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Synthesis of Monocaprin Catalyzed by Lipase

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Abstract

The production of monoglyceride emulsifiers commonly employed in the food, cosmetic, and pharmaceutical industries can be catalyzed by lipases, biocatalysts that are becoming increasingly attractive in the enzyme market. The aim of this study was to produce monocaprin utilizing a commercial immobilized lipase (Lipozyme IM 20) through the direct esterification of capric acid and glycerol. Experiments were performed for 6 h in an open reactor and the products were analyzed by gas chromatography. The parameters investigated were the amount of enzyme, temperature, and molar ratio between the reagents (capric acid/glycerol). The experimental runs followed an experimental design generated using Statistica® software. The results showed that all the parameters were significant and that monocaprin production was enhanced at the lower ranges of the tested variables. The best conditions established were 55°C, 3% (w/w) enzyme concentration, and molar ratio of 1. The final product, obtained after 6 h of reaction, was 61.3% monocaprin, 19.9% dicaprin, and 18.8% capric acid. This composition satisfies the directives of the World Health Organization food emulsifiers, which requires that these mixtures have at least 70% mono- plus diglyceride, and a minimum of 30% monoacylglycerol.

Index Entries: Lipase; monocaprin; capric acid; glycerol; monoglycerides; molar ratio; *n*-hexane; *t*-butanol.

Introduction

The lipases (EC 3.1.1.3) constitute a group of enzymes, that is becoming increasingly attractive for the oil industry. Stability and especially selectivity are the properties that are responsible for their success in

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biotransformations *(1,2)*. These properties have been used in modification of natural fats and oils, or for the production of monoglycerides.

Monoglycerides are nonionic emulsifiers that are widely used in the food, cosmetic, and pharmaceutical industries. These substances are considered Generally Recognized as Safe products by the US Food and Drug Administration. This fact has enhanced the development of new synthetic methods and increased the use of monoglycerides in the food and pharmaceutical industries *(3)*.

Mono- and diglycerides are consumed at an annual level of 85,000,000 kg in the United States, corresponding roughly to 70% of the total emulsifiers used in the food industry *(4)*. Recently, it has been proposed that some fatty acids and monoglycerides, such as monocaprin, may also be used as intravaginal microbiocides for protection against sexually transmitted diseases *(5,6)* and as conservative agents in the dairy industries *(7,8)*.

Presently, industrial-scale production of monoglycerides is carried out by continuous chemical glycerolysis of fats and oils, employing inorganic catalysts (e.g., $Ca[OH]_2$) at high temperatures (220–250°C). This process is characterized by a high energetic consumption and also may cause changes in the sensorial properties of the products, such as color and taste *(9)*.

The enzymatic synthesis of monoglycerides using lipase, besides the advantages already cited, is carried out at lower temperatures, resulting in higher-quality products , lower energy consumption, and effluents that are less aggressive to the environment *(10)*.

Different methods for the enzymatic synthesis of monoglycerides have been investigated, such as selective hydrolysis using 1,3-regiospecific lipases, esterification of fatty esters with glycerol, and glycerolysis of fats or oils *(11)*. The objective of the present work was to study the synthesis of monocaprin by direct esterification of glycerol with capric acid in the presence of a commercial immobilized lipase with and without the use of solvent.

Materials and Methods

Materials

Lipozyme IM-20 (*Mucor miehei* , lipase immobilized on a weak anion-exchange resin) was kindly supplied by Novo Nordisk A/S (Bagsvaerd, Denmark). Capric acid and gas chromatography (GC) standards (mono-, di-, and tricaprin) were purchased from Sigma. (St. Louis, MO). Analytical-grade glycerol, n-hexane, t-butanol, ethyl acetate, ethanol, acetone, lauric acid (99.9%), and molecular sieves (with an effective pore diameter of 3 Å) were purchased from Merck (Darmstadt, Germany).

Measurement of Lipase Activity

The esterification activity of Lipozyme IM-20 was measured according to the method described by Langone and Sant'Anna *(12)*, which determines the consumption rate of fatty acid at 60°C in a reaction system containing glycerol, lauric acid, and a given amount of the commercial enzyme prepa-

Table 1
Variables and Levels of Variation

	Level		
Variable	–1	0	+1
T (°C)	40	55	70
R	0.5	1.0	1.5
E (% [w/w])	1	5	9

ration. One international unit of esterification activity is the quantity of enzyme that consumes 1 µmol of lauric acid/min under the reaction conditions. The enzyme used has an esterification activity of 20 IU/g.

Nonpolar Phase Analysis

Fatty acid and mono-, di-, and tricaprin were analyzed by capillary GC according to the method described by Langone and Sant'Anna *(12)*. All concentrations were calculated as molar fractions from the peak area using calibration curves.

Esterification Experiments

All experiments were performed in duplicate in a 20-mL open-batch reactor with constant stirring and temperature control. The reaction system contained a mixture of capric acid and glycerol and the biocatalyst Lipozyme IM-20. In some experiments, a 1:1 mixture of *n*-hexane:*t*-butanol was added to the reaction medium at different volumes (2.0, 4.0, and 6.0 mL), corresponding to 24, 38, and 48% of the reaction volume, respectively. The reaction's progress was followed by withdrawing 20-µL aliquots at various time intervals and analyzing them by GC, as previously described.

Experimental Design

The effects of different variables on a process can be determined using experimental design methodology, which employs a reduced but meaningful number of experiments *(13)*. A 2^3 factorial design with central point was used to analyze the effects of temperature (*T*), capric acid/glycerol molar ratio (*R*), and enzyme concentration (*E*) on the selectivity of monocaprin. The studied values for each variable are given in Table 1. The variables and levels were chosen as reported in the literature and by preliminary studies *(14)*.

Molecular Sieves

Several tests under the addition of 3-Å molecular sieves (0.07 g/mL) were performed in order to evaluate the effect of water removal on the reaction medium. The reactions were performed at 55°C with a stoichiometric molar ratio of the reagents and enzyme concentrations of 3 and 5% (w/w).

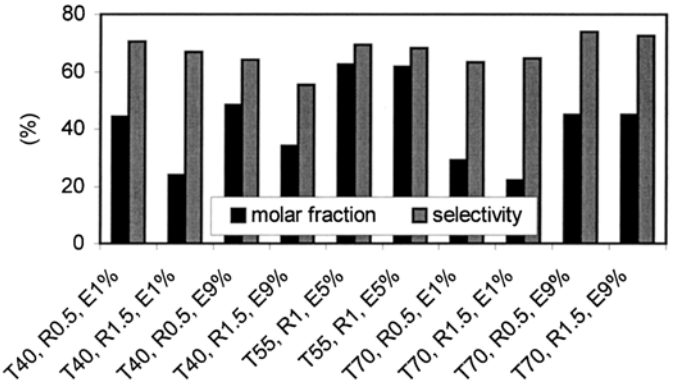

Fig. 1. Effect of variables (T, R, E) on molar fraction and on selectivity in monocaprin after 6 h of reaction.

Glycerol Addition

To test the influence of the reagent molar ratio, glycerol was added to the reaction medium in a fed-batch mode. For the reactions performed with capric acid/glycerol ratios of 0.5 and 1.0, 50% of the necessary glycerol mass was added at the beginning of the reaction ($t = 0$ min), 25% after 30 min of reaction, and the remaining 25% after 1 h of reaction.

Results and Discussion

Experimental Design

The results of the enzymatic synthesis of monocaprin were expressed as a molar fraction of the components in the nonpolar phase (capric acid, monocaprin, dicaprin, and tricaprin). The selectivity parameter, chosen to define the best reaction conditions, was defined as the ratio of monocaprin concentration to the sum of the concentration of the reaction products (mono-, di-, and tricaprin). The results for the reactions are presented in Fig. 1. As can be seen, the best results in terms of molar fraction (61%) and selectivity (70%) of monocaprin were obtained at 55°C, with a reagent molar ratio of 1 and an enzyme concentration of 5% (w/w).

After 4 h of reaction, equilibrium was attained, so the influence of variables on monocaprin selectivity was analyzed at this reaction time. The results were analyzed using Statistica® for Windows, release 5.5, produced by Statsoft. The main effects on selectivity in monocaprin and interactions between factors were determined. Table 2 shows the regression coefficients, standard errors, and effects. Most effects were negative, which agrees with the decrease in monocaprin selectivity observed when the higher level (+1) of each variable (T, R, E) was used (Fig. 1).

To evaluate whether the effects of the variables and their interactions were statistically significant, a student's t-test was employed. In Fig. 2, these

Table 2
Statistical Parameters for Design 2^3 Experimental with Central Point

Factor	Coefficient	SE	Effect
Average	65.495	1.066	65.495
(1) *T*	–4.319	1.192	–8.638
(2) *R*	–6.894	1.192	–13.787
(3) *E*	–3.669	1.192	–7.338
$T \times R$	–0.369	1.192	–0.738
$T \times E$	–0.581	1.192	–1.162
$R \times E$	–2.744	1.192	–5.488
$T \times R \times E$	–4.519	1.192	–9.038

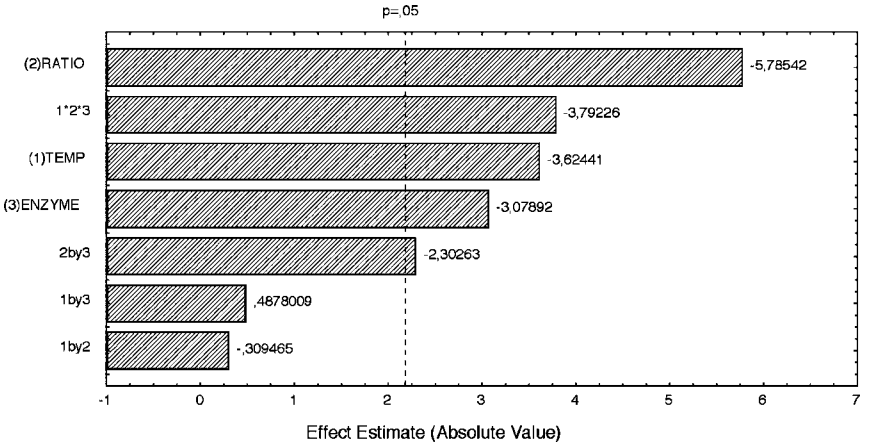

Fig. 2. Pareto's graphic showing different effects of variables and their interactions on monocaprin selectivity after 4 h of reaction.

evaluations are illustrated using a Pareto chart. The vertical line indicates the magnitude of the minimum statistically significant effect for a confidence level of 95%. Values shown in the horizontal columns are student's *t*-test values for each effect. It can be observed that the molar ratio of the reagents is the most significant variable influencing monocaprin selectivity. Temperature, enzyme concentration, the interaction between *R* and *E*, and the interaction between the three variables (*T*, *R*, *E*) are also statistically important. The results showed that all the parameters tested were significant and favored monocaprin production at lower levels. This was further confirmed in the subsequent experiments with varying temperature, molar ratio of reagents, and enzyme concentration.

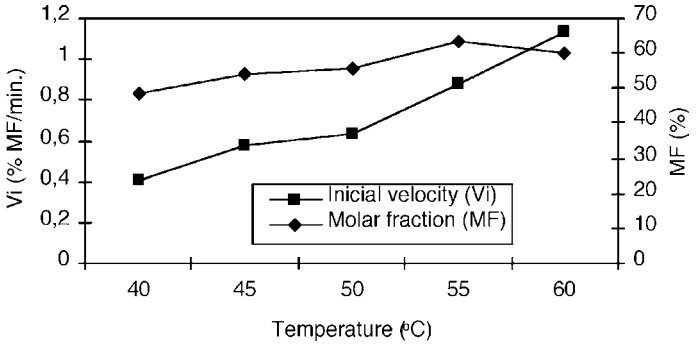

Fig. 3. Effect of temperature on monocaprin molar fraction (%) and on initial velocity (% *MF*/min) of monocaprin's production after 6 h of reaction using E = 5% (w/w) and *R* = 1.

Effect of Temperature

Increased temperature depressed the synthesis of monocaprin, as can be observed in the reactions performed for 40 and 70°C (Fig. 1) and in the statistical analysis. The temperature increase enhanced the synthesis of di- and tricaprin, decreasing monocaprin molar fraction and selectivity. Therefore, the influence of this parameter was studied at a lower range (40–60°C), using an enzyme concentration of 5% (w/w) and the stoichiometric molar ratio of the reagents (Fig. 3).

According to Fig. 3, the best condition for synthesizing monocaprin was obtained at 55°C (63.25%), decreased at higher temperatures. However, the initial speed of monocaprin formation (*Vi*) increased with elevated reaction temperature in the whole range tested (until 60°C). Thus, in spite of the higher initial rate of monocaprin formation at 60°C, the final molar fraction of the monoglyceride was lower at this temperature. This was probably caused by di- and tricaprin formation during the reaction.

The optimum temperature (55°C) was not consistent with the data of Kwon et al. *(15)*, who found that *R. miehei* lipase (Lipozyme IM-20) was not as active over 40°C on the synthesis of medium-chain glycerides (tri-, di-, and monocaprin) in isooctane. They reported that during the esterification of fatty acid and glycerol, water is produced from condensation of the two groups; this might act as a bridge for the exchange of ionization groups. Thus, the conformation of lipase may not be so rigid that the structure of lipase can be destroyed at high temperature. Therefore, considering the results obtained in our work, it can be concluded that the use of a solvent-free system in an open-batch reactor at 55°C was effective at eliminating the water produced in the reaction.

Effect of Enzyme Concentration

As observed in the Pareto chart (Fig. 2), enzyme concentration influences monocaprin synthesis. Therefore, several experiments with different

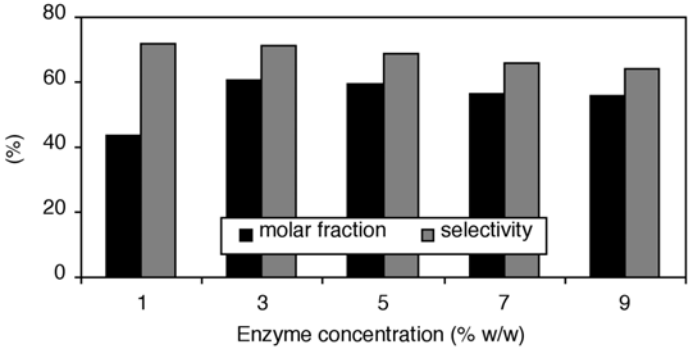

Fig. 4. Effect of the concentration of Lipozyme-IM 20 on monocaprin selectivity and molar fraction at 55°C with R = 1 after 6 h of reaction.

concentrations were proposed, keeping the stoichiometric molar ratio of the reagents and the temperature of 55°C.

The values obtained for the monocaprin molar fraction were similar for the different enzyme concentrations tested (Fig. 4) after 6 h of reaction. The best results were obtained with Lipozyme-IM at 3 and 5% (w/w); thus, an increased enzyme concentration did not significantly increase the monocaprin molar fraction. By contrast, a decreased monocaprin selectivity occurred, probably owing to increased synthesis of di- and tricaprin. Additional advantages favoring lower enzyme concentrations include reduced operating costs and the fact that when 3% (w/w) Lipozyme-IM was used, no tricaprin was produced.

Effect of Molar Ratio of Reagents

The experiments that used the stoichiometric molar ratio of reagents resulted in higher molar fractions of monocaprin. The increase in capric acid concentration (R = 1.5) enhanced the synthesis of di- and tricaprin. Using an excess of glycerol corresponding to the capric acid/glycerol molar ratio of 0.5 did not result in a significant increase in monocaprin selectivity and molar fraction (Fig. 1). The addition of glycerol in the fed-batch mode was done considering that glycerol is a polar substance and could, in a high concentration, inactivate the enzyme *(14)*. As shown in Fig. 5, neither an increase in the capric acid/glycerol molar ratio nor addition of glycerol in the fed-batch mode favored monocaprin synthesis in the reactions performed at 55°C with Lipozyme at 3 and 5% (w/w). Thus, the best molar ratio of the reagents for the synthesis of monocaprin is the stoichiometric molar ratio, with all glycerol added at the beginning of the reaction.

Inhibition by the alcohol substrate was also reported by Wong et al. *(16)*. They investigated the effect of molar ratio of substrates in the reaction mixture on medium-chain glyceride synthesis from capric acid and glycerol by lipase from *C. rugosa*. Excess glycerol in the reaction mixture seemed to in-

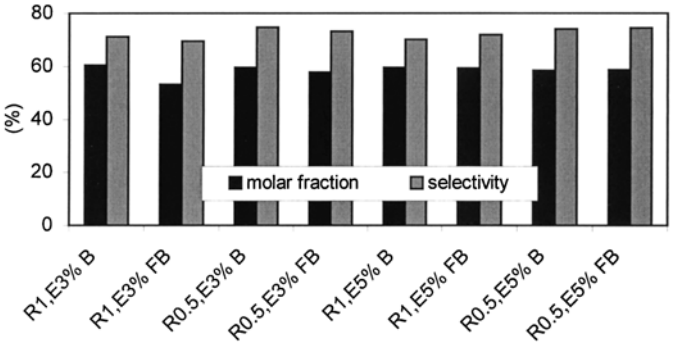

Fig. 5. Effect of addition of glycerol on synthesis of monocaprin after 6 h of reaction at 55°C. B, batch mode; FB, fed-batch mode.

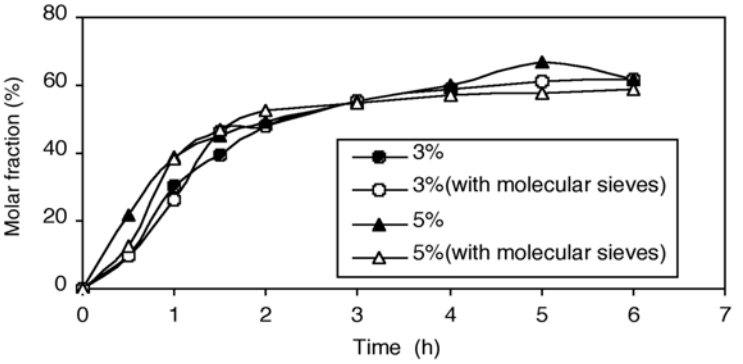

Fig. 6. Effect of addition of molecular sieve on molar fraction in monocaprin for synthesis performed at 55°C using $R = 1$ and Lipozyme IM-20 concentrations of 3 and 5% (w/w).

hibit the activity of the enzyme. During this experiment, high volumes of glycerol caused high solubility of enzyme in glycerol and thus decreased the lipase concentration at the interface, probably decreasing the reaction rate.

Effect of Addition of Molecular Sieves

Molecular sieves have been used to lower the water activity in the reaction medium, thus shifting the equilibrium toward the synthesis of esters *(17)*. Thus, molecular sieves were used in our work to evaluate the efficiency of water removal from the reaction medium in comparison with the free evaporation method. The results showed that monocaprin production was not significantly affected by adding molecular sieves to the reaction medium. Figure 6 presents the reaction progress curves for the two conditions (free evaporation and addition of molecular sieves). The use of molecular sieves for water removal, although effective, did not offer a significant advantage over the simple evaporation technique (open batch stirred reactor at 55°C).

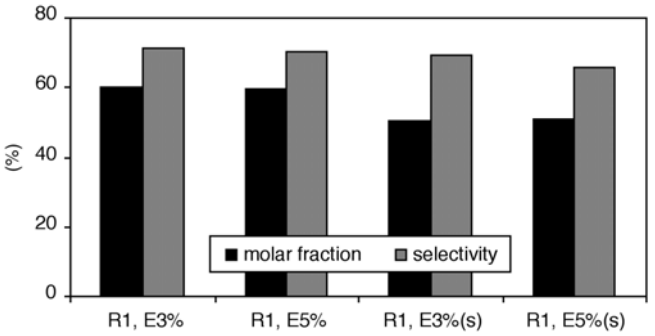

Fig. 7. Effect of adding solvent on monocaprin selectivity and molar fraction at 55°C with $R = 1$ at Lipozyme IM-20 concentrations of 3 and 5% (w/w) after 6 h. (s) = utilization of 2.0 mL of the mixture of 1:1 *n*-hexane/*t*-butanol.

Giacometti et al. *(17)* investigated the esterification of glycerol with oleic acid catalyzed by lipozyme in a solvent system (*n*-hexane) in the presence of a molecular sieve (3000 mg of the 5-Å molecular sieve) at 37°C. Conversions of oleic acid increased more in the solvent system with the molecular sieve than in the solvent system without the molecular sieve.

Again, it can be concluded that in the system used in our work (without solvent, open reactor, at 55°C), water was completely evaporated. However, in a solvent system, at lower temperatures, it is important to use a desiccant.

Effect of Solvent Addition

Several investigators *(18–20)* have reported using organic solvents in enzymatic synthesis of glycerides. Thus, comparison between optimized reaction conditions with and without solvent was also evaluated. The nature of the solvent influences the activity and stability of the enzyme to a large extent. The polarity of the solvent plays a key role. Log *P* has often been used to characterize solvents. Log *P* is defined as the logarithm of the partition coefficient of a substrate in the standard 1-octanol–water two-phase system. Normally, solvents with high Log *P* values (Log $P > 4$) (hydrophobic solvents) cause less inactivation of biocatalysts than more hydrophilic solvents *(19)*. The lipases have higher stability in a hydrophobic organic medium, such as hexane (Log $P = 3.5$). However, glycerol, the reagent of monocaprin synthesis, is a very hydrophilic solvent and has lower solubility in the nonpolar phase of this system. Thus, to obtain a much more polar medium that permits a better glycerol solubilization, we investigated adding *t*-butanol to a medium containing *n*-hexane.

To verify whether a mixture of 1:1 *n*-hexane/*t*-butanol would increase the selectivity in monocaprin, some experiments were performed using increasing volumes of the solvent mixture. Figure 7 shows the reactions with and without solvent, presenting final values of monocaprin selectivity

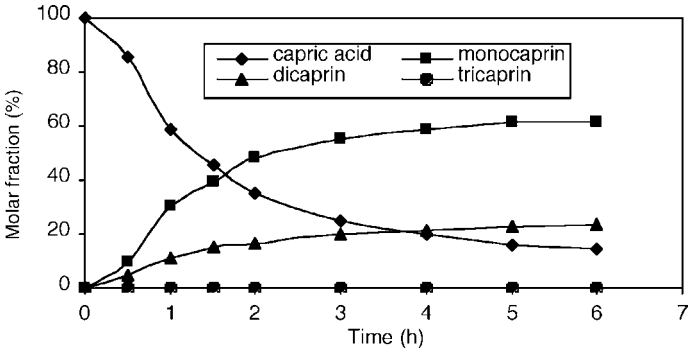

Fig. 8. Time course for monocapin synthesis carries out at 55°C, with a capric acid/glycerol molar ratio of 1 and 3% (w/w) Lipozyme.

and molar fraction. The use of *n*-hexane/*t*-butanol (at a concentration of 24% [v/v] of the total reaction volume) as solvent mixture did not increase the monocaprin selectivity. The best results, in terms of molar fraction (61.3%) and selectivity (71.1%) in monocaprin were obtained with a 3% (w/w) enzyme concentration without solvent. In addition, the use of higher amounts of solvent (equal to 38 and 48% of the reaction volume) did not result in any glyceride synthesis (results not shown). This effect can be explained by the fact that the increase in the quantity of polar solvents, such as *t*-butanol (Log P = 0.32), may deactivate lipase, because they strip off essential water from the enzyme surface, leading to an insufficiently hydrated enzyme molecule, which decreases enzyme activity *(19)*. Arcos and Otero *(20)* also found a less costly and more selective process in the absence of solvents to produce mono- and dioleoylglycerols by direct esterification of glycerol and oleic acid.

Time Course on Best Condition

Considering the results presented, the best conditions for synthesizing monocaprin were 55°C, an enzyme concentration of 3% (w/w), and the use of the stoichiometric molar ratio of reagents. Figure 8 shows the composition of the nonpolar phase for monocaprin synthesis under these conditions.

Conclusion

The factorial design identified the most important parameters determining monocaprin selectivity under the tested conditions. The three parameters studied (temperature, molar ratio of reagents, enzyme concentration) have statistical significance and the synthesis of monocaprin was favored at lower values of these parameters. Using molecular sieves for water removal, although effective, was not better than the simple evaporation technique.

The results obtained in the present work indicate that monocaprin production was feasible in a solvent-free medium. This reaction system avoids the problems of separation, toxicity, and flammability of organic solvents, lowering the cost of the final product and allowing product recovery without further purification steps. The final composition obtained after 6 h of reaction at 55°C with an enzyme concentration of 3% (w/w) and capric acid/glycerol molar ratio of 1 was 61.3% monocaprin, 19.9% dicaprin, and 18.8% capric acid. This composition agrees with the directives of the World Health Organization *(20)* for use as food emulsifiers, which requires that these mixtures have at least 70% mono- plus diglyceride, a minimum of 30% monoacylglycerol, and quantities of glycerol and triglycerides below 10%. The development of monoglyceride production by lipases can be more competitive than the chemical process, because less energy is consumed; simple reaction media are used; and higher productivity, higher selectivity, and higher-quality products are obtained in the bioprocess.

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