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Authors: A. Bódalo, J. Bastida, M.F. Máximo, M.C. Montiel, M.D. Murcia, S. Ortega

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INFLUENCE OF THE OPERATING CONDITIONS ON LIPASE-CATALYSED SYNTHESIS OF RICINOLEIC ACID ESTOLIDES IN SOLVENT-FREE SYSTEMS

AUTHORS: A. Bódalo, J. Bastida, M.F. Máximo, M.C. Montiel*, M.D. Murcia, S. Ortega.

Chemical Engineering Department, University of Murcia, Campus de Espinardo, 30071 Murcia, Spain.

(*) Corresponding author. Telephone number: +34-968-367219. Fax number: +34-968-364148. e-mail address: cmontiel@um.es (M.C. Montiel).

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ABSTRACT

In this work, the synthesis of ricinoleic acid estolides, also named polyricinoleic acid (PR), in two different solvent-free reaction systems, catalysed by immobilised *Candida rugosa* lipase is described. First, the esterification was performed in an open air jacketed batch reactor and the influence of amount of enzyme, temperature and initial water content was tested. The best results were obtained when 5 g of soaked immobilised derivative was used as biocatalyst, and the reaction was carried out at 40 ºC. It was observed that environmental relative humidity plays an important role in the enzymatic synthesis of ricinoleic acid estolides and, given that this parameter takes on a wide range of values depending on the season, it is considered the main cause of the poor reproducibility achieved in the open air reactor. To solve this problem, the ricinoleic acid estolides were synthesised under controlled atmosphere in a vacuum reactor with dry air intake. The optimum drying time of 7 hours was selected. In these conditions, PR

with an acid value of 57.5 mg KOH/g was synthesised in 48 hours of reaction and the results were entirely reproducible.

1. INTRODUCTION

The use of enzymes in non-aqueous environments has increased in the past two decades as an attempt to broaden the applications of biocatalysis (1). In the context of "green chemistry", the technological utility of enzymes can be greatly enhanced by using them in non-conventional media. Currently, there are five main "green" solvent systems: supercritical fluids, fluorinated solvents, ionic liquids, water, and solvent-free systems (SFS) (2). The major advantages of the latter are that the absence of solvents facilitates downstream processing, offering significant cost saving and minimizing the environmental impact (3). The possibility of using enzymes in media containing only the reactants and the enzyme preparation without the addition of other compounds has only been considered for processes using lipase as catalyst and triglycerides as substrate (4).

Estolides are oligomeric molecules that are mostly composed of hydroxy fatty acids. The polymerisation takes place by covalently ester bonds between hydroxyl moiety of one hydroxy acid and the carboxyl moiety of another hydroxy acid molecule (Figure 1). The estolide made from ricinoleic acid ($18{:}1^{9}$ – OH^{12}), is a useful substance with many applications in industry. For example, it is used as a viscosity controller for chocolate and an emulsifier in margarine, as cutting oil base in metal processing and as pigment dispersant in paint, ink and cosmetics (5, 6). The high temperature of the conventional chemical route of polyricinoleic acid synthesis, leads to a lower selectivity, undesired by-products, colouring and malodour of the product. Because of these limitations a new process for the enzyme based production of ricinoleic acid estolides was developed using *Candida rugosa* lipase (5, 7).

The optimisation of some reaction conditions is especially important in an experimental system like the one described. It is known that temperature is a crucial parameter in every enzyme catalysed reactor but in our case, due to the special characteristics of the reaction medium (solvent-free), temperature greatly influences viscosity, mass transport phenomena and, as a consequence, the esterification rate (9). While high temperature favours the medium fluidity, enzyme has to be prevented from thermal deactivation (8, 9).

Another decisive parameter in these processes is the water content. Water plays multiple roles in lipase-catalysed esterifications performed in non-conventional media. It is widely known that water is absolutely necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes (10, 11, 12). On the other hand, in esterification/hydrolysis reactions it is well-known that the water content affects the equilibrium conversion of the reactions. Particularly, in the case of estolides production, the water formed by the reaction must be removed from the reaction mixture if polyricinoleic acid with a high degree of condensation is to be obtained (5).

Usually, enzyme immobilisation improves their operational stability, while preventing contamination of the substrate being converted. In addition, immobilised enzymes can be easily separated from the reaction media for reuse or for use in continuous reactors. For these reasons, efforts have been devoted to obtaining an immobilised derivative with a high immobilised protein percentage and enzymatic activity for the present application (13).

The present study describes a detailed investigation on the synthesis of estolides of ricinoleic acid catalysed by immobilised *Candida rugosa* lipase in a medium containing only the reactant and the enzyme derivative without the addition of solvents not involved in the reaction.

We examine the influence of several key reactions conditions, such as temperature, initial water percentage, and enzyme amount, on the enzymatic reaction rate and the equilibrium conversion. In addition several reactor configurations have been tested.

2. MATERIALS AND METHODS

2.1. Enzyme, carrier and reagents

Microbial lipase from *Candida rugosa* (Type VI) was purchased from Sigma-Aldrich. The crude enzyme has a nominal specific lipolytic activity of 819 U/mg solid (one unit will hydrolyse 1.0 microequivalent of fatty acid from a triglyceride in an hour, at pH = 7.7 and 37 ºC) and contains approximately 15% protein based on the Lowry's method modified by Hartree (14). The immobilisation support, an anionic exchange resin, Lewatit MonoPlus MP 64, and the substrate, ricinoleic acid (~80%), were supplied by Fluka. Other chemicals were of analytical grade and used without further purification.

2.2. Immobilisation procedure

One gram of support was mixed with 10 ml of soybean lecithin suspension (20 mg/ml) in an Erlenmeyer flask and placed in an orbital shaker overnight at room temperature. It was then washed with 10 ml of distilled water and transferred to a jacketed column reactor (2.5 i.d. and 30 cm length). The reactor was equipped with a sinterised glass plate placed 5 cm from the bottom. The enzyme solution (10 ml, 10 mg/ml in acetate buffer 0.01 M, pH=5) was then added to the reactor and recycled for two days at 4ºC. The immobilised derivative was washed twice with the same buffer and stored at 4ºC. The immobilisation method has been optimised elsewhere (13).

2.3. Measurement of the reaction extension

Acid value (AV) (15) was used as an index to show the degree of reaction. The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in one gram of sample. AV corresponds to the carboxyl group concentration in the reaction mixture, which decreases due to the condensation of ricinoleic acid (AV=180 mg KOH/g).

2.4. Viscosity measurement

The viscosity of ricinoleic acid and polyricinoleic acid (AV = 40) was measured by using a rotational viscometer Visco-Star R (100-13,000,000 cP) equipped with different standard spindles. Measurements were carried out at five different temperatures between 25 and 50°C by placing a sufficient amount of ricinoleic acid or polyricinoleic acid in a jacketed glass (total volume 600 ml) connected to a thermostatic water bath. In each case the ratio between shear stress and shear force was registered.

2.5. Humidity measurements

Environmental relative humidity was measured by means of a themohygrometer Testo 645 (Testo AG, Germany).

2.6. Water content measurements

The water content in the reaction mixture was measured by simple titration (16) in a Karl-Fischer equipment Model 701 Titrino KF (Metrohm AG, Herisau, Switzerland).

2.7. Reactor experiments

Unless otherwise stated, the enzymatic reaction was carried out in an open air jacketed batch reactor (250 ml total volume). Complete mixing was achieved by means of a three-bladed propeller stirrer. The reaction temperature was always kept constant at 40ºC (5), except in the experiments where the influence of the temperature was studied. The reaction mixture contained 30 g of ricinoleic acid and five grams of immobilised lipase (40 mg Lowry's protein). The only water in the reaction system was that soaked in the support except in the experiments to study the influence of the initial water content. In all cases, samples were taken from the reactor at given time intervals and AV of the reaction mixture was determined.

2.8. Vacuum reactor

For reactions using a vacuum, a Parr 5100 series low pressure reactor was used. The reaction vessel (100 ml total volume) is made of glass and is equipped with a circulating jacket to heat the vessel. The reactor head is stainless steel and accommodates the reactor controls and instrumentation. The reactor is equipped with a magnetic drive to provide a trouble free internal stirrer, which is a turbine type impeller. The reactor top also includes a vacuum meter, an internal thermocouple, an internal cooling loop, a rupture disk, a liquid sample valve, a gas inlet valve and a gas release valve. Temperature, stirring speed and positive pressure are managed by a controller. The amount of ricinoleic acid, immobilised lipase and water in the reactor at the beginning of the reaction are the same that those reported for the open air jacketed batch reactor. All the experiments were carried out at 40 ºC, and the stirring rate was kept constant at a value of 350 rpm. The pressure was set at 160 mmHg and, in several experiments, an 89 l/h dry air flow was conducted through the reaction mixture to facilitate water removal. The air flow was dried by passing through a silica gel column, therefore its relative humidity was zero.

3. RESULTS AND DISCUSSION

Several experiments were performed in the open air jacketed batch reactor in order to study the influence of three operating variables on the synthesis of ricinoleic acid estolides. The experimental conditions in which the reactions were carried out are summarised in Table 1.

3.1. Influence of the amount of enzyme

For the synthesis of polyricinoleic acid, the influence of different amounts of added immobilised lipase on the degree of polymerisation of the estolides was studied. As shown in Figure 2, when the amount of immobilised enzyme (IME) added to the reactor was increased, the reaction progressed faster and a lower acid value was reached. The differences between the acid values reached led us to think that these were not equilibrium values but that the reaction stops because the enzyme is not active anymore. However, changes in reaction rate and final acid value were more noticeable when the enzyme amount varied from 2.5 g to 5 g than when the enzyme amount was increased to 10 g, meaning that in the last case more enzyme than necessary was being used. On the other hand, 10 g of IME in 30 g of ricinoleic acid was a ratio too high to be easily handle. In consequence 5 g of immobilised derivative was considered as appropriate for further experiments.

3.2. Influence of the temperature

The effect of the temperature on the reaction course was investigated. Temperature was seen to influence the enzymatic reaction rate, the enzyme stability, the velocity of water evaporation from the reaction medium and its viscosity. The lowest temperature chosen was fixed at 40ºC, which is the optimum temperature of *Candida rugosa* lipase (5). Below this temperature stirring the reaction mixture became difficult

because of the high viscosity of the ricinoleic acid and its estolides. The upper temperature limit was fixed at 50ºC to avoid enzyme denaturalisation. When the temperature was raised from 40 to 50 ºC, the viscosity values of ricinoleic acid and polyricinoleic acid fell by 35% (data not shown). Figure 3 shows the evolution with time of the acid value at each temperature. As can be seen, this variable had little influence on the reaction course within the range studied, probably because it was not broad enough and because the different effects (viscosity, reaction rate and enzyme deactivation) are compensated. Nevertheless, a slightly unfavourable effect could be observed at high temperature. Therefore, for further experiments 40ºC was chosen as optimum temperature.

3.3. Influence of the initial water content

As described above, water plays multiple roles on lipase-catalysed esterifications performed in non-conventional media. It is widely known that water is absolutely necessary for the catalytic function of enzymes because it participates, directly or indirectly, in all non-covalent interactions that maintain the conformation of the catalytic site of enzymes (10, 11, 12). However, it has been found that the amount of water necessary for enzyme activity might be very small and, in the case of lipase, just a few layers around the enzyme surface are needed (17).

On the other hand, in esterification/hydrolysis reactions the water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media (18). Particularly, in the case of esterifications, as the water content increased, lower equilibrium conversions were achieved.

In the light of the above considerations, a study on the optimal initial amount of water in the reactor was deemed necessary. With this purpose, four experiments were carried out using the immobilised

derivative as obtained (soaked), adding different amounts of water, and drying the derivative under vacuum at room temperature before use. The time course of these experiments is shown in Figure 4 where the acid value is represented against operation time.

With these experiments it was demonstrated that an optimum in the initial water content exists, although this optimum seems to be quite wide. The same results were obtained when derivative was used as obtained and when small amounts of water were added. However, drying the derivative or adding higher amounts of water led to a lower initial rate (specially the high water content) and a higher final acid value.

All the experiments described above were carried out simultaneously, in an air open tank reactor, within a month of each other. However, when the results were tested for reproducibility, great discrepancy between them was observed, as shown in Figure 5, where a variation of 30% in the AV value was obtained for experiments carried out in different seasons. Measurements of the water content of the reaction medium revealed that, at 40°C, water continuously evaporated and that after 48 hours (approximately) the reactor water content was independent of the initial water content and mainly dependent on environmental relative humidity. The air conditioner/heat pump equipment installed in the laboratory stabilizes the relative humidity at 70% in summer (air conditioner) and at 20% in winter (heat pump). These values correspond with equilibrium water contents of 3600 ppm and 1000 ppm, respectively, which are the cause of the discrepancies in the results obtained in the open air reactor. Obviously, this poor reproducibility of the results is unacceptable if the process is to be applied on an industrial scale.

Therefore, the remaining experiments were carried out in the vacuum reactor described in Section 2 in which the synthesis of ricinoleic acid

estolides can be conducted in a closed system with controlled atmosphere, a suitable level of stirring and low pressure.

3.4. Experiments in the vacuum reactor

First, the influence of pressure on PR synthesis was analysed. The results obtained in the experiment carried out at a constant pressure of 160 mm Hg are illustrated in Figure 6, where it can be observed that, after 50 hours of reaction, the acid value of the estolide obtained under vacuum was only 70 mg KOH/g, which is noticeably higher than the best result obtained in the open air reactor. It was thought that water removal could be improved by using a vacuum (5), but at the end of the experiment (70 hours), it was still higher than 30000 ppm. This might be attributable to the partial condensation of the evaporated water (which consequently drops into the reaction mixture) due to the geometry of the reactor top plate.

In order to enhance dehydration, a current of dry air was passed through the reaction mixture and the results obtained in that experiment are also plotted in Figure 6. As can be seen, a slight improvement in the degree of condensation was achieved, compared with the synthesis carried out without dry air, although the acid value reached at the end of the experiment was still very far from that obtained in the open air reactor. In this case, the reactor water content was only 1500 ppm after 24 hours and the reaction medium was almost anhydrous (0 ppm Karl-Fisher) at the end of the process. These results demonstrate the high rate of dehydration that occurred and consequently the reaction probably stopped probably because the enzyme had insufficient water to maintain the active form and therefore, estolides with very high acid value were synthesised.

To study the influence of dry air intake, several experiments were performed in the vacuum reactor passing the dry gas for different lengths of time, ranging from 2.5 to 21 hours. Samples were taken from the reactor at given time intervals and both the acid value and water content of the reaction mixture were determined. The results obtained are shown in Figure 6, where the acid values reached at 48 hours are plotted in a bar chart. It can be observed that the highest degrees of esterification were reached when drying times of 5, 7 and 9 hours were assayed.

With the objective of analyzing these results in detail, the "average reaction rate" (expressed as the acid value variation per hour of reaction) and the "average rate of water removal" were calculated and are summarised in Table 2. As was expected, higher drying times led to higher rates of water removal; however, long drying periods promoted total water removal from the reaction mixture, which supposedly provoked a slight dehydration of lipase and the consequent loss of activity. This explains the lower reaction rate calculated when drying times of 21 and 9 hours were assayed in comparison with that obtained for the experiment carried out with a drying time of 7 hours. These results agree with the revised literature, in which some authors have reported difficulty in removing water from the reaction medium without dehydrating the enzyme, in an attempt to obtain high conversion yields in esterification reactions catalysed by immobilised lipase (10), as soon as the importance of maintaining a minimum water content for the expression of enzymatic activity (5).

In relation with the reaction rate, the best results were obtained for a drying time of 7 hours, which is sufficient to guarantee a good rate of water removal, without provoking the elimination of all the water contained in the reaction mixture. In this case, the final content of water in the reactor was 3000 ppm, which is suitable to maintain the lipase perfectly hydrated and, consequently, no loss of activity was

observed (5). For the other drying times assayed, lower rates of water removal were obtained, and the final content of water in the reactor was higher. So, esterification reaction progressed more slowly, and a higher acid value was reached.

The ricinoleic acid estolides obtained under the above described reaction conditions had a similar degree of condensation to those synthesised in an open air reactor when the environmental relative humidity was 20%, with the advantage that, in this case, results are totally reproducible.

4. CONCLUSIONS

The ricinoleic acid estolides synthesis can be successfully performed in a solvent-free reaction system, catalysed by immobilised *Candida rugosa* lipase. The experiments carried out in the open air jacketed batch reactor permitted us to establish the optimal value for different reaction conditions (amount of enzyme, temperature and initial water content). However, the equilibrium of the esterification reaction was seriously affected by the environmental relative humidity and, therefore, the operation in an open air reactor cannot be considered a suitable option from the point of view of experimental reproducibility.

The best strategy for avoiding this problem is to carry out estolides synthesis under low pressure (160 mmHg) and passing a current of dry air through the reaction mixture for 7 hours. In these conditions, the water content in the reactor can be adequately controlled and an estolide with a high degree of polymerisation (AV = 57.5 mg KOH/g) can be obtained with only 48 hours of reaction, which is similar to that obtained in the “best” experiment conducted in the open air reactor. In the vacuum reactor, the degrees of the esterificacion between in summer and in winter were not different.

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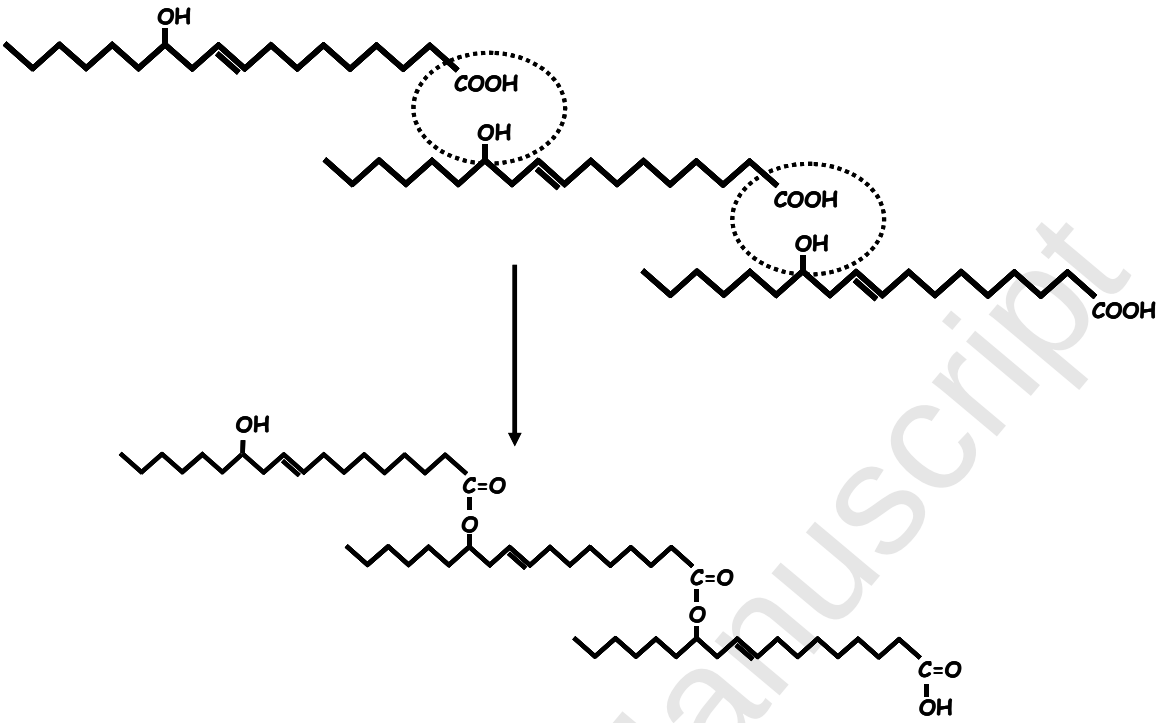

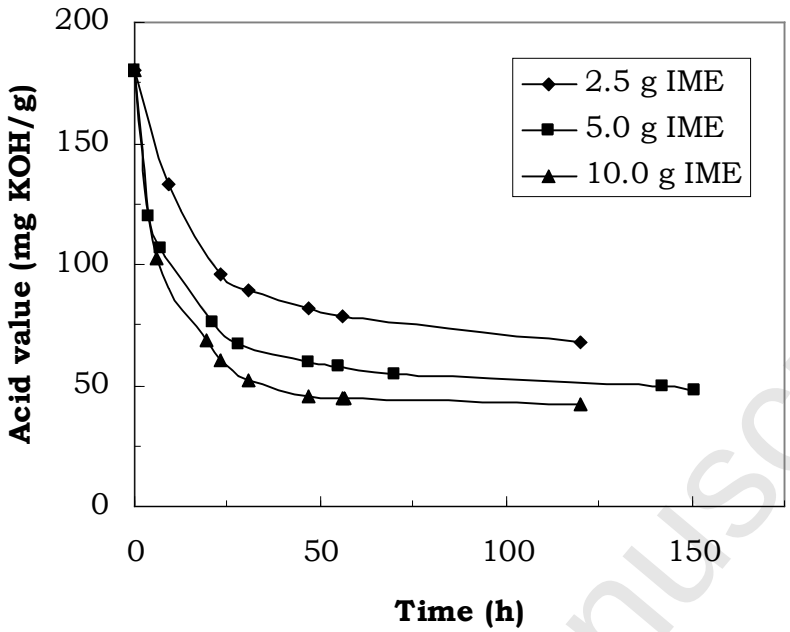

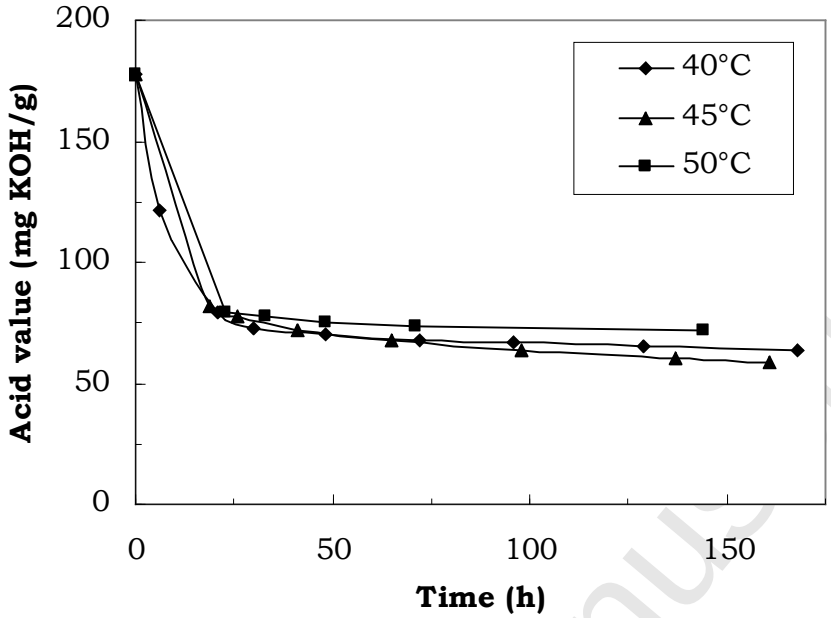

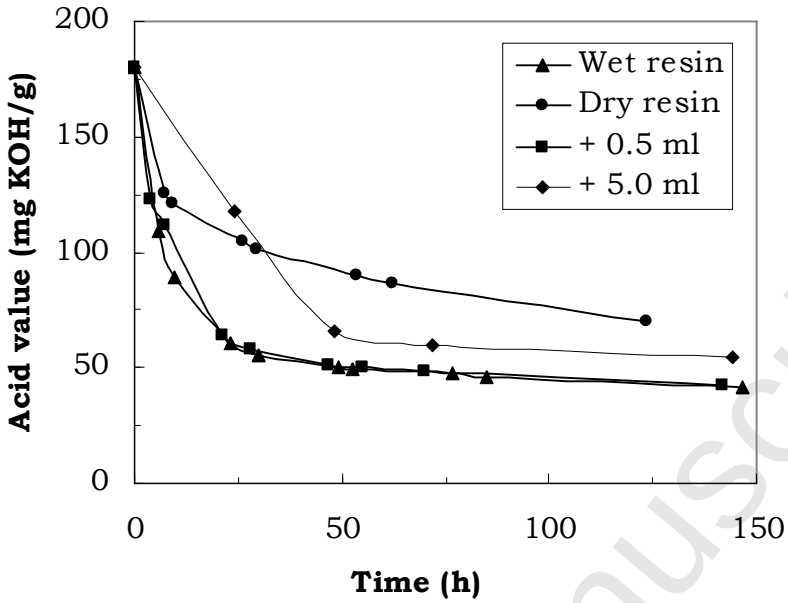

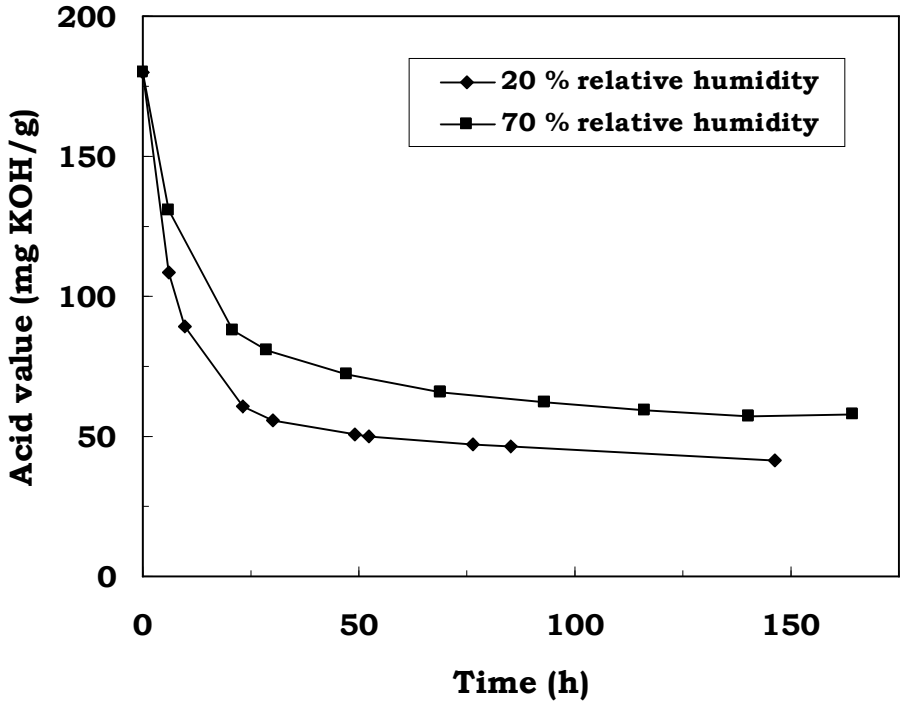

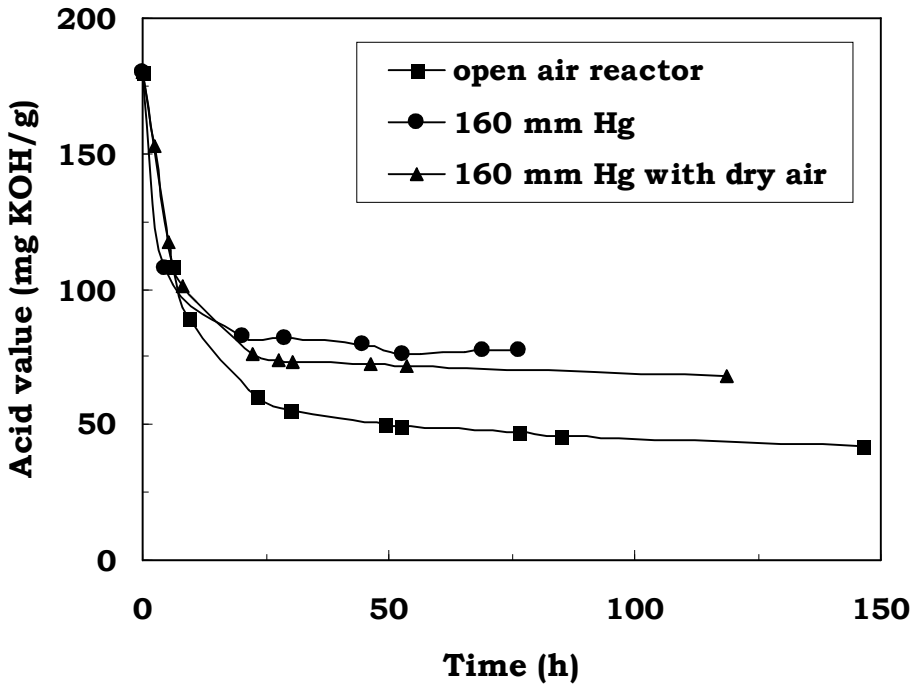

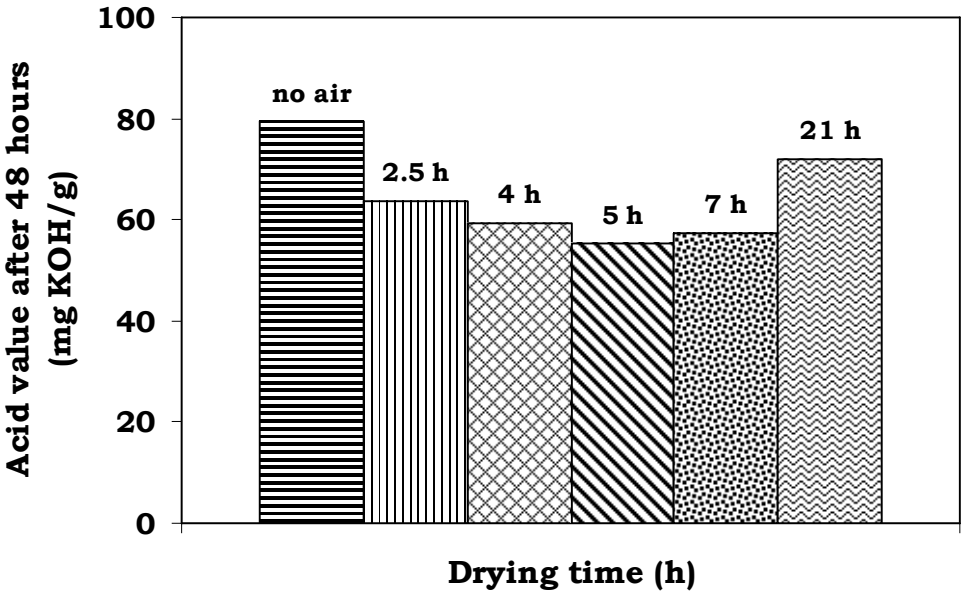

Table 1.- Operating conditions for the lipase-catalysed synthesis of ricinoleic acid estolides in the open air jacketed batch reactor.

Run	IME g	Water ppm	Temperature	Pressure
1	2.5	soaked	40	atmospheric
2	5	soaked	40	atmospheric
3	10	soaked	40	atmospheric
4	5	soaked	40	atmospheric
5	5	soaked	45	atmospheric
6	5	soaked	50	atmospheric
7	5	soaked	40	atmospheric
8	5	dry	40	atmospheric
9	5	+ 0.5 ml	40	atmospheric
10	5	+ 5 ml	40	atmospheric

Table 2.- Summary of results obtained in the experiments carried out to optimise drying time. The operation time has been 48 hours in all the assays.

Drying time (h)	**Δ(AV)/h (mg KOH/g h)**	**Δ[H_2O]/h (ppm/h)**
No air	1.81	403
3	1.94	1567
4	1.96	1689
5	2.08	1747
7	2.21	1787
9	2.10	1824
21	1.93	1912

CAPTION TO FIGURES

Figure 1.- Scheme of reaction for the synthesis of ricinoleic acid estolides.

Figure 2.- Influence of the amount of enzyme on the evolution of acid value with time for the PR synthesis in the open reactor.

Figure 3.- Change in acid value as a function of time for estolide synthesis performed at different temperatures in the open reactor.

Figure 4.- Influence of the initial water content on the evolution of acid value with time for the PR synthesis in the open reactor.

Figure 5.- Comparison of two experiments of PR synthesis carried out in different environmental relative humidity conditions.

Figure 6.- Influence of vacuum and dehydration with dry air on the evolution of acid value with time for the PR synthesis.

Figure 7.- Acid values obtained after 48 hours of PR synthesis in the vacuum reactor, at 160 mmHg, for different values of drying time.