ORIGINAL PAPER

Immobilization in the presence of Triton X-100: modifications in activity and thermostability of *Geobacillus thermoleovorans* CCR11 lipase

M. Guadalupe Sánchez-Otero · Gerardo Valerio-Alfaro · Hugo S. García-Galindo · Rosa María Oliart-Ros

Abstract A partially purified lipase produced by the thermophile *Geobacillus thermoleovorans* CCR11 was immobilized by adsorption on porous polypropylene (Accurel EP-100) in the presence and absence of 0.1% Triton X-100. Lipase production was induced in a 2.5% high oleic safflower oil medium and the enzyme was partially purified by diafiltration (co. 500,000 Da). Immobilization conditions were established at 25 °C, pH 6, and a protein concentration of 0.9 mg/mL in the presence and absence of 0.1% Triton X-100. Immobilization increased enzyme thermostability but there was no change in neither the optimum pH nor in pH resistance irrelevant to the presence of the detergent during immobilization. Immobilization with or without Triton X-100 allowed the reuse of the lipase preparation for 11 and 8 cycles, respectively. There was a significant difference between residual activity of immobilized and soluble enzyme after 36 days of storage at 4 °C ($P < 0.05$). With respect to chain length specificity, the immobilized lipase showed less activity over short chain esters than the soluble lipase. The immobilized lipase showed good resistance to desorption with phosphate buffer and NaCl; minor loses with detergents were observed (less than 50% with Triton X-100 and Tween-80), but activity was completely lost with SDS. Immobilization of *G. thermoleovorans* CCR11 lipase in porous polypropylene is a simple and easy method to obtain a biocatalyst with increased stability, improved performance, with the possibility for re-use, and therefore an interesting potential use in commercial conditions.

Keywords Lipase · Immobilization · *Geobacillus thermoleovorans* · Thermophiles

Introduction

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are ubiquitous enzymes that catalyze in vivo the hydrolysis of triacylglycerols. These enzymes have the ability to catalyze a broad spectrum of bioconversion reactions due to their ability to synthesize soluble or insoluble carboxylic acid esters and other compounds with ester bonds analogs or amido bonds. Lipases have a tremendous biotechnological potential in areas such as food technology, biomedical sciences and fine chemical industry [1–3]. Despite the advantages of these biocatalysts, its use has been limited because of the instability of lipases under industrial conditions that require the use of solvents or moderately high temperatures to dissolve substrates; however, this inconvenience can be circumvented by the use of thermophilic lipases, whose naturally developed resistance to drastic reaction conditions have made them subjects of special interest in biotechnological research [4]. Additionally, enzyme stability can be enhanced by immobilization, a method that also has the advantage of allowing the repeated use of the biocatalyst. This can also favor continuous processing, thereby facilitating process control via manipulation of the feed flow rate. Immobilized enzymes can be easily recovered from the reaction medium, and are potentially useful to obtain a high product yield and purity, and to minimize both downstream processing costs as well as environmental impact [5]. It can also help in the

M. G. Sánchez-Otero · G. Valerio-Alfaro ·
H. S. García-Galindo · R. M. Oliart-Ros (✉)
Instituto Tecnológico de Veracruz,
Unidad de Investigación y Desarrollo en Alimentos,
M. A. de Quevedo 2779, 91897 Veracruz, Mexico
e-mail: roliart@itver.edu.mx

establishment of catalytic sequences. Methods of immobilization can be divided into two major groups: entrapment and surface binding. Among the latter, adsorption, in which the enzyme binds to the support through ionic interactions, Van der Waals forces and hydrogen bonds, has several advantages: simple preparation, low cost, no conformational changes in the enzyme and the generation of stable derivatives [3, 6]. Lipases are commonly activated by interactions with hydrophobic interfaces, due to conformational changes in their secondary structures. Thus adsorption to hydrophobic supports such as polypropylene, have been the most widely used methods for lipase immobilization [7–13].

Recently, it has been shown that lipase activity in aqueous and anhydrous media can be improved in the presence of detergents [14–17] probably due to the breakage of lipase aggregates and/or to the shift on the closed–open equilibrium of the individual lipase molecules [16]. In immobilized lipases, the detergent Triton X-100 is commonly used to desorb the enzyme from the support, but in some instances it has been added during the immobilization procedure resulting in good immobilization yields (60–100%) [18–20].

The purpose of this study was to investigate the effect of immobilization on porous polypropylene, in the presence and absence of Triton X-100, on enzyme activity and stability of a thermoalkaliphilic lipase produced by the thermophilic strain *Geobacillus thermoleovorans* CCR11.

Materials and methods

Bacterial strain

The aerobic thermophilic strain *G. thermoleovorans* CCR11 (EMBL # AJ536599), isolated from "El Carrizal" hot spring in Veracruz, México, was used for thermoalkaliphilic lipase production [15].

Chemicals

Microporous polypropylene powder Accurel EP-100 (particle size 200–1,000 mm) was obtained from Akzo Chemical. Chemical reagents, solvents and culture media were purchased from Sigma-Aldrich, Bioxon, J.T. Baker and Merck; all of them were of analytical grade and used without any further purification.

Lipase production and partial purification

Lipase production was induced in a liquid medium containing 200 mL of nutrient broth 0.325% (w/v), $CaCl_2$ 0.1% (w/v), high oleic safflower oil 2.5%, and arabic gum

1%, pH 6.5 [15]. *Geobacillus thermoleovorans* CCR11 was grown until the stationary phase was reached (38 h), and cells were collected by centrifugation ($14,000 \times g$, 15–20 min, 10 °C). The supernatant was then filtered through a 0.45 μm pore size membrane filter (Millipore) at 25 °C, and then extracted with 200 mL of hexane to remove all the lipid constituents. The crude extract was diafiltered with phosphate buffer 0.05 M, pH 6.5, and concentrated through a polyethersulfone ultrafiltration membrane, co. 500,000 Da (Millipore) in a 200 mL stirred cell (Amicon) [15].

Enzyme assay

Lipolytic activity of free and immobilized lipase preparation was determined by a spectrophotometric assay [21] with slight modifications: 5 mg of the immobilized lipase preparation or 100 mL of the diluted crude enzyme were mixed with 0.9 mL of 0.05 M phosphate buffer, pH 6.5, and 0.1 mL of 0.01 M *p*-nitrophenyl-laurate in ethanol. The hydrolytic reaction proceeded for 30 min at 60 °C, and was stopped by the addition of 250 mL of Na_2CO_3 [15]. One unit of lipase activity was defined as the amount of enzyme that released 1 mMol of *p*-nitrophenol ($e = 4.6$ mM/cm) from *p*-nitrophenyl-laurate in 30 min, under the assay conditions. The enzyme extract and the immobilized lipase were stored at −4 °C.

Protein assay

Protein content was evaluated by Lowry [22] using bovine serum albumin (0.1–1.0 mg/mL) as standard.

Lipase immobilization

Solutions of various lipase concentrations containing 0.16–1.6 mg protein/mL were prepared in phosphate buffer 0.05 M at pH 6.5. Polypropylene powder (Accurel EP-100, 0.025 g) was placed in glass vials and pre-wetted with 0.150 mL of 30% ethanol in water (v/v), which was evaporated for 10 min at 50 °C. Then 1.5 mL of each lipase preparation was added. Blanks of enzyme and support were included, and all the vials were incubated at temperatures 25–60 °C under orbital shaking (250 rpm). The time required to reach adsorption equilibrium for each temperature was determined in preliminary experiments. The immobilized enzyme was separated by filtration (Whatman #2), washed three times with 0.05 M phosphate buffer pH 6.5 and dried under vacuum overnight at room temperature. The amount of protein adsorbed was calculated as the difference between the initial and final protein content in the lipase solution. Langmuir adsorption

parameters were estimated by nonlinear regression analysis.

Immobilization in the presence of detergent Triton X-100

Immobilization in the presence of 0.1% Triton X-100 was carried out at the optimal conditions obtained in the absence of Triton X-100.

Effect of temperature on enzyme activity and stability of free and immobilized lipase

Enzyme activity was assayed at seven temperatures (25–80 °C), as described above. The effect of temperature on enzyme stability was checked by measuring residual activity after 1 h at 4–100 °C.

Effect of pH on enzyme activity and stability of free and immobilized lipase

Lipase activity was assayed in buffers at pH 4.0–12.0. Lipase pH stability was determined by incubating the enzyme preparations in 0.05 M phosphate buffer at pH 4.0–12.0 for 12 h at room temperature. The residual activity was determined by the spectrophotometric assay at pH 6.5.

Substrate specificity of free and immobilized lipase

Substrate specificity was analyzed by the spectrophotometric assay using 0.01 M *p*-nitrophenyl esters (C3, C4, C6, C8, C10, C12, C16 and C18) dissolved in ethanol as substrates, at optimal assay conditions.

Desorption of the immobilized lipase

The leaching of lipolytic activity was studied by incubating the immobilized lipase in 0.05 M phosphate buffer pH 6.5 with either 1.0 M NaCl, 1.0% Triton X-100, 1.0% SDS, 1.0% Tween 80 or 96% ethanol for 12 h at room temperature under orbital shaking (250 rpm). Residual activity was determined by the spectrophotometric assay as described above.

Reutilization of immobilized lipase

Reuse of the immobilized lipase preparation was determined by measuring activity up to 12 cycles. After each cycle, immobilized lipase was washed three times with 0.05 M phosphate buffer pH 6.5 and dried overnight under vacuum.

Storage stability

Immobilized and soluble lipases were stored at either −4 °C or room temperature and residual activity was determined by the spectrophotometric assay.

Results and discussion

Lipase immobilization

After the crude lipolytic preparation of *G. thermoleovorans* was diafiltered (co 500,000 Da), a partially purified lipase was obtained, as demonstrated by Castro-Ochoa and co-workers [15]. This preparation was used for the immobilization experiments with protein concentrations up to 1.5 mg/mL at 25–60 °C. Immobilization time was set at 3 h since after 120 min adsorption reached equilibrium at all temperatures tested. Lipase appeared to bind rapidly to the support; after 30 min at 25 °C as much as 63% of the initial activity disappeared from the solution, which corresponded to 39% of the total protein content (data not shown).

Protein adsorption isotherms data are depicted in Fig. 1, adjusted with the Langmuir isotherm model (Eq. 1) at 25–60 °C. Adsorption equilibrium constants and maximum predicted protein concentrations adsorbed to the support are shown in Table 1.

$$C_{\text{prot ads}} = \frac{C_{\text{prot ads max}} K_{\text{prot}}}{K_{\text{prot}} + C_{\text{prot}}}. \tag{1}$$
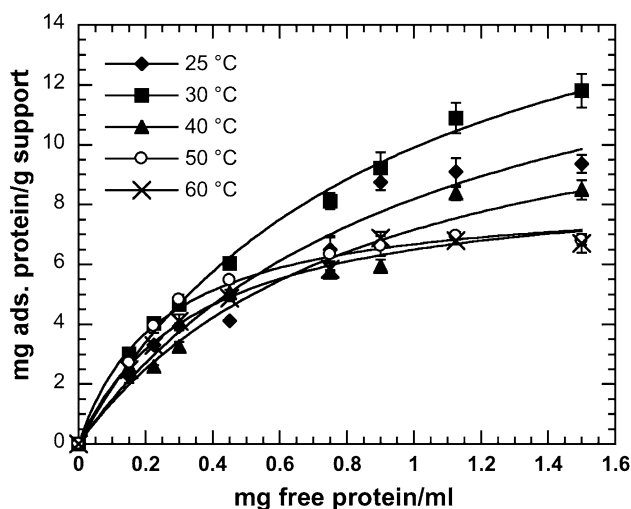

Fig. 1 Experimental data and Langmuir isotherm fits for protein adsorption of the partially purified lipase from *Geobacillus thermoleovorans* CCR11 on microporous polypropylene at different temperatures: (*filled diamond*) 25 °C, (*filled square*) 30 °C, (*filled triangle*) 40 °C, (*open square*) 50 °C, (*times*) 60 °C

Table 1 K_{prot} and $C_{prot\ ads\ max}$ values calculated for the different protein adsorption isotherms

Temperature	25 °C	30 °C	40 °C	50 °C	60 °C
K_{prot} (mg/mL)	1.03	0.92	0.86	0.25	0.35
$C_{protads\ max}$ (mg/g)	16.59	19.03	13.32	8.34	8.81

Table 2 K_{act} and $A_{ads\ max}$ values obtained for the different lipolytic activity adsorption isotherms

	25 °C	30 °C	40 °C	50 °C	60 °C
K_{act} (U/mL)	47,313	31,792	19,585	23,808	16,866
$A_{ads\ max}$ (U/g)	30,284	24,992	13,040	14,970	9,493

As can be seen, the largest amount of protein adsorbed to the support was found at 30 °C and the adsorption became asymptotic at 10.9 mg/g support. Lipase activity adsorption isotherms at 25–60 °C are shown in Fig. 2, which fitted adequately to the Langmuir isotherm model (Eq. 2). Adsorption equilibrium constants and maximum predicted lipolytic activities adsorbed to the support are shown in Table 2. Contrary to protein adsorption behavior, the largest activity adsorption was found at 25 °C. Adsorption became asymptotic at 3.1 E4 U/g support, corresponding to 57% of the total lipolytic activity.

$$A_{ads} = \frac{A_{ads\ max}K_{act}}{K_{act} + A}. \tag{2}$$

Finally, the maximum activity per gram of support was found at 25 °C with 0.925 mg of protein/mL. The amount of protein adsorbed was ca. 8.7 mg protein/g support in accordance to what other authors have reported for lipases on this support [3]. Under these experimental conditions we found the optimum immobilization pH at 6.0, close to the pH reported for the immobilization of lipases produced by *Pseudomonas cepacia*, *Rhizomucor miehei*, *Humicola* sp., *Rhizopus niveus* and *Candida antarctica* onto Accurel EP-100 (pH 7.0) [10, 23]. Consequently all the immobilization procedures were undertaken at 25 °C, pH
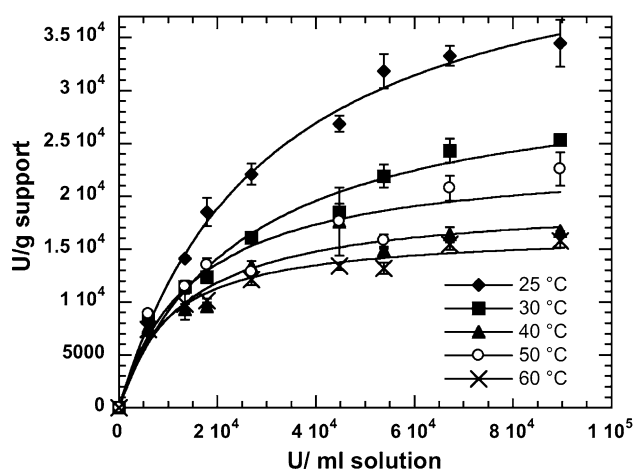
6.0, 250 rpm, and at a concentration of 0.925 mg of protein/mL. Under these conditions, we found up to 40% increase in lipase specific activity after immobilization. The increase in specific activity of lipases immobilized on hydrophobic supports has been reported [8, 9, 24]; the authors propose that this activity increase is caused by a strong interaction of hydrophobic areas of lipases near/on the lid with the support surface, which leads to conformational changes that result in a highly active open form.

Immobilization in the presence of Triton X-100

As previously reported [15], we observed a 5-fold increase on the specific activity of the partially purified soluble lipase in presence of 0.1% Triton X-100, probably due to the disruption of protein aggregates and/or to the shift on the closed/open equilibrium towards the open form of the lipase [16].

When 0.1% Triton X-100 was added to immobilization medium there was no difference in the immobilization yield (52.6 ± 5.3 vs. 58.8 ± 3.1) although a 4–5-fold increase in lipolytic activity per gram of support was observed with respect to the lipolytic activity of immobilized lipase without the detergent.

Effect of temperature on enzyme activity and stability of free and immobilized lipase

When the effect of temperature on enzyme activity was analyzed, a 10 °C increase in the optimum temperature of activity was observed after immobilization on porous polypropylene only when Triton X-100 was not added, from 60 °C (soluble) to 70 °C (immobilized). A similar trend has been reported for other immobilized lipases that showed increases from 1 to 11 °C in their optimal temperatures, depending on the source of the lipase and the immobilization method [8, 13, 25] suggesting that this effect may be attributed to an increase in enzyme thermal resistance due to mobility restrictions.

A significant increase in lipase thermostability was observed when the enzyme was immobilized with or without the detergent (Table 3). Immobilized lipases showed residual activity even after 1 h at 100 °C, when soluble enzyme completely lost activity after 1 h at 80 °C. This result agrees with other reports in which microbial lipases immobilized on hydrophobic supports [19, 25] have

Fig. 2 Experimental data and Langmuir isotherm fits for adsorption of lipolytic activity of the partially purified lipase from *Geobacillus thermoleovorans* CCR11 on microporous polypropylene at different temperatures: (*filled diamond*) 25 °C, (*filled square*) 30 °C, (*filled triangle*) 40 °C, (*open square*) 50 °C, (*times*) 60 °C

Table 3 Effect of temperature on immobilized lipase activity after 1 h of incubation at temperatures above 50 °C

Temperature (°C)	Residual activity ± SD (%) Lipase/wo Triton X-100		Residual activity ± SD (%) Lipase/0.1% Triton X-100	
	Soluble	Immobilized	Soluble	Immobilized
50	100 ± 3.8^a	100 ± 7.2^a	100 ± 2.0^a	100 ± 2.5^a
60	91.5 ± 4.7^b	100 ± 8.1^a	89.6 ± 2.2^a	100 ± 0.4^a
70	48.3 ± 5.9^c	93.2 ± 5.7^b	34.5 ± 3.8^d	100 ± 4.6^a
80	13.0 ± 4.4^e	72.1 ± 7.6^f	8.8 ± 6.3^e	54.2 ± 6.6^c
90	0	56.5 ± 5.3^c	0	15.4 ± 11.7^e
100	0	15.2 ± 5.3^e	0	4.2 ± 2.6^e

Different superscript letters indicate statistical differences between groups

$P < 0.05$

shown greater thermal stability than their free counterparts. They suggest that the restriction on protein conformational motility due to immobilization probably provokes a more rigid lipase structure that impedes the unfolding and the loss of the catalytic properties of the enzyme.

The effect of immobilization on thermal stability was affected by the presence of Triton X-100, since the lost of activity with the increase in temperature was higher in the lipase immobilized with the detergent (Table 3). This is in accordance to [19] who reported that the lipase from *Thermomyces lanuginosa* retained 80% of its initial activity after 30 h at 50 °C, compared to 40%, when it was immobilized in the presence of 0.6% Triton X-100. Probably the presence of oligomers and other lipase aggregates makes the enzyme immobilized without Triton X-100 more resistant to thermal deactivation.

Effect of pH on enzyme activity and stability of free and immobilized lipase

There was no significant change in the optimum pH of activity after immobilization (pH 9), irrelevant to the presence of Triton X-100, as has been reported for other microbial lipases immobilized on hydrophobic supports [8, 25]. Soluble and immobilized lipases showed good pH stability over a wide range of pHs (5–12) (data not shown).

Substrate specificity of free and immobilized lipase

The potential application of a biocatalyst might be determined by the preference for some substrates; it was then important to evaluate the effect of immobilization on the fatty acid specificity of the *G. thermoleovorans* CCR11 lipase by varying the acyl moiety of the *p*-nitrophenyl ester substrates. Both free and immobilized lipases with or without Triton X-100 displayed the highest relative activity towards the acyl C:8 (Fig. 3), although a significant decrease in activity ($P < 0.05$) over the acyl chains C:4, C:6 and C:10
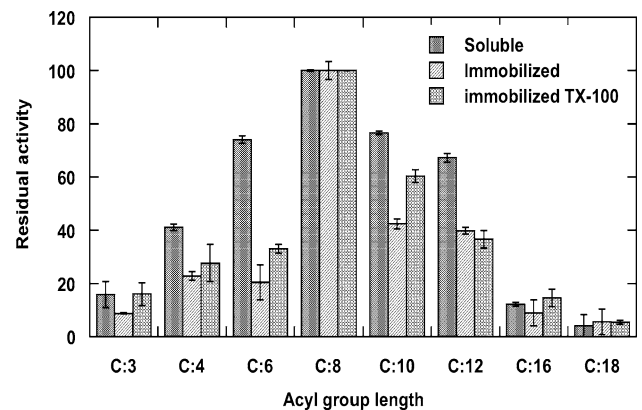

Fig. 3 Effect of immobilization on chain length preference of *Geobacillus thermoleovorans* CCR11 lipase

was observed in immobilized lipases. These changes may be due to modifications in enzyme structure, which may in turn affect substrate-binding sites, and/or cause either substrate diffusional limitations inside the porous support or incompatibility with the hydrophobic nature of the support [11, 26]. The preference over C:8 acyl moiety suggests this biocatalyst as a possible candidate for milk fat modification.

Desorption of the immobilized lipase

Immobilized enzymes can lose activity during its use due to several factors including temperature deactivation, operational pH and water content in the reaction medium. Our results (Fig. 4) suggest that the interaction between the lipase and the polypropylene support was strong, since no change in residual activity was observed in immobilized lipases after washing them with phosphate buffer during 12 h at room temperature and 250 rpm. Previous works have reported that strong hydrophobic forces keep lipase bound to hydrophobic supports [8, 9]. The addition of NaCl in washing buffer did not alter the activity of lipase immobilized without Triton X-100, but the lipase immobilized in the presence of the detergent lost 27.5% of its
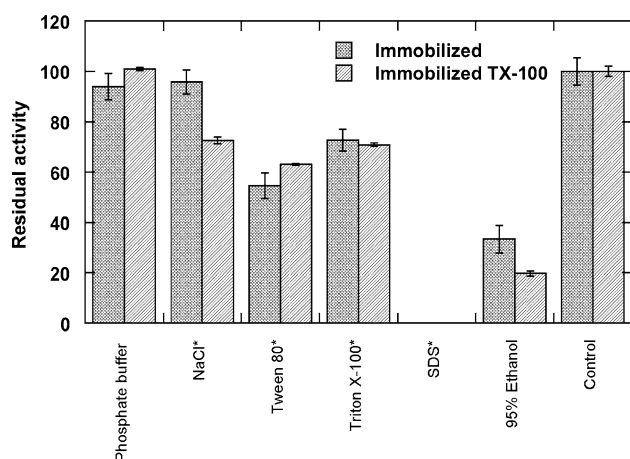
Fig. 4 Effect of detergents and other substances on immobilized lipase activity
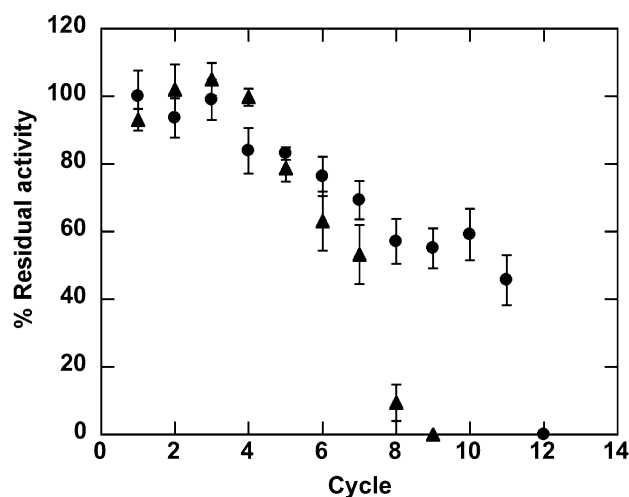

Fig. 5 Residual activity of the lipase from *Geobacillus thermoleovorans* CCR11 immobilized on polypropylene in continuous cycles of hydrolysis of *p*-nitrophenol laureate. *Filled circle* Immobilized (*filled triangle*) immobilized in presence of 0.1% Triton X-100

activity, probably because lipase molecule is in its monomeric form and more evenly distributed on the support surface making it more susceptible to desorption when ionic strength was changed [23].

The detergents SDS, Tween and Triton X-100 are commonly used to disaggregate lipases, to decrease the unspecific protein adsorption during immobilization procedures, to desorb lipases after hydrophobic chromatography, and for the recovery of expensive supports [11, 18–20]. In this work, washing the immobilized lipases in the presence of 1% SDS resulted in a total lost of lipolytic activity probably due to lipase denaturalization, as has been reported by Castro-Ochoa et al. [15]. A different behavior was observed with the other detergents used; there was a 28–45% decrease in lipolytic activity in the presence of Tween 80 and Triton X-100 in both immobilized lipases, contrasting with the results of Pencreac'h et al. [11] who found a total lost of the activity of *Pseudomonas cepacia* lipase immobilized on Accurel EP 100 after 10 min in 0.4% Triton X-100. This suggests that the hydrophobic interaction between the lipase of *G. thermoleovorans* and the polypropylene support is very strong.

The immobilized lipases with and without Triton X-100 washed in 95% ethanol maintained more than 60% of its original activity. Castro-Ochoa et al. [15] reported that the same lipase lost ca. 70% of its activity when exposed to 70% ethanol for 2 h. Consequently the loss of activity observed in our work is most probably due to protein denaturalization than to the wash out from the support.

Reutilization of the immobilized lipase

The chemical nature of the support, the immobilization procedure and the operational conditions are factors that influence the number of possible reutilization cycles for immobilized lipases [11, 12]. One of the main

disadvantages of immobilization through adsorption is that immobilized enzymes display low stability towards reutilization specially when working in aqueous media. In this work, the ability of *G. thermoleovorans* immobilized lipase to be re-used was evaluated in continuous cycles of hydrolysis of *p*-nitrophenyl-laurate. Lipase immobilized in the presence of Triton X-100 showed a total lost of activity after nine cycles of reuse, retaining 53% of its initial activity after seven cycles (Fig. 5), while the immobilized lipase without Triton X-100 lost all of its activity after 12 cycles, retaining 69% of its initial activity after seven cycles. Different levels of resistance to activity loss by reutilization of lipases immobilized in hydrophobic supports have been reported; *Thermomyces lanuginosa* lipase kept only 15% of its initial activity after five cycles of reuse [12], while a lipase from *Bacillus sp.* immobilized on different supports retained 100% of its activity after 25 cycles [25].

Storage stability

Storage stability is an important attribute of immobilized enzyme preparations [3]. In our work, immobilized lipases, with or without Triton X-100, showed high storage stability even after 36 days at 4 °C, as has been previously reported for other lipases [8].

In conclusion, a lipase from *G. thermoleovorans* CCR11 was immobilized on porous polypropylene from a partially purified extract. The presence of 0.1% Triton X-100 in immobilization medium did not interfere with lipase adsorption to the support, although lipase immobilized in the presence of the detergent neither show as much thermostability nor reuse ability as the one immobilized

without it. However, the presence of Triton X-100 resulted in an immobilized biocatalyst with a high activity per gram of support, with a potential use in dairy fat modification.

References

1. Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures and biotechnological applications of lipases. Annu Rev Microbiol 53:315–351. doi:10.1146/annurev.micro.53.1.315

2. Sharma R, Chisti Y, Banerjee UC (2001) Production, purification, characterization and applications of lipases. Biotechnol Adv 19:627–662. doi:10.1016/S0734-9750(01)00086-6

3. Minovska V, Winkelhausen E, Kuzmanova S (2005) Lipases immobilized by different techniques on various support materials applied in oil hydrolysis. J Serb Chem Soc 70:609–624. doi:10.2298/JSC0504609M

4. Leow TC, Rahman RN, Basri M, Salleh AB (2004) High level expression of a thermostable lipase from *Geobacillus sp.* Strain T1. Biosci Biotechnol Biochem 68(1):96–103. doi:10.1271/bbb.68.96

5. Hill CG Jr, Otero C, García HS (2006) Immobilized Enzyme Technology. In: Encyclopedia of Chemical Processing. Tyler and Francis, pp 1367–1380

6. Tischer W, Wedekind F (1999) Immobilized enzymes: methods and applications. Top Curr Chem 200:96–126

7. Malcata FX, Reyes HR, García HS, Hill CG, Amundson CH (1990) Immobilized lipase reactors for modification of fats and oils: a review. J Am Oil Chem Soc 67:890–910. doi:10.1007/BF02541845

8. Malcata FX, García HS, Hill CG Jr, Amundson CH (1992) Hydrolysis of butteroil by immobilized lipase using a hollow-fiber reactor: Part I lipase adsorption studies. Biotechnol Bioeng 39:647–657. doi:10.1002/bit.260390609

9. García HS, Yang B, Parkin KL (1996) Continuous reactor for enzymatic glycerolisis of butteroil in the absence of solvent. Food Res Int 28:605–609. doi:10.1016/0963-9969(95)00051-8

10. Pencreac'h G, Baratti JC (1997) Activity of *Pseudomonas cepacia* in organic media is greatly enhanced after immobilization on a polypropylene support. Appl Microbiol Biotechnol 47:630–635. doi:10.1007/s002530050986

11. Pencreac'h G, Leullier M, Baratti JC (1997) Properties of free and immobilized lipase from *Pseudomonas cepacia*. Biotechnol Bioeng 56:181–189. doi :10.1002/(SICI)1097-0290(19971020)56:2<;181::AID-BIT7>;3.0.CO;2-L

12. Ferrer M, Plou FJ, Fuentes G, Cruces MA, Andersen L, Kirk O et al (2002) Effect of the immobilization method of lipase from *Thermomyces lanuginosus* on sucrose acylation. Biocatal Biotransform 20:63–71. doi:10.1080/10242420210153

13. Deng HT, Xu ZK, Huang XJ, Wu J, Seta P (2004) Adsorption and activity of *Candida rugosa* lipase on polypropylene hollow fiber membrane modified with phospholipid analogous polymers. Langmuir 20:10168–10173. doi:10.1021/la0484624

14. Bano MC, González-Navarro H, Abad C (2003) Long chain fatty acyl-CoA esters induce lipase activation in the absence of a water-lipid interface. Biochem Biophys A 1632:55–61

15. Castro-Ochoa LD, Rodríguez-Gómez C, Valerio-Alfaro G, Oliart-Ros RM (2005) Screening purification and characterization of the thermoalkalophilic lipase produced by *Bacillus thermoleovorans* CCR11. Enzyme Microb Technol 37:648–654. doi:10.1016/j.enzmictec.2005.06.003

16. Fernández-Lorente G, Palomo JM, Cabrera Z, Fernández-Lafuente R, Guisán JM (2007) Improved catalytic properties of immobilized lipases by the presence of very low concentrations of detergents in the reaction medium. Biotechnol Bioeng 97:242–250. doi:10.1002/bit.21230

17. Helisto P, Korpela T (1998) Effects of detergents on activity of microbial lipases as measured by nitrophenyl alkanoate esters method. Enzyme Microb Technol 23:113–117. doi:10.1016/S0141-0229(98)00024-6

18. Rodrigues AR, Cabral JMS, Taipa MA (2002) Immobilization of *Chromobacterium viscosum* lipase on Eudragit S-100: coupling, characterization and kinetic application in organic and biphasic media. Enzyme Microb Technol 31:133–141. doi:10.1016/S0141-0229(02)00087-X

19. Palomo JM, Fuentes M, Fernández-Lorente G, Mateo C, Guisán JM, Fernández-Lafuente R (2003) General trend of lipase to self-assemble giving biomolecular aggregates greatly modifies the enzyme functionality. Biomacromol 4:1–6. doi:10.1021/bm025729+

20. Palomo JM, Segura RL, Fernández-Lorente G, Pernas M, Rua ML, Guisán JM et al (2004) Purification, immobilization and stabilization of a lipase from *Bacillus thermocatenulatus* by interfacial adsorption on hydrophobic supports. Biotechnol Prog 20:630–635. doi:10.1021/bp0342957

21. Nawani N, Dosanjh NS, Kaur J (1988) A novel thermostable lipase from a thermophilic *Bacillus sp*: characterization and esterification studies. Biotechnol Lett 20:997–1000. doi:10.1023/A:1005430215849

22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1955) Protein measurement with the Folin phenol reagent. Department of Pharmacology, Washington University. School of Medicine, St. Louis, Missouri, pp 265–275

23. Bosley JA, Peilow AD (1997) Immobilization of lipases on porous polypropylene: reduction in esterification efficiency at low loading. J Am Oil Chem Soc 74:107–111. doi:10.1007/s11746-997-0153-6

24. Bastida A, Sabuquillo P, Armisen P, Fernández-Lafuente R, Huguet J, Guisán JM (1998) A single step purification immobilization and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. Biotechnol Bioeng 58:486–493. doi :10.1002/(SICI)1097-0290(19980605)58:5<;486::AID-BIT4>;3.0.CO;2-9

25. Dosanjh NS, Kaur J (2002) Immobilization stability and esterification studies of a lipase from *Bacillus sp*. Biotechnol Appl Biochem 36:7–12. doi:10.1042/BA20010070

26. Fernández-Lafuente R, Armisén P, Sabuquillo P, Fernández-Lorente G, Guisán JM (1998) Immobilization of lipases by selective adsorption on hydrophobic supports. Chem Phys Lipids 93:185–197. doi:10.1016/S0009-3084(98)00042-5