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STUDY OF DIFFERENT REACTION SCHEMES FOR THE ENZYMATIC SYNTHESIS OF POLYGLYCEROL POLYRICINOLEATE

Running title:

Study of reaction schemes for the enzymatic synthesis of PGPR

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ABSTRACT

BACKGROUND: Different strategies for the solvent-free enzymatic production of polyglycerol polyricinoleate (PGPR) were explored in an attempt to simplify and improve the process. Besides the conventional procedure (obtaining polyricinoleic acid followed by its esterification with polyglycerol), two alternative methods are proposed: (i) reversing the order of enzymatic synthesis, i.e. first the esterification of polyglycerol with ricinoleic acid and then the condensation of ricinoleic acid with the previously obtained polyglycerol ester, and (ii) the enzymatic synthesis of PGPR in a single-step process.

RESULTS: The reaction sequences were carried out in an open air reactor with free and immobilised lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3): *Candida rugosa* lipase to obtain polyricinoleic acid and *Rhizopus oryzae* lipase for the esterification of polyglycerol with the carboxyl group of ricinoleic or polyricinoleic acid. A co-immobilised derivative containing both lipases was used to catalyse the single-stage scheme. Finally, the three processes were carried out in a vacuum reactor, obtaining in every case a PGPR that complied with the legal specifications of the European Community and with the recommendations provided in the Food Chemical Codex.

CONCLUSION: The results demonstrate that all three protocols are viable for the enzymatic synthesis of PGPR and require similar reaction times, although the single-stage scheme is easier to carry out.

Keywords: Biocatalysis, enzyme immobilisation, fatty acid esters, process optimisation, *Candida rugosa* lipase, *Rhizopus oryzae* lipase.

INTRODUCTION

Food emulsifiers are molecules bearing a hydrophobic part and hydrophilic part. The hydrophobic moiety of the emulsifier often consists of hydrocarbon chains of fatty acids and the hydrophilic part may consist of more polar molecules such as glycerol, lactic acid, citric acid or polyglycerol. Food related industries are very interested in these additives because they can safely be consumed by humans in quantities up to 125 mg/kg body weight per day. They also have useful properties that improve the manufacture of food products, especially confectionery.[1]

Polyglycerol esters have been used as food emulsifiers for many years. From the official point of view, food grade polyglycerol esters are divided into two classes: polyglycerol esters of edible fatty acids (E-number: E-475, also known as "PGFA") and polyglycerol polyricinoleate (E-number: E-476, also known as "PGPR"). Polyglycerol polyricinoleate is used to maintain stable emulsions of oil and water systems with a high water content and as a viscosity modifier. In the chocolate industry, PGPR is used because it considerably diminishes the yield stress of molten chocolate. This allows chocolate to be moulded without any air bubbles, the easier coating of particulate ingredients, and the optimal adjustment of the thickness of chocolate coating. An additional property of PGPR in chocolate is its ability to limit fat-bloom.[2]

Known chemical methods for preparing PGPR involve the autocatalytic condensation of ricinoleic acid and an alkali-catalysed reaction between the condensed ricinoleic acid and polyglycerol. However, these procedures have the disadvantage of requiring very long reaction times and high operating temperature, involving high energy costs. This adversely

affects the quality of the final product because of problems related with coloration and odours.[3]

As an alternative, the authors published a paper on the biotechnological production of PGPR using lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3), which act in mild reaction conditions and produce a final product more suitable for use as a food additive. This enzymatic procedure consisted of two steps: first, the condensation of ricinoleic acid biocatalysed by *Candida rugosa* lipase[4-6] and then the esterification of polyricinoleic acid with polyglycerol to obtain polyglycerol polyricinoleate, PGPR, using either *Rhizopus arrhizus* lipase or *Rhizopus oryzae* lipase.[7-9] All of these reactions are solvent-free systems that can be regarded as a green alternative to the use of organic solvents, which may leave undesirable residues in products. Indeed, many solvents are toxic and are not permitted in processing procedures to make products to be used in food.[10] However, the biocatalytic processes described are time-consuming, while working with two different enzymes represents an additional drawback.

Therefore, it was thought desirable to optimise the previously proposed PGPR production process bearing in mind three fundamental aims: first, the final product must comply with the legal requirements of the European Commission Directive 2008/84/EC;[11] second, the time required to complete the synthesis must be as short as possible; and finally, the process should be simple enough to be carried out it in just one step (one-pot synthesis). To achieve this, alternative reaction sequences were studied (a reverse-reaction and a single-stage scheme) using both free and immobilised lipases, operating in open-air reactors or in a vacuum reactor.

EXPERIMENTAL

Enzymes and substrates

Microbial lipase (E.C. 3.1.1.3) from *Candida rugosa* (Type IV) (CRL) 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 one hour, at pH 7.7 and 37 ºC). Lipase from *Rhizopus oryzae* (55.7 U/mg solid) (ROL) was purchased from Fluka. One unit corresponds to the amount of enzyme which releases 1 µmol of oleic acid per minute at pH 8.0 and 40 ºC using triolein as substrate. Ricinoleic acid (~ 80%) was supplied by Fluka and polyglycerol-3 was a kind gift from Solvay.

Immobilisation carrier and chemicals

The anionic exchange resin Lewatit MonoPlus MP 64 (supplied by Fluka) was used as immobilisation carrier. Soybean lecithin, used as support activator, was of commercial grade from Santiveri S.A., Spain. Other chemicals were of analytical grade and were used without further purification.

Immobilisation by physical adsorption

The support (5 g) was mixed with 50 ml of a soybean lecithin aqueous suspension (20 mg/ml) in an Erlenmeyer flask and placed in an orbital shaker 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 i.d. and 30 cm length) equipped with a sinterised glass plate placed 5 cm from the bottom. The enzyme solution (50 ml, 10 mg/ml in acetate buffer 0.1 M, pH 5) was then added to the reactor and circulated for 2 days at 4 ºC. The immobilised derivative was washed twice with the same buffer and stored at 4 ºC. The amount of protein initially offered and the protein in the wash-liquid after

immobilisation were determined by Lowry's procedure,[12] using bovine serum albumin as standard. The amount of coupled lipase was the difference between the amount of the initial enzyme added and the amount of enzyme in the wash-liquid.

The procedure followed to carry out the co-immobilisation of the two lipases was similar to that described above, except that the amount of support (10 g), the volume of the solution of lecithin (100 ml) and the volume of the enzyme solution (100 ml) were doubled. The enzyme solution (10 mg/ml) contained both lipases in a 1:1 mass ratio.

Atmospheric reactor experiments

The enzymatic reaction was carried out in an open-air glass-jacketed batch reactor (250 ml total volume), maintaining the reaction temperature constant at 40 ºC. Reactions took place in the absence of solvents and complete mixing was achieved by means of a four-bladed propeller stirrer at 450 rpm. For a typical reaction, the reaction mixture contained 30 g of ricinoleic acid (RA) or 30 g of polyricinoleic acid (PR, AV ≈ 50 mg KOH/g) during the first step, and 2.68 g of polyglycerol-3 (PG) in the second step. Unless otherwise stated, 500 mg of lipase was dissolved in 5 ml of distilled water and added to the reactor. When the reaction was catalysed with immobilised lipase, 5 g of immobilised derivative was used and the only water in the reaction system was that soaked in the support (0.6 ml/g approximately). When the reaction was catalysed with co-immobilised lipases, 10 g of immobilised derivative was used.

Vacuum reactor experiments

For the vacuum reaction, a Parr 5100 low pressure reactor was used. The glass reaction vessel (100 ml total volume) was equipped with a circulating water jacket to heat the

vessel. The reactor head of stainless steel accommodated the reactor controls and instrumentation. A magnetic drive internal stirrer acted as a turbine type impeller. The reactor top also included 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 were managed by a controller. The amount of ricinoleic acid, immobilised lipase and water in the reactor at the beginning of the reaction were the same as that reported for the open air jacketed batch reactor. The experiments were carried out at 40 ºC, and the stirring rate was kept constant at 450 rpm. The pressure was set at 160 mmHg and an input of 90 l/h of dry nitrogen facilitated water removal. This input was discontinuous in order to maintain the water content between 1000 ppm and 3000 ppm.

Determination of the reaction extension

Triplicate measurements of the acid value (AV),[13] which represents the number of milligrams of potassium hydroxide necessary to neutralise free acids in 1 g of sample, were carried out to follow the reaction. Here, the AV corresponds to the free carboxyl group concentration in the reaction mixture, which falls as the hydroxy fatty acid is esterified with PG.

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

Measurement of the hydroxyl value

The hydroxyl value of the reaction products was determined in triplicate. This parameter is expressed as the milligrams of potassium hydroxide required to neutralize the acetic acid which combines with 1 g of the sample through acetylation.[13]

Refractive index

The refractive index of the PGPR obtained was determined with an ABBE refractometer (2WAJ model, Optika, equipped with water recirculation and temperature control), at 65 ºC in accordance with the legal specification for the refractive index of this food additive.[11]

Water content

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

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 a 717 PLUS automatic injector and 600 E quaternary-gradient pump. The system was equipped with refractive index detector (model 2414 7.8 mm id x 300 mm GPC Styragel® HR 1 THF column from Waters). The analysis was performed at 35 ºC using THF as solvent at a flow rate of 1 ml/min. A calibration curve (log M_w *vs* retention time), which is used to estimate the molecular weight of samples, was made using the retention times of the resolved peaks for the monomer, dimer, trimer and tetramer of ricinoleic 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:

$$M_n = \frac{\sum A_i M_i}{\sum A_i} \quad (1)$$

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

where the subscript i refers to the i_{th} trapezoid of A_i area and M_i to the average molecular weight of this trapezoid. The ratio of the weight average to the number average gives polydispersity index.

$$r = M_w / M_n \quad (3)$$

RESULTS AND DISCCUSION

The enzymatic synthesis of polyglycerol polyricinoleate (PGPR) from ricinoleic acid (RA) and polyglycerol (PG) has been described by the authors in previous articles.[4-9] The reaction scheme proposed for the biocatalytic synthesis is similar to the traditional chemical procedure and takes place in two steps: first RA condenses to yield polyricinoleic acid (PR) catalysed by *Candida rugosa* lipase (CRL) and then the condensed product is esterified by PG catalysed by *Rhizopus oryzae* lipase (ROL). Both reaction steps were studied and the production of PGPR with immobilised CRL and ROL in a controlled atmosphere reactor was optimised. As a result, PGPR that complies with EC specifications regarding acid value, hydroxyl value and refractive index, was obtained.

As the main problem of this procedure is the time necessary for the reaction to be completed, alternative synthesis protocols were tested in an attempt to shorten the time required to obtain PGPR. Figure 1 shows the three synthesis procedures.

The first procedure, "normal-reaction scheme", has been described elsewhere.[7-9]

The second option consisted of reversing the order of the reaction steps. This synthesis scheme is based on the inability of ROL and most other 1,3-positional selective lipases to use secondary hydroxyl groups as acyl acceptors, which means that it is unable to catalyse the formation of estolide although it can esterify primary hydroxyl groups of polyols.[14] Hence, only the two primary hydroxyl groups of the five hydroxyl groups of PG are esterified by the carboxyl groups of RA chains. In a second step, CRL was added since this random-positional selective lipase is unable to catalyse the esterification of PG and PR,[6] and only catalyses the attachment of ricinoleic groups to the mono-ricinoleyl chains of the PG-RA ester product. The procedure has been named "reverse-reaction scheme" and its main positive point would be that the binding between polyglycerol and ricinoleic acid would have less steric hindrance than the binding between polyglycerol and the polyricinoleic chains.

In the third option, the two reaction steps were performed simultaneously. This protocol was named the "single-stage scheme", in which ricinoleic acid, polyglycerol and both lipases are present in the reactor from the beginning of the process.

Comparison of synthesis protocols using free lipases

It was first checked that is possible to obtain PGPR using the two novel schemes as alternatives to the normal-reaction scheme. Experiments were carried out in a stirred discontinuous tank reactor, using suspended lipases as catalysts. Reaction conditions were those optimised in previous studies.[7-9] The amount of the substrates was calculated based on their average molecular weights (determined by GPC for PR and specified by the supplier in the case of PG) with the aim of obtaining an average of two molecular units of

PR per molecular unit of PG, meaning that the esterification of two of the five hydroxyl groups of polyglycerol had occurred.

When the reverse-reaction and single-stage schemes were carried out, ricinoleic acid and polyglycerol-3 made up the initial mixture, so that the initial acid value (148 mg KOH/g) was lower than in the experiment using the normal-reaction scheme, where the only component in the reactor at the initial time was ricinoleic acid (AV = 161 mg KOH/g).

The first step of the normal-reaction scheme to obtain PR finished when AV reached a value of around 40 mg KOH/g, which meant that reaction sample consisted of oligomers of four units of RA. Even if this value was not reached, the second step was started when the AV remained stable.

However, when the reverse-reaction scheme was studied, the end of the first step of the reaction, the esterification of two units of RA with the primary -OH of PG (RA-PG), was established from the molecular weights of the two reactants. Mass average molecular weight (M_w) of RA (581.71 g/mol) was determined by GPC (following the method described in "Experimental"). This value reflects the presence of di- and tri-oligomers in RA. Furthermore, the average molecular weight of PG (246.66 g/mol) was calculated from its percentage in the different oligomers, as provided by the manufacturer (Product data sheet. Polyglycerol-3 (http://www.solvaychemicals.com)). Taking into account these considerations, the end of the first step was established when the AV reached a value of approximately 90 mg KOH/g.

Figure 2 shows the results obtained in experiments following each of the synthesis protocols. Note that the sharp drop in the measured acid value observed in the normal-reaction scheme at t=214 h (from 63.5 mg KOH/g to 58.4 mg KOH/g) was not due to esterification of –COOH groups but to the dilution effect of adding polyglycerol to the reaction mixture. As can be seen, there was a noticeable decrease in AV as a consequence of esterification between the -COOH groups and the -OH groups for both of the novel protocols proposed in this work. It was evident that the least suitable option was to carry out the reaction according to the reverse-reaction scheme. In the other two operating sequences tested, the same acid value (around 30 mg KOH/g) was achieved at the end of both experiments. But, whereas in the normal-reaction scheme the time required to reach such an acid value was 334 hours, the single-stage scheme only needed 312 hours. In addition, this operational method was easier since all substrates and catalysts were placed in the reactor from the outset.

Although none of the reaction sequences provided a PGPR that met the requirements of the EC,[11] the experiments with lipases in solution were useful for showing that the novel sequences proposed were viable alternatives for improving the procedure for the enzymatic synthesis of PGPR.

Comparison of synthesis protocols using immobilised lipases

In an attempt to improve the results obtained and in light of the well known advantages of using immobilised enzymes, the three reaction sequences were carried out with immobilised lipases. In previous studies the authors described a procedure for immobilising *Candida rugosa* lipase[5,6] and *Rhizopus oryzae* lipase[9] through adsorption on the ion exchange resin, Lewatit MonoPlus MP 64. This immobilisation procedure provided

immobilised derivatives with high activity and stability, which can be used as biocatalysts for the reactions to produce PR and PGPR. These immobilised derivatives were used in the normal-reaction and the reverse-reaction schemes. However, in the case of the single-stage reaction, both lipases were co-immobilised, which is easier than repeating the immobilisation process separately for each enzyme and adding the two immobilised derivatives together to the reactor.

The results obtained for the three immobilisation procedures performed (with CRL, with ROL, and simultaneously with both enzymes) are shown in Table 1, where the amount of initial enzyme in solution, the amount of initial protein (determined by Lowry's method) in solution, immobilisation yield and protein content of the immobilised derivative, can also be observed. As can be seen, commercial preparations of CRL and ROL contained only 18.3% and 14.5% of protein, respectively. When both lipases were dissolved together for the co-immobilisation, this value increased slightly to 21.4%, pointing to an increase in the solubility of one or both lipases. Since the authors have previously described the difficulty of dissolving the CRL enzyme[5], this increase was attributed to the better solubility of CRL as a consequence of the different environment caused by the presence of another enzyme.

In addition, the enzyme load of the co-immobilised derivative was higher than that obtained in each of the individual immobilisation procedures, leading to a higher immobilisation yield. This may have been due to the greater affinity of each lipase for different binding sites in the support: the higher the quantity of support, the greater the area available to retain the lipase. The amount of protein available for immobilisation was higher. Furthermore, a synergistic effect between lipases in the same medium might have existed, promoting immobilisation. However, this increase in the amount of immobilised

protein compared with the protein obtained in individual immobilisations may not be the same for both enzymes and would produce an immobilised derivative in which the proportion of lipases differed substantially. If this is the case, using the co-immobilised derivative to obtain PGPR would result in an incomplete reaction.

Once all the derivatives had been immobilised, the three reaction sequences were carried out using them as catalysts. The results are shown in Figure 3. It can be seen that in the single-stage scheme the acid number of the reaction medium gradually decreased with time, confirming that the co-immobilised derivative contained a sufficient amount of both lipases and neither had been preferentially adsorbed. Furthermore, the acid number reached at the end of the two-step experiments was very similar, unlike in the case of lipases in solution, and was lowest for the single-stage scheme. The main difference between the three procedures lay in the reaction time required to achieve these values. For the normal-reaction and reverse-reaction schemes more than 380 hours were required to reach an acid value of 25 mg KOH/g, whereas only 189 hours were needed to achieve a value of 20 mg KOH/g in the single-stage scheme, reaching 17.8 mg KOH/g after 266 hours.

It was mentioned above that the European Commission Directive 2008/84/EC[11] establishes an acid value lower than 6 mg KOH/g as a requirement for PGPR. In all the experiments described until now the final acid value was far from this objective, which means that the equilibrium had to be shifted towards esterification, which can be carried out using a more anhydrous reaction medium.

Comparison of the synthesis protocols in a vacuum reactor

In order to decrease the water concentration in the reaction medium a vacuum reactor was used. This reactor, which is thermostated with a glass jacket, works over a wide range of pressures and is able to mix the reaction medium despite its high viscosity. The amount of water can be controlled by means of low pressure and the input of dry nitrogen. The drying time for the normal-reaction and reverse-reaction schemes were stabilised taking into account previous studies of the normal-reaction scheme.[6,9] That is, for the lineal esterification of RA catalysed by CRL dry nitrogen was supplied during the first 8 hours, hermetically closing the reactor for the rest of the process. However, in the case of the esterification of RA or PR with PG using ROL, there was a continuous input of dry nitrogen through the experiment. The reason for this difference is the different amount of water needed for each lipase to express its biosynthetic activity. The concentrations of enzyme solutions to immobilise catalysts were higher than those used in the open-air reactors in order to achieve higher reaction rates and conversions. Table 2 shows details of the immobilised derivatives used in the vacuum processes.

In the case of the single-stage scheme, it was more difficult to establish the time during which dry nitrogen had to be supplied because of the different behaviours of the lipases involved in the process and because they were working simultaneously. Although a very anhydrous medium was required for the esterification reaction of PG with RA and PR molecules, it could not be too anhydrous for CRL to maintain its activity. Accordingly, drying was carried out discontinuously with a time distribution based on maintaining the amount of water in the reaction medium lower than 3000 ppm (allowing the reaction to proceed towards esterification) but higher than 1000 ppm (to avoid any loss of CRL activity). Moreover, at the start of the reaction the drying time should be longer due to the

high amount of water from the immobilised derivative and that produced because of the faster initial rate of the reaction.

Figure 4 shows the results of PGPR synthesis using both immobilised lipases and the co-immobilised derivative following the three reaction sequences and operating in the vacuum reactor. It can be seen that all three of the proposed reaction protocols produced a PGPR that complied with EC specifications[11], that is, an acid value lower than 6 mg KOH/g. The time required to obtain PGPR following the normal-reaction and single-stage schemes was very similar (approximately 290 hours), while for the reverse-reaction scheme a longer reaction time (approximately 320 hours) was needed.

The main disadvantage of the one-stage process was the difficulty of estimating the extent of each reaction, whereas the normal two-step process permits the progress of each stage to be determined with greater confidence.

Characterization of the PGPR obtained

Polyglycerol polyricinoleate as a food additive has to comply with a series of purity specifications set by the EC,[11] which are listed in Table 3. The same table shows the acid number, hydroxyl value and refractive index at 65 ºC of the PGPR obtained in this work using the three reaction sequences. As can be seen, the PGPR obtained met all of the legal specifications. In addition to the specifications established by the EC for this food additive, the European Society of Manufacturers of Emulsifiers has its own recommendations of purity. These recommendations (among them the iodine value) are included in the Food Chemicals Codex (FCC) of 2004.[13] The iodine value indicates the degree of unsaturation of the final product and is connected with its emulsifying properties. The iodine value does

not change during the reaction to obtain PGPR because the double bonds in the fatty acid chain do not play any part in PR production or in its esterification with PG. The iodine value stated in the FCC and those obtained for the PGPR produced in this work are listed in Table 2. It is evident that, under the operational conditions used for the three reaction sequences, the double bond of PR was not altered. Therefore, there were no side reactions, such as cyclation reactions or oxidation of the double bond, which would have produced secondary products and imparted undesirable organoleptic characteristics to the final product.

Gel permeation chromatography. Average molecular weight

Gel permeation chromatography (GPC) was used to determine the number average molecular weight, weight average molecular weight and polydispersity index of several reaction samples obtained during PGPR production using the three reaction sequences.

Figure 5 shows the superimposed chromatograms of three samples taken as the normal-reaction scheme progressed. The first peak from the right in Figure 5A corresponds to the RA and the first peak from the left is PR (reactant and product of the first step, respectively). In Figure 5B, the first peak from the right corresponds to PR and the first peak from the left is PGPR (representing the second step of the process). Likewise, Figure 6 shows the relevant chromatograms for the reverse-reaction scheme, the first step in Figure 6A and the second step in Figure 6B. Finally, Figure 7 shows chromatograms of compounds obtained in the single-stage scheme, where the first peak from the right represents RA and the first from the left PGPR. Table 4 shows the average molecular weights (M_n and M_w), as well as the polydispersity index, determined for every reactant and product involved in the different protocols (RA, PR, RA-PG and PGPR).

As shown in Figure 5A and Figure 7, the ricinoleic acid used as substrate predominantly contained monomer, but also other oligomers such as dimer and trimer. The proportion of each oligomer was determined from the areas under the curves. It was seen that the commercial RA had 74% of monomer, which was slightly lower than the value indicated by the supplier (80%). From the GPC data of PR (Fig. 5A), M_n and M_w were obtained (Table 3). The M_w was used, as mentioned above, to estimate the mass ratio of substrates as well as the end point of the first stages for both the normal-reaction scheme and the reverse-reaction scheme.

In the chromatograms of the second stage of the normal-reaction scheme (Figure 5B), the peaks were not resolved by the column used, but the main peak shifted towards lower retention time, which reflects the increase in the average molecular weight of the polymer, whose final value is shown in Table 3.

As regards the chromatograms obtained from samples taken during the first stage of the reverse-reaction scheme (esterification of PG by one or more molecules of RA) (Figure 6A), the polymers obtained, whose M_n and M_w are shown in Table 3, had a high polydispersity index (r = 1.46). On the other hand, in Figure 6B, which shows the chromatograms corresponding to the second stage of this procedure, it is observed that, as the reaction products had a more homogeneous composition, and the final product obtained had a polydispersity index, r = 1.17, slightly higher than in the normal-reaction scheme.

Chromatograms from the single-stage scheme (Figure 7) showed the great variety of compounds that were originated during the first few hours of the reaction, which may be

oligomers of RA and/or PG-RA esters. As the reaction progressed, these compounds were combined to form a polymer with M_n and M_w values higher than those obtained for products synthesized by the two-step sequences.

CONCLUSION

In an attempt to improve the two-step biosynthesis of the food additive PGPR, previously described by the authors, two novel reaction procedures are proposed. The results presented in this paper illustrate the potential of producing PGPR following the reverse-reaction scheme (esterification of PG with RA using *Candida rugosa* lipase, followed by lineal esterification of RA with *Rhizopus oryzae* lipase) as well as the single-stage scheme (adding both substrates and both lipases at the beginning). The success achieved with these two schemes was undoubtedly due to the specificity of the two different lipases used. In previous studies, it was shown that CRL is not capable of catalysing the esterification of RA or PR with PG, while ROL, as most fungal lipases, is 1,3-specific and so incapable of catalysing the condensation of RA.

Studies carried out using lipases in solution underline the potential of the alternative sequences although it would clearly be more profitable to operate with immobilised enzymes if the operation is scaled up. Both lipases are immobilised by adsorption on Lewatit MonoPlus MP 64 and co-immobilised to carry out the one-step reaction more easily.

The three reaction sequences with immobilised enzymes provided encouraging results, since in all cases the acid number decreased, and in the single-stage scheme a value of 18 mg KOH/g was reached after 350 hours of reaction. However, none of the products

obtained using open-air reactors, complied the standards established by the European Commission. Therefore, a vacuum reactor with a drying nitrogen flow is necessary to shift the equilibrium towards synthesis. Using this reactor, it was demonstrated that each lipase has its own water-content requirements (higher for CRL and lower for ROL) although the water content of the reaction medium should be maintained in all cases between 1000 and 3000 ppm. Under these conditions, PGPR reaches AV, HV and n_{65}^{D} values within the limits established by the European Directive. Furthermore, the IV pointed to no secondary reactions which would result in the rupture of the double bond present in the fatty acid, hence fulfilling recommendations provided by the FCC.

The results obtained under controlled conditions in the vacuum reactor showed that none of the three reaction sequences tested is significantly better than the others. In fact, when the reverse-reaction scheme was first proposed, it was considered that the esterification of molecules of PG by RA would be less sterically hindered than by molecules of PR and that the subsequent lineal esterification of RA with the produced PG-RA would be straightforward since it would be possible to obtain polymers with higher molecular weight. However, the results reported in this paper show that this reaction procedure neither increases the reaction rate nor reduces the final acid number. Furthermore, the single-stage scheme does not provide any improvement over the normal sequence reaction apart from the easier operational process.

Finally, GPC analyses of intermediate and final products from each reaction sequence show that the synthesised products have weight average molecular weights between 3000 and 3500 g/mol, with polydispersity indexes approaching unity, indicating that the

polymers obtained are uniform. The highest molecular weight was obtained with the single-stage scheme.

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Table 1. Immobilised derivatives obtained for use as catalysts in open-air reactions, corresponding to individual immobilisations (5 g of support and 50 ml of enzyme solution, 10 mg/ml) and to co-immobilisation (10 g of support and 100 ml of enzyme solution, 10 mg/ml). Values show mean±SD.

Lipase	Protein content (%)	Immobilisation yield (%)	Immobilised protein (mg prot/g support)
CRL	18.3 ± 0.6	40.3 ± 3.3	7.37 ± 0.41
ROL	14.5 ± 0.5	40.7 ± 5.8	5.90 ± 0.41
CRL + ROL	21.4 ± 0.5	45.2 ± 4.5	9.68 ± 0.61

Table 2. Immobilised derivatives obtained for use as catalysts in vacuum reactions, corresponding to individual immobilisations (5 g of support and 25 ml of enzyme solution, 50 mg/ml) and to co-immobilisation (10 g of support and 50 ml of enzyme solution, 50 mg/ml). Values show mean±SD.

Lipase	Protein content (%)	Immobilisation yield (%)	Immobilised protein (mg prot/g support)
CRL	18.6 ± 0.5	38.5 ± 0.2	17.90 ± 0.72
ROL	15.1 ± 1.1	39.1 ± 1.3	14.76 ± 0.6
CRL + ROL	20.9 ± 0.9	44.3 ± 0.2	23.13 ± 1.13

Table 3. Characterisation of PGPR obtained in vacuum reactions following each of the three processes studied and values specified for this food additive. Experimental values show mean±SD.

	European Directive	FCC	PGPR "normal-reaction sequence"	PGPR "reverse-reaction sequence"	PGPR "one-stage reaction"
AV (mg KOH/g)	≤ 6	≤ 6	5.31 ± 0.36	5.11 ± 0.06	3.62 ± 0.12
HV (mg/KOH/g)	80 - 100	80 - 100	89.16 ± 13.17	87.03 ± 12.17	91.52 ± 18.02
$\mathbf{n_{65}^D}$	1.4630 - 1.4665	1.4630 - 1.4670	1.4655	1.4655	1.4655
IV (g I_2/100 g)	-	72 - 103	74.96 ± 3.47	76.04 ± 5.24	77.91 ± 4.81

Table 4. Number and weight average molecular weights and polydispersity index corresponding to substrates and products of every enzymatic reaction involved in the three sequences studied to obtain PGPR (RA, PR, RA-PG and PGPR).

"Normal-reaction sequence"	First reaction step						Second reaction step		
	RA			PR			PGPR		
	M_n	M_w	r	M_n	M_w	r	M_n	M_w	r
	438	582	1.33	1685	2103	1.25	2728	3051	1.12

"Reverse-reaction sequence"	First reaction step						Second reaction step		
	RA			RA-PG			PGPR		
	M_n	M_w	r	M_n	M_w	r	M_n	M_w	r
	438	582	1.33	745	1089	1.46	2642	3102	1.17

"One-stage reaction"	Single step					
	RA			PGPR		
	M_n	M_w	r	M_n	M_w	r
	438	582	1.33	2983	3539	1.19

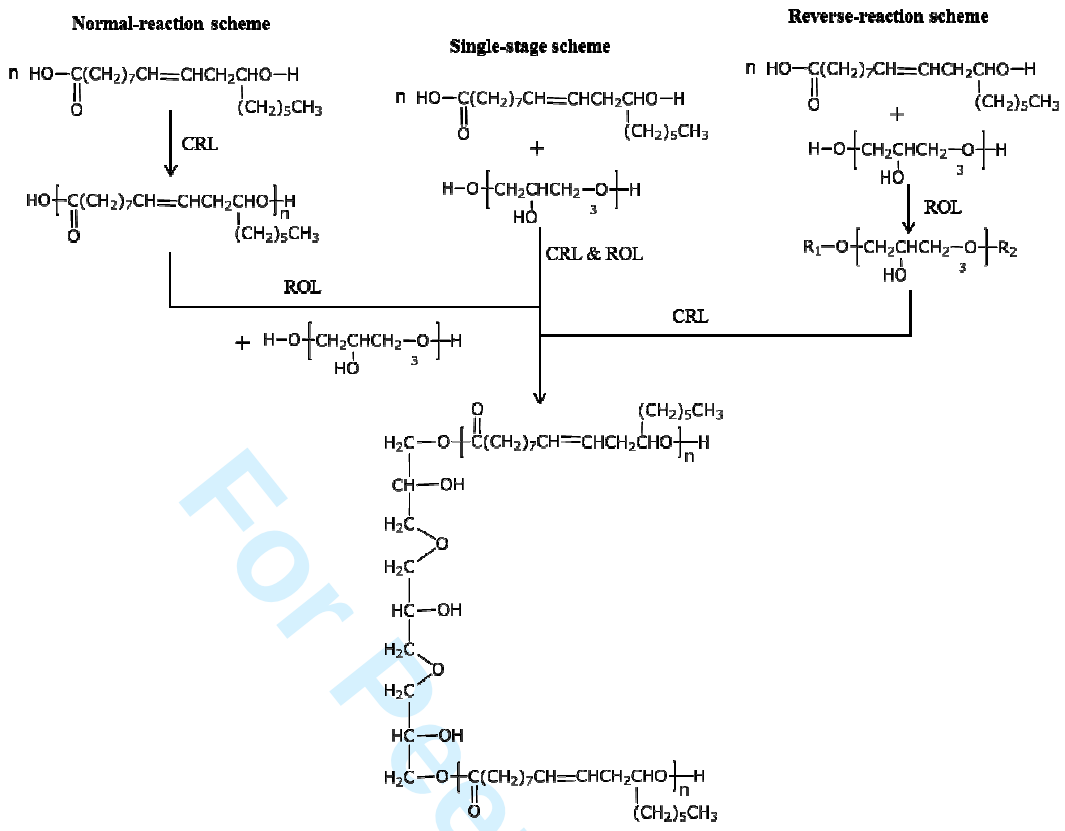


Figure 1. Schematic representation of every reaction sequence studied for the enzymatic production of PGPR.

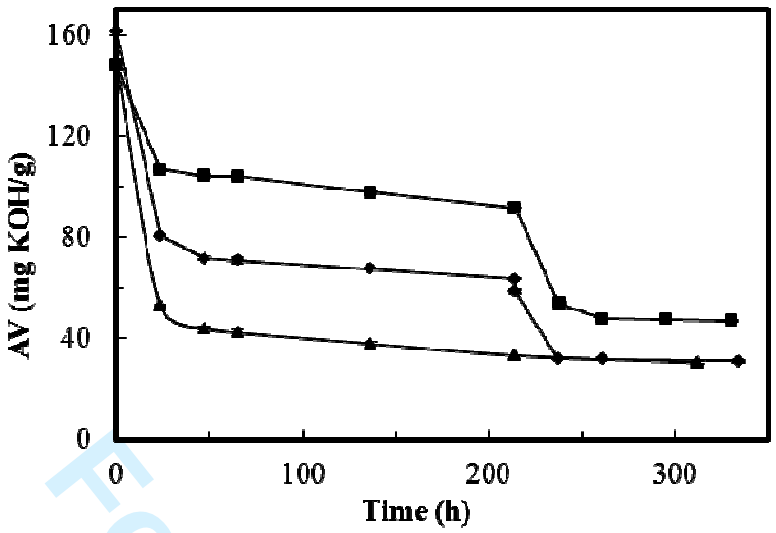


Figure 2. Time course of PGPR synthesis in open-air reactors, using free lipases (0.1 g/ml): (♦) normal-reaction scheme, (■) reverse-reaction scheme, (▲) single-stage scheme. The errors bars are not clearly visible due to the very low value of the standard deviation.

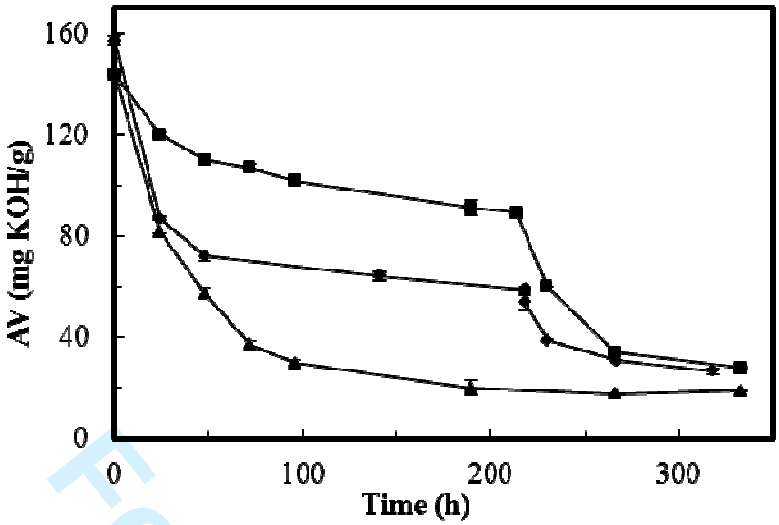


Figure 3. Time course of PGPR synthesis in open-air reactors, using immobilised lipases (characteristics of immobilised derivatives shown in Table 1): (♦) normal-reaction scheme, (■) reverse-reaction scheme, (▲) single-stage scheme.

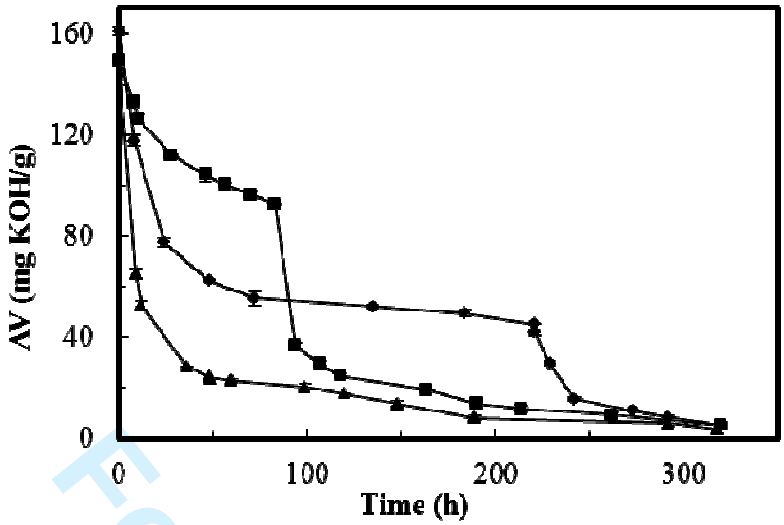


Figure 4. Time course of PGPR synthesis in a vacuum reactor, using immobilised lipases (characteristics of immobilised derivatives shown in Table 2): (♦) normal-reaction scheme, (■) reverse-reaction scheme, (▲) single-stage scheme.

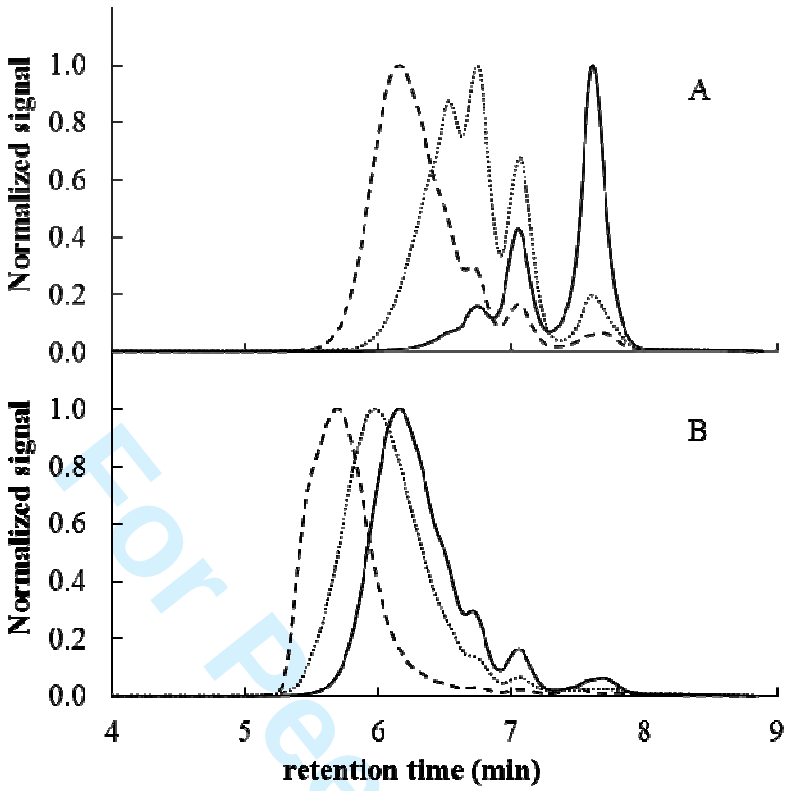


Figure 5. Superimposed chromatograms of three reaction samples for each step of the normal-reaction scheme carried out in a vacuum reactor. (A) First step: continuous line corresponds to ricinoleic acid, dotted line to a sample taken after 26 h and dashed line to a sample from the final first step (PR, 240 h). (B) Second step: continuous line corresponds to PR, dotted line to a sample taken after 264 h and dashed line to a sample from the final PGPR (after 331 h).

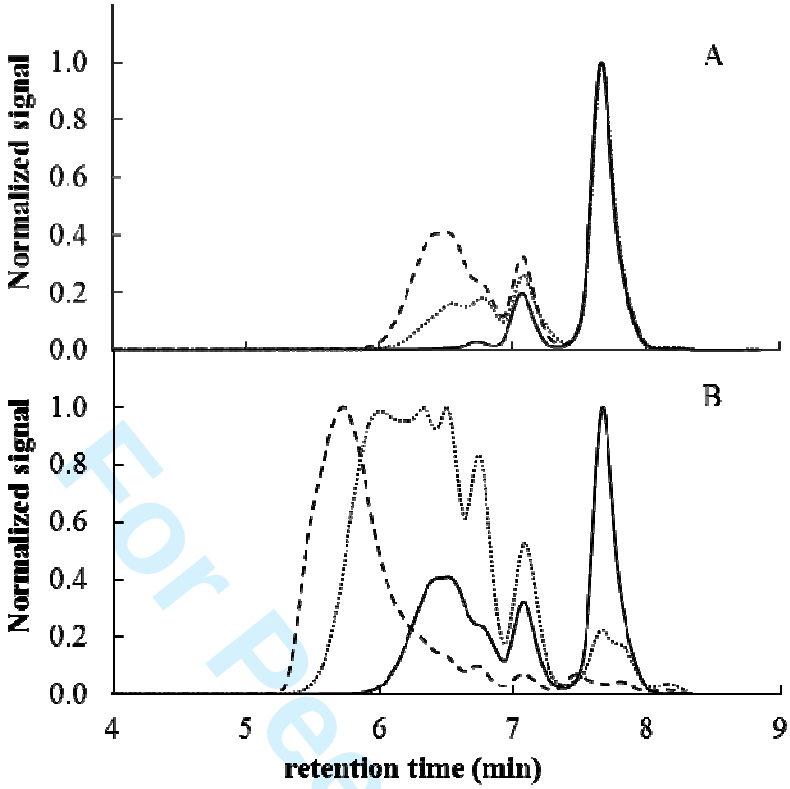


Figure 6. Superimposed chromatograms of three reaction samples for each step of the reverse-reaction scheme carried out in a vacuum reactor. (A) First step: continuous line corresponds to ricinoleic acid, dotted line to a sample taken after 28 h and dashed line to a sample from the final first step (82.5 h). (B) Second step: continuous line corresponds to the final sample from the first step, dotted line to a sample taken after 107 h and dashed line to a sample from the final PGPR (after 320 h).

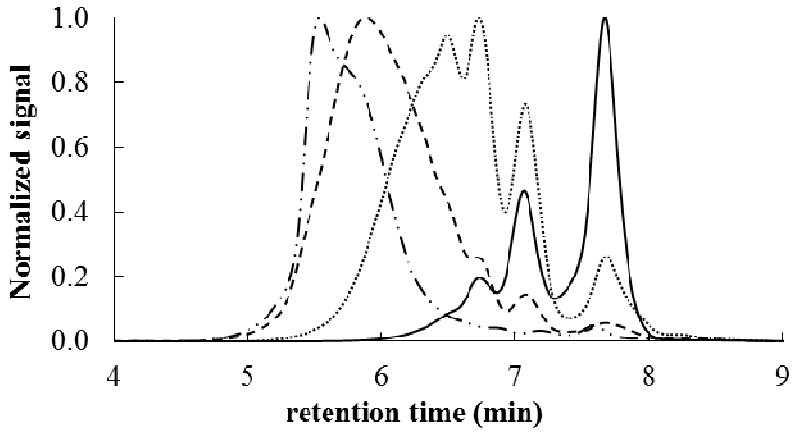
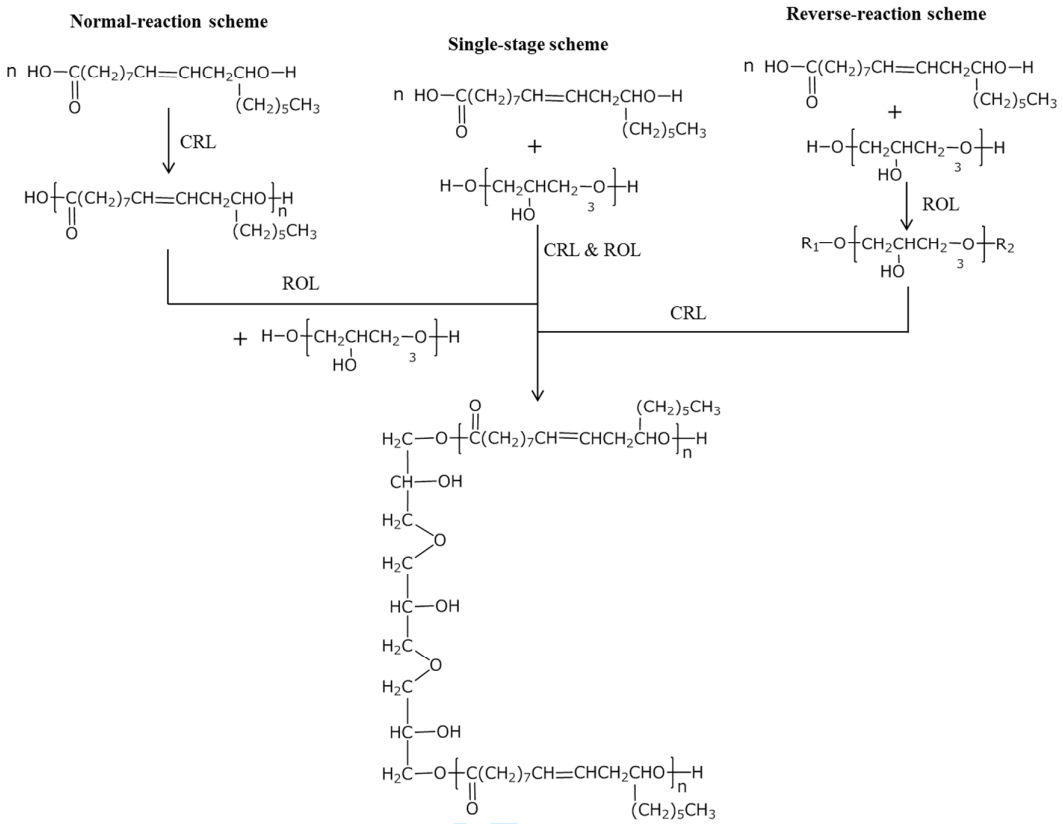


Figure 7. Superimposed chromatograms of four reaction samples for the single-stage scheme carried out in a vacuum reactor. Continuous line corresponds to ricinoleic acid, dotted line to a sample taken after 7.5 h, dashed line to a sample after 60 h and double-dotted-dashed line to the final PGPR (after 318 h).



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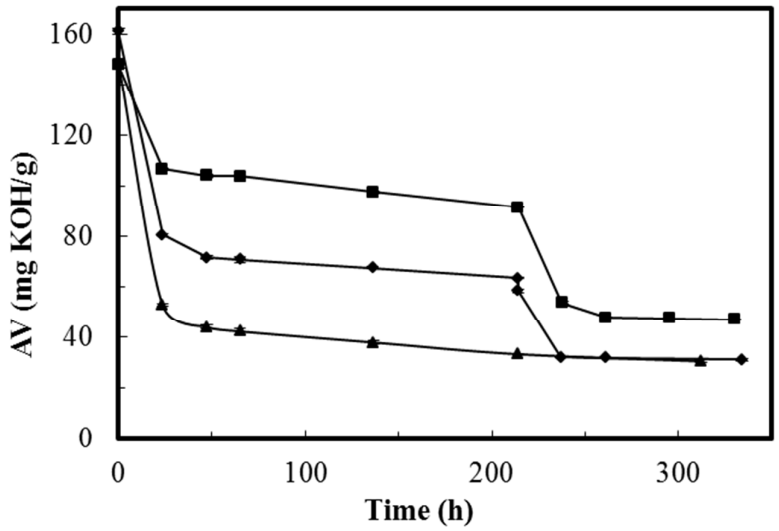

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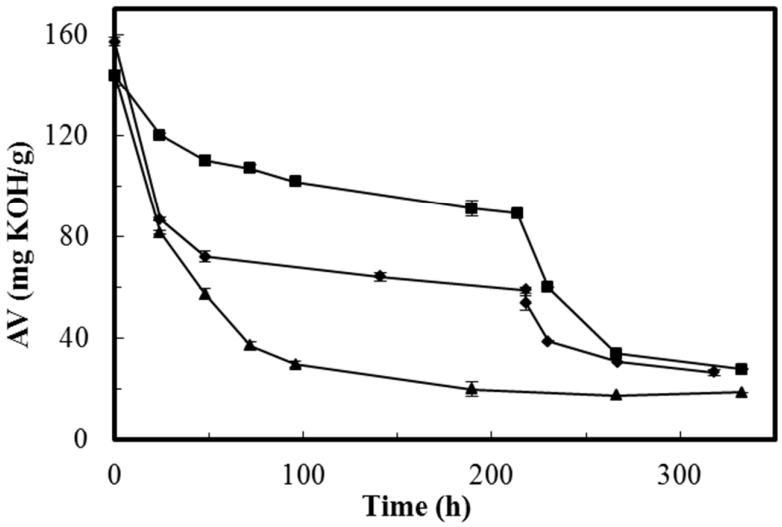

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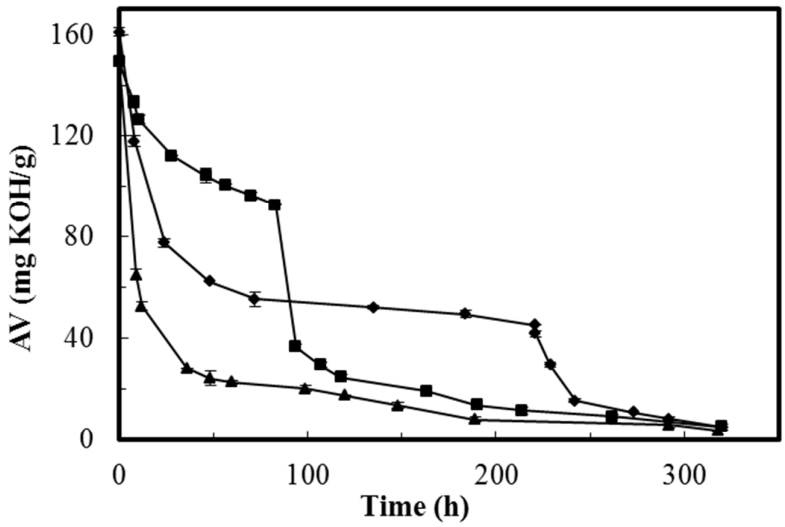
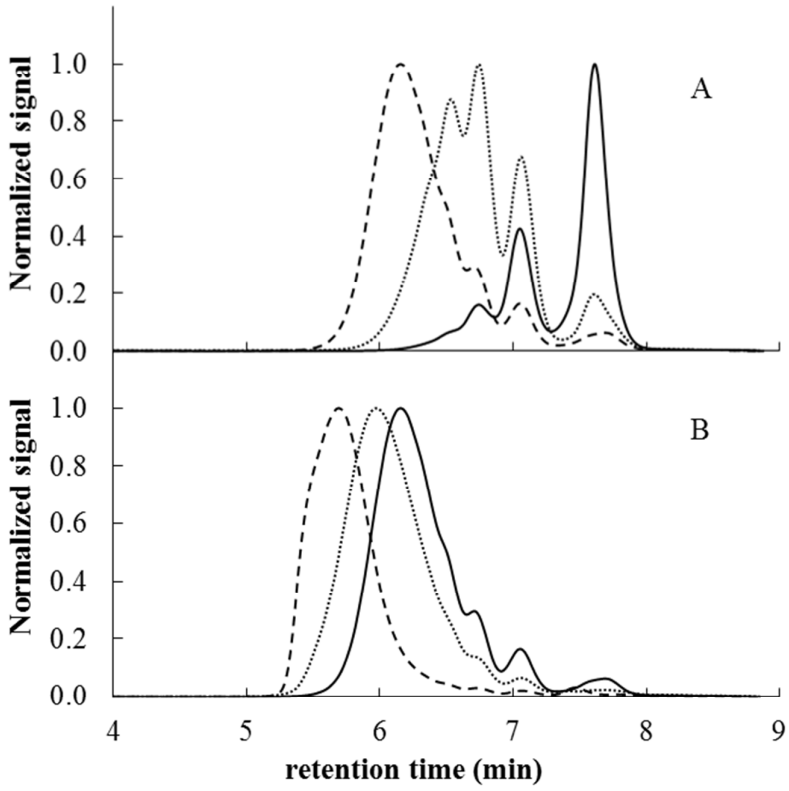


Figure 4. Time course of PGPR synthesis in a vacuum reactor, using immobilised lipases (characteristics of immobilised derivatives shown in Table 2): (♦) normal-reaction scheme, (■) reverse-reaction scheme, (▲) single-stage scheme.



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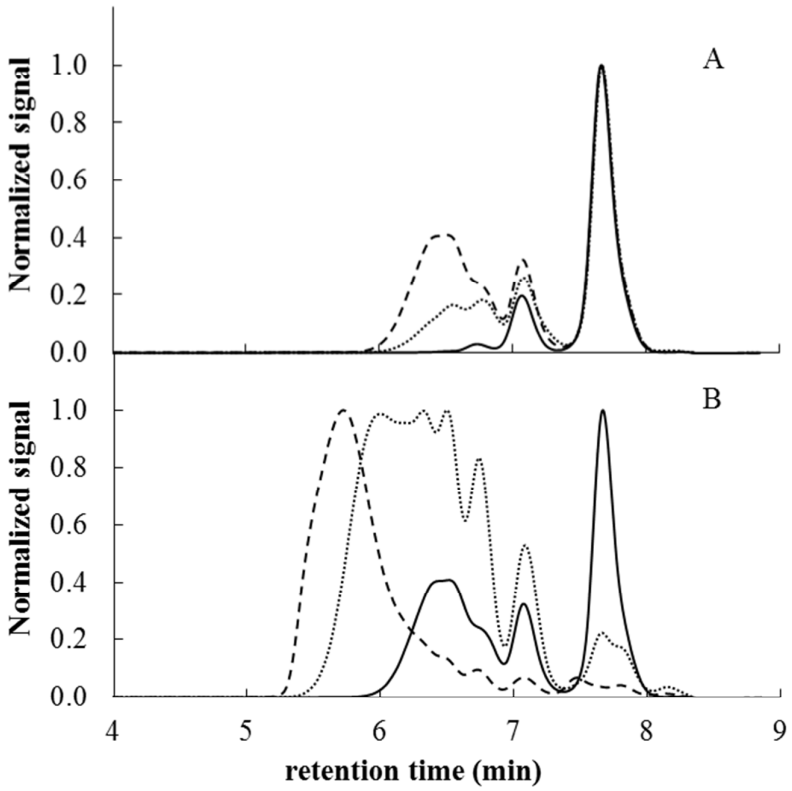

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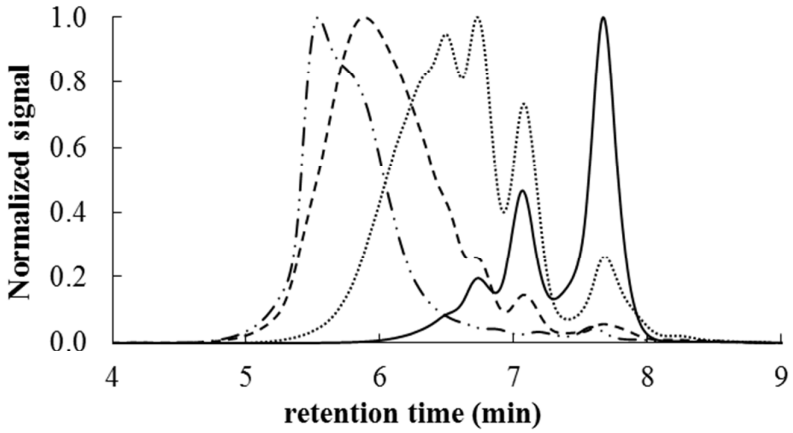

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