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Improving lipase activity in solvent-free media by interfacial activation-based molecular bioimprinting

Herminia González-Navarro *, Lorenzo Braco

Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, Universitat de Valencia, E-46100 Burjassot, Valencia, Spain

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Abstract

Nine lipases of mammalian, fungal and bacterial origin and two different model reactions, direct esterification and transesterification (alcoholysis), have been used to probe the potential in solvent-free media of the recently reported strategy of interfacial activation-based molecular (bio)imprinting (IAMI) [Mingarro et al., *Proc. Natl. Acad. Sci. USA*, 92 (1995) 3308]. The results demonstrate that the imprinting treatment permits nonaqueous rate accelerations which are lipase-dependent and span in some cases up to higher than two orders of magnitude. For several lipases, the method allows conversion yields after short reaction times (in either of the model reactions assayed) which are remarkably higher than those obtained with either the corresponding powdered commercial sample, the pH-optimized control lipase or even the lipase 'protected' by conventional means such as the use of lyoprotectants (sugars, polyols) or inorganic salts. Therefore, IAMI is proposed as a straightforward, convenient approach to improve lipase performance in more practical solvent-free-based applications.

Keywords: Lipase; Nonaqueous media; Solvent-free; Interfacial activation; Conformation

1. Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) have attracted enormous interest during the last years, particularly from the biotechnological point of view [1] and the fact that they account for about one fourth of all current enzyme-mediated industrial applications [2]. In most of these cases, lipases have been used in nonaqueous media (for general, recent reviews on nonaqueous enzymology see e.g. [3–7]), which is easily understandable as these enzymes naturally function at oil–water interfaces and especially because their substrates are typically water-insoluble. Therefore, efforts to improve lipase activity in these water-restricted environments are warranted and desirable.

Among the various, general strategies developed to enhance the nonaqueous activity of an enzyme (summarized in [8]), the so-called molecular (bio)imprinting [9–12] is a promising though so far relatively unexplored approach. In particular, imprinting of lipases was an exciting challenge given the wealth of structural and mechanistic information unraveled in the last years mainly from crystallographic studies [13–16], which have shown the existence of two relevant conformational states of a lipase, namely, 'closed' (inactive or poorly active) and

* Corresponding author. Fax: (+346) 3864635; E-mail: herminia.gonzalez@uv.es

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'open' (active) forms. More specifically, for most (but not all) lipases, their activation at interfaces seems to be the result of important conformational rearrangements, including opening of a lid covering the active site (thus increasing substrate accessibility) and/or formation of an 'oxyanion hole' (thus adjusting the catalytic machinery).

In this context, we have recently implemented a rational strategy to imprint lipolytic enzymes and hence activate them for operation in nonaqueous media [8,17]. Briefly, the approach, named interfacial activation-based molecular (bio)imprinting (IAMI), is based on the following rationale: an activating conformational change (presumably lid opening and/or fitting of the catalytic machinery) is induced in the lipase in aqueous solution upon binding to amphiphile interfaces (acting as a template), then trapped by rapid freeze-drying and further retained (after washing out of the amphiphile with an anhydrous solvent) in the lyophilized powder by virtue of the known greatly enhanced conformational rigidity of proteins in anhydrous media [18–20]. The immediate practical consequence of lipase imprinting is a remarkably higher nonaqueous reaction rate of the activated enzyme relative to that of its nonimprinted (control) counterpart.

Lipase-mediated conversions in solvent-free media (as a particular case of nonaqueous media) have undoubtedly many advantages, for instance, substrate concentration can be maximized, toxic and expensive organic solvents can be avoided ('clean' conversions) and downstream processing reduced. In fact, just to name a few examples, lipases have recently been used in solvent-free systems (either in direct esterifications or in transesterifications) in the resolution of racemic drugs [21] or in the synthesis of flavor esters [22,23], sugar esters [24], waxes [25], phospholipids [26] or glycerides [27–29]. Given the possibilities opened up by the IAMI strategy [8,17], the aim of the present work is to explore for the first time the consequences of lipase imprinting in solvent-free media. For this purpose, nine lipases of mammalian, fungal and bacterial origin have been assayed using different model reactions.

2. Experimental procedures

2.1. Materials

Porcine pancreatic lipase (ppL), *Candida rugosa* lipase (CrL), oleic acid (99% purity), 1-butyl caprylate (99% purity), n-octyl-β-D-glucopyranoside (n-OG), lactose and sucrose were purchased from Sigma. *Rhizopus niveus* lipase (RnL), *Rhizopus delemar* lipase (RdL), *Rhizopus javanicus* lipase (RjL), *Aspergillus niger* lipase (AnL), *Penicillium cyclopium* lipase (PcL), *Humicola lanuginosa* lipase (HlL) and *Pseudomonas fluorescens* lipase (PfL) were generously provided by Amano. 1-Hexanol (for synthesis) and all other chemicals were from Merck or Aldrich and of analytical grade or purer. All the organic solvents were of analytical or HPLC grade (Merck). The term 'anhydrous' solvent refers to a solvent desiccated with 3 Å molecular sieves (Merck) to bring its water content below 0.01%, as determined by Karl–Fisher titration.

2.2. Preparation of imprinted and nonimprinted enzyme samples

The protocol followed was, with slight modifications, the same as in a preliminary study where a screening of experimental conditions was carried out for optimization of the method [17]. Briefly, each lipase was incubated (before freeze-drying) for 5 min at 4°C in either 10 mM Tris–HCl buffer pH 7.5 (pH 9.0 in the case of HlL) *in the presence* of micellar n-OG (30 mM) (for the imprinted enzyme), or in the same buffer *in the absence* of amphiphile (for the nonimprinted, control enzyme). During and after freeze-drying (typically overnight), both imprinted and nonimprinted samples were rigorously treated in an identical manner. After a washing step (to remove the amphiphile) with benzene/ethanol, 90:10 (v/v), repeated three

times, both samples were vacuum dried for at least 2 h.

2.3. Solvent-free assays

Two solvent-free reactions were selected as a model: (i) the direct esterification of oleic acid and 1-hexanol, and (ii) the transesterification (alcoholysis) of 1-butyl caprylate and 1-hexanol. Substrates were mixed in either case at a 1:1 mole ratio (as a typically reported optimal stoichiometry in this type of conversions). A given amount of washed lipase powder (either imprinted or nonimprinted) was suspended in an Eppendorf tube in a given volume of the mixture of the substrates (1.0 mmol each for the esterification reaction and 1.2 mmol each for the transesterification), sonicated for 10 s and incubated at 25°C with stirring with a magnetic bar at 250 rpm; periodically, 10 μl aliquots were withdrawn from the reaction mixture and analyzed by high performance liquid chromatography (HPLC).

For every lipase assayed, the nonaqueous enzyme concentration was optimized to maximize the rate enhancement obtained by imprinting. The activation factor (AF), as defined elsewhere [17], refers to the ratio of the nonaqueous initial reaction rate for the imprinted relative to the nonimprinted lipase.

2.4. HPLC determinations

A Waters liquid chromatograph, equipped as previously described [17], was used in the analysis of nonaqueous reactions. The column, a reverse-phase Lichrospher 100 RP-18 (Merck), was isocratically eluted with HPLC grade methanol, and detection was performed using a Waters 410 differential refractometer. Routinely, 10 μl of reaction mixture was immediately diluted 1/15 (v/v) with the chromatographic eluent, vortexed for 10 s, centrifuged for 3 min at 12000 *g*, and 10 μl of the supernatant injected onto the column. Simultaneous substrate(s) disappearance and product appearance was followed.

As in previous work [8,17], in the case of ppL, colipase was not added since bile salts were not used in any experiment. Typically, the results shown are representative or correspond to the mean of two independent experiments. Other details are given in the corresponding legends to Figs. 1–4.

3. Results

Fig. 1 summarizes the three different ways of preparing a powdered lipase for use in a nonaqueous conversion. It can be (1) directly taken 'straight from the bottle', (2) freeze-dried from a solution buffered at its optimal pH, or (3) subjected to the imprinting procedure. Although simple and practical, case 1 usually offers little control on the sample. In case 2, the sample can be pH-optimized ('pH memory') [30] and in principle can be more reproducibly prepared; optionally, lyoprotectants (sugars, polyols) can be added to the lyophilization buffer. But since amphiphile interfaces are *absent* in this buffer, one should expect that the lipase is freeze-dried (captured) and hence further used in the anhydrous medium in a closed, to be rather inactive, far from optimal conformation (which it has in solution just prior to freezing). On the contrary, in case 3, an activated conformation is generated and maintained in the lipase powder by imprinting; indeed, not only a pH memory but also an 'interface memory' seem logical requisites for an optimal preparation of the sample.

In the present investigation, micellar n-OG was selected as a template since it was revealed to be among the best print molecules in a preliminary screening using several lipases [17]. As in previous work [8,17], purposely no water was added to the powdered enzyme samples (e.g. by routine preequilibration procedures), not only to maximize the conversion yields in the nonaqueous reactions, but especially as a requisite inherent in the molecular bioimprinting

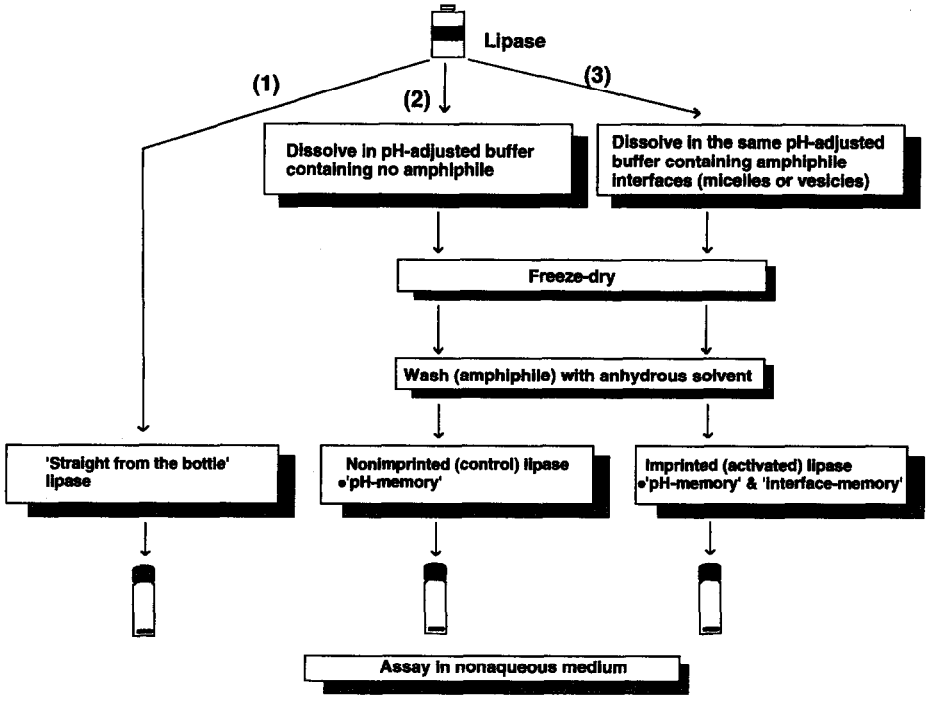

Fig. 1. Scheme of different protocols of preparing a powdered lipase sample for use in nonaqueous media.

strategy [8–12,17]; indeed, it was previously shown that the imprinted lipase 'memory' was gradually lost as the water content of the enzyme sample or the reaction medium was increased [17], plausibly as a result of protein conformational relaxation facilitated by the 'flexibilizing' effect of water [18–20].

3.1. Consequences of lipase imprinting on solvent-free direct esterification

Fig. 2 compares, for three lipases as an example, the time course of esterification obtained with either the imprinted or the nonimprinted (control) enzyme. In either of these cases the

Table 1
Comparison between control and imprinted lipase in the solvent-free esterification of oleic acid and 1-hexanol [a]

Lipase	Activation factor	Initial rate (nmol/min mg lipase powder)		Yield (%) 3 h		24 h	
		Control	Imprinted	Control	Imprinted	Control	Imprinted
ppL	50.4	0.45	22.7	< 0.5	7.5	1.5	20.1
CrL	105.0	0.47	49.3	< 0.3 (2.5) [b]	42.5	1.2 (78.0)	78.2
RnL	38.7	0.89	34.4	0.3 (0.2)	15.8	4.1 (1.6)	63.3
RdL	109.0	12.8	1400	1.8	37.3	19.7	62.2
RjL	16.1	70.3	1125	9.1	40.6	49.1	63.8
AnL	6.7	0.35	2.4	< 0.2	1.0	1.4	9.9
PcL	3.0	3.3	9.8	0.6	1.9	9.3	27.4
HIL	5.3	0.32	1.7	n.d. [c]	0.4	0.9	530
PfL	47.0	3.7	174	1.3 (3.5)	50.2	13.5 (70.3)	93.7

[a] The reaction mixture contained in all cases 1.0 mmol of each substrate, and the following amounts of washed lipase powder, in mg: 25 (ppL); 25 (CrL); 25 (RnL); 8 (RdL); 8 (RjL); 25 (AnL); 20 (PcL) ; 20 (HIL); 20 (PfL).
[b] The numbers in parentheses corresponds to yields for the lipase 'straight from the bottle'.
[c] n.d. = not detected.

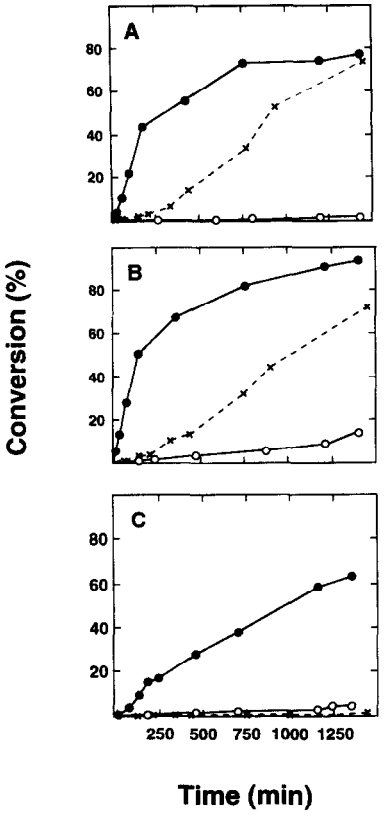

Fig. 2. Comparison of the time course of solvent-free esterification of oleic acid and 1-hexanol mediated by imprinted (●) or control (○) lipase. (A) CrL, (B) PfL and (C) RnL. Each reaction mixture contained 1.0 mmol of each substrate, and 25 (A), 20 (B) or 25 mg (C) of washed lipase powder (either control or imprinted). Samples were incubated at 25°C in closed reactors. The dashed curves correspond to the kinetic profile for the same amount of every lipase used as 'straight from the bottle' (without pH optimization and lyophilization).

imprinting treatment resulted in a remarkable rate acceleration and a dramatic yield improvement in the time interval studied. Therefore, the benefits of the IAMI strategy seem valid, as anticipated, not only for organic solutions of the substrates [8,17] but also for solvent-free mixtures of these substrates.

Table 1 summarizes, for nine lipases of diverse origin, the AF values obtained by imprinting as well as the conversion yield for either the control or the imprinted enzyme after given reaction times. It can be seen that, in general, imprinting caused a rate acceleration and an increase in the reaction yield, although the response was markedly lipase-dependent.

Fig. 2 also shows for comparison the time course of esterification by the same lipases used as powders taken 'straight from the bottle' (without pH optimization and lyophilization). In the case of CrL and PfL, although conversion after 24 h was close to that obtained with the imprinted enzyme, after short reaction times (e.g. 3 h, see Table 1) there was a marked difference in the yields. In principle, the higher activity for these two lipases used directly 'from the bottle' relative to their nonimprinted (pH-adjusted and lyophilized) counterparts might be attributed to one or several of the following reasons: (i) a difference in the initial powder water content; (ii) the (known) detrimental effect of lyophilization on enzyme samples [31], which would affect the control lipase; or (iii) the possibility that in the preparation of the commercial sample precipitation of the lipase

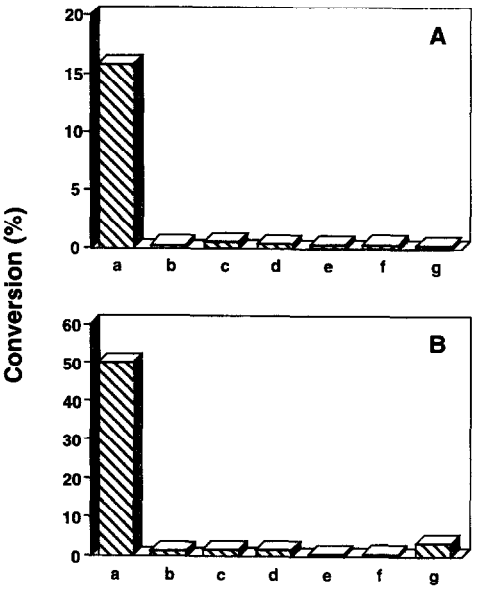

Fig. 3. Comparison of solvent-free production (after 3 h) of 1-hexyl oleate (by direct esterification), obtained using RnL (A) or PfL (B) freeze-dried from different solutions: a, buffer (10 mM Tris–HCl, pH 7.5) in the presence of 30 mM n-OG (imprinted); b, buffer alone (control); c, 2% sucrose in the buffer; d, 2% lactose in the buffer; e, 5 mg/ml KCl in the buffer; f, 25 mg/ml KCl in the buffer; g, lipase taken 'straight from the bottle'. The rest of the conditions as in Fig. 2.

with organic solvent had been employed. Interestingly, in this respect, it has been documented (in the particular case of CrL) that a moderate proportion of a cosolvent in the crystallization medium was sufficient to induce crystallization of an 'open' form of the lipase [32]. In any case, the behavior for CrL and PfL (i.e., the lipase 'from the bottle' is more active than the control) cannot be generalized, since for RnL the activity of both samples (nonimprinted as well as 'from the bottle') was negligible as compared to that of the imprinted lipase (Fig. 2C).

3.2. Comparison of activities of imprinted lipase and lipase 'protected' by conventional means

Different strategies have been reported to cause in general enzymatic rate enhancements in nonaqueous media, by virtue of a protective effect on the enzyme against denaturation by freeze-drying or aggression by organic solvents; in particular, the addition to the lyophilization buffer of conventional lyoprotectants (sugars or polyols) [33,34] or inorganic salts [35]. When several of these additives were independently incorporated to the (amphiphile-free) lyophilization buffer for either of two selected lipases (Fig. 3), only a poor increase in the esterification rate was observed, in all cases marginal relative to the activation obtained by imprinting. This result greatly supports that although amphiphiles may indeed contribute to a protection of the enzyme during imprinting, the activation observed seems not merely the result of this protection, but rather the consequence of the acquisition (and maintenance) by the enzyme of an optimized (more opened and/or better adjusted) conformation. On the other hand, given the peculiar mechanism of action of lipolytic enzymes, it is not surprising that the aforementioned conventional means of protection are insufficient to provide in the case of lipases the full activation achieved by imprinting (i.e., the nonimprinted lipase may be indeed protected but still closed and therefore rather inactive).

3.3. Consequences of lipase imprinting on solvent-free transesterification

Fig. 4 depicts, for three selected lipases, the comparison of the time course of transesterification obtained with either the imprinted or the control enzyme. Again, as for direct esterification, the imprinting treatment resulted in a remarkable rate enhancement and a considerable increase in the yield in the time interval studied.

Table 2 summarizes for the nine lipases the AF values and the conversion yields after given reaction times. The general comments in this case are similar to those presented above for

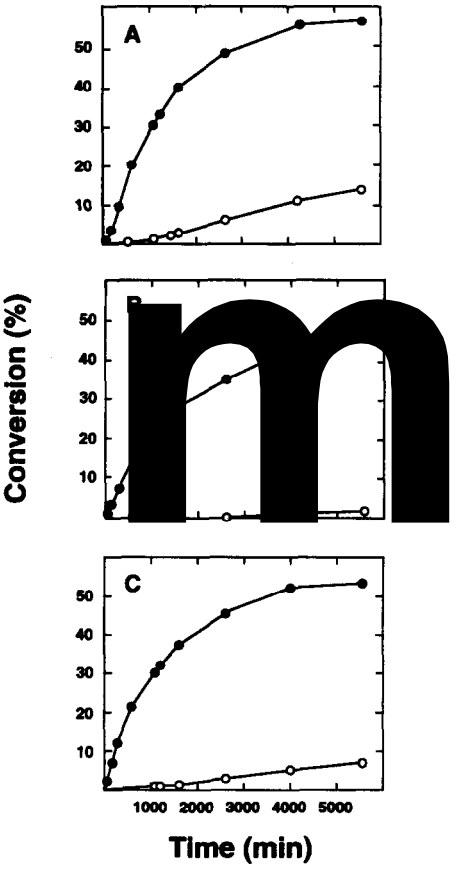

Fig. 4. Comparison of the time course of solvent-free transesterification of 1-butyl caprylate and 1-hexanol mediated by imprinted (●) or control (○) lipase. (A) ppL, (B) CrL and (C) RnL. Each reaction mixture contained 1.2 mmol of each substrate, and 30 mg of washed lipase powder (either control or imprinted). The rest of the conditions as in Fig. 2.

Table 2
Comparison between control and imprinted lipase in the solvent-free transesterification of 1-butyl caprylate and 1-hexanol [a]

Lipase	Activation factor	Initial rate (nmol/min mg lipase powder)		Yield (%) 24 h		48 h	
		Control	Imprinted	Control	Imprinted	Control	Imprinted
ppL	25.3	0.48	12.2	2.4	37.4	7.6	50.0
CrL	117.5	0.08	9.4	< 0.2	27.1	0.4	37.8
RnL	42.7	0.67	15.8	1.4	36.4	3.5	48.1
RdL	34.6	7.5	260.1	3.7	44.1	6.7	52.7
RjL	44.5	5.8	258.5	2.0	42.9	6.1	52.6
AnL	13.3	0.15	2.0	0.6	6.4	2.3	13.7
PcL	2.1	1.4	2.8	5.2	9.8	11.8	17.4
HIL	9.1	0.8	7.2	4.6	21.8	10.9	40.4
PfL	9.0	24.7	216.5	23.8	52.6	36.6	58.3

[a] The reaction mixture contained in all cases 1.2 mmol of each substrate, and the following amounts of washed lipase powder, in mg: 30 (ppL); 30 (CrL); 30 (RnL) ; 3 (RdL); 3 (RjL); 30 (AnL); 30 (PcL); 30 (HIL); 10 (PfL).

Table 1. Notice, for instance, that in the case of ppL, RnL or RjL a reasonable conversion close to 40% was obtained after 24 h with the imprinted enzyme, in contrast to only a marginal yield with the nonimprinted counterpart (about 2%). In order not to be reiterative, it can be concluded that the general benefits observed for direct esterification are also valid in the case of the transesterification reaction.

4. Discussion

One of the advantages foreseen in our preliminary work [8,17] was that the IAMI strategy should be expected, in principle, to be applicable in improving lipase performance in a variety of 'nonaqueous media' in a broad sense. This means not only organic solvents but solvent-free mixtures of substrates or even supercritical fluids or gases; as has been stated before [25], the absence of water in the reaction medium does not imply the use of organic solvents. Our present results demonstrate in particular and for the first time, the feasibility of successfully exploiting interfacial activation-based lipase imprinting in solvent-free systems, in either direct esterifications or transesterifications, and for a repertoire of enzymes from varied origin. At the same time, they expand the range of proven areas of application of the approach.

An overview of recent literature about lipase-mediated conversions in solvent-free media reveals a number of experimental conditions or strategies which are quite common practice in order to enhance reaction rates and improve conversion yields (see, e.g. [21–29]). For instance, the reaction temperature has been typically maintained in the range from moderate to high (40–80°C), to increase the enzymatic rate and diminish the (usually elevated) viscosity of the mixture. Also, lipases have been often used as immobilized on varied supports such as macroporous resins, dextran-based gels or glass beads, which minimizes substrates (and products) diffusional limitations and permits an easy recovery and reuse of the biocatalyst; indeed, immobilized commercial preparations such as Lipozyme-IM20™ (*Rhizomucor miehei*) or Novozym-435™ (*Candida antarctica*) have been often successfully used in solvent-free conversions. On the other hand, in some instances the water activity of the enzyme preparation has been preadjusted to an optimal value. Finally, diverse procedures for eliminating in situ produced water have been applied to improve the yield in solvent-free esterifications, e.g., addition of molecular sieves to the reaction mixture, use of 'open reactors' (water is removed by simple evaporation), application of vacuum or more sophisticated strategies not worth being mentioned here.

Nevertheless, although these practices are in most cases undoubtedly useful, our purpose in the present investigation was to test imprinted lipases in solvent-free media under deliberately simple (in a sense quite unfavorable) reaction conditions: (i) relatively low temperature (25°C), which apart from being cost-effective minimizes background hydrolysis of the products; (ii) no immobilization on support, which is sometimes tedious; (iii) no optimization of the enzyme sample water activity, as a requisite inherent in the imprinting methodology and to minimize the loss of the acquired 'memory'; and (iv) no water removal ('closed reactor').

The present results show that even under these conditions and for varied lipases, imprinting can provide remarkable productivities in solvent-free conversions, in the range of those obtained with proven very efficient, immobilized, commercial preparations used under more optimal reaction conditions. On this basis, the IAMI approach may be particularly advantageous to exploit the selectivities of other lipases traditionally discarded because of their 'reluctance' or marginal activity. Of course, there is no reason a priori not to combine activation by imprinting with a higher reaction temperature and/or with elimination of water formed in situ in order to improve the results in terms of both reaction rate and yield.

Finally, having established with simple model reactions the potential of lipase imprinting on conversions in solvent-free systems, the next logical step would be to test the benefits of the approach on more practical applications, such as the synthesis of flavoring esters or waxes, or the production of tailor-made triglycerides. Work in this direction is in progress in our laboratory.

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