, 2011), the lipase used and the water present in the reaction medium.

Synthesis of triacylglycerols

Due to the low goodness of fit of a second-order polynomial model (R^2=0.74) to describe the percentage mole fraction of TAG, it was decided to consider a third-order model that included the combined effect of all factors and their respective cubic terms. The terms with the greatest effect in the model are the quadratic and cubic terms of the molar ratio ($p < 0.01$). The equation that describes the % TAG is:

$$\%\,TAG = -0.844\,M^3 - 3.695\,t^3 + 0.059\,M\,T\,t + 7.550\,M^2 - 0.009\,T^2 + 17.97\,t^2 - 0.153\,M\,T - 4.139\,M\,t - 8.384\,M + 1.042\,T - 22.073\,t + 5.029 \quad (6)$$

TAGs are the products generated at the last stage of the enzymatic esterification reaction; this is reflected in their low concentration obtained in the experimental results due to the reversibilities present during the reaction. The model obtained through RSM indicates that higher molar fractions of TAG are obtained by increasing the reaction time and with n molar ratio of approximately 4.5 mol PUFA/mol G, reaching a molar fraction close to 26 % (Fig. 5a). The low TAG conversions achieved in the experimental runs may also be due to the reversible effects caused by the water produced during the esterification reaction, for this reason, many investigations seek to eliminate or isolate the water present in the medium to optimize the process and obtain better performances in the formation of TAG (Rodrigues and Fernandez, 2010). Noriega *et al*. (2013)-studied the yield of TAG formation in the enzymatic esterification of PUFA catalyzed by the NV-435 lipase. After a 24 h reaction they only reached yields close to 12 %, so they added molecular meshes in order to isolate the water in the reaction medium and after another 24 h they achieved yields close to 80 %; this implies that the water caused strong reversible effects in the formation of TAG. The MSR graph of temperature versus molar ratio (Fig. 5b), at t=1.5 h, reflects that higher TAG conversions can be obtained at temperatures close to 50°C.

It is recommended that the model obtained be considered only within the operating range established in the experimental design because, although a good fit (R^2=0.92) is observed, certain inconsistencies occur when extrapolating the study range. Studying a process through an experimental design is very effective to contemplate the effect of factors

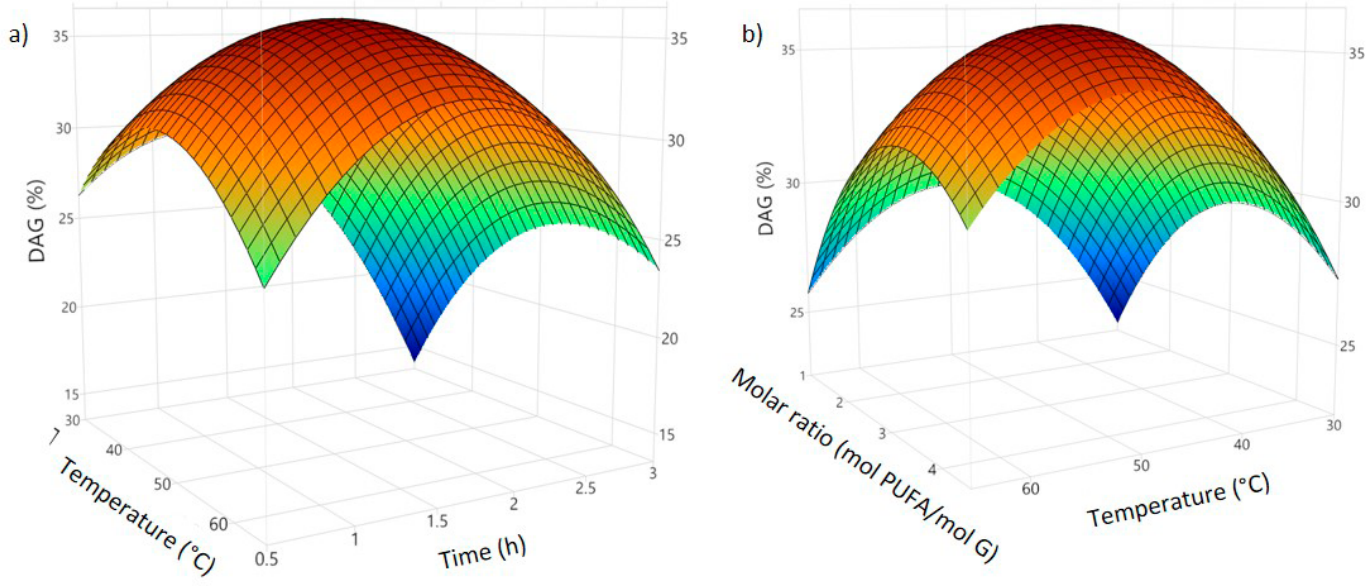

Figure 4. Response surface for diacylglycerols (DAG) in the PBR with recirculation. a) versus temperature and time at molar ratio =3.26, b) versus temperature and molar ratio at t=1.5 h.

Figura 4. Superficie de respuesta para diacigliceroles (DAG) en el RLE con recirculación. a) contra la temperatura y el tiempo a una relación molar =3.26, b) contra la temperatura y la relación molar a un t=1.5 h.

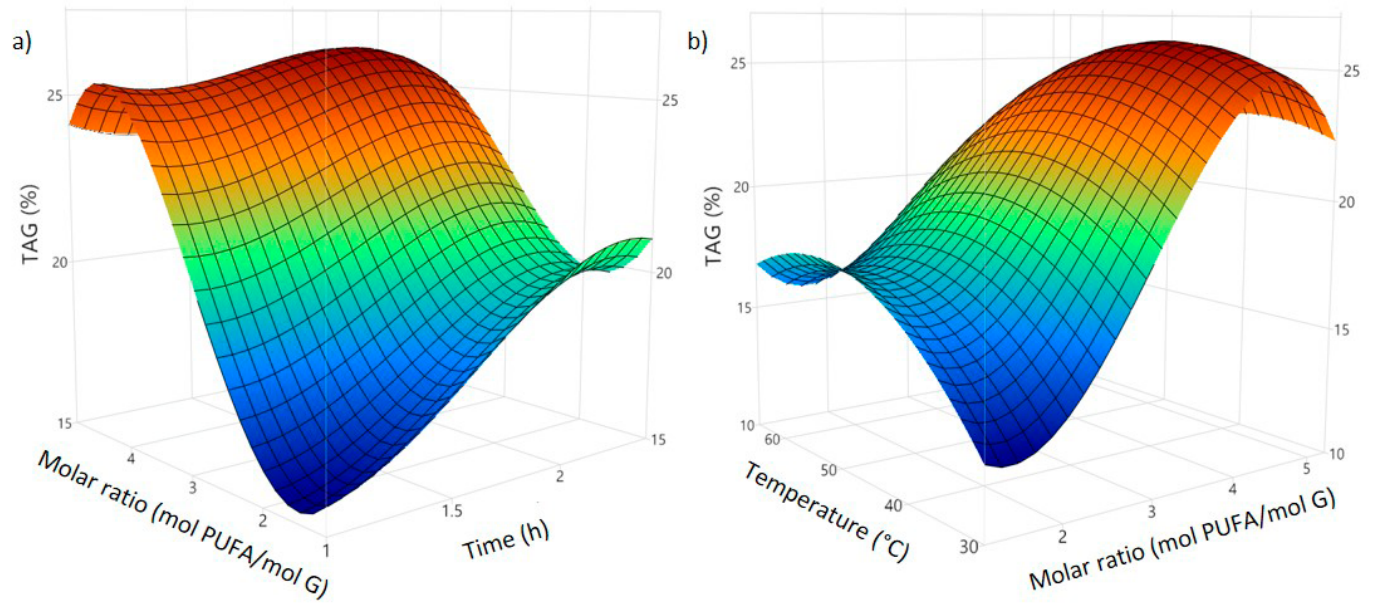

Figure 5. Response surface for triacylglycerols (TAG) in the PBR with recirculation. a) versus molar ratio and time at T = 50°C, b) versus temperature and molar ratio at t = 1.5 h.
Figura 5. Superficie de respuesta de triacilgliceroles (TAG) en el RLE con recirculación. a) contra la relación molar y el tiempo a T = 50°C, b) contra la temperatura y la relación molar a t = 1.5 h.

on the responses analyzed, but the models are usually limited to values established at the treatment levels (Montgomery, 2019).

CONCLUSIONS

The operation of the PBR with recirculation presented multiple benefits compared to an intermittent reactor, such as high degrees of esterification at relatively short operating times, the possibility of reusing the immobilized lipase which, throughout the experiments, did not present apparent activity loss and in addition, resulted in an easy products separation. The experimental design considered to evaluate the effect of the ratio of substrates, temperature and operation time on the synthesis of structured acyglycerols allows the optimization of reaction conditions depending on the desired product. The analysis of variance showed that the main factors of the models presented a significant effect on the different responses ($p < 0.05$), and the established models had a good fit to the experimental data ($R^2 > 0.92$). With RSM, it was determined that a GE close to 80 % can be achieved when operating with a molar ratio of 0.5 mol PUFA/mol G, 70°C and 2.75 h of reaction. To maximize the production of MAG, it is recommended to operate with a molar ratio of 0.5 mol PUFA/mol G and 30°C, for 2.5 h of reaction, obtaining 80 % mol MAG. The maximum formation of DAG, close to 36 mol %, is reached with a ratio of 3.26 mol PUFA/mol G, 50.3 °C and 1.5 h of reaction. As for TAG, a molar fraction of 26 % was achieved when operating with a ratio of 4.5 mol PUFA/mol G, 50 °C and 2 h of reaction. The water produced during the reaction caused considerable reversible effects during the reaction, which may favor the production of MAG, but impair the formation of TAG. This study can be considered as a reference for the design and scaling of similar bioprocesses.

Abbreviations

AG	Acylglycerol
ANOVA	Analysis of variance
DAG %	Percent molar fraction of diacylglycerols
DAG	Diacylglycerol
FFA	Free fatty acids
GE	Global esterification
G	Glycerol
MAG %	Percent molar fraction of monocylglycerols
MAG	Monoacylglycerol
PBR	Packed bed reactor
PUFA	Polyunsaturated fatty acid
RCCD	Rotatable central composite design
RSM	Response surface methodology
TAG %	Percent molar fraction of triacylglycerols
TAG	Triacylglycerol

Acknowledgments

Author Correa-Leyva gratefully acknowledges financial support for his PhD studies (scholarship 590674) from CONAHCYT (Mexico).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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