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Role of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase used for biodiesel synthesis

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ABSTRACT

The present work investigates the influence of the support surface on the loading and the enzymatic activity of the immobilized *Pseudomonas fluorescens* lipase. Different porous materials, polypropylene (Accurel), polymethacrylate (Sepabeads EC-EP), silica (SBA-15 and surface modified SBA-15), and an organosilicate (MSE), were used as supports. The immobilized biocatalysts were compared towards sunflower oil ethanolysis for the sustainable production of biodiesel. Since the supports have very different structural (ordered hexagonal and disordered) and textural features (surface area, pore size, and total pore volume), in order to consider only the effect of the support surface, experiments were performed at low surface coverage. The different functional groups occurring on the support surface allowed either physical (Accurel, MSE, and SBA-15) or chemical adsorption (Sepabeads EC-EP and SBA-15–R-CHO). The surface-modified SBA-15 (SBA-15–R-CHO) allowed the highest loading. The lipase immobilized on the MSE was the most active biocatalyst. However, in terms of catalytic efficiency (activity/loading) the lipase immobilized on the SBA-15, the support that allowed the lowest loading, was the most efficient.

1. Introduction

Almost 25 years ago Zaks and Klibanov firstly found that enzymes, in particular lipases, could work in non-aqueous media [1], even though with a catalytic activity usually lower by comparison with that in conventional water media. Considerable efforts have been made in order to decrease the activity gap between aqueous and non-aqueous media. *Biocatalyst engineering* aims to develop different enzyme formulations – soluble or insoluble – able to improve enzyme performance towards specific applications [2]. Among different approaches, enzyme immobilization on porous materials seems to be very effective, because they are able to spread enzyme molecules on their high surface areas. This is of fundamental importance in non-aqueous media since enzyme powders tend to aggregate; thus only a small fraction of enzyme molecules, those present on the aggregate surface, can work [2]. The materials used as supports generally affect enzyme performance. Both enzyme loading and activity in non-aqueous media depend on the morphological features (surface area and pore size) and the surface nature of the support [3]. The surface area can be fully used only if pore size is larger than enzyme size. Surface coverage is an important parameter that indicates how much of the available support surface is used by the immobilized enzyme. High surface coverage may result in a high activity per support mass unit, nevertheless, since surface area increases with pore size decreasing, limitations due to substrate diffusion inside the pores may occur. The surface nature of the support can affect the enzyme activity through both direct and indirect effects. The former are due to the type, strength, and orientation of enzyme–support interactions. The latter are due to the substrate/product interaction with the support, thus affecting substrate/product partitioning between the medium and the enzyme substrate-binding site. More importantly, the partitioning of water between the enzyme and the medium due to the support hydrophilic/hydrophobic character should be considered [3].

Lipases (EC 3.1.1.3) are surface-active enzymes whose natural function is triglyceride hydrolysis. They belong to the large structural family (α/β-hydrolases) that comprises a wide variety of enzymes (proteases, esterases, etc.) whose activities rely mainly on a catalytic triad usually formed by Ser, His, and Asp residues. Bacterial lipases have been classified into eight families on the basis of the amino acid sequences and some fundamental biological properties [4]. Lipases display a common feature that is a high hydrophobic surface in proximity of the active site that, in water solution, is buried by an amphiphilic amino acid chain (the lid) [5]. The first step of the catalytic path involves a substantial conformational change of the lipase architecture (lid opening) that leads to the adsorption of the lipase at the oil/water interface through the hydrophobic

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surface. Thus, lipases prefer water-insoluble substrates and display high affinities towards hydrophobic surfaces. Lipases are among the most used enzymes in biotechnology because of their high versatility mainly in non-aqueous media [6]. Among these applications biodiesel synthesis is receiving great attention as demonstrated by the high number of recent papers in the field [7–31]. Biodiesel is a mixture of fatty acid methyl (or ethyl) esters that is currently used as a substitute of diesel fuel coming from petrol. Current production processes are energy consuming and produce unwanted by-products (i.e. soaps) that complicate the purification steps [32,33]. Instead, the production of biodiesel through biocatalysis is a sustainable method that does not produce any waste. Moreover the process is carried out at low temperature (i.e. 30 °C) and atmospheric pressure [34].

The present work is aimed to investigate the role of the nature of the support surface on the loading and the activity of *Pseudomonas fluorescens* lipase. The immobilized biocatalysts were compared towards the ethanolysis of sunflower oil for biodiesel production. *P. fluorescens* lipase was chosen since it seems to be one of the best enzymes for this application [31,35]. The materials – namely: SBA-15 (pure silica), SBA-15–R-CHO (surface-modified SBA-15), MSE (organosilicate), Accurel MP1004 (polypropylene) and Sepabeads EC-EP (polymethacrylate carrying surface epoxy groups) – were used as supports for enzyme immobilization. All these materials carry different surface functional groups that can affect the type of enzyme–support interactions and thus the catalytic activity. As it will be shown below, the morphological features of the used materials are very different, therefore, a low enzyme surface coverage was used to focus on the effect of the surface functional groups only. Consequently a small fraction of the available surface area, likely mainly the external part, was involved so that the effects due to pore size and extent of surface area would be negligible.

2. Experimental

2.1. Chemicals

Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3) from *P. fluorescens* (lipase AK) was purchased from Amano enzymes (Japan). Tetraethylorthosilicate (98%), Pluronic copolymer 123, 3-aminopropyltrimethoxysilane (97%) and glutaraldehyde (50%) were purchased from Aldrich. Bradford reagent and bovine serum albumin (98%) were from Sigma. Buffer salts, Na_2HPO_4 (99%) and NaH_2PO_4 (99%), acetonitrile and methylene chloride (HPLC grade) were from Merck. Ethanol (99.9%) was purchased from J.T. Baker. Karl-Fischer solution was purchased from Riedle de Haen. Accurel MP1004 polypropylene powder and Sepabeads EC-EP powder were kind gifts of Membrana GmbH Accurel Systems (Obernburg, Germany), and Resindion SRL, Mitsubishi Chemical Co. (Milan, Italy) respectively.

2.2. Characterization of supports

SBA-15 and MSE mesoporous materials were synthesised according to what previously reported [36,37]. The surface modification of SBA-15 mesoporous silica was carried out in two steps [38]: first the amino function – reaction with aminopropyltrimethoxysilane – then the aldehyde function – reaction with glutaraldheyde – were introduced. Upon modification with glutaraldehyde, the colour of the support changed from white to red.

Textural analysis of supports was carried out on a Thermoquest-Sorptomatic 1990, by determining the N_2 adsorption/desorption isotherms at 77 K. Before analysis, samples were out-gassed overnight at 40 °C prior to measurement. The specific surface area, the total pore volume and the pore size distribution were assessed by the *Brunauer-Emmett-Teller* (BET) [39] and the *Barret-Joyner-Halenda* (BJH) [40] methods, respectively.

2.3. Pseudomonas fluorescens lipase immobilization

Enzyme immobilization was carried out by suspending 125 mg of the preliminary sieved support powder (120 mesh) in 10 mL of an enzyme solution (5 mg/mL) in phosphate buffer (0.1 M, pH 8). The suspension was kept under gentle stirring at constant temperature (298 K), until the equilibrium of the process was reached. The suspension was centrifuged and washed twice with 5 mL of phosphate buffer (0.1 M, pH 8). The amounts of lipase adsorbed by the different supports were quantified in terms of loading, that can be expressed both as mg protein/g support, L_P, and LU/g (1 LU = 1 μmol/min) support, L_A, by determining the protein content or the lipase activity of the enzyme solution at the beginning and at the end of the adsorption process, respectively. These two different ways to express the loading were used since different proteins generally occur in the commercial preparation. Thus, L_P measures the behaviour of all the proteins in the immobilising solution, while L_A focuses only on the target enzyme, that is the lipase.

Determination of protein content was carried out according to the Bradford method [41]. A volume of 50 μL of the lipase solution was mixed with 950 μL of phosphate buffer solution 0.1 M at pH 8 and 1 mL of the Bradford reagent. After exactly 6 min, absorbance was read by mean of a Cary 50 spectrophotometer at λ = 595 nm. The blank was obtained by mixing 1 mL of the buffer solution with 1 mL of the Bradford reagent. Protein content was estimated by mean of a calibration curve obtained using BSA (98%) as protein standard.

Lipase activity was measured by the pH-stat method, using a 718 Stat Titrino equipment from Metrohm (Herisau, Switzerland). A sample of 100 μL of lipase solution was added to a gum arabic-stabilised emulsion of tributyrin in distilled water at 25 °C. The pH was maintained at 7.0 by titration with 10 mM sodium hydroxide solution. The substrate emulsion was prepared by homogenising a mixture of tributyrin (3 mL), distilled water (47 mL) and an emulsification reagent (10 mL) at 18,000 rpm for 1 min by an Ultra-Turrax homogeniser. The emulsification reagent was prepared by dissolving gum arabic (6.0 g), glycerol (54 mL), NaCl (1.79 g) and KH_2PO_4 (0.041 g) in distilled water (40 mL) [42]. The enzymatic activity expressed as LU (lipase units) is defined as the amount of enzyme that produces 1 μmol of butyric acid per min.

2.4. Pre-equilibration of substrates and enzymes at the desired water activity

Pre-equilibration was carried out by putting for several days the vials containing sunflower oil, ethanol, and the enzyme preparations inside closed vessels containing saturated salt solutions at 25 °C. The chosen water activities (a_w) were 0.113 (LiCl), 0.328 ($MgCl_2$), 0.529 ($Mg(NO_3)_2$), 0.708 ($SrCl_2$), and 0.973 (K_2SO_4).

Water content of the pre-equilibrated reagents was measured through a Karl–Fischer 737 KF Coulometer from Metrohm (Herisau, Switzerland). The knowledge of the water content of the pre-equilibrated ethanol was necessary to calculate the amount of wet ethanol to be weighed to keep constant the molar ratio between the reagents.

2.5. Biocatalytic ethanolysis of sunflower oil

A typical substrate mixture was obtained by mixing 2 g (2.28 mmol) of sunflower oil, a suitable amount of ethanol in the molar ratio (alcohol:oil) 8:1, both pre-equilibrated at the desired

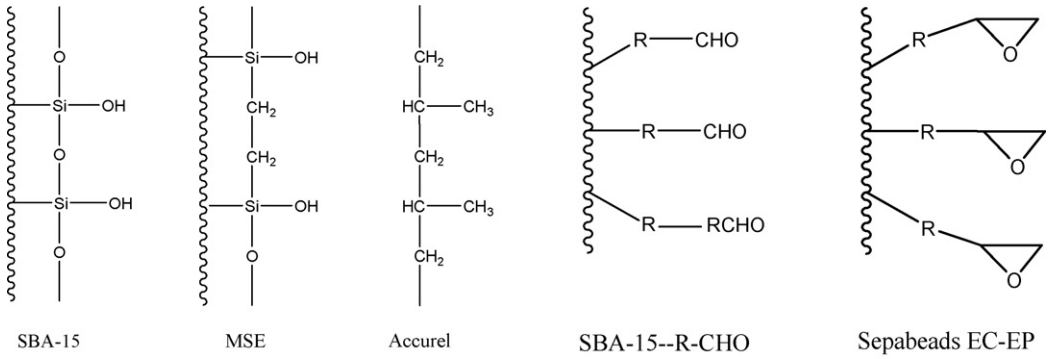

Fig. 1. Functional groups occurring on the surface of the supports used for lipase immobilization.

water activity, in 4 mL screw-capped vials with Teflon-lined septa. The reactions were carried out at 30 °C and started by adding 125 mg of immobilized lipase to the substrates mixture. Reaction vials were shaken through a horizontal shaking water bath at 80 oscillations min^{-1}. Samples (5 μL) were withdrawn at different times and 40 μL of the internal standard solution (trilaurin in hexane 3×10^{-2} M) was added. The resulting mixtures were diluted to the final volume of 1.2 mL with hexane and analyzed by HPLC. All reactions were performed at least in duplicate.

2.6. HPLC analysis

HPLC analysis was performed using a Lichrospher 100 RP-8 end capped, 5 μm column (Merck, Darmstadt, Germany) and monitored by an evaporative light scattering detector Sedex 75 (Sedere, France). Analyses were carried out at 35 °C, at constant flow of 1.6 mL/min with the following solvent gradient. After 2 min of running pure acetonitrile, a linear gradient to 20% methylene chloride was achieved over 3 min. This mixture was run for 5 min when the gradient to pure acetonitrile was achieved over 4 min. This was run for one additional minute. A good separation was obtained for triglycerides coming from sunflower oil (five peaks: 8.7; 9.4; 10.1; 11.1 and 12.0 min), trilaurin (7.0 min); mono- and diglycerides (three peaks: 4.3; 5.0 and 5.8 min) and the fatty acid ethyl esters (two peaks: 2.3 and 2.4 min). Sunflower oil conversion and ethyl ester yields were calculated according to calibration curves obtained with the internal standard (trilaurin) method. All analyses were performed in triplicate with reproducibility within 3%.

3. Results and discussion

In consideration of the different nature of the materials used to immobilize the enzyme, some characterizations had to be preliminarily performed. This was done in order to choose the best operational conditions which should emphasize the role of the surface on the immobilization and on the catalytic performance. Particles of the same size, obtained through preliminary sieving (120 mesh) were used, then the supports were characterized in terms of surface area and pore size in order to choose a low value of loading. Thus, only the external surface of the mesoporous materials is assumed to be involved in the adsorption of the enzyme. The indirect effect due to water partitioning was minimised comparing the biocatalysts performance at the optimal water activity.

3.1. Supports characterization

All the materials used in the present work (SBA-15, surface modified SBA-15, MSE, Accurel MP1004, and Sepabeads EC-EP) were previously used as supports for enzyme immobilization [38,43–45]. By the chemical point of view, SBA-15 is an inorganic material since is constituted by pure silica, two are organic polymers (Accurel polypropylene and Sepabeads EC-EP polymethacrylate), and MSE has a hybrid nature being an organosilicate. However it should be remarked that, rather than the chemical composition of the bulk, the functional groups occurring on the surface are important for the present work. As shown in Fig. 1, these are silanols for the SBA-15 surface, methyl and methylene groups at the Accurel surface, whereas those at the surface of MSE are both silanols and methylene groups. Aldehydic groups occur at the surface of functionalized SBA-15 and epoxy groups at that of Sepabeads EC-EP. Table 1 reports the specific surface area (A_{BET}), the maximum of the pore size distribution and the total pore volume of the materials obtained by the N_2 adsorption/desorption isotherms.

3.1.1. Mesoporous materials

SBA-15 was first synthesized by Stucky and coworkers [36]. It is a silica-based mesoporous structure constituted by cylindrical channels organized with a hexagonal pattern. The surface area, determined by the BET method [39] was 766 m^2/g; the total pore volume was 2.14 cm^3/g (Table 1). The pore size distribution was mono-modal with a maximum centered at the value of 6.9 nm.

Table 1
Morphological properties of the mesoporous materials determined through N_2 adsorption/desorption isotherms.

Support	A_{BET} (m^2/g)[a]	Maximum of pore size distribution (nm)[b]	Total pore volume (cm^3/g)	Maximal surface coverage (%)	Actual surface coverage (%)
SBA-15	766	6.9	2.1	0.53	0.13
Modified SBA-15	213	3.6	0.4	2.1	1.1
MSE	1400	3.9	1.5	0.32	0.12
Accurel polypropylene	28	Macroporous	0.5	16	8.0
Sepabeads EC-EP polymethacrylate	86	2–80	0.4	5.2	2.6

[a] Calculated by the BET method [39].
[b] Calculated by the desorption branch by mean of BJH method [40].

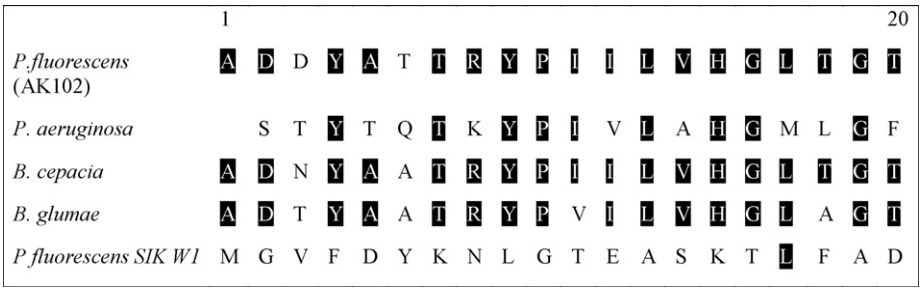

Fig. 2. Comparison among the first twenty amino acid sequence of *Pseudomonas fluorescens* lipase and other bacterial lipases from subfamilies I1, I2 and I3.

The SBA-15 surface was modified following a previously reported procedure [38]. This consists of two steps, where at first the $-NH_2$ functional group is introduced (reaction of the silanols with aminopropyltrimethoxysilane), then the reaction with glutaraldehyde introduces the –CHO functional group. The functionalizing procedure alters the SBA-15 morphology significantly. Both pore size, surface area and total pore volume decreased being 3.6 nm, 213 m^2/g and 0.4 cm^3/g, respectively.

The synthesis of MSE organosilicate was reported for the first time by Bao et al. [37]. The texture of this material is similar to that of SBA-15 since it has the same type of uniform cylindrical pores arranged through a hexagonal pattern. Differently from SBA-15, MSE silicon atoms are alternatively connected by means of –Si–O–Si– and $-Si-CH_2-CH_2-Si-$ groups (Fig. 1). The presence of the methylene groups confers a partial hydrophobic character to the material. The pore size distribution, displayed a very narrow peak at 3.9 nm. The surface area was 1400 m^2/g, and the total pore volume was 1.45 cm^3/g (Table 1).

3.1.2. Macroporous materials

Accurel polypropylene is a commercial support whose characterization was previously reported [44]. Here it is recalled that both macropores and mesopores occur. The maximum of the pore size distribution is around 6 μm for macropores and 9.0 nm for mesopores. The specific surface area (A_{BET}) was around 28 m^2/g and the total pore volume was around 0.5 cm^3/g.

The Sepabeads EC-EP is a commercial polymeric support (polymethacrylate) carrying surface epoxy groups. Hence it is able to bind enzymes covalently through the amino groups of lysine residues occurring at the enzyme surface. The polymer had a very wide distribution of pore size (3–50 nm). The surface area of this material was 86 m^2/g, and the total pore volume was about 0.4 cm^3/g (Table 1).

3.2. Information available in the literature about lipase AK (*Pseudomonas fluorescens* lipase): an attempt of classification

Microbial lipases are generally inactivated by short-chain alcohols, i.e. methanol or ethanol used for biodiesel synthesis. The transesterification activity of *Candida antarctica* lipase B decreased when the methanol:oil molar ratio exceeded 3:2 [46]. Previous studies showed that *Pseudomonas* lipases have high tolerance towards methanol and ethanol used in triglyceride alcoholysis [8,20,35,47]. We have recently shown that lipase AK – a commercial preparation of *P. fluorescens* lipase (Pfl) – immobilized on polypropylene was able to work with a 8:1 (methanol:oil) molar ratio [35]. Moreira et al. found that the same lipase, immobilized on silica–PVA composite was still active although a very high (18:1) ethanol:oil molar ratio was used [31]. The different behaviour of lipases coming from different microbial sources with respect to the inhibition caused by short-chain alcohols should be searched in the different enzyme structures. Unfortunately the structure of Pfl has not yet been resolved, thus no speculation about structure/activity can be done at the present time. Nevertheless, starting from the information available in the literature a classification of this enzyme may be attempted.

This enzyme was characterized by the Amano researchers that isolated the strain No. 924—identified as *P. fluorescens* AK102. A molecular weight of about 33 kDa was estimated [48]. The isoelectric point p*I* = 4, the pH stability range 4 < pH < 10, and the optimum pH of activity in the range 8 < pH < 10 were determined [48]. As additional information the N-terminal sequence of the first 20 amino acids was reported (ADDYATTRYPIILVHGLTGT) [48].

Fig. 2 reports the comparison among the sequences of the first twenty amino acids of Pfl and other four bacterial lipases from three different subfamilies. Eighteen of them follow the same sequence homology as those of lipase from *Burkolderia cepacia* (formerly *Pseudomonas cepacia*), 15 of those from *Burkholderia glumae* (formerly *Chromobacterium viscosum*) lipase, 9 of those from *Pseudomonas aerouginosa* lipase, and only 1 of those from *P. fluorescens* SIK W1 lipase.

The first two lipases belong to subfamily I.2, the third to subfamily I.1 the fourth to subfamily I.3 (Table 2). The sequence homology of Pfl, although limited to the first 20 amino acids, is very high if compared to those of lipases from *Burkholderia cepacia* and *Burkholderia glumae*. Moreover, as reported in Table 2, the lipases of the subfamily I.2 are characterized by having almost the same number of amino acids (~320) and a molecular weight of 33 kDa [4]. Hence, on the basis of the available data, that is the molecular weight (33 kDa) and the first 20 amino acid sequence homology, it may be hypothesised that Pfl belong to subfamily I.2 of microbial lipases.

Table 2
Classification of some bacterial lipases.

	Sub-family	Structure	Pdb file	Number of amino acids	MW (kDa)	Ref.
Pseudomonas aeruginosa	I.1	Yes	1EX9	285	30	[56]
Pseudomonas fluorescens (AK102)	–	No	–	–	33	[57]
Burkholderia cepacia	I.2	Yes	3LIP	320	33	[5]
Burkholderia glumae	I.2	Yes	2ES4	319	33	[58]
Pseudomonas fluorescens SIK W1	I.3	No	–	449	50	[59]

3.3. Estimation of support surface coverage

Morphological features can affect both the immobilization and the catalytic processes. This is particularly important for mesoporous supports since the pore and the enzyme sizes are similar. On the contrary, Accurel and Sepabeads EC-EP are not affected by this problem since they have very large pores (Table 1).

It is known from structural data that *Burkholderia cepacia* lipase (Bcl) is a globular enzyme with approximate dimensions of 3 nm × 4 nm × 5 nm [5]. Since Pfl and Bcl have the same molecular weight the dimensions of Pfl are likely to be similar to those of Bcl. Thus, assuming that Pfl is spherical with an enzyme diameter (d_e) equal to 5 nm, A_{enzyme} – the specific surface area of the support, occupied by the immobilized enzyme (m^2/g) – can be calculated according to Eq. (1):

$$A_{enzyme} = \pi \left(\frac{d_e}{2}\right)^2 \times n_{enzyme} \quad (1)$$

where $\pi(d_e/2)^2$ is the surface occupied by a single enzyme molecule and n_{enzyme} is the number of enzyme molecules per gram of support obtained according to Eq. (2):

$$n_{enzyme} = \frac{L_p}{1000 \times MW_{enzyme}} N_A \quad (2)$$

here L_P is the protein loading is (12.4 mg/g); MW_{enzyme} is the molecular weight of Pfl (33,000 g × mol^{-1}), N_A is the Avogadro number. This allows to estimate the surface coverage – $S_{cov}(\%)$ – of the immobilized lipase:

$$S_{cov}(\%) = \frac{A_{enzyme}}{A_{BET}} \times 100 \quad (3)$$

here A_{BET} is the specific surface area (m^2/g) of the supports. The results of the calculations are reported in the fifth column of Table 1. The mesoporous supports: MSE, SBA-15, and SBA-15–R-CHO have very large surface areas and thus very low surface coverages, being 0.32%, 0.53%, and 2.1%, respectively. Accurel and Sepabeads EC-EP have low surface areas (28 and 86 m^2/g), thus surface coverages were equal to 15.9% and 5.2%, respectively.

On the basis of these surface coverage values, Pfl is expected to be bound mainly on the external surface of the mesoporous supports. Indeed, it is reasonable that during the adsorption process enzyme macromolecules first interact with free external adsorption sites. It can reasonably be assumed that the structure does not affect the loading and the activity of the immobilized lipase. As a consequence, the chemical nature of the support surface could be the main parameter involved in the immobilization and catalysis processes.

3.4. Immobilization of Pseudomonas fluorescens lipase

The Pfl was immobilized on the five characterized supports. Fig. 3 reports the comparison of the loadings – L_A and L_P – obtained from the quantification of the immobilization experiments on the different supports. The higher the loading, in particular L_A, the higher the enzyme–support affinity. The affinity of Pfl in terms of both L_A (Fig. 3a) and L_P (Fig. 3b) for the five supports follows the series: SBA-15–R-CHO > Accurel > Sepabeads EC-EP > MSE > SBA-15.

Depending on the functional groups located on the support surface, different kind of interactions between the enzyme and the support surface should take place. On the basis of the nature of their surface, the supports SBA-15, Accurel polypropylene, and MSE are expected to interact through physical (electrostatic, hydrogen bonds, dipole–dipole and hydrophobic) interactions. Accurel polypropylene is the most hydrophobic material whereas the silica

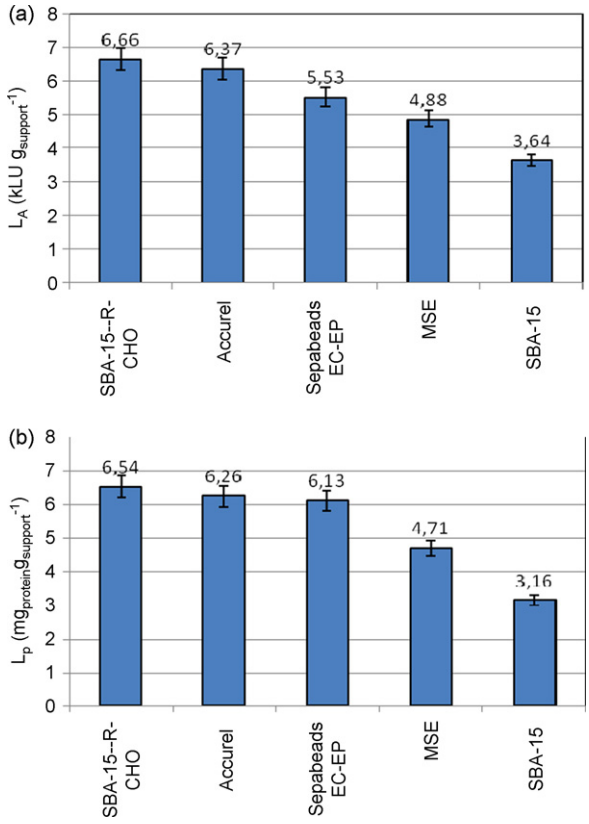

Fig. 3. Comparison among loadings of Pfl immobilized on different supports. (*a*) L_A and (*b*) L_P. Enzyme immobilization was carried out by suspending 125 mg in 10 mL of an enzyme solution (5 mg/mL) in phosphate buffer (0.1 M, pH 8). The suspension was kept under gentle stirring at constant temperature (298 K), until the equilibrium of the process was reached.

based SBA-15 is the most hydrophilic (Fig. 1). The organosilicate MSE, due to the simultaneous presence of both polar –OH and apolar –CH_2– groups, has an intermediate hydrophobic/hydrophilic character. As shown in Fig. 3a, L_A decreases as hydrophilicity increases, confirming the affinity of lipases toward hydrophobic surfaces [44,49–52].

The low loading obtained for SBA-15 can be explained by its hydrophilic nature and by the surface electrical charges carried by both the support and the enzyme. These charges depend on both the isoelectric points (p*I*) of the enzyme and of the support and on the pH of the immobilizing solution [38,43]. Since Pfl has a p*I* = 4 and SBA-15 has a p*I* = 3.7 [43], both the support and the lipase are expected to be negatively charged at pH 8. Hence, unfavourable electrostatic interactions are likely to occur. The immobilization pH was chosen on the basis of the optimal pH for catalytic activity. Indeed, in non-aqueous media the enzyme is expected to maintain the 'memory' of the pH of the immobilising solution. In other words, the enzyme amino acid residues retain the same charges they had in the immobilizing solution [53]. The chosen pH should allow for a high activity even though, not necessarily, for a high loading. In any case, although the net charge of the enzyme at pH 8 is negative, there might be some regions of it where positive charges are still present, thus allowing the formation of favourable electrostatic interactions with the SBA-15 surface. In addition, other kinds of forces, such as hydrogen bonds and dipole–dipole interactions should be responsible of the reached loading.

The intermediate loading measured for Pfl on MSE mesoporous organosilicate agrees with the simultaneous presence of both hydrophilic and hydrophobic groups on MSE surface [54].

The groups occurring on the surface of the SBA-15–R-CHO (aldehydic) and Sepabeads EC-EP (epoxy) can establish covalent bonds with the $-NH_2$ groups of the lysine residues occurring at the enzyme surface. A slightly higher value of L_A was reached by the SBA-15–R-CHO compared to Sepabeads EC-EP. A less net distinction was observed in terms of L_P, being the differences within the error bars.

On the basis of these results, it can be concluded that, at the investigated loadings, the textural properties (surface area and pore size) do not affect the immobilization process significantly. Indeed a high loading is observed for Accurel, that has the lowest surface area (A_{BET} = 28 m^2/g). Moreover, comparing L_A of physisorbed lipases on mesoporous materials, MSE loading is higher than that measured for SBA-15, although the pore size of the latter (6.9 nm) is larger than that of the former (3.9 nm). This might confirm that enzymes do not deeply penetrate the pores, and the external surface is mainly involved in the adsorption. Similar considerations can be done if L_P instead of L_A is considered.

3.5. Catalytic measurements

3.5.1. Effect of water content on transesterification enzymatic activity

The ethanolysis of sunflower oil was the reaction chosen to evaluate the role of the support. The reactions were carried out in solvent-free conditions, with a molar ratio ethanol:oil = 8:1, T = 30 °C, atmospheric pressure, and the same mass of immobilized biocatalyst (125 mg).

In this environment the hydration level of the enzyme is one of the fundamental parameters to be considered, since it strongly affects the enzyme activity [53]. Water allows for the internal enzyme flexibility due to polypeptide chain motion, which is needed by the enzyme to be catalytically active. In the case of immobilized enzymes, the partitioning of water among the components of the system (enzyme, support and reagent mixture) strongly depends on the support hydrophilic/hydrophobic balance. Therefore, to avoid problems related to water partitioning, before starting the reactions, the different components of the system were pre-equilibrated to fixed values of water activity (a_w) [53]. Table 3 reports the water content of the pre-equilibrated ethanol as determined by Karl-Fischer coulometric measurements. The water content ranged between 5.9% (a_w = 0.113) and 32% (a_w = 0.973). After pre-equilibration, the enzyme activity as a function of water activity was determined by adding to the reagents mixture a weighed amount of the pre-equilibrated Pfl immobilized on Accurel polypropylene. Fig. 4 shows the results of this study.

At low a_w values, Pfl displayed a low activity; as water activity increased, also enzyme activity increased until a maximum (104 μmol min^{-1}) at a_w = 0.529 was reached. A further increase of a_w produced an enzyme activity decrease. Therefore a_w = 0.529 was used in the experiments aimed to compare the effect of the support surface on the biocatalyst performance.

Table 3
Water content of pre-equilibrated ethanol at different water activities (a_w) determined through Karl–Fischer coulometric measurements.

Salt	a_w	Water content ± S.D. (%)
LiCl	0.113	5.9 ± 0.4
$MgCl_2 \cdot 6H_2O$	0.328	13 ± 1
$Mg(NO_3) \cdot 6H_2O$	0.529	19 ± 1
$SrCl_2 \cdot 6H_2O$	0.708	27 ± 2
K_2SO_4	0.973	32 ± 2

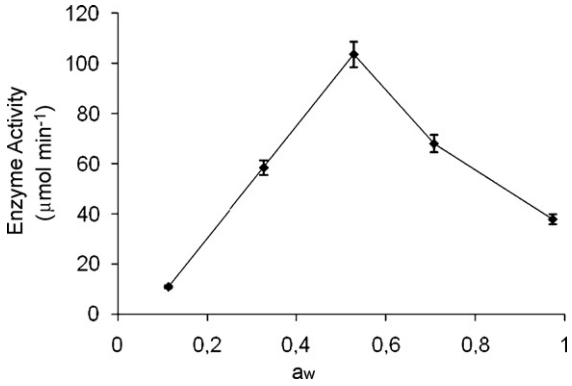

Fig. 4. Enzymatic activity of Pfl immobilized on polypropylene vs. water activity (a_w). Sunflower oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilized Pfl (125 mg) was added to the substrates mixture pre-equilibrated at different a_w values, and incubated at 30 °C with a constant shaking (80 oscillations min^{-1}).

3.5.2. Comparison among immobilized biocatalysts towards biodiesel synthesis

The comparison among the different biocatalytic systems was made by following ethyl esters yield (Fig. 5) as a function of time. All the immobilized biocatalysts were active towards sunflower oil ethanolysis. However the different biocatalysts showed different performance. For instance, considering ethyl esters yields at 8 h of reaction time, the mol% values are 91 for Pfl-MSE, 88 for Pfl-Sepabeads EC-EP and 84 for the others biocatalysts. The different yield curves presented different slopes, and thus different biocatalyst activities. Activity values were calculated considering the initial rate of formation of the ethyl esters. As shown in Fig. 6, the series of immobilized biocatalysts activity followed the order: Pfl-MSE > Pfl-SBA-15 > Pfl-Sepabeads EC-EP > Pfl-Accurel > Pfl-SBA-15–R-CHO. Remarkable differences are observed in the catalytic activity of Pfl immobilized on the different supports. For instance the Pfl-MSE is almost twice more active than the Pfl-SBA-15–R-CHO. It is evident that the enzymatic activity can be modulated by the different surface–enzyme intermolecular interactions.

3.6. Role of support surface on catalytic efficiency

The catalytic activities reported in Fig. 6 were obtained using the same mass of immobilized preparation. But, this does not take into account the different loadings. Thus, in order to compare the direct effects of functional groups of the different supports on enzyme performance the catalytic efficiency (activity/mg of loaded pro-

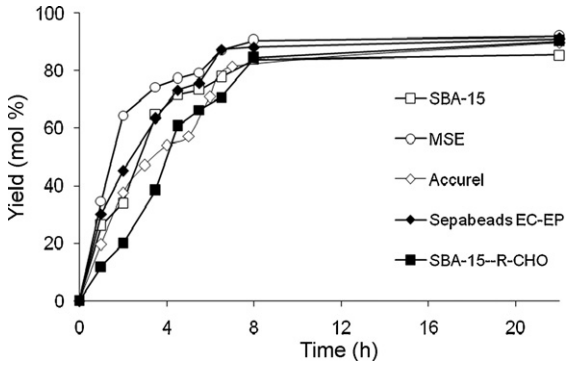

Fig. 5. Time course of enzymatic biodiesel synthesis. Sunflower oil (2 g) and methanol were mixed in the stoichiometric ratio 1:8. Immobilized Pfl (125 mg) was added to the substrates mixture pre-equilibrated at a_w = 0.529, and incubated at 30 °C with a constant shaking (80 oscillations min^{-1}).

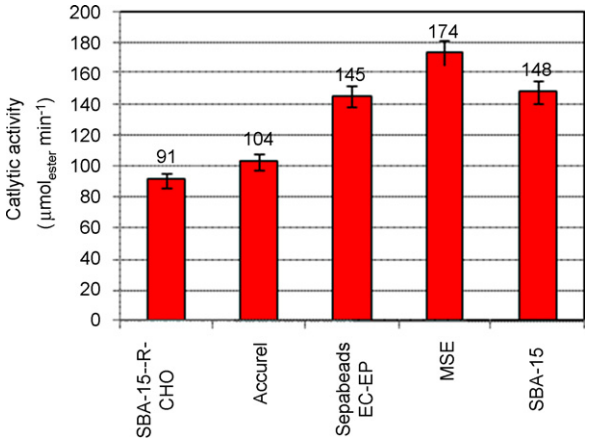

Fig. 6. Comparison among transesterification activities of Pfl immobilized on the different supports.

tein) of the immobilized preparations was calculated. Results are reported in Fig. 7.

In principle, a high activity for a high loading would be expected, since more enzyme molecules are immobilized in the same mass of support. In fact, the Pfl-SBA-15–R-CHO biocatalyst, that reached the highest loading, shows the lowest catalytic efficiency (112 μmol min^{-1} mg$^{-1}_{loaded\ protein}$). More striking, the Pfl-SBA-15 biocatalyst, that reached the lowest loading, shows the highest catalytic efficiency (375 μmol min^{-1} mg$^{-1}_{loaded\ protein}$). The catalytic efficiencies of the other biocatalysts follows the series: Pfl-MSE > Pfl-Sepabeads EC-EP > Pfl-Accurel. Substantially, the series of catalytic efficiency follows exactly the opposite order of the loading (see Fig. 3).

A possible explanation to understand this apparently counter-intuitive result is related to the nature of the interactions. A high loading means a high enzyme–support affinity, due to a very strong interaction. But strong enzyme–surface interactions can distort the tertiary structure of the enzyme with a consequent partial deactivation. Previous studies have shown that, at low loading, the hydrophobic interactions between lipases and polypropylene are strong enough to cause inactivation of a significant fraction of enzyme molecules [55]. A similar effect may also be suggested for covalently bound enzymes on Sepabeads EC-EP and SBA-15–R-CHO. It is noteworthy that these two supports showed similar enzymatic loading but a rather different catalytic behaviour. This could be due to the type of chemical bond between the support and the enzyme that in turns affects the enzyme flexibility. Strong covalent interactions with these supports may also restrict the self-

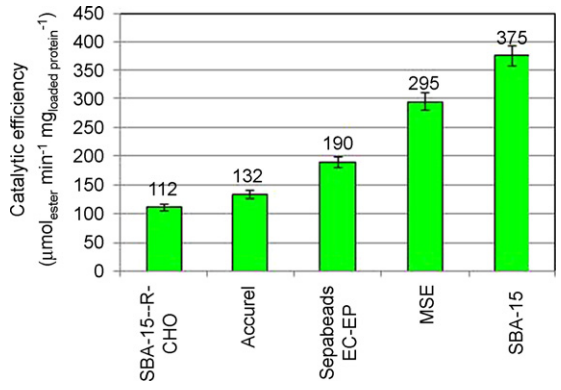

Fig. 7. Comparison among catalytic efficiencies of Pfl immobilized on the different supports.

dynamics of the bound enzyme molecules required for catalytic activity. On the contrary, the weak interactions occurring in the case of SBA-15 are likely to preserve the enzyme in its active conformation, thus favouring the high catalytic efficiency, despite the low loading. The intermediate loading and catalytic efficiency observed for MSE are clearly related to the nature of the surface that contains both hydrophobic and hydrophilic groups.

4. Conclusions

In conclusion, the different supports used for Pfl immobilization affect the biocatalyst performance according to the functional groups occurring on their surface. They may influence the type and the strength of enzyme–support surface interactions, thus affecting enzyme loading and activity. Hence, the SBA-15–R-CHO allowed the highest loading, whereas the Pfl-MSE was the most active biocatalyst. However, if catalytic efficiency is considered, the Pfl-SBA-15 was the best biocatalyst for sunflower oil ethanolysis.

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