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Title: SCREENING AND SELECTION OF LIPASES FOR THE ENZYMATIC PRODUCTION OF POLYGLYCEROL POLYRICINOLEATE

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Keywords: Polyglycerol polyricinoleate; lipase; immobilized enzyme; biocatalyst; biosynthesis

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Abstract: We have demonstrated, for the first time, that polyglylcerol polyricinoleate (PGPR) can be synthesised using lipases as biocatalyst with very good results. Of the twenty one lipases screened for their ability to catalyse PGPR production from a mixture of polyricinoleic acid and polyglycerol-3, only twelve lipases were able to catalyse the reaction. All of them were from microbial sources (bacteria and fungi) and were 1, 3-specific or "random" lipases. The selection procedure was based not only on the enzymatic activity but also on economic criteria. Lipases from Mucor javanicus, Rhizopus arrhizus and Rhizopus oryzae were finally chosen, and all three enzymes were successfully immobilized by adsorption onto an anion exchange resin where they showed their suitability to catalyse the synthesis of PGPR. This represents a promising starting point for developing an industrial process for the green production of polyglycerol polyricinoleate.

Dear Editor:

In reference to the submission BEJ-D-09-00216 these are the answer to the reviewer 1:

1. Materials section: the average degree of polymerization for oligo(ricinoleic acid) should be given in Sect 2.3 Similarly, for the reaction between triglycerol and oligo(ricinoleic acid), the (average) mole ratio between the two substrates should be given (Sect. 2.6)

Average degree of polymerisation has been included in sections 2.3 and 2.6.

2. Sect 2.2: What specifically is meant by "activation"?

The immobilization method was previously described and optimised in reference 17. In the previous article it was established that, when support is treated with an aqueous solution of soy lecithin, the resulting enzymatic derivative does not contains more enzyme but this is more active.

3. Sect. 2.5: Why was a pH of 5 chosen? Please add details to the manuscript.

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4. Footnotes for tables: For Table 1, please include a footnote to explain "Added amount (mg)" (Added to "what"?) for Table 3, add a footnote to explain "[i]mmobilization yield". Is this on a "per mg of protein", or a "per mg of enzyme preparation" basis? I also suggest that "lipase(s) be replaced by "free lipase(s)" in the titles to Tables 1+2 to add clarity.

In Table 1 “Added amount (mg)” has been substituted by “Enzyme added to the reactor (mg)”.

In Table 3 the footnote has been added.

In Tables 1 and 2 the word “free” has been added to the title.

5. For the Fig 1 caption, why are the amounts of lipase added given for some, but not all, of the lipases?

In caption to Figure 1, when two lipases from the same source are used, the activity is given to identify them. It is the case of *Pseudomonas fluorescens* and *Pseudomonas* sp.

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The sentence has been changed in the sense that the reviewer suggests.

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SCREENING AND SELECTION OF LIPASES FOR THE ENZYMATIC PRODUCTION OF POLYGLYCEROL POLYRICINOLEATE

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KEYWORDS: Polyglycerol polyricinoleate; lipase; immobilized enzyme; biocatalyst; biosynthesis.

ABSTRACT

We have demonstrated, for the first time, that polyglylcerol polyricinoleate (PGPR) can be synthesised using lipases as biocatalyst with very good results. Of the twenty one lipases screened for their ability to catalyse PGPR production from a mixture of polyricinoleic acid and polyglycerol-3, only twelve lipases were able to catalyse the reaction. All of them were from microbial sources (bacteria and fungi) and were 1, 3-specific or "random" lipases. The selection procedure was based not only on the enzymatic activity but also on economic criteria. Lipases from *Mucor javanicus, Rhizopus arrhizus* and *Rhizopus oryzae* were finally chosen, and all three enzymes were successfully immobilized by adsorption onto an anion exchange resin where they showed their suitability to catalyse the synthesis of PGPR. This represents a promising starting point for developing an industrial process for the green production of polyglycerol polyricinoleate.

1. INTRODUCTION

Enzymes are widely used as biocatalysts for a wide range of biotechnological applications [1]. Lipases, for example, are enzymes (EC 3.1.1.3), that are frequently used to catalyze the hydrolysis of fatty acid esters in an aqueous environment. The lipase-catalyzed hydrolysis in water can be easily reversed in non-aqueous media or in media with a very low water content, in ester synthesis or transesterification reactions [2]. It has been reported that polyesters of polyricinoleic acid and polyol acyl acceptors can be synthesized enzymatically by the action of several lipases [3], but the literature contains no mention of the enzymatic synthesis of polyglycerol polyricinoleate.

Polyglycerol polyricinoleate, PGPR, is an additive (E-476) widely known as an excellent water-in-oil emulsifier in the food industry, because it forms very stable emulsions even when the water content is very high, such as 80%. Therefore, PGPR is used as emulsifier in tin-greasing emulsions for the baking trade [4], and for the production of low-fat spreads [5, 6]. However, the main application of PGPR is in the chocolate industry, where, besides its action as an emulsifier, it also has important properties as a viscosity modifier, and thus improves the moulding properties of the molten chocolate [7]. An additional property of PGPR in chocolate is its ability to limit “fat bloom” [8-10].

Known chemical methods for preparing this emulsifier involve the autocatalytic condensation of ricinoleic acid and alkali-catalysed reaction between the condensed ricinoleic acid and polyglycerol to give polyglycerol polyricinoleic fatty acid esters. However, these reactions require long reaction times and thus involve a large outlay in the form of high energy costs. This, together with the high operating temperatures, adversely affect the quality of the final product, which could, in turn, lead to problems of coloration and odors that could make it inadvisable for the food industry [11-15].

As an alternative, the authors of this contribution are developing the enzymatic synthesis of PGPR by the catalytic action of one or more lipases, which act in mild reaction conditions of temperature and pressure, neutral pH and in a solvent-free system, which makes the process environmentally friendly. The enzymatic procedure consists of two steps. First the ricinoleic acid is polymerized by the action of a *Candida rugosa* lipase to obtain the estolide (also called polyricinoleic acid, PR), in a process that has been optimized by the authors [16-18]. Then, the polyricinoleic acid obtained is esterified with polyglycerol-3 (PG-3). It was hypothesised that this second step could also be catalysed by a lipase, and so, the first aim of this work was to identify the best lipase to carry out the esterification reaction. Since the enzymatic synthesis of polyglycerol polyricinoleate has not been described elsewhere, it was necessary to exhaustively screen lipases by testing the greatest possible number from different sources, including both 1,3-specific and random lipases. In this paper, 21 lipases from different sources have been used: 9 from fungi, 6 from bacteria, 4 from yeasts, 1 from plants and 1 of mammalian origin. The selection criteria were based on enzymatic activity and on the price of the commercial enzyme preparations.

2. MATERIALS AND METHODS

2.1. Enzymes

"Lipase basic kit" was purchased from Fluka. This kit contains lipases from different sources: *Aspergillus* (culture not specified) (0.2 U/mg solid), *Candida antarctica* (2.9 U/mg solid), *Candida cylindracea* (3.85 U/mg solid), *Mucor miehei* (1.4 U/mg solid), *Pseudomonas cepacia* (46.2 U/mg solid), *Pseudomonas fluorescens* (36 U/mg solid), *Rhizopus arrhizus* (9.18 U/mg solid), *Rhizopus niveus* (1.7 U/mg solid) and porcine pancreas (20.6 U/mg solid).

"Lipase extension kit" was acquired from Fluka. This kit includes lipases from different sources: *Aspergillus oryzae* (48 U/mg solid), *Candida lipolytica* (0.0011 U/mg solid), *Mucor javanicus* (11.6 U/mg solid), *Penicillium roqueforti* (0.65 U/mg solid), *Pseudomonas fluorescens* (309 U/mg solid), *Rhizomucor miehei* recombinant from *Aspergillus oryzae* (0.51 U/mg solid), wheat germ (0.1 U/mg solid), *Chromobacterium viscosum* (2711 U/mg solid), *Pseudomonas* sp. (2324 U/mg solid) and *Pseudomonas* sp. (Type B) (256 U/mg solid).

Lipase from *Candida rugosa* (Type VI) (819 U/mg solid) was supplied by Sigma-Aldrich. The lipase from *Rhizopus oryzae* (58.4 U/mg solid) was purchased from Fluka.

2.2. Immobilisation support and activator

The immobilisation support, an anionic exchange resin, Lewatit MonoPlus MP 64 was supplied by Fluka and was activated with soybean lecithin of commercial grade from Santiveri.

2.3. Substrates and reagents

The ricinoleic acid estolide (AV≤50 mg KOH/g, which corresponds with an average degree of polymerization ≥ 3.7), also called polyricinoleic acid (PR) was obtained by enzymatic polymerization of ricinoleic acid as described previously [16-18]. Polyglycerol-3 (PG-3) was kindly donated by Solvay and it is a glycerol oligomer based on an average of three glycerol groups (average MW = 250 g/mol). It contains minimum 80% di-, tri- and tetraglycerol and has very low levels of cyclic byproducts. More information about polyglicerol-3 can be found on its product data sheet [19]. Other chemicals were of analytical grade and were used without further purification.

2.4. Measurement of the reaction extension

Acid value (AV) [20] as used as an index to show the reaction degree. The acid value is the number of milligrams of potassium hydroxide necessary to neutralize the free acids in 1 gram of sample. AV corresponds to the carboxyl group concentration in the reaction mixture, which decreases as a result of the esterification of the polyricinoleic acid with the polyglycerol-3.

2.5. Immobilisation of lipases by physical adsorption

One gram of support was mixed with 10 ml of soybean lecithin suspension (20 mg/ml) in an Erlenmeyer flask and placed in an orbital shaker overnight at room temperature. It was then washed with 10 ml of distilled water and transferred to a jacketed column reactor (2.5 i.d. and 30 cm length). The reactor was equipped with a sinterised glass plate placed 5 cm from the bottom. The enzyme solution (10 ml, 10 mg/ml in acetate buffer 0.1 M, pH=5) was then added to the reactor and recycled for two days at 4ºC. The immobilised derivative was washed twice with 25 ml of the same buffer and stored at 4ºC. The immobilisation method has been optimised elsewhere [17]. The protein initially offered and in the wash-liquid after immobilization was determined by Lowry's procedure modified by Hartree [21], using bovine serum albumin as standard. The coupled lipase was the difference between

the initial enzyme added and the enzyme in the wash-liquid. The immobilized derivative soaks 0.6 gram of solution buffer per gram of dry support.

2.6. Synthesis of polyglycerol polyricinoleate with free lipase

The reactions were carried out in a 250 ml jacketed batch reactor at 40ºC and the mass transfer was promoted by a powerful four-bladed impeller stirrer, which was used as mixing device. First, a certain amount of lipase was placed to the reactor and 5 ml of distilled water was poured over the lipase. Next, 30 g of PR (AV≤50 mg KOH/g, which corresponds with an average degree of polymerization ≥ 3.7) and 6 g of PG-3 were added to the reactor, so that the mass ratio PR/PG was 15 (which means that three of the five hydroxyl groups of the polyglycerol could be esterified). As can be seen, the reaction occurs in the absence of solvent and with a limited initial amount of water. Samples were taken from the reactor at given time intervals and the AV of the reaction mixture was determined. All experiments were left to progress for approximately seven days.

2.7. Synthesis of polyglycerol polyricinoleate with immobilized lipase

The enzymatic reactions were carried out in the same batch reactor and under the experimental conditions described in the previous section. The reaction mixture contained 30 g of polyricinoleic acid (AV≤50), 6 g of polyglycerol-3 (mass ratio PR/PG=15) and 5 grams of immobilized derivate. The only water in the reaction system was that soaked in the support (0.6 g of water per g of dry resin). Samples were taken and AV was determined.

3. RESULTS AND DISCUSSION

3.1. Selection of lipases

As mentioned above, the main objective of this work was to find the most suitable lipase to carry out the esterification reaction between polyricinoleic acid and polyglycerol to obtain polyglycerol polyricinoleate, PGPR.

In previous works [16, 22], lipase from *Candida rugosa* was selected to carry out the autocondensation of ricinoleic acid to obtain the estolide, which is the first step in PGPR synthesis. It would obviously be very convenient if the same lipase could serve as catalyst for the two reaction steps, so that, the first lipase used to catalyse the esterification reaction between the polyricinoleic acid and polyglycerol was the lipase from *Candida rugosa.* The obtained results were not satisfying; after 48 hours the decrease of acid value was only 12 mg KOH/g (AV≈42 mg KOH/g at the beginning of the reaction), and the final acid value reached was 30 mg KOH/g. This value is very far from specific purity criteria for PGPR established by the European Commission Directive 98/86/EC [23], in which it is reported that for PGPR to be used as food additive, the acid value must not be higher than 6 mg KOH/g. In light of this result, the lipase from *Candida rugosa* was considered unsuitable for synthesising of PGPR and therefore others lipases were assayed for this purpose.

A further twenty lipases from different sources were used and the corresponding experiments of PGPR synthesis were performed as described in Section 2.6. Table 1 shows the lipases tested, their specific activities (as declared by the manufacturer) and the amounts of protein used in each experiment. It is important to note that many of the lipases were part of two kits and the amount available was limited. In such cases, the total available protein was added to the reactor.

The evolution of the acid value with time during for the enzymatic production of PGPR with the above mentioned lipases is plotted in Figure 1 (A to D). In a first selection, eight lipases were rejected because they were not able to reach acid values lower than 15 mg KOH/g in seven days; they are lipases from *Aspergillus* sp., *Candida antarctica, Candida cylindracea, Candida lipolytica, Penicillium roqueforti*, porcine pancreas, *Rhizopus niveus* and wheat germ.

The lipase from wheat germ exhibited a particular behaviour. When it was tested to produce PGPR, the acid value of the reaction mixture increased, which indicates that polyricinoleic acid is being hydrolysed and, therefore, under the experimental conditions, the hydrolytic activity of this lipase is greater than its synthetic activity.

None of the twelve remaining lipases were able to produce a PGPR with an acid value lower that 6 mg KOH/g, which is the threshold criterion of the European Commission Directive [23], although we considered that, after applying appropriate optimization procedures, one or more of these enzymes might be able to efficiently catalyse the enzymatic synthesis of PGPR.

The twelve chosen lipases were all from microbial sources, being some 1,3-specific and others “random” lipases. It was thought that any acid value decay in the reaction mixture might be due to two possible reactions: (i) the synthesis of estolides with a higher polymerisation degree and (ii) the esterification of polyricinoleic acid with polyglycerol-3 (the desired process). It has been described in the literature that the enzymatic synthesis of estolides can only be successfully catalysed by lipases that lack 1,3-positional selectivity [2, 24, 25], so that lipases from *Chromobacterium viscosum* and from *Pseudomonas* (which are “random” lipases and show the best results, Figure 1 D) should be capable, in theory at least, of catalysing the first step of the enzymatic synthesis of PGPR. However, it was experimentally demonstrated that, under the assayed experimental conditions, these lipases are not capable of catalysing the production of

estolides with an acid value lower than 50 mg KOH/g (data not shown), so that, the noticeable decrease of the acid value observed in the above described experiments can be attributed mainly to the esterification reaction between polyricinoleic acid and polyglycerol. In the case of the reactions catalysed by the remaining lipases tested, there is no doubt about the cause of the decrease of acid value, because they are 1,3-specific and cannot act on secondary -OH groups of hydroxy fatty acids [2].

On the other hand, it may surprise that *Mucor javanicus* and *Rhizopus* sp lipases (1,3 specific) performed so well. If polyglycerol-3 is a linear molecule only two of the five hydroxyl groups available as acyl acceptor groups are primary, and the acid value reached when these lipases are used indicates that more than two hydroxyl groups has been esterified. This fact can be explained if condensation of glycerol takes place between secondary-primary or secondary-secondary hydroxyl groups. In that case more than two primary hydroxyl groups may remain available as acyl acceptor groups.

As can be seen in Figure 1, satisfactory results were obtained when the twelve mentioned lipases were used to catalyze the production of PGPR and some graphs are indistinguishable. Table 2 shows the acid values reached after 7 days of reaction, which permits a better comparison of the obtained results. It can be observed that the lowest acid values were reached when lipases from *Pseudomonas* (3 enzymes) and *Chromobacterium viscosum* were used. However, some of the lipases used in the present work are very expensive, which is an aspect that should be carefully considered if the long-term purpose is to develop an industrial procedure for PGPR production. Therefore, in order to finally choose one or more of these lipases, we took into account not only kinetic aspects (reaction rates and final acid value of the reaction mixture) but also the cost of the procedure.

In order to evaluate this economic aspect of the enzymatic biosynthesis of PGPR and because lipase is the most expensive material involved in the reaction, the cost of biocatalysts that cause a decrease of one unit of the acid

value was calculated and the results are showed in the last column of Table 2. It can be observed that the cheapest procedures were those catalysed by lipases from the fungi *Rhizopus oryzae*, *Rhizopus arrhizus*, *Mucor javanicus*, *Rhizomucor miehei* and *Rhizopus niveus*, with which the decrease of one unit in acid value costs less than 1 €. These results, together with those shown in Figure 1, led us to select lipases from *Rhizopus oryzae*, *Rhizopus arrhizus* and *Mucor javanicus* for further experiments.

3.2. Immobilization of the selected lipases

Although the above selected lipases are not very expensive, to develop an industrial procedure for synthesising PGPR it is desirable to use immobilized enzymes because of the well known advantages: continuous operation of reactors and/or the reusability of the immobilized enzymes, both of which diminish operational costs. Therefore, the three chosen lipases were immobilized by physical adsorption onto an anion exchange resin (Lewatit Monoplus MP64). The authors have previously optimized the immobilization process of *Candida rugosa* lipase (which is the biocatalyst used in the synthesis of polyricinoleic acid, first step of the production of PGPR) [17] and, as a preliminary attempt, the same technique was used in this work in order to compare the behaviour of these three lipases. In further studies the immobilization process should be optimized.

Thus, three immobilized derivatives were prepared following the method described in Section 2.5 and the results are shown in Table 3, where the protein content of the commercial lipases, the immobilization yields and the enzyme loadings of all the immobilized derivatives are summarized, all data being based on the protein concentration values provided by Lowry's method [21]. It should be mention here that the protein content of the three commercial preparations was quite low, although that of the lipase from *Rhizopus arrhizus* was slightly higher than the others. However, the percentage of immobilized protein obtained with this lipase was approximately half that obtained with the other two lipases, and so the

enzyme loading factor of this immobilized derivative was the lowest (8.59 mgE/g support). The highest immobilization yield was achieved when the lipase from *Rhizopus oryzae* was adsorbed; in this case an immobilized derivative with adequate enzyme content was obtained in spite of the low Lowry protein content of the commercial enzyme. The immobilized derivative of lipase from *Mucor javanicus* had the higher enzyme loading, 14.11 mgE/g support.

The above results did not differ sufficiently to permit us to decide at this stage which of the three lipases should be selected. Therefore the immobilized derivatives were tested for activity, using them to catalyse the synthesis of PGPR following the procedure described in Section 2.7. Figure 2 shows the variation of the acid value of the reaction mixtures with time. As can be seen, all the immobilized derivatives showed their ability to catalyse the esterification between polyricinoleic acid and polyglycerol-3. The use of the lipase from *Mucor javanicus* should not be totally discarded because reasonably good results were obtained when it was used as biocatalyst and a PGPR with an acid value of 13 mg KOH/g was reached at the end of the experiment.

The highest reaction rates were achieved when lipases from *Rhizopus arrhizus* and *Rhizopus oryzae* were used and, in these cases, PGPRs with lower acid values were produced. Comparing these results with those obtained with the soluble enzymes, it can be observed that the acid values reached with the immobilized derivatives (10.42 mg KOH/g with lipase from *Rhizopus arrhizus* and 9.22 mg KOH/g with lipase from *Rhizopus oryzae*) were similar to those obtained with the soluble lipases (11.04 mg KOH/g and 13.94 mg KOH/g, respectively), even though the amounts of soluble enzymes added to the reactors (500 mg in both cases) were higher than those used in the experiments with immobilized enzymes (42.95 mg lipase from *Rhizopus arrhizus* and 64.4 mg lipase from *Rhizopus oryzae*). These results suggest that immobilization had a beneficial effect on the activity and stability of both lipases.

It is important to notice that the behaviour of *Rhizopus arrhizus* and *Rhizopus oryzae* lipases was very similar throughout this work. This could be explained by the fact that, taxonomically, *Rhizopus arrhizus* is synonymous with *Rhizopus oryzae* [26, 27]. Therefore, for further studies we will use both lipases indistinctly.

4. CONCLUSIONS

A new enzymatic process to obtain polyglycerol polyricinoleate (PGPR) has been developed. It has been demonstrated that the esterification between polyricinoleic acid (PR) and polyglycerol-3 can be catalysed by several lipases from different sources, either 1, 3-especific lipases or "random" lipases. However, after the screening and selection processes, it was concluded that the most suitable enzymes for PGPR production are the lipases from *Mucor javanicus*, *Rhizopus arrhizus* and *Rhizopus oryzae*. These lipases were successfully immobilized by adsorption onto an anion exchange resin, Lewatit MonoPlus MP 64, and all of them showed their ability to catalyse the reaction under study. The best results were obtained with lipases from *Rhizopus arrhizus* and from *Rhizopus oryzae*. Further studies on the optimization of PGPR biosynthesis are now being carried out with both lipases.

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Table 1.- Free lipases tested to catalyse the enzymatic production of PGPR.

	Enzyme	Source	Activity (U/mg solid) (as declared by the manufacturer)	Enzyme added to the reactor (mg)
BASIC KIT	1	*Aspergillus* sp.	0.20(1)	100
	2	*Candida antarctica*	2.9(2)	50
	3	*Candida cylindracea*	3.85(2)	1000
	4	*Mucor miehei*	1.4(2)	100
	5	*Pseudomonas cepacia*	46.2(2)	100
	6	*Pseudomonas fluorescens*	36(2)	50
	7	*Rhizopus arrhizus*	9.18(3)	500
	8	*Rhizopus niveus*	1.7(4)	1000
	9	Porcine pancreas	20.6(5)	1000
EXTENSION KIT	10	*Aspergillus oryzae*	48(2)	100
	11	*Candida lipolytica*	0.0011(2)	1000
	12	*Mucor javanicus*	11.6(5)	500
	13	*Penicillium roqueforti*	0.65(5)	500
	14	*Pseudomonas fluorescens*	309(2)	50
	15	*Rhizomucor miehei* recombinant from *Aspergillus oryzae*	0.51(2)	50
	16	Wheat germ	0.1(1)	500
	17	*Chromobacterium viscosum*	2711(2)	25
	18	*Pseudomonas* sp.	2324(2)	10
	19	*Pseudomonas* sp. (Type B)	256(6)	50
	20	*Rhizopus oryzae*	58.4(7)	500

(1) 1 Unit corresponds to the amount of enzyme which liberates 1 μmol acetic acid per minute at pH 7.4 and 40 ºC, using triacetine as substrate.
(2) 1 Unit corresponds to the amount of enzyme which liberates 1 μmol oleic acid per minute at pH 8.0 and 40 ºC, using triolein as substrate.
(3) 1 Unit corresponds to the amount of enzyme which liberates 1 μmol butyric acid per minute at pH 8.0 and 40 ºC, using tributyrin as substrate.
(4) 1 Unit corresponds to the amount of enzyme which liberates 1 μmol fatty acid from a triglyceride per minute at pH 7.7 and 37 ºC, using olive oil as substrate.
(5) As (4) but at pH 8.0.
(6) 1 Unit corresponds to the amount of enzyme which liberates 1 μmol oleic acid per minute at pH 8.0 and 37 ºC, using cholesteryl oleat as substrate.
(7) As (4) but at pH 7.2.

Table 2.- Selection of free lipases based on kinetic and economic aspects.

Enzyme(1)	**Source**	**Final AV** (mg KOH/g) after 7 days	**Δ AV**(2)	**Enzyme cost**(3) (€)	**€/unit AV**(4)
4	*Mucor miehei*	8.0	34.0	46.8	1.4
5	*Pseudomonas cepacia*	8.1	33.9	35.7	1.1
6	*P. fluorescens* (36 U/mg solid)	7.2	34.8	35.3	1.0
7	*Rhizopus arrhizus*	11.0	31.0	22.9	0.7
10	*Aspergillus oryzae*	11.4	30.6	85.2	2.8
12	*Mucor javanicus*	8.7	33.3	22.0	0.7
14	*P. fluorescens* (309 U/mg solid)	9.6	32.4	110.5	3.4
15	*Rhizomucor miehei*	9.3	32.7	27.4	0.8
17	*Chromobacterium viscosum*	7.1	34.9	43.4	1.2
18	*Pseudomonas* sp. (2324 U/mg solid)	7.7	34.3	44.6	1.3
19	*Pseudomonas* sp. Type B (256 U/mg solid)	7.6	34.4	43.0	1.2
20	*Rhizopus oryzae*	13.9	28.1	1.8	0.06

(1) Enzyme identification numbers are the same that those used in Table I.

(2) Calculated as the difference between initial AV (42.0) and final AV (3rd column)

(3) Estimated from commercial price lists.

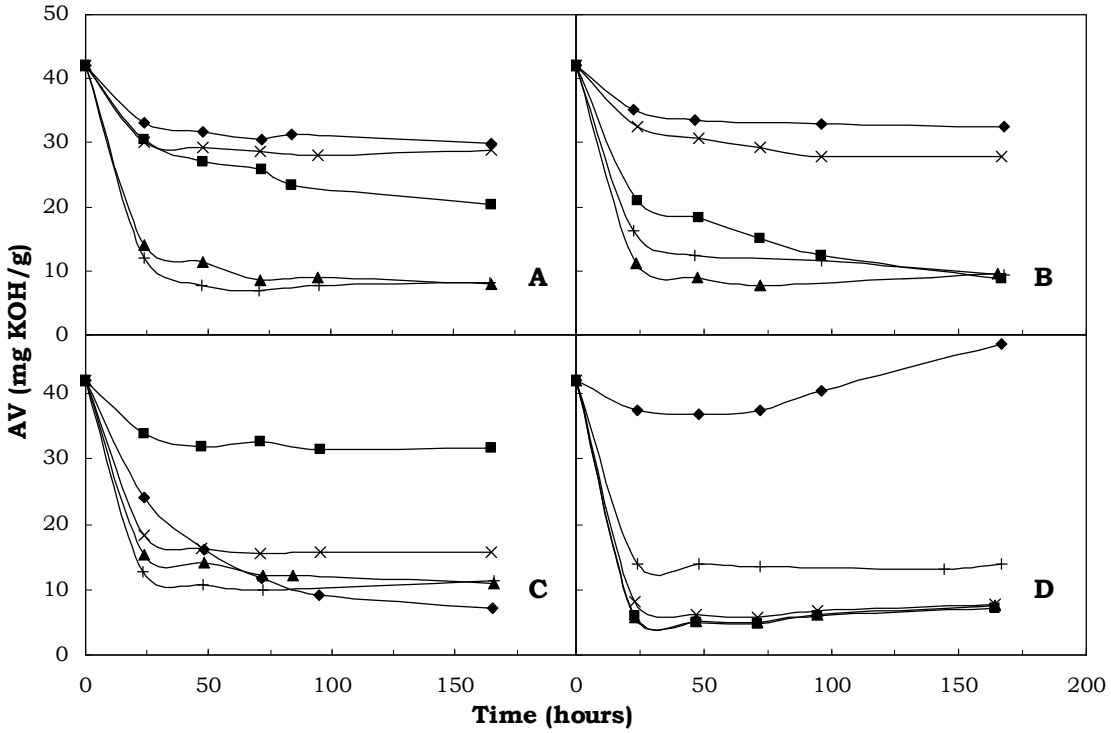
(4) Calculated as column 5 divided by column 4.

Table 3.- Coupling parameters for the immobilization of lipases onto Lewatit Monoplus MP64.

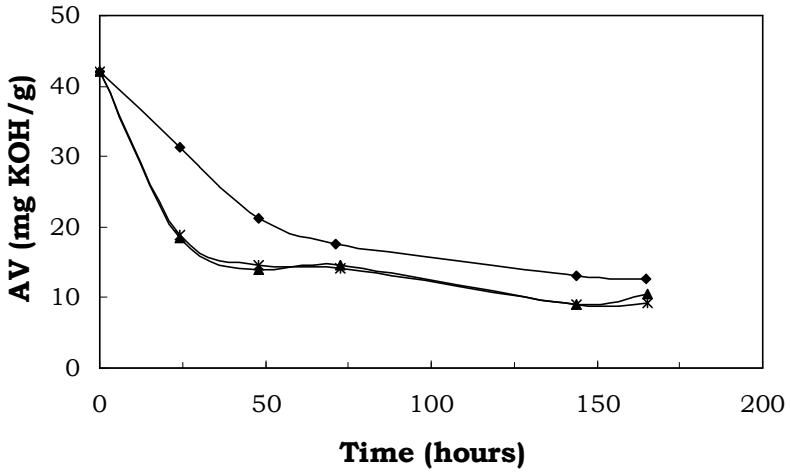
Lipase source	**Protein content of the commercial lipase** (%)	**Immobilization yield (1)** (%)	**Enzyme loading (1)** (mgE/g support)
Mucor javanicus	22.07	63.90	14.11
Rhizopus arrhizus	26.35	32.59	8.59
Rhizopus oryzae	19.80	65.03	12.88

(1) Calculated on the basis of Lowry protein.

Figure(s)



Figure(s)



CAPTION TO FIGURES

Figure 1.- Evolution of acid value with time for the PGPR synthesis catalysed by free lipases from different sources.

(A) ♦ *Aspergillus* sp.; ■ *Candida antarctica;* × *Candida cylindracea*; ▲ *Mucor miehei;* + *Pseudomonas cepacia.*

(B) ♦ *Candida lipolytica*; ■ *Mucor javanicus;* × *Penicillium roquefortii*; ▲ *Pseudomonas fluorescens* (300 units/mg solid)*;* + *Rhizomucor miehei.*

(C) ♦ *Pseudomonas fluorescens* (40 units/mg solid); ■ Porcine pancreas*;* × *Rhizopus niveus*; ▲ *Rhizopus arrhizus;* + *Aspergillus oryzae.*

(D) ♦ Wheat germ; ■ *Chromobacterium viscosum;* × *Pseudomonas* sp. (1200 units/mg solid); ▲ *Pseudomonas* sp Tyme B (≥160 units/mg solid)*;* + *Rhizopus oryzae.*

Figure 2.- Evolution of acid value with time for the PGPR synthesis catalysed by immobilized lipases from different sources. (♦) *Mucor javanicus,* (▲) *Rhizopus arrhizus* and (×) *Rhizopus oryzae.*

Reviewer Suggestions

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