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Covalent Immobilization of *Pseudomonas stutzeri* lipase on a porous polymer: an efficient biocatalyst for a scalable production of enantiopure benzoin esters under sustainable conditions.

Antonio Aires-Trapote,[†,‡] Pilar Hoyos,[†] Andrés R. Alcántara,[†] Aitana Tamayo,[§] Juan Rubio,[§] Angel Rumbero,[‡] and María J. Hernáiz.*[†]*

[†]Department of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, Complutense University of Madrid, Campus de Moncloa, 28040 Madrid, Spain. Fax: (+34) 9139 41822; Tel: (+34) 91139 41821

[‡]Department of Organic Chemistry, Faculty of Science, Autonoma University of Madrid, Campus de Cantoblanco.28049-Madrid. Spain.

[§]Department of Chemistry Physics of Surfaces and Processes, Instituto de Cerámica y Vidrio (CSIC), Kelsen, nº 5, 28049-Madrid. Spain.

KEYWORDS: Covalent immobilization, Lipase, *Pseudomonas stutzeri*, Benzoins, Dynamic Kinetic resolution, 2-MeTHF

ABSTRACT:

The immobilization of lipase from *Pseudomonas stutzeri* (Lipase TL®) by covalent bonding to a porous polymer is described for the first time. The immobilized enzyme was characterized in terms of optimal pH and thermal stability, and its catalytic efficiency was tested in the kinetic resolution (KR) of symmetrical and unsymmetrical benzoins (1,2-diaryl-2-hydroxyethanone structures). Reactions were performed in a green solvent 2-MeTHF, reaching maximum conversions and enantiomeric excesses, with a significant increase of productivity due to the possibility of reuse of the catalyst. Moreover, the immobilization allowed the development of an adequate scaling up of this KR process permitting a further rise in the catalytic efficiency. Finally, the dynamic kinetic resolution of benzoin (DKR) was carried out by the combination of the immobilized lipase and a ruthenium catalyst (Shvo´s catalyst) in 2-MeTHF, reaching conversions up to 90%, maintaining its excellent enantioselectivity during 6 catalytic cycles.

INTRODUCTION

Optically pure benzoins (1,2-diaryl-2-hydroxyethanone structures) are very useful building blocks in the synthesis of different drugs as antidepressant, astringent, anti-inflammatory, carminative, diuretic, expectorant, sedatives and antibacterial.[1, 2]Benzoins and its derivatives are used as intermediates for the synthesis of organic compounds and as a catalyst in photo polymerization, which are used as anticratering in powder coating. It has been reported that the benzoin can be used in skin disorders as an antibacterial and antifungal agent.[3] Benzoin has also been used as photo initiators in polymeric reaction. Benzoin also used as a starting material for preparing complexes like Schiff base compound. Schiff base complexes of transition metals are

of greater importance in medicine, biochemistry and industries among others.[4] Due to the increasing interest towards these molecules, different strategies have been developed for obtaining benzoin derivatives in an enantioselective manner. In this context, although several attempts have been carried out by using asymmetric synthesis,[5] biocatalysis is a real alternative, because more sustainable processes can be implemented by using this approach[6] In this sense, enzymatic kinetic resolution (KR) of racemic mixtures has proven to be a useful method to obtain these chiral benzoins.[7] Additionally, the coupling of enzymes and metal catalysts in a dynamic kinetic resolution (DKR) process has overcome the intrinsic limitation of the maximum theoretical yield of 50% obtained in a KR process, making possible the complete transformation of racemic substrates to optically pure products.[8]

As it is well known, the application of lipases in the resolution of racemic mixtures is widely spread, due to their regio-, stereo- and chemoselectivity and their stability in organic solvents.[9] Recently we have reported the DKR of benzoins, a special type of bulky secondary alcohols, by using a commercial Lipase TL® (from *Pseudomonas stutzeri*) coupled to an *in situ* racemization of the remnant substrate through a ruthenium catalyzed redox process (Shvo's catalyst), obtaining high conversions and excellent enantiomeric excess.[7,10]

On the other hand, it is widely accepted that enzyme immobilization onto solid materials offers a great number of advantages over native enzymes such as the enhanced stability, simplicity in separation, and the capability in recovery and reuse,[11] thus making these immobilized biocatalysts very useful specially for industrial applications.[12] There are many examples on lipase immobilization in literature, being covalent bonding to a solid support one of the preferred methodologies;[12a] nevertheless, for lipase TL, only physical adsorption on hydrophobic support[13] and entrapment in silicon spheres[14] had been described so far.

Thus, in this work we described the first example of a covalent immobilization of lipase TL from *Pseudomonas stutzeri* onto a new porous polymer activated with epoxy groups, showing how the best immobilized derivative can be usefully applied as a recoverable and reusable catalyst in the green bio-solvent 2-MeTHF for KR and DKR of benzoins with high yield and stereoselectivity. The excellent activities displayed by the immobilized biocatalyst allowed an initial scaling-up the process, testing two types of bioreactors (batch and column), and opening the way for further implementation of the methodology.

RESULTS AND DISCUSSION

Thus, we have carried out the first covalent immobilization of commercial lipase from *Pseudomonas stutzeri* (Lipase TL®). In these study two different supports were tested, commercial Eupergit C, used for comparative purposes, and poly(GMA-co-HDDMA). Eupergit C is a spherical acrylic polymer (surface area: 4.5 m^2/g; pore size: 10 nm; pore volume: 0.06 mL/g), made by copolymerization of *N,N'*-methylen-bis(methacrylamide), glycidyl methacrylate, allyl glycidyl ether, and methacrylamide. This epoxide-containing copolymer (0.6 mmol epoxy groups per g dry weight[15]), is particularly suitable for covalent immobilization of enzymes for industrial applications because of its reactor-compatibility.[16] The methacrylic copolymer described in this paper, Poly(GMA-*co*-HDDMA, surface area: 27 m^2/g; pore size: 26 nm; pore volume: 0.15 mL/g), also contains epoxide groups as the reactive components, although displaying a higher density of reactive epoxy groups (4.3 mmol epoxy groups per g dry weight) compared to Eupergit C.

Synthesis and characterization of the porous poly(GMA-*co*-HDDMA).

Porous poly(GMA-*co*-HDDMA) copolymer was synthesized by solution polymerization as previously described[17], and characterized using different techniques:

i) Porosity Volume Distribution Analysis (PVD). Nitrogen adsorption provides a good estimation of the Specific Surface Area (SSA), Pore Volume (PV) and Median Pore Size (MPS) of any support, however it is also possible to determine its pore volume distribution (PVD) for a given pore size (radius) range. The porosity analysis (see Table S1 in supporting information) showed that the presence of macro pores contributes significantly to the volume of the support porosity.

ii) FT-IR spectrum. The infrared spectrum of the synthesized copolymer was used to verify the presence of epoxy groups (Fig. S1a in supporing information). From the FT-IR spectra, epoxy groups are clearly present in the copolymer as indicated by the occurrence of typical stretching vibrations of epoxy groups at 1258, 908, and 844 cm^{-1}. On the other hand, those stretching vibrations of C=O and C–O at 1720 and 1144 cm^{-1}, respectively, confirms the presence of ester groups in the structure of the copolymer coming from GMA monomer.

iii) Thermal characterization. Thermo gravimetric analysis (TGA) shows that this copolymer has excellent thermal stability up to 190 ºC, while for higher temperature the material begins to decompose and weight loss becomes evident (see Fig. S1b in supporting information).

iv) Scanning electronic microscopy (SEM). From the SEM photographs (Fig. 1) the internal structure presents numerous interconnected cavities (pores) of different sizes that are aggregated in larger clusters.

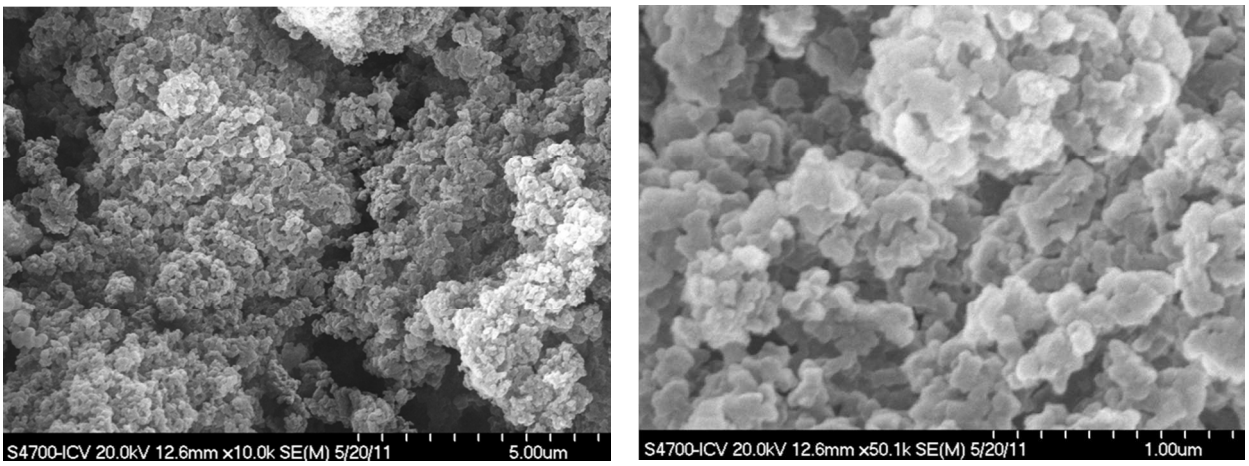


Figure 1. SEM photographs showing the internal morphology of porous poly(GMA-co-HDDMA).

Enzyme Immobilization

Optimization of enzyme loading

The first variable studied which could affect the immobilization efficiency was the relative amounts of polymer and enzyme used in the coupling process. Thus, different enzymatic amounts were used, keeping a fixed quantity of supporting copolymer (Table S2 in supporting information). As can be seen, using a 1:100 ratio of protein to polymer, 100% binding of protein to poly(GMA-*co*-HDDMA) copolymer was observed. Nevertheless, when the amount of enzyme added to the immobilization mixture was increased, up to 40% of the protein added did not get linked to the support. On the other hand, for Eupergit C at the optimum molar ratio of enzyme to polymer (1:100), around 30% less protein was bound compared to poly(GMA-*co*-HDDMA) copolymer. This finding could be explained taking into account the lower density of reactive groups for Eupergit C already mentioned, so that a larger quantity of polymer should be used to achieve the same enzymatic loading.

Increasing the initial amount of enzyme from 1 to 2 mg (entry 2) led to a derivative (TL-PGcH-2) with higher enzymatic loading but displaying approximately the same stationary effectiveness

factor. Nevertheless, this value decreased when the amount of enzyme was increased up to 3 mg (entry 3). This fact is suggesting the presence of diffusional problems for the substrate upon reaching the enzyme molecules on the derivative having a higher enzymatic loading, maybe as consequence of enzyme clustering. Anyhow, all the derivatives on poly(GMA-*co*-HDDMA) were more active than that one obtained with Eupergit C (TL-EuC), which only showed 45% of catalytic efficiency compared to the native enzyme, and for which diffusional restrictions have been frequently described.[15,18] Based upon results. all the following experiments were conducted with the best immobilized derivative, TL-PGcH-2 (entry 2).

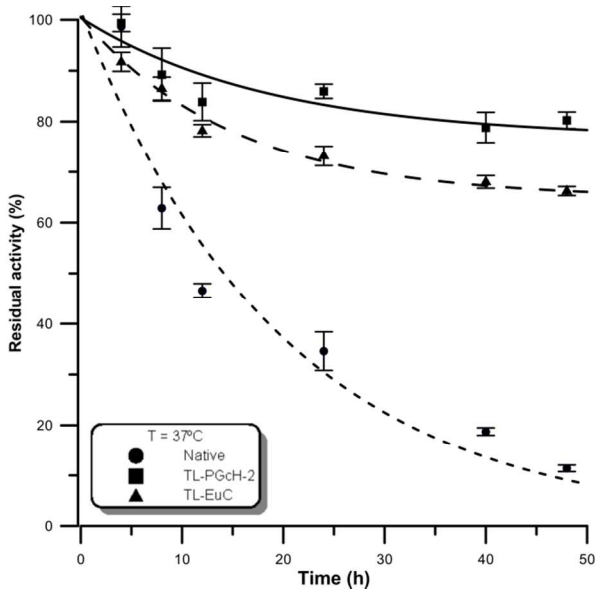
Effect of pH on enzyme activity

The study of pH effect on the activity of free lipase from *Pseudomonas stutzeri* and TL-PGcH-2 was performed in the pH range from 6 to 10 (Fig.S2 in supporting information). TL-PGcH-2 exhibited a shift in the optimal pH of about 0.5 units toward acidic pH values, indicative of the activated matrix behaving as a polycation. Similar results have been reported for different enzymes immobilized on Eupergit C, such as pectinlyase,[19] galactosidases[18] or lipases.[20]

Thermal stability of free and immobilized enzyme

The immobilized enzyme preparations can be stored at 4°C for at least one month without appreciable deactivation (less than 5%). Thermal stability experiments were performed as described in the experimental section, and Figure 2 shows the thermal stability behavior of the commercial and immobilized enzymes.

a)



b)

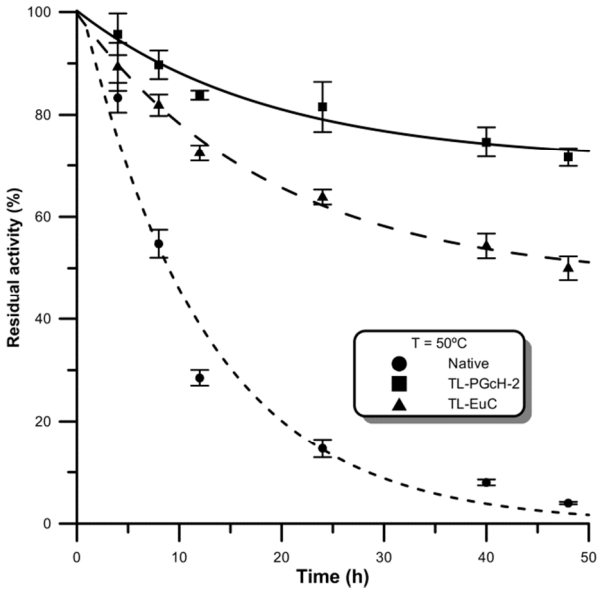


Figure 2 Thermal stability of free and immobilized lipase TL from *Pseudomonas stutzeri*. a) 37°C b) 50 °C.

As can be seen, native enzyme shows a clear deactivation with half-life times of 14 and 9 h at 37 and 50 ºC, respectively. This loss of activity upon heating at 50 ºC had been previously described by Hoyos et al.[20a] Equally, those derivatives on Poly(GMA-*co*-HDDMA) show a higher thermostability compared to those obtained with Eupergit C. It has been described that enzymes are immobilized on Eupergit C® through their different reactive groups (amino, sulfhydryl, hydroxyl, phenolic), and this fact can block the substrate accessibility to the active site, or may establish multipoint-binding or can even lead to enzyme denaturation[20a] but this deleterious effect is not observed in our immobilized derivatives.

Kinetic Resolution and Dynamic Kinetic Resolution of benzoins.

Substrates employed in the enzymatic resolution (Table 1) were purchased from Sigma Aldrich or synthesized previously by our research group.[20]

Kinetic Resolution of benzoins

Lipase TL-catalyzed benzoin kinetic resolution (KR) was selected as test reaction to measure the activity of both immobilized and free enzyme (Scheme 1).

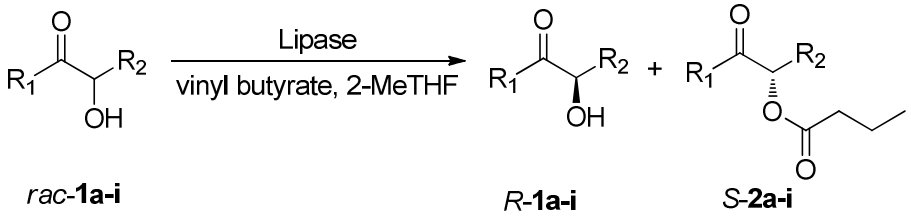
Substrates (**1a-i**) were chosen because of the different size of the substituent at both sides of the acyloin core, in order to study the ability of the immobilized enzyme to distinguish between them. The enzymatic kinetic resolution of compounds **1a-i** was carried out in 2 Me-THF at room temperature, employing vinyl butyrate as acyl donor and crude or immobilized lipase. The reaction progress was monitored by HPLC in order to calculate the initial rate of the enzymatic process, as reported in the experimental section.

Table 1. Kinetic resolutions of different benzoins[a].

substrate	R_1	R_2	biocatalyst	reaction time (h)	Conv.[b] (%)	ee_p[b] (%)	E[c]
1a	Phenyl	Phenyl	free enzyme	6	50	>99	>200
			TL-PGcH-2	6	50	>99	>200
1b	4-Methoxyphenyl	4-Methoxyphenyl	free enzyme	8	50	>99	>200
			TL-PGcH-2	8	50	>99	>200
1c	4-Isopropylphenyl	4-Isopropylphenyl	free enzyme	8	49	>99	>200
			TL-PGcH-2	8	48	>99	>200
1d	Phenyl	4-Ethoxyphenyl	free enzyme	8	50	>99	>200
			TL-PGcH-2	8	49	>99	>200
1e	4-Ethoxyphenyl	Phenyl	free enzyme	8	49	>99	>200
			TL-PGcH-2	8	48	>99	>200
1f	Phenyl	3,4-dichlorophenyl	free enzyme	8	50	>99	>200
			TL-PGcH-2	8	49	>99	>200
1g	3,4-dichlorophenyl	Phenyl	free enzyme	8	49	>99	>200
			TL-PGcH-2	8	49	>99	>200
1h	2-Furyl	2-Furyl	free enzyme	8	48	95	113
			TL-PGcH-2	8	47	95	125
1i	3-Thienyl	3-Thienyl	free enzyme	8	48	>99	>200
			TL-PGcH-2	8	48	>99	>200

[a]Reaction conditions: 0.094 mmol of substrate were solved in 1.5 mL of 2-MeTHF and vinyl butyrate (0.3 mmol) and Lipase TL (20 mg of free enzyme (0.336 U) or 200 mg of immobilized enzyme (0.269 U)) were added. The mixture was stirred at room temperature under inert atmosphere. Enzyme units were calculated through hydrolysis of *p*-nitrophenyl-palmitate as described in the experimental section. [b]Determined by HPLC analysis using Chiralcell OD® column. [c]Enantiomeric ratio, ratio of specific constants (apparent second-order rate constant k_{cat}/K_m) for both enantiomers.[7,10]

Scheme 1. Kinetic resolutions of different benzoins catalyzed by lipase from *Pseudomonas stutzeri.*



rac-**1a-i** *R*-**1a-i** *S*-**2a-i**

As shown in Table 1, in most cases maximum conversions (around 50%) were reached after 8 h; in all cases, excellent enantiomeric excess values were obtained both with the crude enzyme and TL-PGcH-2, clearly indicating that the immobilization procedure had not altered the exquisite enantioselectivity of this lipase in the stereorecognition of benzoins. Thus, enantiomerically pure butyrates **(*S*)-2a-i** were easily isolated and purified by silica column chromatography, and absolute configurations were assigned to be *S* according to a correlation of the positive optical rotations values of these compounds with data from literature.[21]

Re-use of the immobilized lipase TL in the kinetic resolution of benzoin.

The potential for the re-use of the supported enzyme in the KR of benzoin was investigated. After the first assay, TL-PGcH-2 was recovered, washed and re-assayed with fresh substrate mixture under the same experimental conditions, and this procedure was repeated 15 times, always using the green solvent 2-MeTHF, in which we have already reported the highest yields for KR of benzoins.[7,10]

The experimental results shown in Figure 3 illustrate the excellent reusability in 2-MeTHF for the immobilized enzyme, which retained 74% of its initial activity after the 15th catalytic cycle (Figure 3).

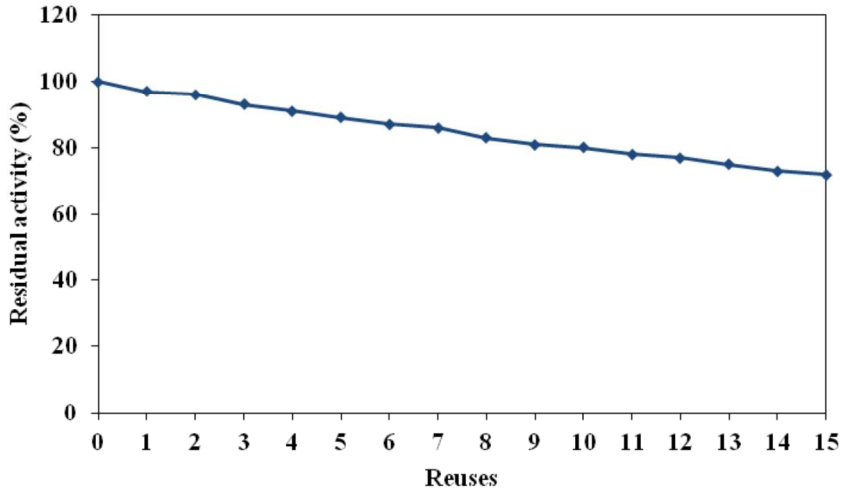


Figure 3. Operational stability of TL-PGcH-2.

Scale up of the kinetic resolution of benzoin

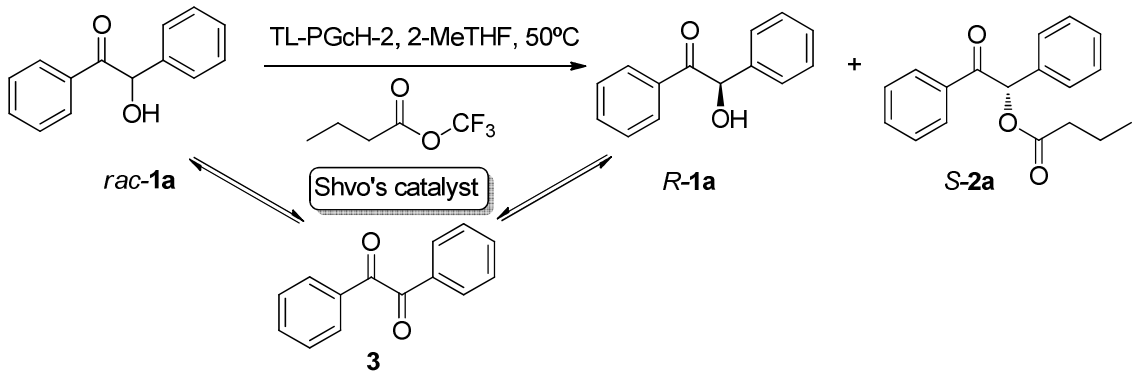
Based on the excellent results obtained with the immobilized lipase, KR of benzoin was scaled-up 5 times (from 20 to 100 mg) up to 10 ml volume, testing two types of bioreactors (batch and column). For both experiments, 3 g of TL-PGcH-2 were used. In the case of the batch reactor, a final conversion of 49% after 2 h was observed, with an enantiomeric excess of the product over 99%, resulting in a productivity of $2.46 \cdot 10^{-3}$ mmol product$\cdot h^{-1}$ (mg enzyme)$^{-1}$. Similar results were obtained in the same process on a smaller scale ($2.5 \cdot 10^{-3}$ mmol product$\cdot h^{-1}$ (mg enzyme)$^{-1}$), therefore confirming the absence of any diffusional limitation for the immobilized derivative.

For the KR protocol in a column reactor (see experimental section), the retention time observed for the reaction mixture in the column was 60 minutes, yielding a 50% conversion with an enantiomeric excess above 99%. The optimal reaction time was 90 minutes (after that time, product **(*S*)-2a** and remnant **(*R*)-1a** were not detected in the reaction eluent). In this kinetic

resolution process the productivity obtained was $3.3 \cdot 10^{-3}$ mmol product$\cdot h^{-1}$ (mg enzyme)$^{-1}$, slightly higher than that one obtained employing in a batch reactor. Very recently, benzoin DKR in batch and continuous mode has been reported with Accurel-adsorbed lipase TL[22] not only in 2-MeTHF, but also in other green biosolvents such as 1,2-dioxolane and remarkably cyclopentyl methyl ether (CPME, a very attractive new alternative for replacing traditional organic solvents), also with excellent results[22].

Dynamic Kinetic Resolution of benzoins

Once the optimal conditions were selected for the kinetic resolution, and it was proven that TL-PGcH-2 derivative showed a good thermostability at 50 ºC (Figure 2b), the dynamic kinetic resolution (DKR) was the next logical step to confirm the real capability of the immobilized enzyme. Nevertheless, in order to avoid the described interference of acetaldehyde produced from vinyl esters during KR with the hydrogen transfer catalyst,[23] trifluoroethyl butyrate was used as acyl donor, as depicted in Scheme 2. The Shvo's catalyst, (1-hydroxytetraphenylcyclopentadienyl (tetraphenyl-2,4-cyclopentadien-1-one)-α-hydrotetracarbonyldiruthenium (II)), was chosen as the catalyst of the *in-situ* racemization *via* a reversible oxidation of the remnant benzoin **(*R*)-1a** into bencil **3** and its ulterior reduction to regenerate **(*rac*)-1a.**



Scheme 2. Dynamic kinetic resolution process.

As previously commented, we had formerly studied the combination of the Shvo´s catalyst and native lipase from *Ps. stutzeri* in the resolution of symmetrical[24] and asymmetrical benzoins,[7] developing a three-step DKR in order to minimize lipase deactivation at 50 ºC. This temperature, relatively high for a biocatalytic process mediated by a mesophylic lipase, is needed to promote the required splitting of the Shvo's catalyst into the two monomers required for the *in situ* racemization of the remnant substrate.[10a] Therefore, immobilization was the rational procedure for increasing thermostability of the lipase: in that sense we initially described how the entrapment of Lipase TL in silicon elastomer (LipTL-SS derivative) allowed the development of a one-pot DKR at 60 ºC in THF leading to 87% yield an 99% ee_p in the first catalytic cycle and a four-times recycling of the catalyst.[24b]

In order to improve these results, we have now developed a *one pot* process (Scheme 2) to afford a rapid DKR of **(*rac*)-1a** in 20 h at a lower temperature (50 ºC), finding an almost quantitative conversion (95%) of product with an enantiomeric excess higher than 99% (although some traces of the correspondent dicarbonyl intermediated **3** were detected by HPLC analysis). For reusing TL-PGcH-2 derivative in consecutive DKR reactions, the immobilized enzyme matrices were

washed with 2-MeTHF until no remaining substrate or product was detectable in the solvent and another DKR cycle was started. The results obtained in the re-use of immobilized enzyme in benzoin DKR process are shown in the Figure 4.

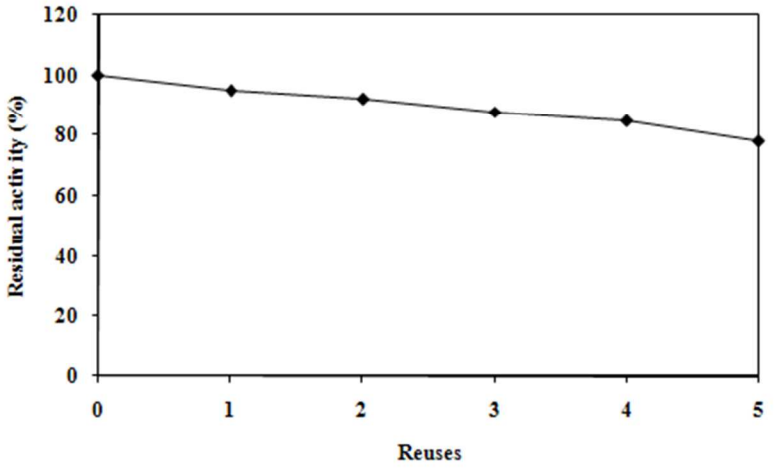


Figure 4. Repetitive DKR of benzoin with trifluoroethyl butyrate catalyzed by TL-PGcH-2.

As can be seen, the new immobilized enzyme was recycled five times (6 catalytic cycles), so this mean that the same amount of TL-PGcH-2 was working 114 h (24 h, initial cycle, plus (5x18h), 5 reuses) at 50ºC, still retaining a 78% of residual activity. To calculate the operational productivity of TL-PGcH-2, in Table 2 we show the results of the catalytic performance of TL-PGcH-2 compared to both native and silicon elastomer-trapped lipase TL (LipTL-SS).

As can be seen, although the overall productivity obtained with covalent derivative TL-PGcH-2 is slightly lower than that obtained with LipTL-SS, in general terms TL-PGcH-2 results a better catalyst considering the lower operational temperature required (50 vs 60º C) and the improved mechanical properties of the covalent immobilized derivative, making it suitable for both batch

and column engineering designs. Ansorge-Schumacher and coworkers[14] also reported a good stability of their immobilized derivative in the same repetitive reaction using 2-MeTHF as solvent, better than using toluene, although only three reaction cycles were carried out.

Table 2. Repetitive DKR of benzoin with trifluoroethyl butyrate

		Reaction parameters			
Cycle	**Catalyst**	**Conversion (%)**	**ee_p**	**Productivity[a]**	**Overall productivity[b]**
1	commercial LipTL, THF[c]	92	99	2	2
	commercial LipTL, 2-MeTHF[d]	85	99	4	4
	LipTL-SS, THF[e]	87	99	15	15
	TL-PGcH-2, 2-MeTHF	95	99	8	8
2	LipTL-SS, THF[e]	81	99	14	29
	TL-PGcH-2, 2-MeTHF	90	99	7.5	15.5
3	LipTL-SS, THF[e]	80	99	14	43
	TL-PGcH-2, 2-MeTHF	87	99	7.3	22.8
4	LipTL-SS, THF[e]	78	99	13	56
	TL-PGcH-2, 2-MeTHF	82	99	6.9	29.7
5	TL-PGcH-2, 2-MeTHF	80	99	6.7	36.4
6	TL-PGcH-2, 2-MeTHF	74	99	6.5	42.9

[a] mg of product/mg protein of the individual catalytic cycle. For immobilized derivatives, the amount of protein is calculated from the enzymatic loading

[b] mg of product/mg protein considering also the previous cycles

[c] T = 50ºC (data taken from literature[22]

[d] T = 50ºC (data taken from literature [7]

[e] T = 60ºC, THF as solvent (data taken from literature [10b])

Chemometric Parameters

To calculate the usual chemometric parameters to quantify the "greenness" of the reactions, equations defined in literature were used[25] using always racemic benzoin 1a as standard substrate. These parameters are E-Factor (E)[26] E-factor Based Molecular (E_{MW})[27], Atom Economy (AE)[28], Mass Intensity (MI)[29], Material Recovery Parameter (MRP)[27], Stoichiometric Factor (SF)[27], and, finally, Reaction Mass Efficiency (RME)[27], which can be used as a threshold metric for gauging the true "greenness" of reactions, considering the "golden ratio", a RME value ≥ 0.618[30].

Benign index (BI) has been recently described to also assess the overall "greenness" of chemical reactions and synthesis plans[25b], and takes into account the following potentials for environmental harm: acidification−basification (ABP), ozone depletion (ODP), global warming (GWP), smog formation (SFP), inhalation toxicity (INHTP), ingestion toxicity (INGTP), inhalation carcinogenicity (INHCP), ingestion carcinogenicity (INGCP), bioconcentration (BCP), abiotic resource depletion (ARDP), cancer potency (CPP), persistence (PER), and endocrine disruption (EDP). Although the software automatically calculates BI with data implemented, not many of the parameters included in it can be calculated for the (D)KR of benzoins, so that we have only considered those parameters directly derived by implementing data obtained from logP and H (Henry's law constant, HLC), as reported from ChemDraw (Table S3 and Figures S3 and S4 in supporting information).

As can be seen, RME value is smaller than 0.618[30], the golden value for real sustainability. Also, MI parameter for KR increased from 2.8 up to 91.3 (E-factor from 1. 8 to 90.3, 32,6-fold rise) upon committing all reaction solvents, catalysts, and byproducts, while for DKR MI increased from 2.8 to a very similar value of 87.9 (31.4 rise). This means that moving from KR

to DKR does not denote a significant growth of production of waste material, while the productivity enhance is really noteworthy. Furthermore, we should also take into account that, because of the good reusability of TL-PGc-2H both in KRs and DKRs, really the immobilized enzyme cannot be considered as "normal waste", because it can be employed in several successive reaction runs**.**

Scale up of the dynamic kinetic resolution of benzoin

The DKR of benzoin was scaled up from 40 mg to 400 mg in order to study the applicability of the lipase-copolymer particles in a batch reactor. As it has been previously described, increasing the reaction volume allows the decrease of the ruthenium catalyst loading, as the presence of traces of molecular oxygen, which causes its deactivation, is reduced at larger scales. Thus, catalyst loading was decreased from 10 % mol to 2.5 % mol. After 24 h, 92 % yield was achieved, maintaining an excellent 99 % enantiomeic excess.

CONCLUSION

All results presented in this work shows how lipase from *Pseudomonas stutzeri* (Lipase TL®) immobilized on poly(GMA-*co*-HDDA) polymer could be usefully applied as a recoverable and reusable biocatalyst for the KR and DKR of benzoins in 2-MeTHF with high yield and enantioselectivity in all cases. The first scaling up presented here is a proof-of-concept of the applicability of this immobilized derivative, which can be easily operated both in batch and column design.

EXPERIMENTAL SECTION

General

Lipase TL® (from *Pseudomonas stutzeri*) was purchased from Meito & Sangyo Co., Ltd. Shvo's catalyst (1-hydroxytetraphenylcyclopentadienyl(tetraphenyl-2,4-cyclopentadien-1-one)-α-hydrotetracarbonyldiruthenium (II), 98%) was obtained from Strem Chemicals Inc. (Newburyport, MA, USA).). Glycidyl methacrylate (GMA), 1,6-hexanediol dimetacrylate (HDDMA), cyclohexanol and 2,2´-azobis(isobutyronitrile) (AIBN) were purchased from Sigma-Aldrich. All other chemicals were from analytical grade. UV–visible spectra were recorded on a UV-2401 PC Shimadzu. HPLC analyses were performed with a chiral column Chiralcel® OD-H (UV detector) at room temperature; mobile phase *n*-hexane/2-propanol 90/10, at a flow rate of 1 mL/min. NMR spectra were performed on a Bruker AC-300 MHz: chemical shifts (δ) are reported in parts per million (ppm) relative to $CHCl_3$ (^{1}H: δ 7.27 ppm) and $CDCl_3$ (^{13}C: δ 77.0 ppm). **Polymerization procedure.** Porous poly(GMA-*co*-HDDMA) copolymer was synthesized by solution polymerization as previously described[17] in a cylindrical reactor (15x160 mm) at 70 rpm under argon overlay at 50ºC for 16 h. Cyclohexanol was employed as porogenic diluent (60% respect the reaction mixture). The GMA/HDDMA ratio employed was 60/40 and the free-radical initiator percentage (in the reaction mixture) was 5%. After the reaction was completed, the polymeric product was filtered under vacuum and washed with acetone, water and methanol several times to remove low-molecular-weight products and then dried in at 70 ºC in vacuum till constant weight.

Characterization of epoxy-functionalized polymers

i) *Surface Area and Pore Size Analysis*. Specific Surface Area (SSA), Pore Volume (PV) and Median Pore Size (MPS) were determined by nitrogen gas adsorption-desorption a 77 K by using a Tri-Star 3000 instrument (Micromeritics, USA). Samples were degassed at 120 ºC for 18 h. SSA values were calculated using the BET equation[14] in the nitrogen partial pressure range of 0.05-0.35. PV and MPS were obtained by using the adsorption branch of the nitrogen isotherms according to the BJH method in the nitrogen partial pressure range of 0.35-0.99.[31]

(ii) *FT-IR spectroscopy*. A Perkin-Elmer 681-Fourier transform infrared spectrophotometer with a resolution of 1 cm^{-1} in the transmission mode was used to study the infrared absorption. The synthesized polymers (2.0 mg) were milled with potassium bromide (100 mg) and pressed into a solid disk of 1.2 cm diameter prior to the infrared measurement.

(iii) *Scanning electron microscopy (SEM)*. Surface morphology of copolymers particles was observed by using Field-Emission Scanning Electron Microscopy (FE-SEM). Specimen preparation was done as follows: dried copolymers particles were mounted on stubs and sputter-coated with gold. Micrographs were taken on a Hitachi-S4700 FE-SEM instrument.

(iv) *Thermal Characterization.* Thermo Gravimetric Analysis (TGA) was performed using an AQ-500 TA Instruments equipment. For each essay 4-5 mg of polymer were used. The heating rate was set at 5 ºC/min and all the experiments were carried out under a constant nitrogen flow of 20 ml/min.

(v) *Polymers epoxidation degree*. Quantitative determination of epoxide groups in the polymers was carried out by chemical titration.[32] The polymer (2.0 g) was re-suspended in CH_2Cl_2 (30

mL) and treated with a 20 wt % solution of tetraethylammonium bromide in glacial acetic acid, prepared previously. After addition of 6-8 drops of crystal violet indicator solution in acetic acid, the mixture was titrated with 0.1 N perchloric acid solution in acetic acid. Hydrobromic acid formed *in situ* by the exchange reaction between perchloric acid and tetraethylammonium bromide reacted instantaneously with the epoxide group, leading to bromohydrin formation. The end point of the titration was determined by the change of the color of the solution from a sharp blue to green.

Enzyme immobilization. Poly(GMA-*co*-HDDMA) (100 mg) was added to the lipase solution (different concentrations were tested; 1, 2 and 3 mg) in 1 mL of 0.05 M phosphate buffer, pH 7. The reaction was carried out for 24 h at 20 ºC with gentle shaking (200 rpm). After 24 h of incubation, polymer particles were collected on a sintered-glass filter and the solution was removed by suction under vacuum. The copolymer particles were washed thoroughly on the same filter with 10 ml of 0.05 M phosphate buffer, pH 7 (the volume was divided into five aliquots and one aliquot was used for each washing step). The excess of epoxy groups on the matrix were blocked by incubation with 3 M glycine solution for 16 h at 25 °C. The immobilized lipase-copolymer samples were stored at 4 ºC until use.

Determination of the amount of immobilized lipase. Protein concentration was determined using Bradford's method[33] following the manufacturer's protocol (Bio-Rad) and bovine serum albumin as standard. The coupling yield (%) of the lipase was determined by the difference between the initial lipase amount present in the lipase coupling solution and the final lipase amount present in the remaining coupling solution.

Enzyme assays. Hydrolytic activity of free and immobilized enzyme were analyzed spectrophotometrically measuring the increment in the absorption at 410 nm promoted by the hydrolysis of *p*-nitrophenol palmitate (pNPP). The reaction mixture consisted of 0.1 mL of diluted enzyme sample (or 10 mg immobilized copolymer particles), 2.0 mL of 0.05 M sodium phosphate buffer (pH 7.0) and 0.25 mL 10 mM pNPP in 2-propanol, incubated at 25ºC for 1 min. The absorbance was measured at 410 nm. A lipase unit was defined as the amount of enzyme necessary to hydrolyze 1 μmol of pNPP per minute under the described conditions.

pH and thermal stability of free and immobilized enzyme. The pH stability of free and immobilized lipase was studied by incubating the enzyme at 25 ºC in buffers of varying pH in the range of 6-10 for 20 min and then determining the catalytic activity. Residual activities were calculated as the ratio of the activity of enzyme after incubation to the activity at the optimum reaction pH.

Thermal stability experiments were performed with free and immobilized enzymes which were incubated in the absence of substrate at 37 °C and 50 °C. The immobilized enzymes were placed in 100 mM sodium phosphate buffer (pH 6.0) and the specific enzymatic activities were measured at different storage times.

General procedure for benzoin kinetic resolution (KR). Substrate (20 mg, 0.094 mmol) was dissolved in 1.5 mL of dry 2-MeTHF. Lipase TL (20 mg of the commercial preparation or 200 mg of lipase–copolymer particles) and vinyl butyrate (38 μl, 0.3 mmol) were added to the solution and the mixture was stirred at room temperature for 10 h. At fixed reaction times, samples (20 μL) were taken from the reaction medium, re-dissolved in 1 mL of n-hexane, and filtered through Millex®-GV (PVDF, 0.22 μm pore size) to stop the enzymatic reaction. As 2-

MeTHF (polar solvent) may damage the chiral column (Chiralcel OD-H®), each sample was evaporated under vacuum and the solid collected was re-dissolved in 1 mL of *n*-hexane/2-propanol (50:50, v/v) before analyzing by HPLC to measure conversion and enantiomeric excess values. After 10 h the reaction mixture was filtered, and 2-MeTHF was evaporated before products were purified by column chromatography (SiO_2, *n*-hexane/ ethyl acetate, 5:1). The following compounds were isolated and characterized.

For evaluating potential biocatalyst reuses for KR, 400 mg of lipase-copolymer particles were used, choosing 3 hours as final time. Lipase-copolymer particles were washed with 2-MeTHF until no remaining product was detected by HPLC analysis, and these recycled lipase-copolymer particles were used in a new KR process.

Analysis of reaction products (*S*)-2-oxo-1,2-diphenylethyl butyrate (**2a**), (*S*)-1,2-bis(4-methoxyphenyl)-2-oxoethyl butyrate (**2b**), (*S*)-1,2-bis(4-isopropylphenyl)-2-oxoethyl butyrate (**2c**), (*S*)-1-(4-methoxyphenyl)-2-oxo-2-phenylethyl butyrate (**2d**), (*S*)-2-(4-methoxyphenyl)-2-oxo-1-phenylethyl butyrate (**2e**), (*S*)-1-(3,4-dichlorophenyl)-2-oxo-2-phenylethyl butyrate (**2f**), (*S*)-2-(3,4-dichlorophenyl)-2-oxo-1-phenylethyl butyrate (**2g**), (*S*)-1,2-di-2-furyl-2-oxoethyl butyrate (**2h**) and (*S*)-2-oxo-1,2-di-3-thienylethyl butyrate (**2i**), was carried out by NMR, and all data were in accordance with those ones previously published (NMR data in Supporting information).[34]

Scale-up of the kinetic resolution process of the benzoin. The process of scale-up was carried out in two different types of reactors, batch reactor and column reactor, using an initial substrate concentration 5 times higher than that one used for analytical scale. Therefore, also biocatalyst amount was increased from 200 mg up to 3 g of lipase-copolymer catalyst.

Scale-up in batch reactor. Racemic benzoin (100 mg, 0.47 mmol) was dissolved in 10 mL of dry 2-MeTHF. Lipase–copolymer particles (3 g) and vinyl butyrate (190 µl, 1.5 mmol) were added to the solution and the mixture was stirred at room temperature for 10 h. At fixed reaction times, 20 µL aliquots of supernatant were taken from the reaction medium, redissolved in 1 mL of *n*-hexane, and filtered through Millex®-GV (PVDF, 0.22 µm pore size) to stop the enzymatic reaction an analyzed as described above.

Scale-up in column reactor. Lipase-copolymer particles (3 g) were packed with 2-MeTHF in a chromatography column (40 cm x 1 cm), giving rise to a filling length of 21 cm. Then, this column reactor was loaded with racemic benzoin (100 mg, 0.47 mmol) dissolved in 10 mL of dry 2-MeTHF. The column reactor was set up with a peristaltic pump to run the reaction and the eluent passed through the column where a fraction collector at the end of the column collected the eluted samples. 20 µL of each sample were evaporated under vacuum and the solid collected was re-dissolved in 1 mL of *n*-hexane/2-propanol (50:50, v/v) before analyzing by HPLC to measure conversion and enantiomeric excess values.

Dynamic Kinetic Resolution of benzoin. Racemic benzoin (40 mg, 0.19 mmol), lipase-copolymer particles (400 mg) and Shvo´s catalyst (20 mg, 0.02 mmol) were added to a 25 mL flask. Anhydrous 2-MeTHF (1,5 ml) was incorporated and the reaction was started by the addition of trifluoroethyl butyrate (92 µl, 0.6 mmol). The mixture was stirred at 50 ºC under argon atmosphere for 24 h. Conversion and enantiomeric excess were determined by HPLC analysis, following a similar protocol to that described in the previous section. After 24 h the reaction was finished and the mixture was filtered. In the case of the reuses, the final time was 18 hours and then the reaction mixture was filtered and the lipase-copolymer particles were washed with 2-MeTHF until no remaining product was detected by HPLC analysis, and those recycled

lipase-copolymer particles were used in a new benzoin DKR process. 2-MeTHF was evaporated and the products were purified by column chromatography (SiO_2, *n*-hexane/ ethyl acetate, 5:1) and characterized.

Scale-up in batch reactor. Racemic benzoin (400 mg, 1.88 mmol), lipase copolymers particles (6 g) and Shvo´s catalyst (51 mg, 0.047 mmol) were added to a 50 mL flask. Anhydrous 2-MeTHF (15 mL) and trifluoroethyl butyrate (851 μL, 5.64 mmol) were added, and the reaction was stirred at 50 ºC under argon atmosphere. After 24 h the reaction mixture was filtered, conversion and enantiomeric excess were determined by HPLC analyses and the product **(*S*)-2a** (488 mg, 1.72 mmol) was purified as described above.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*María J. Hernáiz: mjhernai@ucm.es (Tel: +34 913941821)

Angel Rumbero: angel.rumbero@uam.es

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors contributed equally.

Notes

The authors declare no competing finalcial interest.

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