Article

LIPASE FROM *Rhizopus oryzae* IMMOBILIZED ON CORN COB POWDER: A NEW APPROACH FOR DIETETIC TRIGLYCERIDE SYNTHESIS IN A FIXED BED REACTOR

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This work explores the possibility of using corn cob powder as a carrier for immobilization by physical adsorption of lipase from *Rhizopus oryzae* used in acidolysis reaction of grape seed oil with capric acid (C10) to synthesize dietary triglycerides (TAGs) formed from medium-long-medium chain (MLM) fatty acids in a fixed bed reactor. First, enzymatic loading was investigated to improve hydrolytic activity and the immobilized biocatalyst was characterized in terms of biochemical parameters and thermal stability. An enzyme load of 30 mg mL^{-1} was quite adequate, as about 90% of the immobilization yield was achieved. The central composite rotatable design (CCRD) experimental design revealed that, for the immobilized enzyme, the optimal hydrolytic activity was obtained at pH 7.75 and 41 °C. Stirred tank reactions were optimized in CCRD statistical analysis revealing the most desirable conditions for molar ratio of 1:9.36 (oil:acid), 45 °C and 15% biocatalyst, obtaining a maximum ID value of 86.93 ± 2.25%. In a fixed bed reactor, the synthesis showed an average ID of 51.13 ± 0.77%, demonstrating the potential of this configuration. It is concluded that corn cob powder as an immobilization carrier is a promising alternative for the enzymatic synthesis of MLM triacylglycerols.

Keywords: dietary triglycerides; grape seed oil; immobilization; corn cob powder; fixed bed reactor.

INTRODUCTION

Lipases (EC 3.1.1.3), belonging to the class of hydrolases, are enzymes that catalyze reactions of fats and oils, and are also capable of catalyzing various reactions in both aqueous and non-aqueous media. These biocatalysts are commonly present in plants, animals and microorganisms and are responsible for catalyzing a series of chemical reactions, such as complete or incomplete triacylglycerol hydrolysis and chemical reactions of esterification, interesterification and transesterification in lipids, being widely applied in the food industry, detergent industry, wastewater treatment, cosmetic industry, pharmaceutical industry, biomedical testing and in the bioenergy sector.[1] The specificity of lipases is a significant factor for their industrial applications. Depending on this characteristic, lipases can be categorized into three types: sn-1,3 specificity, sn-2 specificity and non-specificity. Specific lipases 1,3, such as lipases from *Rhizopus oryzae* (ROL), hydrolyze the acylglycerols present in the sn-1 and sn-3 positions of the TAG, producing free fatty acids, 2-monoacylglycerols and 1,2- or 2,3-diacylglycerols, preventing final production of glycerol. Fungal lipases have been widely studied given the known potential of these native microorganisms to produce enzymes and their remarkable unique catalytic properties. On an industrial scale, one of the most used lipase-producing fungi belongs to the genera *Rhizopus* sp., and, specifically, according to data from the Web of Knowledge, lipase from ROL is one of the most studied enzymes of this fungus, due to the relevance of the lipolytic activity of this enzyme.[2]

Enzymatic catalysis is a promising modification method; however, the cost of enzymes is still the main obstacle to fully exploiting their potential. An alternative is to immobilize this biocatalyst, since its immobilization leads to greater stability and easy recovery from the reaction medium, in addition to allowing its reuse. An enzyme should be immobilized when bound or confined to a material, otherwise known as carrier, which is incapable of moving freely in the solution.[3] Each immobilization method has advantages and disadvantages; therefore, it is worth highlighting that there is more than one immobilization method or carrier applicable to all enzymes, regardless of the properties of the substrate, the physicochemical characteristics of each enzyme and the applications of the products obtained. The immobilization process defines the efficiency of the lipase, influencing its properties in a specific reaction and its catalytic properties. Immobilization by physical adsorption is a simple, easy and cheap technique, although it presents weak interactions between the enzyme and the carrier.[4] The main advantage of this method is that there are no noticeable changes in the structural configuration of the lipase caused by this binding mode, maintaining a high activity of the immobilized lipase.[5] The increasing of industrial and agricultural production leads to greater generation of agro-industrial waste, which is around 200 million tons worldwide. As a result, short-term solutions to reuse these wastes are fundamental, as the use of agro-industrial wastes as immobilization carriers will reduce process costs and improve performance, in addition to offering a new ecologically friendly use.[6] Corn production is one of the most important agricultural activities around the globe, and corn cob is one of its main residues. In addition to being a biodegradable and low-cost material, due to its physical and chemical characteristics it can be reused as a carrier to immobilize enzymes.[7,8] There are some works in literature[6-9] on the use of corn cob powder as immobilization carrier, thus its use is rather attractive as an innovative carrier in the immobilization of lipase from *R. oryzae*. In previous studies by the group,[10-13] commercial lipases were applied, corroborating the deepening of results using the enzyme immobilized in this innovative carrier.

Still, in addition to substantial scientific findings on the effects of food on human health, an increased search for quality of life is evidently observed, as there is greater consumer demand for healthier products nowadays. Oils and fats are the most common basis of food, but they can trigger several diseases when consumed in excess, which in turn afflicts millions of individuals with obesity, diabetes, high cholesterol, cardiovascular diseases, among other conditions.[11]

In this context, dietary triglycerides stand out in comparison with conventional ones, since they not only prevent such diseases, but also promote a beneficial effect to the body due to their lower caloric value,

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greater solubility and facilitated metabolism. Among these, MLM-type triglycerides stand out as they are characterized as having a medium-chain fatty acid (M) conformation in the sn-1 and sn-3 positions and a long-chain fatty acid (L) in the sn-2 position, thus enhancing the nutraceutical properties of triacylglycerol. This modification has as main advantages its therapeutic, functional and nutritional effects, such as increasing satiety and disposition, they are quickly metabolized, which contributes to controlling obesity and reducing fat deposition, also in pancreatic insufficiency and metabolic diseases, in addition to improve the technological properties of lipids, with better fluidity, elasticity, melting point or high temperature behavior.[11,12]

Such a positive effect of dietary triglycerides on health arouses interest in their use for treating and preventing diseases, as there have been studies aimed to find healthier alternative sources to commonly used oils.[14] Substrates with a high content of long-chain polyunsaturated fatty acids have been explored for new technologies such as for the synthesis of MLM triacylglycerols, with grape seed oil (GSO) being a rich source containing 58-78% linoleic acid. GSO, a valuable byproduct generated mainly from the wine industry, is rich in vitamin E, antioxidants and essential oils, in addition to being used in various branches of industry with great potential for application in the pharmaceutical, cosmetic and food industries,[15] thus corroborating the importance of developing technologies to disseminate their use. The synthesis of these products involves the process of acidolysis, where a fatty acid or a fatty acid ester is used as an acyl donor and the lipase promotes the modification of the TAGs of the substrate. These regiospecific changes in the glycerol structure of TAG produce specific restructured end products, with altered physicochemical properties, in addition to the formation of byproducts, such as MAGs and DAGs of interest.[16]

Selection of proper reactor configurations and operating conditions is critical to achieving high reaction yields. The synthesis of dietary triglycerides can be performed both in reactors operating in batch mode and in continuous, semi-continuous mode.[12,17] The fixed bed reactor (FBR) is most commonly used in industrial-scale enzymatic processes, as it facilitates the recovery of the biocatalyst, in addition to ensuring efficiency and allowing mass production. In general, the use of FBR presents advantages such as simple operation, construction and maintenance, low cost, in addition to intrinsic kinetic advantages in relation to stirred tank reactors for most types of bioprocesses, guaranteeing higher rates and lower risk of disintegration of the support by mechanical shear, as the biocatalyst particles are fixed.[18] This reactor configuration is still not extensively explored, particularly in the synthesis of TAGs,[10,11,19] thence demonstrating its innovative character.

Therefore, this work was aimed to synthesize dietary MLM-type triglycerides through an acidolysis reaction of grape seed oil with capric acid (C10) catalyzed by lipase from *R. oryzae* immobilized by physical adsorption on corn cob powder in a fixed bed reactor. Its results are going to allow an advance in the area of biotechnology, as it offers good prospects for the production of MLM-type dietary triglycerides. For this reason, this paper provides a vision of perspectives, presenting the progress achieved, seeking a state-of-the-art industrial scenario that envisages an environmentally benign environment and economically competitive waste-based bioprocesses for enzyme immobilization.

EXPERIMENTAL

Materials

The experiments were carried out using food-grade lipase from *R. oryzae*, kindly provided by Prozyn (São Paulo, Brazil). Corn cob powder was used as immobilization carrier, acquired from the company Sagran Indústria e Comércio de Ingredientes Rações Ltda, Andira, PR. Grape seed oil (Distriol, São Paulo), capric acid - C10 (Sigma Aldrich, Germany), commercial olive oil (purchased locally), hexane (Sigma-Aldrich, Germany), ethanol 98°GL (Sigma-Aldrich, Germany) and acetone (Sigma-Aldrich, Germany) were used in the reactions. Other reagents were of analytical grade.

Carrier treatment and immobilization by physical adsorption

The carrier was sieved using a 42/60 mesh, collected and autoclaved at 120 °C for 20 min with the addition of 70% ethanol in the ratio of 1:10 (m v^{-1}). Then, distilled water was added in the same proportion and left overnight. Afterwards, the carrier was filtered and treated with 2 mol L^{-1} sodium hydroxide solution in a 1:10 ratio, cob:solution (m v^{-1}), stirred for 24 h at room temperature (25 °C on average), and then washed thoroughly with warm distilled water.[10]

For immobilization, hexane was added to the treated carrier in a ratio of 1:10 (m v^{-1}), and then kept under gentle stirring for 2 h at room temperature (25 °C on average). Next, 20 mL of a lipase solution (phosphate buffer 0.1 mol L^{-1} pH 7.0) was added for each gram of carrier. It was once more left under gentle stirring for 2 h at room temperature (25 °C on average). After this period, suspension was incubated for 24 h under refrigeration at approximately 9 °C. Subsequently, the immobilized biocatalyst was filtered using a paper filter and a vacuum pump, and then stored under refrigeration. The hydrolytic activity of the enzyme preparations was determined using the oily emulsion method of olive oil, water, phosphate buffer (0.1 mol L^{-1} and pH 7.0) and gum arabic. The analysis was carried out in a shaking bath at 37 °C for 5 min and was stopped by adding 10 mL of the water:acetone:alcohol solution (1:1:1, v/v/v), adding 3 drops of phenolphytalein, as an indicator and 10 mL of 0.05 M potassium hydroxide. The released fatty acids were titrated with 0.05 M hydrochloric acid, previously standardized, and calculated based on a methodology adapted from Soares *et al.*[20]

The calculation of hydrolytic activity was carried out according to Equation 1:

$$A\left(\frac{U}{g}\right) = \frac{(Vb - Va) \times M \times 1000}{t \times ms} \quad (1)$$

where: Vb is the volume of hydrochloric acid used in the blank titration (mL); Va: volume of hydrochloric acid used to titrate the sample (mL); M: molarity of the titrant (mol L^{-1}); t: reaction time (5 min); ms: dry mass of the enzyme (g), expressed by Equations 2 and 3.

For the free enzyme:

$$ms(g) = \frac{mt \times 1\ mL \times 100}{vt \times (100 - u)} \quad (2)$$

where: mt is the total mass of enzyme used (g); vt: volume of buffer used in the enzyme solution (mL); u: humidity of the free enzyme (%).

For the immobilized enzyme:

$$ms(g) = \frac{mu_{imob} \times 100}{100 - u_{imob}} \quad (3)$$

where: mu is the wet mass of enzyme used (g); u_{imob}: humidity of the immobilized enzyme (%).

The Figure 1 shows the preparation scheme for lipase immobilization.

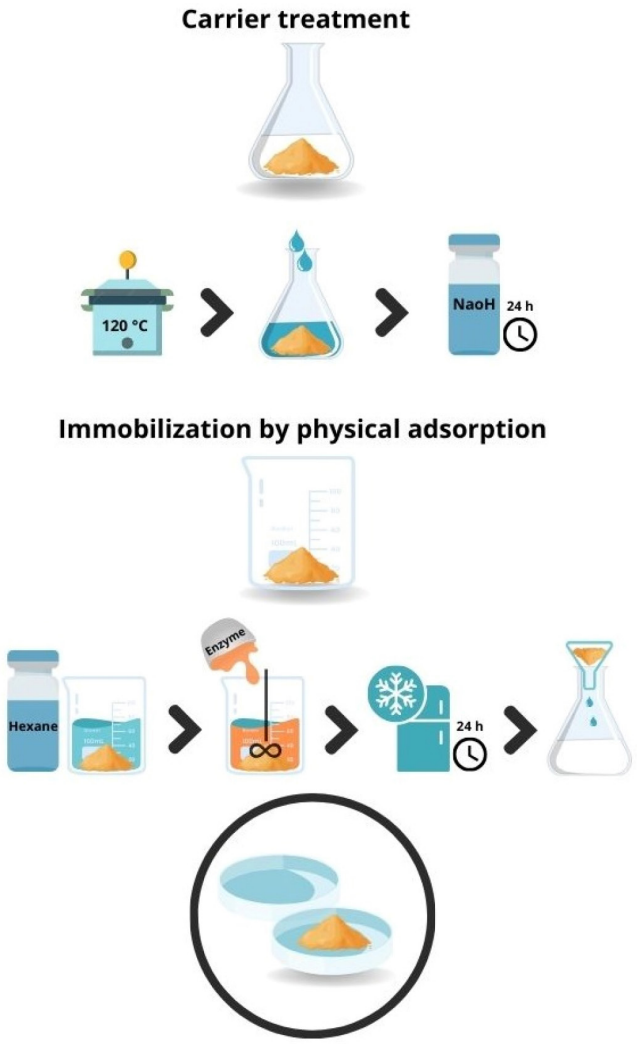

Figure 1. *Scheme of the preparation of enzymes process; carrier treatment and immobilization by physical adsorption*

Enzyme loading test

Enzyme solutions were prepared in phosphate buffer in 0.1 mol L^{-1} at pH 7.0 and different concentrations, *i.e.* 10, 15, 20, 30 and 35 mg mL^{-1}. A 1 g carrier sample was used for immobilization by varying the enzyme solution concentration. The suspension was separated in a funnel to collect the supernatant, and then it underwent filtration and drying. Hydrolytic activities of the control (without enzyme), supernatant and immobilized enzyme were measured. For comparison purposes, their fixed asset yield and recovered activity were observed and calculated according to Lima *et al.*[21]

The fixed asset yield (AY) was calculated using Equation 4:

$$AY\,(\%) = \frac{At}{A0} \times 100 \quad (4)$$

To calculate the recovered activity (RA) using Equation 5:

$$RA\,(\%) = \frac{Ader}{At} \times 100 \quad (5)$$

where: At is the theoretically immobilized activity (U g^{-1}); Ader: activity of the immobilized derivative (U g^{-1}).

A_0 is the activity offered during immobilization, measured in the control and calculated by Equation 6:

$$A_0\ (U/g) = \frac{At_0 \times me}{ms} \quad (6)$$

where: At_0 is the activity of the enzyme solution (U g^{-1}); me: enzyme mass (g); ms: support mass (g). At: is the theoretically immobilized activity, calculated using Equation 7:

$$At(U/g) = A_0 - Af \quad (7)$$

Af is the non-immobilized activity, measured by the supernatant, calculated using Equation 8:

$$Af(U/g) = \frac{Atf \times v}{ms} \quad (8)$$

where: Atf is the final activity of the supernatant (U mL^{-1}); v: final volume of the supernatant (mL); ms: support mass (g).

Immobilized enzyme characterization

Optimal pH and temperature

Optimal pH and temperature of the immobilized enzyme were defined by carrying out a 2^2 central composite rotational design (CCRD) using the software Protimiza Experimental Design®.[22] Tests were performed at different incubation bath temperatures and pH conditions for the phosphate buffer in 0.1 mol L^{-1} solution used in the enzymatic solution. The response variable was hydrolytic activity.[20] Table 1 shows the values selected for each variable under analysis.

Table 1. Real and coded values of independent variables analyzed using a central composite rotatable design for the immobilized enzyme characterization

Variables	Level				
	–1.41	–1	0	+1	+1.41
Temperature (°C)	24	30	45	60	66
pH	5.0	5.5	7.75	10.0	10.5

Thermal stability

To evaluate the immobilized enzyme thermal stability, 0.25 g samples were incubated in a 2 mL phosphate buffer (0.1 mol L^{-1} and pH 7.0) and then placed in a bath at different temperatures (35, 41 and 45 °C) under agitation. For comparative purposes, the same tests were performed for the free enzyme using an enzyme solution of 0.5 mg mL^{-1}. At each pre-established time (0, 15, 30, 45 and 60 min), samples were immediately cooled in an ice bath, then a 1 mL aliquot of the enzyme solution and immobilized enzyme sample was removed, in addition to a 2.5 mL olive oil emulsion so as to measure the hydrolytic activity[20] of samples.

Kinetics and thermodynamics

Kinetic and thermodynamic deactivation parameters were determined for free and immobilized enzymes. The thermal inactivation constant k_d (min^{-1}) and half-life time $t_{1/2}$ (min) of free and immobilized enzyme were calculated by performing a nonlinear regression of the relative hydrolytic activity as a function of time,[23] according to Equations 9 and 10:

$$A_R = A_{R0} \times e^{-k_d t} \quad (9)$$

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (10)$$

where: A_R is the relative enzyme activity (%) at incubation time t (min); A_{R0}: initial relative enzyme activity (%).

Deactivation energy E_d (J mol^{-1}) was obtained by a linear regression of k_d as a function of temperature T (K) calculated according to Equation 11:[23]

$$k_d = A \times e^{-\frac{E_d}{RT}} \quad (11)$$

where: R is the universal gas constant (8.314 J K^{-1} mol^{-1}); A: Arrhenius' constant.

The stability factor SF (adimensionaless) determines immobilization efficiency, which is calculated by Equation 12:[23]

$$SF = \frac{t_{1/2immobilized}}{t_{1/2free}} \quad (12)$$

where: $t_{1/2immobilized}$ is the immobilized enzyme half-life time (min); $t_{1/2free}$: that of the free enzyme (min).

The enthalpy ΔH (J mol^{-1}), Gibbs free energy ΔG (J mol^{-1}) and entropy ΔS (K = kJ mol^{-1}) required for enzyme thermal denaturation was calculated according to Equations 13, 14 and 15, respectively:[24]

$$\Delta H = E_d - RT \quad (13)$$

$$\Delta G = -RT\ln \frac{k_d h}{k_B T} \quad (14)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (15)$$

where: h is the Planck's constant (11.04 × 10^{-36} J min^{-1}); k_B: Boltzmann's constant (1.38 × 10^{-23} J k^{-1}).

Nonlinear regressions were performed using the OriginPro 2022® software (Origin Lab Corporation, Washington, USA).[25]

Grape seed oil characterization

Grape seed oil was characterized based on its peroxide index (PI) defined according to the American Oil Chemist's Society Cd 8b-90 method.[26]

Synthesis of triglycerides in batch stirred-tank reactions (BSTR)

For synthesizing the triglycerides, a reaction medium containing grape seed oil and capric acid (C10) was prepared in different proportions to obtain a final volume of 20 g. Then, an amount of biocatalyst was added to the mixture and placed in a stirred tank reactor with 6 cm in height and 3 cm in diameter operating in batch mode whose temperature was controlled by a thermostatic bath. Reactions were maintained for 24 h under magnetic stirring at 200 rpm. Ultimately, the medium and enzyme were separated, and then the medium was stored under refrigeration at approximately 7 °C.

To define optimal molar ratio parameters for capric acid, reaction temperature and amount of biocatalyst to be used in the synthesis of triglycerides, a 2³ central composite rotational design (CCRD) was carried out using the software Protimiza Experimental Design®.[22] Table 2 shows the values selected for each variable under analysis.

Table 2. Real and coded values of independent variables analyzed using a central composite rotatable design of batch reactions

Variables	Level				
	–1.68	–1	0	+1	+1.68
Molar ratio (v v^{-1})	2.64	4	6	8	9.36
Temperature (°C)	28.18	35	45	55	61.82
Biocatalyst (% m m^{-1})	6.59	10	15	20	23.41

Hydrodynamic characterizations of the fixed bed reactor

The mixture was characterized through the determination of axial dispersion described by Zanin and Moraes.[27] Space-time, τ (min), was calculated according to Levenspiel.[28] Residence time distribution was experimentally determined by pulse-type injection of a Rosa Mix™ dye solution (São Paulo, Brazil, purchased from a local market) into a jacketed acrylic reactor with 20.5 cm in a height and 2.2 cm in diameter to be subsequently used in the reactions. Tracer concentration was measured spectrophotometrically (545 nm) at the reactor outlet (GENESYS 10S UV-Vis; Thermo Fisher Scientific, San Jose, CA, USA) as a function of time. Residence time distribution (DTR) function, E(t), was calculated according to Equation 16 considering constant volumetric flow:

$$E(t) = \frac{C(t)}{\int_0^\infty C(t)dt} \quad (16)$$

where: C(t) is the concentration at each time; $\int_0^\infty C(t)dt$: area under the tracer concentration curve as a function of time data by the integral.

Mean residence time, tm (min), was calculated according to Equation 17.

$$tm = \int_0^\infty E(t) \times t\, dt \quad (17)$$

Equations 8 and 9 were calculated using the OriginPro 2022® software (Origin Lab Corporation, Washington, USA).[25]

The recalculated space-time, τ* (min), is demonstrated by Equation 18.[29]

$$\tau^* = \frac{V_{total} + V_c}{v} \quad (18)$$

where: V_{total} is the total reactor volume (mL); V_c: volume of connections (mL); v: flow (mL min^{-1}).

Synthesis of triglycerides in fixed bed reactor (FBR)

Regarding the synthesis of TAGs in a fixed bed reactor, a jacketed acrylic reactor with 20.5 cm in height and 2.2 cm in diameter was used and operated at a temperature of 41 °C, and the reaction was kept at a feed rate of 1 mL min^{-1} in flow rising for 96 h. The reaction medium consisted of grape seed oil and capric acid (C10) in a molar ratio of 1:3 and the reactor bed was filled with 15 g of the immobilized biocatalyst. Aliquots were collected at 0, 1, 2, 4, 8, 24, 46, 72 and 96 h.

Triglyceride separation, methylation and determination of the fatty acid composition

Once the reactions were carried out, the reaction medium was

neutralized using a KOH hydroalcoholic solution at 0.8 mol L^{-1} (30% ethanol) and TAGs were then separated as described by Wang *et al.*,[30] TAGs were methylated according to ISO 12966-2[31] with a few adaptations. The composition profile of produced fatty acid was determined by gas chromatography according to ISO 12966,[32] i.e., by replacing helium gas with nitrogen gas.

The incorporation degree, ID (%), which determines the amount of acid incorporated into the triglyceride molecule, was calculated according to Equation 19 with a few modifications:[33]

$$ID\,(\%) = \frac{MFA}{MT} \times 100 \quad (19)$$

where: MFA is the number of moles of medium chain fatty acids (C10:0), MT: number of total moles of fatty acids in triglyceride.

The obtained ID values (%) were normalized according to Equation 20. As the lipase used is 1.3 specific, the replacement of fatty acids only occurs in the sn-1 and sn-3 positions of triglyceride, resulting in a maximum incorporation degree of 66.7%.

$$ID\,(\%) = \frac{X \times 100}{66.7} \quad (20)$$

where: X is the non-normalized ID value obtained.

Enzyme reuse

The possibility of reusing the immobilized enzyme was evaluated by relative activity after use in the reaction. The immobilized lipase was separated from the reaction by filtration, washed thoroughly with hexane and dried with nitrogen. The relative activity (R) was calculated through the relationship between the hydrolytic activities[20] before and after the reaction, using Equation 21:

$$R\,(\%) = \frac{A}{A_0} \times 100 \quad (21)$$

where: A is the post-reaction hydrolytic activity (U g^{-1}), A_0: initial hydrolytic activity (U g^{-1}).

RESULTS AND DISCUSSION

Immobilization by physical adsorption

As regards the immobilization of *R. oryzae* lipase, the best enzymatic loading was explored with the aim of reaching optimal biocatalyst performance without overusing it. Table 3 presents the results of fixed asset yield (AY), recovered activity (RA) and fixed asset activity (AA) for each enzyme solution (ES).

In terms of AY, there was no marked difference between enzyme loadings, as values higher than 90% were found, except for ES 20 mg mL^{-1}. Otherwise, increased enzyme loading has led to RA improvement, reaching higher values of 6.1 and 6.5% at ES 30 and 35 mg mL^{-1}, respectively. The same behavior was observed for AA at 251.6 and 273.8 U g^{-1} using ES 30 and 35 mg mL^{-1}, respectively. Based on these results, an enzyme loading of 30 mg mL^{-1} was the best result, as good immobilization yield (90.8%) and considerable recovered activity (6.1%) were achieved, with a load of approximately 33.23 mg of protein *per* g of carrier. RA demonstrates the amount of active enzyme on the carrier. Negative factors such as binding of the enzyme's active center to the carrier, loss of catalytic structure, enzyme desorption or low substrate accessibility to the enzyme may contribute to low RA. Nonetheless, there was an increase in enzyme activity, possibly due to the selected method of carrier immobilization,[34] which is a factor of greater interest and decisive to monitor the presence of active lipase, moreover, it remained unchanged and close to that presented by the solution having the highest concentration.

In fact, the properties of agricultural residues such as high porosity and high surface area favor potential applications in immobilization. Furthermore, the presence of different chemical groups (amino, hydroxyl, carboxyl, thiol and phosphate groups) can be involved in several complex mechanisms, such as surface adsorption. In this way, enzyme industries can benefit from the advantages of using agro-industrial waste, aligned with the requirements of green technologies and also significant economic benefits.[7]

Zhang *et al.*[35] reported immobilization yield of 93.0 ± 0.2% and enzyme load of 28.1 ± 0.3 mg g^{-1} using lignin derived from corn cob residue as carrier to immobilize lipase B from *C. antarctica*. Machado *et al.*[36] achieved maximum immobilization yields of 55% through lipase from *Thermomyces lanuginosus* (TLL) immobilized via interfacial activation on silica particles from rice husks functionalized with triethoxy(octyl)silane. Therefore, it is worth emphasizing the true potential of using corn cob as a carrier, as well as the selected immobilization method. Furthermore, results show that greater loading is not necessary, in addition to the fact that it might have reached or is close to carrier saturation.

Immobilized enzyme characterization

Optimal pH and temperature

The immobilized enzyme was characterized as for optimal pH and temperature conditions using an experimental design, and hydrolytic activity was selected as response variable. Table 4 presents the experimental design matrix to evaluate the influence of temperature and pH on the immobilized enzyme hydrolytic activity.

Runs 9, 10 and 11 (central points) presented higher activity values, 154.44, 158.47, 158.41 U g^{-1}, respectively, evidencing that 45 °C and pH 7.75 were optimal conditions for enhancing the immobilized enzyme performance. The Protimiza Experimental Design® software[22] allowed carrying out a statistical analysis of results so as to find regression coefficients, standard errors, and calculated *t* and *p*-values for significant variables (Table 5). *p*-Values indicate that temperature (linear and quadratic) and pH (quadratic) conditions were significant at 5% level. Negative values of coefficients reveal that lower pH and temperature values increased hydrolytic activity.

Table 3. Fixed asset yield, recovered activity and fixed asset activity of *R. oryzae* lipase immobilization for different enzyme concentrations

Parameters	ES (mg mL^{-1})				
	10	15	20	30	35
AY (%)	90.4 ± 0.9	90.6 ± 0.9	82.3 ± 0.2	90.8 ± 0.5	90.6 ± 0.2
RA (%)	2.8 ± 0.2	2.4 ± 0.2	5.5 ± 0.4	6.1 ± 0.3	6.5 ± 0.1
AA (U g^{-1})	91.1 ± 3.6	99.2 ± 5.1	175.5 ± 11.6	251.6 ± 10.3	273.8 ± 11.2

Fixed asset yield (AY). Recovered activity (RA). Fixed asset activity (AA). Enzyme solution (ES).

Table 4. Experimental design matrix for the characterization of immobilized lipase from *R. oryzae* regarding the influence of temperature and pH on the hydrolytic activity of the immobilized enzyme

Run	Coded variables		Real variables		Response variable
	T (°C)	pH	T (°C)	pH	Activity (U g^{-1})
1	–1	–1	30	5.5	93.45
2	1	–1	60	5.5	83.10
3	–1	1	30	10	121.52
4	1	1	60	10	83.19
5	–1.41	0	23.79	7.75	112.14
6	1.41	0	66.21	7.75	95.01
7	0	–1.41	45	4.57	104.79
8	0	1.41	45	10.93	83.01
9	0	0	45	7.75	154.44
10	0	0	45	7.75	158.47
11	0	0	45	7.75	158.41

Temperature (T). Hydrolytic activity of the immobilized enzyme (activity).

Table 5. Regression coefficients, standard errors, calculated *t* and *p*-values for the evaluation of temperature and pH effects on hydrolytic activity of the immobilized enzyme (significance level of 5%, *p*-value < 0.05)

Name	Coefficient	Standard error	*t*-calculated	*p*-value
Mean	157.11	5.94	26.47	0.0000
x_1	–9.11	3.64	–2.51	0.0406
x_1^2	–27.62	4.33	–6.38	0.0004
x_2^2	–32.46	4.33	–7.50	0.0001

Linear effect temperature (x_1). Quadratic effect temperature (x_1^2). Quadratic effect pH (x_2^2).

An analysis of variance (ANOVA) was performed and its calculated values are shown in Table 6. Although the model showed lack of fit, as $F_{calc} > F_{table}$ and $R^2 = 92.11\%$, the model can be considered statistically significant, given the fact that experimental values fit predicted ones.

The experimental design results lead to obtaining the mathematical model expressed by Equation 22:

$$Y_1 = 157.11 - 9.11x_1 - 27.62x_1^2 - 32.46x_2^2 \quad (22)$$

where: Y_1 is the enzyme activity (U g^{-1}); x_1: temperature (°C); x_2: pH.

From the mathematical model, a response surface plot of enzyme activity as a function of temperature and pH was drawn and shown in Figure 2.

Figure 2 shows excellent hydrolytic activity at temperatures ranging between 35 and 50 °C and pH values from 6.7 to 8.9, whose optimal point is at 41 °C and pH 7.75. Experiments were performed in triplicate at optimal conditions and an average hydrolytic activity of 158.4 ± 20 U g^{-1} was obtained, which is very close to the value predicted by the model, *i.e.* 157.57 ± 6.02 U g^{-1}. These values can be found in literature, such as Brígida[37] who has found that lipases are active in pH values ranging from 4 to 10, mostly between 7 and 9, and temperatures from 20 to 50 °C, whose optimal values are

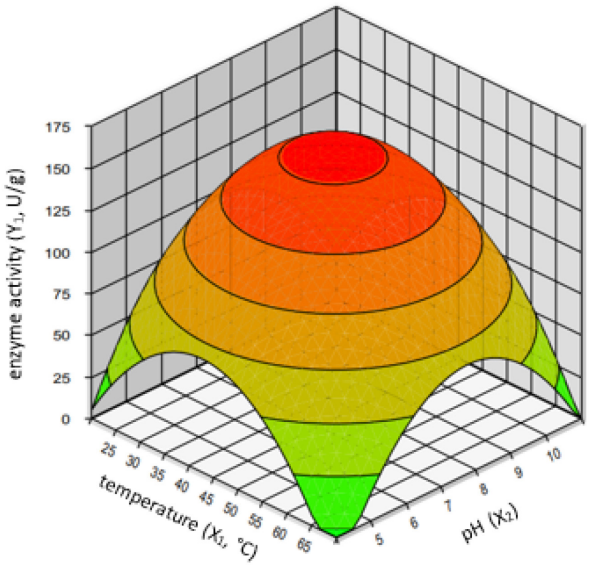

Figure 2. *Response surface plot of immobilized enzyme hydrolytic activity as a function of temperature and pH*

Table 6. Results of variance analysis (ANOVA) for the assessment of temperature and pH effects

Source of variation	Sum of squares	Degrees of freedom	Medium square	F_{calc}	*p*-value
Regression	8633.6	3	2877.9	27.2	0.00031
Residue	740.0	7	105.7	-	-
Lack of fit	729.3	5	145.9	27.3	0.03565
Pure error	10.7	2	5.3	-	-
Total	9373.6	10	-	-	-

found between 35 and 45 °C. This improvement is also attributed to the greater rigidity of its structure, acquired after immobilization, increasing the temperature and pH range carried. It is known that increasing the average temperature accelerates the reaction rate of an enzyme. However, the enzyme can denature, simultaneously with the loss of enzymatic activity, which usually decreases significantly under unfavorable conditions. Furthermore, each enzyme reaches its highest catalytic activity at a certain ideal pH value. This depends on the effect of on the protonation/deprotonation balance of functional groups at the active site of the enzyme, which can modify both substrate recognition (affecting K_m value) and the catalytic mechanism (affecting the k_{cat} value). In general, depending on the type of immobilization carrier, it can cause pH shift due to its surface and residual charges, reducing the electrostatic forces that limit the binding of the substrate to the active site of the enzyme.[3,38]

Furthermore, Girelli and Chiappini[39] listed several studies using lipases immobilized by physical adsorption on natural carriers, where the optimum pH is 6.5-8 and the optimum temperature 37-50 °C. Pashangeh *et al.*[40] also reported maximal catalytic activity of *R. oryzae* lipase immobilized on magnetic nanoparticles at pH 8.0 and 40 °C.

Thermal stability

The thermal stability of free and immobilized enzymes was assessed at 35, 41 (optimal) and 45 °C. Thermal deactivation profiles and nonlinear adjustments are shown in Figure 3.

Decayed relative activity was evidenced during incubation time at all studied temperatures (Figure 3). Such decay of relative activity increases as temperature rises, thus indicating that temperatures of over 45 °C accelerate enzyme inactivation, destroying the lipase structure, leading to a decrease in enzymatic activity. This increase in thermal stability can also be explained by the increase in surface hydrophobic interactions between the lipase-carrier and the reduction of lipase conformational changes compared with free lipase. The presence of secondary interactions (hydrogen bonds, polar and ionic interactions) between the enzyme and the transporter is also an important factor in increasing thermal stability.[41] In general, the immobilized enzyme shows a greater temperature tolerance, which is related to the increase in the rigidity of the secondary structure of the immobilized lipase.[42]

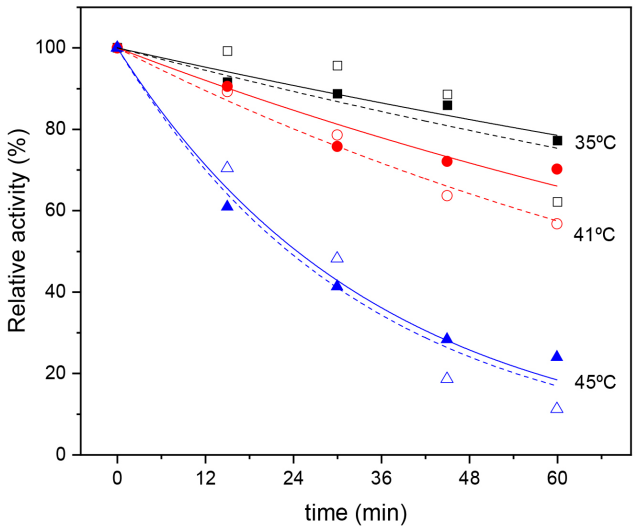

Figure 3. *Thermal deactivation profile of free (□○△) and immobilized (■●▲) enzymes at 35, 41 and 45 °C. Nonlinear adjustments of free (dash-dotted) and immobilized (solid dot) enzyme*

At the end of 1 h, the relative activities of immobilized enzyme at 35, 41 and 45 °C were 15.07, 13.48 and 12.82% higher, respectively, if compared to the free lipase, therefore it is found that the immobilization process confers thermal stability to the enzyme. Nematian *et al.*[43] also found similar results while the residual activities of *R. oryzae* lipase immobilized on magnetic nanoparticles (Fe_3O_4) and magnetic graphene oxide after 60 min incubation and at 60 °C were 13.09 and 27.20%, respectively.

Kinetic and thermodynamic parameters of thermal inactivation

Estimating kinetic and thermodynamic parameters is extremely important in enzymatic processes, as it assists in understanding how enzyme denaturation might behave. Kinetic and thermodynamic parameters of thermal inactivation of both the free and immobilized enzyme were determined according to the influence of temperature on their stability. These parameters are described in Table 7.

In general, results show that higher temperatures accelerate the

Table 7. Kinetic and thermodynamic parameters of thermal inactivation of free and immobilized enzyme of the influence of temperature on its stability

Parameter	Enzyme	Temperature (°C)		
		35	41	45
k_d (min^{-1})	free	0.0061 ± 0.0024	0.0096 ± 0.0000	0.0307 ± 0.0041
	immobilized	0.0038 ± 0.0000	0.0066 ± 0.0010	0.0275 ± 0.0021
R^2	free	0.7075	0.9878	0.9686
	immobilized	0.9516	0.9317	0.9873
$t_{1/2}$ (min)	free	113.63	72.20	22.58
	immobilized	177.73	106.64	25.21
SF		1.56	1.48	1.12
E_d (kJ mol^{-1})	free		125.51 (R^2 = 0.8680)	
	immobilized		151.27 (R^2 = 0.8538)	
ΔH (kJ mol^{-1})	free	125.51	125.51	125.51
	immobilized	151.27	151.27	151.27
ΔG (kJ mol^{-1})	free	99.11	99.90	98.13
	immobilized	100.32	100.88	98.42
ΔS (kJ mol^{-1} K^{-1})	free	0.0857	0.0815	0.0861
	immobilized	0.1654	0.1584	0.1661

Thermal deactivation constant (k_d). Half-life time ($t_{1/2}$). Stability factor (SF). Deactivation energy (E_d). Enthalpy (ΔH). Gibbs free energy (ΔG). Entropy (ΔS).

thermal deactivation of the enzyme in its free or immobilized form, since there was an increase in thermal deactivation constant (k_d). In addition, compared to the free enzyme, there was a decrease in k_d values for immobilized forms, which demonstrates that the immobilization process conferred more thermal stability to the enzyme.

Half-life time ($t_{1/2}$) corresponds to the time required to halve the initial enzyme activity.[28] The immobilization process makes lipase more stable, and higher values of half-life times are found as a consequence. This behavior was also demonstrated by the stability factor (SF), wherein the immobilized enzyme showed 1.56, 1.48 and 1.12 times more stability in comparison with its free form at temperatures of 35, 41 and 45 °C, respectively. Faria *et al.*[23] also reported stability factor values in the range of 1.4 to 2.5 for immobilized FTase enzyme.

An increase in deactivation energy (E_d) for the immobilized enzyme (151.27 kJ mol^{-1}) indicates that it requires more energy to initiate the thermal inactivation process when compared to the free lipase (125.51 kJ mol^{-1}), thus evidencing enzyme thermostability improvement. E_d represents the energy barrier to be overcome for enzyme inactivation, therefore, the higher it is, the more energy is required and better stability is conferred.[44]

Enthalpy (ΔH), i.e., heat exchange, provides the number of non-covalent bonds broken, and represents a measure of the energy barrier to be overcome for molecules to start reacting.[45] Thus, it was evidenced that the immobilized enzyme, which presented higher values of ΔH needs more energy for stretching, compressing, or breaking bonds to undergo a change from a native to a denatured state. Entropy (ΔS) measures the disorder of the enzyme structure and it is associated with the transition state formation. Positive values of ΔS indicate that system disorder increases as the enzyme denatures.[44] According to Rigo *et al.*[46] values of ΔS close to zero indicate that thermal inactivation caused no relevant change in the tertiary structure of the enzyme, i.e., most hydrogen bonds responsible for the active structure of the catalytic site of the enzyme are still present in the activated complex. The authors also reports low ΔS values (around 0.097 and 0.149 for free and immobilized enzymes, respectively) and that there was no clear variation in temperature. In terms of enzyme stability, there is a relationship between the values of ΔH and ΔS , in which high ΔH and low ΔS values reveal an increase in enzyme stability.[47]

Gibbs free energy (ΔG) expresses the spontaneity of enzyme's loss of stability, as lower values reveal greater tendency to denaturation. Higher Gibbs free energy values for immobilized lipase indicate that immobilization conferred greater stability to the enzyme. Positive values of ΔG showed that the process of enzyme thermal inactivation is thermodynamically non-spontaneous.[47] Results found for ΔG in this study agreed with the values of ΔG (98.6-104.9 kJ mol^{-1}, from 40 to 70 °C) reported by Ortega *et al.*[47] A decrease in ΔG at temperatures from 41 to 45 °C may be related to decreased availability of reactive sites by denaturation and a change in its initial conformation.[48]

Therefore, according to these results, lipase immobilization conferred thermal stability to the enzyme, such as an increase in its half-life time.

Stirred tank batch reactions

In order to optimize reaction conditions for triglycerides synthesis, the influence of reaction parameters, such as acid molar ratio (MR) (acid-to-oil), temperature, T (°C), and amount of biocatalyst, Biocat (m m^{-1}), (mass of biocatalyst to mass of oil, in %) on the normalized incorporation degree, ID (%), was evaluated through a CCDR experimental design. Table 8 presents the experimental design matrix.

It was observed that the ID of C10 in grape seed oil ranged from 42.62 (exp 9) to 86.93% (exp 10). The degree of incorporation ID (%), determines the amount of acid incorporated into the triglyceride molecule, and is an important parameter for the efficiency of acidolysis. The difference between higher and lower values was due to acid molar ratio, indicating the influence of this parameter

Table 8. Experimental design matrix for evaluating the influence of acid molar ratio, temperature and amount of biocatalyst on the normalized degree of incorporation of capric acid in triacylglycerols in a stirred tank batch

Run	Coded variables			Real variables			Response
	MR	T (°C)	Biocat (%)	MR	T (°C)	Biocat (%)	ID (%)
1	–1	–1	–1	4	35	10	55.38 ± 1.82
2	1	–1	–1	8	35	10	72.16 ± 3.47
3	–1	1	–1	4	55	10	49.85 ± 1.49
4	1	1	–1	8	55	10	73.15 ± 0.80
5	–1	–1	1	4	35	20	54.33 ± 4.26
6	1	–1	1	8	35	20	81.27 ± 2.06
7	–1	1	1	4	55	20	58.16 ± 1.73
8	1	1	1	8	55	20	84.68 ± 2.14
9	–1.68	0	0	2.63	45	15	42.62 ± 0.17
10	1.68	0	0	9.36	45	15	86.93 ± 2.25
11	0	–1.68	0	6	28.18	15	61.83 ± 2.15
12	0	1.68	0	6	61.81	15	66.07 ± 2.63
13	0	0	–1.68	6	45	6.59	59.94 ± 0.94
14	0	0	1.68	6	45	23.40	72.10 ± 2.06
15	0	0	0	6	45	15	66.34 ± 0.91
16	0	0	0	6	45	15	64.41 ± 1.73
17	0	0	0	6	45	15	66.85 ± 2.63

Molar ratio (MR). Temperature (T). Amount of biocatalyst (Biocat). Normalized incorporation degree (ID).

on ID values, which may be associated with the fact that there was more fatty acid in the reaction medium. In addition, the results from the experimental design matrix demonstrate that molar ratio is highly significant, as well as the amount of biocatalyst, which is corroborated by the results shown in Table 9 revealing the striking effects of variables at 5% significance (p-value < 0.05). Molar ratio (x_1) and biocatalyst amount (x_3) linear were significant effects, in addition to their interaction ($x_1.x_3$) and that between molar ratio and temperature ($x_1.x_2$). Positive values indicate that an increase in these variables leads to higher ID values.

An analysis of variance (ANOVA) was performed and its results were described in Table 10.

As $F_{calc} > F_{table}$ and $R^2 = 98.24\%$, the model is statistically significant, *i.e.* experimental values fit the predicted values. The model presented no lack of fit and the mathematical model generated by the experimental design is expressed by Equation 23:

$$Y_1 = 65.65 + 12.30x_1 + 3.54x_3 + 1.67x_1x_3 + 1.47x_2x_3 \quad (23)$$

where: Y_1 is the enzyme activity (U g^{-1}); x_1: molar ratio; x_2: temperature (°C); x_3: amount of biocatalyst (%).

Figure 4 shows a response surface plot of the ID as a function of molar ratio and amount of biocatalyst. It was evidenced that higher values of molar ratio and amount of biocatalyst promote increased ID values, as indicated by the ANOVA. Certainly, higher molar ratios promote greater incorporation of fatty acids, shifting the reaction equilibrium towards product formation. However, it is important to observe the use of a large amount of substrate, not only because of the possibility of an impediment occurring in the presence of too much free fatty acid, inactivating the biocatalyst, but also due to the costs of operation on an industrial scale.[13] A similar behavior was found in literature by Bassan *et al.*,[13] who have obtained TAGs with incorporation degree (ID) ranging from 34.40 to 54.36% using grape seed oil and C10 catalyzed by the commercial lipase Lipozyme RM IM, and once again molar ratio also exerted greater influence. Kavadia *et al.*[16] also described the higher molar ratio of substrates and higher enzymatic load as the most influential factors for incorporation of caprylic acid and reduction of tristearin in the acidolysis process using the soluble lipase *Thermomyces lanuginosus*. Yet, Costa *et al.*,[49] immobilized ROL lipase on Amberlite IRA96 for carrying out a reaction of grape seed oil with medium chain fatty acids, thus achieving ID of 52.4 ± 6.6%.

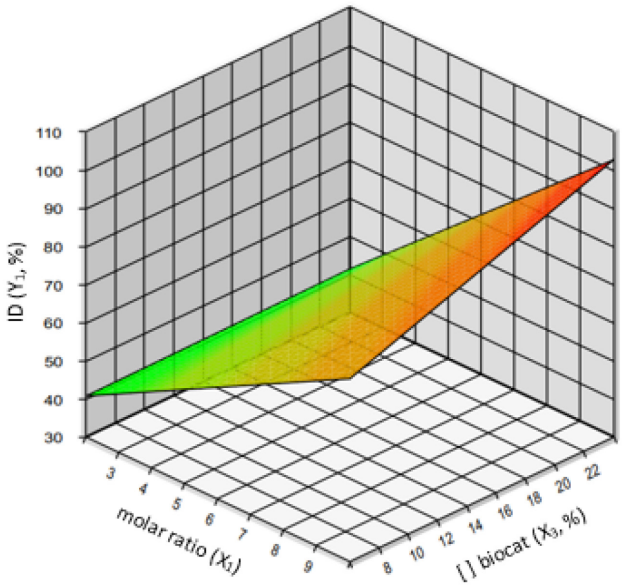

Figure 4. *Response surface plot of degree of incorporation (ID) of capric acid as a function of grape seed oil/capric acid molar ratio and amount of immobilized biocatalyst in a stirred tank reactor*

Fixed bed reaction

Hydrodynamic characterization of the fixed bed reactor

After injecting dye into the reactor's feed line, samples were collected at the reactor output and their absorbances were measured. A correlation between absorbance A (nm) and dye concentration C (mg mL^{-1}) is expressed by Equation 24:

$$A = 1.1475C \quad (24)$$

Concentration as a function of time C(t) was determined and the coefficient of determination ($R^2 = 98.39\%$) indicated good model fit to experimental data.

Table 9. Regression coefficients, standard errors, calculated t and p-value for the evaluation of acid molar ratio, temperature, and amount of biocatalyst effects on incorporation degree (significance level of 5%, p-value < 0.05)

Name	Coefficient	Standard error	t-calculated	p-value
Mean	65.65	0.45	146.96	0.0000
x_1	12.30	0.50	24.69	0.0000
x_3	3.54	0.50	7.10	0.0000
$x_1.x_3$	1.67	0.65	2.57	0.0246
$x_2.x_3$	1.47	0.65	2.26	0.0433

Linear effect molar ratio (x_1). Linear effect amount of biocatalyst (x_3). Interaction between molar ratio and amount of biocatalyst ($x_1.x_3$). Interaction between molar ratio and temperature ($x_1.x_2$).

Table 10. Results of variance analysis (ANOVA) for the assessment of acid molar ratio, temperature, and amount of biocatalyst effects

Source of variation	Sum of squares	Degrees of freedom	Medium square	F_{calc}	p-value
Regression	2278.7	4	569.7	167.9	0.00000
Residue	40.7	12	3.4	-	-
Lack of fit	37.4	10	3.7	2.2	0.34680
Pure error	3.3	2	1.7	-	-
Total	2319.4	16	-	-	-

To calculate the E(t) function, the denominator of Equation 16 was determined from the area under the curve of the C(t) plot. The E(t) function was calculated for each time period and the area below/at the bottom of the residence time distribution graph (Figure 5) was determined, and average residence time was obtained through Equation 17. All plots and areas were drawn using the OriginPro 8® software.[25]

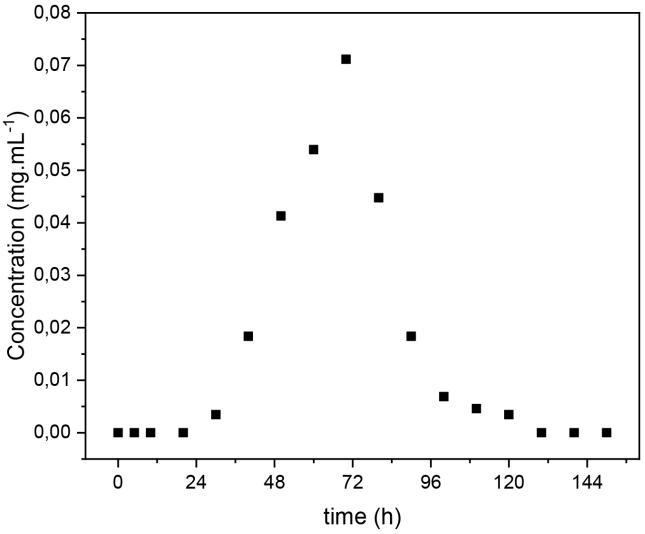

Figure 5. *Distribution of residence time of the fixed bed reactor packed with corn cob powder*

Average residence time (tm) was 67.46 min, and a working reactor volume of 51.11 mL, flow rate of 1 mL min^{-1}, and space-time (τ) of 51.11 min was found. It is worth mentioning that the concept of space-time only considers the volume occupied by the catalytic bed, thus disregarding the remaining volume and connections of the reactor. The recalculated space-time (τ*) was 68 min. A similarity between times indicates absence of preferential paths or stagnation zones, as well as good bed packing and good reactor hydrodynamics.[29,50]

Fixed bed reactions in total recycling

After TAGs synthesis has been optimized in stirred tank reactions, TAGs results in a fixed bed reactor were also evaluated. Due to the operational difficulties observed in this reactor configuration due to the fact that the reaction medium had higher ratio of acid solidified which blocked the passage, it was impossible to use such higher molar ratio of acid. Therefore, a molar ratio of 1:3 (oil:acid) was selected for the reactions. To compare the effect of biocatalyst amount, two reactions were performed using trice the amount of U of immobilized biocatalyst per gram of reaction medium. Average values of ID of acidolysis reactions are shown in Figure 6.

Truly noteworthy, MLM-type TAGs have grand nutritional properties. From a dietary point of view, the main advantage is the rapid metabolization as energy sources, which avoids storage in adipose tissues, preventing diseases associated with weight gain.[12]

ID is such an important indicator of reaction efficiency, as it demonstrates how much acid was incorporated into the oil molecule. As observed previously, higher values of ID were reached by the second reaction whose ID was 56.59 ± 0.02% at 72 h and 51.13 ± 0.77% on average at the end of the reaction. The first reaction showed a linear ID increase, indicating that the reaction has not reached stabilization, thus obtaining ID average of 37.72 ± 0.28%. On the other hand, the second reaction reveals a marked increase up to 8 h, which afterwards tended to achieve a steady state. Akil *et al.*[51] showed a degree of incorporation between 21.51 ± 6.09 and 30.02 ± 3.28%

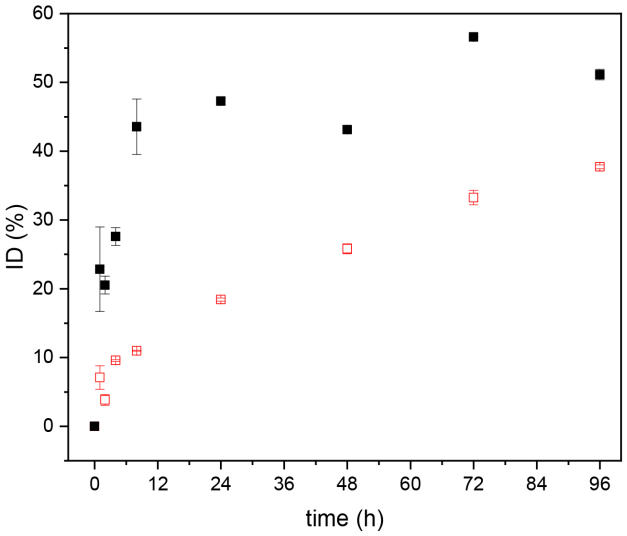

Figure 6. *Average degree of incorporation of capric acid in grape seed oil at a molar ratio of 1:3 and the immobilized biocatalyst of reactions 1 (■) and 2 (□) containing triple U in a fixed bed reactor in total recycling*

in the acidolysis of lauric acid with olive oil using *Y. lipolytica* lipase microencapsulation in chitosan-alginate beads. Cozentino *et al.*[11] performed acidolysis between grape oil and capric acid in a fixed bed reactor catalyzed by immobilized lipase Lipozyme RM IM and achieved a mean ID of 39.91 ± 2.77%.

One of the purposes of immobilization is to improve efficiency, stability of the biocatalyst, and enable easy recovery and reusability. Reuse is essential, considering the high cost of enzymes, for their efficient industrial applications, reducing production costs by reusing the same enzyme several times. In addition to thermal and pH stability, other factors that promote loss of biocatalyst durability can be improved after immobilization such as operational stability and storage stability, overcoming such disadvantages.[52]

The immobilized biocatalyst hydrolytic activities were quantified before and after the acidolysis reactions took place. After the 96 h reaction, 91.7 and 82.7% of the hydrolytic activity of the biocatalyst were maintained in the first and second reactions, respectively, showing immobilization advantages. It is noteworthy that the immobilized lipase can be recovered and reused in new cycles.

In the work by Dash and Banerjee,[53] *Rhizopus oryzae* lipase immobilized on celite maintained approximately 90% residual activity in 3 cycles of 30 h. Costa-Silva *et al.*[54] used lipase from *Cercospora kikuchii* immobilized on corn cob activated with glutaraldehyde, sodium periodate or epichlorohydrin and retained an average of 57.42% of the initial enzymatic activity after five reuse cycles.

The determination of the peroxide index (PI) indicates whether an oil can be considered suitable for use in food or as a raw material for the generation of other products. This parameter in oils is of great importance, from which unsaturated fatty acids with broken double bonds are quantified. PI of grape seed oil was evaluated before and after the reactions took place. The used oil had initial PI of 1.04 ± 0.45 mEq kg^{-1}, but the product of the first acidolysis presented PI of 2.32 ± 0.39 mEq kg^{-1} and the second one presented PI of 1.64 ± 0.54 mEq kg^{-1} after 96 h of reaction. These results are in accordance with that permitted by ANVISA for refined and cold-pressed oils, *i.e.* 10 mEq kg^{-1}, such as that for grape seed oil.[55]

CONCLUSIONS

Results obtained in this study evidence the potential of *R. oryzae*

lipase immobilized on corn cob to be used as biocatalyst in MLM-type triglycerides production. The immobilization procedure increased enzyme stability, which has been confirmed by kinetic and thermodynamic studies revealing higher half-life time and deactivation energy values. The enzymatic acidolysis reaction of grape seed oil and capric acid carried out in stirred-tank reactors in batch mode was optimized, suggesting that ID increases gradually according to the amount of acid and biocatalyst present in the reaction medium. Under optimal conditions, *i.e.* molar ratio 1:9.36 (oil:acid) at 45 °C and 15% of biocatalyst, a maximum ID of 86.93 ± 2.25% was reached. Reactions were carried out in a fixed bed reactor in recycling and ID of 51.13 ± 0.77% was obtained, with lower values of PI (< 10 mEq kg^{-1}), thus indicating that the process is capable of producing MLM triglycerides within the standards established by ANVISA. And the biocatalyst maintained higher hydrolytic activity (82-91%) even after 96 h of reaction, which corroborates enzyme stability.

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