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Optimization of 2-ethylhexyl Palmitate Production Using Lipozyme RM IM as Catalyst in a Solvent-Free System

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Abstract This work reports the application of a lipase in the 2-ethylhexyl palmitate esterification in a solvent-free system with an immobilized lipase (Lipozyme RM IM). A sequential strategy was used applying two experimental designs to optimize the 2-ethylhexyl palmitate production. An empirical model was then built so as to assess the effects of process variables on the reaction conversion. Afterwards, the operating conditions that optimized 2-ethylhexyl palmitate production were established as being acid/alcohol molar ratio 1:3, temperature of 70°C, stirring rate of 150 rpm, 10 wt.% of enzyme, leading to a reaction conversion as high as 95%. From this point, a kinetic study was carried out evaluating the effect of acid:alcohol molar ratio, the enzyme concentration and the temperature on product conversion. The results obtained in this step permit to verify that an excess of alcohol (acid to alcohol molar ratio of 1:6), relatively low enzyme concentration (10 wt.%) and temperature of 70°C, led to conversions next to 100%.

Keywords 2-ethylhexyl palmitate · Esterification · Lipozyme RM IM · Solvent-free system · Kinetic evaluation

Introduction

The enzymatic synthesis, an alternative to chemical process, has some well-defined advantages as higher reaction yields at near ambient temperatures, leading to high-quality products obtained by low-cost energy and reduced formation of secondary products [1–4].

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Fatty acid esters from 2-ethyl hexanol, such as 2-ethylhexyl palmitate, show interest due to their applications in cosmetics, pharmaceutics, and food and chemical industries. They are used, for example, as low temperature plasticizer for polyvinyl chloride, vinyl chloride, copolymers, polystyrene, ethyl cellulose, and synthetic rubber, and also used in making lubricants water-resistant or as solvents [5].

Actually, the commercial production of 2-ethylhexyl palmitate is based on chemical esterification, which has a series of disadvantages, for example, formation of many side-products and high energy consumption [6, 7].

Some techniques have been employed to produce 2-ethylhexyl palmitate by lipase-catalyzed reactions, both in organic medium and in solvent-free systems, in a few works presented in the literature [6, 7].

Concerning the techniques mentioned above, the present work reports part of a project aiming at building a platform to allow developing new processes for fatty acids esters production by enzyme-catalyzed esterification. Here, the main objective is to evaluate the effects of process parameters on the production of 2-ethylhexyl palmitate by a sequential strategy of experimental designs. A kinetic study was also performed with the main objective of obtaining the effect of acid to alcohol molar ratio, enzyme concentration, and temperature on product conversion.

Related to the specific literature, it is important to cite that there is a lack of results regarding the enzymatic esterification of esters obtained from 2-ethyl hexanol in a solvent-free system. However, it is worth mentioning that the results obtained in this work and the inconvenience of using organic solvents as reaction media may attest to the relevance of the present study.

Material and Methods

Materials

The substrates used in esterification reactions were commercial palmitic acid (Vetec, 99% purity), 2-ethyl hexanol (Merck, 98%). Lipozyme RM IM, a lipase from *Rhizomucor miehei* purchased from Novozymes S/A., was used as catalyst. Acetonitrile and methanol of HPLC-grade were obtained from Vetec and J. T. Baker. The standards of 2-ethylhexyl palmitate were kindly donated by Jovii Cosmecêutica (Cachoeirinha, RS, Brazil).

Sequential Strategy of the Experimental Designs

With the objective of determining the best reaction values for substrates molar ratio, enzyme concentration, and temperature, two experimental designs were employed, as one can see in Table 1. Three replications at the central point of each experimental design were carried out in order to calculate the experimental error. In all experiments, the stirring rate and reaction time was fixed at 150 rpm and 6 h, respectively. Enzymatic esterification reactions were carried out in an orbital shaker.

For the first experimental planning, the levels of the variables investigated were defined from preliminary experiments performed by our research group. After analyzing the results of the first experimental design, a second CCRD (Central Composite Rotatable Design) was carried out for conversion optimization, adjusting the substrates molar ratio and enzyme concentration (wt.%, based on the substrates).

Table 1 Ranges of the factors investigated in the two experimental designs.

Level	−1.41	−1	0	+1	+1.41
First experimental design					
Temperature (°C)	–	40	55	70	–
Molar ratio (acid/alcohol)	–	1:1	1:2	1:3	–
Enzyme concentration (wt.%)	–	1	5.5	10	–
Second experimental design					
Molar ratio (acid/alcohol)	1:1	1:1.5	1:3	1:5	1:6
Enzyme concentration (wt.%)	3	5	10	15	17

After analyzing the results of the experimental designs, reaction kinetic experiments were performed in acid palmitic to 2-ethyl hexanol molar ratio of 1:1, 1:3, 1:5, 1:7, and 1:10 , enzyme concentration [*E*], of 1, 5, 10, 15, and 20 wt.% (based on the total amount of substrates—palmitic acid and 2-ethyl hexanol), and temperature range from 30 to 70°C. Samples were taken from the bulk reactive system at 0, 5, 10, 15, 30 min, and 1, 2, 3, 4, 5, 6, 7, and 8 h. One important issue when dealing with esterification is to assure safe sample withdrawals of the whole content of the reacting mixture. For this purpose, a set of preliminary tests were carried out for some experimental conditions with the actual reaction system (palmitic acid and 2-ethyl hexanol in the presence of the enzyme), performing destructive experiments and comparing with sampling results. In all tested experimental conditions, excellent agreement was found thus assuring the reliability of the sampling system. The software used for statistical analysis of the experimental designs was Statistica 6.0 (Statsoft Inc, Tulsa, OK, USA).

Products Quantification

Quantitative analyzes of the products were conducted using an HPLC system from Agilent Series, equipped with a refractive index. The following instrumentation and conditions were used: Zorbax C_{18} column (4.6 m×250 mm, 5 μm), flow rate of 1.0 mL/min, column temperature of 35°C; the mobile phase, acetonitrile:methanol:H_2O (75:25:5, *v*/*v*/*v*). Acetonitrile:methanol:H_2O (75:25:5, *v*/*v*/*v*) was used as a sample dissolving solvent, and the injection volume was 20 μL. Quantification was carried out using authentic standards of 2-ethylhexyl palmitate and palmitic acid, the limiting reactant.

Calibration curves for both compounds were built with the following concentrations 1,410; 2,820; 5,640; 11,280; 16,920; 22,560; 28,200; and 33,840 ppm, showing a R^2 of 0.9945 and 0.9943, respectively. Reaction conversion was calculated based on the content of 2-ethylhexyl palmitate in the analyzed sample and on the reaction stoichiometry.

Results and Discussion

Optimization of the Reaction Conversion

To assess the effects of acid to alcohol molar ratio, enzyme concentration, and temperature on the 2-ethylhexyl palmitate production, a 2^3 experimental design with central point triplicate was adopted. The matrix of the experimental design, with coded levels and the

response in terms of 2-ethylhexyl palmitate conversion is presented in Table 2. From this table, one can see that very distinct conversions were obtained as a function of the variables levels studied.

Results obtained in the first experimental design were statistically analyzed and permitted to generate an empirical coded model for 2-ethylhexyl palmitate conversion as a function of substrates molar ratio, temperature, and enzyme concentration. This model was validated by analysis of variance (ANOVA). The R squared (coefficient of determination) value (0.78) and the F test for regression (calculated value about three times the listed one) prove that the model Eq. 1 is capable of well representing the experimental data of 2-ethylhexyl palmitate conversion in the range of factors investigated. In Eq. 1, T represents the variable temperature, RM the acid to alcohol molar ratio and E the enzyme concentration.

$$\text{Conversion}(\%) = 67.40 + 9.00 \times T + 7.66 \times \text{RM} + 15.92 \times E - 4.89 \times \text{RM} \times E \quad (1)$$

As can be observed in the above-presented equation, all variables presented positive significant main effect ($p<0.05$) on 2-ethylhexyl palmitate conversion. Higher values of temperature, enzyme concentration, and an alcohol excess seem to promote a good reactional system. As an example, experiment 8 of the first experimental design led to a conversion of 85.6% in 6 h of reaction (acid:alcohol molar ratio of 1:3, enzyme concentration of 10 wt.% and temperature of 70°C).

According to the results obtained in the first experimental design, a second one (CCRD) was carried out aiming to optimize the process conversion. As the higher conversions were achieved at 70°C, this value was kept constant in the second experimental design. On the other hand, the enzyme concentration and substrates molar ratio variables were changed to upper levels, as these variables presented a positive effect.

Table 3 presents the CCRD matrix and the obtained 2-ethylhexyl conversions where one can, at a first moment to observe that higher conversions (about 95%) were obtained in the central point. At experimental conditions of lower substrates molar ratio, lower conversions were observed. In these cases, the quantity of 2-ethylhexanol used in the reactional system could be insufficient to that necessary to shift the reaction equilibrium in the product way.

Table 2 Matrix of the first experimental design (coded values) with responses in terms of 2-ethylhexyl palmitate conversion.

Trial	Temperature (°C)	Acid:alcohol molar ratio	Enzyme concentration (wt.%)	Conversion (%)
1	−1	−1	−1	22.37
2	1	−1	−1	44.84
3	−1	1	−1	46.62
4	1	1	−1	70.79
5	−1	−1	1	67.36
6	1	−1	1	83.09
7	−1	1	1	75.94
8	1	1	1	85.58
9	0	0	0	78.31
10	0	0	0	83.73
11	0	0	0	82.82

Table 3 Matrix of the second experimental design (coded values) with responses in terms of 2-ethylhexyl palmitate conversion.

Trial	Acid:alcohol molar ratio	Enzyme concentration (wt.%)	Experimental conversion (%)	Predicted conversion (%)	RED (%)[a] $\text{RED} = \left\lvert \frac{Y_{\text{exp}} - Y_{\text{model}}}{Y_{\text{exp}}} \right\rvert \times 100$
1	−1	−1	91.12	89.26	2.04
2	1	−1	96.40	96.65	−0.26
3	−1	1	89.14	89.26	−0.13
4	1	1	96.93	96.65	0.29
5	−1.41	0	84.51	85.51	−1.18
6	1.41	0	96.15	95.93	0.23
7	0	−1.41	93.11	95.22	−2.26
8	0	1.41	96.76	95.22	1.59
9	0	0	94.98	95.22	−0.25
10	0	0	96.46	95.22	1.29
11	0	0	94.22	95.22	−1.06

[a] *RED* relative error deviation

After the statistical analysis of the results obtained in the second experimental design, an optimized coded model for 2-ethylhexyl palmitate production was validated. The analysis of variance (ANOVA) led to values of F test for regression calculated higher than the listed one and an R squared (coefficient of determination) value of 0.91. This implies a satisfactory representation of the process by the empirical model, within the range evaluated for each variable, conform is illustrated by the predicted conversion (fifth column of Table 3) and relative error deviation (RED) (sixth column of Table 3). The coded model fitted by regression analysis is given by Eq. 2.

$$\text{Conversion}(\%) = 95.27 + 3.70 \times \text{RM} - 2.27 \times \text{RM}^2 \quad (2)$$

Figure 1 presents the response surface showing the influence of enzyme concentration and substrates molar ratio on the 2-ethylhexyl palmitate conversion. In this second experimental design (CCRD), the enzyme concentration and acid to alcohol molar ratio that optimized 2-ethylhexyl palmitate production were obtained in trials 9, 10, and 11, corresponding to the central point (acid palmitic:2-ethyl hexanol molar ratio 1:3, 10 wt.% of enzyme, 70°C, 150 rpm and 6 h of reaction) of the design, reaching maximum values of 94.98, 96.46, and 94.22% (95.22 %—mean of the three replicates). From Fig. 1 we can observe that the enzyme concentration did not present a significant effect on the reaction conversion within the studied range (3 to 17 wt.%), since substrates molar ratio of 1:3 to1:6 be used.

In an attempt to compare the results obtained in this work with some presented in the literature, we found only one work related to enzymatic esterification of 2-ethylhexyl palmitate in solvent-free system. He et al. evaluated the enzymatic synthesis of 2-ethylhexyl palmitate from palmitic acid and 2-ethyl hexanol as substrates, using an immobilized lipase from *Candida* sp. 99–125 as catalyst. In a solvent-free system, 10 wt.% of enzyme (based on the total amount of substrates), 2-ethyl hexanol/palmitic acid molar ratio of 1:1 and 40°C the authors obtained conversions of about 84% [6]. Some other works presented in the literature relate the enzymatic esterification in solvent-free system using different fatty acids

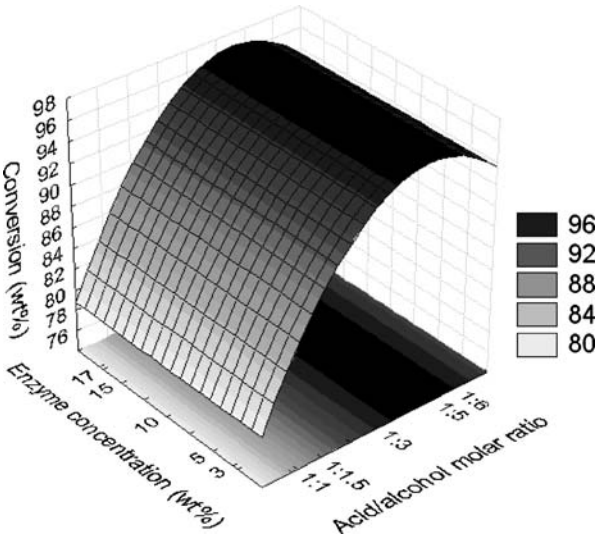

Fig. 1 Response surface for 2-ethylhexyl palmitate production as a function of enzyme concentration and acid to alcohol molar ratio

and alcohols, compared with those used in the present work. Kumar et al. investigated the ethyl palmitate synthesis in solvent-free system and obtained conversions of 97% [8]. Güvenç et al. studied the enzymatic production of isoamyl acetate in solvent-free system and reached 80% of conversion using 5 wt.% of Novozym 435, in 6 h of reaction, acid to alcohol molar ratio of 1:2 at 30°C and 150 rpm [9]. Using the commercial immobilized lipase Novozym 435, Santos et al. evaluated the synthesis of butyl esters in solvent-free system, where higher conversions (about 49%) were obtained when butyric acid was employed as acyl group donor [10]. Chang et al., in the esterification reaction for hexyl laurate production, using Lipozyme IM 77 as catalyst in a solvent-free system, obtained, after 40.6 min of reaction, at 58.2°C, an enzyme concentration of 25.4 mg/vol (0.196 BAUN) and pH of 5.9, conversions of around 69.7% [11].

The inconvenience of using organic solvents as reaction media and the fact that good 2-ethylhexyl palmitate conversions were obtained in this work may attest the relevance of the present study. In the presence of organic solvent, as the main objective of comparing the results obtained here, the work of He et al. [6] can be cited. The authors studied the enzymatic synthesis of fatty acids esters, mainly 2-ethyl palmitate, catalyzed by an immobilized lipase from *Candida* sp. 99–125. In petroleum ether system, 10 wt.% of lipase, 2-ethyl hexanol molar ratio of 1:1, 40°C and silica gel as adsorbent, conversions of 91% were achieved [6]. With the objective of reuse, the immobilized lipases for several batches, Tan et al. evaluated a method for enzymatic synthesis of 2-ethylhexyl palmitate by a lipase immobilized onto membranes. Conversions of 95% were obtained at 40°C, acid to alcohol molar ratio of 1:1.3 to 1:1.5, lipase activities of 5,000 to 6,000 IU/g of membrane, in petroleum ether system [7]. In a recent work, Richetti et al. [12] showed that the sequential strategy involving three full experimental designs proved to be useful in optimizing the conditions for 2-ethylhexyl palmitate conversion in solvent-free system using Novozym 435 as catalyst. The optimum concentrations for the production of 2-ethylhexyl were found to be: acid to alcohol molar ratio of 1:5.5, stirring rate of 150 rpm, 70°C, enzyme concentration of 10.5 wt.% at 6 h of reaction, resulting in a 2-ethylhexyl palmitate conversion of about 93%.

Kinetic Study of Enzymatic Production of 2-ethylhexyl Palmitate

The effects of acid to alcohol molar ratio, temperature, and enzyme concentration were investigated on the kinetics of 2-ethylhexyl palmitate production. As presented earlier, the execution of two experimental designs with the above-mentioned variables revealed the achievement of good conversions in 6 h of reaction at 70°C, enzyme concentration of 10.5 wt.%, acid to alcohol molar ratio of 1:5.5 and 150 rpm. It may be important to mention that the kinetic results subsequently presented in this work are in fact mean values of triplicate runs, which resulted in an overall absolute deviation in terms of reaction conversion of around 5%.

Effect of Acid to Alcohol Molar Ratio

In order to evaluate the effect of acid to alcohol molar ratio on 2-ethylhexyl palmitate conversion, temperature was kept fixed at 70°C, enzyme concentration at 10 wt.% and 150 rpm, making possible to build conversion versus time curves, as presented in Fig. 2.

From this figure, one can observe that at the molar ratios of 1:3, 1:5, 1:7, and 1:10, up to 15 min of reaction, reaction conversions were quite similar. At 2 h reaction, an enhancement in conversion was observed for the molar ratios of 1:5 and 1:7, reaching maximum values of 95.23 and 98.63%, respectively. At molar ratios of 1:1 and 1:3, lower conversions were obtained.

Dörmo et al. [13] evaluated the effect of acid to alcohol molar ratio on esterification for isoamyl oleate production at 40°C, 0.5 wt.% of Novozym 435, 150 rpm and 7 h of reaction, in a solvent-free system. Testing molar ratios of 1:1, 1:2, 1:5, and 2:1, higher conversions (70% to 80%) were obtained after 4 h of reaction using molar ratios of 1:2 and 1:5.

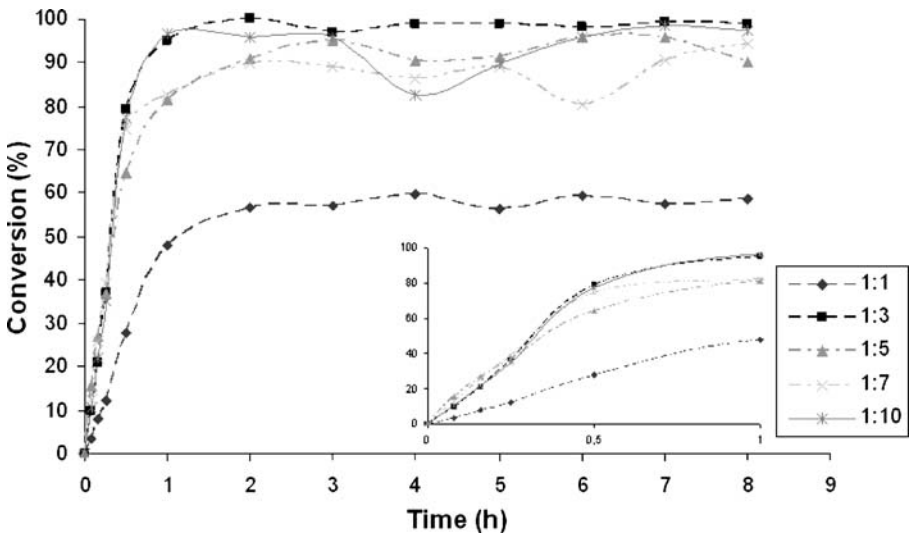

Fig. 2 Kinetics of 2-ethylhexyl palmitate production at varying acid to alcohol molar ratio: temperature of 70°C, enzyme concentration of 10 wt.% and 150 rpm in solvent-free system. Initial reaction rates, r (min^{-1}): 0.86, 2.21, 2.72, 2.51, and 2.50 for acid:alcohol molar ratio of 1:1, 1:3, 1:5, 1:7, and 1:10, respectively

He et al. [6] also investigated the effect of acid to alcohol molar ratio on enzymatic synthesis of 2-ethylhexyl palmitate using petroleum ether as solvent, immobilized *Candida* sp. 99–125 lipase (10 wt.%) as catalyst at 40°C. Molar ratios of 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:4 were tested and conversions as high as 86% were obtained at ratios of 4:1 and 2:1 [6].

It is well known that the substrates molar ratio is usually one of the most important parameters in enzymatic esterification reactions. Since the reaction is reversible, an enhancement on the concentration of one reactant (particularly, the alcohol) can displace the chemical equilibrium, resulting in higher conversions. On the other side, high alcohol concentrations may reduce the reaction rate due to the inhibition effect.

Effect of Enzyme Concentration

The effect of enzyme concentration on 2-ethylhexyl palmitate conversion was evaluated at 70°C keeping constant the acid to alcohol molar ratio of 1:6 and 150 rpm, varying the enzyme concentration of 1, 5, 10, 15, and 20 wt.% (based on the substrates amount). The kinetic curves obtained at this step are presented in Fig. 3. Using 5, 10, 15, and 20 wt.% enzyme in terms of total amount of the substrates we can observe that high reaction rates were obtained. Using 1 wt.% of enzyme in the reactional medium, lower 2-ethylhexyl palmitate conversion was achieved. The highest conversion in this experimental condition (89.71%) was obtained only after 8 h of reaction.

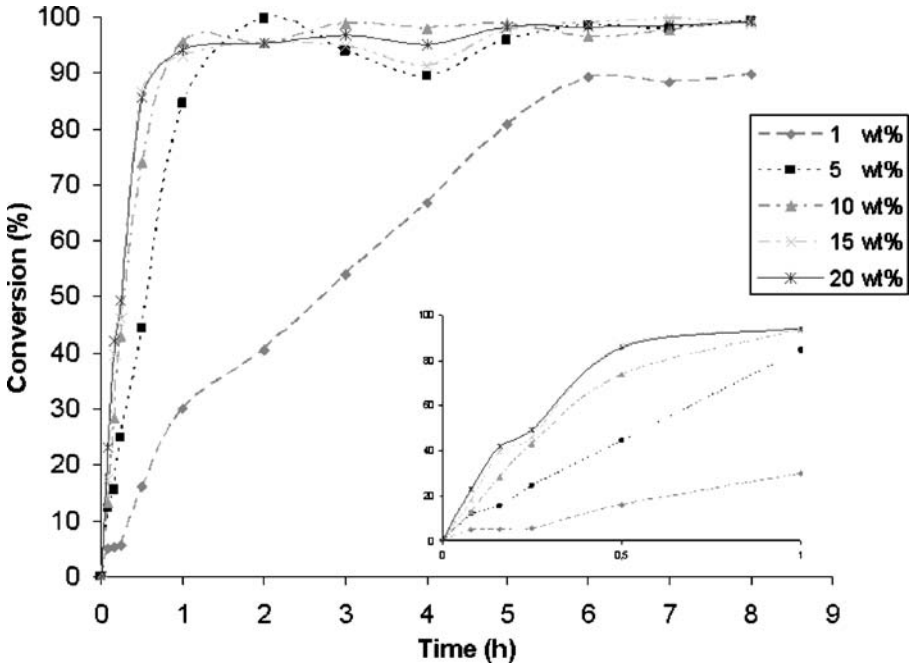

Fig. 3 Kinetics of 2-ethylhexyl palmitate production at varying enzyme concentration: temperature of 70°C, acid:alcohol molar ratio of 1:6 and 150 rpm in solvent-free system. Initial reaction rates, r (min^{-1}): 0.54, 1.53, 2.57, 3.02, and 2.98 for enzyme concentration of 1, 5, 10, 15, and 20 wt.%, respectively

As one can notice from Fig. 3, there is a tendency in reaching high conversions with 30 min of reaction when using enzyme concentration in the range of 5 to 20 wt.%. The use of 15 and 20 wt.% of Novozym 435 does not present significant difference on 2-ethylhexyl palmitate production, indicating that the optimal enzyme concentration for this system is within the range of 5 to 10.5 wt.% (based on the substrates).

A possible explanation for this fact might be related to the fact that an excess of enzyme in the reactional medium could not contribute to the conversion enhancement, since high enzyme concentration may form aggregates, thus not making the enzyme active site available to the substrates. The enzyme molecules on external surface of such particles are exposed to high substrate concentrations but the mass transport could drastically limit the substrate concentration inside the particles. Lower activities of the biocatalyst reduce the efficiency of the enzyme, not enhancing the reaction conversion [14].

Effect of Temperature

In order to evaluate the effect of temperature (30, 40, 50, 60 and 70°C) on 2-ethylhexyl palmitate conversion, the molar ratio of acid to alcohol was kept fixed at 1:6, enzyme concentration at 10 wt.% and 150 rpm, making possible to follow the course of the reaction conversion, as presented in Fig. 4.

It is well known that temperature presents two important roles in this kind of reactional system. Firstly, an increase in temperature can reduce mixture viscosity, enhance mutual solubility, and improve diffusion process of substrates, thus reducing mass transfer limitations and favoring interactions between enzyme particles and substrates. Further, enzymes generally have an optimal working temperature value, and in the case of Novozym 435, it is situated in the range of 40 to 65°C [15–17]. In this sense, Güvenç et al. [9] studied

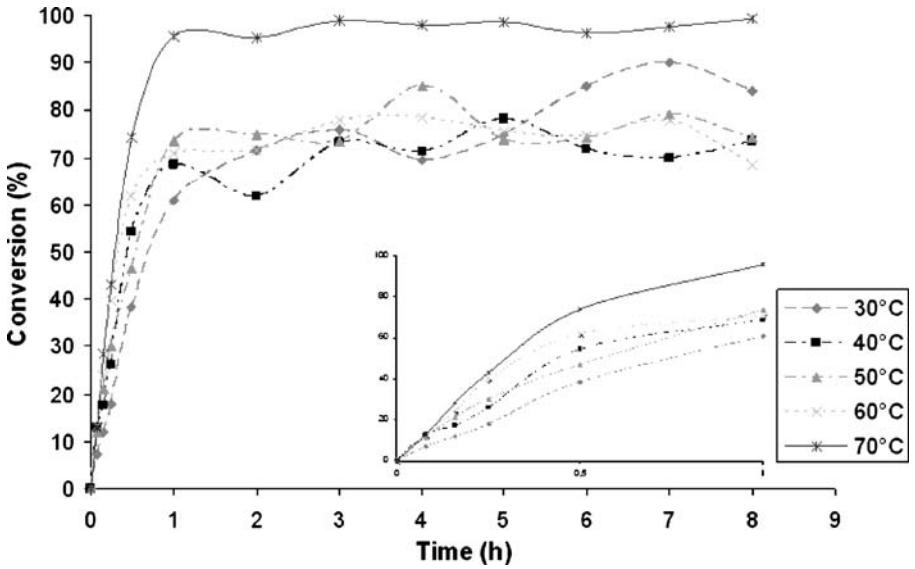

Fig. 4 Kinetics of 2-ethylhexyl palmitate production at varying temperature: acid:alcohol molar ratio of 1:6, enzyme concentration of 10 wt.% and 150 rpm in solvent-free system. Initial reaction rates, r (min^{-1}): 1.26, 1.81, 2.07, 2.20, and 2.57 for temperature of 30, 40, 50, 60, and 70°C, respectively

the esterification of isoamyl acetate catalyzed by Novozym 435 and Lipozyme RM IM in solvent-free system. Acid to alcohol molar ratio of 1:2 using Novozym 435 led to the reaction equilibrium after 6 h at 30, 40, and 50°C with conversions of about 81 %. The use of Lipozyme RM IM and acid to alcohol molar ratio of 1:1, after 48 h of reaction, conducted to conversions of 20% at 50°C and 13% at 30°C [9].

From Fig. 4, it can be verified that at 70°C and 1 h of reaction, conversion of about 95.66% was obtained, leading, this temperature, to higher initial reaction rates. He et al., using a lipase from *Candida* sp. 99–125, in petroleum ether system, studied the effect of temperature on 2-ethylhexyl palmitate production. The authors observed that controlled temperature in the range of 40–50°C, conducted to an enhancement on 2-ethylhexyl palmitate. On the other side, when temperatures were increased from 50 to 70°C, the reaction yield decreased [6]. Reaction kinetics of 2-ethylhexyl palmitate production was also evaluated by Richetti et al. [12]. The authors concluded that very satisfactory reaction conversions (~80%) can be achieved in short reaction times (30 min) using Novozym 435 as catalyst. Similar results were obtained in the present work.

Finally, it may be relevant to mention that measurements of enzyme activity before (fresh) and after (used) reaction experiments revealed no important changes in residual lipase activity, thus suggesting possible enzyme reuse. In an attempt to better understand the esterification reaction in solvent-free system, further experiments using other commercial and non-commercial enzymes, are underway within our working group.

Conclusions

The sequential strategy involving two full experimental designs was useful in optimizing the conditions for 2-ethylhexyl palmitate conversion in a solvent-free system using Lipozyme RM IM as catalyst. The optimum concentrations for the production of 2-ethylhexyl were found to be: acid to alcohol molar ratio of 1:3, stirring rate of 150 rpm, temperature of 70°C and enzyme concentration of 10 wt.%, resulting in a 2-ethylhexyl palmitate conversion of about 95.22%. The kinetics of 2-ethylhexyl palmitate production showed that very satisfactory reaction conversions (~80%) can be achieved in short reaction times (30 min). In general, high initial reaction rates were observed for all experimental conditions investigated with a positive effect verified of all process variables studied in almost the entire time range covered.

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