BIOCATALYTIC SYNTHESIS OF POLYGLYCEROL POLYRICINOLEATE: A COMPARISON OF DIFFERENT COMMERCIAL LIPASES

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Department of Chemical Engineering.University of Murcia.30071 Murcia. Spain.

(*) Corresponding author.

Tel: +34 868 88 7219.Fax: +34 868 884148. E-mail: cmontiel@um.es.

ABSTRACT

In this work, the studies were carried out to select the most suitable lipase as catalyst for the esterification of polyglycerol with polyricinoleic acid to yield polyglicerol polyricinoleate (PGPR), a valued-added, biobased food emulsifier are described. The enzymes assayed were lipases from *Rhizopus arrhizus*, *Rhizopus oryzae* and *Mucor javanicus*, previously selected because of their suitable activity and moderate cost. First, reaction was catalyzed by free lipases in a batch reactor and the influence of different operating conditions (initial water content, amount of enzyme and temperature) on the progress of the reaction was studied. Next, the three lipases were immobilized by physical adsorption on the anion exchange resin, Lewatit MonoPlus MP 64, providing derivatives with a high activity and stability. Recovery of the immobilized derivative from the reaction medium was conducted with very good yields (≥ 99%) and no loss of activity of the derivative with successive uses was proved. Finally, a high performance reactor, which operates at low pressure and with a dry atmosphere, was used to synthesise PGPR using the immobilized enzymes. Both *Rhizopus arrhizus* and *Rhizopus oryzae* lipases allowed the production of a PGPR which fulfils the "specific purity criteria on food additives other than colours and sweeteners" established by the Commission of the European Communities (A.V. ≤ 6 mg KOH/g), with an acid value of 4.91 and 5.31 mg KOH/g respectively.

Keywords: Polyglycerol polyricinoleate, lipase, immobilized enzymes, heterogeneous reaction, enzyme biocatalysis, vacuum reactor.

LIST OF SYMBOL

AV - Acid Value (mg KOH/g).

EC – European Communities.

GPC – Gel Permeation Chromatography.

HV – Hydroxyl Value (mg KOH/g).

i.d. – Internal Diameter.

IV – Iodine Value (g I_2/100 g).

M_n – Number-averaged molecular weight (g/mol).

M_W – Weight-averaged molecular weight (g/mol).

PG-3 – Polyglicerol-3.

PGPR - Polyglicerol polyricinoleate.

ppm – parts per million (w/w)

PR - Polyricinoleic acid.

r – Polydispersity index (dimensionless).

1. Introduction.

Polyglycerol polyricinoleate (PGPR, E-476) is a powerful water-in-oil emulsifier [1-5] used to manufacture stable pan release agents for the bakery industry and to stabilize low fat margarine systems with a high water content. However, its main application is in the chocolate and confectionery industry[6, 7], since it improves the flow properties of chocolate and vegetable fat coatings by lowering the friction between the particles suspended in the liquid fat phase. In this way, the yield stress value is reduced and the liquid chocolate mass flows easily even at a low total fat content. Moreover, PGPR decreases the susceptibility of solidified chocolate to suffer fat bloom [8-10], a physical defect that appears during chocolate storage as a greyish-white film on the surface of the product. This has been a significant problem in chocolate ever since the industry began, since it leads to sensory defects.

Known methods for preparing this compound involve two steps: the autocatalytic condensation of ricinoleic acid (or castor oil fatty acids) and the alkali-catalyzed reaction between the condensed ricinoleic acid and polyglycerol to give polyglycerol polyricinoleic fatty acid esters[1]. However, these methods have the disadvantage of requiring very long reaction times and thus involve a large financial outlay and high energy costs. The long reaction times also adversely affect the quality of the final product, which presents problems of coloration and odour[11].

Enzymatic synthesis might be regarded an alternative for overcoming these problems, because, the literature shows enzymes act favourably in mild temperature and pressure conditions and at neutral pH. For this reason an enzymatic PGPR synthesis process is being developed by our research group using lipase (E.C.3.1.1.3) as catalyst. The enzymatic procedure consists of two steps. Firstly, the ricinoleic acid is polymerized by the action of

Candida rugosa lipase to obtain the estolide. The optimization of this reaction step was described in previous manuscripts [12-14]. Secondly, the obtained polyricinoleic acid (PR) is esterified with polyglycerol through the action of a different lipase (Figure 1). In a previous work, twenty-four lipases were tested to catalyze this second step and three of them were selected because of their good results and moderate cost: *Rhizopus arrhizus* lipase, *Rhizopus oryzae* lipase and *Mucor javanicus* lipase[15]. The objective of the present study was to compare the behaviour of *Rhizopus oryzae* and *Mucor javanicus* lipases with that of *Rhizopus arrhizus* lipase, published previously[16], in order to choose the most suitable for the production of PGPR, while complying with the "specific purity criteria on food additives other than colours and sweeteners" established by the Commission of the European Communities.

2. Materials and methods.

2.1. Enzymes and substrates.

Lipases from *Mucor javanicus* (10 units/mg solid), *Rhizopus arrhizus* (10 units/mg solid) and *Rhizopus oryzae* (≥30 units/mg solid) were purchased from Fluka. The ricinoleic acid estolide, also called polyricinoleic acid (PR)(with an acid value (AV) ≤50mg KOH/g, which corresponds to an average degree of polymerization of around 3.7, and with a weight-averaged molecular weight (M_W)≥1838 g/mol) was obtained by enzymatic polymerization of ricinoleic acid as described previously [12-14]. Polyglycerol-3 (PG-3), kindly provided by Solvay, is a glycerol oligomer based on an average of three glycerol groups (average MW= 246.66 g/mol). It contains a minimum of 80% di-, tri- and tetraglycerol and has a very low level of cyclic by-products. More information about polyglycerol-3 can be found in its product data sheet [17].

2.2. Immobilization support and reagents.

The anionic exchange resin Lewatit MonoPlus MP 64 (Fluka) was used as immobilization carrier. The soybean lecithin used as support activator was of commercial grade from Santiveri S.A., Spain. Other chemicals (acetic acid, sodium acetate, bovine serum albumin, sodium carbonate, sodium hydroxide, Folin-Ciocalteu reagent, potassium-sodium tartrate, cupric sulfate pentahydrate, potassium hydroxide, ether, ethanol, phenolphthalein, anhydride acetic, pyridine, sodium thiosulfate, starch wheat, chloroform, potassium dichromate, Wijs solution, potassium iodide, Hydranal® Composite 5, Hydranal® Methanol Dry and tetrahydrofuran) were of analytical grade and were used without further purification.

2.3. Immobilization by physical adsorption.

Enzymes were immobilized in an anion exchange resin (Lewatit Monoplus MP 64) following the immobilization protocol previously optimized and described[13]. Five grams of support were mixed with 50 mL of a soybean lecithin suspension in distilled water (20 mg/mL H_2O) in an Erlenmeyer flask and placed in an orbital shaker (120 rpm) overnight at room temperature. The activated support was washed with 50 mL of distilled water and then transferred to a jacketed column reactor (2.5 cm i.d. and 30 cm length) equipped with a sinterized glass plate placed 5cm from the bottom. The enzyme solution (50 mL, 10 mg/mL in 0.1 M acetate buffer, pH 5) was then added to the reactor and recirculated for 2 days at 4ºC. The immobilized derivative was washed twice with the same buffer and stored at 4ºC in the acetate buffer. The amount of protein initially offered and the protein remaining in the supernatant and wash-liquid was determined by Lowry's procedure, as modified by Hartree [18], using bovine serum albumin as standard. The amount of coupled protein was the difference between the amount of the initial protein added and the amount of protein in the supernatant and in the wash-liquid. The immobilisation yield describes the percentage of total coupled protein from the initial amount of protein.

2.4. Atmospheric reactor experiments.

The enzymatic reaction was carried out in an open-air glass-jacketed batch reactor (250 mL total volume). Complete mixing was achieved by means of a four-bladed propeller stirrer. The amount of polyricinoleic acid added in each experiment was 30 g, and the amount of polyglycerol was adjusted, depending on the polyricinoleic acid molecular weight, in order to keep the PR/PG molar ratio at around 3 (which means that approximately three of the five hydroxyl groups of the polyglycerol could be esterified). The relative humidity was about 40-50%. Unless otherwise stated, when the reaction was carried out with free lipase, 500mg of lipase (*Mucor javanicus*/*Rhizopus arrhizus*/*Rhizopus oryzae*) were dissolved in 5 mL of

137 distilled water and added to the reactor before the substrates. This implies a ratio of 10 µl
138 H_2O/mg enzyme in the reactor at the beginning of the reaction. To optimize the water content,
139 the initial amount of water added to the reactor was adjusted to obtain ratios of 1.5, 10 and 20
140 µl H_2O/mg enzyme. All the experiments of optimization of reaction conditions were carried
141 out by using free lipase. Additional experiments with immobilized lipase were conducted by
142 using 5 g of immobilized derivative and the only water in the reaction system was that soaked
143 into the support (0.6 g of water per g of dry resin). All these experiments were carried out at
144 40 ºC. When influence of temperature was studied, temperatures of 50 and 60 ºC were tested.
145
146 **2.5. Vacuum reactor experiments.**
147 For reactions carried out under controlled moisture conditions using a Parr 5100 series low
148 pressure reactor. The reaction vessel (100 mL total volume) is made of glass and is equipped
149 with a water circulating jacket to heat the vessel. The stainless steel reactor head
150 accommodates the reactor controls and instrumentation. The reactor is equipped with a
151 magnetic drive to provide an internal stirrer, which is a turbine-type impeller. The reactor top
152 also includes a vacuum meter, an internal thermocouple, an internal cooling loop, a rupture
153 disk, a liquid sample valve, a gas inlet valve and a gas release valve. Temperature, stirring
154 speed and positive pressure are managed by a controller. The amounts of polyricinoleic acid,
155 polyglycerol-3 and immobilized lipases in the reactor at the beginning of the reaction were the
156 same as those reported for the atmospheric jacketed batch reactor with immobilized lipases
157 (Section 2.4). All the experiments were carried out at 40ºC, and the stirring rate was kept
158 constant at 350 rpm. The pressure was set at 160 mmHg and dry air (90 l/h) was blown
159 through the reaction mixture to facilitate water removal. Relative humidity of the air flow was
160 reduced by passing atmospheric air through a silica gel column.
161

2.6. Recovery of the immobilized derivative.

When immobilized derivatives were tested for reusability, the reactor content was placed in a sinterized glass filter (Pyrex®, number 0) to separate the derivative from the product. After 8 h at room temperature, the immobilized derivative was placed in the reactor for a new reaction cycle without further purification.

2.7. Measurement of the acid value.

The acid value (AV) [19] is the number of milligrams of potassium hydroxide necessary to neutralize the free acids present in 1 g of sample. AV corresponds to the carboxyl-group concentration in the reaction mixture.

2.8. Measurement of the hydroxyl value.

The hydroxyl value (HV) is expressed as the milligrams of potassium hydroxide required to neutralize acetic acid coupled with the hydroxyl group of the substance when 1 g of sample is acetylated. It is determined by acetylating the hydroxyl group with acetic acid and titrating the remaining acid against KOH, following the standard method described in [19].

2.9. Determination of iodine value.

As regards the iodine value (IV), PGPR has to comply with the recommendations given in the Food Chemical Codex [19], i.e., IV = 70-90 $gI_2/100g$, as determined following the protocol described in the same Codex[19]. This parameter is a measure of unsaturation and guarantees its emulsifying properties. In this work it was evaluated as a check to confirm the absence of side reactions.

2.10. Refractive index.

The refractive index of the obtained PGPR was measured with an ABBE refractometer (2WAJ model, Optika, equipped with water recirculation and temperature control), at 65ºC according to the legal specification of the refractive index for this food additive [20].

2.11. Determination of water content.

The water content of reactor samples was measured with a Karl-Fischer automatic titrator (701 KF, Metrohm), using Hydranal® composite 5, from Riedel-De-Häen.

2.12. Determination of relative humidity.

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

2.13. Gel permeation chromatographic analysis.

Gel permeation chromatographic (GPC) analysis was carried out to determine the average molecular weight of products using a modular system from Waters, with an automatic injector model 717 PLUS and a 600 E quaternary-gradient pump. The system was equipped with a refractive index detector (model 2414) and a 7.8 mm id x 300 mm GPC Styragel® HR 1 THF column from Waters. The analysis was performed at 35ºC using THF as solvent at a flow rate of 1 mL/min. The calibration curve (logMW*vs*retention time), which is used to estimate the molecular weight of samples, was constructed using the retention times of the resolved peaks for the monomers, dimers, trimers, and tetramers of ricinoleic and polyricinoleic acid. The number- and weight- averaged molecular weight (M_n and M_w, respectively) were calculated by dividing the chromatogram area into trapezoids and using the formulae:

$$M_n = \frac{\sum A_i M_i}{\sum A_i} \quad (1) \qquad M_w = \frac{\sum A_i M_i^2}{\sum A_i M_i} \quad (2)$$

where the subscript *i* refers to the *ith* trapezoid of A_i area and M_i to the average molecular weight of this trapezoid. The M_w/M_nratio is the polydispersity index, PDI.

$$r = M_w / M_n$$

3. Results and discussion.

The reaction course in all the experiments was monitored by measuring the acid value, which decreased due to the esterification of polyricinoleic acid (AV≤50) by polyglycerol-3. According to the standard EC specifications of PGPR, this parameter has to be lower than 6 mgKOH/g.

3.1. Biocatalytic synthesis of polyglycerol polyricinoleate with free lipases.

Preliminary studies were carried out in the atmospheric reactor, in which several experiments were catalyzed by free lipases in order to optimize the water content, biocatalyst concentration and temperature. The effect of these operating variables was studied following the decrease in the Acid Value (AV) during the esterification reaction.

3.1.1.- Influence of initial water content.

The literature describes the important role of water on the catalytic activity of lipases, since water participates in all non-covalent interactions that maintain the conformation of the catalytic site of the enzymes [21-23]. However, in esterification reactions, the water produced in the reaction medium can negatively affect the equilibrium as well as the distribution of products in the medium. Therefore, optimization of the initial amount of water to be added to the reaction mixture was deemed necessary.

For this purpose, experiments were carried out for each assayed lipase (*Rhizopus arrhizus* ,*Rhizopus oryzae* and *Mucor javanicus)* varying the initial amount of water added to the reactor (1.5, 10 and 20 µl H_2O/mg enzyme), as indicated in Section 2.4. The progress of the reaction was quantified by AV measurements, and no differences were observed for the different initial amounts of water assayed with each lipase (Figure 2). In the three experiments

using *Rhizopus oryzae* lipase as catalyst, a decrease in the acid value of 17 mg KOH/g in 24 h was observed, while the corresponding decreases for *Rhizopus arrhizus* lipase and *Mucor javanicus* lipase were 21 mg KOH/g and 15 mg KOH/g respectively. Taking into account these results, it can be stated that the activity of each lipase is independent of the amount of water initially added. However, the acid value of the PGPR obtained is still far from the EC specification.

As described in previous works [14, 16], relative humidity influences the equilibrium of this esterification process. In this case, additional experiments conducted with different values of relative humidity (from 25% to 80%) showed that lower relative humidity values led to higher acid values decreased, and the differences observed were about 25%, in the same controlled-experimental conditions (data not shown).

As a consequence and to avoid this effect, all the experiments to optimize an individual variable were carried out simultaneously, at a relative humidity of 40-50%.

3.1.2.- Influence of the amount of enzyme.

For the synthesis of polyglycerol polyricinoleate, the influence of different amounts of added lipase on the esterification reaction was studied in five experiments using 1.39, 2.78, 6.94, 13.89 and 27.28 mg enzyme/g substrate. The results obtained are shown in Figure 3.

In the case of *Rhizopus arrhizus* lipase, small differences in the final acid value can be seen between experiments carried out with enzyme/substrate ratios lower than 6.94 mg enzyme/g substrate. However, no differences were noticed when higher ratios were studied; so that 6.94 mg enzyme/g substrate can be considered as optimal ratio. In regards to *Mucor javanicus*

lipase and *Rhizopus oryzae* lipase it can be said that the optimal ratio is 13.89 mg enzyme/g substrate.

3.1.3.- Influence of the temperature.

The temperature influences the enzymatic reaction rate, enzyme stability, the velocity of water evaporation from the reaction medium and its viscosity. Consequently, the effect of temperature on the reaction course was investigated, by carrying out experiments at three different temperatures: 40, 50 and 60ºC. The results obtained are shown in Figure 4.

First, it can be seen that 60°C is not a suitable temperature for carrying out the esterification of polyglycerol with polyricinoleic acid catalyzed by *Mucor javanicus* lipase or *Rhizopus arrhizus* lipase[16], because the reaction progressed slowly and the acid value reached was quite high. These results clearly show the thermal deactivation of these lipases due to their denaturation. It is well known that most proteins tend to denaturalize at temperatures above 50ºC. The most common cause for the inactivation of enzymes at high temperatures is loss of the native, catalytically active conformation through thermodenaturation. In contrast, the higher the temperature used with *Rhizopus oryzae* lipase, the lower the acid value obtained, indicating that this enzyme is more thermostable. These results demonstrate differences in the thermal stability of the lipases studied. Thus, lipases from *Rhizopus arrhizus* and *Mucor javanicus* should not be used at temperatures above 50°C, because higher temperatures provoke the thermal deactivation of these lipases and a decrease of catalytic activity. However, *Rhizopus oryzae* lipase is still active at 60°C, and can be used at this temperature.

The bibliography contains different references to the optimum operating temperature for each of the biocatalysts used. For *Rhizopus arrhizus* lipase, operating temperatures of 30-40°C, with maximum activity at 35°C,have been mentioned [24, 25]. However, in our case, using a

291 temperature below 40ºC would be unsatisfactory, because of the negative effect on the
292 viscosity of the reaction medium[14]. For the lipase from *Mucor javanicus*, a temperature of
293 50ºC was found to be optimal [26]. For both the above lipases the results obtained in our study
294 are very similar to those reported in the literature. However, in the case of *Rhizopus oryzae*
295 lipase, the optimum temperatures of 40ºC [27] and 37ºC [28]and drastic decrease in activity
296 when used above 50ºC reported by other authors, differ from the results obtained here.
297
298 The use of relatively high temperatures in this process may lead to the appearance of side
299 products due to the degradation of fatty acids. Therefore, the products obtained in
300 experiments at different temperatures were analysed to determine their degree of
301 unsaturation[19]. The iodine values corresponding to the end products, such as those calculated
302 theoretically from substrates values, are shown in Table 1. The differences observed between
303 the experimental and theoretical values obtained for this parameter suggested that 60ºC
304 caused the partial degradation of the double bond of the fatty acid so that this temperature
305 must be considered unsuitable in the experimental conditions used in these assays. Given that
306 the presence of air can strongly affect degradation, this problem could be avoided by using a
307 sealed reactor with an inert atmosphere. Since lower temperatures decreased the tendency to
308 develop side reactions, 40ºC was considered a suitable temperature for further investigations.
309 In addition, from the standpoint of its industrial application, this temperature would be a
310 better choice because it implies lower energy costs and simplification of the process, since
311 this is the temperature used in polyricinoleic acid production, the first stage of the process.
312
313 Comparing processes catalyzed by these three free lipases in an atmospheric reactor at the
314 selected temperature (40ºC), no significant differences were found. However, *Rhizopus*

315 *arrhizus* and *Rhizopus oryzae* lipases were slightly better catalysts than *Mucor javanicus*
316 lipase.
317
318 **3.2. Immobilization of lipases by physical adsorption.**
319 Based in the study carried out with the free lipases, operational conditions (initial amount of
320 water, amount of lipase and temperature) were optimized, but the final product obtained was
321 far from the EC specification concerning the acid value. In previous works on the synthesis of
322 ricinoleic acid estolide [12-14] and the screening of lipases for PGPR production [15], it was
323 demonstrated that the use of immobilized lipases improves the results and can be considered
324 advantageous because it permits continuous operation of the reactors and/or reusability of the
325 immobilized enzymes, both of which diminish operational costs. Therefore, the three chosen
326 lipases were immobilized by physical adsorption onto an anion exchange resin (Lewatit
327 Monoplus MP 64), as described in Section 2.3. The immobilization of *Mucor javanicus* and
328 *Rhizopus oryzae* lipase led to higher yields and enzyme loading than were obtained with
329 *Rhizopus arrhizus* lipase[15].
330
331 To study the activity of the immobilized lipases, experiments catalyzed by free and
332 immobilized lipases were carried out under the same reaction conditions, using the same
333 amount of total protein as catalyst; that is, one experiment was performed with the
334 immobilized derivative of each lipase and the other was carried out with an amount of protein
335 equivalent to that adsorbed on the support, dissolved in 3 mL of buffer, a volume that
336 corresponds to the volume of water soaked into the 5 g of support.
337
338 As can be seen in Figure 5, both immobilized and free lipases from *Rhizopus oryzae* and
339 *Rhizopus arrhizus*[16] produced the same decrease in the acid value. However, when

340 immobilized *Mucor javanicus* lipase was used, a lower final acid value was reached than with
341 free lipase, which reflects an increase in stability. Variations in enzyme stability as well as in
342 such properties as activity and selectivity as a result of immobilization are described in the
343 bibliography [29, 30].
344
345 **3.3. Re-use of immobilized derivatives.**
346 To further examine the effect of immobilization on enzyme stability and to determine the
347 possibility of reusing the immobilized derivative, three successive PGPR synthesis
348 experiments catalyzed by each of the immobilized derivatives were performed, maintaining
349 the conditions constant in the three experiments. The biocatalysts were recovered from
350 products as described in Section 2.6. The results show that all three immobilized derivatives
351 studied were very stable, with no substantial loss of activity, and almost the same change in
352 the acid value was obtained for the three runs of each lipase (Figure 6). Moreover, since no
353 buffer was added before re-use, the amount of water that remains in the support can be
354 considered sufficient to maintain the protein catalytically active.
355
356 **3.4. Vacuum reactor experiments.**
357 As mentioned above, the European Commission Directive 2008/84/EC [20] establishes as a
358 requirement for PGPR an acid value lower than 6mg KOH/g. In all the experiments described
359 above, the final acid value was far from this objective and, as a consequence, it was necessary
360 to shift the equilibrium towards the esterification pathway by improving the removal of water
361 from the reaction medium.
362
363 On the other hand, it was observed that environmental relative humidity played an important
364 role in the enzymatic synthesis of PGPR in the atmospheric reactor and, as this parameter

takes on a wide range of values depending on the air-conditioned system used, it was considered to be the main cause of the poor reproducibility obtained in the atmospheric reactor. To solve this problem and to compare these three immobilized lipases (*Mucor javanicus*, *Rhizopus arrhizus* and *Rhizopus oryzae* lipase) more accurately, PGPR was synthesized under a controlled atmosphere in a vacuum reactor, in which, the amount of water in the reaction medium can be controlled and reduced through pressure and the entry of dry air, rendering it independent of environmental conditions. Measurements of the water content in the reaction medium showed that it decreased as the reaction progressed, reaching a final value of about 1300-1900 ppm.

As can be seen from Figure 7, the higher final acid value was obtained when using *Mucor javanicus* lipase as biocatalyst; furthermore, that value was far from that specified by EC (≤6 mg KOH/g). However, both *Rhizopus arrhizus*[16] and *Rhizopus oryzae* lipases were able to catalyze the reaction to give acid values lower than 6 mg KOH/g. Therefore, both of them can be considered suitable for obtaining PGPR and the yield of the reaction, calculated from the initial and final acid value, was higher than 90%. The main difference between these two enzymes lies in the reaction time required to reach the lowest acid value: around 100 h in the case of *Rhizopus arrhizus* lipase and around 225 h in the case of *Rhizopus oryzae* lipase. However, as regards the cost, lipase from *Rhizopus arrhizus* costs 10 times more than lipase from *Rhizopus oryzae,* which would mean a high impact on the overall process costs.

Additional studies with *Rhizopus oryzae* lipase were carried out to check the possibility of shortening the reaction times by increasing the loading of the immobilized derivative. An immobilized derivative was obtained from a 50 mg/mL enzyme solution, and the immobilization yield was 67.62%, the derivative containing 39.4 mg protein/g support. PGPR

production was carried out in the vacuum reactor and the results were compared with those obtained in the reaction carried out using an immobilized derivative with 16.36 mg protein/g of support, described above. Figure 8 shows the evolution of the acid value for both experiments, and points to a notable reduction in the reaction time needed. In addition, if we compare these results with those obtained with *Rhizopus arrhizus* lipase[16] (Figure 7), it is seen that the process catalyzed by *Rhizopus oryzae* lipase is economically more feasible than using *Rhizopus arrhizus* lipase. Since the reaction time using the high-loading derivative of *Rhizopus oryzae* lipase was slightly shorter than the reaction time for *Rhizopus arrhizus* lipase, the enzyme consumption was five times higher, but the price per mg of enzyme was ten times lower, so the overall cost was still lower.

3.5. Characterization of PGPR obtained using lipases from *Rhizopus arrhizus* and *Rhizopus oryzae* as catalysts.

From the study developed until now, it can be concluded that both *Rhizopus arrhizus* lipase[16] and *Rhizopus oryzae* lipase are suitable enzymes for catalyzing the esterification of polyglycerol with polyricinoleic acid to yield a PGPR which complies with European specifications concerning the acid value. Therefore, the complete characterization of these products was carried out in order to prove the purity and quality of the PGPR obtained.

As a food additive, PGPR has to comply with several purity specifications, besides its acid value, that are set in European directives. These purity specifications, as well as the results obtained in the characterization of the final products synthesized are shown in Table 2. As can be seen, the PGPR obtained from the esterification of polyricinoleic acid with polyglycerol catalyzed with *Rhizopus arrhizus* lipase or with *Rhizopus oryzae* complied with these

414 specifications, demonstrating that the enzymatic process developed in this research work is
415 suitable for producing PGPR for use as food additive.
416
417 GPC analyses demonstrated that the PGPR obtained using these lipases had an almost
418 identical composition, a low level of low molecular weight compounds and high purity. From
419 the values of the weight-averaged molecular weight and taking into account the molecular
420 weight of polyricinoleic acid, it can be established that the final product was a mixture of
421 mono and di-esters, in which polyricinoleic acid is attached to –OH end groups of
422 polyglycerol due to the selectivity of the lipases used. The polydispersity indexes were 1.11
423 and 1.13, respectively, values close to unity, confirming the high uniformity of the obtained
424 products.
425

4. Conclusions.

This contribution describes the studies carried out to select the most suitable lipase for catalysing the esterification of polyglycerol with polyricinoleic acid to yield polyglycerol polyricinoleate, a valued-added, biobased food emulsifier. The enzymes assayed were lipases from *Rhizopus arrhizus*, *Rhizopus oryzae* and *Mucor javanicus*, previously selected because of their proved suitability to catalyze the studied reaction and their moderate cost. Experiments carried out in the atmospheric reactor and using free lipases demonstrated that *Rhizopus oryzae* lipase was the most thermostable one, reaching lower acid value in shorter reaction time when used at 60ºC. *Mucor javanicus* lipase had an optimum temperature of 50ºC, while *Rhizopus arrhizus* lipase behaved identically at 40º and 50ºC, being unable to catalyze this reaction at 60ºC. *Rhizopus arrhizus* lipase showed higher specific activity because similar acid values were reached using lower amounts of biocatalyst. Immobilization yields were higher for *Mucor javanicus* lipase and *Rhizopus oryzae* lipase. However, the best acid value results were obtained for *Rhizopus arrhizus* lipase even though its immobilization yield was the lowest.

When the process was catalyzed by immobilized *Rhizopus arrhizus* or *Rhizopus oryzae* lipases in a high performance reactor, the obtained PGPR complies with the purity criteria set by the EC. It is worth mentioning that the acid value decreased faster when the biocatalyst was *Rhizopus arrhizus* lipase. However, using an immobilized derivative of *Rhizopus oryzae* lipase with a higher enzyme loading, the reaction time was shortened. Despite the higher biocatalyst consumption, the overall cost for using *Rhizopus oryzae* lipase instead of *Rhizopus arrhizus* lipase was still lower due to its lower price.

GPC analysis, the number- and weight- averaged molecular weights determined and the polydispersity index indicated the high uniformity and purity of the obtained products, which contained a low concentration of low molecular weight compounds.

454 **ACKNOWLEDGEMENTS**

455

456 This study was partially supported by grant CTQ2011-24091, MICINN, Spain.

457

458 **REFERENCES**

459

460 [1] Wilson, R., Van Shie, B.J., Howes, D.,Food Chem. Technol. 36 (1998) 711-718.

461 [2] Velde, F. van de, Weinbreck, F., Edelman, M.W., Linden, E. van der, Tromp, R.H.,
462 Colloid. Surface. B 31 (2003) 159–168.

463 [3] Kobayashi, I., Lou, X., Mukataka, S., Nakajima, M, J. Am. Oil Chem. Soc. 82 (2005),
464 65-71.

465 [4] Su, J., Flanagan, J., Hemar, Y., Singh, H, Food Hydrocolloid. 20 (2006) 261–268.

466 [5] Surh, J., Vladisavljevic, G.T., Mun, S., McClements, D.J. J.Agr. Food Chem. 55
467 (2007) 175-184.

468 [6] Emulsifiers for chocolate and confectionery. PGPR.

469 http://www.palsgaard.com/products/confectionery/pgpr/

470 [7] Kaiser, J.M., Van Gestel, A.M.J.C., Vercauteren, J.A. (Mars, Inc) EP 1069831, 14
471 May 2003.

472 [8] Polyglycerols in Food Applications. Application Data Sheet.

473 http://www.solvaychemicals.com/Chemicals%20Literature%20Documents/Polyglycer
474 ols/APP-2800_food_x3.pdf, 2013.

475 [9] Peschar, R., Pop, M.M., Ridder, D.J.A. de, Van Mechelen, J.B., Driessen, R.A.J.,
476 Schenk, H., J. Phys. Chem. B 108 (2004) 15450-15453.

477 [10] Schenk, H., Peschar, R., Radiat. Phys. Chem.71 (2004) 829-835.

478 [11] Denecke, P., Börner, G., Von Allmen, V., 1981. UK Patent 2073232.

479 [12] Bódalo, A., Bastida, J., Máximo, M.F., Montiel, M.C., Murcia, M.D., Biochem. Eng.
480 J. 26 (2005)155-158.

[13] Bódalo, A., Bastida, J., Máximo, M.F., Montiel, M.C., Gómez, M., Murcia, M.D., Biochem. Eng. 39 (2008) 450–456.

[14] Bódalo, A., Bastida, J., Máximo, M.F., Montiel, M.C., Murcia, M.D., Ortega, S., Biochem. Eng. J. 44 (2009) 214–219.

[15] Bódalo, A., Bastida, J., Máximo, M.F., Montiel, M.C., Gómez, M., Ortega, S., Biochem. Eng. J.46 (2009)217–222.

[16] Gómez, J.L., Bastida, J., Máximo, M.F., Montiel, M.C., Murcia, M.D., Ortega, S., Biochem. Eng. J. 54 (2011) 111-116.

[17] Polyglycerol-3. Product Data Sheet. http://www.solvaychemicals.com/Chemicals%20Literature%20Documents/Polyglycerols/PDS-2820-0007-W-EN_WW_.pdf, 2013.

[18] Hartree, E.F., Anal. Biochem. 42 (1972) 422-427.

[19] Committee on Food Chemicals Codex, 2012. Food Chemicals Codex, 8th ed. The United States Pharmacopeial Convention, Rockville, MD.

[20] Commission Directive 2008/84/EC of 27 August 2008 laying down specific purity criteria on food additives other than colours and sweeteners [Official Journal L253/1, 20.9.2008].

[21] Dang, H.T., Obiri, O., Hayes, D.G., J. Am. Oil Chem. Soc. 82 (2005) 487–493.

[22] Goldberg, M., Thomas, D., Legoy, M.D., Eur. J. Biochem. 190 (1990) 603–609.

[23] Yahya, A.R.M., Anderson, W.A., Moo-Young, M., Enzyme Microb. Tech. 23 (1998) 438–450.

[24] Niu, W.N., Li, Z.P., Zhang, D.W., Yu, M.R., Tan, T.W., J. Mol. Catal. B –Enzym. 43 (2006) 33-39.

504 [25] Dobrev, G., Zhekova, B., Nedelcheva, P., Chochkov, R., Krastanov, A., Biotechnol.
505 Biotec. Eq. 25 (2011) 2295-2300.
506 [26] Liu, B., Quian, X., Wu, Q., Lin, X., Enzyme Microb. Tech. 43 (2008) 375-380.
507 [27] Méndez, J.J., Canela, R., Torres, M., Acta biológica colombiana 14 (2009) 161-172.
508 [28] Razak, C.N.A., Musani, R., Basri, M., Salleh, A.B., J. Am. Oil Chem. Soc. 76 (1999)
509 171-174.
510 [29] Gianfreda, L., Scarfi, M.R., Mol. Cell. Biochem. 109 (1991) 97-128.
511 [30] Klibanov, A.M., Anal.Biochem., 93 (1979) 1-25.
512

513 **Table 1.** Experimental and calculated iodine values corresponding to products
514 obtained at different temperatures (Fig. 4) using three lipases (*Mucor*
515 *javanicus*, *Rhizopus arrhizus, Rhizopus oryzae*).
516

	***M. javanicus* lipase**		***R. arrhizus* lipase**		***R. oryzae* lipase**	
T^a **(ºC)**	$I.V._{theor}$	$I.V._{exp}$	$I.V._{theor}$	$I.V._{exp}$	$I.V._{theor}$	$I.V._{exp}$
40	73.8	71.9	73.8	71.6	73.8	72.3
50	73.8	70.2	73.8	70.6	73.8	69.8
60	73.8	53.7	73.8	50.3	73.8	52.5

517 $I.V._{theor}$: Iodine value calculated from iodine values of reagents.
518 $I.V._{exp}$: Iodine value determinedanalytically.
519

520 **Table 2.-** Characterization of polyglycerol polyricinoleate obtained in the vacuum reactor
521 with immobilized lipases from *Rhizopus arrhizus* and *Rhizopus oryzae*.
522 Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; temperature,
523 40ºC; catalyst, 5 g of the immobilized derivative and the amount of water soaked
524 into the support (3 ml); pressure, 160 mmHg; dry air flow, 90 l/h.
525

	EC specification	***Rhizopus arrhizus* lipase**	***Rhizopus oryzae* lipase**
Acid Value (mg KOH/g)	< 6	4.91	5.31
Hydroxyl Value (mg KOH/g)	80-100	90.47	89.16
Iodine Value (g I_2/100 g)	70-90	74.35	74.96
Refractive Index	1.4630-1.4665	1.4655	1.4655
M_n (g/mol)	-	2879	2728
M_w (g/mol)	-	3242	3016
PDI (dimensionless)	-	1.13	1.11

526
527

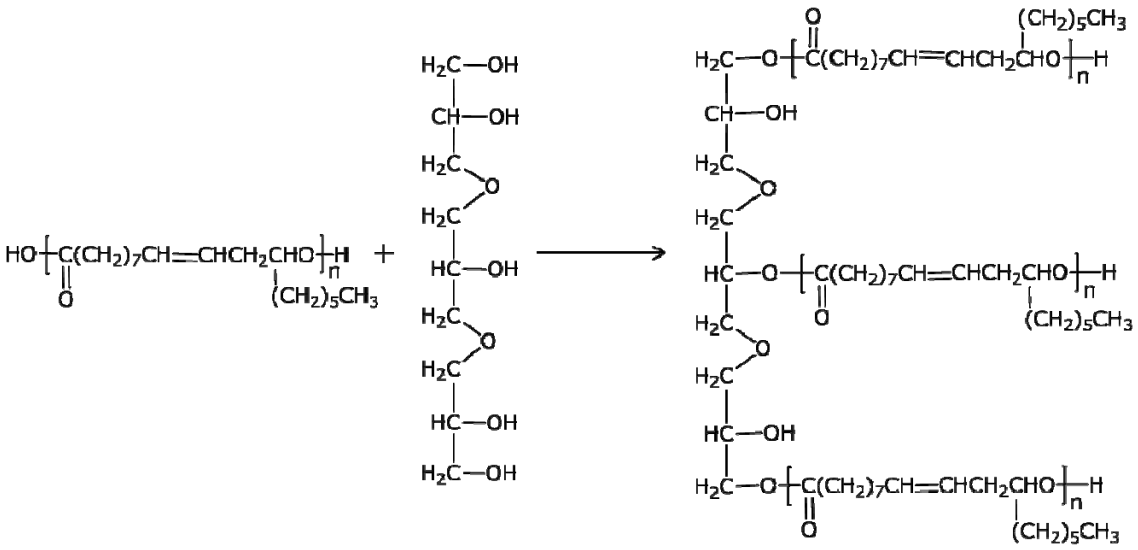

Figure 1

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

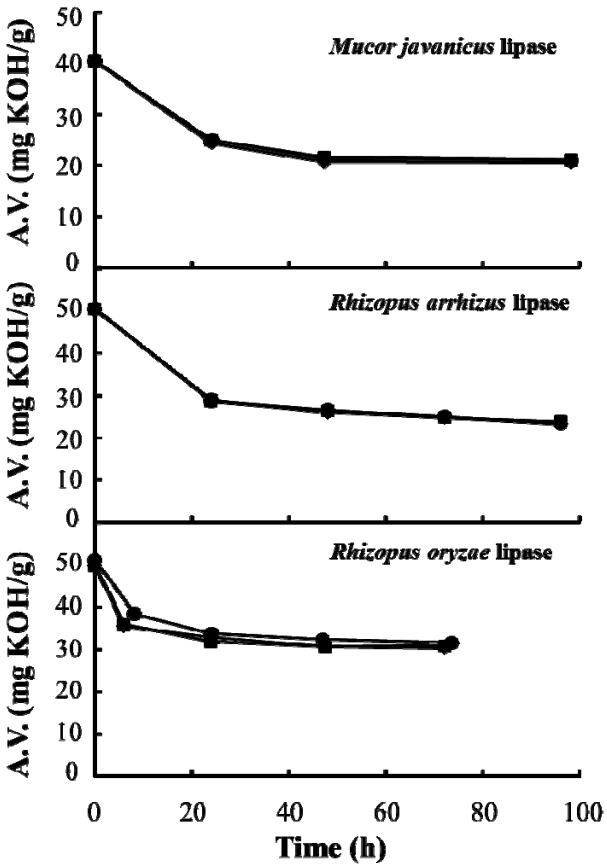

Figure 2

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

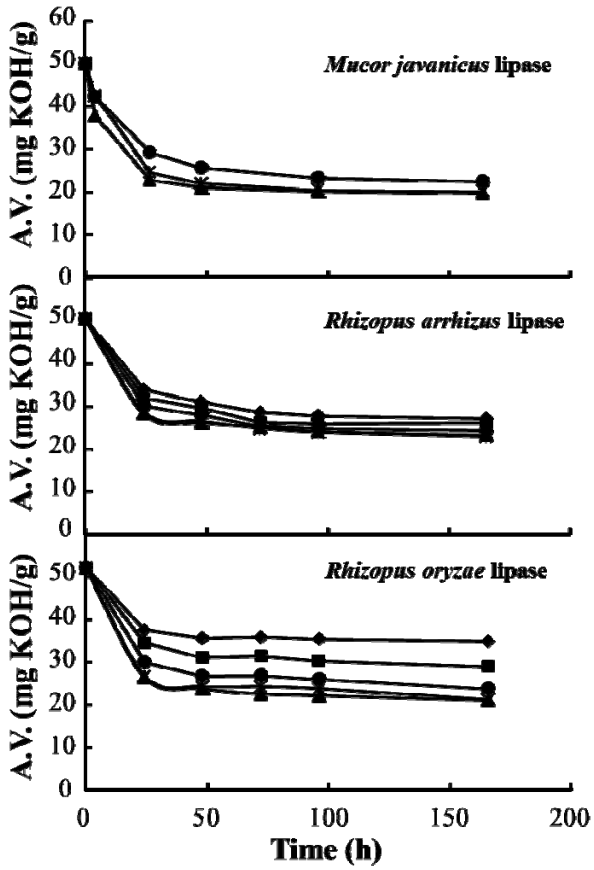

Figure 3

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

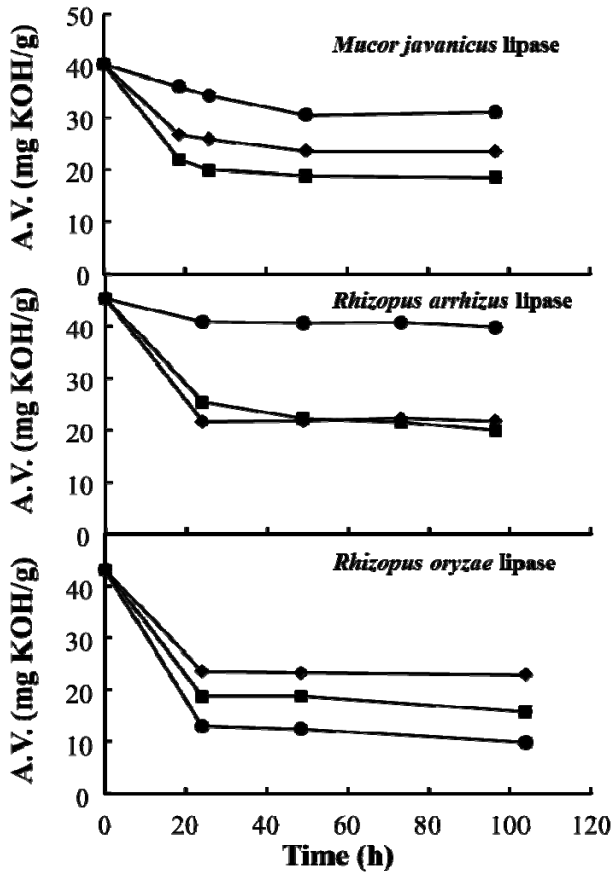

Figure 4

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

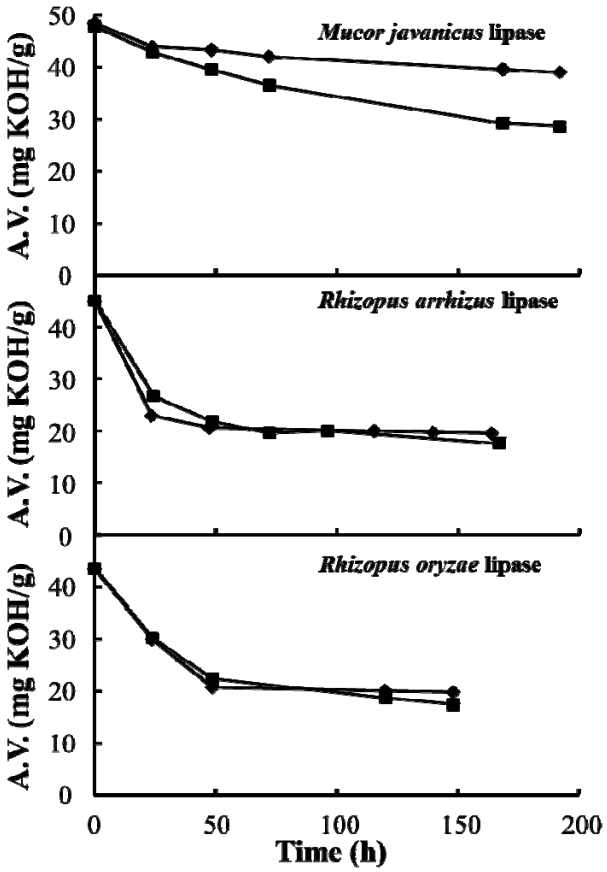

Figure 5

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

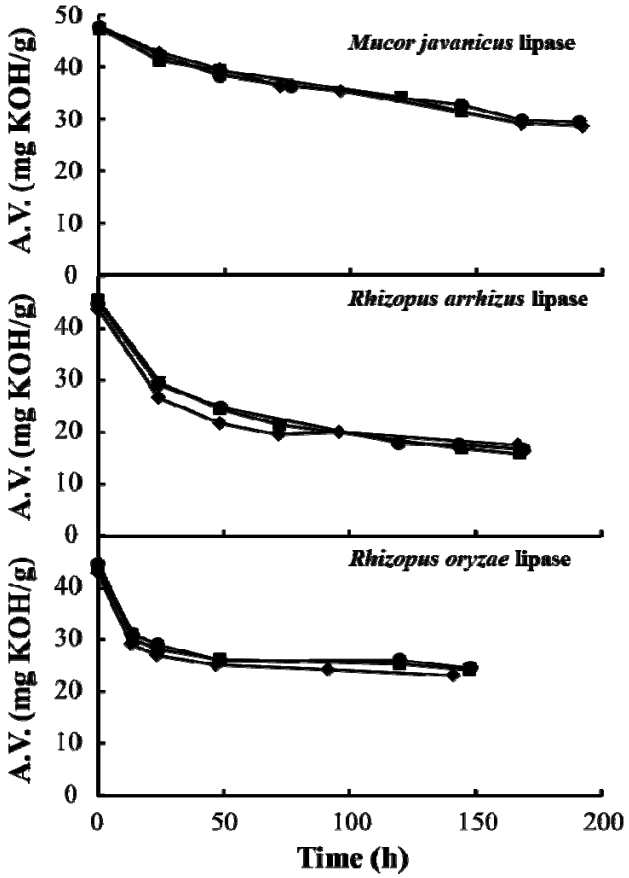

Figure 6

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

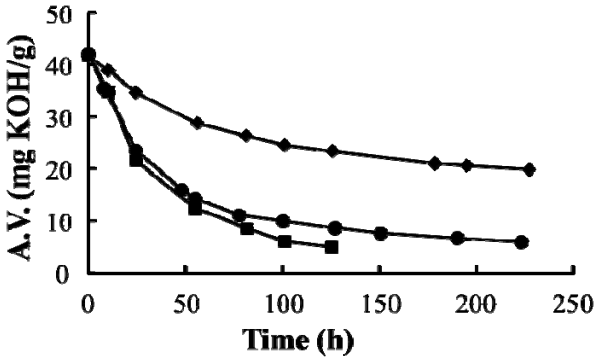

Figure 7

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

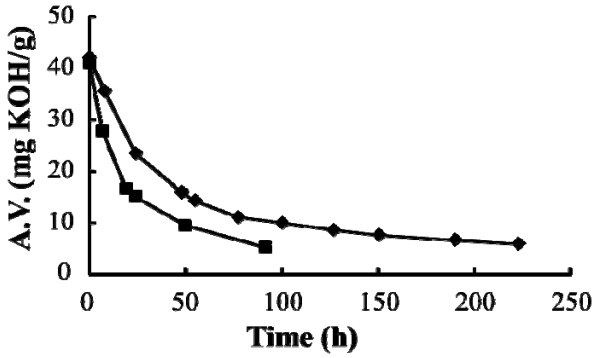

Figure 8

Ortega S, Gómez JL, Bastida J, Máximo MF, Montiel MC*, Gómez M.

Biocatalytic synthesis of polyglycerol polyricinoleate: a comparison of different commercial lipases.

Figure 1. Reaction scheme of the synthesis of polyglycerol polyricinoleate (PGPR)

Figure 2. Change in acid value as a function of time for the esterification of polyglycerol with polyricinoleic acid in an open air reactor using different free lipases, with different amounts of initial water added. Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; temperature = 40ºC; enzyme, 0.5 g. (♦) 1.5 µl H_2O/mg enzyme, (■) 10 µl H_2O/mg enzyme, (●) 20 µl H_2O/mg enzyme.

Figure 3. Influence of the amount of biocatalyst on the acid value in the esterification of polyglycerol with polyricinoleic acid in an open air reactor using different free lipases. Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; temperature = 40ºC; enzyme, (♦) 1.39 mg enzyme/g, (■) 2.78 mg enzyme/g, (●) 6.94 mg enzyme/g, (∗) 13.89 mg enzyme/g, (▲) 27.28 mg enzyme/g; initial amount of water, 10 µl H_2O/mg enzyme.

Figure 4. Change in acid value as a function of time in the esterification of polyglycerol with polyricinoleic acid in an open air reactor using different free lipases at different temperatures. Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; enzyme, 0.5 g dissolved in 5 ml of water. (♦) 40ºC, (■) 50ºC, (●) 60ºC.

Figure 5. Change in acid value as a function of time in the esterification of polyglycerol with polyricinoleic acid performed by free and immobilized

lipases, in an open air reactor. Experimental conditions: reaction mixture, molar ratio PR/PG ≈3; temperature, 40ºC; catalyst (♦) 0.5 g of free lipase dissolved in 5 ml of distilled water, (■) 5 g of immobilized derivative and the amount of water soaked into the support (3 ml).

Figure 6. Acid-value results obtained for esterification of polyglycerol with polyricinoleic acid catalyzed by new and re-used immobilized lipases. Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; temperature, 40ºC; catalyst, 5 g of the immobilized derivative and the amount of water soaked into the support (3 ml). Reaction cycle: (♦) "New immobilized derivative", (■) First re-use, (●) Second re-use.

Figure 7. Comparison of esterification of polyglycerol with polyricinoleic acid in a vacuum reactor, catalyzed by different immobilized lipases. Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; temperature, 40ºC; catalyst, 5 g of the immobilized derivative from (♦) *Mucor javanicus* lipase, (■) *Rhizopus arrhizus* lipase, (●) *Rhizopus oryzae* lipase and the amount of water soaked into the support (3 ml); pressure, 160 mmHg; dry air flow, 90 l/h.

Figure 8. Evolution of acid value in the esterification of polyglycerol with polyricinoleic acid in the vacuum reactor, using different enzyme-loading derivatives of *Rhizopus oryzae* lipase. Experimental conditions: reaction mixture, molar ratio PR/PG ≈ 3; temperature, 40ºC; pressure, 160 mmHg; dry air flow, 90 l/h; catalyst, 5 g of the immobilized derivative (♦) 16.36

112 mg protein/g support, (■) 39.4 mg protein/g support and the amount of
113 water soaked into the support (3 ml).