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High catalytic performances of *Pseudomonas fluorescens* lipase adsorbed on a new type of cyclodextrin-based nanosponges

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ABSTRACT

Lipases are water-soluble enzymes that catalyze the hydrolysis of triacylglycerols (in aqueous media) or trans-esterification reactions (in microaqueous media) and are involved in a number of industrial applications. As a limit to lipase application is represented by the need for interfacial activation, the search for suitable solid supports able to fulfill this requirement is always ongoing. In the present work, we report the preliminary characterization of a system obtained by adsorbing *Pseudomonas fluorescens* lipase on a newly synthesized cyclodextrin-based carbonate nanosponge (CD–NS–1:4). The activity and structural stability of lipase adsorbed on this new support were evaluated by checking the effect of temperature, pH changes and organic solvents (methanol) on the enzyme structure and function, which were compared with those of the free enzyme in solution. Our data show that the non-covalent interaction of *Ps. fluorescens* lipase with CD–NS–1:4 results in enzyme structural and functional stabilization, as it was still active after 66 days of incubation at $T \sim 18\,^{\circ}$C. Stabilization with respect to *T*, pH and the presence of organic solvent was observed as well as, unlike the solubilized enzyme, the adsorbed lipase was active at $T > 40\,^{\circ}$C, at pH 5 and after 24-h incubation with 70% (v/v) methanol (13% residual activity).

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1. Introduction

Enzyme immobilization is advantageous as it allows enzyme recycling and facilitates the separation and recovery of the reaction products from the reaction environment. In addition, it often results in increased thermal and operational stability of the biocatalysts [1,2], thus allowing their employment in a wider set of experimental conditions as compared to solubilized enzymes. Hydrolytic enzymes, such as proteases, amylases, esterases and lipases, are among the most widely used enzymes for industrial purposes. Lipases are triacylglycerol hydrolases (EC 3.1.1.3) that either catalyze the hydrolysis of triacylglycerols (in aqueous media) or trans-esterification reactions (in microaqueous media). They have found wide usage in a growing range of industrial applications such as detergents, food processing, pharmaceutical, cosmetic, textile, leather and paper industry, biotransformations, waste treatment and bioremediation. Lipases have also medical applications as drugs or biosensors [3,4]. One of the most intriguing industrial application of lipases is the synthetic process of methyl- and ethyl-esters of long-chain fatty acids (biodiesel) starting from oils and methanol (or ethanol) [5]; although, it must be said that the level of performance provided by lipases is not yet comparable to that provided by basic catalysis, in the presence of NaOH or KOH [6].

The issue of enzyme immobilization is particularly relevant for lipases, as it improves their stability and modulates properties such as enantioselectivity and reaction rates [7]; as a consequence, the demand for new solid supports, suitable for this family of enzymes, is constantly growing. Lipase stabilization and activation is partly related to the peculiar structural features of lipases, i.e. the presence of a mobile domain acting as a “lid” for the enzyme active site, which is able to modulate the entrance of substrates into the catalytic pocket. In the presence of a hydrophylic environment the lid is closed and the active site almost unaccessible. The contact with hydrophobic interfaces or the presence of drops of hydrophobic substances induces a conformational movement of such mobile domain and the consequent exposure of wide hydrophobic patches that surround the active site. The catalytic site becomes accessible to substrates and the enzyme turns to its activated form [8]. Such complex mechanism makes the modulation and control of lipase-catalyzed organic synthesis processes quite difficult, both on the laboratory and the industrial scale; notwithstanding these problems, it may be exploited to design activated enzyme forms whose properties may be modulated by changing the immobilization conditions *ad hoc* [9]. Hydrophobic supports generally mimics the presence of substrates, thus acting as triggers for interfacial

Abbreviations: p-NP, para-nitrophenol; p-NPD, para-nitrophenol dodecanoate; CD, β-cyclodextrin; NS, nanosponges; CD–NS, β-cyclodextrin-based nanosponges.

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activation: in fact, several examples of lipase activation through the adsorption on hydrophobic supports are now available in the literature; *Pseudomonas fluorescens* lipase adsorbed on octyl-agarose has shown 6-fold activation and a sharp increase of its stereospecificity [7]. 300-Fold activation has been observed in organic media on lipases immobilized on hydrophobic sol–gels [10]. Alternatively, trapping of lipases in hydrophobic gel fibers prevents enzyme inactivation [11]. In general, hydrophilic supports, like cellulose acetate, are not as good for lipases and cannot achieve the same degree of enzyme activation that has been observed with hydrophobic beads [12].

Aim of this study was to check the suitability of a new type of β-cyclodextrin-based nanosponges as a support for lipase. β-Cyclodextrins, with their typical toroidal form, are characterized by an external hydrophylic surface (hydrophilicity is determined by the presence of OH groups from glucose moieties) and a relatively hydrophobic internal cavity [13]. They are often used to solubilize organic molecules in aqueous media (e.g., drugs) as they can host hydrophobic moieties inside their cavity, while the external surface is solvated. Cyclodextrins can be cross-linked through the reaction between OH groups and a chemical linker to form a water insoluble material, with emergent properties as compared to the original building blocks. Their interaction with enzymes may result in enzyme stabilization [1].

The catalytic performances of lipase co-lyophilized with α-, β-, γ-cyclodextrins [14–16] or covalently immobilized on β-CD polymers containing urethane groups have been reported in the literature [1,17,18]. Conversely, the effect of non-covalent immobilization of lipase on carbonate cross-linked CD–NS has not yet been reported.

In the present study we investigated the effect of the interaction of *Ps. fluorescens* lipase with carbonate CD–NS 1:4 on the enzyme properties (namely, the activity, the chemical and thermal stability and the persistency of the enzyme catalytic proficiency vs. time). *Ps. fluorescens* lipase is produced by a psychrophilic organism; it undergoes interfacial activation, as described above and prefers small- or medium-chain fatty acids [4]; its application in biodiesel production has been proven successful [19–22]. As a first step, we compared the interaction of *Ps. fluorescens* lipase with three different types of nanosponges: the newly synthesized carbonate-linked CD–NS 1:4 and CD–NS 1:2 (characterized by distinct degree of cross-linkages) and the urethane-linked CD–NS 1:8 (Fig. 1). As the CD–NS 1:4 support provided the best balance between loading capacity and catalytic proficiency of the adsorbed enzyme, we investigated and compared the differential response of solubilized and immobilized lipase from *Ps. fluorescens* with respect to pH, temperature changes and mixed organic/aqueous media.

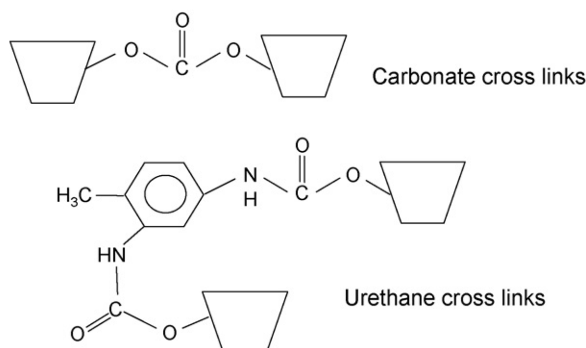

Fig. 1. A graphical representation of the two kind of chemical linkages found in the cyclodextrin-based nanosponges employed in this work.

2. Materials and experimental methods

2.1. Enzyme and reagents

Ps. fluorescens lipase, para-nitrophenol dodecanoate (p-NPD), mono- and dihydrogen sodium phosphate, sodium hydroxide, 2-propanol, sodium acetate, Triton X-100, aceton, methanol and ethanol were purchased from Sigma–Aldrich. Analytical grade boric acid was purchased from Merck. Hydrochloric acid 37% (w/w) was purchased from Carlo Erba. Cyclodextrin-based nanosponges CD–NS 1:2 and CD–NS 1:4 (carbonate) were synthesized as reported by Trotta and coworkers [23,24] with minor modifications, whereas NS 1:8 (urethane) were synthesized as reported by Li and Ma [25]. Aliquots of a mother solution of *Ps. fluorescens* lipase 10 mg/ml dissolved in 100 mM phosphate buffer pH 7 + Triton X-100 0.1% (v/v) were kept at −20 °C and thawed out before use.

2.2. Immobilization protocol of Ps. fluorescens lipase on CD–NS supports

In order to remove possible residues of by-products, supports were washed with ethanol and with 100 mM phosphate buffer pH 7 or 8 + Triton X-100 0.1% (v/v), as specified in the text. Immobilization was carried out by incubating either 50, 200 or 500 mg of solid support with a solution of 0.625 mg/ml *Ps. fluorescens* lipase in 10–100 mM phosphate buffer pH 8 + Triton X-100 0.1% (v/v); samples were incubated for variable timelengths (2–20 h) at RT. Enzyme adsorption was monitored during incubation by measuring the residual hydrolase activity of lipase in the supernatant. Finally, the mixture was centrifuged at 4500 rpm and the functionalized support was washed and resuspended in 100 mM phosphate pH 7 or 8 + Triton X-100 0.1% (v/v), as specified in the text. In order to check possible enzyme desorption, the activity of the supernatant was checked periodically after immobilization.

2.3. Activity measurements on Ps. fluorescens lipase in solution; effect of pH, methanol and Triton X-100

Activity measurements were carried out according to the protocol reported by Kosaka et al. [12] with little changes. A mother solution of p-NPD was prepared by dissolving 22 mg/ml of substrate in the measuring buffer (100 mM phosphate buffer pH 7, aceton (0.07%, v/v), 2-propanol (10%, v/v), Triton X 100 (0.2%, v/v)). Due to its low water solubility, p-NPD was first dissolved in a small volume of aceton and subsequently diluted with the above-described mixture.

873 μl of *p*-NPD solution were put into a 3-ml cuvette, diluted with measuring buffer up to a final concentration of 6.4 μg/ml and kept under stirring at the desired T (10–60 °C) for 2 min. 12 μl of lipase solution 0.625 mg/ml were then added and the formation of the reaction product *p*-NP was monitored spectrophotometrically ($\Delta A_{400\,nm}$) for 6 min ($\varepsilon_{400\,nm}$ = 13,800 M^{-1} cm^{-1}) [12].

The pH dependence of the enzyme activity was checked according to the above-mentioned protocol, by using 100 mM buffers in the 5–10 pH range (pH 5, sodium acetate; pH 6–8, sodium phosphate; pH 9–10, sodium borate. All buffers contained Triton X-100 0.1%, v/v). The activity of each sample was measured in triplicate, after 30-min and 24-h incubation at the desired pH.

The effect of methanol on the hydrolase activity of lipase was checked according to the above-described protocol, by using 100 mM phosphate buffer pH 7 + Triton X-100 0.1% (v/v) in the presence of distinct methanol percentages (0–93%, v/v range). The activity of each sample was measured in triplicate, after 30-min and 24-h incubation.

The effect of Triton X-100 on the enzyme activity was checked according to the above-mentioned protocol, by using 100 mM

phosphate buffer pH 7 + Triton X-100 in the 0–0.5% (v/v) range. The activity of each sample was measured in triplicate, after 30-min and 24-h incubation.

Optical measurements were performed on a double-beam spectrophotometer Unicam UV300 Thermospectronic equipped with a Peltier device.

2.4. Activity measurements on immobilized Ps. fluorescens lipase in batch; effect of methanol

Activity measurements in batch were perfomed in triplicate at pH 7 or 5 (as specified in the text) according to the following protocol: ~4 mg NS (containing ~7.5 μg lipase) were mixed in a 3-ml cuvette with *p*-NPD dissolved in the measuring buffer (final concentration = 6.4 μg/ml); blank measurements were done accordingly, in the absence of lipase. Samples were incubated for 30 min at 30 °C; the reaction was stopped by putting the reaction mixture into an ice bath, the mixture was centrifuged and the amount of *p*-NP found in the supernatant was determined spectrophotometrically ($\lambda_{400\,nm}$ = 13,800 M^{-1} cm^{-1} at pH 7; $\varepsilon_{312\,nm}$ = 9500 M^{-1} cm^{-1} at pH 5) [12,26].

The effect of methanol on the activity of the immobilized enzyme was checked after 24-h incubation of the "lipase–NS 1:4" system in the presence of 70% (v/v) methanol. This percentage was chosen as it was found to induce complete inactivation of the free enzyme. Activity measurements were performed according to the above-described protocol.

2.5. Activity measurements on immobilized Ps. fluorescens lipase under continuous-flow conditions

~0.12 mg lipase adsorbed on 50 mg support (~2.0 mg enzyme/g of support) were introduced in a thermostated column (Ø = 1 cm, height = 4 cm) and washed with 100 mM phosphate buffer pH 7 + Triton X-100 0.1% (v/v) or with the suitable measuring buffer, while kept under stirring (slurry conditions). A substrate solution (22 mg/ml p-NPD dissolved in the measuring buffer pH = 7) was streamed through the column at a flow ~5–7 ml/h. The amount of reaction product was monitored spectrophotometrically at 3- or 45-min intervals along 8 h at 30 °C ($\varepsilon_{400\,nm}$ = 13,800 M^{-1} cm^{-1}). The effect of T on the enzyme activity was checked in the 10–65 °C range, according to the same protocol, in the absence of stirring.

3. Results and discussion

Cyclodextrin-based nanosponges are solid supports obtained by cross-linking β-CD moieties: the chemical nature of the linker is crucial as it provides the polymer with peculiar properties. Typically, urethane-bridged CD–NS have been widely reported [1,17], whereas carbonate-bridged CD–NS represent a novelty in this field (Fig. 1). CD–NS exhibit much higher inclusion constants as compared to β-CD and have turned out to be suitable supports for enzyme immobilization, as they are capable of preserving (and sometime enhance) the catalytic proficiency and stability of the immobilized enzymes [27]. Aim of this study was to monitor the structural and functional properties of *Ps. fluorescens* lipase after immobilization on a new type of β-cyclodextrin-based nanosponges (CD–NS 1:4) characterized by carbonate bridges.

As a first step, we compared carbonate CD–NS 1:4 with two other cyclodextrin nanosponges: carbonate CD–NS 1:2 (with a lower degrees of cross-linkage) and urethane CD–NS 1:8. Our goal was to identify the support able to provide the best balance between loading capacity and catalytic proficiency of the adsorbed enzyme.

Table 1
Loading efficiency of CD–NS 1:2, CD–NS 1:4 (carbonate) and CD–NS 1:8 (urethane). Activity was measured during incubation of the enzyme with the loading support; aliquots of solution were drawn during incubation, centrifugated (in order to remove any residual solid particle) and the activity was checked according to the protocol described in Section 2.

	Residual enzyme activity (% scale) at					
	0 h	1 h	2 h	3 h	4 h	20 h
Control solution	100	98	96	99	96	73
NS 1:2	100	99	97	100	100	61
NS 1:4	100	96	99	100	100	67
NS 1:8	100	100	97	100	96	0

3.1. Loading capacity of the CD–NS supports

Ps. fluorescens lipase was incubated with each of the above-cited supports for a fixed timelength. The amount of enzyme adsorbed on each support was assessed by measuring the residual enzyme activity in the supernatant, before and after incubation, based on the assumption that it is inversely proportional to the adsorbed enzyme. Table 1 shows the decrease of the hydrolase activity of *Ps. fluorescens* lipase in the supernatant vs. time, during incubation at RT with each of the three CD–NS supports. At relatively short timelengths (up to 4 h) adsorption was negligible and the activity in the supernatant kept almost unchanged. After 20-h incubation in the presence of CD–NS 1:8 no residual hydrolase activity was found: this indicates that the totality of the enzyme had been adsorbed on the urethane support and corresponds to a loading capacity of 12.5 mg lipase/(g of support) in these experimental conditions. Much higher residual activities were found in the presence of NS 1:2 and 1:4 after 20-h incubation: this indicates that the kinetic of the adsorption process is slower compared to urethane CD–NS. In all cases, the adsorption of lipase was stable as the enzyme was not desorbed neither after 24 h at RT and several washings nor during continuous-flow conditions. No enzyme release had been detected from CD–NS 1:2 and 1:4 supports even after 66 days from the immobilization experiment. Most surprisingly, these samples were still enzymatically active under continuous-flow conditions, although a direct comparison with the activity of the freshly immobilized enzyme was not feasible.

Activity of the immobilized lipase was checked under continuous-flow conditions upon stirring (slurry conditions) and normalized with respect to the amount of immobilized enzyme. It was expressed as μmol of product released by the system working at full capacity and followed the trend: CD–NS 1:4 > CD–NS 1:2 > CD–NS 1:8 (Table 2). Data normalized with respect to the amount of support follow a similar trend. This shows that urethane CD–NS greatly diminish lipase catalytic proficiency, although they are more effective in enzyme loading (6.6-fold with respect to carbonate CD–NS 1:4). It is worth noting that this is a relative rather than an absolute value and might be even higher; in fact, the experiment was done by incubating a same amount of enzyme with a given amount of each support. We cannot exclude that higher

Table 2
Activity of lipase immobilized on CD–NS 1:2, CD–NS 1:4 and CD–NS 1:8. Activity was measured under slurry conditions, as described in the text. The plateau was reached after: 2 h (CD–NS 1:2), 2 h (CD–NS 1:4) and 12 h (CD–NS 1:8).

	μmol p-NP produced per hour by 1 mg of *Pfl* lipase adsorbed on CD–NS[a]	μmol p-NP produced per hour by 1 g of CD–NS[a]
NS 1:2	114	217
NS 1:4	600	1140
NS 1:8	35	437

[a] System working at full capacity (flux = 6 ml/h).

quantities of lipase could have been adsorbed on the same amount of CD–NS 1:8 employed in this experiment.

We also checked the dependence of the activity of the immobilized enzyme on the presence of soluble β-CD (not cross-linked): activity measurements were carried out at a fixed enzyme concentration in the presence of various enzyme/β-CD weight ratios (from 1/1 to 1/22 mg enzyme/mg β-CD). The activity was unaltered up to a 10-fold excess; 22-fold excess results in ∼30% inactivation. This finding agrees with that reported by Ghanem [28], who found that co-lyophilization of lipase with peracetilated β-CD resulted in enhanced reaction rates, whereas the addition of β-CD to a lipase solution did not cause the same effect. Based on SEM analysis, they conclude that the intimate contact of β-CD and lipase during co-lyophilization brings about morphological changes in the enzyme, that are responsible for the reactivity changes.

All in all, these data show that: (i) all the three supports adsorb *Pfl* firmly; (ii) the loading capacity of urethane vs. carbonate CD–NS is markedly different; (iii) the enzyme catalytic proficiency of *Pfl* is better preserved by the interaction with carbonate CD–NS rather than with urethane support. The explanation of such behaviour at a molecular level is not straightforward, although some speculations can be made, based on the available information concerning CDs and CD-based nanosponges.

The adsorption stability of the enzyme on CD–NS relies on two different interaction mechanisms: the ability of CD cavities to host aromatic and hydrophobic side chains of aminoacid residues sitting on the protein surface [29]; the presence of a complex network of tertiary contacts between the protein residues, the OH groups exposed on the exterior of CD moieties and the carbonate or the carbamate moieties, that may stabilize the enzyme *via* entropy changes [2].

As these mechanisms are shared by both kinds of CD–NS, the differences in loading capacity and catalytic proficiency must raise from other features. In this respect, a further element needs to be taken into account: the chemical nature of the linker. As shown in Fig. 1, the linker found in the urethane CD–NS employed in this study includes an aromatic moiety; this is not the case with carbonate CD–NS. In addition, urethane NS are highly cross-linked. It is likely to hypothesize a strong and diffuse interaction between such phenyl rings and the aromatic residues of lipase, which may induce molecular deformation and result in conformational rigidity that hamper interfacial activation. This would explain both the higher enzyme loadings and the lower lipase activity found in the presence of urethane CD–1:8 as compared to carbonate CD–NS. Conversely, the higher catalytic proficiency recorded in the presence of carbonate CD–NS suggests a higher level of conformational freedom for the adsorbed lipase. It's worth noting that the effectiveness of carbonate CD–NS in non-covalent immobilization of enzymes has already been reported by Di Nardo et al. in their study on 1,2-catechol dioxygenase [27]. These speculations are supported by the results of the experiments made in the presence of soluble CDs: in fact our findings suggests that the architecture of CD–NS rather than the effect of their elementary building blocks is crucial in modulating the activity of the adsorbed enzyme. Notwithstanding, the mechanism of lipase adsorption and stabilization on CD–NS 1:4 needs to be explored and a structural and morphological characterization of the "lipase-CD–NS 1:4" system is in progress.

It has to be borne in mind that an ideal support should be characterized by a subtle balance between the affinity towards the protein (that provides the driving force for protein binding) and the absence of critical structural distortions (that affects the protein stability and activity). In such a perspective, too high affinities are counterproductive in that they mimic the effect of a mild denaturant. This behaviour is particularly critical for lipase, whose catalytic proficiency is strongly dependent on the structural plasticity of the protein molecule. As the best compromise between loading efficiency and catalytic proficiency of the adsorbed enzyme was found in the presence of CD–NS 1:4, all subsequent experiments were carried out on the "lipase-NS 1:4" system.

3.2. The effect of temperature and pH on the enzyme in solution

In order to optimise the immobilization protocol, we determined the *T* and pH values corresponding to the highest catalytic efficiency of *Ps. fluorescens* lipase in solution.

Activity was checked in the 10–50 °C range. A classical bell-shaped trend with a maximum around 30 °C was found (Fig. 2, closed squares, left scale). All subsequent activity measurements, performed in batch or in solution, were done at 30 °C.

The effect of pH was checked in the 5.0–9.0 range (Fig. 3A) at 30 °C. Again, a bell-shaped curve with a maximum at pH 8 was found. At pH 5, complete enzyme inactivation was observed after only 30 min-incubation. Conversely, 24-h incubation was required to reduce the activity by 15% at pH 9 with respect to pH 8. Spontaneous hydrolysis of the substrate (denounced by the appearance of a yellowish colour) increases with pH; at pH ≥10 this process is almost instantaneous and prevents a reliable estimation of the enzyme activity. Although, the bell-shaped trend allows to expect a substantial decrease of enzyme activity at such pH values. All subsequent immobilization experiments were carried out at pH 8.

3.3. Effect of detergents on the activity of the enzyme in solution

When lipase is immobilized on porous supports, its interaction with drops of substrates may be strongly limited and is influenced by the degree of porosity of the solid support. This may result in insufficient interfacial activation. In order to obviate this drawback, the enzyme may be immobilized in its activated form by introducing small quantities of detergent in the immobilization buffer. Non-ionic detergents have been reported to increase the activity of different kinds of lipase (e.g., *Ps. fluorescens* and *Candida antartica*) both in the solubilized and immobilized form [30,31]. Detergents trigger enzyme activation through two distinct mechanisms of action: (i) breakage of protein dimers, that form spontaneously in aqueous media, and consequent enzyme solubilization; (ii) shifting of the equilibrium from the inactive to the active form, by opening the "lid" that protects the substrate binding site. It has to be pointed out that large detergent excess or inappropriate choice of detergent

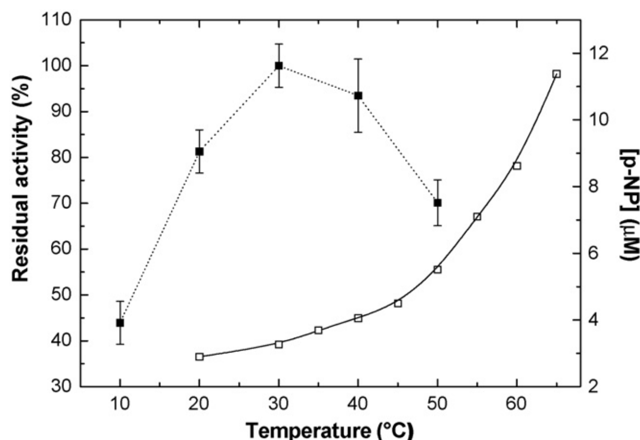

Fig. 2. Comparison between the activity of *Ps. fluorescens* lipase in solution (closed squares) and immobilized on CD–NS 1:4 (open squares). In the former case, measurements were performed in batch; the activity is expressed on a % scale, based on the concentration of reaction product measured in 6 min (left scale). In the latter case, measurements were performed under continuous-flow conditions and the activity is expressed as concentration of reaction product in the eluate (right scale). The two scales are not directly comparable. Blank subtracted.

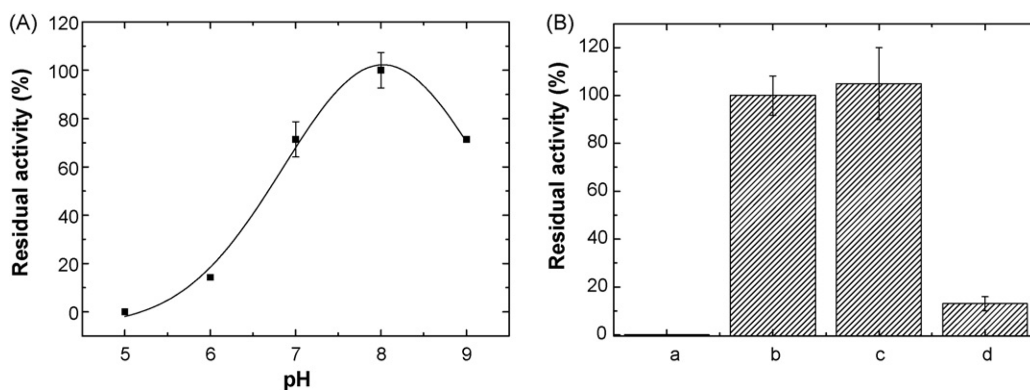

Fig. 3. Effect of pH on the activity of *Ps. fluorescens* lipase in solution (panel A) and immobilized on CD–NS 1:4 (panel B). Panel B shows a comparison between the activity of the solubilized enzyme at pH 5 (a) and the immobilized enzyme at pH 7 (b) and 5 (c); column d reports the activity of the immobilized enzyme after 24-h incubation at pH 7 in the presence of 70% (v/v) methanol. The contribution given by spontaneous hydrolysis of p-NPD at these pH values has been subtracted.

may compromise dramatically the enzyme stability or may carry out a dual effect, by acting as an activator upon short incubation timelengths and as a denaturant upon longer incubation. Testing different detergents and distinct concentrations of each is crucial to identify the best operational conditions for immobilization and usage of the enzyme.

Triton X-100 is a non-ionic detergent successfully employed with *Ps. fluorescens* lipase both on the solubilized and immobilized form [31]. In order to spot the concentration of detergent able to ensure an optimal degree of enzyme activation during the immobilization process of *Ps. fluorescens* lipase, distinct concentrations of Triton X-100 (in the 0–0.5% (v/v) range) were checked at different incubation timelengths. The best balance between interfacial activation and structural destabilization (denaturation) of the enzyme was obtained in the presence of 0.1% (v/v) detergent. In these experimental conditions, enzyme activity is doubled compared to the absence of detergent (data not shown); although higher quantities (>0.1%, v/v) of Triton X-100 may still induce interfacial activation of lipase, it has been observed that, on longer timescales, inactivation takes place; this might be due to the occupancy of the catalytic site by the hydrophobic moiety of Triton X-100 [31]. Consequently, all subsequent immobilization experiments have been performed in the presence of 0.1% (v/v) Triton X-100.

3.4. Effect of ionic strength on enzyme immobilization

In order to check the effect of ionic strength on the yield of the immobilization process, *Ps. fluorescens* lipase dissolved in phosphate buffer 10, 50 or 100 mM pH 8 (in the presence of Triton X-100 0.1%, v/v) was incubated with CD–NS 1:4 for 24 h. Bastida et al. [9] have shown that adsorption on hydrophobic supports in the presence of low ionic strength is fast and selective, to the point that it allows enzyme purification, activation and immobilization in a single step. Enzyme adsorption occurred efficiently in all three cases, although the use of 10 mM buffer assured a quick (2-h incubation) and quantitative adsorption of the enzyme. This allowed to carry out a reliable estimate of the quantity of protein adsorbed on the solid support. As a consequence of these findings, all subsequent immobilization experiments were carried out in 10 mM phosphate buffer pH 8.0, in the presence of 0.1% (v/v) Triton X-100.

3.5. Activity of the immobilized enzyme. Stability vs. time and the effect of temperature

The activity of the immobilized enzyme was checked either in batch or under continuous-flow conditions plus stirring (slurry conditions) and compared with the activity of the enzyme in solution. *Ps. fluorescens* lipase immobilized on NS 1:4 retains ~80% activity as compared to the enzyme in solution. Most remarkably, adsorption is irreversible. Experiments performed at RT, under slurry conditions (Fig. 4), have shown that the system takes ~1 h to reach a stationary state; after such initial phase, the enzyme activity keeps almost unaltered for at least 8 h and the enzyme is still proficient after 3 days in a state of flux; no evidence of enzyme release or decreased reaction yields were observed during that time. Experiments under slurry conditions were carried out by introducing the “lipase-CD–NS 1:4” system in a chromatographic column and by streaming the substrate solution across it. Eluate was checked spectrophotometrically (in the 250–700 nm wavelength range) and the presence of the reaction product was highlighted by the appearance of an absorption band at 400 nm ($\varepsilon_{400\,nm}$ = 13,800 M^{-1} cm^{-1}, pH 7) [12]. Spontaneous hydrolysis of the substrate was taken into account by carrying out blank measurements in the same experimental conditions, without lipase, and subtracting this contribution from the original spectra.

The same experiment was carried out at different temperatures, in the 20–60 °C range on a packed column (without stirring) by sampling the eluate every 45 min and the results were compared with the behaviour of the enzyme in solution. The two profiles are shown in Fig. 2 (the contribution given by the spontaneous hydrolysis of

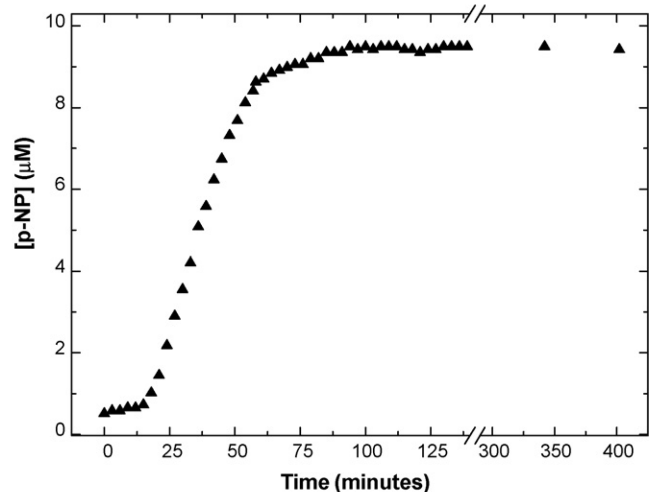

Fig. 4. Measurements of *Ps. fluorescens* lipase activity under slurry conditions. Activity (expressed in terms of mmol of reaction product monitored under a flow of 6 ml/h) was followed during 8 h. ~0.125 mg *Ps. fluorescens* lipase were adsorbed on ~50 mg di CD–NS 1:4; the column was supplied with a solution of p-NPD (22 mg/ml) and the reaction was carried out at T = 25 ± 3 °C. Blank subtracted.

p-NPD has been substracted). The solubilized *Ps. fluorescens* lipase follows a bell-shaped curve with maximum activity at ∼30 °C; a 30% loss of activity was found at 50 °C (Fig. 2, closed squares, left scale). Conversely, enzyme immobilization results in an astonishingly different trend, as a progressive activation of the immobilized lipase was observed between 20 and 65 °C (Fig. 2, open squares, right scale): the amount of eluted product increased by 4-fold within this *T* range. Such an abrupt change cannot be ascribed to simple kinetic reasons: if this was the case, we would have expected the solubilized enzyme to follow a similar tendency, on a shifted *T* range. It is thus tempting to speculate about a structural stabilization effect brought about by the interaction of the enzyme with the CD–NS scaffold; interestingly, a similar stabilizing effect has been observed on *Candida rugosa* lipase adsorbed on a cyclodextrin-based solid support [1,32].

3.6. Catalytic proficiency of Ps. fluorescens lipase immobilized on NS 1:4 at pH 5

Lipase-catalyzed hydrolysis of esters provides fatty acids and alcohol as the reaction products: both compounds are of commercial interest. The increase of fatty acids concentration that occurs during enzymatic hydrolysis results in increased acidity of the reaction medium which, in turn, may decrease the activity of lipase and compromise the final yield of the process. For this reason, basic conditions are usually preferred for lipase-catalyzed reactions [33]. In fact, complete inactivation of solubilized *Ps. fluorescens* lipase was observed after 30-min incubation at pH 5; conversely, the "lipase–NS 1:4" system was still active at that pH value (Fig. 3B). The residual activities associated with the "lipase–CD–NS 1:4" system at pH 5 and pH 7 were comparable and corresponded to 80% of the activity measured on the free enzyme at pH 7, based on the amounts of product formed during the reaction. pH-induced inactivation on the free enzyme was almost instantaneous. Persistence of the activity in the immobilized enzyme as a consequence of the interaction of lipase with CD–NS might be explicated by structural changes that somehow preserve the lipase active site from being damaged or – as an alternative – prevent protonation of aminoacid residues directly involved in the catalytic mechanism. In fact, the catalytic site of *Pfl* and of many other lipases is characterized by the presence of the catalytic triad Ser-His-Asp (or Glu) [4]. Specific interactions between these three residues maintain the enzyme in a catalytically active form: more in details, the imidazole ring interacts with the Ser residue and modulates the nucleophilicity of the alcoolic function, which is directly involved in the catalytic process. The Asp (or Glu) residue is involved in H-bonding with Ser as well. As the pKa of His is 6.5, one may understand why the optimal working-pH for *Pfl* in solution is >7.0. Immobilization of lipase widens the pH range for optimal lipase activity, allowing the enzyme to function at a pH as low as 5: this is consistent with polarity changes in the environment of the catalytic triad, that may affect the pKas and may result from the interaction between the protein and the solid surface.

3.7. Effect of methanol on free and immobilized Ps. fluorescens lipase

Ps. fluorescens lipase was solubilized in 10 mM phosphate buffer pH 8 and incubated at RT for 20 h in the presence of different percentages (0–93%, v/v) of methanol. Activity measurements were subsequently performed on pre-treated samples, in order to check the effect of methanol on the catalytic proficiency of lipase. *Ps. fluorescens* lipase turned out to be quite stable, even in the presence of relatively high % of methanol. Data reported in Fig. 5 show that 40% (v/v) methanol resulted in 20% activity loss, whereas a drop of the activity was observed in the presence of higher methanol percentages (40–60%, v/v). Activity in the presence of 50% (v/v) methanol was 40% that measured in the absence of organic solvent. Finally, methanol percentages higher than 60% (v/v) resulted in complete enzyme inactivation. The effect of methanol is irreversible, unlike reported by Coggon et al. for *C. antartica* lipase [20]. In fact, samples of *Ps. fluorescens* lipase treated with 70% (v/v) methanol and subsequently diluted to a final 30% (v/v) do not recover their catalytic proficiency, even after 24-h incubation. Once again, immobilization of the enzyme on NS 1:4 is accompanied by a stabilization effect. Immobilized lipase pre-incubated for 24 h in the presence of 70% (v/v) methanol retains 13% of its activity, as compared to the immobilized enzyme in the absence of organic solvent (Fig. 3B).

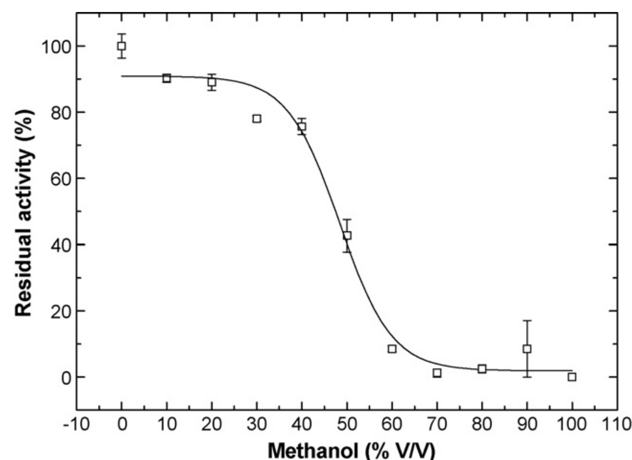

Fig. 5. Effect of methanol on the catalytic activity of free *Ps. fluorescens* lipase. Activity measured in the absence of methanol was taken as a reference and equalized to 100%; all other values are expressed as % of the reference value.

4. Conclusions

Aim of this preliminary study was to evaluate the feasibility of a new type of β-cyclodextrin-based nanosponges as a support for lipase, by checking the catalytic performances of the enzyme in different experimental conditions. Cyclodextrin-based nanosponges are an original support, as they share both hydrophylic and hydrophobic properties. In fact, the cyclodextrin building blocks of CD–NS are characterized by internal hydrophobic cavities and external hydrophylic surfaces that give rise to an intrinsically amphiphilic structure. Based on this peculiar properties, a number of studies on the interaction of CDs and enzymes have been carried out [15,16,28]; much less information is available on cyclodextrin-based nanosponges as support for enzyme immobilization, although this topic raises an increasing attention [1,17,27] due to the peculiar features of these nanomaterials, whose architecture may be modulated by varying the degree of cross-linking between the CD building blocks and the chemical nature of the linker; this provides the solid support with emergent properties, that are not observed at a lower level of structural organization.

The main problem one has to confront with during the employment of lipases for industrial purposes is interfacial activation, that relates with the presence of a mobile protein domain (lid) that protects the substrate binding site: in order to get an active enzyme, the lid must lie in an "open" conformation. Literature data [9] reports that hydrophobic supports may induce interfacial activation, thus assuring adsorption of the enzyme in its activated form; the activating effect of certain kinds of urethane CD–NS on lipase has also recently been reported, but this is the first time that carbonate CD–NS are employed with this purpose. The amphiphilic

structure of these nanomaterials conjugate the hydrophobic character required for enzyme activation with a hydrophylic character, that opens the door to their employment in polar media.

The experimental data shown in the previous section allows to reach the following conclusions:

- The loading capacity of CD–NS depends strongly on the kind of bonds found inside the CD–NS, being the urethane CD–NS the best. However, only carbonate CD–NS seem to preserve the right conformation of the enzyme, increasing its stability and preserving the catalytic proficiency. In this frame, the newly synthesized solid support CD–NS 1:4 has been shown to provide the optimal balance between these two requirements (loading capacity and catalytic proficiency) as compared to NS 1:2 and 1:8 (urethane).
- The adsorption of *Ps. fluorescens* lipase on CD–NS–1:4 results in slightly reduced activity (80%) with respect to the free enzyme. Nevertheless, this partial activity loss is counterbalanced by an increased functional and structural stability of the catalytic system: in fact, the enzyme keeps fully proficient for 3 days under continuous-flow conditions; further, no evidence for enzyme release was ever detected.
- The interaction of lipase with NS 1:4 confers to the enzyme additional stability to T and pH. In particular, adsorbed lipase was found to be active until 65 °C, whereas the free enzyme underwent denaturation at much lower T values. In addition, adsorbed lipase was fully active in those acidic conditions that quickly inactivate the free enzyme.
- The stability and catalytic proficiency of the adsorbed lipase are maximised by the presence of low % of detergents; low ionic strength conditions allows a fast, quantitative adsorption of the enzyme.

The overall picture that comes out from this preliminar investigation allows to formulate hypothesis on the molecular mechanism that lies behind the improved features of the lipase–CD–NS 1:4 system as compared to the free lipase. In particular, the high level of residual activity found in the adsorbed lipase, together with its improved resistance to extreme pH and T conditions suggests that the interaction of lipase with CD–NS 1:4 assures a good amount of interfacial activation; the good accessibility of the active site seems to go along with a structural stabilization, probably due to the establishment of specific interactions between aminoacid residues and the support scaffold. These findings establish carbonate CD–NS 1:4 as a promising solid support for industrial application of lipases; its functionalization with hydrophobic groups might improve the level of enzyme activation and deserves to be explored.

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