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# Hydrolysis and synthesis reactions catalysed by *Thermomyces lanuginosa* lipase in the AOT/Isooctane reversed micellar system

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**Abstract**

The kinetics of hydrolysis of triolein and tributyrin and of the synthesis of ethyl-laurate by the lipase of *Thermomyces lanuginosa*, contained in a commercial preparation (Lipolase®), were studied in AOT/Isooctane reverse micelles. Lipolytic activity against triolein depended strongly on the water content in the system ($W_0 = [H_2O]/[AOT]$), in a bell-shaped manner, with a maximum at a $W_0$ of 15. The best conditions for enzyme activity were pH 8.0 and 37 °C. The enzyme did not show Michaelis–Menten kinetics for the hydrolysis of either triolein or tributyrin. The enzyme was unstable at temperatures of 37–60 °C, losing approximately 50% of its activity after 30 min. The catalysis of ethyl-laurate synthesis by *T. lanuginosa* lipase in reverse micelles was studied using factorial designs to optimize the reaction conditions. The most important variables were pH and temperature and their combined effect. The best conditions for ester synthesis were a $W_0$ of 10, a pH of 5.6, a molar ratio of alcohol to acid of 5.0 and a temperature of 30 °C. The specific enzymatic activity under these conditions was 220 U mg$^{-1}$ and the ester yield 92% after 60 min of reaction. This high yield, obtained in a relatively short time, justifies further exploration of the potential of this system in biocatalysis.


*Keywords:* Lipases; Reverse micelles; *Thermomyces lanuginosa*; Hydrolysis; Synthesis reactions

## 1. Introduction

Lipases (triacyl glycerol acyl hydrolases, E.C. 3.1.1.3), which are typically thought of in terms of their hydrolysis of triacylglycerols to glycerol and free fatty acids, possess the unique feature of acting at an interface between aqueous and non-aqueous phases. Furthermore, they have exceptional characteristics for catalysis of various types of reaction with high specificity, including those of hydrolysis of esters and organic synthesis reactions such as esterification, transesterification and regioselective acylation, these synthesis reactions requiring restricted water-environments in order to occur [1–7].

The low water content necessary to favour the synthesis reactions can be achieved by carrying out the reaction in organic solvents, either by adding the enzyme directly to the organic solvent or by microencapsulation into reverse micelles. The AOT (sodium bis 2-ethylhexyl sulfosuccinate)/Isooctane system is one of the most suitable systems for enzymatic catalysis [8–11], since the reverse micelles formed by this surfactant are very stable over a wide range of concentrations in the absence of co-surfactants. The reversed micellar AOT system is particularly attractive for lipases because it provides a high interfacial area of contact, allowing the enzyme to anchor at the aqueous side of the AOT interface, and also because it enables the use of hydrophobic substrates, since these will be readily soluble in the bulk organic phase. Besides, some enzymes have been reported to be highly stable in reverse micelles, which is the case of *Chromobacterium viscosum* lipase and α-chymotrypsin [12].

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*Thermomyces lanuginosa* (previously *Humicola lanuginosa*) lipase (TLL) is the enzyme responsible for the lipolytic activity of Lipolase®, a commercial lipase preparation (Novozymes Latin America Ltd.) that has important industrial applications. In aqueous media the enzyme is very stable, being active over the pH range of 7.0–11.0. It maintains activity reasonably well at 55–60 °C although the recommended temperature for applications is between 30 and 40 °C [13]. However, its suitability for application in organic media, especially in reversed micellar systems, has been little explored. The current work aims to characterize the kinetics of hydrolysis and synthesis reactions catalysed by Lipolase® in the AOT/Isooctane reversed micellar system in order to evaluate the potential of this enzyme for biocatalysis in organic media.

## 2. Materials and methods

### 2.1. Materials

Lipolase® 100 L EX (*T. lanuginosa*) lipase was supplied by Novozymes Latin America (Araucaria, Paraná, Brazil) as an aqueous solution and was used without further purification. Dioctyl sulfocuccinate sodium salt (AOT) of 99% purity was purchased from Sigma Chemicals (St. Louis, USA) and used as received. Triolein (glyceryl trioleate, 65%) and lauric acid (dodecanoic acid, 99%) were also from Sigma and absolute ethanol was from Carlo Erba (São Paulo, Brazil).

### 2.2. Preparation of reversed micellar solutions

The standard micellar enzymatic assay was carried out in a stirred vessel, which initially contained 8 ml of 100 mM AOT and 150 mM of triolein in isooctane, at the stated temperature. The micelles were prepared by the injection method [12], in which, for a $W_0$ ([$H_2O$]/[AOT]) of 15, 216 μl of an enzymatic solution, containing 312 μg of protein in 50 mM phosphate buffer at the stated pH (i.e. 39 μg of protein per ml of micellar solution), was added. The mixture was strongly agitated in a vortex to give a clear micellar solution [12] and samples were taken at given time intervals. Note that the concentrations refer to the total volume of the micellar solution except the buffer molarity, which considers only the aqueous phase. Also the pH values quoted are those for the buffer solution in which the enzyme was diluted to prepare the aqueous phase.

The effect of $W_0$ (5–20) was evaluated with the same protein concentration (39 μg $ml^{-1}$), but the solutions were prepared, at 30 °C, in different volumes of 50 mM phosphate buffer at pH 7.0 to give the required $W_0$ values. The optimisation of protein concentration was done at 30 °C over the range of 39–160.5 μg $ml^{-1}$, at a $W_0$ of 15, with 50 mM buffer solution, pH 7.0. In the pH studies, done at 30 °C and with a $W_0$ of 15, the original enzyme solution was diluted with the following buffers to give the desired pH values: acetate, pH 4.0–5.0; phosphate, pH 5.5–8.0; Tris–HCl, pH 8.0–9.0, and glycine, pH 10.0. The effect of temperature was studied in the range of 23–58 °C, using a $W_0$ of 15 and 50 mM buffer solution, pH 8.0. To study the effect of substrate concentration, which was done at 37 °C with a $W_0$ of 15, using 50 mM buffer solution, pH 8.0, either triolein (10–250 mM) or tributirin (10–170 mM) were used.

### 2.3. Hydrolysis and synthesis studies

The reverse micelles were prepared as described in the previous section and the reaction was started by adding the appropriate amount of enzyme solution, except for the esterification reactions, which were started by the addition of the substrates. At fixed intervals, 200 μl samples of the mixture were collected from the reaction medium. The concentration of free fatty acid produced in the system was determined according to the Lowry–Tinsley method [14], for both hydrolysis and synthesis reactions. One unit of enzyme activity was defined as the amount of enzyme required to produce (hydrolysis) or consume (synthesis) 1 μmol free fatty acid per min, under the conditions of the assay. The yield of the reaction in terms of ester production was calculated by consumption of free fatty acid from the reaction medium.

The experiments were undertaken in such a manner to enable determination of the initial velocities of the reaction. Unless otherwise stated, the activities cited for the experiments done in this work are based on these initial velocities.

### 2.4. Optimisation of the conditions for ester synthesis

To optimize the conditions for the synthesis of ethyllaurate, we used a $2^{4-1}$ factorial design [15]. The variables studies were water content, $W_0$ (5 and 10), pH (5.6 and 10.0), temperature (30 and 45 °C) and the molar ratio of alcohol to acid (3:1, being 213 mM alcohol and 71 mM acid and 5:1, being 355 mM alcohol and 71 mM acid). The enzyme concentration was constant at 72 μg protein in 8 ml of reaction medium. After obtaining the optimal conditions in the above experiment, a second experiment with a $2^2$ factorial design was done, with $W_0$ fixed at 10 and the molar ratio of alcohol to acid fixed at 5:1.

From these factorial designs, the contrast coefficients ($C_i$), which allow the determination of the effect of each parameter, were calculated according to Eq. (1):

$$C_i = \frac{\sum EL + \sum EL_2}{4} \quad (1)$$

where $C_i$ is the contrast coefficient of factor $i$, $E$ the esterification rate and $L_1$ and $L_2$ are the levels of parameter $i$ (+ or −).

### 2.5. Validation of the use of the Lowry–Tinsley method

In order to show that the use of the Lowry–Tinsley method to follow the production of ester in the synthesis reactions is valid even though it only measures the disappearance of the acid, ester production under optimised reaction conditions (a $W_0$ of 10, a molar ratio of alcohol to acid of 5:1, a pH of 5.6 and a temperature of 30 °C) was followed by both the Lowry–Tinsley method [14] and high-performance liquid chromatography (HPLC). A number of replicate reactions were done, each with 8 ml of micellar medium. At different times (0, 30 and 60 min) a reaction vessel was sacrificed with the reaction being stopped by the addition of 8 ml of acetone. The samples were centrifuged for 10 min at 5000 g, evaporated, and then purified by elution with hexane:ethyl ether (99:1) in a column containing 75 $cm^3$ of activated silica gel 60 (Merck) with a granulometry of 0.063–0.200 mm, this being necessary in order to remove the AOT, because in unpurified samples the AOT peak obtained by HPLC overlapped with the ethyl-laurate peak. For each sample eluted, eight fractions of 2 ml were collected. The solvent was removed and the samples were analyzed by HPLC. The ethyl-laurate standard for the HPLC was prepared as follows. One gram of lauric acid (Sigma, 99% pure) and thionyl chlorine were incubated together for 90 min at 65 °C. After this, ethanol was added and the mixture left standing in an ice-bath for 1 h. Cold water and hexane were then added. The experiment was done twice.

The Lowry–Tinsley method estimated ester synthesis with sufficient accuracy: after 60 min, the yield calculated on the basis of the HPLC analysis was 94.7 ± 1.6% while that calculated on the basis of the Lowry–Tinsley method was 90.5 ± 1.7%.

### 2.6. Temperature stability studies

Various tubes containing 100 mM AOT/Isooctane reverse micelles were prepared with 39 μg enzyme per ml of micellar solution, 50 mM phosphate buffer, pH 8.0, and a $W_0$ of 15. The tubes were incubated at the desired temperature. At different times tubes were removed, the contents added to the reaction vessel at 37 °C, and triolein started to initiate the reaction. Samples were removed and analyzed by the Lowry–Tinsley method [14].

### 2.7. Analytical methods

HPLC was performed in a Shimadzu Model LC 10 AD chromatograph equipped with a Micropak MCH 10-$C_{18}$ column and a refraction index detector (RID 10 A). Integration was carried out using the CLASS 10 software (Shimadzu). For the analysis of ethyl-laurate, a 20 μl aliquot of the sample was injected and acetonitrile/acetone 9:1 (v/v) was used as the mobile phase, at a flow rate of 0.9 ml/min. The column was maintained at 40 °C. The samples were quantified based on a calibration curve established with chemically synthesized ester, at concentrations ranging from 0.05 to 2.0 mg/ml.

Protein was determined by the method of Bradford [16].

## 3. Results and discussion

### 3.1. Kinetic characterization of lipase in hydrolysis reactions

#### 3.1.1. Effect of water content

The effect of water content ($W_0$) on triolein hydrolysis by *T. lanuginosa* lipase (TLL) in a 100 mM AOT/Isooctane reversed micellar system was determined by changing the $W_0$ value from 5 to 20, while keeping the other conditions constant (Fig. 1). TLL showed maximum activity (1092 U $mg^{-1}$) at a $W_0$ of 15. On either side of this optimum the activity decreased markedly with changes in $W_0$, reaching approximately 50% of the maximum activity at the lower $W_0$ of 5 and the upper $W_0$ of 20. Many authors have reported similar bell-shaped profiles with optimum $W_0$ values of 10–15 for hydrolysis reactions with a range of lipases produced by filamentous fungi in related reversed micelle and emulsion systems (Table 1). Crooks et al. [19] reported a different optimum $W_0$ of 5 for *Humicola lanuginosa* (*T. lanuginosa*) lipase, but the substrate (p$NPC_4$, *p*-nitrophenyl butyrate), the AOT concentration (200 mM), the solvent (*n*-heptane) and the pH (9.3) were different from those that we used, so the difference is not surprising.

The optimum $W_0$ value for enzyme activity depends on several factors, amongst which two of the most important are the dependence of the reaction rate on the water concentration and the effect of the water on the size of the micelles within the system. The type of reaction to be catalyzed is also decisive, since hydrolytic reactions usually require higher $W_0$ values than do synthetic reactions. However, these factors have not been studied sufficiently to allow theoretical estimations of optimum water contents for hydrolysis. The

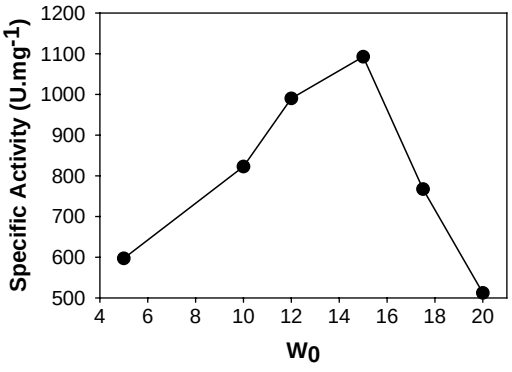


Fig. 1. Effect of the water content ($W_0$) on triolein hydrolysis by *T. lanuginosa* lipase in reverse micelles. Conditions: 100 mM AOT in Isooctane; pH 7.0, 50 mM phosphate buffer; triolein as substrate; $T = 30$ °C and [protein] = 39 μg $ml^{-1}$ micellar solution.

Table 1
Comparison of the optimum values for lipolytic activity of $W_0$, pH, temperature and activation energy for *T. lanuginosa* lipase (TLL) with values reported in the literature for related systems

| Lipase source | $W_0$ | pH | $T$ (°C) | $E_a$ (kJ/mol) | System | Substrate | Reference |
|---|---|---|---|---|---|---|---|
| *T. lanuginosa* | 15 | 8.0 | 37 | 77.4 | Reverse micelles AOT/Isooctane | Triolein | This work |
| *Penicillium citrinum* | 10–15 | 8.0 | 45 | 56.1 | Reverse micelles AOT/Isooctane | Triolein | [17] |
| *C. viscosum* | 11–15 | 7.0–7.5 | 35 | 82.2 | Reverse micelles AOT/Isooctane | Triolein | [18] |
| *C. viscosum* | 11–15 | 7.0–7.5 | 42 | 42.0 | Aqueous (emulsion) | Triolein | [18] |
| *H. lanuginosa* | 5 | 9.3 | 40 | 54.4 | Reverse micelles AOT/*n*-heptane | $pNPC_4$ | [19,20] |
| *H. lanuginosa* | 5 | 10.4 | 40 | 62.8 | Reverse micelles AOT/*n*-heptane | Tributyrin | [19,20] |
| *Rhizopus arrhizus* | 11–15 | 7.0 | 30 | 29.3 | Reverse micelles AOT/Isooctane | Palm kernel olein | [21] |
| *C. cylindracea* | 11 | 6.5 | 55 | 131.2 | Reverse micelles lecithin/Isooctane | Milk fat | [22] |
| *C. cylindracea* | 11–15 | 6.3–7.3 | – | – | Reverse micelles AOT/Isooctane | Olive oil | [23] |
| *C. cylindracea* | 11–15 | 7.8 | – | – | Emulsion system | Olive oil | [23] |

literature only contains empirical results obtained in other systems [12,17].

### 3.1.2. Effect of the protein concentration

The hydrolytic activity of TLL within the AOT/Isooctane reversed micellar system decreased with increasing enzyme concentration (Fig. 2). Such behaviour is quite different from aqueous systems, in which the reaction rate is typically first-order in enzyme concentration. This type of behaviour was also found by Prazeres et al. [18]. They proposed that the high enzyme concentration in the system could lead to an aggregation of the molecules, which hinders catalysis and consequently decreases the specific activity. Besides, the protein concentration interferes with the optimal $W_0$; high enzyme concentrations normally require high $W_0$ values in order to accommodate the protein in the core of the micelle [24].

### 3.1.3. Effect of pH on activity

The effect of the pH on the hydrolysis of triolein by TLL in the 100 mM AOT/Isooctane reversed micellar system was determined over the range of 4.0–10.0. The behavior of the enzyme was quite different from that reported for aqueous media, in which, at 30 °C and using tributyrin as the substrate, the enzyme maintains a high activity from pH 7.0–11.0 [13]. In the micellar system, using triolein as the substrate, we observed a much sharper activity peak, with a maximum of 1474 U mg$^{-1}$ at pH 8.0 (Fig. 3). Perhaps in aqueous systems the interaction of the enzyme with water is stronger and minimizes conformational changes in response to internal ionizations caused by pH changes, while in reversed micellar systems, with their much lower water content, the stabilizing effect of water is lower, such that conformational changes with pH are more pronounced [12]. However, there have not been any studies done to provide the experimental evidence about enzyme conformations that would be needed to support this idea.

Both lower and higher pH optima have been reported for fungal lipases in related reversed micellar and emulsion systems (Table 1). The lowest optima reported are pH 6.5 for the hydrolysis of milk fat by *Candida cylindracea* lipase in a soybean lecithin/Isooctane reversed micellar system [22], pH 6.3–7.3 for the hydrolysis of olive oil in an AOT/Isooctane reversed micellar system and pH 7.8 for the hydrolysis of olive oil in an emulsion system by the lipase of *C. cylindracea* [23]. The highest pH optima reported are 9.3 for the hydrolysis of $pNPC_4$ and 10.4 for tributyrin hydrolysis in AOT/*n*-heptane microemulsions by the lipase of *H. lanuginosa*, which is equivalent to TLL [19,20]. Note that these

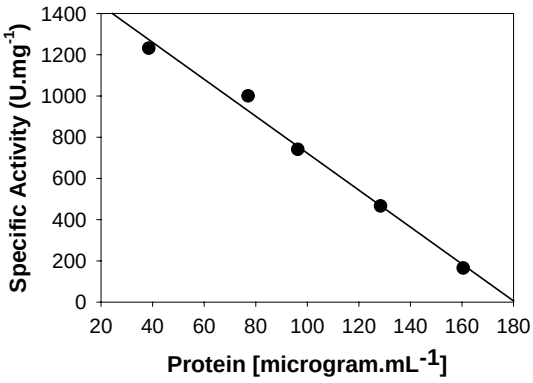


Fig. 2. Effect of protein concentration on triolein hydrolysis by *T. lanuginosa* lipase in reverse micelles. Conditions: 100 mM AOT in Isooctane; pH 7.0, 50 mM phosphate; $W_0 = 15$; triolein as substrate and $T = 30$ °C.

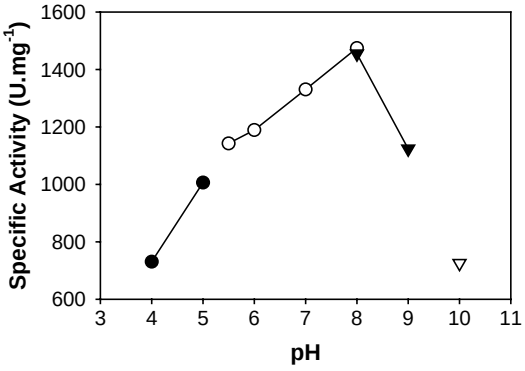


Fig. 3. Effect of pH and different buffers on lipolytic activity of the lipase of *T. lanuginosa*. Conditions of assay: triolein as substrate, 30 °C; [protein] = 39 μg ml$^{-1}$ micellar solution; $W_0 = 15$; buffer 50 mM: (●) acetate (4.0, 5.0); (○) phosphate (5.5, 6.0, 7.0, 8.0); (▼) Tris–HCl (8.0, 9.0), (▽) Glycine (10.0).

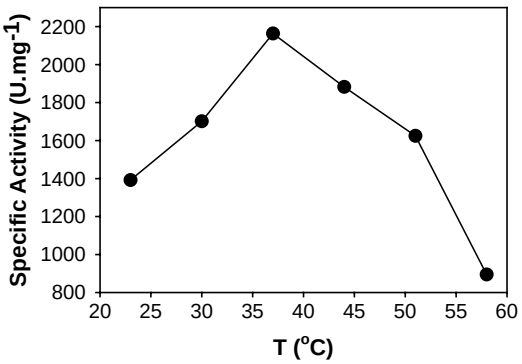


Fig. 4. Effect of temperature on triolein hydrolysis by *T. lanuginosa* lipase in reverse micelles. Conditions: 100 mM AOT in Isooctane; $W_0 = 15$; pH 8.0, 50 mM phosphate; triolein as substrate and [protein] = 39 μg ml$^{-1}$ micellar solution.

results for TLL were obtained in reaction systems different from that used in the current work.

In these studies it is assumed that the pH of the water pools within the reverse micelles corresponds to the pH values of the aqueous enzyme solutions from which the micelles are prepared. However, the pH inside the micelles might be different from the expected value due to partitioning of ionic species. A lower intramicellar pH was reported for reverse micelles formed by AOT [9,12,22].

### 3.1.4. Effect of temperature on hydrolysis activity

The effect of the temperature of the 100 mM AOT/Isooctane reversed micellar system on triolein hydrolysis by TLL was studied over the range of 23–58 °C. The enzyme presented maximum activity at 37 °C (Fig. 4). This optimum is typical of fungal lipases in emulsions and in AOT reversed micellar systems (Table 1). At higher temperatures, the interface is more fluid and disordered, which could cause a faster inactivation, with low or no activity detected after the microencapsulation.

The energy of activation ($E_a$) for the hydrolysis reaction, calculated from the Arrhenius plot of the three temperatures for which the activity was increasing (Fig. 5), was

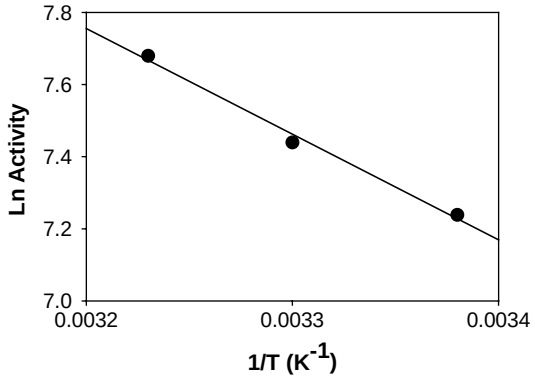


Fig. 5. Arrhenius plot of *T. lanuginosa* lipase activity in reverse micelles. Conditions: 100 mM AOT in Isooctane; $W_0 = 15$; pH 8.0, 50 mM phosphate; triolein as substrate and [protein] = 39 μg ml$^{-1}$ micellar solution.

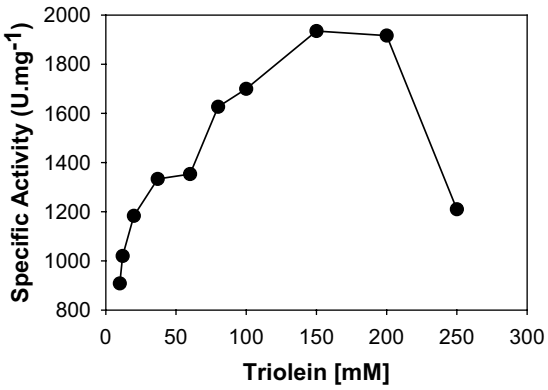


Fig. 6. Effect of substrate concentration on the rate of hydrolysis of triolein by *T. lanuginosa* lipase in reverse micelles. Conditions: 100 mM AOT in Isooctane; pH 8.0, 50 mM phosphate; $W_0 = 15$; 37 °C and [protein] = 39 μg ml$^{-1}$ micellar solution.

77.4 kJ mol$^{-1}$. This activation energy falls within the values (22.2–100.8 kJ mol$^{-1}$) reported in emulsion systems, and is similar to that (82.2 kJ mol$^{-1}$) obtained by Prazeres et al. [18] for the lipase of *C. viscosum* in AOT/Isooctane reversed micellar system (Table 1).

### 3.1.5. Effect of substrate concentration

The effect of substrate concentration on lipolytic activity in 100 mM AOT/Isooctane reverse micelles was studied using triolein and tributyrin as substrates. The maximum activities for triolein (Fig. 6) and tributyrin (Fig. 7) hydrolysis were obtained at substrate concentrations of 150 mM (1935 U mg$^{-1}$) and 120 mM (1485 U mg$^{-1}$), respectively. In the case of triolein, the activity decreased at substrate concentrations above 200 mM. This could be caused either by the direct influence of the substrates on the lipase or by a change in the micellar structure. It has been reported that triglycerides can act as co-surfactants and cause a decrease in micellar size independently of the $W_0$ value [9].

Literature data show that the kinetics of reactions catalysed by enzymes microencapsulated in reverse micelles

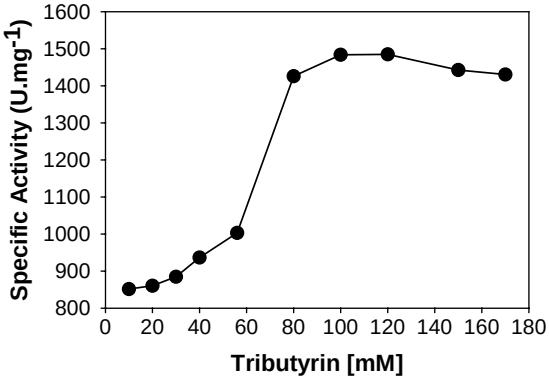


Fig. 7. Effect of substrate concentration on the rate of hydrolysis of tributyrin by *T. lanuginosa* lipase in reverse micelles. Conditions: 100 mM AOT in Isooctane; pH 8.0, 50 mM phosphate; $W_0 = 15$; 37 °C and [protein] = 39 μg ml$^{-1}$ micellar solution.

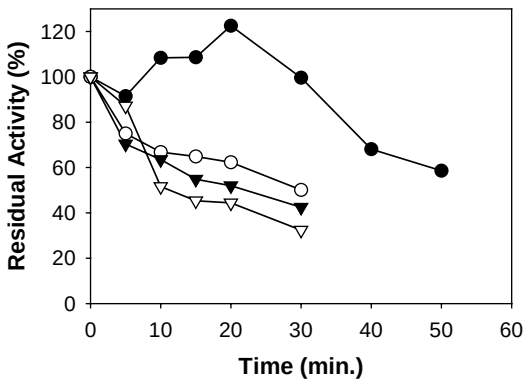


Fig. 8. Loss of enzyme activity at temperatures from 30 to 60 °C. Incubation conditions: 100 mM AOT in Isooctane; pH 8.0, 50 mM phosphate; [protein] = 39 μg ml$^{-1}$ micellar solution; $W_0$ = 15 and temperatures: (●) 30 °C, (○) 37 °C, (▼) 50 °C, (▽) 60 °C.

generally obey the classical Michaelis–Menten model. However, in our experiments, TLL did not present a Michaelis–Menten behavior for either substrate. The interpretation of the kinetic behaviour of lipases microencapsulated in reverse micelles is rather difficult. Even if it is possible to calculate the constants of the Michaelis–Menten equation, they differ from the ones observed with the same enzyme in aqueous media due to partition effects and diffusional limitations, and also due to alterations in the conformational structure of the enzyme. Therefore, the values of the kinetic parameters obtained from studies in these systems must be treated as apparent values [12,18].

*3.1.6. 3.1.6. Temperature stability*

Fig. 8 shows the residual activities as a function of time for incubation of the enzyme in the reverse micelle system at temperatures between 30 and 60 °C. Loss of enzyme activity was not described by a simple first order model, nor by the more flexible sequential deactivation model of Henley and Sadana [25], therefore it was not possible to calculate rate constants for the denaturation reaction. However, given that in aqueous solution at pH 8.0 the enzyme retains 95% of its activity after 2 h incubation at 55 °C and 80% of its activity after 2 h incubation at 60 °C [13], it is possible to conclude that the thermostability of the enzyme is reduced in the micellar system, since even after only 30 min at 37 °C the residual activity was only 60%.

### 3.2. Optimisation of the conditions for ester synthesis

Using experimental conditions extracted from a preliminary study (37 °C, a $W_0$ of 5, 50 mM phosphate buffer at pH 8.0), a screening for an appropriate esterification reaction was carried out with the alcohols ethanol, propanol and butanol and the acids ethanoic, propanoic, butyric and lauric in the AOT/Isooctane reversed micelle system. Under these experimental conditions only the reaction between ethanol and lauric acid occurred. In fact, ethyl-laurate is of some interest, firstly, because lauric acid can be obtained easily and at low cost in Brazil, from babassu oil, and secondly, because esters of lauric acid such as ethyl-laurate, butyl-laurate and isoamyl-laurate are produced and sold commercially as fruit aromas in Europe [26].

Table 2
$2^{4-1}$ Factorial design to optimize the production of ethyl-laurate using *T. lanuginosa* lipase: experimental conditions and results

| Run | $W_0$ | pH | $T$ (°C) | M.R.[a] | Ester yield at 60 min (% of theoretical maximum) |
|---|---|---|---|---|---|
| 1 | 5 | 5.6 | 30 | 3.0 | 83 |
| 2 | 10 | 5.6 | 30 | 5.0 | 92 |
| 3 | 5 | 10 | 30 | 5.0 | 32.5 |
| 4 | 10 | 10 | 30 | 3.0 | 37 |
| 5 | 5 | 5.6 | 45 | 5.0 | 91 |
| 6 | 10 | 5.6 | 45 | 3.0 | 73 ± 2 |
| 7 | 5 | 10 | 45 | 3.0 | 0 |
| 8 | 10 | 10 | 45 | 5.0 | 0 |

[a] M.R. = Molar ratio (alcohol:acid), Run 6 was done in triplicate.

In the preliminary experiments for the synthesis of ethyl-laurate, only 50% of the acid was esterified after 60 min, representing an average specific activity of 66 U mg$^{-1}$. To maximize the esterification rate and to evaluate the effect of the main experimental parameters involved in the esterification process ($W_0$, pH, temperature and alcohol:acid molar ratio, MR), a fractional factorial design $2^{4-1}$ was used (Table 2). The contrast coefficients ($C_i$) obtained from these results are shown in Table 3. The most important variables were pH ($C_i = -67$), temperature ($C_i = -20$) and the combined effect of pH and temperature ($C_i = -15$). The highest specific activity (220 U mg$^{-1}$) and reaction yield (92% at 60 min) occurred at 30 °C, a $W_0$ of 10, with acetate buffer pH 5.6 and a molar ratio of ethanol to lauric acid of 5.

Given that a significant combined effect was observed between pH and temperature, and that $W_0$ and MR did not have a significant effect on the response, a specific $2^2$ factorial design (Table 4) was carried out, fixing $W_0$ at 10 (Level +) and the molar ratio of acid to alcohol at 1:5 (Level +). The contrast coefficients obtained for this second factorial experiment (pH, $C_i = -52$; $T$, $C_i = -38$; and pH × $T$, $C_i = +24$) confirmed that for high ester yields, low pH (5.6) and temperature values (30 °C) must be used.

Table 3
Contrast coefficients ($C_i$) calculated from the factorial design $2^{4-1}$ of Table 2

| Variables | Level | | $C_i$ |
|---|---|---|---|
| | $L_1$ (−) | $L_2$ (+) | |
| $W_0$ (1) | 5 | 10 | −1 |
| pH (2) | 5.6 | 10 | −67 |
| $T$ (3) | 30 | 45 | −20 |
| M.R.[a] (4) | 3 | 5 | +6 |
| pH × $T$ | – | – | −15 |

[a] M.R. = Molar ratio (alcohol:acid).

Table 4
Experimental results of the second factorial design $2^2$ for the production of ethyl-laurate by *T. lanuginosa* lipase: experimental conditions and results at fixed values of $W_0$ (10) and molar ratio[a] (5)

| Run | pH | $T$ (°C) | Ester yield at 60 min (% of theoretical maximum) |
|---|---|---|---|
| 1 | 5.6 | 30 | 89 |
| 2 | 10 | 30 | 28 |
| 3 | 5.6 | 45 | 56 |
| 4 | 10 | 45 | 0 |
| 5 (duplicate) | 5.6 | 30 | 90 ± 3 |

[a] M.R. = Molar ratio.

The best conditions for ester production were identical with those identified in the previous factorial design experiment (Table 2), with an 89% yield of ester. This yield of approximately 90% was confirmed when the same reaction was repeated and analyzed by HPLC, as described in Section 2.5.

The results for ethyl-laurate synthesis obtained in the present work (60 min for a yield of 90%) are superior to those reported in the literature either for micellar systems or for other systems used for ester synthesis. Reaction times of 24 h have been reported for a 95% reaction yield in the production of butyl-laurate by the lipase of *C. cilindracea* in the AOT/Isooctane reversed micelle system [27] and for a 90% reaction yield in the production of octyl-oleate by the lipase of *Rhizopus delemar* in a microemulsion system [28]. Even longer times have been reported. Alvarez–Macarie and Baratti [29] obtained a yield of 95% for the synthesis of ethyl-caproate with the esterase of *Bacillus licheniformis* in *n*-heptane at 45 °C after 168 h of reaction, while Krishna et al. [30] obtained a 95% reaction yield for the synthesis of isoamyl acetate in isooctane with an immobilized lipase of *R. miehei* in *n*-heptane at 40 °C after 72 h of reaction.

## 4. Conclusions

In this work, we have shown that *T. lanuginosa* lipase (TLL) can be used in reversed micellar systems for both the hydrolysis of triacylglycerides and the synthesis of esters, although not all ester synthesis reactions are catalyzed. Hydrolysis of triacylglycerides in this system does not follow Michaelis–Menten kinetics and the kinetics of this reaction deserve further attention. Synthesis of ethyl-laurate was catalyzed, with yields of over 90% being obtained within 60 min under optimized conditions. This high yield in a relatively short time justifies further exploration of the potential applications of TLL in reversed micelle systems.

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## References

[1] L. Steenkamp, D. Brady, Enzyme Microbiol. Technol. 32 (2003) 472.
[2] N. López, R. Pérez, S. Vázquez, F. Valero, A. Sánchez, J. Chem. Technol. Biotechnol. 77 (2002) 175.
[3] R. Sharma, Y. Chisti, U.C. Banerjee, Biotechnol. Adv. 19 (2001) 627.
[4] Y. Cajal, A. Svendsen, V. Girona, S.A. Patkar, M.A. Alsina, Biochemistry 39 (2000) 413.
[5] J.L. Del Río, G. Caminal, M. Fité, I. Faus, J. Bladé, C.J. Solá, Chem. Technol. Biotechnol. 75 (2000) 991.
[6] S.H. Krishna, B. Manohar, S. Divakar, S.G. Prapulla, N.G. Karanth, Enzyme Microb. Technol. 26 (2000) 131.
[7] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, J. Mol. Catal. B Enzymatic. 9 (2000) 113.
[8] J.R.S. Alves, L.P. Fonseca, M.T. Ramalho, J.M.S. Cabral, Biochem. Eng. J. 15 (2003) 81.
[9] C.M.L. Carvalho, J.M.S. Cabral, Biochimie 82 (2000) 1063.
[10] N. Krieger, M.A. Taipa, E.H.M. Melo, J.L. Lima-Filho, M.R.Aires-Barros, J.M.S. Cabral, Bioprocess. Eng. 20 (1999) 59.
[11] N. Krieger, M.A. Taipa, M.R. Aires-Barros, E.H.M. Melo, J.L. Lima-Filho, J.M.S. Cabral, J. Chem. Biotechnol. 69 (1997) 77.
[12] E.P. Melo, M.R. Aires-Barros, J.M.S. Cabral, Biotechnol. Ann. Rev. 7 (2001) 87.
[13] Novozyme A/S, Lipolase 100 L, B 434 d-GB 500, Bagsvaerd-Denmark, 2001.
[14] R.R. Lowry, J.I. Tinsley, J. Am. Oil Chem. Soc. 53 (1976) 470.
[15] R.H. Myers, Response Surface Methodology, Allyn and Bacon, Boston, MA, 1971.
[16] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[17] N. Krieger, M.A. Taipa, E.H.M. Melo, J.L. Lima-Filho, M.R. Aires-Barros, J.M. S Cabral, Appl. Biochem. Biotechnol. 67 (1997) 87.
[18] D.M.F. Prazeres, F.A.P. Garcia, J.M.S. Cabral, J. Chem. Techn. Biotechnol. 53 (1992) 159.
[19] G.E. Crooks, G.D. Rees, B.H. Robinson, M. Svensson, G.R. Stephenson, Biotechnol. Bioeng. 48 (1995) 78.
[20] G.E. Crooks, G.D. Rees, B.H. Robinson, M. Svensson, G.R. Stephenson, Biotechnol. Bioeng. 48 (1995) 190.
[21] K. Taegeum, C. Kiomin, Enzyme Microb. Technol. 11 (1989) 528.
[22] J.-P. Chen, K.-C. Chang, J. Ferment. Bioeng. 76-2 (1993) 98.
[23] S. Koichiro, I. Makoto, B. Yoshinari, K. Yoshinobu, K. Ryoichi, K. Isao, J. Ferment. Bioeng. 81 (1995) 143.
[24] K. Martinek, N.L. Klyachko, A.V. Kabanov, Y.L. Khmelnitski, A.V. Levashov, Biochem. Biophys. Acta 981 (1989) 161.
[25] J.P. Henley, A. Sadana, Enzyme Microb. Technol. 7 (1985) 50.
[26] G.A. Macedo, G.M. Pastore, Ciência Tecnol. Alimentos 17 (1997) 115.
[27] D.G. Hayes, E. Gulari, Biotechnol. Bioeng. 35 (1990) 793.
[28] K. Nagayama, Matsu-Ura, Syn-Ichi, T. Doi, M. Imai, J. Mol. Catal. B Enzymatic 4 (1998) 25.
[29] E. Alvarez-Macarie, J. Baratti, J. Mol. Catal. B Enzymatic 10 (2000) 377.
[30] S.H. Krishna, B. Manohar, S. Divakar, S.G. Prapulla, N.G. Karanth, J. Biotechnol. 87 (2001) 191.