

Enzymatic Synthesis of Monolaurin

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Abstract

The aim of this study was to produce monolaurin utilizing a commercial immobilized lipase (Lipozyme IM-20; Novo Nordisk, Bagsvaerd, Denmark) through the direct esterification of lauric acid and glycerol in a solvent-free system. The influence of fatty acid/glycerol molar ratio, temperature, and Lipozyme (IM-20) concentration on the molar fraction of monolaurin were determined using an experimental design. The best conditions employed were 55°C, lauric acid/glycerol molar ratio of 1.0, and 3.0% (w/w) enzyme concentration. The final product, obtained after 6 h of reaction, was 45.5% monolaurin, 26.8% dilaurin, 3.1% trilaurin, and 24.6% lauric acid. The reusability of the enzyme was also studied.

Index Entries: Monolaurin; immobilized lipase; esterification; experimental design; solvent-free medium.

Introduction

Monolaurin, a monoglyceride resulting from the reaction between lauric acid and glycerol, is a nonionic surfactant with important applications in pharmaceuticals, food, and cosmetics production (1,2). Recently, the potential of monolaurin and other monoglycerides to inhibit the growth of bacterial spores and vegetative cells was demonstrated, including that of *Clostridium botulinum* and *Bacillus cereus* (2–4). Chemical preservation is widely used to extend food shelf life and to delay or inhibit the growth of pathogenic microorganisms. However, the questionable safety of some chemical food preservatives has stimulated efforts to develop alternatives based on natural substances.

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Monolaurin has also been studied for its bactericidal activity against *Helicobacter pylori*, which is responsible for chronic gastritis and duodenal ulceration, also a known risk factor in gastric cancer owing to inflammatory mechanisms (5). The best available treatments with combinations of drugs result in between 70 and 100% eradication of the bacterium, but adverse reactions, side effects, and the developing resistance of bacterium to available antibiotics are continuing problems (6). Therefore, new methods to prevent and treat gastrointestinal infections caused by *H. pylori* have been studied. In studies on antibacterial activity of monoglycerides, Bergsson et al. (5) found monocaprin and monolaurin to be the most active agents against *H. pylori* for short incubation times.

Monolaurin has demonstrated a large spectrum of activity against fungi and viruses. Beyond the antiviral activity, monolaurin increases an organism's defenses against virus attack, potentializing immunologic reactions initiated by agents such as antigens.

Presently, monoglycerides are manufactured on an industrial scale by continuous chemical glycerolysis of fats and oils at high temperatures (220–250°C), employing inorganic alkaline catalysts under a nitrogen gas atmosphere. The product obtained by this route has several drawbacks such as low yield, dark color, and burnt taste. In addition, the process is not very selective toward monolaurin and is intensive on energy consumption. Recently, some studies involving alternative heterogeneous catalytic routes have been reported, such as glycerol esterification with fatty acids using different catalysts: cationic resins, zeolitic molecular sieves, functionalized mesoporous materials, and oxides such as MgO and ZnO (1). Alternative methods have been developed as substitutes for conventional chemical processes. In the last decade, many approaches have been investigated for the enzymatic synthesis of monoglycerides using lipase (7). The advantages of enzymatic synthesis are mild reaction conditions, resulting in products of higher quality and lower energy consumption; the selectivity of lipases; and fewer environmental problems (8).

The objective of the present work was to study the synthesis of monolaurin by direct lipase-catalyzed esterification between glycerol and lauric acid without any solvent or surfactant. The effects of lauric acid/glycerol molar ratio, enzyme concentration, and temperature were studied using an experimental design. The reuse of the commercial immobilized lipase, to reduce the process cost, was also investigated.

Materials and Methods

Chemicals

Lipozyme IM-20 (*Mucor miehei* lipase immobilized on a weak anion-exchange resin) was kindly supplied by Novo Nordisk (Bagsvaerd, Denmark). The gas chromatography (GC) standards (mono-, di-, and trilaurin) were obtained from Sigma (St. Louis, MO). Analytical-grade glycerol, lauric acid (99.9%), *n*-hexane, ethyl acetate, acetone, and ethanol 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 (9), 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 preparation. 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

Lauric acid and mono-, di-, and trilaurin were analyzed by capillary GC according to the method described by Langone and Sant'Anna (9). All concentrations were calculated as molar fractions from the peak area using calibration curves.

Esterification Experiments

All experiments were performed in a 20-mL open batch reactor with constant stirring and temperature control. The reaction system contained a mixture of lauric acid and glycerol and the biocatalyst Lipozyme IM-20. 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 (10). The statistical analysis of monolaurin molar fraction (Y_{mon}) was made by means of a two-level-three-factors central composite design with six star points and six central points. The experimental data were analyzed using STATISTICA® for Windows, release 5.5, produced by Statsoft.

Recovery of Immobilized Enzyme

After completion of each esterification reaction, the medium was centrifuged, and the solid phase (immobilized enzyme) was recovered according to the method described by Langone and Sant'Anna (11). Then, the experiments were performed reusing the enzyme recovered after each batch reaction assay.

Results and Discussion

Experimental Design

An experimental design technique was used to identify the best conditions to produce monolaurin. The basic idea was to devise a small set of

Table 1
Coded Levels and Corresponding Actual Values Employed

Variables	Code	Actual factor level at coded factor levels of				
		-1.682	-1	0	+1	1.682
Acid/glycerol molar ratio	<i>R</i>	0.5	0.7	1.0	1.3	1.5
Temperature (°C)	<i>T</i>	48	51	55	59	62
Enzyme Concentration(%w/w)	<i>E</i>	0.5	1.5	3.0	4.5	5.5

experiments accounting for all pertinent factors (10). The subsequent analysis of data identified optimal conditions, factors that most influenced the results as well as those that did not, and the presence of significant interactions. A model relating the dominating factors to the response variable (monolaurin molar fraction Y_{mon} %) was obtained.

A central composite design for three factors was used to generate 20 combinations. The effects of independent variables—acid/glycerol molar ratio (*R*), temperature (*T*), and enzyme concentration (*E*)—on the response (i.e., the monolaurin molar fraction at 4 h) were investigated. The upper and lower limits of each variable were chosen based on published data and preliminary studies (12,13). Actual independent variables or factors and their corresponding coded levels are presented in Table 1.

The enzymatic synthesis of monolaurin was quantified in terms of molar fraction of each component in the nonpolar phase (lauric acid, mono-, di-, and trilaurin), and monolaurin molar fraction was chosen to define the best reaction conditions. The selectivity parameter, defined as the ratio between concentrations of monolaurin and total concentration of reaction products (mono-, di-, and trilaurin), was also evaluated.

The full factorial central composite design includes factorial points, star points, and center points. The corresponding model is the complete quadratic surface between the response and the factors, as given by Eq. 1:

$$Y_{pred} = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2 + b_{12}x_{12} + b_{13}x_{13} + b_{23}x_{23} \quad (1)$$

in which Y_{pred} is the predicted response (monolaurin molar fraction), $x_1 = R$, $x_2 = T$, $x_3 = E$, $x_{12} = RT$, $x_{13} = RE$, $x_{23} = TE$, b_1 through b_{23} are the respective coefficients of variables and interactions, and b_0 is the mean.

Table 2 presents the analysis of the main effects and interactions of the factors (linear and quadratic) for the chosen response and their statistical significance (*p* value). It can be observed that the main factor *E* (linear and quadratic) and two main factors (*R* and *T* quadratic) are the important ones with a statistically significant effect on the response of monolaurin molar fraction ($p < 0.05$).

Substituting the coefficients b_i in Eq. 1 by their values from Table 2 on obtains:

$$Y_{mon} = 40.1 - 0.2R + 0.9T + 7.6E - 1.2R^2 - 3.2T^2 - 4.5E^2 + 0.2RT + 0.4RE - 0.1TE \quad (2)$$

Table 2
Estimated Factor and Interaction Effects, Coefficients
for Predictive Mathematical Models, and *p* Values

Variable	Coefficient	SEC ^a	Effect	SEE ^b	<i>t</i> -test	<i>p</i> Value
Mean	40.10 (<i>b</i> ₀)	0.52	40.1	0.52	76.87	0.0000
R—linear (<i>x</i> ₁)	−0.20 (<i>b</i> ₁)	0.35	−0.4	0.69	−0.59	0.5775
R—quadratic (<i>x</i> ₁ ²)	−1.19 (<i>b</i> ₁₁)	0.34	−2.58	0.68	−3.48	0.0177
T—linear (<i>x</i> ₂)	0.93 (<i>b</i> ₂)	0.34	1.86	0.68	2.73	0.0411
T—quadratic (<i>x</i> ₂ ²)	−3.20 (<i>b</i> ₂₂)	0.31	−6.40	0.63	−10.16	0.0001
E—linear (<i>x</i> ₃)	7.61 (<i>b</i> ₃)	0.35	15.21	0.69	21.92	0.0000
E—quadratic (<i>x</i> ₃ ²)	−4.50 (<i>b</i> ₃₃)	0.34	−9.00	0.68	−13.18	0.0000
<i>RT</i> (<i>x</i> ₁₂)	0.22 (<i>b</i> ₁₂)	0.45	0.45	0.90	0.50	0.6395
<i>RE</i> (<i>x</i> ₁₃)	0.44 (<i>b</i> ₁₃)	0.45	0.88	0.87	0.98	0.3722
<i>TE</i> (<i>x</i> ₂₃)	−0.12 (<i>b</i> ₂₃)	0.45	−0.24	0.90	−0.27	0.7970

^aSEC, standard error of coefficient.

^bSEE, standard error of effect.

After estimating the factors' main effects, other significant factors affecting the monolaurin molar fraction were established by analysis of variance (ANOVA), as shown in Table 3. From the *p* values, defined as the smallest level of significance leading to rejection of the null hypothesis, it appears that the main effect of each factor (linear and quadratic) and the interaction effects are statistically significant when *p* < 0.05. The effects of *E* and *E*², and *T*² were of higher statistical significance. The effects statistically not significant were *R*, *RT*, *RE*, *TE*.

A useful plot for identifying factors that are important is a Pareto chart. The graph in Fig. 1 shows the *t*-test values in the horizontal axis and also includes a vertical line to indicate the *p* value (an effect that exceeds the vertical line may be considered significant). As observed in the Pareto chart, enzyme concentration is the most significant variable influencing monolaurin molar fraction.

Based on *F*-test and student's *t*-test, some effects were discarded, because they did not exhibit any statistical significance. The resultant model can be represented by

$$Y_{mon} = 40.1 + 0.9T + 7.6E - 1.2R^2 - 3.2T^2 - 4.5E^2 \quad (3)$$

Figure 2 shows the three-dimensional plot for the response of monolaurin molar fraction vs temperature ratio and enzyme concentration at a molar ratio of 1.0 and reaction time of 4 h. The highest *Y*_{mon} values were reached at temperatures between 54 and 56°C and an enzyme concentra-

Table 3
Analysis of Variance^a

Factor	Sum of squares	Degree of freedom	Mean of squares (MS)	F_0	p Value
R	0.578	1	0.578	0.3545	0.577516
R^2	19.727	1	19.727	12.0903	0.017714
T	12.187	1	12.187	7.4689	0.041136
T^2	168.332	1	168.332	103.1681	0.000159
E	784.152	1	784.152	480.5950	0.000004
E^2	283.391	1	283.391	173.6861	0.000045
RT	0.405	1	0.405	0.2482	0.639470
RE	1.566	1	1.566	0.9601	0.372172
TE	0.120	1	0.120	0.0736	0.797041
Lack of fit	36.939	5	7.388	4.5279	0.061487
Pure Error	8.158	5	1.632	—	—
Total sum of squares	1255.291				

^a $F_0 = MS_{\text{FACTOR}}/MS_{\text{ERROR}}$; $R^2 = 0.9641$; R^2 adj. = 0.9317.

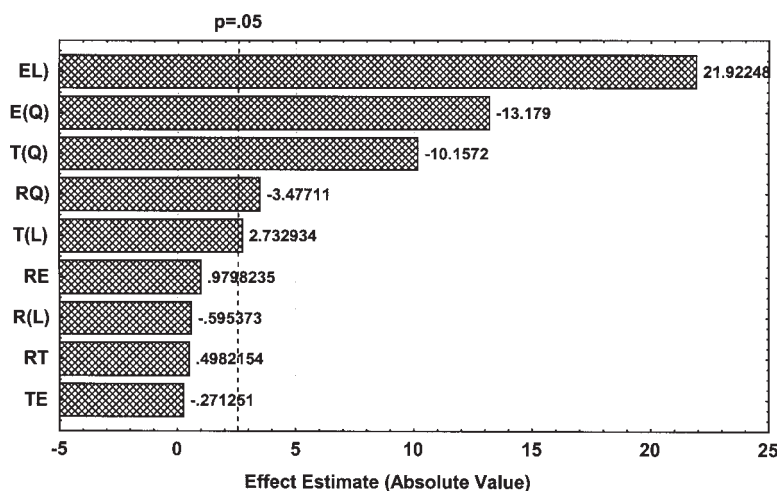


Fig. 1. Pareto chart of effects.

tion in the range of 4.0 to 5.0% (w/w). These observations are consistent with the experimental data in Fig. 3. The best measured results in terms of molar fraction of monolaurin were obtained at 55°C, with a reagent molar ratio of 1 and an enzyme concentration of 3% (w/w).

Data analysis led to the optimal conditions: 55.5°C, molar ratio of 1.0, and enzyme concentration of 4.3% (w/w) corresponding to monolaurin molar fraction (43.3%). The best measured values are closer than those obtained from the statistical analysis. ANOVA demonstrated that modeling was successful with a coefficient of determination (R^2) of 0.964. The plot

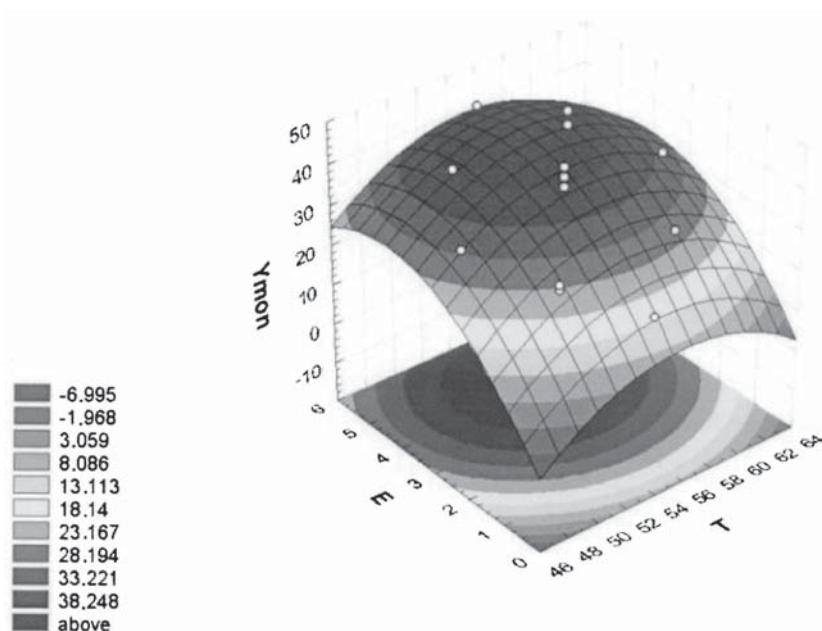
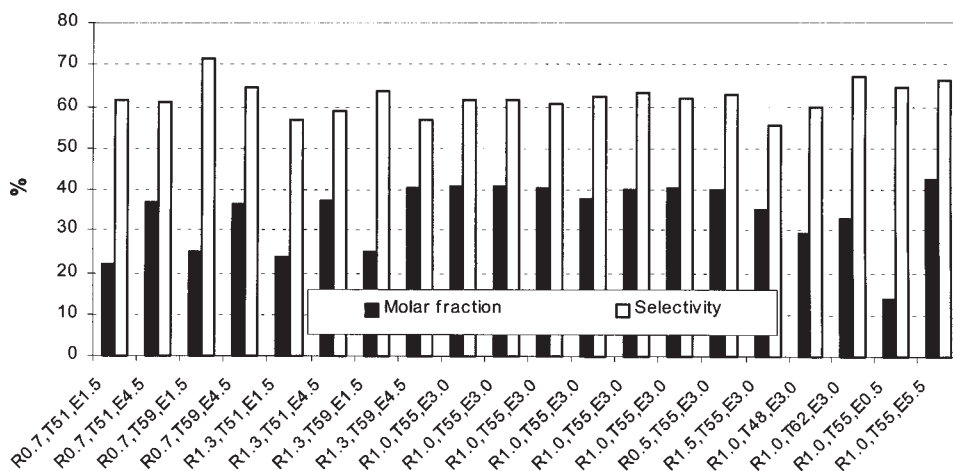


Fig. 2. Response surface for monolaurin molar fraction.

Fig. 3. Influence of variables (R , T , and E) on monolaurin molar fraction and monolaurin selectivity after 4-h reaction.

of experimental vs predicted values is shown in Fig. 4. Clearly the model represents the experimental range of studied variables adequately.

Effects of Enzyme Concentration

According to Fig. 1, the enzyme concentration is the variable presenting higher statistical importance on monolaurin synthesis. Therefore,

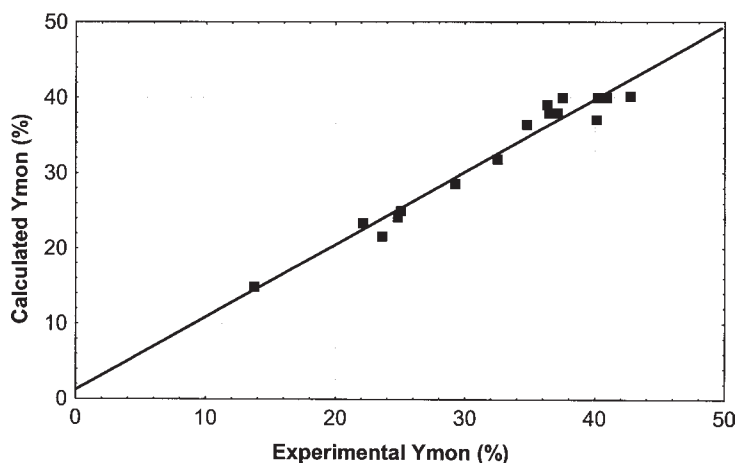


Fig. 4. Comparison of experimental and calculated values of monolaurin molar fraction.

experiments with different concentrations were proposed, keeping the stoichiometric molar ratio of reagents and temperature of 55°C; results are presented in Fig. 5. The best measured results were obtained with Lipozyme IM-20 at 3.0–5.8 % (w/w). Enzyme concentration affected the initial reaction rate but did not significantly affect the final (after 6 h) molar fraction. Figure 5 confirms the data in Fig. 3 and in the response surface (Fig. 2). Considering the advantages of reduced operating costs, the best concentration of enzyme preparation was 3.0% (w/w).

Wong et al. (14) also studied the effect of the amount of enzyme (lipase from *Candida rugosa*; Sigma) on monocaprin synthesis in isooctane at 37°C. They observed that monocaprin molar fraction increased when the amount of lipase was increased, but no significant increase in monocaprin yield (conversion of capric acid equal to 35%) was observed for a lipase loading of more than 100.0 mg (corresponding to 16.4% [w/w]).

Ferreira-Dias et al. (15) tested Lipozyme IM-20 for the glycerolysis of olive oil residue in *n*-hexane aimed at the production of monoglycerides and diglycerides. The highest monoglyceride production was in the range of 43–45% (w/w), on the basis of total fat, with about 26% Lipozyme IM-20.

It can be concluded that in the system used in our work (without solvent, open reactor, at 55°C), it is possible to obtain better conversions (up to 70%) using lower enzyme concentration.

Effect of Temperature

The effect of temperature on monolaurin synthesis was also statistically significant. The best temperature tested was 55°C. Figure 6 shows the effect of temperature on monolaurin molar fraction during 6 h of reaction using a concentration of enzyme preparation of 3.0% (w/w) and substrate molar ratio of 1.0. An increase in temperature (above 55°C) did not favor

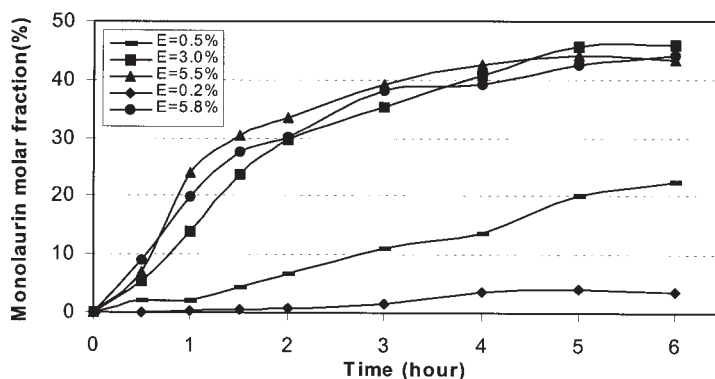


Fig. 5. Effect of Lipozyme IM-20 concentration on molar fraction in monolaurin for synthesis performed at 55°C using $R = 1.0$.

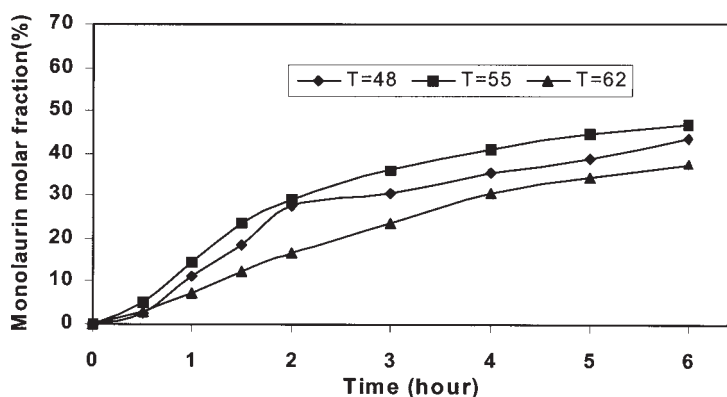


Fig. 6. Effect of temperature on monolaurin synthesis (molar fraction) carried out for 6 h with lauric acid/glycerol molar ratio of 1 and 3.0% (w/w) Lipozyme IM-20.

monolaurin synthesis. This can be confirmed by the lesser values of monolaurin molar fraction during the reaction course at 62°C (Fig. 6).

Langone et al. (13) observed a lower thermal stability of Lipozyme IM-20 in the synthesis of monoglycerides (monomiristin and monolaurin) compared with the synthesis of the respective triglycerides. The synthesis of triglycerides (direct esterification between an excess of fatty acid and glycerol: $R = 5.0$) in a solvent-free system was possible at temperatures near 100°C, allowing a triglyceride selectivity of 100% after 24 h. For the synthesis of monoglycerides (using $R = 1.0$), Langone et al. (13) showed that an increase in temperature from 60 to 90°C resulted in a decrease in monolaurin molar fraction of 30%. Thus, temperature itself is not the unique factor affecting lipase denaturation; the higher glycerol concentration required for the synthesis of monolaurin seems to reduce the enzyme activity.

Effect of Molar Ratio of Reagents

As shown in Fig. 3, using an excess of glycerol corresponding to the lauric acid/glycerol molar ratio of 0.5 and 0.7 did not result in a significant increase in monolaurin molar fraction. Wong et al. (14) also investigated the effect of substrate molar ratio on monocaprin synthesis catalyzed by a lipase from *C. rugosa*. They concluded that an excess of glycerol on the reaction mixture inhibited enzyme activity. This can be explained by the fact that glycerol is a polar substance and in excess can be adsorbed by the immobilized enzyme support, removing the catalyst water layer that is necessary for lipase stabilization causing enzyme inactivation. On the other hand, an increase in lauric acid concentration ($R = 1.3$; $R = 1.5$) enhanced the synthesis of di- and trilaurin. Therefore, the best lauric acid/glycerol molar ratio tested for the monolaurin synthesis was the stoichiometric molar ratio ($R = 1$).

Time Course on Best Condition

Considering the results presented, the best experimental conditions used for monolaurin synthesis were 55°C, an enzyme concentration of 3% (w/w), and use of the stoichiometric molar ratio of reagents. Figure 7 shows the nonpolar phase for monolaurin synthesis under these conditions. The final product, obtained after 6 h of reaction, was 45.5% monolaurin, 26.8% dilaurin, 3.1% trilaurin, and 24.6% lauric acid.

Bellot et al. (16) investigated monoglyceride synthesis by *Rhizomucor miehei* lipase (Lipozyme) via direct esterification between glycerol and oleic acid in organic solvents. In pure *n*-hexane, the monoglyceride represented only 6 molar % of the total products at the thermodynamic equilibrium (34 and 60% for di- and triglyceride, respectively). The use of a mixture of *n*-hexane/2-methyl-2-butanol enables a product mixture to be obtained containing 94% monoglycerides at equilibrium. However, this positive effect is counterbalanced by a decrease in both initial velocities and substrate conversion at thermodynamic equilibrium.

Again, it can be concluded that the system used in our work, without solvent, is a less costly and more selective process to produce mono- and diglycerides by direct esterification of glycerol and fatty acid. Such a system avoids the problems of separation, toxicity, and flammability of organic solvents, permitting recovery of product without further complex purification or evaporation steps and lowering the cost of the final product.

The results presented here are close to those obtained by chemical glycerolysis of fat and oils at high temperatures, employing inorganic alkaline catalysts. The product is a mixture that contains 35–60% monoglycerides, 35–50% diglycerides, 1–20% triglycerides, and 1–10% free fatty acids and their alkali metal salts (8).

Machado et al. (1) studied the synthesis of monolaurin from lauric acid and glycerol employing commercial Beta, Y, and Mordenite zeolites as catalysts. The optimized conditions for the monolaurin synthesis

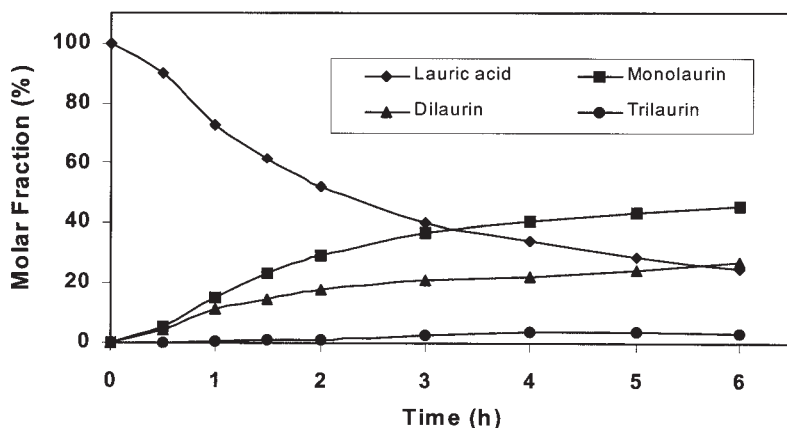


Fig. 7. Time course for monolaurin synthesis carried out at 60°C with lauric acid/ glycerol molar ratio of 1 and 3.0% (w/w) Lipozyme IM-20.

were 110°C, stoichiometric ratio of reagents, catalyst concentration of 5.0% (w/w), and reaction time of 24 h. The best results were obtained by using zeolite Beta as catalyst, and monolaurin yields of 20% with selectivities higher than 65% were achieved.

A comparison of these methods shows that the development of monoglyceride production by enzymatic method could be considered as compared to the chemical process, since milder reaction conditions are utilized and less energy is consumed in a shorter time.

Reusability of Enzyme

The results of the production of monolaurin at 55°C with an enzyme concentration of 3.0 (w/w) and substrate molar ratio of 1.0, in several sequential experiments performing enzyme recover and reuse are shown in Fig. 8. The test was realized in triplicate (medium 1, 2, and 3). The enzyme could be reused, keeping its performance, two times for the synthesis of monolaurin. Langone and Sant'Anna (11) investigated the reutilization of immobilized lipase in triglyceride synthesis. They observed that the enzyme remained stable even when reused 16 times. The small number of enzyme reuses obtained in monolaurin synthesis can be explained considering the greater amount of glycerol in the reaction medium, which, as previously described, can inactivate the enzyme.

Conclusion

A fully central composite design was applied to optimize monolaurin synthesis. A three factorial design was proven effective to establish the influence of the variables on the monolaurin synthesis. The central composite design procedure was adopted to optimize variables affecting the monolaurin molar fraction.

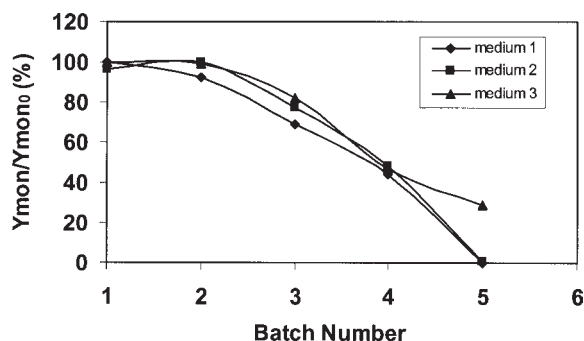


Fig. 8. Effect of enzyme reutilization—Lipozyme IM-20 3.0% (w/w)—on monolaurin synthesis (molar fraction). Successive 6-h batches were performed using a lauric acid/glycerol molar ratio of 1 at 55°C.

Statistical analysis showed that within the experimental range considered, the most important factor was enzyme concentration. The temperature also affected the monolaurin molar fraction. This factor had a negative influence. According to these results, the maximum monolaurin molar fraction (43.3%) was achieved at 55°C, a molar ratio of 1.0, and an enzyme concentration of 4.3% (w/w) within the range studied.

The results of our work illustrate the technical feasibility of producing monoglycerides in a solvent-free medium, although conversions (defined as the percentage of consumed lauric acid) near 70% were attained, with monolaurin being the main product. Such a system avoids problems of separation, toxicity, and flammability of organic solvents, lowering the cost of the final product and permitting recovery of product without further purification or evaporation steps. Furthermore, the results obtained are in accordance with the directives of the World Health Organization. The requirements for utilization of these mixtures as food emulsifiers are (1) to have at least 70% mono- and diglycerides, (2) to have a minimum of 30% monoglycerides, and (3) to present concentrations of both glycerol and triglycerides below 10% (17). Therefore, the results indicate that direct esterification of lauric acid and glycerol under catalysis by immobilized lipase (Lipozyme IM-20) is a very efficient method, allowing production of an appreciable amount of monolaurin with good selectivity.

The possibility of immobilized enzyme reuse was confirmed for monolaurin synthesis. This aspect, not often investigated, certainly increases the economical viability of the process.

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