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# Production of monoglycerides by glycerolysis of olive oil with immobilized lipases: effect of the water activity

**S. Ferreira-Dias, M.M.R. da Fonseca**



**Abstract** The production of monoglycerides by glycerolysis of olive oil catalyzed by lipases from Candida rugosa, Chromobacterium viscosum and Rhizopus sp. immobilized in a hydrophilic polyurethane foam was investigated. The effect of the amount of aqueous phase used for foam polymerization on the competing reactions of glycerolysis and hydrolysis was studied. The highest monoglyceride production was achieved with the C. rugosa lipase which was thus selected for subsequent studies.

The extent to which hydrolysis and glycerolysis occur and the influence of the initial $a_w$ of the system on both reactions were also investigated. In glycerolytic reaction systems, initial rates of fatty acid release were always higher than in hydrolytic systems. At $a_w$ values lower than 0.43, hydrolysis was completely repressed, although glycerolysis still occurred. This suggests that hydrolysis of the ester bond in the glyceride, promoted by glycerol, is the first reaction step.

In glycerolysis, initial rates of FFA and DG production increased exponentially with the initial $a_w$ of the system.

The lowest total conversion (in terms of % TG consumed) at 48 hours was obtained at intermediate $a_w$ values; higher conversions at extreme $a_w$ indicated an increase in hydrolytic and glycerolytic rates, at high and low $a_w$, respectively.

The yield of MG increased with decreasing $a_w$. The highest yield of MG (~70%, w/w) was obtained at the lowest $a_w$ used (0.23). The initial $a_w$ of the reaction system is an important parameter in glycerolysis.

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**List of symbols**

| | |
|---|---|
| $a_w$ | thermodynamic activity of water |
| $C_{12:0}$ | lauric acid |
| $C_{14:0}$ | myristic acid |
| $C_{18:1}$ | oleic acid |
| DG | diglyceride (s) |
| FAME | fatty acid methyl ester (s) |
| FFA | free fatty acid (s) |
| FID | fire ionization detector |
| Gly | glycerol |
| $n$ | number of replicates |
| MG | monoglyceride (s) |
| PCA | principal component analysis |
| PU | polyurethane |
| $r$ | correlation coefficient |
| TG | triglyceride (s) |
| TO | triolein |
| $\alpha$ | significance level |

## 1 Introduction

In food and pharmaceutical industries, monoglycerides (MG) are among the most important emulsifiers. Current processes for monoglyceride production are based either on direct esterification of glycerol with fatty acids or on interesterification of triglycerides with glycerol (glycerolysis) in the presence of inorganic catalysts at high temperatures (200–250 °C) [1]. These reactions are nonselective and consume large amounts of energy.

To obtain highly pure monoglycerides (>90%), the synthesized mono-diglycerides mixture (40–60% of MG) has to undergo molecular distillation, which considerably increases their manufacturing cost [2].

The replacement of inorganic catalysts by lipases (E.C.3.1.1.3.) in monoglyceride synthesis avoids side product formation and is less polluting and energy consuming because of the mild conditions used.

The esterification of glycerol with free fatty acids (FFA) by lipases to produce primarily MG was reported by several authors [3–10].

Monoglycerides were also obtained by the hydrolysis of the triglycerides from palm oil catalyzed by 1,3-selective lipases in microemulsions [11].

Other ways of producing MG involve the interesterification of TG with a FFA (acidolysis) [12], with an alcohol, e.g. glycerol (alcoholysis), or even the transesterification of glycerides with fatty acids methyl esters [10].

The enzymatic alcoholysis of vegetable oils with *n*-butanol [13] and of fish oil with isopropanol [14] was also reported.

The glycerol alcoholysis, glycerolysis, has been achieved with several lipase preparations. Lipase-catalyzed glycerolysis of TG seems to be a rational route to produce monoglycerides in various types of systems: reversed micelles [15]; liposomes in reversed micelles [16], in the presence of solvent with the catalyst in an immobilized form [17] and in the absence of solvent with free [18–19] or immobilized [20] catalyst.

In this study, three different lipases were immobilized in a hydrophilic biocompatible polyurethane foam and used to catalyze the production of monoglycerides by glycerolysis of olive oil in *n*-hexane.



The influence of water activity on the reaction kinetics and on the competing hydrolytic reaction was investigated in order to maximize the yield of monoglycerides and to make a preliminary assessment of the glycerolysis mechanism.

The following aspects have to be considered in this reaction system:

(1) The hydrophobic character of glycerides vs. the hydrophilic character of glycerol, which prevents the solubilization of glycerol in the organic medium;

(2) The hydrophilic phase (pure glycerol) has a high viscosity ($\sim$ 1490 cP at 20 °C) and density (1.26 g cm$^{-3}$ at 20 °C), leading to glycerol accumulation in the bottom of the reaction vessel or its aggregation to the enzyme (when this is used in a freeze-dried powdered form);

(3) The use of a solvent for the olive oil is advantageous in order to favour (i) the contact between immobilized lipase and glycerides, (ii) the emulsification of glycerol in the organic medium and (iii) product recovery. Because *n*-hexane is widely used for the extraction and processing of fats and oils, it was the solvent chosen for this system.

(4) MG display a low solubility in *n*-hexane, at room temperature. This is a desirable situation both (i) kinetically, because less monoglyceride is available for the reverse reaction and a displacement of the reaction equilibrium in the direction of monoglyceride production is favoured and (ii) from a process point of view, because downstream processing is facilitated [17].

(5) The chemical equation for fatty glycerolysis is as follows:

$$TG + Gly \rightarrow DG + MG, \quad (1)$$

$$DG + Gly \rightarrow 2MG, \quad (2)$$

Thus, the overall, simplified equation is:

$$TG + 2Gly \rightarrow 3MG, \quad (3)$$

i.e., the stoichiometric ratio of Gly : TG in glycerolysis is 2 : 1. In the enzymatic esterification of glycerol with oleic acid, an excess of glycerol was able to increase MG production [21]. Thus, glycerol was used in excess in the present glycerolysis studies.

(6) The mechanism of lipase-catalyzed interesterification appears to consist of fatty acid release during the first step, followed by the esterification of FFA with the glycerol residue [4, 14, 22–24].

(7) The hydrolytic activity of lipases is not completely suppressed even in reaction media with low water content [12, 24–25], because hydrolysis competes with the interesterification reactions [22, 25].

(8) The use of a 1,3-selective lipase to prevent the hydrolysis of the 2-MG formed, is unnecessary since MG show a low solubility in *n*-hexane.

(9) The immobilization of the lipase in a macroporous matrix allows the reuse of the biocatalyst and promotes a large interface area between the water immiscible reagents and the aqueous phase containing the biocatalyst.

(10) The use of a hydrophilic immobilization support improves the enzyme operational stability [26] and promotes the penetration of glycerol.

## 2
## Materials and methods

### 2.1
### Materials

The crude lipase from Chromobacterium viscosum was purchased from Toyo Jozo, Japan; Candida rugosa lipase (lipase AY) and Rhizopus sp. lipase (lipase N), (1,3 regioselective), were a gift from Amano, Japan.

The lipases were immobilized in a hydrophilic polyurethane foam (Hypol FHP 2002), kindly donated by Hampshire Chemical GmbH, Germany.

Refined olive oil was a gift from Empresa Fabril de Moura, Moura, Portugal. Triolein, trimyristin, diolein (mixed isomers), monoolein, oleic acid and glycerol (99%) of analytical grade were purchased from Signa, USA. All other chemicals were of analytical grade and obtained from various sources.

### 2.2
### Hydrolytic enzyme activity assay

One cm$^3$ of olive oil was mixed with 10 cm$^3$ of a phosphate buffer solution (pH 7.0) in a conical flask and sonicated for 10 minutes. This emulsion was placed in a reciprocal shaker (230 strokes/min) at 30 °C, and 50–100 mg of the lipase powder were added. After 30 minutes, the reaction was stopped by addition of 25 cm$^3$ of a mixture of acetone-methanol (1 : 1, v : v). The FFA were titrated with a 0.1N NaOH aqueous solution.

One lipase unit was defined as the amount of lipase which liberates one micromole of FFA per minute under the above conditions.

### 2.3
### Immobilization

Lipase immobilization in the hydrophilic polyurethane foam was concomitant with the polymerization, as previously described [27].

Foams prepared with aqueous phosphate buffer solution (pH 7.0) to Hypol pre-polymer ratios of 1 : 2 and 1 : 1 were tested for both glycerolysis and hydrolysis. These foams (with a porosity of about 0.70) were subsequently cut into small cuboids ($\sim$0.07 cm$^3$) and introduced in the reaction medium.

### 2.4
### Glycerolysis reaction

The immobilized lipase was added to a biphasic system consisting of 12 cm$^3$ of a solution of refined olive oil in

*n*-hexane (11%, w/v) and 0.5 $cm^3$ of glycerol (molar ratio Gly/TG = 4.5). The olive oil had been previously treated with alumina to remove DG, MG, FFA, oxidation products and traces of water eventually present.

The reaction was carried out in a thermostated cylindrical glass vessel closed with a rubber stopper, at 30 °C, under magnetic stirring. In each experiment 108 lipase units were used. Samples were taken during the time-course of the reaction and TG and products analyzed.

Initial rates were calculated by linear regression on the first 4 data-points (substrate or product concentration vs. time).

### 2.5
### Hydrolysis reaction

The protocol followed for the hydrolysis experiments was similar to the glycerolysis, except that no glycerol was added.

### 2.6
### Analytical methods

The TG and DG, after separation by thin layer chromatography and methylation, were assayed as fatty acid methyl esters (FAME) by gas chromatography in a Hewlett Packard 5890 gas chromatograph equipped with a SGE capillary column (25QC2/HT5 0.1; 25 m × 0.22 mm; fused silica) and a FID, as previously described [17].

The FFA were assayed using the Lowry and Tinsley's colorimetric method [28] with benzene replaced by *n*-heptane [17].

Since monoglycerides showed a low solubility in *n*-hexane, their quantification was achieved via an indirect method [17].

In the glycerolysis experiments the TG, DG and FFA were analyzed, while in hydrolysis experiments only the FFA were analyzed.

### 2.7
### Evaporation correction

Since *n*-hexane is a rather volatile solvent (vapour pressure of 200 mm Hg at 31.6 °C), its evaporation during long course batch reactions has usually to be taken into account, even in vessels closed with rubber stoppers. Thus, a correction factor was calculated from blank experiments. Foams (1 : 2) were prepared with 0.6 g of pre-polymer, without addition of enzyme, and introduced in 12 $cm^3$ of organic medium containing oleic acid (1.56%, w/v) and 0.5 $cm^3$ of glycerol, under magnetic stirring at 30 °C. Aliquots were taken at different times for 48 hours and assayed for their content in FFA.

Assuming that the solvent was the sole compound to evaporate, a linear relationship was found between the observed concentration of a given compound, $C_t$, at time $t$ (hours) and the real concentration if no solvent evaporation had occurred, $C_r$:

$$C_r = (-0.0033\, t + 0.9656)\, C_t. \quad (4)$$

(14 data-points; $r = -0.9809$)

### 2.8
### Estimation of partition coefficients matrix/organic solvent

The (1 : 2) foams, prepared with 0.8 g of pre-polymer, were soaked in 12 $cm^3$ of solutions of triolein (0.854%, w/v), diolein (0.578%, w/v), oleic acid (1.188%, w/v) or monoolein (0.156%, w/v), in *n*-hexane, under the same conditions used for the enzymatic reactions. After one hour aliquots were withdrawn from the organic medium and the concentration of each compound was measured (see "analytical methods"). The partition coefficients [(1 : 2) foam/*n*-hexane] for these compounds were estimated according to Fukui *et al.* equation [29], as previously described [27].

### 2.9
### Lipase screening

The 3 lipases were immobilized in polyurethane (1 : 1) and (1 : 2) foams (see "immobilization") and used as biocatalysts for the glycerolysis reaction. The immobilized biocatalyst preparations were prepared with a weight ratio prepolymer/lipase powder of 2.5.

To select the best performer for olive oil glycerolysis, a "Principal Component Analysis" (PCA) on the data matrix containing the reaction medium composition after 24 hours, was carried out.

With this technique, the initial *m*-dimensional space (*m* variables) may be reduced to *n* dimensions ($n < m$) without considerable loss of information [30]. The initial system of *m* axis is replaced by another system where the new axis are the principal components [31–32]. The first component shows the maximum correlation with all the variables and explains the highest proportion of the global variance [33].

This method allows the geometric representation of the original objects (lipases) in a space of reduced dimensions defined by a new set of axis. It may also provide a particular interpretation of the components and subsequently of the original variables.

For olive oil hydrolysis, the performance of the 3 lipases (in triplicate), immobilized under the same conditions as for the glycerolysis reaction, was also evaluated. The amounts of FFA released after 24 hours of reaction were analyzed by a one way analysis of variance test (ANOVA) and post-hoc multiple comparison tests (Fisher PSLD and Scheffé F). Significance was set at the level $\alpha \leq 0.05$.

### 2.10
### Mass of immobilization polymer

A fixed mass of Candida rugosa lipase (240 mg) was immobilized in (1 : 2) foams (A, B and C) prepared with 0.4, 0.6 and 0.8 g of "FHP 2002", respectively, to assess the influence of different amounts of free water on the hydrolysis reaction and the extent of diffusion limitations due to the compactation of the lipase molecules.

### 2.11
### Water activity equilibration

The C. rugosa immobilized lipase (in a (1 : 2) foam), suspended on a plastic net, was previously equilibrated, for 5 days at 22 °C, with the vapour phase of saturated salt solutions of known water activities ($a_w$): $KCH_3COO$, ($a_w = 0.23$); $K_2CO_3$, ($a_w = 0.43$); $Mg(NO_3)_2$, ($a_w = 0.53$); NaCl, ($a_w = 0.75$); KCl ($a_w = 0.84$); $KNO_3$, ($a_w = 0.94$). The solvent was also adjusted to the desired

$a_w$, under the same conditions, but in a separate sealed container.

The time reported for enzyme preparations to attain equilibrium with saturated salt solutions varies widely [23, 34–37]. Thus, it had to be established for our system prior to the experiments: the foams were contacted with the equilibration solutions and the $a_w$ of the matrices monitored daily.

The traces of water present in the olive oil were removed by the alumina treatment. Since the equilibration of the substrates with saturated salt solutions takes at least 2 weeks [37], the olive oil was not pre-equilibrated to prevent the formation of oxidative by-products which could affect the activity of the enzyme preparation [38–40].



Glycerol, also, was not pre-equilibrated with saturated salt solutions, because of its very low $a_w$, approximately 0.17 [41]. Glycerol is a powerful water binder, often added to intermediate moisture foods and other products to depress the water activity [42].

### 2.12
### Water activity assay

Before starting the reaction, the $a_w$ of the enzyme preparations was measured at 25 °C in a ROTRONIC HYGROSKOP DT humidity sensor (DMS-100H).

### 2.13
### Reactions under an initially controlled water activity

After equilibration of the immobilized enzyme and the solvent at an initial pre-established $a_w$ value, the glycerolysis and hydrolysis reactions followed the same protocol as described above. All the experiments were carried out in duplicate or triplicate.

## 3
## Results and discussion

### 3.1
### Partition coefficients matrix/solvent

Partition coefficients for triolein, diolein, monoolein and oleic acid were found to be zero. The hydrophilic nature of the polyurethane foam used (i) leads to the accumulation of the final and intermediate hydrophobic products of glycerolysis and hydrolysis in the organic phase and (ii) promotes the capture of glycerol.

### 3.2
### Lipase screening

PCA allows the plotting of the immobilized lipases and the glycerolysis products on a plane formed by the first and the second principal components, $F_1F_2$ (Fig. 1). This plane accounts for 92.6% of the variance explained by the original data. MG and DG productions appear to be highly correlated with the first principal component, $F_1$ ($r=0.85$ and $r=-0.94$, respectively). MG production increases along the positive side of this axis against DG production. The second principal component, $F_2$, can be identified with FFA production ($r=0.87$).

A 50% reduction in the water content of the polyurethane matrix resulted in a decrease in FFA production with all the enzymes under screening. The major effect was found with C. rugosa lipase.

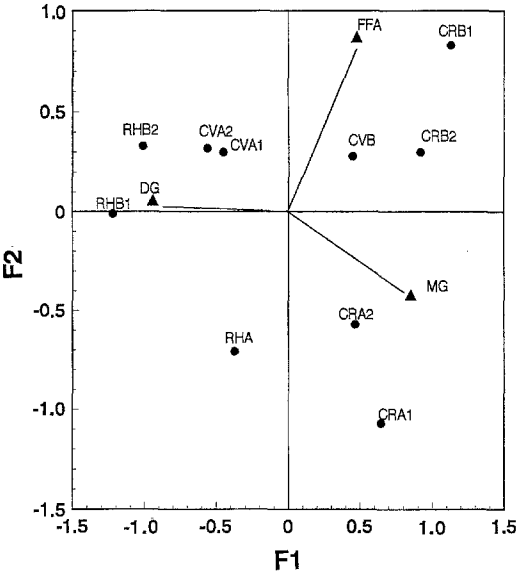


Fig. 1. Lipase screening for glycerolysis by PCA. Lipases (●) (CR – C. rugosa, CV – Chromobacterium viscosum, RH – Rhizopus sp.), immobilized in (1:2, A) and (1:1, B) PU foams, and original variables (glycerolysis products, ▲) are plotted on a $F_1F_2$ (first and second principal components) plane

The Rhizopus sp. lipase showed the highest DG production and the lowest MG levels, both with (1 : 1) and (1 : 2) foams.

The highest MG concentration was attained with the C. rugosa lipase.

The amount of FFA released by the 3 types of lipases immobilized in (1 : 1) and (1 : 2) foams, after 24 hours of hydrolysis, was compared to assess the extent of the hydrolytic reaction (Fig. 2). No significant differences were found in the case of the (1 : 1) foams. A considerable reduction in FFA production was observed with all the lipases entrapped in the (1 : 2) foams. The lowest levels of FFA were observed with C. rugosa and Rhizopus sp. lipases in a (1 : 2) foam. The lipase from Chromobacterium viscosum, in the same type of foam, showed a significantly higher production of FFA.

Since the highest MG concentration was achieved with the C. rugosa lipase, this enzyme, immobilized in (1 : 2) polyurethane foams so as to restrain hydrolysis, was selected for subsequent studies.

As expected, a lipase with positional specificity over the triglycerides gave no advantage, since monoglycerides become inaccessible to the reverse hydrolytic reaction due to their low solubility in *n*-hexane.

### 3.3
### Mass of polyurethane pre-polymer

The hydrolytic rates increased with the amount of polyurethane pre-polymer used (Fig. 3), even though the increase in the $a_w$ of the foams was rather small (*A*, $a_w=0.849$; *B*, $a_w=0.885$; *C*, $a_w=0.891$, $t=25$ °C).

When the foam was prepared with 0.8 g of "FHP 2002", its volume was too large to be immersed in the reaction medium. Some of the foam cuboids were projected against the reactor

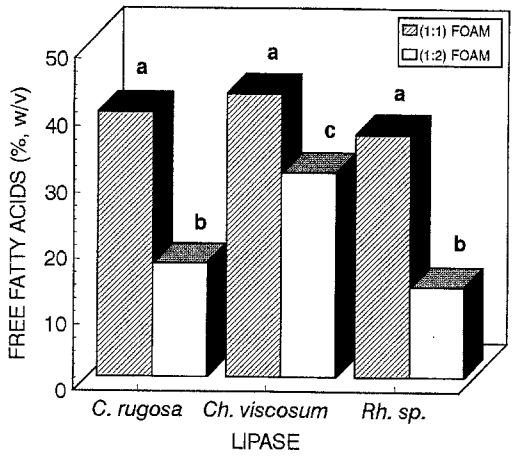


Fig. 2. Production of FFA by hydrolysis of olive oil catalyzed by three different lipases immobilized in (1:1) and (1:2) PU foams. Values represent means for each group ($n=3$). Letters which are different from one another are different at $\alpha \leq 0.05$

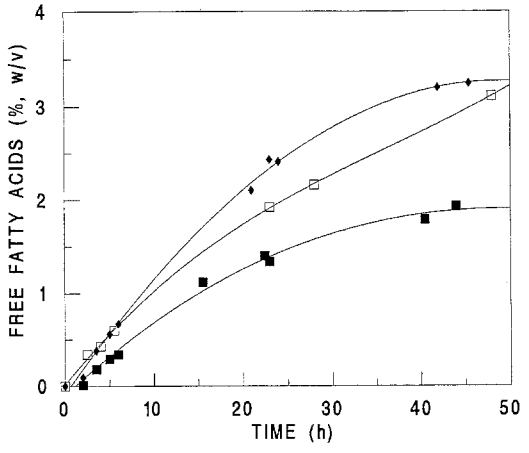


Fig. 3. Production of FFA during the hydrolysis of olive oil by the lipase from C. rugosa immobilized in different amounts of (1:2) PU foams: A, 0.4g (■); B, 0.6g (□); C, 0.8g of pre-polymer (◆)

walls, over the liquid level, where they remained inaccessible to the substrates. According to the manufacturer, the solid load incorporated in "FHP 2002" should not exceed 80%. With 0.4 g of pre-polymer (60% load), hydrolysis occurred to a less extent. Nevertheless, a proportion of "FHP 2002" to lipase of 2.5 : 1 (0.6 g FHP 2002 : 0.24 g lipase) was selected for the subsequent experiments, to prevent enzyme losses and internal diffusion limitations.

### 3.4
### Reactions under initially controlled water activity

Hydrolysis and glycerolysis are competing reactions. Since the non pre-equilibrated enzyme preparations have a high initial $a_w$ (~0.89), the production of FFA during the course of both reactions was compared and found to be considerably higher in the presence of glycerol (Fig. 4).

To further investigate, glycerolysis and hydrolysis of olive oil were carried out under different initial $a_w$ values (Figs. 5–12).

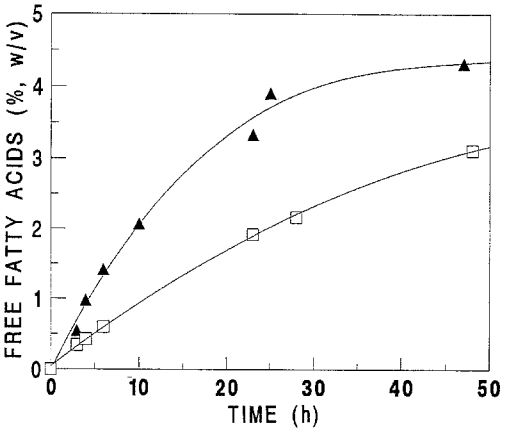


Fig. 4. Production of FFA during the hydrolysis (□) and glycerolysis (▲) of olive oil by the lipase from C. rugosa immobilized in a (1:2) PU foam

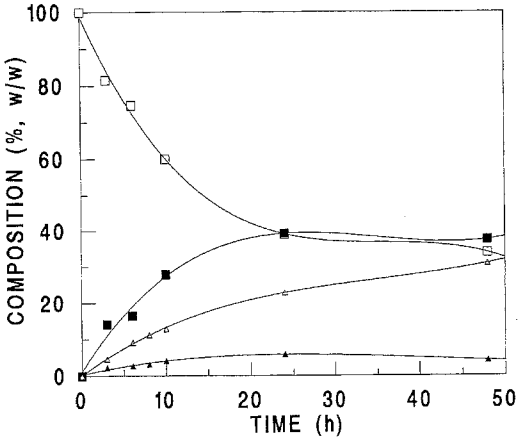


Fig. 5. Olive oil glycerolysis catalyzed by the lipase from C. rugosa immobilized in a (1:2) PU foam, with no $a_w$ control: TG (□), DG (▲), MG (■), FFA (△)

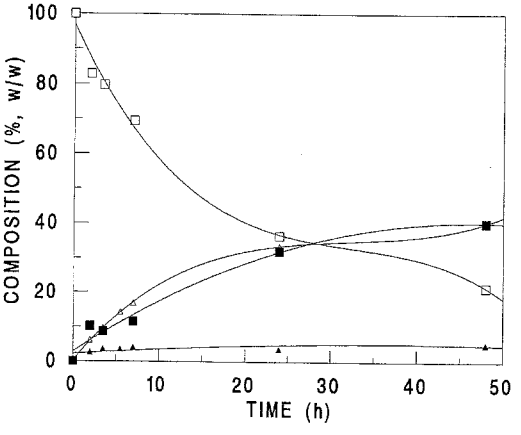


Fig. 6. Olive oil glycerolysis at an initial $a_w$ value o 0.92, catalyzed by the lipase from C. rugosa immobilized in a (1:2) PU foam: TG (□), DG (▲), MG (■), FFA (△)

The kinetics of glycerolysis depended on the initial $a_w$ of the reaction system: as the $a_w$ decreased, FFA and DG productions decreased and the final conversion into MG increased (Figs. 5–9).

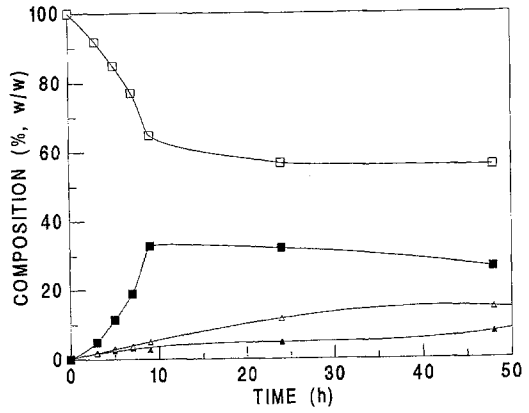


Fig. 7. Olive oil glycerolysis at an initial $a_w$ value of 0.75, catalyzed by the lipase from C. rugosa immobilized in a (1:2) PU foam: TG (□), MG (▲), DG (■), FFA (△)

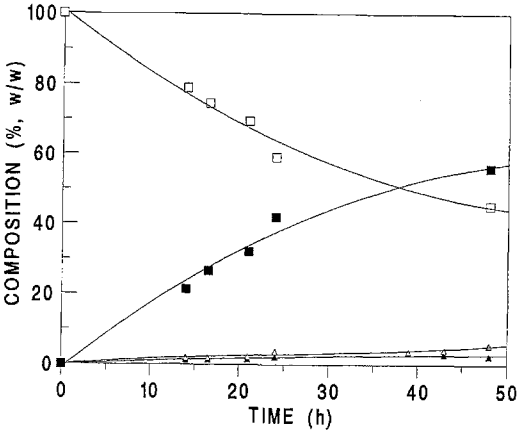


Fig. 8. Olive oil glycerolysis at an initial $a_w$ value of 0.56, catalyzed by the lipase from C. rugosa immobilized in a (1:2) PU foam: TG (□), MG (▲), DG (■), FFA (△)

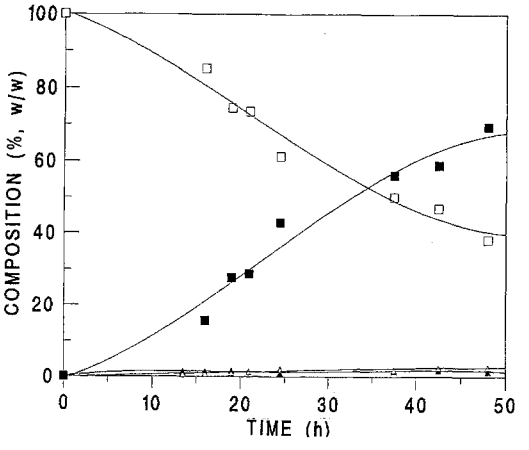


Fig. 9. Olive oil glycerolysis at an initial $a_w$ value of 0.23, catalyzed by C. rugosa lipase immobilized in a (1:2) PU foam: TG (□), DG (▲), MG (■), FFA (△)

The initial rates of fatty acids produced both by hydrolysis and glycerolysis showed an exponential dependence on the $a_w$ of the system (Fig. 10). The curves probably reflect the pattern of water adsorption to protein molecules [43], or to the

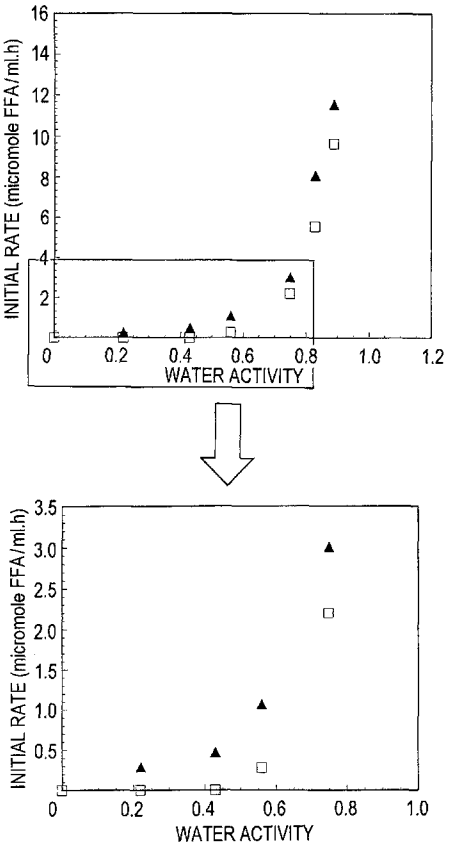


Fig. 10. Initial rates of FFA production during olive oil glycerolysis (▲) and hydrolysis (□). Reactions were catalyzed by C. rugosa lipase immobilized in a (1:2) PU foam and carried out at different initial $a_w$

polyurethane matrix, and can be compared to the adsorption multilayer isotherms [44].

In glycerolytic reaction systems, the initial rates of fatty acid production were higher than in hydrolytic systems. At $a_w$ values lower than 0.43, hydrolysis was completely repressed, although glycerolysis still occurred.

The presence of FFA, even in low water activity glycerolytic systems, suggests that glycerolysis is not a direct interesterification, but rather the hydrolysis of the ester bond in the glyceride, followed by esterification of FFA to Gly. The same general mechanism was proposed for lipase-catalysed interesterification reactions [22].

However, because the addition of glycerol depresses the $a_w$ of the reaction medium, there is less water available for hydrolysis in a glycerolytic system than in a hydrolytic system. Thus, a lower production rate of FFA would have been expected in glycerolysis.

In low water activity systems, the water available for hydrolytic reactions has been reported to be essential for enzyme activation [22–23, 34]. This result is sustained by Miller's et al. [45], who suggested that hydrolysis may be the rate-controlling step in interesterification. Conversely, Heisler et al. [24] concluded that in the isomerization of 1,2-dipalmitin into the 1,3-isomer catalyzed by 1,3-selective lipases, the hydrolysis of the DG was the fastest reaction in the system.

According to our knowledge, the present study is the first on the effect of the $a_w$ on the rate of fatty acids release in

glycerolysis and hydrolysis. In previous works, the presence of FFA in the reaction media was ascribed to the hydrolytic step.

A mechanism for glycerolysis involving the breaking of the ester bond and fatty acid release by glycerol would explain the differences found between the kinetics of glycerolysis and hydrolysis. The water monolayer bound to the lipase molecule ($a_w \sim 0.2$–$0.3$) appears to be enough to keep the lipase active in the glycerolysis reaction. No hydrolysis occurred at these values of $a_w$ because free water was not available as a substrate. In fact, Tanaka *et al.* [46] reported in a U.S. patent that the water required to activate dry interesterification catalysts can be replaced by glycerol or various diols.

Like for FFA, the initial rate of diglyceride production increased exponentially with the initial $a_w$ of the system (Fig. 11). As the water available for hydrolytic reactions increases, lipase activation probably occurs [22]. The DG produced result mainly from hydrolysis, since the $a_w$ is too high to promote the esterification reaction [34, 47–48]. A direct relationship between DG production and $a_w$ was also found in other systems [25, 35–36].

The highest initial rate of monoglyceride production was observed for an $a_w$ value of 0.75 (Fig. 12), while MG production increased as the $a_w$ decreased (Fig. 13). The highest MG production (70%, w/w) was obtained at the lowest $a_w$ tested (0.23), after 48 hours of reaction.

However, the time to attain reaction equilibrium was longer at lower $a_w$. For $a_w \leq 0.56$, the equilibrium was not reached in 48 hours. Thus, higher MG productions can be expected after prolonged reaction times under low $a_w$.

The solubilization of glycerol becomes more difficult at decreasing water activities, because of water depletion in the microenvironment. Nevertheless, low moisture contents restrain hydrolysis and allow the esterification step to proceed, leading to high yields of MG.

A lag period, at low $a_w$, was also observed in the synthesis of esters (heptyl octanoate and heptyl oleate) catalyzed by C. rugosa lipase in a free form and in the absence of organic solvents [39]. The occurrence can be probably related to the limited amount of water present, prior to the activation effect promoted by the water resulting from the esterification, or to

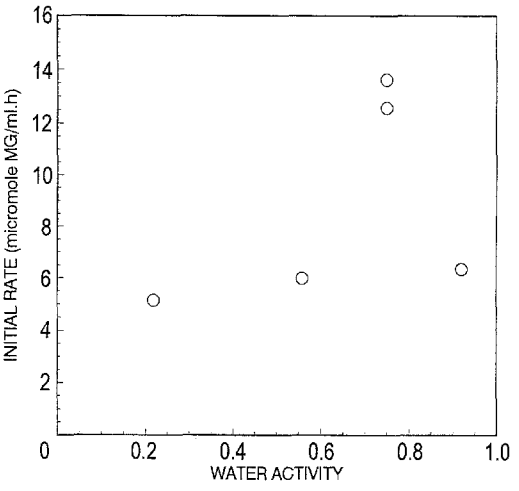


Fig. 12. Initial rates of MG production during olive oil glycerolysis. Reactions were catalyzed by C. rugosa lipase immobilized in a (1:2) PU foam and carried out at different initial $a_w$

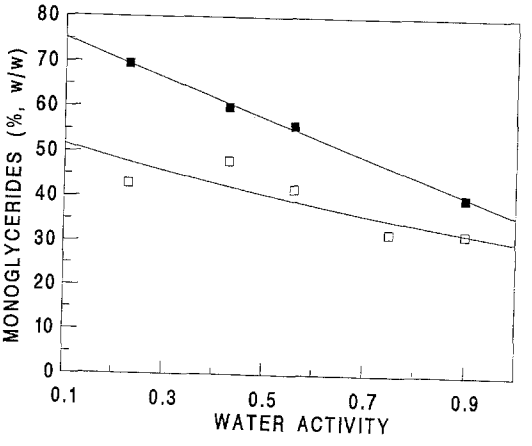


Fig. 13. The yeld of MG (% MG/TG) during olive oil glycerolysis (24h, □; 48h, ■). Reactions were catalyzed by C. rugosa lipase immobilized in a (1:2) PU foam and carried out at different initial $a_w$

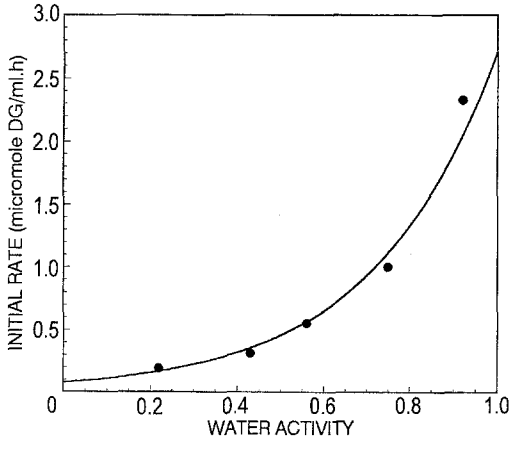


Fig. 11. Initial rates of DG production during olive oil glycerolysis. Reactions were catalyzed by C. rugosa lipase immobilized in a (1:2) PU foam and carried out at different initial $a_w$

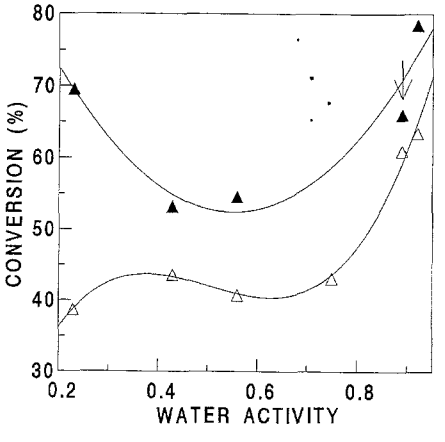


Fig. 14. The total conversion (% TG consumed) during olive oil glycerolysis (24h, △; 48h, ▲). Reactions were catalyzed by C. rugosa lipase immobilized in a (1:2) PU foam and carried out at different initial $a_w$. The arrow indicates the experiment without $a_w$ control

**Table 1.** Performance of various bioreaction systems aimed at the production of MG.

| Reaction | Lipase source | Time (h) | V ($cm^3$) | Conversion (%, w/w) | Volumetric productivity ($mg/cm^3 \cdot h$) | Lipase units (U) | Specific productivity ($mg/h \cdot U$) | Ref. |
|---|---|---|---|---|---|---|---|---|
| GLYCEROLYSIS | | | | | | | | |
| Gly + olive oil (in hexane; $a_w = 0.23$) B | C. rugosa (immob.) | 48 | 12.5 | 58 | 1.6 | 108 | 0.19 | Present work |
| Gly + TO (in isoctane) B | Chr. viscosum (micelles) | 40 | 10.0 | 55 | $9.8 \times 10^{(-2)}$ | ? | ? | [15] |
| Gly + beef tallow (solvent free) B | Chr. viscosum (powder) | 6 | ~16.5 | 58 | 92.7 | 7000 | 0.22 | [18] |
| Gly + olive oil (solvent free) B | Pseudomonas fluorescens (powder) | 96 | ~16.5 | 75 | 7.5 | 6535 | 0.02 | [19] |
| Gly + mutton tallow (solvent free) B | Rhizomucor miehei (Lipozyme) | 72 | ~11.4 | 16 | 2.3 | ? | ? | [20] |
| Gly + olive oil (in isooctane) C | Chr. viscosum (liposome in micelles) | – | 20 | 48 | 1.3 | ? | ? | [16] |
| ESTERIFICATION | | | | | | | | |
| Gly + $C_{18:1}$ (solvent free) | Penicillium sp. (immob.) | | | 14.2 | 38.0 | | | [14] |
| B | | 3.3 | ~0.16 | | | ? | ? | |
| | Rh. miehei (Lipozyme) | | | 5.0 | 13.3 | | | |
| Gly + $C_{14:0}$ (in isooctane) | Rh. delemar (micelles) | 24 | | 55 | 0.7 | | | [5] |
| B | | 10 | ? | | | ? | ? | |
| | | | | 50 | 1.5 | | | |
| Gly + $C_{12:0}$ (in isooctane) | Rh. delemar (micelles) | 50 | ? | 45 | 0.3 | ? | ? | [6] |
| B | | 140 | | 60 | 0.1 | | | |
| Gly + $C_{18:1}$ (in hexane) | Penicillium sp. (immob.) | | ~3 | 35 (40 °C) | 5.4 | 5100 | $3.20 \times 10^{(-3)}$ | [7] |
| B | | 24 | ~3.5 | 98.5 (25 °C) | 4.4 | 8415 | $1.83 \times 10^{(-3)}$ | |
| Gly + $C_{18:1}$ (in tButOMe) B | Chr. viscosum (powder) | 72 | 50 | 66 | 0.7 | ? | ? | [8] |

B – Batch operation
C – Continuous operation
tButOMe – methoxy-*t*-butane

some structural changes at the interface, upon which lipase activity is generally strongly dependent [49].

At high $a_w$ ($\geq 0.75$), reaction equilibrium in glycerolysis was attained in 24 hours, with a low conversion into MG, probably because a steady-state between esterification and hydrolysis is attained, i.e. this seems to be a true reversible reaction limited [50].

The total conversion (in terms of % TG consumed) at 48 hours showed a minimum at intermediate $a_w$ values (Fig. 14). Higher conversions at extreme $a_w$ indicated an increase in the hydrolytic and glycerolytic rates, at high and low $a_w$, respectively.

According to the literature, the highest activity of lipases in the esterification and interesterification of lipids is usually observed at $a_w$ between 0.20 and 0.50, depending both on the biocatalyst and on the reaction itself.

For instance, the best results in the transesterification of coconut oil with methyl stearate [51], of phosphatidylcholine with FFA [52], of triolein with palmitic acid or ethyl palmitate [35], of sunflower seed oil with ethyl stearate [36] and in the esterification of decanol with decanoic acid [37], were obtained with the Rhizomucor miehei immobilized lipase (Lipozyme™), at an $a_w$ of about 0.4–0.5.

With regard to transesterification reactions [51] and the esterification between linoleic acid and glycerol and several glycols [41] with Rhizopus arrhizus lipase, the optimum $a_w$ was 0.3. When this enzyme was immobilized on a polypropylene support and used as a catalyst for the transesterification of phosphatidylcholine, the highest yield was obtained at an $a_w$ of 0.064 [52].

Optimum $a_w$ values of 0.3 were also observed with the porcine pancreatic lipase in transesterification reactions carried out in a packet-bed gas-solid reactor [53] and with the C. deformans lipase in the transesterification of coconut oil with methyl stearate [51].

The C. rugosa lipase was active in the esterification of heptanol with octanoic acid under low (0.13) and intermediate (0.69) water activities [34]. In the transesterification between *n*-octanol and vinyl butyrate catalyzed by the lipase from Pseudomonas cepacia (as a powder or immobilized onto celite), the Michaelis constant for the nucleophile increased with increasing $a_w$ and the highest maximum rate was attained at $a_w$ values of 0.11–0.38 [54].

Since an increase in MG production was observed as the $a_w$ decreased, and some lipases exhibit the highest activity under very low $a_w$, it is worthy attempting to carry out glycerolysis at $a_w$ below 0.23.

The addition of pairs of salt hydrates to the organic medium, proposed by Halling [55], is an alternative way to control the $a_w$ during the enzymatic reactions. Since the hydrate pairs are able to take up or release water, they can buffer the $a_w$, maintaining the optimal conditions as the reaction proceeds. With this methodology, it is possible to control on-line the $a_w$ of the whole system. Plus, the initial equilibration period of substrates and enzyme with saturated salt solutions is superfluous, which is an advantage in terms of enzyme activity.

The presence of salt hydrates in the reaction was successfully applied to peptide synthesis by chymotrypsin in hexane [56], to the esterification of butanoic acid with butanol by C. rugosa lipase in hexane [57], and to the control of tyrosinase activity in chloroform and toluene [58]. It would be worth trying this attractive way of $a_w$ control in future research related to olive oil glycerolysis. It will be necessary, though, to find the right pair of salt hydrates that fixes the desired $a_w$ value, without giving side-products, nor affecting the activity and the stability of the biocatalyst.

The results obtained in the present study indicated that the initial $a_w$ of the reaction system is an important parameter in glycerolysis.

The comparison between results obtained in the system under study and those reported by other authors is not straightforward because data have been presented in different modes. An attempt on the conversion of results to a common format is presented in Table 1.



Besides conversion into MG (TG% (w/w) consumed for MG production), volumetric (grams of MG produced per $cm^3$ of reaction medium and hour) and specific (grams of MG produced per hour and lipase unit) productivities were calculated whenever possible. Specific productivity could only be calculated in a few cases, since different methods to assay for enzymatic activity were followed in the various reactions. With regard to batch glycerolysis results, the conversion into MG doesn't seem to be the best way to evaluate the process. In fact, the highest conversions don't necessarily give the maximum productivity. In spite of a conversion of the same order of magnitude achieved both in solvent and solvent free systems [present work, 15, 18], the lowest volumetric productivity was obtained in a reversed micelle system [15], probably because of the low substrate concentrations used. Volumetric productivities obtained in the various systems vary within a wide range. The specific productivity in our case is comparable to that of McNeill et al. [18], in a solvent free system, in spite of the considerable difference in volumetric productivities between the two systems. However, the large quantities of lipase used in solvent free systems may represent the majority of costs in the bioprocess [59]. Thus, the alternative costs of substrate dilution and subsequent solvent recovery should be considered.

When MG were produced by direct esterification of glycerol with FFA, the lowest volumetric productivities were also obtained in the reversed micelle systems. From the results compiled in Table 1 it is not clear which is the best route for MG production. The system under study may become a feasible process option if the lipase preparation has a high operational stability. This aspect will be addressed in a forthcoming article.

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