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Optimized hybrid nanospheres containing Rhizomucor miehei Lipase for Chiral Biotransformation.

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The immobilization of Lipase from *Rhizomucor Miehei* into hybrid nanospheres containing a liposomal core, where enzyme is confined, was reported. Organic Liposomal-enzyme phase was protected by inorganic silica matrix obtained with and without surfactant that stabilizes the internal organic phase, isolates and protects the bioactive molecules. The optimized heterogeneous biocatalysts prepared was used for enantioselective esterification of (R,S)-ibuprofen. The influence of several catalytic parameters on the activity of hybrid nanospheres (type of solvent, nature of the alcohol, reaction temperature), was investigated. The best catalytic performances of heterogeneous biocatalysts were showed at 37°C, using iso-octane as solvent and 1-propanol as alcohol (ester yield value ranging between 78 and 93%). A strong activity and stability (up to 9 reaction cycles) of immobilized enzyme into hybrid nanospheres, with respect to the free form, was observed: ester yield of free Lipase is only the 25% in the same reaction conditions. *Rhizomucor miehei* lipase, both in its free and immobilized form, only reacts with the S (+) enantiomer of (R, S)–Ibuprofen, in all the reaction conditions tested.

Keywords: Rhizomucor miehei Lipase; Liposome; Optimized Heterogeneous hybrid-biocatalysts; (R,S)-ibuprofen; biotransformation.

1. Introduction

The enzymes are very active and highly specific biocatalysts that make enormous efforts of current research to obtain catalytic systems with comparable behavior to this type of natural catalysts [1,2]. Attempts to imitate the enzymatic action through the synthesis of artificial homogeneous or heterogeneous catalysts have largely been carried out [3-6]. However, the achieved specificity with these solid porous catalysts is not comparable with the well-known and reported catalytic capacity of enzymatic systems which exhibit high sterospecificity and regioselectivity due to their characteristic shape, charge and hydrophobic-hydrophilic properties [7,8]. The direct use of natural enzymes or their derivatives could be a serious alternative to perform several reaction processes with high specificity and reactivity. On the other hand, the poor physico-chemical stability of enzymes seriously limits their employ as effective catalysts because the reaction media conditions favor their denaturalization and, consequently, their inactivity. Another associated problem would be the impossibility to recover and reuse the enzymatic systems in successive catalytic cycles [9-10]. To overcome these obstacles would come from the isolation and stabilization of active enzymes into inorganic matrixes or organic systems which presumably provide an elevated protection, allowing the enzymatic action and preserving their associated catalytic activity and selectivity [11-23]. Among these, Liposome can be used to largely been used to encapsulated enzyme [24-27]. However, Liposomes show important limitations regarding to their poor hydrothermal and chemical stability that would favor the rapid denaturation of encapsulated enzymes [28-31]. The alternative approach could be the protection of the liposomal phase with external inorganic shells, such as porous silica [32-35]. In this case, the hydrothermal stability of silica would protect the internal enzymatic – liposomal system and its external porosity would allow the interaction with the reaction media. Furthermore, the associated structural role of organized phosphatidylcholine micelles, which are forming the liposomes, as surfactants, would facilitate the generation of external porous silica layers around of spherical liposomes. Specifically, these

nanospheres would be composed of a purely organic internal liposomal phase in which bioactive enzymes are encapsulated. Covering this part, an external self-assembled porous silica shell would be present, stabilizing the internal liposomal phase and, consequently, isolating and protecting the enzymes [36]. This methodology was successfully used to obtain organic-inorganic nanospheres with responsive external molecular gates, localized in the outer silica shell, for effective drug storage and controlled release [37].

Recently we describe a effective biocatalysts prepared through the encapsulation of liposome – lipase systems onto porous silica nanoparticles for biodiesel production [38]. In spite of previous work, in this study we optimized the synthesis procedure of heterogeneous biocatalyst in order to improve the stability of immobilized Rhizomucor miehei Lipase. The influence of several experimental factors, such as silica/liposome weight ratio and mixing time between liposome and lipase, was studied. At this point, the additional use of templating agent (Hexadecylamine) during the core liposome coverage by silica, has made possible the creation of mesoporous silicic shell with properties to allow reagent and product diffusion.

The catalytic performance and the stability of optimized heterogeneous catalysts was evaluated in a enantioselective esterification of racemic ibuprofen (Figure 1), in order to produce active enantiomer from racemic product. In the last year, there was an increasing trend toward the use of Lipase for a production of enantiomerically pure compounds [39-48], particularly in a chiral resolution of (R,S)-ibuprofen [49-59]. In particularly, Immobilized Rhizomucor miehei lipase shows to have adequate stability and biosynthetic capabilities for a chiral resolution of (R,S)-ibuprofen [60-62].

2. Materials and Methods

2.1. Materials

Organic nanospheres preparation. For the preparation of organic parts of nanospheres, L-α-phosphatidylcholine has been used as lecithin liposome precursor, purchased from Sigma Aldrich, while commercial lipase solution, PALATASE 20000L (Novo Nordisk Denmark), has been used as enzyme. This enzyme is a purified 1,3-specific lipase (EC 3.1.1.3) from *Rhizomucor miehei* (RML).

Silica porous shell preparation. For the preparation of silica porous shell, tetraethyl orthosilicate (99%), as silica source, hexadecylamine (98%), as template, sodium fluoride (99%), as mineralizing agent, have been used. All of these products were purchased from Sigma Aldrich.

Reactants for Catalytic Test: For the reaction tests, different alcohols have been tested: methanol (99.9%), 1-Propanol (99.9%), 1-butanol (99.9%). While isooctane (99.9%) and dimethylformamide (99%), have been tested as reaction solvents. Racemic Ibuprofen (98 %) and its pure enantiomers (R and S), have been purchased from Sigma-Aldrich.

2.2. Hybrid Nanospheres Synthesis Method: Enzyme immobilization procedure.

The synthesis of hybrid nanospheres take place through two consecutive steps. In the first step preparation of liposomal phase containing Rhizomucor miehei lipase was carried out and the influence of two important synthesis parameters was evaluated: silica/liposome weight ratio and mixing time of liposome/lipase solution. In the second step, formation of porous silica shell around the liposomal phase was performed: an amount of TEOS was added in the liposome/lipase solution at room temperature for 24 hours. After this time, an amount of sodium fluoride (7.1 mg) was incorporated to initializing the condensation of silane groups and the stirring was maintained for 48 h at room temperature. In order to synthesized different sample, templating agent was used during the core liposome coverage

by silica. An amount of hexadecylamine (tetraethyl orthosilicate/hexadecylamine molar ratio equal to 4) was dissolved in a 40 ml of ethanol. The hexadecylamine solution was added drop-wise 24 h after the addition of TEOS, with vigorous stirring at room temperature for 24 hours. 7.1 mg of NaF was incorporated and stirring for 48 h at room temperature. Later on, the sample was centrifugated and the recovered solid was washed with distillated water and dried at 30 °C overnight. All prepared catalysts were activated by washing with 100 ml of solvent (isooctane) and 900 ml of distillated water, and dried at 30 °C overnight.

The total protein concentration of the initial and final solution was calculated using UV absorption method at 235/280 nm [63], and the quantity of protein adsorbed on the support was determined by a mass balance between initial and final solution.

2.3. Catalyst Characterization

Thermogravimetric and Differential Thermal Analysis. TGA-DTA curve were recorded in nitrogen stream with a Metler Toledo TGA/SDTA 851E instrument. Measurements were effectuated in a temperature range between 20 and 800°C, with heating rate of 10°C/min and in synthetic air stream with a flow of 50mL/min.

Fluorescence Confocal Microscopy. LEICA TCS-SL is the imaging core's point-scanning laser confocal system and it was used to clarify with greater accuracy the exact position of the enzyme inside the nanospheres. In order to perform the analysis, during the nanospheres synthesis process, lipase was mixed with a fluorescent compound (fluorescein isothiocyanate (99%)) for 2 hours.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) micrographs were obtained with a Philips CM10 electron microscope operating at 100 KeV.

^{13}C NMR and ^{29}Si NMR. Spectra have been recorded at room temperature under magic angle spinning (MAS) in a Bruker AV-400 spectrometer. The single pulse ^{29}Si spectra were

acquired at 79.5 MHz with a 7 mm Bruker BL-7 probe using pulses of 3.5 μs corresponding to a flip angle of 3/4 π radians, and a recycle delay of 240 s. Pulses of 0.5 μs to flip the magnetization π/20 rad, and a recycle delay of 2 s were used. The ^{13}C spectra were recorded with a 7 mm Bruker BL-7 probe and at a sample spinning rate of 5 kHz. ^{13}C and ^{29}Si were referred to adamantine and tetramethylsilane, respectively.

N_2 adsorption/desorption. Nitrogen adsorption isotherms were measured at -196 ºC with a Micromeritics ASAP 2010 volumetric adsorption analyser. Before analyses, all samples were calcinated at 600°C under vacuum condition for 8 hours, and outgassed for 12 h at 100 ºC.

Powered X-ray Diffraction (XRD). This analysis were performed by powered X-ray diffraction technique using Philips X'PERT diffractometer. Data were collected stepwise over the $2° \leq 2\vartheta \leq 20°C$ angular region, with steps of 0.01° 2ϑ, 20-s/step accumulation time and Cu Kα (λ= 1.54 Å) radiation.

2.4. Catalytic Test

2.4.1 Enantioselective esterification procedure.

The standard reaction mixture was composed of organic solvent (10 mL), racemic ibuprofen (66 mM) and alcohol (66 mM), without addition of water. The reaction started by the addition of prepared heterogeneous biocatalyst (7%wt of lipase with respect to ibuprofen) to the solution and carried out in 50 mL conical flask, under orbital magnetic stirring at 135 rpm and at different temperature. Samples of 50 μL of the solution were withdrawn at different times and diluted in 50 μL of isooctane. The amount of ester (ester yield) formed during the reaction and the enantiomeric excess were determined by gas chromatography and chiral gas chromatography, respectively.

2.4.2. Analytical procedures of reaction products.

<u>*Gas Chromatography Analysis.*</u> This technique has been performed in an Agilent 7890A, equipped with flame ionization (FID) and mass spectrometry detectors. The column used was a BP5MS with low polarity phase (5%-phenyl-95%-polysilphenylene-siloxane; 30m x 250 µm x 0.25 µm). The injector and the detector temperatures have been, respectively, of 280°C and of 300°C. The carrier gas was nitrogen, with a flow rate of 25 mL/min. The temperature program of the column was: 2 min at 50°C and 30°C/min until 280°C. Ester yield was calculated in accordance with the equation (1):

$$\text{ester yield \%} = \left[\frac{(A_E/PM_E)}{\left(\frac{A_E}{PM_E}\right)+\left(\frac{A_{ib}}{PM_{ib}}\right)}\right] * 100 \qquad (1)$$

Where the A_E and PM_E are the peak area an molecular weight of the (S)-ester of ibuprofen (desired product) respectively, while A_{ib} and PM_{ib} are respectively, the peak area and molecular weight of ibuprofen. The quantitative analysis to measure formed ester and remaining acid was carried out using internal standardization method.

Turnover number (TON) and turnover number of frequency (TOF) were also calculated in correspondence with a reaction time equal to 1 hours (Eq (2) and (3)).

$$TON = \left(\frac{\%ester\ yield}{100}\right) * \left(\frac{mol\ iburpofen}{mol\ enzyme}\right) \qquad [/] \qquad (2)$$

$$TOF = \frac{TON}{Time} \qquad [h^{-1}] \qquad (3)$$

<u>*Chiral Analysis*</u>**.** .This analysis was performed using a chiral gas chromatograph (Agilent 8000S) equipped with FID and with a BETADEX^{TM}120 column (35%-phenyl-65%-dimethylsiloxane; 30m x 0.25 mm x 0.25µm). The injector and the detector temperatures have been, respectively, of 280°C and of 300°C. The carrier gas has been nitrogen, with a flow rate of 25 mL/min. The temperature program of column was 20 minutes at 50°C; 5°C/min until 140°C; 20 minutes at 140°C; 5°C/min

until 210°C and 20 minutes at 210°C. The retention times observed Enantiomeric excess (ee) was calculated according to the equation (4):

$$ee\ \% = \frac{R-S}{S+R} * 100 \qquad \text{For R > S} \qquad (4)$$

where R is the peak area for the R(-) enantiomer of ibuprofen (retention times equal to 76 min) and S is the peak area for the S(+) enantiomer of ibuprofen (retention times equal to 76.4 min).

3. Results and Discussion

3.1 Characterization catalysts results

The influence of two important synthesis parameters, silica/liposome weight ratio and mixing time of liposome/lipase solution, on the quantity of immobilized enzyme and on the morphology of nanospheres, were evaluated. Table 1 summarizes these specifications, for each prepared catalysts. The tendency of enzyme immobilization quantity towards liposome/lipase solution mixing time, for each liposome/silica ratio tested, was reported in Figure 2. It is immediately evident that the mixing time between liposome and lipase strongly affects the amount of immobilized enzyme (Figure 2a). Particularly, after only 2 h, for each Silica/Liposome ratio tested, the highest Lipase immobilized amount was obtained. Increasing the mixing time, the amount of retained enzyme decrease probably due to the damage/break of liposomal shell. Moreover, for the same liposome/lipase mixing time, the specific amount of immobilized enzyme strongly increased when the SiO_2 amount used was lower, as expected (Figure 2b).

As Shown by TEM analysis result (Figure 3), also the morphology of the samples was strongly affected by the two changed parameters. The equal o lower amount of SiO_2 with respect to the liposome (NS2 and NS3 samples) does not permits the complete single liposome sphere coverage by the silica shell (Figure 3a), in fact more spheres are included in an unique silica shell. Increasing time mixture between lipase and liposome, the damage/break and the opening of liposomal shells occurs (Figures 3b and 3c, respectively for NS2 and NS3 samples).

On the contrary, perfect coverage of liposomal cells by the silica shell can be observed where the amount of silica is double respect to the amount of Liposome (sample NS1), both for low (2 hours) and high (12 hours) mixing time between liposome and lipase (Figure 4(a) and (b)**,** respectively). However, in this last case, the quantity of immobilized enzyme is very low, probably because only few liposome cells remain unaltered after this long mixing time.

It is possible to conclude that the optimal hybrid nanospheres were obtained by a double amount of silica with respect to liposome quantity and after only 2 hour of mixing time between liposome and lipase (Figure 4(a)), represented by NS1 sample that was selected as reference catalyst and analyzed with more details. First of all, in order to clarify more accurately the exact position of the enzyme inside the optimized nanospheres of sample NS1, *fluorescence confocal microscopy analysis* was carried out. This analysis confirm that, the enzyme is distributed inside the internal liposomal phase and the organic liposome/lipase phase has been protected by inorganic matrix (Figure 5).

The optimized procedure (SiO_2/Liposome weight ratio equal to 2 and the optimum liposome/lipase solution mixing time equal to 2 hours) was used also to prepare different hybrid nanospheres (NSH1 sample), using surfactant to create silica shell. In Table 2 the synthesis conditions are reported. The use of surfactant does not affect the final conformation of the nanospheres and the position of the enzyme, which results perfectly confined inside the liposomal (Figure 6). Moreover, to get more information about the external silica structure of this sample, XRD and TEM analysis was carried out (For XRD results see ***Supporting Information: Section A*)**. With respect to the nanospheres synthesized without surfactant (in which the external silica shell is amorphous, as corroborate by XRD pattern – results not shown), the silica shell of NSH1 sample exhibits a typical worm-hole structure characterized by parallel channels to the support surface (Figure 7).

Other characterization tests were carried out in order to give more information about optimized biocatalysts structure (**Supporting Information: Section A**).

3.2. Catalytic test results

3.2.1. Influence of catalyst composition

Catalytic test to evaluate the role of Liposome and Hexadecylamine in the reaction, were carried out. See **Supporting Information: Section B** for more details. The results confirm that no reaction between ibuprofen and liposome or ibuprofen and hexadecylamine occurs.

3.2.2. Influence of the alcohol

It is recognized that lipase from *Rh*izomucor *miehei* works better in esterification of primary alcohols, whereas its activity is lower with secondary alcohols and is inactive with tertiary alcohols [64]. With the aim of studying the effect of alcohol nature on our esterification reaction, the attention was directly focused on the study of the influence of primary alcohols with different chain lengths, i.e, methanol, 1-propanol and 1-butanol. The results are shown Table 3. The stereobias (S-(+)-preference) is the same for all the nucleophiles tested. However the catalyst performance is strong influenced by the length of the used alcohol**.** With 1-butanol and methanol, immobilized *Rhizomucor miehei* lipase shows low activity, probably due to the different substrate specificity of the lipase and/or to the different substrate solvation of the alcohol, as also suggested by previous studies [65]. The highest enantiomeric excess (ee), ester yield and turnover numbers (TON and TOF) are obtained in the reaction where 1-propanol is used as alcohol. In the **Supporting Information**: **Section B**, the progress and the whole time profile of the reaction carried out in presence of 1-propanol, monitored by chiral gas chromatography, was reported.

3.2.3. Influence of the Temperature.

The effect of temperature, in the range from 27 to 80 °C, on the enzyme activity in the enantioselective esterification of (R,S)-ibuprofen was examined. As consequence of previous results, all catalytic tests were carried out in presence of 1-propanol as alcohol. At all temperature

tested Rhizomucor miehei Lipase shows S-(+)-enantiorecognition. The results were reported in Table 4**.** We can observe that the highest ester yield and turnover numbers was obtained at 37°C, decreasing at higher temperature due to the modification of the active center geometry of enzyme. In order to avoid the evaporation of the organic solvent and to obtain a higher ester yield of the desired product, the optimum temperature in the esterification of Ibuprofen was fixed at 37 °C.

3.2.4. Influence of the Solvent nature.

The influence of the solvent nature on the catalytic efficiency of enzymes was also studied. Beyond using a-polar isooctane solvent, the activity of immobilized *Rhizomucor miehei* Lipase in the presence of polar solvent, dimethylformamide (DMF), was tested. The polar DMF totally deactivates the enzyme contained into NS1 catalyst (Figure 8), due to the removal of the necessary water for maintaining the native and active conformation of the enzyme. On the contrary, hydrophobic solvents, due to their lower tendencies to strip essential water in the micro-environment of enzyme, allow preserving their activity.

3.4. Stability of catalysts

After selection of the best reaction conditions: temperature 37°C; 1-propanol as alcohol; iso-octane as a solvent, a "leaching test" was carried out in order to verify if there is a leaching after the first catalytic use of the optimized catalysts. So, after 1 hour, the reaction was stopped, the catalyst was separated from the liquid reaction media and the reaction was again carried out without catalyst. The time reaction profile was compared with time reaction profile of standard reaction (see **Supporting Information: Section C**). When the NS1 catalyst was removed, the ester yield does not increase significantly after the first hour of reaction (lower than 5 %). So, no significant enzyme leaching occurs.

At this point, the residual esterification activity under optimal reaction conditions of NS1 and NSH1 catalyst, after repeated catalytic uses, was analyzed**.** In Figure 9, the stability results after 9 reaction cycles for both catalysts, were reported. Immobilized lipase in the NS1 catalyst retains its activity with moderate loss: lower than 3% after 4 reaction cycles; lower than 11% after 5 reaction cycles. After nine cycles, the lipase immobilized into NS1 catalyst retains almost the 45% of its initial esterification activity. For NSH1 sample the activity loss was lower than 3% after 4 reaction cycles; lower than 18% after 8 reaction cycles. After nine cycles, the lipase immobilized into NSH1 catalyst retains almost the 60% of its initial esterification activity and the external mesoporous structure of NSH1 catalyst was maintained (Figure 10). The NSH1 show the best catalytic performance most probably because the mesoporous structure of its external silica shell facilitates the mass transfer of substrate and products. However, for both sample, immobilized is stable after repeated catalytic cycles and the enzyme remains linked to the phospholipids layer inside the liposome and is still present in the nanospheres (Figure 11).

3.3. Comparison with Free Lipase.

Comparison between free and immobilized enzyme performances, was carried out. The results are reported in Figure 12(a) and (b). Both catalysts, NS1 and NSH1, show better performance with respect to the free lipase. Particularly, NSH1 sample shows the best one probably because the mesoporous structure of its external silica shell facilitates the mass transfer of substrate and products. Rhizomucor miehei Lipase reacts better only with the S-(+) enantiomer of (R,S)-ibuprofen both in it immobilized and free form.

4. Conclusion

Novel organic–inorganic nanospheres formed by a hybrid silica shell (with an internal liposomal core) containing bioactive molecule (lipase enzyme) were successfully synthesized. The highest

immobilized enzyme amount and the best nanospheres morphology were obtained using a SiO_2/Liposome ratio equal to 2 and a Lipase/Liposome mixing time equal to 2 hours.

Hybrid nanospheres with mesoporous external silica shell have also been synthesized by use of hexadecylamine as surfactant in the polymerization of silica shell. Heterogeneous biocatalysts were used in the enantioselective esterification of ibuprofen. Many factors affect the enzyme activity in the esterification reaction. The optimal conditions for the esterification of ibuprofen using hybrid nanospheres containing *Rhizomucor miehei* Lipase as biocatalyst are: temperature = 37°C, alcohol = 1-propanol, solvent = iso-octane and ibuprofen/alcohol molar ratio equal to 1.

Catalyst with mesoporous external silica shell shows better catalytic performance with respect to that one synthesized without surfactant. Most probably, the mesoporous channel facilitates the substrate and product mass transfer during the reaction. Finally, the stability of immobilized lipase is very high: up to 9 reaction cycles, at least. The high stability of the heterogeneous biocatalyst produces an immobilized enzyme productivity c.a. 15÷16 times higher than that of its free form.

Optimized catalysts and selected best reaction conditions lead a simple and efficient enantioselective synthesis of (S)-enantiomer of ibuprofen, being a potential application in the pharmaceutical industry. The high-efficiency catalytic system here proposed could be used in a next future for various enzymatic/chemical processes, like multi-enzymatic cascade reaction, drug and gene delivery and biosensors.

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