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Process Development for Production of Medium Chain Triglycerides Using Immobilized Lipase in a Solvent-Free System

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Abstract

The synthesis of tricaprylin, tricaprin, trilaurin, and trimyristin in a solvent-free system was conducted by mixing a commercial immobilized lipase with the organic reagents (glycerol and fatty acid) in a 20-mL batch reactor with constant stirring. The effects of temperature, fatty acid/glycerol molar ratio, and enzyme concentration on the reaction conversion were determined. The reactions were carried out for 26 h and the nonpolar phase was analyzed by gas chromatography. Appreciable levels of medium chain triglycerides were achieved, except for tricaprylin. The higher selectivity values for the production of triglycerides were attained under the following conditions: a fatty acid/glycerol molar ratio of 5; enzyme concentration of 5 or 9% (w/w); and temperatures of 70°C (tricaprin), 80°C (trilaurin), and 90°C (trimyristin). After completion of the esterification reaction under these conditions, the recovery of the triglyceride and fatty acids, and the reusability of the enzyme were studied. The unreacted fatty acid and the produced triglyceride were satisfactorily recovered. The commercial immobilized lipase was used in 10 consecutive batch reactions at 80°C, with 100% selectivity in the trilaurin and trimyristin synthesis. The possibility of enzyme reuse and the recovery of residual fatty acid are relevant results that contribute to increasing the viability of the process.

Index Entries: Medium chain triglycerides; immobilized lipase; esterification.

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Introduction

An increased interest in the chemistry and biotechnology of fats and oils has emerged in recent years. The utilization of enzymes, mainly lipases (triacylglycerol lipases; EC 3.1.1.3), in organic syntheses has become widespread. These enzymes have been widely used for the conversion of natural fats and oils into value-added products, such as medium-chain triglycerides, which are specialty fats composed primarily of medium carbon-chain fatty acids *(1)*. The chemical structure of medium chain triglycerides affords them unique properties over regular vegetable oils and other food-grade solvents in many food applications. Medium chain triglycerides are also used as carriers for flavors, colors, vitamins, and pharmaceuticals *(2,3)*.

The large-scale manufacturing process of medium chain triglycerides involves the direct esterification of medium-chain fatty acids and glycerol at high temperature and high pressure *(4)*. The product, obtained at low yield, has some undesirable properties such as dark color and burnt taste. Enzyme-catalyzed esterification reactions have advantages over the conventional chemical esterification methods, because reactions are conducted under mild conditions, exploiting the high catalytic efficiency and selectivity of lipases and leading to purer products. For the enzymatic production of medium chain triglycerides, several methods are described in the literature.

Some investigators *(4–10)* have shown that such reactions could be performed in a medium solely composed of substrates in the presence of immobilized lipase without any solvent or surfactant. Such a system reduces the phase-separation problems; eliminates the handling of toxic and flammable organic solvents, enables product recovery without further purification or evaporation steps; and, consequently, reduces product cost.

One of the major advantages of immobilized enzymes is reutilization, which is a key factor for process economics. Furthermore, the use of immobilized lipase reactors reduces the risks of product contamination by the residual enzyme and often enhances enzyme stability. A better process and product quality control may be achieved in such reactors *(11–13)*.

Although the use of lipase for the synthesis of medium chain triglycerides is an area of increasing interest and research, in general, there is little information about the recovery of the triglycerides produced, the unreacted fatty acid, and the immobilized lipase in such reaction systems.

The aim of the present work was to study the effect of the reaction temperature, fatty acid/glycerol molar ratio, and enzyme concentration on the synthesis of tricaprylin, tricaprin, trilaurin, and trimyristin in solvent-free media. The recovery of the product (triglyceride) and reagent (fatty acid) and the reuse of the commercial immobilized lipase, aspects that are not often investigated, were focused on for the production of trilaurin and trimyristin.

Materials and Methods

Materials

Lipozyme IM-20 (*Mucor miehei* lipase, immobilized on a weak anion-exchange resin) was a donation from Novo Nordisk A/S (Bagsvaerd, Denmark). All fatty materials (substrates and gas chromatography [GC] standards) were purchased from Sigma (St. Louis, MO). Analytical grade glycerol, *n*-hexane, ethyl acetate, ethanol, acetone, and chloroform were purchased from Merck (Darmstadt, Germany).

Determination of Lipase Activity

The esterification activity of the enzyme was measured according to the method described by Langone and Sant' Anna *(10)*, which determines the rate of consumption of fatty acid at 60°C in a reaction system containing glycerol, lauric acid, and a given amount of the commercial enzyme preparation.

Nonpolar Phase Analysis

The fatty acids and mono-, di-, and triglycerides were analyzed by capillary GC according to the procedure proposed by Legier-Deyris et al. *(14)* adapted to the conditions of this work. Each 20 µL sample was diluted (1000×) in a 1:1 hexane/ethyl acetate mixture, and 1 µL was injected into a Chrompack CP 9000 gas chromatograph with a flame ionization detector. The GC was fitted with a 10 m × 0.25 mm × 0.12 µm Chrompack CP Sil 5CB column. Helium was used as the carrier gas with a flow rate of 2 mL/min. The detector and injector temperatures were set at 350°C. The column temperature was set at 80°C for 1 min and was then programmed at 20°C/min to 320°C, which was maintained constant for 2 min. For caprylic acid and caprylic glycerides, after injection of the samples, the temperature of the column was kept constant for 1 min (65°C), then linearly increased to 300°C (25°C/min) and kept at this temperature for 2 min. All concentrations were calculated as molar fractions from the peak area using calibration curves.

Esterification Experiments

All experiments were carried out in a 20-mL batch reactor with constant stirring, using a magnetic stirrer. The reactor (jacketed beaker) was kept at the selected temperature by a thermostatic water bath. Given quantities of glycerol and fatty acid were mixed together and preincubated at the selected temperature, followed by the addition of the immobilized lipase. The progress of the reaction was followed by withdrawing 20-µL aliquots at various time intervals, and then analyzing them by the GC method already described. The water produced in the reaction was spontaneously removed by free evaporation from the reaction medium. The reactions were carried out for 26 h.

The following reaction conditions were investigated: temperature ranging from 50 to 100°C, fatty acid/glycerol molar ratios of 1, 3, 5, and 7; and commercial enzyme amounts corresponding to 1, 5, and 9% (w/w).

Recovery of Triglyceride, Fatty Acid and Immobilized Enzyme

After completion of each esterification reaction, the medium was centrifuged. The solid phase (immobilized enzyme) was resuspended in 10 mL of dry hexane to extract any substrate or products, centrifuged, further washed with an additional 10 mL of hexane, recovered by filtration over filter paper, and dried under vacuum as suggested by Castro et al. *(15)*. It was stored for 24 h in a desiccator for subsequent reuse. Fifty milliliters of chloroform and 25 mL of sodium carbonate 5% (w/v) in water were employed to perform the extractive separation of the liquid phase. The solution containing the analyte was extracted successively with two portions of fresh chloroform and sodium carbonate 5% (w/v). An ordinary separation funnel was used. The final product (triglyceride) was obtained by evaporation of the chloroform from the organic phase in a rotary vacuum evaporator. Acetic acid was added to the aqueous solution (until pH 5.0) to recover the fatty acid, which was crystallized when the solution was refrigerated. The recovered solids were further dried under vacuum.

Results and Discussion

Commercial Enzyme Preparation Activity

A linear relationship between the initial rate of lauric acid consumption and the commercial enzyme concentration (% [w/w]) was observed for the range studied (1–9% [w/w]). Taking into account the definition of activity formulated by Langone and Sant'Anna *(10)* and the linear relationship, it can be stated that the commercial lipase preparation used in our experiments presented an activity of 0.03 U/g. For consistency, the enzyme amount per volume will hereinafter be expressed on a weight/weight percentage basis.

Triglyceride Synthesis

Experimental results of triglyceride synthesis are summarized in Figs. 1–4. The results are expressed as a molar fraction of the component in the nonpolar phase (fatty acid; mono-, di-, and triglyceride). The selectivity parameter, chosen to define the best reaction conditions, was defined as the ratio between triglyceride and the total glyceride (mono-, di-, and triglyceride) content in a molar basis.

Trilaurin

Maximum selectivity values were only attained when molar ratios equal to or greater than 5 were used in the experiments, conducted at 80°C (Fig. 1). Since the use of a molar ratio of 7 did not promote an appreciable

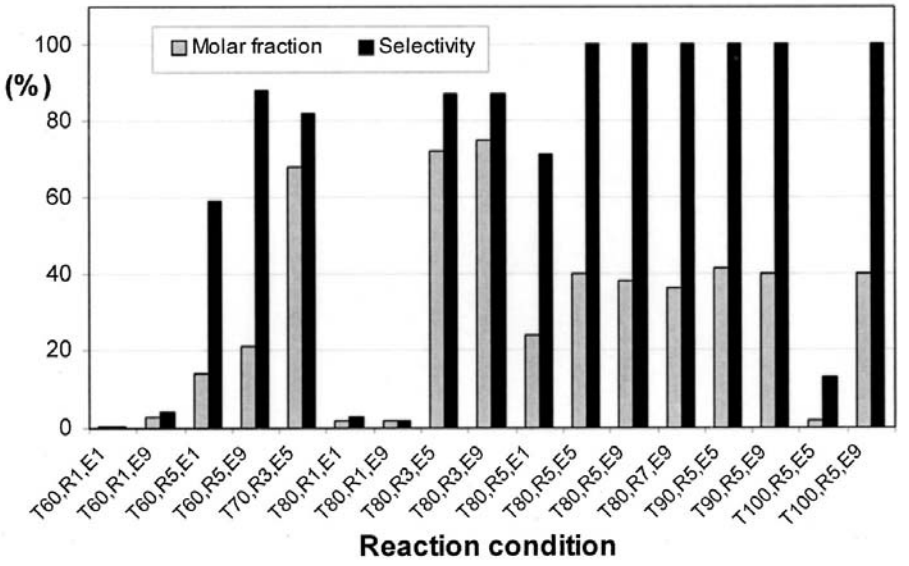

Fig. 1. Effect of temperature (*T*), fatty acid/glycerol molar ratio (*R*), and enzyme concentration (*E*) on trilaurin synthesis carried out for 26 h.

increase in trilaurin production rate, the ratio of 5 was taken as an optimal value. As expected, the utilization of the stoichiometric ratio (molar ratio = 3) increased the molar fraction of trilaurin in the reaction medium. However, the production of diglyceride (dilaurin) reached molar fractions in the range of 10–20%, reducing the selectivity of the reaction toward the triglyceride.

Note that the molar fraction attained in the experiments presenting 100% selectivity (excess of fatty acid) was higher than the theoretical value (33%). This difference perhaps can be attributed to the following factors: evaporation of lauric acid under reactions conditions (80–90°C, 26-h reaction); fatty acid adsorption on the enzyme immobilization support, reducing its concentration in the bulk phase; analytical error associated with the low fatty acid concentration found at the end of the reaction.

A surplus of lauric acid, which leads to the production of trilaurin as the sole product, can be used, from a process point of view, if the unreacted fatty acid can be recovered at the end of the reaction. The utilization of an excess of glycerol (molar ratio fatty acid/glycerol = 1) favored the synthesis of monolaurin, as expected, and as also observed by Ergan et al. *(5)*.

Temperature had a marked effect on trilaurin synthesis. High selectivity values were only attained at 80 and 90°C. Higher temperatures promote free water evaporation, shifting the reaction equilibrium toward the formation of the triester. Under these conditions, the reactions were accomplished after 15 h. At 100°C, enzyme denaturation was severe, leading to lower selectivity values, when the amount of enzyme corresponded to 5% (w/w). Selectivity toward trilaurin increased when the enzyme amount was raised

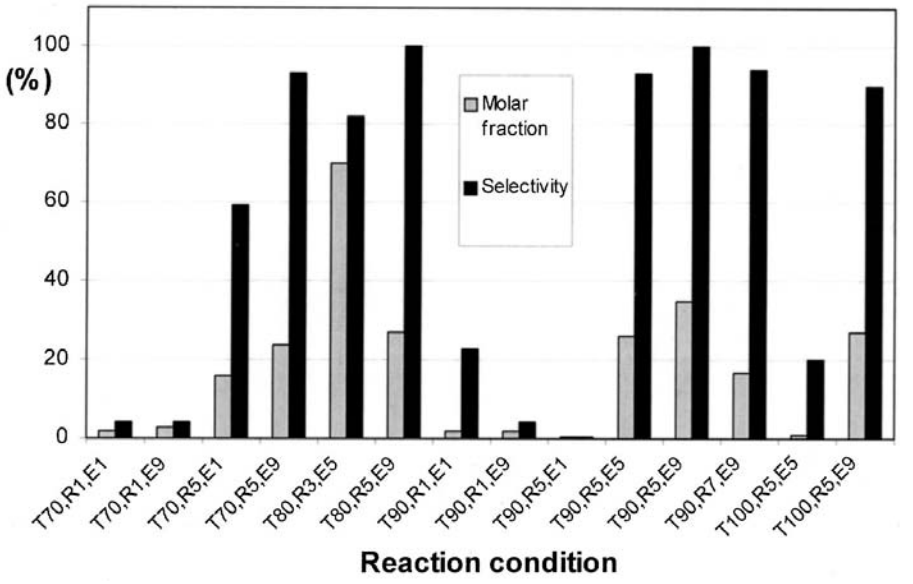

Fig. 2. Effect of temperature (*T*), fatty acid/glycerol molar ratio (*R*), and enzyme concentration (*E*) on trimyristin synthesis carried out for 26 h.

from 1 to 5%. However, a further increase in enzyme concentration did not result in a significant improvement in selectivity.

For the reaction condition leading to the higher (100%) selectivity, further tests were carried out to recover trilaurin, residual lauric acid, and the immobilized enzyme. Solids (commercial enzyme) were recovered from the reaction medium as already described. The recovery of lauric acid from the reaction medium was performed with a yield of 76% and presented trilaurin as the main impurity. The term *yield* is the ratio between the mass recovered and the theoretical mass that should be obtained from the initial concentrations of reactants. Recovery of trilaurin presented a yield of 59% and a purity of 95%. A selectivity value of 95% was reached after 20 h of reaction in a specific experiment conducted with the recovered enzyme and the recovered lauric acid. This is an important result in terms of process viability.

Trimyristin

The results shown in Fig. 2 are very similar to those obtained for trilaurin synthesis. The best conditions for trimyristin synthesis were 90°C, molar ratio of 5, and commercial enzyme amount of 9% (w/w). Under these conditions, myristic acid was recovered with a yield of 76% and purity of 90%, while trimyristin was obtained with a purity of 93% and a recovery yield of 63%. Utilization of the recovered myristic acid and the commercial enzyme, in a further experiment, led to a selectivity value of 92%, after 20 h of reaction.

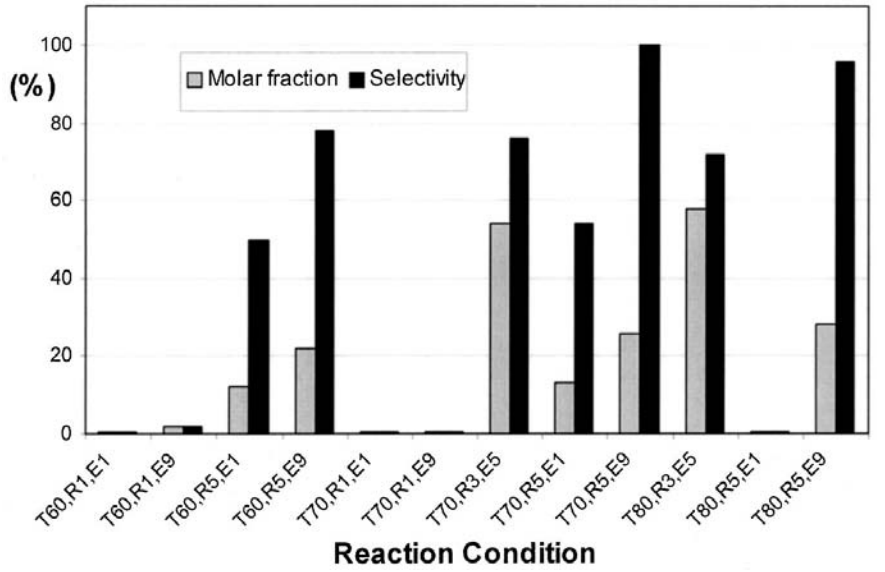

Fig. 3. Effect of temperature (*T*), fatty acid/glycerol molar ratio (*R*), and enzyme concentration (*E*) on tricaprin synthesis carried out for 26 h.

Tricaprin

Moderate levels of this triglyceride were obtained in the set of experiments shown in Fig. 3. At 90°C, tricaprin was not produced and the synthesis of mono- and dicaprin was markedly reduced, probably as a consequence of the thermal instability of lipozyme in the presence of capric acid, as observed by Kwon et al. *(11)*. Although in a few experiments a high selectivity was observed, a large amount of capric acid remained at the end of the reaction, indicating that the reaction conversion was low, when compared with trilaurin and trimyristin synthesis reactions. Reaction equilibrium was only reached after 20 h. Tricaprin was also produced in small amounts by Kim and Rhee *(4)* after 80 h of reaction, using lipozyme in a similar reaction system.

Recovery of product and reactants was performed in experiments conducted under the best selected conditions (70°C, molar ratio of 5, and enzyme concentration of 9% [w/w]). The following results were achieved: recovery yield of 78 and 72% and purity of 94 and 82% for capric acid and tricaprin, respectively. A selectivity value of 78% (after 20 h of reaction) was attained in an experiment conducted with the recovered enzyme and the recovered capric acid.

Tricaprylin

Figure 4 shows data confirming that tricaprylin was not produced in significant amounts in most of the experiments. Tricaprylin synthesis did not occur at 70 and 80°C, and the utilization of 1% (w/w) of lipozyme was not effective for triglyceride production. The increase in lipozyme content

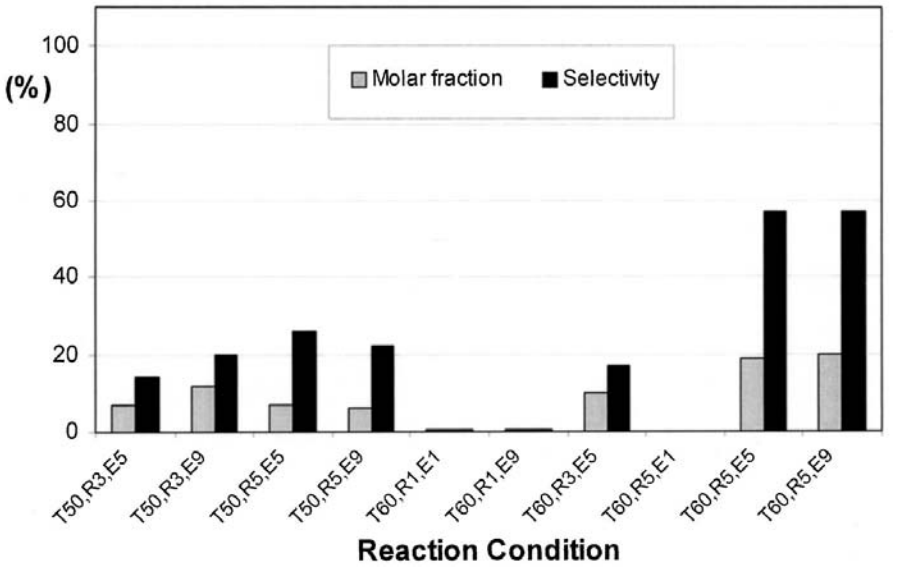

Fig. 4. Effect of temperature (*T*), fatty acid/glycerol molar ratio (*R*), and enzyme concentration (*E*) on tricaprylin synthesis carried out for 26 h.

Table 1
Best Conditions for Synthesis of Medium Chain Triglycerides

Triglyceride	Temperature (°C)	Fatty acid/glycerol molar ratio	Enzyme amount (% [w/w])	Molar fraction (%)	Selectivity (%)
Tricaprylin	60	5	9	20	57
Tricaprin	70	5	9	26	100
Trilaurin	80	5	5	40	100
Trimyristin	90	5	9	35	100

to 5 and 9% (w/w) did not enhance the production of tricaprylin. The highest selectivity (57%) and molar fraction (20%) values for tricaprylin synthesis were attained at 60°C in experiments employing a molar ratio of 5 and an enzyme content of 9% (w/w). Selmi et al. *(8,9)*, working with a similar reaction system, obtained a higher content of tricaprylin in 72-h experiments.

The best conditions for the synthesis of medium chain triglycerides are summarized in Table 1. The amounts of triglyceride produced and the optimum temperature values varied with the fatty acid chain length.

The commercial enzyme preparation shown to be more effective in the synthesis of triglycerides presenting long chain fatty acids, as reported elsewhere *(16–18)*. Considering the characteristics of lipases, mainly their preferential action on interfaces, it is understandable that lipozyme showed a certain selectivity toward the less water-soluble long chain fatty acids (lauric and myristic).

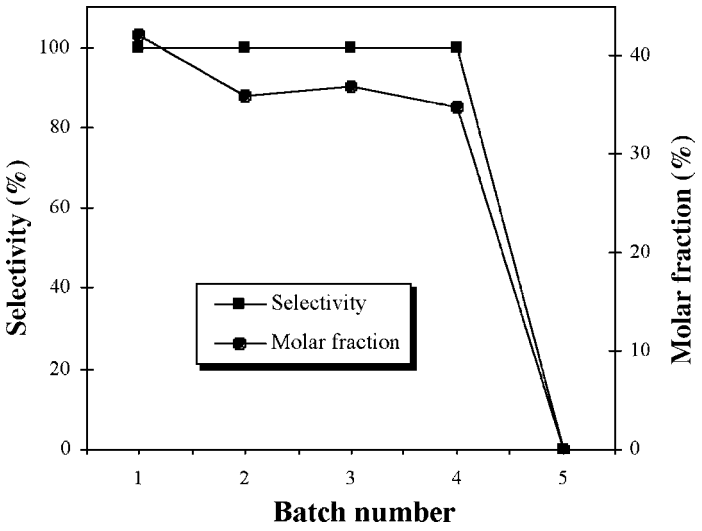

Fig. 5. Effect of enzyme reutilization (9% lipozyme [w/w]) on trilaurin synthesis. Successive 26-h batches were performed using an initial fatty acid/glycerol molar ratio of 5 and a temperature of 90°C.

Reutilization of Immobilized Lipase

The experiments showed that the residual fatty acid and the triglyceride product could be recovered, after the completion of the reaction, with appreciable yields. Since the viability of the recovery procedure is obviously higher for the triglycerides produced in larger amounts (trilaurin and trimyristin), experiments were performed reusing the enzyme recovered after each batch reaction assay.

The results of the production of these two triglycerides, at 90°C, in several sequential experiments, performing enzyme recovery and reuse are shown in Figs. 5 and 6. It can be observed that the enzyme can be reused and keep its performance, three times and two times for the synthesis of trilaurin and trimyristin, respectively. However, a sharp drop in the triglyceride production was observed for both products beyond the fourth (trimyristin) and the fifth (trilaurin) reutilization of enzyme.

The drop in lipase activity has been investigated by several researchers *(4,15,19–21)* who have suggested some of the factors affecting enzyme stability: reaction temperature, mechanical abrasion of the immobilized enzyme matrix or support, and modification of the hydration state of the matrix. To confirm that a lower temperature should reduce the decline in enzyme activity, a set of experiments (synthesis of trilaurin and trimyristin) was conducted at 80°C. The results shown in Figs. 7 and 8 confirm that the enzyme remained stable even when reused 16 times. The high values of selectivity and triglyceride molar fraction obtained confirm the viability of enzyme reutilization at 80°C. In the last experiment (sixteenth reutilization), the selectivity value was >80%, while the molar fraction decreased to

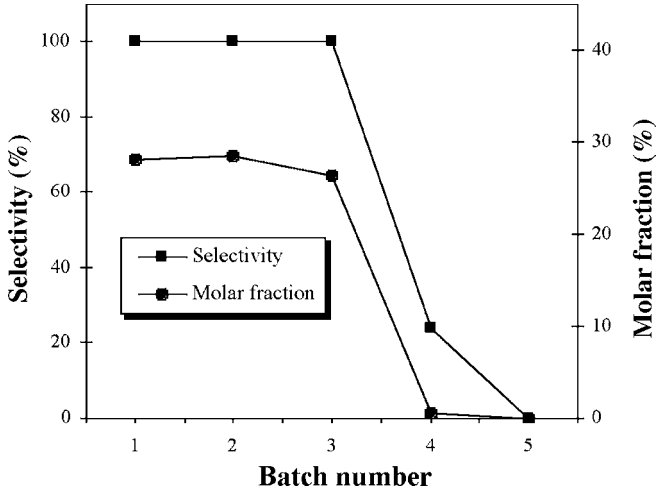

Fig. 6. Effect of enzyme reutilization (9% lipozyme [w/w]) on trimyristin synthesis. Successive 26-h batches were performed using an initial fatty acid/glycerol molar ratio of 5 and a temperature of 90°C.

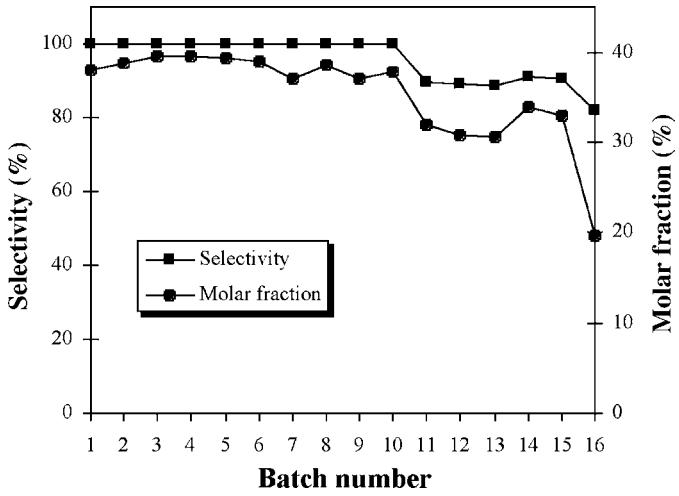

Fig. 7. Effect of enzyme reutilization (9% lipozyme [w/w]) on trilaurin synthesis. Successive 26-h batches were performed using a fatty acid/glycerol molar ratio of 5 and a temperature of 80°C.

50%. However, up to the tenth experiment, for both triglycerides produced, the molar fraction was constant and the selectivity value was 100%. Thus, the reduction in the temperature from 90 to 80°C had a marked effect on enzyme stability. Operation at 80°C led to selectivity values of 100% and molar fractions similar to those obtained at 90°C.

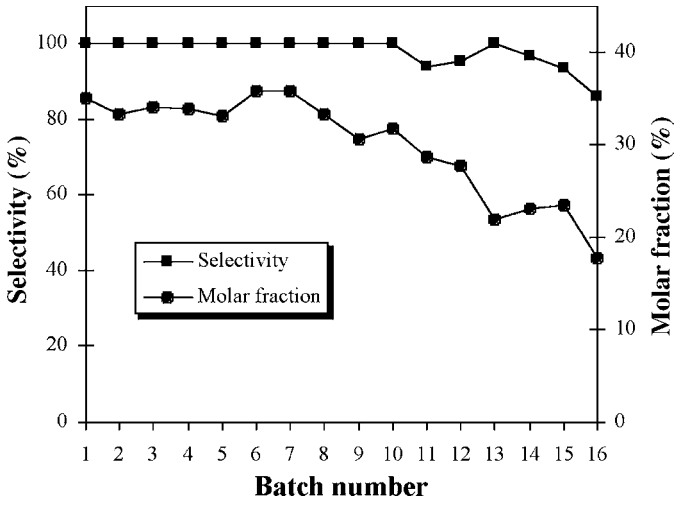

Fig. 8. Effect of enzyme reutilization (9% lipozyme [w/w]) on trimyristin synthesis. Successive 26-h batches were performed using a fatty acid/glycerol molar ratio of 5 and a temperature of 80°C.

Mechanical abrasion, as suggested by some researchers *(4,19)*, did not have a detectable effect on the decline in enzyme activity, since the same immobilized enzyme matrix was used 15 times at 80°C, in experiments with the same degree of agitation as those performed at 90°C.

Although the effect of support hydration was not specifically investigated in our work, it is unlikely that it would have influenced our results. Reduction in temperature causes a lower water evaporation rate. Thus, the increased water content of the medium could have two major effects: reduction of the reaction rate (product accumulation) and hydration of the immobilized enzyme system content. In fact, the magnitude of these effects was not high enough to affect the experimental results. The enzyme recovery procedure, which utilizes *n*-hexane, might lead to a severe reduction in the water content of the immobilized enzyme. However, specific investigations would be required in order to properly evaluate the possible deleterious effect of dehydration on the immobilized enzyme's stability.

Conclusions

The synthesis of triglycerides may be accomplished in a solvent-free system, leading to high molar fraction and selectivity values. Temperature has a marked effect on the reaction and the best results were achieved at 60, 70, 80, and 90°C for the synthesis of tricaprylin, tricaprin, trilaurin, and trimyristin, respectively. The best temperature values increased in accordance with the fatty acid chain length. Enzyme content affected the rate of reaction, but its influence on the ultimate molar fraction and the selectivity toward the product was not so significant. The best results were attained with enzyme contents of 9 or 5% (trilaurin).

The possibility of enzyme reuse and recovery of residual fatty acid was confirmed for trimyristin and trilaurin synthesis. To overcome the problem of enzyme instability at 90°C, the reaction temperature was reduced to 80°C and the immobilized enzyme was used at least 10 times, in sequential experiments, without any appreciable loss of final molar fraction and the selectivity toward the product. The fatty acid remaining at the end of the reaction can be recovered and easily reused. These results confirm the process viability of the synthesis of triglycerides in a solvent-free medium.

Acknowledgments

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