Universidad Autónoma
de Madrid

Biblos-e Archivo
Repositorio Institucional UAM

Repositorio Institucional de la Universidad Autónoma de Madrid
https://repositorio.uam.es

Esta es la **versión de autor** del artículo publicado en:
This is an **author produced version** of a paper published in:

Applied Catalysis A: General 450 (2013): 204-210

DOI: https://doi.org/10.1016/j.apcata.2012.10.021

Copyright: © 2012 Elsevier B.V. This manuscript version is made available under the CC-BY-NC-ND 4.0 licence http://creativecommons.org/licenses/by-nc-nd/4.0/

El acceso a la versión del editor puede requerir la suscripción del recurso
Access to the published version may require subscription

Hybrid composites octyl-silica-methacrylate agglomerates as enzyme supports

Oscar Fernández[a,b], Isabel Díaz[a], Carlos F. Torres[b], Montserrat Tobajas[c], Víctor Tejedor[a,c], Rosa M. Blanco[a,*]

[a] Instituto de Catálisis y Petroleoquímica, CSIC, c/Marie Curie 2, 28049 Madrid, Spain

[b] Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, Nicolás Cabrera 9, 28049 Madrid, Spain

[c] Sección de Ingeniería Química, Universidad Autónoma de Madrid, 28049 Madrid, Spain

A R T I C L E I N F O

Article history:
Received 21 June 2012
Received in revised form 2 October 2012
Accepted 14 October 2012
Available online xxx

A B S T R A C T

The use of immobilized enzymes as catalysts may be limited by particle size which must be larger than the mesh that retains them in the reactor. Octyl-silica (OS) beads of 70 µm average size were agglomerated to obtain hybrid organic–inorganic composites with particle sizes between 100 and 200 µm. The agglomeration process has been achieved by polymerization of methacrylate from glycidyl methacrylate and ethylene dimethacrylate in the presence of silica beads and further functionalization of the composite with octyl groups.

Methacrylate content of the composite (20%) is high enough to stick OS beads, and low enough to preserve the advantages of these particles as supports. The properties of the octyl silica particles for lipase immobilization have been very closely reproduced with the octyl-silica-methacrylate (OSM) composite. Enzyme loading of 210 mg lipase per gram of support has been achieved on OSM vs 230 mg/g on OS. Also catalytic activity values are close for both catalysts, OSM-lipase remaining fully active and stable after 15 cycles in acetonitrile.

© 2012 Published by Elsevier B.V.

1. Introduction

Current application of immobilized lipases is of doubtless interest in fields like the syntheses of pharmaceuticals or food additives and nutraceuticals [1]. Industrial use of these biocatalysts requires optimization of catalytic activity and stability in a number of reaction cycles [2]. In this regard, the choice of a suitable support is of utmost relevance to obtain catalysts with the best properties [3–5].

Leaving aside the chemical nature, the main limiting factors are the textural and morphological features of the support and indeed the real possibilities of application for the same enzyme can vary a lot as a function of this material. The distribution of the enzyme along inner surfaces and therefore the loading capacity of the support may be a challenge especially when using materials with large particle size and/or narrow pore diameter. The diffusion of substrates and products through the porous network of the support is also highly dependent on the particle size [6] and particle size distribution [7]. Although larger particles contributes to easier handling, the diffusion of the enzyme or substrates and products through internal pores may be limited and the enzyme loading and the apparent catalytic activity can be diminished compared to the smaller ones. Particle size may become a controlling step for catalysts in reactors where they must be retained within a mesh, and it must be large enough to enable an easy design of the reaction. But it must be small enough to prevent serious mass transfer restrictions. A large pore diameter may contribute to minimize this unwished effect [8], and indeed, it has been described [3] that this effect disappears when pores are larger than 100 nm diameter.

Gross et al. [9,10] have recently established that the enzyme-support affinity may also significantly affect immobilization of lipases on supports with different bead sizes: for high affinity no effect of the particle size on the immobilization rate was observed, but the distribution of the enzyme is uneven and limited to external bark. However, with moderate affinity between enzyme and support, immobilization rates are higher on the smaller particles; in this case the enzyme distribution is homogeneous only in particles of 75 µm diameter or less. In particles over this size the enzyme can only diffuse along the external shell.

Amorphous meso-macroporous silica MS3030 has been successfully used to immobilize lipases upon grafting with octyl groups. The textural properties of the silica combine high surface area and a high average pore diameter (of 27 nm), which is almost four fold the diameter of the molecule of lipase from *Candida antarctica* B [11]. By pre-wetting this octyl-silica (OS) with ethanol the restrictions of the aqueous enzyme solution [12] to diffuse through the hydrophobic environment of pore channels efficiently

* Corresponding author. Tel.: +34 91 5854636; fax: +34 91 5854760.
E-mail address: rmblanco@icp.csic.es (R.M. Blanco).

0926-860X/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.apcata.2012.10.021

Please cite this article in press as: O. Fernández, et al., Appl. Catal. A: Gen. (2012), http://dx.doi.org/10.1016/j.apcata.2012.10.021

decrease. Thus, the enzyme can reach most of the internal surfaces of the particle, and as a result, high values of enzyme loading and catalytic efficiency have been obtained. However, their average particle size is not large enough to keep them retained in 100 µm meshes. Based on these materials, the objective of this work is to preserve high enzyme loading and similar values of catalytic activity with larger size particles. Most of the work in this field is focused on the use of isolated large particles, such as immobilization on monoliths from silica or organic polymers with different shapes and prepared from different precursors [13]. Some works can also be found in the literature describing the formation of larger particles, for example, through crosslinking of lipase immobilized on ordered mesoporous materials with chitosan by means of reaction with glutaraldehyde [14]. We propose the formation of agglomerates of octyl silica particles by sticking them to each other through a minimal contact surface as the strategy to increase the final size of the catalyst

2. Materials and methods

2.1. Chemicals

n-Octyltriethoxysilane (TCI Europe, Belgium), glycidyl methacrylate (from now GMA) 97%, ethylene dimethacrylate (from now EGDMA) 98%, 1,1′-azobis (cyclohexane-carbonitrile) 98%, cyclohexanol, 1-tetradecanol, poly(vinylpolypyrrolidone), glyceryltributyrate (tributyrin), oleic acid and butanol were purchased from Aldrich (St. Louis, USA). Ethanol (HPLC grade), di-sodium hydrogen phosphate and toluene were purchased from Panreac (Barcelona, Spain). All chemicals were of analytical grade. MS3030 silica was kindly donated by Silica PQ Corporation (Valley Forge, PA, USA). Lipase from *C. antarctica* B (Lypozyme, CaLB) was donated by Novozymes (Denmark). p-Nitrophenyl acetate (pNPA) was purchased from Sigma.

2.2. Synthesis of organic resin

The organic resin (M) was synthesized as described in the literature [15]. The main procedure was as follows: the monomer phase containing the monomer mixture (3.5 g of GMA and 2.3 g of EGDMA), 0.15 g 1,1′-azobis (cyclohexane-carbonitrile) as an initiator and 7.5 g of inert component (6.78 g of cyclohexanol and 0.7 g of tetradecanol), was suspended in the aqueous phase consisting of 40 g of water and 0.6 g of poly (vinyl pyrrolidone) (PVP). The copolymerization was carried out at 70 °C for 2 h and then at 80 °C for 6 h with a stirring rate of 200 rpm. After completion of the reaction, the copolymer particles were washed with water and ethanol, kept in ethanol for 12 h and then dried in a vacuum oven at 45 °C for 24 h.

2.3. Synthesis of hybrid composite

Different amounts of MS3030 silica were added to the aqueous phase before polymerization of organic resin. The mixture was stirred to form a homogeneous suspension. Next, the monomer phase of organic resin was added to initiate polymerization. The final octyl-silica-methacrylate composites will be called OSM-*x*, being *x* the grams of silica added to the synthesis mixture.

OSM hybrids were gently crushed in a mortar and sieved to separate a fraction with diameters ranging between 100 and 200 µm.

2.4. Support functionalization

The functionalization of the support was carried out as described by Blanco et al. [11]. 1 g of silica previously degassed at 80 °C under vacuum for 12 h was suspended in a 10 mL solution of octyltriethoxysilane in toluene (1:4, v/v). The suspension was gently stirred for 48 h at 80 °C. After that, the suspension was filtered and washed twice with dry toluene, and three times with hexane and acetone, and finally exhaustively vacuum dried. This support is referred to as octyl-silica (OS). The same procedure was followed for the functionalization of silica-methacrylate (SM) agglomerates to obtain the composites referred as OSM-10.

2.5. Characterization of the solids

Nitrogen isotherms were measured at the temperature of liquid N_2 with a Micromeritics ASAP 2000 apparatus. Samples were previously degassed at room temperature for 20 h. The surface areas were determined following the BET method. Thermogravimetric analyses (TGA) were carried out on a Mettler Toledo TGA/SDTA 851e apparatus. Typically, 5 mg of the sample was heated from 25 to 800 °C at a rate of 20 °C/min under air flow (200 ml/min). Scanning electron microscopy (SEM) micrographs were taken with a Hitachi TM-1000 at 15 kV and without coating.

2.6. Immobilization of lipase

Protein content of the enzyme extract (3.5 mg/ml) was determined according to the Bradford method [16]. SDS-PAGE electrophoresis of this extract showed a unique band so all the protein can be attributed to the lipase.

Different amounts of the enzyme extract were dissolved in 50 mM phosphate buffer, pH 7.0, up to a total volume of 20 mL. After assaying the esterasic activity of these solutions, 100 mg of the corresponding support previously wet with ethanol were added and maintained in suspension with a helical stirrer. Aliquots from suspension and supernatant were withdrawn at 10–240 min to analyze their esterasic activities.

Final time is determined by the lack of activity, or low constant activity of the supernatant. After that, suspensions were filtered and washed three times with 10 mL volumes of 200 mM phosphate buffer. The derivatives were washed twice with 10 mL dry acetone, filtered out and vacuum dried for at least 30 min to ensure a complete drying of the catalyst.

2.7. Determination of enzyme activity. Hydrolytic activity

2.7.1. Esterasic activity

Despite an assay for pNPA hydrolysis activity is not a specific test for lipase activity, this assay was selected for use as a routine assay because it is easy to conduct via spectrophotometric measurements and it provides a rapid assessment of relevant enzymatic activity. Hydrolysis of p-NPA was followed at 348 nm in an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies) equipped with stirring device and constant temperature capability. The cell contained 1.9 mL of substrate solution at 25 °C (0.4 mM p-NPA in 50 mM sodium phosphate buffer, pH 7.0). Aliquots of the suspension were diluted in different proportions in 50 mM phosphate buffer (pH 7.0) prior to being added to the cell (50 µL) to facilitate the analysis. Aliquots from the supernatant were not diluted: 50 µL were added directly to 1.9 mL substrate solution. One esterase unit corresponds to consumption of 1 µmol p-NPA/min (ε p-NP = 5150 M^{-1} cm^{-1}).

2.7.2. Tributyrin activity

The hydrolysis of tributyrin measures lipase activity by the liberation of butyric acid. The reaction was monitored titrimetrically in a Mettler DL50 pH-stat, using 100 mM sodium hydroxide. A 48.5 mL potassium phosphate buffer solution (10 mM, pH 7.0) was incubated in a thermostated vessel at 25 °C and stirred sufficiently. Then, 1.47 mL tributyrin were added and the pH-stat was started

to keep the pH at 7.0. When the pH stabilizes, 5 mg catalyst were added and the consumption of NaOH was determined. One lipase unit corresponds to consumption of 1 μmol NaOH/min.

2.8. Kinetics and diffusional studies

The hydrolysis of tributyrin catalyzed by OSM-lipase at different stirring rates (1140, 1900, 2280 and 2660 rpm) was carried out in order to study external mass transfer. No effect of stirring rate over 1900 rpm on catalytic activity was found, which indicates that external diffusion limitations are negligible. Only a lower value of activity was detected when the reaction was stirred at the slowest rate. This effect seems to be related to the poor dispersion of tributyrin under not-enough stirring conditions. No significant decrease in particle size due to the stirring was observed in any case. Thus, the kinetic parameters (V_{max} and K_M) for the hydrolysis of tributyrin (0.1–1 mM) catalyzed by lipase immobilized on OS and OSM-10 were performed at 1900 rpm.

In order to obtain the values of the non diffusion-limited rate constants the immobilized enzyme particles were crushed to a fine powder. To achieve this, a suspension of 50 mg OS-lipase in 5 ml phosphate buffer 50 mM pH 7.0 was put under vigorous magnetic stirring in an icebath (4 °C) to prevent enzyme inactivation. Aliquots of the suspension were assayed at different times and the increase of activity with the abrasion was followed (tributyrin hydrolysis). Once a maximum and constant value of activity was achieved, the stirring was stopped and assays for determination of kinetic parameters were performed.

Average particle size of OSM agglomerates, OS and OS after abrasion was calculated through SEM observation of different preparations of the three classes of beads: crushed OS: 15 μm, OS: 70 μm, and OSM: 120 μm.

2.9. Determination of enzyme activity. Synthesis activity

The synthetic activity of immobilized lipases was tested by the esterification of oleic acid and butanol as a model reaction. A mixture of 75 mg of oleic acid and 50 mg of butanol was stirred in an orbital shaker at 200 rpm and 30 °C. A known amount of enzyme was added to the mixture. The reaction was stopped after 5 min with 50 μl of water.

The analyses were performed on a Kromasil silica 60 column (250 mm × 4.6 mm, Análisis Vínicos, Tomelloso, Spain) coupled to a CTO 10A VP 2 oven, a LC-10AD VP pump, a gradient module FCV-10AL VP, a DGU-14A degasser, and an evaporative light scattering detector ELSD-LT from Shimadzu (IZASA, Spain). The column temperature was maintained at 35 °C. The method consisted of a ternary gradient of trimethylpentane, trimethylpentane/methyl tert butyl ether 1:1, and methyl tert butyl ether/propan-2-ol 1:1 previously reported by Torres et al. [17]. One unit of activity corresponds to consumption of 1 μmol of oleic acid/min.

2.10. Stability in organic media

300 mg of biocatalyst containing 120 mg lipase per gram were incubated in 10 ml acetonitrile, maintained 35 °C and stirred at 200 rpm in an orbital shaker. After 2 h incubation, the catalyst was washed with acetone three times and then filtered out and vacuum dried. Then the dry biocatalyst was weighted and assayed in tributyrin hydrolysis to evaluate its activity. A new reaction was then started, and this was repeated up to 15 reaction cycles.

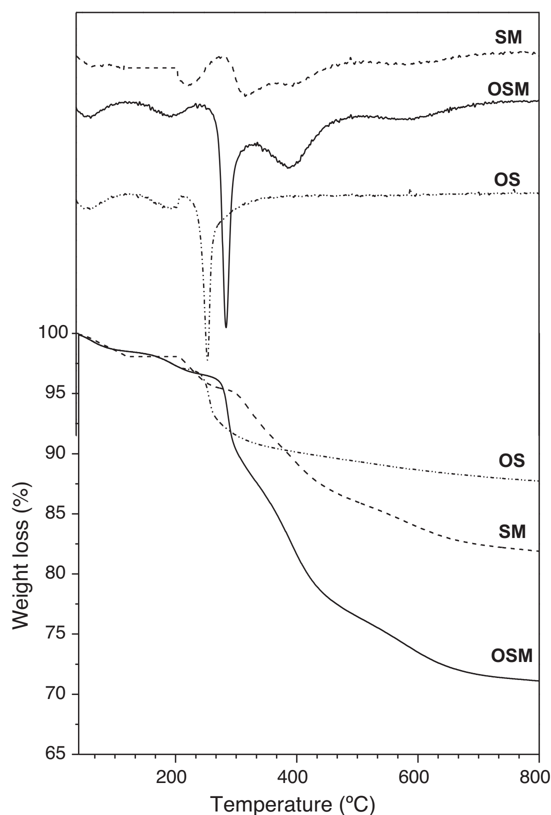


Fig. 1. Thermograms and derivatives of non functionalized hybrid composite (SM), octyl-silica methacrylate (OSM) and octyl silica (OM).

3. Results and discussion

3.1. Synthesis and characterization of the hybrid composites

In a first step, an organic resin based on acrylic reagents was prepared following previous works reported in the literature [15,18]. This material had poor surface area and hardly any pore volume, consequently a low enzyme loading of 58 mg per gram was achieved (see Table 1). The high catalytic efficiency of the lipase-resin suggests that the enzyme is probably anchored only on the external surfaces of the beads, compared to lipase-octyl silica where activity loss and the presumed internal diffusional restrictions are associated with the location of the lipase within the pore channels. Therefore, in order to search for an alternative support which may reproduce the results of OS while having a higher particle size, several studies were performed. First, polymerization of methacrylate in the presence of OS particles was tested with reaction mixtures containing 2 and 3 g of octyl-silica per 8 g of final support. Hybrid octyl-silica-methacrylate agglomerates (OSM-2, and OSM-3 respectively) with particle sizes between 100 and 200 μm were obtained. Textural properties of the resulting hybrid composites are shown in Table 1. It is worth mentioning here that OS beads have a mesoporous structure whereas the methacrylate seems to be composed by nonporous fibers tightly assembled. Therefore the results are somehow difficult to compare. Thermogravimetric analyses revealed that almost 80% of the hybrid composites are composed by nonporous organic polymer (Fig. 1). Besides, the surface area and pore volume values are not significantly higher than those of the organic resin. Thus, most pores of OS beads seem to be buried inside this nonporous material.

Higher OS to methacrylate ratios were tested in order to search for a more porous hybrid composite. But these syntheses yielded macroscopically heterogeneous materials in all the cases, meaning

Table 1
Textural properties and capacities as supports for lipase of the composites.

Composition	S_{BET} (m^2/g)	P_V (cm^3/g)	Inorganic matter (%)	Enzyme load (mg/g)[a]	TB activity (U/g)[b]	Cat. efficiency (U/mg)[c]
M (organic resin)	61	0.32	0	58	8400	145
OS (octyl silica)	254	2.20	92	230	21,700	93
SM	248	2.04	–	–	–	–
OSM-2	100	0.34	20	105	12,500	119
OSM-3	100	0.34	22	104	11,900	114
OSM-10[d]	212	1.54	70	210	20,300	96.6

[a] mg of lipase immobilized per gram of support.
[b] Lipase units in tributyrin hydrolysis assay per gram of biocatalyst.
[c] Catalytic efficiency: activity per mg of immobilized lipase.
[d] OSM composite prepared agglomerating pure silica particles and further functionalization with octyl groups.

that polymerization did not succeed to agglomerate OS particles. These results suggest that there is a limit amount of OS over which methacrylate polymerizes independently of the particles and it cannot agglomerate them.

Methacrylate polymerization was then carried out in the presence of pure silica, non-functionalized, particles. Nitrogen adsorption isotherm of this silica-methacrylate (SM) composite was similar to that of mesoporous OS isolated particles, and both were different to the isotherm of pure resin M. This new ungrafted hybrid composite which was macroscopically homogeneous was obtained by using higher mass of silica (10 g) than the formers OSM-2 and OSM-3 using the same amount of reagent.

The only difference between both syntheses was the presence or absence of octyl groups grafting silica beads in the polymerization process. Hydrophobic interactions between octyl groups and hydrophobic medium seemed to be established, provided that the process was carried out in the presence of hydrophobic solvents and reagents. These interactions might interfere with the polymerization by driving different orientations of the molecules involved.

Grafting with octyl triethoxysilane was performed after the agglomeration process yielding the final optimum octyl-silica-methacrylate (OSM-10) composite. The successful introduction of octyl groups was checked by TG analysis. Fig. 1 shows the weight loss evolution with temperature. Non-functionalized silica-methacrylate support (SM) remained stable until 300 °C, showing a slow and broad weight loss above that temperature due to the decomposition of the methacrylate resin. After octyl functionalization, the profile of the thermogram changed showing a sharp and intense weight loss at 280 °C followed by a broad peak centered at c.a. 400 °C. The first one corresponds to 9% of organic matter and it is related to the grafted octyl groups, since it shows exactly the same profile and percent weight as that of isolated OS particles. The shift of this peak from 250 °C in OS to 280 °C in OSM could be due to different kinetics of the combustion process when changing from discrete particles to larger agglomerates.

Fig. 3 shows the SEM micrographs. Methacrylate displays a compact structure (Fig. 3a), whereas octyl silica particles are spherical (Fig. 3b). OSM-10 (Fig. 3c) shows a minimal presence of methacrylate just enough to stick together the silica particles. Thus, most of the material is porous and hence most of the pores should be available for enzyme immobilization.

The total weight loss in the OSM-10 hybrid composite corresponds to 30% of organic matter, 20% corresponding to methacrylate, and 9% to octyl groups approximately. These groups are mostly located on the internal surfaces of the pore channels of silica beads, as demonstrated by the decreasing of the surface area of the grafted OSM composite compared to the non-functionalized one SM (Table 1 and Fig. 2). Textural and catalytic properties of OSM-10 are given in Table 1. Surface area and pore volume values of OS and OSM-10 are very close, whereas OSM-2 and OSM-3 show significantly lower values. Provided the low organic content in OSM-10 and the similarities with OS, the final properties of OSM-10 seem to be mostly due to the octyl silica of the agglomerate. Therefore the behavior as lipase support should be also very close to octyl silica regarding enzyme loading, activity and catalytic efficiency.

3.2. OSM behavior as lipase support and characterization of the biocatalyst

By sieving the hybrid composites, 80% of the particles of OSM were retained in a mesh size between 100 and 200 μm. The supports were loaded with lipase (CaLB) under the conditions described in Section 2. As shown in Table 1, the maximum loading capability of OSM-2 and OSM-3 was 105 and 104 mg/g respectively. In contrast, the enzyme loading achieved with OSM-10 was 210 mg/g, which is almost as high as in isolated octyl-silica particles (230 mg/g). Since enzyme loading data are given per gram

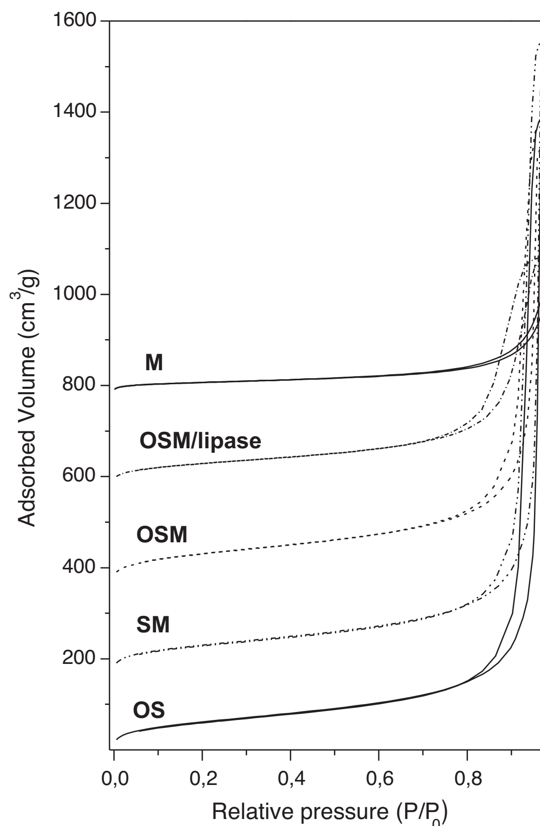


Fig. 2. N_2 adsorption/desorption isotherms of organic resin (M), non functionalized silica-methacrylate composite (SM), octyl-silica (OS), octyl-silica methacrylate (OSM-10), and OSM-10 after enzyme loading (OSM/lipase). Isotherms have been displaced for clarity.

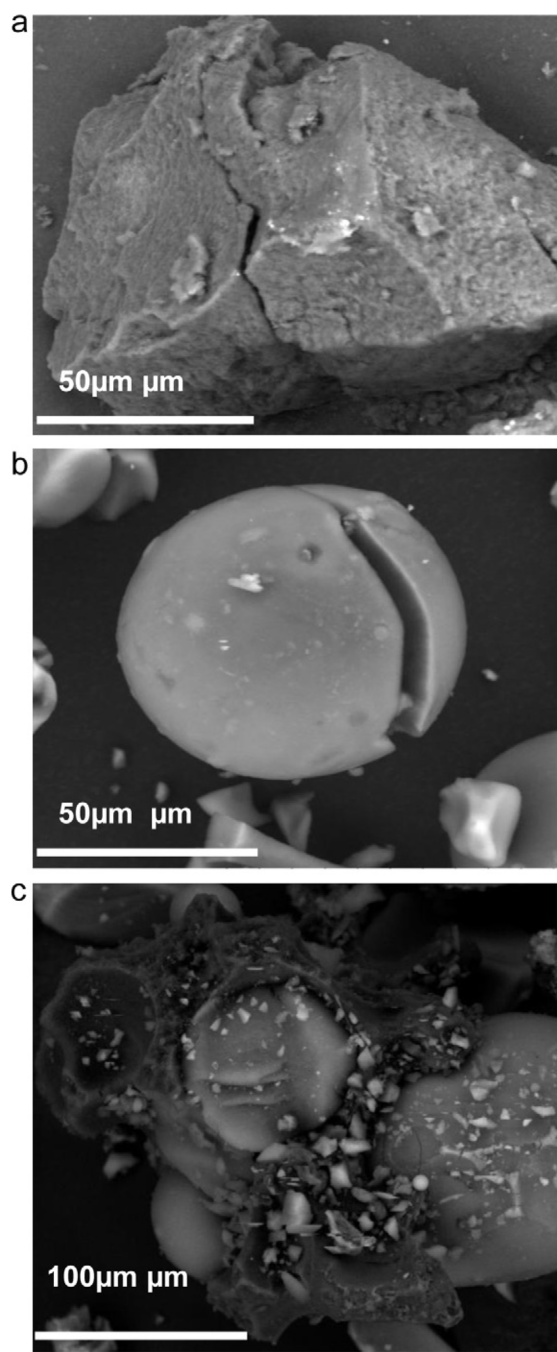


Fig. 3. SEM micrographs of methacrylate resin (a), octyl silica isolated particles (b) and octyl-silica methacrylate hybrid composite, OSM-10 (c).

of solid, and provided that OSM-10 contains around 20% of resin, the small difference in enzyme loading on OS and OSM-10 is most likely due to the different content in silica particles. Their respective activities in tributyrin hydrolysis were also very close: 20,300 U/g vs 21,700 U/g, as well as their catalytic efficiencies (96.6 U/g vs 93 U/mg) as can be seen in Table 1.

The immobilization of lipase within the pore channels in OSM-10 could be confirmed by the decrease in the surface area and pore volume of the biocatalyst compared to the OSM support (Fig. 2).

Table 2
Catalytic activity in butyl oleate synthesis of lipase on different supports.

Support	Units/g support	Cat. efficiency (U/mg)
Novozym 435	2825	–
OSM-2 105 mg/g	1587	15.1
OSM-10 210 mg/g	3455	16.4
Octyl silica	3840	16.7

3.3. Activity in condensation reaction

Octyl silica-lipase (lipase loading 230 mg/g) and commercial Novozym 435 were compared to OSM catalysts (OSM-2 and OSM-10) with enzyme loadings of 105 and 210 mg lipase per gram of support respectively. The catalysts were tested in esterification reaction to obtain butyl oleate and the results are displayed in Table 2. Noteworthy, the activity of the worldwide used Novozym 435 (2825 U/g) was significantly surpassed by OS-lipase (3840 U/g), This excellent result was almost reached by our OSM-10-lipase (3455 U/g), Catalytic efficiencies of lipase immobilized on OSM-10 and OSM-2 are close, whereas the activity of OSM-2 catalyst is half of OSM-10 one. Since the enzyme loading in OSM-2 is also half of OSM-10, this decreased activity must be due to the lower enzyme content in this composite.

The small difference in activity between the lipase immobilized on OSM-10 and OS might also be related to the similar enzyme loading achieved with both materials. Indeed, catalytic efficiencies are 16.4 and 16.7 U/mg respectively. Therefore, it seems that at least in this esterification reaction, diffusional limitations or mass transfer problems do not significantly increase due to the larger particle size of agglomerates. However, in order to quantify this effect, kinetic studies were performed.

3.4. Kinetics of tributyrin hydrolysis and diffusion limitation

The effect of diffusion limitation caused by particle size (isolated OS particles and OSM aggregates) on the catalytic rate of tributyrin hydrolysis was analyzed. For intact particles as well as enzyme powder from this fraction, reaction rate was calculated for several substrate concentrations with the aim of calculating the kinetic parameters by using the Michaelis–Menten equation:

$$ = \frac{V_{max} S}{K_M + S} \quad (1)$$

where V_{max} is the maximum reaction rate (mmol ml^{-1} min^{-1}), K_M is the Michaelis constant (mmol ml^{-1}) and S is the substrate concentration (mmol ml^{-1}).

The influence of internal diffusion limitation on the enzymatic reaction rate is usually described in terms of the effectiveness factor, , which can be calculated as the ratio of the diffusion-limited and the non-diffusion-limited rate constants:

$$ = \frac{K_{diff}}{K} \quad (2)$$

where

$$K_i = \frac{V_{max}}{K_M} \quad (3)$$

The values of the non-diffusion-limited rate constants can be determined by grinding the immobilized enzyme particles to a fine powder. Table 3 shows the values of the kinetic parameters for the different catalysts. Those kinetic parameters were introduced in Eq. (3) to evaluate the presence of diffusion limitation.

The hydrolysis of tributyrin by lipase immobilized in OS and OSM is nearly no limited by diffusion. As can be seen in Table 3, although the value of K_{powder} is higher than those of K_{OS} and K_{OSM}, the difference in the Michaelis constant (from 0.031 to

Table 3
Kinetic parameters and efficiency factor for the hydrolysis of tributyrin by lipase immobilized in OS and OMS-10.

	V_{max} (mmol ml^{-1} min^{-1})	K_M (mmol ml^{-1})	K (min^{-1})	Efficiency factor ( )
OS	27.87	0.034	809.82	0.903
OSM-10	27.50	0.037	743.22	0.829
Powder catalyst	27.89	0.031	896.40	

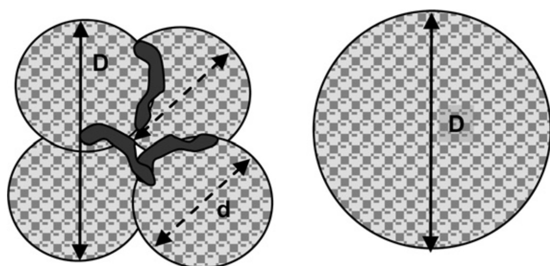
0.037 mmol ml^{-1}) is within standard error in enzyme kinetics. These close values mean that the agglomeration of octyl silica beads hardly affects internal diffusional limitation. Indeed, the effectiveness factor of OS is only 8% higher than the one of OSM, whereas particle size increases from 70 (OS) to 120 µm (OSM).

This apparently short increase in bead size is enough to allow working with 100 µm meshes in reactors. More interestingly, it enables to maintain similar properties in the isolated and larger agglomerated particle catalysts.

Scheme 1 displays a possible scenario to explain these results. Despite the large external diameter of the agglomerate (D), the substrate or product molecules do not diffuse through the distance D, but only across the smaller (d), which is the diameter of every single silica particle in the agglomerate. One particle with the same diameter D as the agglomerate OSM would probably cause higher diffusional restrictions. Moreover the presence of void areas of support, without enzyme, would be very likely found in this homogeneously large particle. Nevertheless, diffusion restrictions of substrates or products through smaller paths (d) to reach the whole internal surface of the porous network are quite similar in isolated OS and agglomerated OSM-10 particles. This can be also applied to enzyme diffusion, where the decrease of the hydrophobic environment during the immobilization of lipase due to the presence of ethanol improved the enzyme distribution [12]. This is in accordance with the effect of enzyme-support affinity on lipase distribution described by Gross et al. [9,10]. Thus, the distribution of the enzyme through the porous network should be favored also in larger beads obtained through agglomeration of individual ones.

3.5. Stability

The stability of the biocatalyst in organic medium is an interesting parameter to test provided that lipases are enzymes acting mainly on hydrophobic substrates. With this aim, OSM-10-lipase was incubated in acetonitrile at 35 °C as described in Section 2 and stirred in an orbital shaker. After 2 h incubation, tributyrin activity was assayed. After the reaction, the catalyst was washed in order to remove traces of products, dried and weighted to catalyze the next cycle. No significant loss in catalytic activity was detected after 15 incubation cycles, as seen in Fig. 4. This not only means that the biocatalyst is very stable, but also that no leaching of the enzyme had occurred. Again, similar stability results were previously obtained for lipase immobilized on octyl silica [11,12]: after incubation in acetonitrile for 1 h at 40 °C OS-lipase kept activities between 100 and 80% for 15 cycles. This excellent operational stability can be once more successfully reproduced with the enzyme immobilized on the OSM composite.



Scheme 1. Diameter of large particles or agglomerates (D) is longer than diameter of each single octyl silica particle in the agglomerate (d). Diffusion of enzyme, substrates or products in the agglomerates is only through shorter distance (d). ~~D: diameter of the whole particle; d: diameter of each octyl silica particle.~~

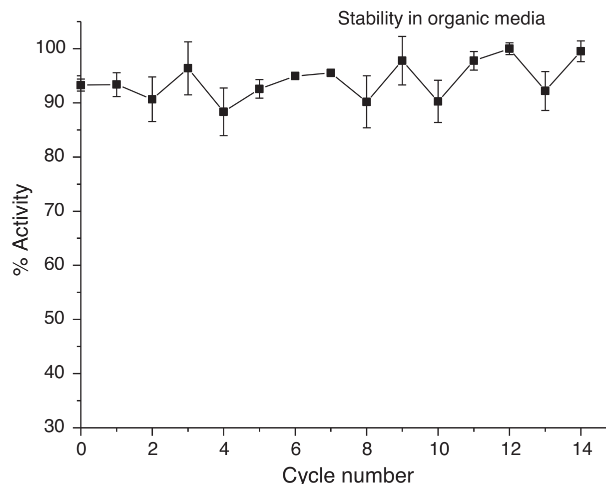


Fig. 4. Cycles of esterification of oleic acid with butanol catalyzed by OSM-10 lipase.

4. Conclusions

Starting from a siliceous material with excellent properties as support for lipase, a new hybrid organic-siliceous composite was obtained and optimized. The polymerization of a minimum amount of methacrylate around silica beads enabled to obtain larger support beads. Octyl-silica particles remain stuck to each other through small amount of polymer, so their individual sizes do not change, and keep their porosity and surface area almost intact. Consequently agglomeration does not significantly interfere with their properties and thus no differences regarding enzyme loading and distribution, immobilization rate, diffusion of substrates or activity and stability in organic solvents were found compared to the behavior of isolated OS-lipase particles. Therefore, this octyl-silica-methacrylate agglomerate with larger particle size seems to be an excellent support for lipase and very likely also a good candidate for industrial application of immobilized lipase.

Acknowledgements

The authors thank Dr. Carlos Marquez from Institute of Catalysis, CSIC, for valuable discussions. The companies Novozymes Spain and Silica PQ (PA, USA) are gratefully acknowledged for their kind gifts of lipase and silica MS3030 respectively. This work has been supported by the Spanish project MAT-2009-13569.

References

[1] F. Hasan, A.A. Shah, A. Hameed, Enzyme Microb. Technol. 39 (2006) 235–251.
[2] C. Spahn,.S.D. Minteer, Recent Patents Eng. 2 (2008) 195–200.
[3] J.A. Bosley, J.C. Clayton, Biotechnol. Bioeng. 43 (1994) 934–938.
[4] H.H.P. Yiu, P.A. Wright, J. Mater. Chem. 15 (2005) 3690–3700.
[5] R.A. Sheldon, Adv. Synth. Catal. 349 (2007) 1289–1307.

[6] P. Valencia, L. Wilson, C. Aguirre, A. Illanes, Enzyme Microb. Technol. 47 (2010) 268–276.
[7] P. Valencia, S. Flores, L. Wilson, A. Illanes, Biochem. Eng. J. 49 (2010) 256–263.
[8] Y. Li, F. Gao, W. Wei, J-B. Qu, G-H. Ma, W-Q. Zhou, J. Mol. Catal. B: Enzym. 66 (2010) 182–189.
[9] B. Chen, E.M. Miller, L. Miller, J.J. Maikner, R.A. Gross, Langmuir 23 (2007) 1381–1387.
[10] B. Chen, M.E. Miller, R.A. Gross, Langmuir 23 (2007) 6467–6474.
[11] R.M. Blanco, P. Terreros, M. Fernández-Pérez, C. Otero, G. Díaz-González., J. Mol. Catal. B: Enzym. 30 (2004) 83.
[12] R.M. Blanco, P. Terreros, N. Muñoz, E. Serra, J. Mol. Catal. B: Enzym. 47 (2007) 13.
[13] J. Krenkova, F. Svec, J. Sep. Sci. 32 (2009) 706–718.
[14] S. Gao, Y. Wang, X. Diao, G. Luo, Y. Dai, Bioresour. Technol. 101 (2010) 3830–3837.
[15] N. Miletic, Z. Vukovic, A. Nastasovic, K. Loos, J. Mol. Catal. B: Enzym. 56 (2009) 196–201.
[16] .M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[17] C.F. Torres, L. Vazquez, F.J. Señorans, G. Reglero, J. Chromatogr. A 1078 (2005) 28–34.
[18] N. Miletic, R. Rohandi, Z. Vukovic, A. Nastasovic, K. Loos, React. Funct. Polym. 69 (2009) 68–75.