Biocatalytic Synthesis of Polymeric Esters Used as Emulsifiers

S. Ortega-Requena, M. Serrano-Arnaldos, M. C. Montiel, F. Máximo, J. Bastida, and M. D. Murcia*

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

*Corresponding author. Tel.: +34868889101; fax: +34868884148

md.murcia@um.es

ABSTRACT

Polyglycerol polyricinoleate (PGPR) is a polymeric ester widely used as emulsifier in the food industry. In this work, PGPR biocatalytic synthesis was carried out in a one-step solvent-free enzymatic process using lipase CALB immobilized in Lewatit®MonoplusMP 64 by adsorption. The optimal immobilization conditions were determined: initial enzyme concentration of 13 mg of Lowry protein per mL phosphate buffer pH 7, and ricinoleic acid as a support activator. An immobilized derivative with 35.93 ± 4.90 mg of Lowry protein per g of dry support was obtained. It was used as a catalyst for PGPR production in open air and vacuum batch reactors, and the results obtained showed that only when the reaction equilibrium was shifted towards ester production by means of water removal, the PGPR produced fulfilled the European legislation (acid value ≤ 6 mg of KOH per g of product).

Keywords

biocatalysis, enzymes, esterification, immobilization, lipases

INTRODUCTION

Emulsifiers are molecules that have a polar (hydrophilic) and a nonpolar (hydrophobic) part that are used in creams, sauces, and lotions formulation to keep the W/O or O/W emulsions stable and thus they have wide applicability in the cosmetic and food industries. Typical emulsifiers are usually fatty acid esters, such as sucrose esters or polyglycerol esters. Among them, the strongly lipophilic emulsifier polyglycerol polyricinoleate is highlighted (PGPR, E-476). This compound is a clear and highly viscous liquid, insoluble in ethanol and soluble in fats and oils, and is used as a viscosity reducing agent for chocolate.[1]

Currently, PGPR is produced by chemical methods that involve a reaction in two stages: the autocatalytic condensation of ricinoleic acid, and the esterification between polyricinoleic acid and polyglycerol.[2] This method of synthesis has many disadvantages, the main one being the long reaction time required, which implies high energy costs. This fact, together with the high operating temperature, can negatively affect the final product quality causing problems related to colour and odour, which makes it unsuitable for use in the food industry.[3]

As an alternative, the authors of this article proposed the PGPR biotechnological production using lipases from different sources as catalysts, which, acting under mild operating conditions, provide a final product much more suitable for use in the food industry. Firstly, a promising process in two stages was proposed, where each of the steps was catalysed by a different lipase.[4-9] In addition, the fact that the reaction takes place in the absence of solvents ("solvent free") makes this process highly attractive

within the field of “green chemistry”. This process also offers significant savings in production costs, allows working with small volumes, and improves separation operations. On the other hand, the use of two different biocatalysts is a disadvantage to possible future industrial application. For this reason, in other works, the polymeric ester biocatalytic synthesis was carried out in a single step using the two lipases co-immobilized[10] or using the widely known immobilized *Candida antarctica* lipase B (CALB) commercially known as Novozym® 435.[11] Although in both cases good results were obtained, the results obtained with Novozym® 435 were significantly better, requiring only 55 h of reaction to produce PGPR that fulfils the strict specifications of the European Commission regulation,[12] which states that CALB is a non-specific lipase capable of catalysing the two stages of the reaction synthesis. However, when using Novozym® 435, it is necessary to centrifuge the preparation in order to separate the immobilized enzyme from the product, due to the low density of the biocatalyst (0.4 g cm^{-3}).[11,13]

In order to solve this problem, lipase CALB derivatives immobilized on different kinds of supports are needed. In literature, several studies describe the immobilization of lipase CALB by covalent attachment on core-shell magnetic nanoparticles[14] or on pore-expanded SBA-15.[15] But most of the available papers in the literature have studied the immobilization of lipase CALB by adsorption on different supports, such as silica,[16-19] chitosan[20] or kaolin.[21]

In the present work, lipase CALB was immobilized by adsorption on different supports with adequate physical characteristics to improve the separation operations. The obtained immobilized derivative was used in the biocatalytic synthesis of the polymeric

ester PGPR in a single step solvent-free system, and operating in a controlled atmosphere of dry N_2 and vacuum. In these conditions, the obtained PGPR fulfilled the European legislation. Besides, with the purpose of its possible application on an industrial scale, the reuse of immobilized derivative in a different number of cycles has been studied.

Fig. 1 depicts the PGPR biocatalytic synthesis reaction scheme, where it can be observed that several hydroxyl groups can be esterified in the final product because CALB is a non-specific lipase that can esterify both primary and secondary alcohols. Moreover, the condensed ricinoleic acid chains length is also variable.

Materials and methods

Chemicals

Lipozyme® CALB L (liquid solution of *Candida antarctica* lipase B, 26 mg of Lowry protein per mL) was kindly provided by Novozymes Spain S.A. Immobilization supports were Lewatit® MonoPlus MP 64, Lewatit® Monoplus MP 500, and Dowex 50x8, all from Fluka. The substrates, ricinoleic acid (~80 %), and polyglycerol-3 were supplied by Fluka and kindly gifted by Solvay, respectively. The activators used in the immobilization, soybean lecithin of commercial grade and oleic acid were supplied by Santiveri and Fluka, respectively.

Immobilization of Lipozyme® CALB L

A standard immobilization procedure for all the assayed supports was carried out following these steps. Firstly, for the support activation (when necessary) 5 g of support were mixed with 50 mL of a 20 mg mL^{-1} activator suspension, and stirred at 120 rpm in

an orbital shaker for 24 hours. Secondly, in the immobilization procedure the activated support was transferred to a jacketed column reactor (2.5 cm i.d. and 30 cm length), provided with a sintered glass plate placed 5 cm from the bottom. The support was then washed twice with 25 mL of deionized water. The corresponding volume of Lipozyme® CALB L and buffer (total volume 25 mL) was added to the support and circulated for 48 h at 4 °C. The supernatant solution was recovered and the immobilized derivative was rinsed twice with 25 mL of the same buffer in order to remove unbound enzyme. Finally, it was washed twice with 12.5 mL of acetone, air dried, and stored at 4 °C.

The amount of immobilized protein was determined from the difference between the protein in the enzymatic solution and that in the supernatant and washings. The protein quantification was made by Lowry's method.[22]

Enzymatic synthesis of PGPR

To carry out PGPR synthesis, both substrates (30 g of ricinoleic acid and 2.5 g of polyglycerol-3, corresponding to an initial acid value of 160 ±8 mg KOH g^{-1}) were placed together in the reactor with 3.6 g of the immobilized derivative, and all the experiments were conducted at 70 °C and 350 rpm.

Two different reactors were used:an open-air glass-jacketed batch reactor (250 mL, total volume) provided with an overhead stirrer with a two-bladed propeller (axial flow), and a Parr 5100 series low-pressure glass-jacketed reactor (100 mL, total volume) equipped with a crossed blade impeller of 4 flat blades. The pressure in the second reactor was

kept at 213 hPa, and dry N_2 (25 $cm^3 s^{-1}$) was passed through the vessel so that the water content was maintained around 2000 ppm.

The enzymatic reaction extent was followed by measurement of acid value (AV) (ASTM D974-02e1),[23] which represents the number of mg of potassium hydroxide necessary to neutralize free acids in 1 g of sample.

The conversion based on acid value was calculated as:

$$\text{Conversion } (\%) = \frac{(\text{AV})_{t=0} - (\text{AV})_t}{(\text{AV})_{t=0}} \cdot 100$$

Results are presented graphically as the mean of the three measurements and include the error bars (± standard deviation).

Results and discussion

Use of different supports for immobilization

Based on previous studies[5,7-9] ion exchange resins were used as supports for the immobilization of *Candida antarctica* lipase (Lipozyme® CALB). In particular, Lewatit® MonoPlus MP 64 (weakly basic), Lewatit® Monoplus MP 500 (strongly basic), and Dowex 50x8 (strongly acidic) were studied. These supports were chosen based on the particle size (0.6 mm approximately)[24-29] similar to that of the commercial immobilized enzyme Novozym® 435. Their density (1.04, 1.06, and 0.80 g cm^{-3}, respectively) is higher than that of Novozym® 435 (0.40 g cm^{-3}).[13] The resins are based on styrene-divinylbenzene matrices which were hydrated with distilled water before use. Three different immobilized derivatives were obtained following the standard

procedure described in the Materials and methods section without the support activation step. The highest amount of immobilized protein was obtained with Lewatit® Monoplus MP 64 as a support, in which 24.33 ± 2.67 % of protein was adsorbed. A percentage of 19.30 ± 2.07 % was immobilized on Lewatit® Monoplus MP 500, and 13.30 ± 1.28 % on Dowex 50x8.

The results obtained in this work are slightly lower than those in the previous reports. Lewatit® Monoplus MP 64 was used as support for the immobilization of lipases from different sources.[5,7-9] The difference can be attributed to different origin of the immobilized lipases (*Candida rugosa*, *Rhizopus arrhizus* and *Rhizopus oryzae*). The immobilized derivatives described here show adequate physical characteristics to facilitate their separation from the reaction media, PGPR. In another report[30], CALB L lipase (commercial solution of lipase stabilized with glycerol and sorbitol diluted in buffer[13]) was successfully immobilized on hydrophobic supports and has been used as a catalyst in the synthesis of esters with cosmetic application; the main problem was its high cost.

The esterification activity of the three immobilized derivatives was tested in the synthesis of polyglycerol polyricinoleate. The AV decreased by 9 % with the immobilized derivative of Lewatit® Monoplus MP 64 and by 6 % with that of Lewatit® Monoplus MP 500, while a decrease of only 2 % was obtained with the immobilized derivative of Dowex 50x8. These negative preliminary results showed that, although the immobilized derivatives contained lipase, it was not in its active conformation. Lewatit® Monoplus MP 64 showed slightly better results, so this support was selected for subsequent optimization studies.

Optimization of the immobilization procedure

Influence of pH

The influence of pH of the buffer used to dilute Lipozyme® CALB L for its immobilization on Lewatit® Monoplus MP 64 has been studied. This study is important because pH influences the electrostatic forces, and the pH changes over the isoelectric point of lipase might have a great impact on the amount of bound protein.[31,32]

In this case, Lipozyme® CALB L was diluted in different buffers, 0.1 M acetate buffer and 0.1 M phosphate buffer, covering pH values ranging from 4.0 to 8.0; the rest of the immobilization process was as described in Materials and methods section. The results presented in Fig. 2 show that pH variations influence the immobilization yield, which confirms the hypothesis that electrostatic forces are the driving force of the immobilization process. However, hydrophobic interactions could also be involved in the adsorption process. The maximum percentage of immobilized protein was obtained using phosphate buffer pH7. Therefore, the pH value of 7 was chosen for the remainder of the study.

Influence of lipase concentration

For the purpose of increasing the immobilization yield and obtaining an immobilized derivative with a high enzyme content, experiments using Lipozyme® CALB L solutions with concentrations between 2.6 and 26 mg of Lowry protein per mL were conducted. These experiments were carried out following the general procedure described in Materials and Methods section, and with the previously selected conditions: Lewatit® Monoplus MP 64 as support without activation, and pH 7. The

results are shown in Table 1, where mass of immobilized protein per gram of dry support and immobilization yield are specified. Measurements revealed that 5.00 ± 0.05 g of support had a water content of 28 %, resulting in 3.60 ± 0.05 g of dry support. This water content differs from the value (61-66 %) for commercial specifications of Lewatit® Monoplus MP 64.

As may be seen, as the concentration of the immobilization solution increases, the loading of protein also increases but the percentage of immobilized protein decreases. From the obtained results, it seems that for lipase solution concentrations higher than 13 mg of Lowry protein per mL, the increase in the amount of adsorbed protein does not justify the increase in the offered protein, so this concentration was selected for the remainder of the experiments.

Study of different activators of the support

The selected immobilized derivative (Lewatit® Monoplus MP 64 as support without activation, pH 7, and with initial enzyme concentration of 13 mg of Lowry protein per mL) was used for the synthesis of PGPR in a jacketed reactor following the procedure described in Materials and methods section. After 4 days, no notable decrease in the acid value was observed. These results can be attributed to the insufficiently hydrophobic enzyme microenvironment in the immobilized derivative. Based on different studies[5,7-9,33,34] it is known that the use of support activators improves lipase activity of the immobilized derivatives. Therefore, different immobilization experiments were conducted using Lewatit® Monoplus MP 64 as support at the optimal conditions determined previously, and soybean lecithin, ricinoleic acid or oleic acid as support activators.

The results presented in Table 2 show that, when the support was activated with very hydrophobic substances (oleic and ricinoleic acid), the immobilization yield increased considerably, suggesting that *Candida antarctica* lipase adsorption process is also controlled by hydrophobic interactions and not only by electrostatic forces, and that the support activation with a fatty acid modifies the support hydrophobicity and improves its adsorption capacity. On the other hand, activation with a phospholipid, soybean lecithin seemed not to be as effective, at least with respect to immobilization yield. These results are in contrast with those obtained previously[5] for *Candida rugosa* lipase immobilization on the same support, where it was observed that its activation reflected no increase in the amount of adsorbed enzyme.

The abovementioned immobilized derivatives were used as biocatalysts in the synthesis of PGPR in open air tank reactors, following the protocol described in Materials and methods. Firstly, the specific activities of the four immobilized derivatives (expressed as Δ(AV) per h per g of Lowry protein) were determined from these experiments, and are shown in the last column of Table 2. The results presented in Fig. 3 reveal that the biocatalysts obtained using hydrophobic compounds (oleic and ricinoleic acid) as activators exhibit great catalytic activity in the synthesis of PGPR, reaching conversions around 70 %, much higher than that observed when the immobilized enzyme was used without support activation or treated with soybean lecithin. These results are, again, in contrast with those previously mentioned[5] in which no noticeable difference in enzyme activity was observed between the activators, and in which soybean lecithin was found to be the best of them. These differences can be attributed, as previously commented, to the fact that the enzyme source is different. Since both hydrophobic activators have the

same effect on the process, both in the immobilization and in PGPR synthesis, the more logical choice is ricinoleic acid, since it is one of the reaction substrates and thus the use of a compound strange to the reaction is avoided.

Thus, when operating under optimal immobilization conditions, 129.35 ± 16.80 mg of Lowry protein was adsorbed by the support when 325 ± 42.3 mg of Lowry protein was offered to immobilization, which represents an immobilization yield of 39.80 ± 5.17 %. The specific protein content of the immobilized derivative is referred to as g of dry support as previously described (35.93 ± 4.90 mg of Lowry protein per g of dry support).

PGPR synthesis in batch reactors

The legislative requirements for PGPR determine that this product should present an AV ≤ 6 mg of KOH per g of product, which can only be obtained by forcing the equilibrium towards the ester (right-hand) side of the esterification reaction equation. In order to achieve complete consumption of substrates and shift the equilibrium towards the product, the water produced in the reaction was removed using a vacuum reactor with a continuous dry N_2 inflow. The results obtained by operating in open air and vacuum reactors under the same operating conditions are compared in Fig. 4.

As may be observed, the use of a controlled atmosphere reactor provides higher conversion degrees. In fact, with open air reactor, PGPR with AV ≤ 6 mg(KOH) g^{-1} was not produced. The minimum acid value after almost 170 h of operation reached around 40 mg(KOH) g^{-1}, whereas with vacuum reactor reached AV of 3.8 mg(KOH) g^{-1} in 159 hours. This reaction time is significantly higher than when Novozym® 435 was used as

a catalyst (55 h).[11] However, the final choice of the best immobilized lipase should be based on economic studies of the entire process. Namely, with the new derivative, the downstream processes would be cheaper because centrifugation could be avoided.

Finally, it is important to note that the obtained PGPR maintained the acid value for at least one week when stored at room temperature, which evidenced that lipase desorption in the reaction media was insignificant.

Immobilized derivative reuse study

One of the main advantages of using immobilized biocatalyst instead of a free one is the possibility of obtaining a final product free of catalyst residues, thus enabling the reuse of the immobilized derivative. This fact usually results in a significant decrease in the overall costs of the production process. It is therefore necessary to explore the possibility of separating the derivative from the reaction medium in order to verify if its reuse is viable in practice, and determine the possible loss of activity after successive reuses.

In a previous study,[11] the commercial immobilized derivative Novozym® 435 was used for the PGPR production, but due to the high viscosity of the reaction product and the small density of the commercial enzyme, the biocatalyst was removed from the final product by centrifugation followed by decantation. The advantage of using the new immobilized derivative is that the density of the macroporous resin, on which *Candida antarctica* lipase is immobilized, is higher than that of the commercial Novozym® 435, so the centrifugation step to separate both phases is not required. Thus, to separate the obtained PGPR from the immobilized derivative, it is only necessary to wait less than a

minute for the resin to settle, and with the help of a pipette, to extract the product from the top. After pipetting the reaction product, the biocatalyst is washed with acetone in order to remove the excess of the product. Finally, when immobilized derivative is completely dry and free of acetone, it is ready to catalyse a new reaction. More than 97 % of the biocatalyst activity is recovered.

In this work, the immobilized derivative was used three times in a vacuum reactor reaction under the same experimental conditions. The results are shown in Fig. 5. The first analysis of Fig. 5 showed that final conversion values were the same in the three runs, although the time required was slightly higher in the case of the third run. Therefore, it may be affirmed that the activity loss of the biocatalyst had no significant influence on the reduction of the acid number, since the AV of the final product was practically the same.

On the other hand, it should be noted that the first reaction run was slower that the two others. This may be explained by the assumption that, after the immobilization process, the immobilized derivative still contained some buffer that soaked it, which interfered with the reaction course. In the successive runs, the immobilized derivative was sodden with the product itself and/or washing acetone, which created a hydrophobic environment that favoured the course of the reaction.

Conclusion

In this work, a polymeric emulsifier food additive has been biotechnologically obtained with Lipozyme® CALB L lipase, immobilized on different supports, in a solvent-free and one-step process. Among the assayed supports, the resin Lewatit® Monoplus MP 64

presented the best results in protein immobilization, but the immobilized derivative was inactive without previous activation of the support with a hydrophobic agent. For the immobilization, phosphate buffer of pH 7, initial enzyme concentration of 13 mg of Lowry protein per mL, and ricinoleic acid as support activator were chosen. Experiments carried out under the optimum conditions in an open air batch reactor with vertical stirring, produced a PGPR with AV = 40 mg(KOH) g^{-1} in 170 h, which does not fulfil the acid value requirements prescribed in the European Commission regulation (below 6 mg(KOH) g^{-1} for its use in food industry). When PGPR was synthesized in a vacuum reactor operating under nitrogen atmosphere, AV = 3.8 mg(KOH) g^{-1} was reached after 159 hours. Besides, this immobilized derivative proved to conserve the same activity for at least three successive runs, which allows the scaling up of the process.

ACKNOWLEDGEMENTS

This work has been funded with support from the Spanish MINECO (CTQ2015-66723-R). M. D. Murcia and S. Ortega were beneficiaries, both from MINECO, of Ramon y Cajal contract (RYC-2016-20211) and Torres Quevedo (PTQ-15-08015) grant, respectively. We wish to acknowledge D. Ramiro Martínez Gutiérrez (Novozymes Spain S.A.) for kindly supplying the biocatalyst.

References

1. *Bódalo, A., Bastida, J., Máximo, M. F., Montiel, M. C. Gómez, M. Ortega, S.,* Screening and selection of lipases for the enzymatic production of polyglycerol polyricinoleate, Biochem. Eng. J. **46** (2009) 217.
doi: https://doi.org/10.1016/j.bej.2009.05.004

2. *Wilson, R., Van Shie, B. J., Howes, D.,* Overview of the preparation, use and biological studies on polyglycerol polyricinoleate (PGPR), Food Chem. Technol. **36** (1998) 711.
doi: https://doi.org/10.1016/S0278-6915(98)00057-X
3. *Denecke, P., Börner, G., Von Allmen, V.,* Method of preparing polyglycerol polyricinoleic fatty acids esters, UK Patent 2,073,232, 1981.
4. *Bódalo, A., Bastida, J., Máximo, M. F., Montiel, M. C., Murcia, M. D.,* Enzymatic biosynthesis of ricinoleic acid estolides, Biochem. Eng. J. **26** (2005)155.
doi: https://doi.org/10.1016/j.bej.2005.04.012
5. *Bódalo ,A., Bastida, J., Máximo, M. F., Montiel, M. C., Gómez M., Murcia, M. D.,* Production of ricinoleic acid estolide with free and immobilized lipase from *Candida rugosa*, Biochem. Eng. J. **39** (2008) 450.
doi: https://doi.org/10.1016/j.bej.2007.10.013
6. *Bódalo, A., Bastida, J., Máximo, M. F., Montiel, M. C., Murcia, M. D., Ortega, S.,* Influence of the operating conditions on lipase-catalyse synthesis of ricinoleic acid estolides in solvent-free system, Biochem. Eng. J. **44** (2009) 214.
doi: https://doi.org/10.1016/j.bej.2008.12.007
7. *Gómez, J. L., Bastida, J., Máximo, M. F., Montiel, M. C., Murcia, M. D., Ortega, S.,* Solvent-free polyglycerol polyricinoleate synthesis mediated by lipase from *Rhizopus arrhizus,* Biochem. Eng. J. **54** (2011) 111.
doi: https://doi.org/10.1016/j.bej.2011.02.007
8. *Ortega, S., Máximo, M. F., Montiel, M. C., Murcia, M. D., Arnold, G., Bastida, J.,* Esterification of polyglycerol with polycondensed ricinoleic acid catalysed by immobilised *Rhizopus oryzae* lipase, Bioproc. Biosyst. Eng. **36** (2013) 1291.
doi: https://doi.org/10.1007/s00449-012-0874-2

9. *Ortega, S., Gómez, J. L., Bastida, J., Máximo, M. F., Montiel, M. C., Gómez, M.,* Biocatalytic synthesis of polyglycerol polyricinoleate: A comparison of different commercial lipases, Chem. Biochem. Eng. Q. **27** (2013) 439.
10. *Ortega, S., Gómez, J. L., Bastida, J., Máximo, M. F., Montiel, M. C., Murcia, M. D.,* Study of different reaction schemes for the enzymatic synthesis of polyglycerol polyricinoleate, J. Sci. Food Agric. **94** (2014) 2308.
doi: https://doi.org/10.1002/jsfa.6559
11. *Ortega, S., Bódalo, A., Bastida, J., Máximo, M. F., Montiel, M. C., Gómez, M.,* Optimized enzymatic synthesis of the food additive polyglycerol polyricinoleate (PGPR) using Novozym®435 in a solvent free system, Biochem. Eng. J. **84** (2014) 91.
doi: https://doi.org/10.1016/j.bej.2014.01.003
12. Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council.
13. Novozymes, Product Data Sheet Novozym® 435. (Supplied by Novozymes).
14. *Meshrasbi, M. R., Mohammadi, H., Peyda, M., Mohammadi, M.,* Covalent immobilization of *Candida antarctica* lipase on core-shell magnetic nanoparticles for production of biodiesel from waste cooking oil, Renew. Energ. **101** (2017) 593.
doi: https://doi.org/10.1016/j.renene.2016.09.022
15. *Rios, N. S., Pinheiro, M. P., dos Santos, J. C. S., Fonseca, T. S., Lima, L. D., de Mattos, M. C., Freire, D. M. G., da Silva Júnior, I. J., Rodríguez-Aguado, E., Gonçalves, L. R. B.,* Strategies of covalent immobilization of a recombinant *Candida antarctica* lipase B on pore-expanded SBA-15 and its application in the kinetic resolution of (R,S)-phenylethyl acetate, J.Mol. Catal. B-Enzym. **133** (2016) 246.

doi: https://doi.org/10.1016/j.molcatb.2016.08.009

16. *Rios, N. S., Pinheiro, M. P., Lima, M. L. B., Freire, D. M. G., da Silva Júnior, I. J., Rodríguez-Castellón, E., de Sant'Ana B., Macedo, A. C., Gonçalves, L. R. B.*, Pore-expanded SBA-15 for the immobilization of a recombinant *Candida antarctica* lipase B: Application in esterification and hydrolysis as model reactions, Chem. Eng. Res. Eng. **129** (2018) 12.
doi: https://doi.org/10.1016/j.cherd.2017.10.032

17. *De Souza, S. P., de Almeida, R. A. D., Garcia, G. G., Leão, R. A. C., Bassut, J., de Souza, R. O. M. A., Itabaina Jr, I.,* Immobilization of lipase B from *Candida antarctica* on epoxy-functionalized silica: Characterization and improving biocatalytic parameters, J. Chem. Technol. Biot. **93** (2018) 105.
doi: https://doi.org/10.1002/jctb.5327

18. *Zdarta, J., Klapiszewski, L., Jedrzak, A., Nowicki, M., Moszynski, D., Jesionowski, T.,* Lipase B from *Candida antarctica* immobilized on a silica-lignin matrix as a stable and reusable biocatalytic system, Catalysts **7** (2017) 1.

19. *Mittersteiner, M., Machado, T. M., de Jesus, P. C., Brondani, P. B., Scharf, D. R., Wendhausen Jr. R.,* Easy and simple SiO_2 immobilization of lipozyme CALB-L: Its use as a catalyst in acylation reactions and comparison with other lipases, J. Brazil. Chem. Soc. **28** (2017) 1185.

20. *Dos Santos, J. C. S., Bonazza, H. L., de Matos, L. J. B. L., Carneiro, E. A., Barbosa, O., Fernandez-Lafuente, R., Gonçalves, L. R. B., de Sant' Ana, H. B., Santiago-Aguiar, R. S.,* Immobilization of CALB on activated chitosan: Application to enzymatic synthesis in supercritical and near-critical carbon dioxide, Biotechnology Reports **14** (2017) 16.
doi: https://doi.org/10.1016/j.btre.2017.02.003

21. *Tanasković, S. J., Jokić, B., Grbavčić, S., Drvenica, I., Prlainović, N., Luković, N., Knežević-Jugović, Z.*, Immobilization of *Candida antarctica* lipase B on kaolin and its application in synthesis of lipophilic antioxidants, Appl. Clay Sci. **135** (2017) 103. doi: https://doi.org/10.1016/j.clay.2016.09.011

22. *Hartree, E. F.*, Determination of protein: A modification of the Lowry method that gives a linear photometric response, Anal. Biochem. **42** (1972) 422. doi: https://doi.org/10.1016/0003-2697(72)90094-2

23. Standard Test Method for Acid and Base Number by Color-Indicator Titration (ASTM D974-02e1).

24. Product information Lewatit®MonoPlus MP 64 (Supplied by Lenntech).

25. Product information Lewatit®MonoPlus MP 500 (Supplied by Lenntech).

26. Product information Dowex 50x8 (Supplied by Lenntech).

27. *Severac, E., Galy, O., Turon, F., Pantel, C. A., Condoret, J. S., Monsan, P., Marty, A.*, Selection of CALB immobilization method to be used in continuous oil transesterification: Analysis of the economic impact, Enzyme. Microb. Tech. **48** (2011) 61. doi: https://doi.org/10.1016/j.enzmictec.2010.09.008

28. *Mei, Y., Miller, L., Gao, W., Gross, R. A.*, Imaging the distribution and secondary structure of immobilized enzymes using infrared microspectroscopy, Biomacromolecules **4** (2003) 70. doi: https://doi.org/10.1021/bm025611t

29. *Poojari, Y., Clarson, S. J.*, Thermal stability of *Candida antarctica* lipase B immobilized on macroporous acrylic resin particles in organic media, Biocatal. Agric. Biotechnol. **2** (2013) 7. doi: https://doi.org/10.1016/j.bcab.2012.10.002

30. *Serrano-Arnaldos, M., Ortega-Requena, S., Montiel, M. C., Máximo, F., Bastida, J., Murcia, M. D.,* Preliminary economic assessment: A valuable tool to establish biocatalytic process feasibility with an in-lab immobilized lipase, J. Chem. Technol. Biot. (2018) In press: DOI10.1002/jctb.5784.
doi: https://doi.org/10.1002/jctb.5784

31. *Gitlesen, T., Bauer, M., Adlercreutz, P.,* Adsorption of lipase on polypropylene powder, BBA-Lipid Lipid Met. **1345** (1997) 188.
doi: https://doi.org/10.1016/S0005-2760(96)00176-2

32. *Rodrigues, A. S., Cavalcante, G. P., Ferrei, A. L. O., Goncalves, L. R. B.,* Immobilization of *Candida antarctica* lipase type B by adsorption on activated carbon, Chem. Biochem. Eng. Q. **22** (2008) 125.

33. *Yoshida, Y., Kawase, M., Yamaguchi, C., Yamane, T.,* Enzymatic synthesis of estolides by a bioreactor, JAOCS. **74** (1997) 261.
doi: https://doi.org/10.1007/s11746-997-0133-x

34. *Montiel, M. C., Serrano, M., Máximo, M. F., Gómez, M., Ortega-Requena, S., Bastida, J.,* Synthesis of cetylricinoleate catalyzed by immobilized Lipozyme® CALB lipase in a solvent-free system, Catalysis Today **255** (2015) 49.
doi: https://doi.org/10.1016/j.cattod.2014.09.015

Figure captions

Figure 1. PGPR biocatalytic synthesis reaction scheme

Figure 2. Experimental results obtained in the immobilization of Lipozyme® CALB L on Lewatit®Monoplus MP 64 to study the influence of pH using different buffers (●) Acetate, and (▼) Phosphate buffer

Figure 3. Influence of the activator on the evolution of conversion with reaction time in the synthesis of PGPR. Reaction conditions: open air batch reactor, 30 g ricinoleic acid, 2.5 g polyglycerol-3, 3.6 g immobilized derivative, 70 °C, 350 rpm. (●) Without activator, (▼) soybean lecithin, (■) ricinoleic acid, and (♦) oleic acid

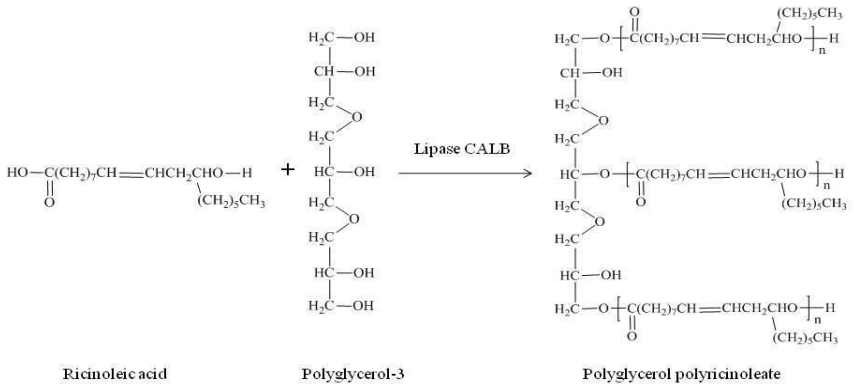
Figure 4. Conversion variation with time in the synthesis of PGPR in two different reactors, an open air and a vacuum reactor. Reaction conditions: 30 g ricinoleic acid, 2.5 g polyglycerol-3, 3.6 g immobilized derivative, 70 °C, 350 rpm. (●) Vacuum reactor, and (▼) open air reactor

Figure 5. Conversion variation with time for PGPR synthesis in the vacuum reactor after first, second, and third use. Reaction conditions: 30 g ricinoleic acid, 2.5 g polyglycerol-3, 3.6 g immobilized derivative, 70 °C, 350 rpm. (●) First, (▼) second, and (■) third use

Table captions

Table 1. Experimental results obtained in the immobilization of Lipozyme® CALB L on Lewatit Monoplus MP 64 to study different lipase solution concentrations.

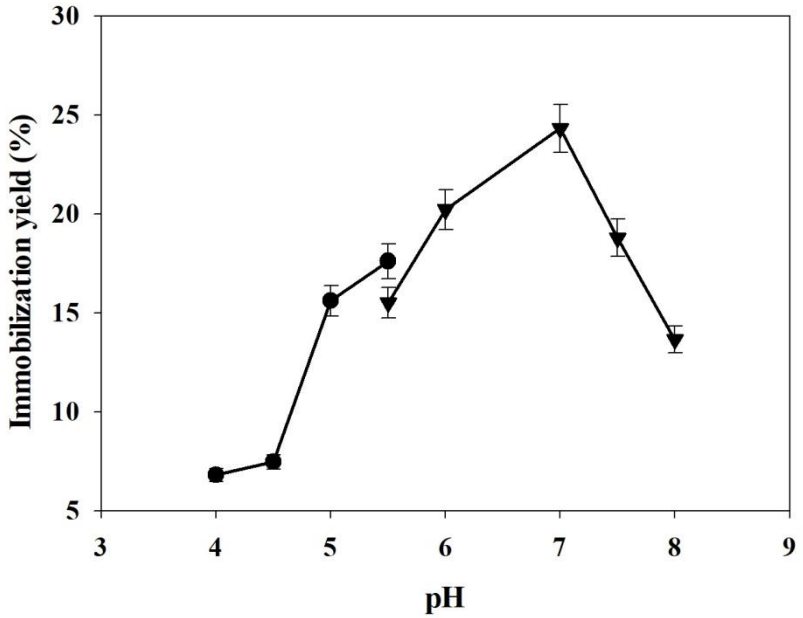
Table 2. Experimental results obtained in the immobilization of Lipozyme® CALB L on Lewatit Monoplus MP 64 to study the influence of different activators



1

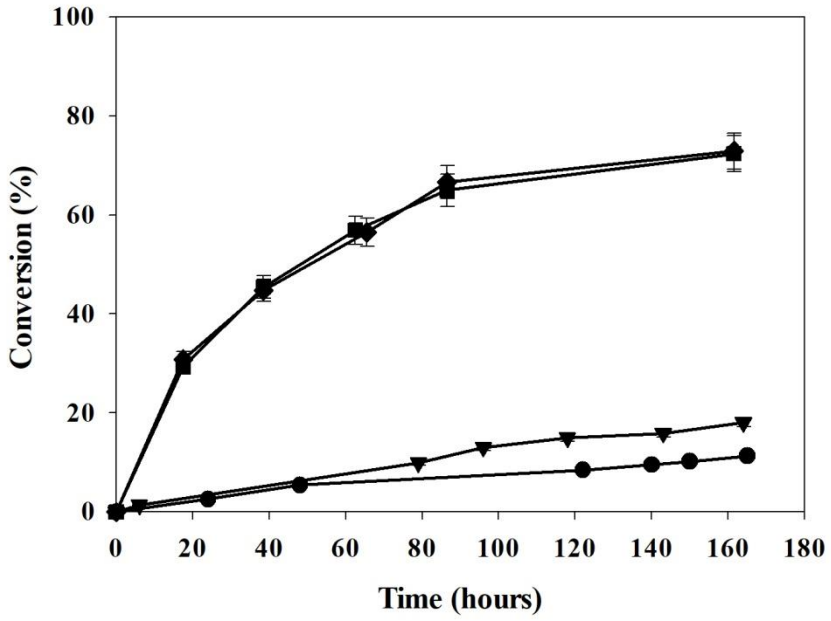
2 Fig. 1

3



4

5 Fig. 2



1

2 Fig. 3

3

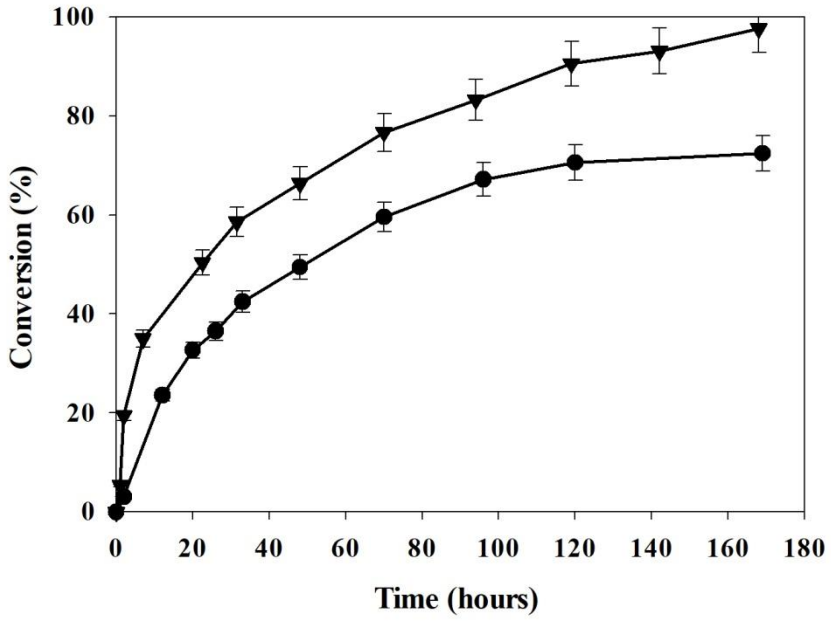


Fig. 4

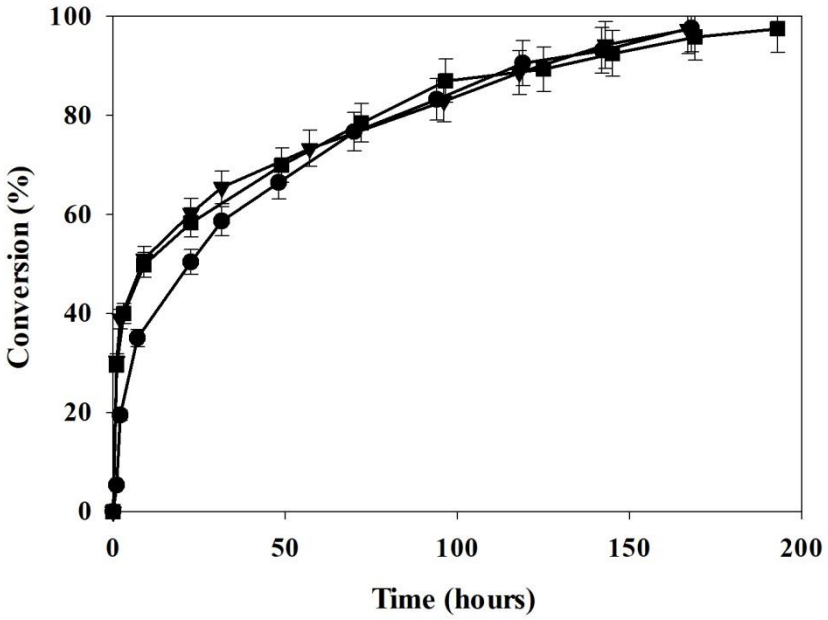


Fig. 5

Table 1

Lipase concentration in immobilization solution (mg of Lowry protein per mL)	Immobilized protein (mg of protein per g of dried support)	Immobilization yield (%)
2.60 ± 0.26	5.90 ± 0.59	32.68 ± 3.30
3.90 ± 0.40	7.27 ± 0.73	26.84 ± 2.68
6.50 ± 0.65	12.80 ± 0.13	28.36 ± 2.84
13.00 ± 1.43	21.96 ± 2.42	24.33 ± 2.67
19.50 ± 0.20	25.31 ± 0.25	18.69 ± 1.87
23.40 ± 0.23	26.59 ± 0.27	16.36 ± 1.64
26.00 ± 0.26	27.38 ± 0.27	15.16 ± 1.52

Table 2

Activator	Immobilized protein (mg protein per g of dry support)	Immobilization yield (%)	Specific activity Δ(AV) per h per g of Lowry protein)
Without activation	21.96 ± 2.42	24.33 ± 2.67	2.04 ± 0.22
Soybean lecithin	19.42 ± 1.94	21.55 ± 2.16	5.12 ± 0.51
Ricinoleic acid	35.93 ± 4.90	39.80 ± 5.17	19.18 ± 2.49
Oleic acid	37.61 ± 3.80	41.66 ± 4.20	19.17 ± 0.19