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Enhancement of operational stability of chloroperoxidase from *Caldariomyces fumago* by immobilization onto mesoporous supports and the use of co-solvents

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ABSTRACT

Chloroperoxidase (CPO) from *Caldariomyces fumago* is a versatile enzyme because of its ability to catalyze different reactions, with high regio- and enantio-selectivities, and among them the styrene epoxidation is of especial industrial interest. However, the use of CPO at industrial scale has been limited mainly due to their low operational stability. In this work, two fundamental aspects are addressed: the selection of a suitable co-solvent to improve biocatalytic epoxidation reaction of hydrophobic substrates such as styrene and some of its derivatives, and CPO immobilization in mesoporous materials in order to increase the enzyme operational stability. Co-solvents tested were acetonitrile, 2-methyl-2-propanol and 2-methyl-2-butanol. In addition, CPO immobilization onto three mesoporous materials was assayed: two siliceous mesocellular foams (MCF and MSU-F) and titanium dioxide (TiO_2, anatase). The CPO/MCF preparation showed 3-times higher catalytic activity for styrene epoxidation than the free enzyme. In addition, the total turnover numbers (TTN) were determined for styrene, 3-chlorostyrene and 3-nitrostyrene epoxidations, both with free and immobilized enzyme. The CPO immobilization in mesoporous materials together with use of co-solvent increased the operational stability of CPO in 51% in the case of styrene, 41% for 3-chlorostyrene and 246% for 3-nitrostyrene, when compared to the corresponding values obtained with the free enzyme. These results clearly indicated that CPO immobilization onto nanostructured materials and the use of co-solvent significantly enhances the enzymatic epoxidation reactions of styrene derivatives.

1. Introduction

Epoxides and vicinal diols are important synthetic intermediates in the preparation of pharmaceutical compounds with biological activity. The synthetic versatility of the epoxides is due to the oxirane function, which can be opened by a variety of nucleophiles yielding a broad range of valuable intermediates [1]. Despite various methods for the synthesis of these compounds [2], the biocatalytic synthesis appears as an attractive alternative because its high stereo-selectivity and regio-selectivity, mild reaction conditions, and it is considered an environmental friendly process compared to a large number of chemo-catalysis process, among other reasons [1].

The enzyme chloroperoxidase from the fungus *Caldariomyces fumago* (CPO; E.C. 1.11.1.10) has been proposed for the biocatalytic synthesis of epoxides. This enzyme catalyzes the asymmetric epoxidation of a wide variety of olefinic substrates with high enantiomeric excess (ee) and yields [3–6]. Among the major products of this CPO-catalyzed reaction are the vicinal diols that are generated by the hydrolysis of epoxides in aqueous medium, which maintain their stereogenic center and thus retain their importance as chiral synthetic synthones. CPO-mediated biocatalytic epoxidation shows several advantages because this enzyme is the fastest peroxidase known so far, it does not need cofactors, and the final electron acceptor is the hydrogen peroxide. Moreover, the enzyme can be produced in large quantities and it is stable to store conditions. However, there are two main limitations for the implementation of large-scale production of epoxides using CPO. First, the low solubility of hydrophobic substrates in aqueous system, in which CPO reactions are carried out and the generated epoxides undergo hydrolysis. Secondly, as all peroxidases, CPO stability is very low

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at catalytic concentrations of hydrogen peroxide [7–10], showing poor operational stability, which is a key limiting problem for its biocatalytic performance [11].

To overcome these drawbacks, different strategies have been developed. The addition of organic co-solvent in reaction mixture has been used to enhance the solubility of hydrophobic substrates and minimizing diffusional problems related with the substrate along with the stabilization of reaction products [12,13]. Ionic liquids, as cosolvents, have been studied for the enhancement of enzyme activity, stability, and enantioselectivity, as well for the increase of substrate solubility [14,15]. In the case of CPO, ionic liquids have been used for the transformation of 1,2-dihydronaphthalene [16], phenylmethylsulfide [17], indole and thionisole [18] showing that ionic liquids are suitable cosolvents for these transformations, yielding high enantiomeric excess and good conversion rates.

On the other hand, hydrogen peroxide dosage and enzyme immobilization have shown be useful strategies to minimize CPO inactivation [11]. A diversity of solid supports has been assayed for the enzyme immobilization including organic polymers, chitosan, hydrogels, silica gels, zeolite, nanomaterials and mesoporous materials [19,20]. Among them, mesoporous silica materials have proved to be good supports for enzyme immobilization due to their high surface area, special pore structure, thermal stabilization and biocompatibility [21]. Siliceous mesopourous materials such as mesostructured cellular foams (MCF) and Michigan State University silica (MSU-F) are among the most promising supports for successful enzyme immobilization, including CPO [22–25]. In addition, these mesoporous materials are attractive for their robustness, well-defined and interconnected pore structure, ultra large pores sizes (24–42 nm), which facilitate the substrate diffusion, and their hydrothermal stability. However, it is interesting to point out that MSU-F materials have been little employed as support for enzyme immobilization, despite having similar physical and chemical properties than MCF. TiO_2 is another material that could have mesoporous structure with good mechanical and chemical properties, thermal stability, biocompatibility, environmentally safe, and widely used in catalytic applications. However, no information is available on enzyme immobilization on mesoporous TiO_2. This material has been widely used as supports in Au catalysts for heterogeneous catalysis of alkene epoxidation [26] and as an electrode modifier for horseradish peroxidase (HRP) immobilization for electrochemical biosensors [27–31]. Recently, TiO_2 nanoparticles were used for HRP immobilization by physical adsorption [32], and the relationship between the geometric topography of the mesopores of TiO_2 and adsorption of some proteins (bovine serum albumin, myoglobin and lysozyme) at their isoelectric point has been studied [33].

In this work, the operational parameters on the biocatalytic epoxidation using immobilized CPO have been evaluated. In addition, the effect of organic co-solvent in reaction medium on the catalytic activity and operational stability of CPO was determined. The biocatalyst preparations were assayed in combination with a dosed addition of H_2O_2 in order to enhance the operational stability of CPO, which was characterized in terms of the total turnover number (TTN). Styrene, 3-chlorostyrene and 3-nitrostyrene were used as substrates, and the potential application for large-scale industrial processes is discussed.

2. Experimental

2.1. Chemicals

Hydrogen peroxide 30% (w/w) solution, 2-methyl-2-propanol (2M2P) and 2-methyl-2-butanol (2M2B) were obtained from Merck KGaA (Darmstadt, Germany). Titania (99.7% anatase) was purchased from Alfa Aesar® and MSU-F mesoporous silica was obtained from Sigma–Aldrich (St. Louis, MO). Mesocellular foam (MCF) was obtained for as previously described [24] and chloroperoxidase from *C. fumago* was obtained and purified as previously reported [34]. The enzyme preparation contained 6530 U mL^{-1}, with a R_Z = 1.0 and an enzyme concentration of 289 μmol L^{-1}. All other chemicals, unless otherwise stated, used in this work were supplied by Sigma–Aldrich.

2.2. Characterization of mesoporous materials

Specific areas were obtained from Nitrogen adsorption isotherms at 77 K performed in an automatic Micromeritics apparatus Model ASAP 2010 in the 0.05–0.30 relative pressure range. The Barrett–Emmett–Teller (BET) method was utilized to calculate the surface areas from a linear part of the BET plot according to IUPAC recommendations. The pore volume and pore size distributions were calculated from the desorption branches of the isotherms using the Barrett–Joyner–Halenda (BJH) method. Zeta-potential measurements were carried out in a Zeta Meter 3+, using 20 mg of sample dispersed in 200 mL of a solution containing 1 mmol L^{-1} KCl. The pH was adjusted with 1 mmol L^{-1} KOH or HCl solutions. The isoelectric point (IEP) was taken at zero rate of migration.

2.3. Enzyme activity

Peroxidase activity of CPO was determined spectrophotometrically using guaiacol as substrate at pH 6.0 and measuring the increase in absorbance at 470 nm. The transformation rate was estimated by using an extinction coefficient of 26,600 M^{-1} cm^{-1}, as it was converted to tetraguaiacol [35]. Halogenase activity of CPO was determined using the standard monochlorodimedone (MCD) assay [36] in acetate buffer solution, pH 3.0, containing KCl (20 mM) and H_2O_2 (1 mmol L^{-1}), in which chlorination of MCD is followed by absorbance diminution at 278 nm (ε = 12,200 M^{-1} cm^{-1}). In both cases, one unit of activity (U) is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of substrate per min. For experiments with continuous dosage of hydrogen peroxide a Series I Metering HPLC Pump (Scientific Systems, Inc.) was used.

2.4. Determination of catalytic parameters of CPO

Epoxidation reactions were performed in a 60 mmol L^{-1} phosphate buffer, pH 3.0 and pH 6.0, containing substrate (0.10–10.0 mM), 15% (v/v) co-solvent and 800 nmol L^{-1} CPO final concentration. The reaction was started by adding hydrogen peroxide solution (40 mM) at a rate of 0.2 μmol min^{-1} flow until reach 1 mmol L^{-1} of final concentration (saturating concentration). The reactions were stopped after 5 min by adding 1 mL acetonitrile and rapid cooling in ice/water bath. Then, the reaction mixtures were analyzed in a Knauer high-resolution liquid chromatography (HPLC) equipped with *Smartline* Photodiode Array (PDA) Detector 2850 (HPLC-PDA). The elution was performed with a mobile phase of 65:35 (v/v) acetonitrile–water, with a flow of 1.0 mL min^{-1}, through a reverse phase C_{18} column 5 μm Eurospher 100–5 (250 mm × 4.6 mm). The detection wavelengths were set at 207 nm and 216 nm to measure the generated diols and epoxides, respectively. All extracts obtained from different experiments were filtered using 0.22 μm nylon syringe filters (Membrane Solutions), prior to their analysis by HPLC. Enzyme assays and other UV–vis experiments were performed with UV-VIS spectrophotometer UV2310II (Techcomp).

Prior to catalytic tests, calibration curves for each substrate and their corresponding diols and epoxides were obtained. Hill's

equation (Eq. (1)) was used as adjustment model to determine the catalytic parameters of free and immobilized enzymes [37].

$$\frac{v}{E_t} = K_{cat}\left(\frac{[S]^n}{K\prime + [S]^n}\right) \quad (1)$$

where v is the reaction rate calculated as total concentration produced of diols and epoxides per minute, E_t is the total concentration of enzyme and k_{cat} represents the catalytic rate constant. The initial concentration of substrate is defined by [S], n corresponds to the substrate cooperativity to the enzyme and K' is a constant including different interaction factors and the intrinsic dissociation constant K_S of enzyme–substrate complex. CPO specific activity (U_{SP}) is defined as v/E_t ratio, expressed in min^{-1} units.

2.5. Organic co-solvent evaluation on catalytic activity of CPO

Initially, three co-solvents at 15% (v/v) concentration in reaction medium were evaluated, acetonitrile (ACN), 2-methyl-2-propanol (2M2P) and 2-methyl-2-butanol (2M2B). In this case, styrene was used as substrate in a concentration ranging from 0.1 to 3.0 mM. For the co-solvent in which the best catalytic activity was found (2M2P), different solvent concentrations were tested: 5, 15, 30, 60 and 90% (v/v), and three selected substrates were assayed. The pH of the buffer–solvent mixtures has been adjusted before experiments. Hydrogen peroxide (40 mmol L^{-1}) was dosed in all experiments at a rate of 0.2 μmol min^{-1} until to reach 1.0 mmol L^{-1} final concentration. The reaction time was 5 min.

2.6. Immobilization of CPO in mesoporous materials

CPO immobilization was carried out in 60 mmol L^{-1} phosphate buffer either at pH 3.0 for MSU-F and MCF or at pH 5.0 for TiO_2. A CPO solution (30 μmol L^{-1}) prepared with the corresponding buffer were added at each support (5.0 mg), stirred at 400 rpm and incubated at 4 °C for 8 h. Thereafter, suspensions were centrifuged at 6000 rpm for 4 min and the supernatant removed. Then, the solid biocatalysts was washed many times with 1 mL of their respective buffer until the CPO leaching in washings was less at 1% of the initial peroxidase activity of the enzyme. The resulting biocatalysts was resuspended in fresh buffer and stored at 4 °C until use. The amount of immobilized CPO onto different materials was estimated by measuring the absorbance difference at 398 nm (CPO ε = 85,000 M^{-1} cm^{-1}) between the initial CPO solution and supernatants after immobilization, including all washings.

The CPO immobilization efficiency was estimated in two ways: the immobilization yield (I_Y) and the immobilization efficiency (I_E), which were calculated according Sheldon and van Pelt [38]. The immobilization yield (Eq. (2)) describes the percentage of total enzyme activity that is immobilized from the free enzyme solution, while the immobilization efficiency (Eq. (3)) describes the percentage of enzyme activity that is observed in the biocatalyst:

$$I_Y(\%) = \left(\frac{U_0 - U_S + w}{U_0}\right) \times 100\% \quad (2)$$

$$I_Y(\%) = \left(\frac{U_{biocat}}{U_0 - U_{S+W}}\right) \times 100\% \quad (3)$$

where U_0 is the total starting activity of CPO solution, U_{S+W} is the total residual enzyme activity that remains in the enzyme solution after immobilization (supernatant and washings) and U_{biocat} is the activity measured for the biocatalyst. Parallel blank experiments were carried out to compensate for free enzyme deactivation under the immobilization conditions.

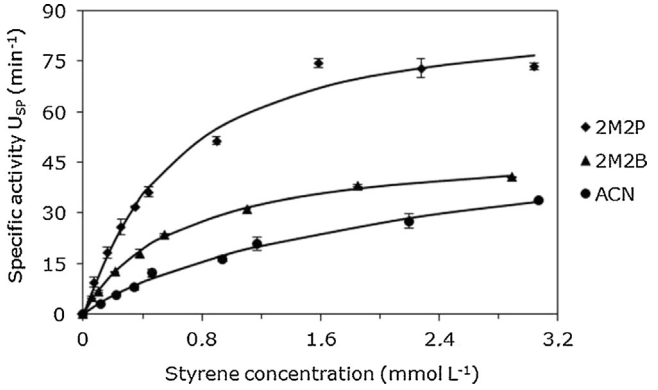

Fig. 1. Effect of co-solvent type on specific activity curves of CPO for the epoxidation reaction of styrene: acetonitrile (ACN), 2-methyl-2-butanol (2M2B) and 2-methyl-2-propanol (2M2P). Co-solvent content used was 15%, v/v.

Table 1
Kinetic parameters of styrene epoxidation reactions catalyzed by CPO, using 15% (v/v) of organic co-solvent in reaction medium.[a]

Co-solvent	k_{cat} (min^{-1})	K' ($mmol^n$ L^{-n})	n	Model adjustment (R^2)
ACN	66 ± 1	2.7 ± 0.1	0.9 ± 0.1	0.986
2M2B	51 ± 3	0.7 ± 0.1	1.0 ± 0.1	0.992
2M2P	89 ± 3	0.6 ± 0.1	1.1 ± 0.1	0.987

[a] For experimental conditions see numeral 2.5.

2.7. Determination of enzyme operational stability

The total turnover number (TTN) is related to the enzyme operational stability. The TTN values were determined by quantifying the number of moles of product (as epoxides and diols) that were generated by one mole of enzyme until its inactivation occurs. Products were followed and quantified at 207 nm (diols) and 216 nm (epoxides) by HPLC-PAD, after calibration. Hydrogen peroxide was added at a continuous flow of 25 nmol s^{-1}. To verify the enzyme inactivation, the halogenase CPO activity was periodically measured in the reaction medium until no activity was detected.

3. Results and discussion

In order to extent the lifetime of CPO by minimizing the inactivation effect of hydrogen peroxide during the reactions, the addition of 40 mmol L^{-1} H_2O_2 was supplied at a low flow of 0.2 μmol min^{-1}. It is well know that high concentrations of H_2O_2 inactivates the CPO by oxidative degradation of heme group [9,10], and thus a dosed addition of hydrogen peroxide during CPO reactions improves the catalytic efficiency [39,40]. With this variable controlled, we proceeded to select a co-solvent by favoring epoxidation reactions with hydrophobic substrates such as styrene and some of its derivatives.

3.1. Effect of co-solvent type

Three co-solvents were assayed; acetonitrile (ACN), 2-methyl-2-propanol (2M2P) and 2-methyl-2-butanol (2M2B). ACN [24,41] and 2M2P [42,43] have been previously used in enzymatic reactions catalyzed by CPO. While, 2M2B solvent has been used in biocatalytic applications with different enzymes, including lipases [44–46], beta-galactosidase [47] and different hydrolases [48]. Fig. 1 shows the styrene epoxidation in a medium containing 15% of different organic co-solvents. 2M2P clearly favors CPO catalytic activity for epoxidation reaction while the ACN showed the lowest V_{max} value. The kinetic data fitted the Hill equation and the catalytic parameters k_{cat} and K' of the CPO were estimated (Table 1). The 2M2P

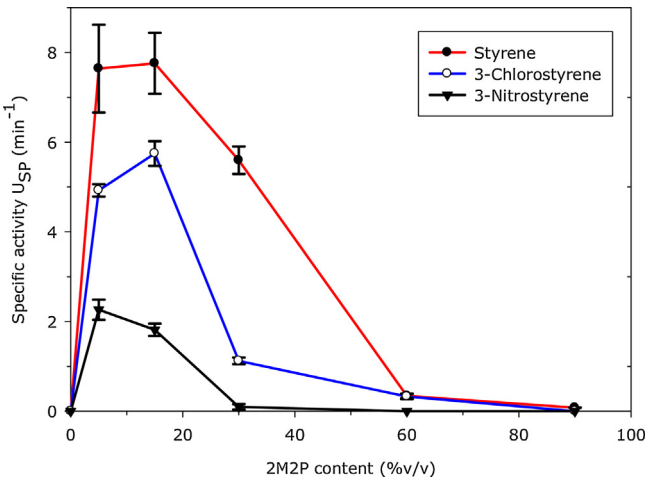

Fig. 2. Effect of 2M2P content on specific activity of CPO for epoxidation reaction carried out at pH 6.0. Reaction condition: CPO 800 nM, substrate 0.2 mM, 1 μmol of hydrogen peroxide (total amount added), 293 K, 400 rpm and 5 min of reaction time.

solvent showed the highest catalytic constant for styrene. On the other hand, the *K'* values were similar for 2M2P and 2M2B, whereas with ACN its value was at least 4 times higher. It could be considered that the values of *K'* are equivalent to the Michaelis constant K_m because the values of *n* are close to 1 for the kinetic models from the three solvents. Thus, the values of *K'* may indicate a greater affinity of the enzyme–substrate complex with the first two solvents compared to ACN.

Subsequently and in order to reduce the mass transfer limitations of the water insoluble substrate, different 2M2P concentrations in the reaction medium were evaluated (Fig. 2). It is well known that the presence of organic co-solvents in the reaction mixture is an important factor affecting the general stability of enzymes [13]. The solvent effect was evaluated for three substrates; styrene, 3-chlorostyrene and 3-nitrostyrene. The CPO enzymatic activity for all substrates increased in 2M2P concentrations from 5 to 15% (v/v). Maximal activities were found between 10 and 15% of co-solvent. The results also clearly showed that enzyme is inactivated at higher co-solvent concentrations (>20%, v/v), presumably by conformational disruption of the active site [49]. The denaturing action exerted by the organic co-solvent is attributed to a conformational loss of the active site [49]. Even if there are some reports regarding the use of aqueous co-solvent mixtures in CPO catalyzed reactions [3,24,41,42], the rational election of organic co-solvents has not been easy, and some attempts to establish a thermodynamic rational [43–48] have been reported.

Table 2
Characterization of mesoporous materials used as support for CPO immobilization.

Support	BET surface area[a] ($m^2\ g^{-1}$)	Total pore volume[b] ($cm^3\ g^{-1}$)	$D_{P(BJH)}$ (nm)	IEP
MCF	588	2.00	12.1	2.3
MSU-F	225	1.50	29.9	2.2
TiO_2	146	0.30	8.3[c]	5.8

Pore diameter, D_P, was determined from the desorption branch.
[a] Calculated using Brunauer–Emmett–Teller BET methods.
[b] Calculated using Barrett–Joyner–Halenda BJH methods.
[c] Bimodal distribution of pores (4.8 and 12.4 nm, respectively).

3.2. CPO immobilization in mesoporous materials

CPO was immobilized on three different mesoporous supports to enhance enzyme its stability with the use of co-solvent. The three materials used were MCF, MSU-F and TiO_2. MCF and MSU-F are siliceous mesostructured cellular foams. These materials possess three-dimensional, continuous ultra-large pore mesoporous structures with large spherical cells interconnected by uniform windows [50]. Despite having similar physicochemical characteristics, only the MCF has been widely used for enzyme immobilization [22,24,51], while the MSU-F has not been employed as support for biomolecules and no related research with enzyme immobilization could be found in the literature. On the other hand, TiO_2 in anatase phase is also a mesoporous material with defined crystal structure. The TiO_2 interest is mainly related to their optical and electronic properties, chemical stability and low toxicity. The application of TiO_2 as a support in heterogeneous catalysis is abundant, but their applications in biocatalysis are scarce.

The dimensions of the pores are crucial for adsorption into the mesopore silica networks. The protein immobilization on silica surface involves only weak interactions such as hydrogen bonding, acid-base interactions and van der Waals attraction [52]. The enzyme adsorption can be improved and the leaking can be minimized if there are electrostatic interact ions between oppositely charged residues on the enzyme molecules and the silica surface. The overall charge on the enzyme surface and silica surface can be controlled by varying the pH of the solution. An attractive interaction between enzyme and silica is obtained at a pH somewhere in-between the point of zero charge of silica and the isoelectric point of the specific enzyme. Desorption of the protein in the presence of ammonium sulfate indicates that electrostatic interactions are predominant, whereas hydrophobic interactions are more predominant with the more positively charged silica.

Table 2 summarizes the main textural characteristics and the isoelectric points (IEP) of the materials. All materials seem to be suitable for CPO immobilization, since the dimensions of the enzyme (5.3 nm × 4.6 nm × 6.0 nm) are smaller than the pore

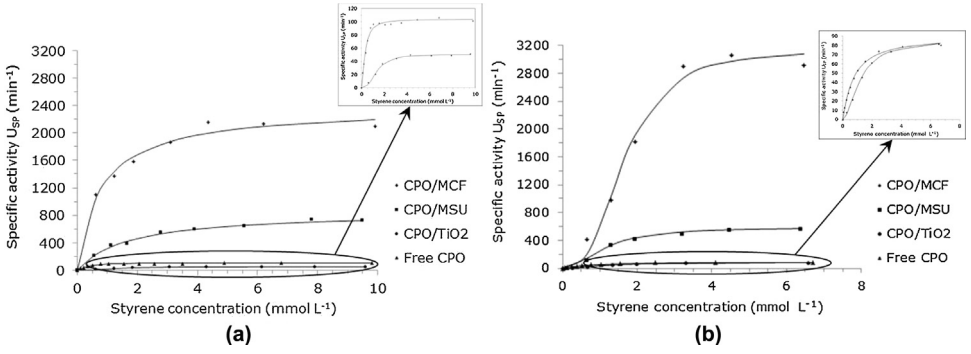

Fig. 3. Specific activity curves of different biocatalysts for the epoxidation reaction of styrene carried out at (a) pH 3.0 and (b) pH 6.0. The solid lines correspond to the fit of the experimental data to the Hill equation used as model. Reaction conditions were phosphate buffer 60 mM, 18 U of biocatalyst, 15% (v/v) of 2M2P as co-solvent, hydrogen peroxide (0.2 μmol min^{-1}), 293 K, 400 rpm and 5 min of reaction.

Table 3
Biocatalytic characterization of obtained biocatalysts using mesoporous materials.

Biocatalyst	Load ($nmol_{CPO}\ g^{-1}{}_{SUP}$)	Specific activity[a] ($nmol\ min^{-1}\ mg^{-1}{}_{SUP}$)	I_Y (%)	I_E (%)
CPO/MCF	3184	1301	9	9
CPO/MSU-F	6807	6406	19	18
CPO/TiO_2	1781	260	5	3

[a] Calculated as peroxidase activity. Units reported for specific activity are equivalents to $U\,g^{-1}{}_{SUP}$.

diameter of these materials, allowing the enzyme diffusion into pores [53,54]. Furthermore, enzyme has an IEP close to 4.0 [55], which is different to those of materials, and thus allows the enzyme immobilization by electrostatic interaction by just adjusting the medium pH to an intermediate value between enzyme and supports IEP. According to the adsorption/desorption isotherms of N_2 at 77 K (Fig. S1, supplementary material) and considering the IUPAC isotherms classifications [56], both MCF as MSU-F showed a type IV adsorption isotherms with H1 type hysteresis loops in a range of relative pressures of 0.5–1.0 and 0.8–1.0, respectively, indicating that they show compacted agglomerates of spherical pores with uniform diameters, being a typical behavior of mesocellular foam materials [25,57]. Both materials showed a wide unimodal distribution of pore diameters with maximum values around 12 and 30 nm, respectively. Unlike the MCF and MSU-F mesoporous silicates, the TiO_2 has a bimodal pore diameter distribution with an average value of 8.3 nm, with a narrow band that presents a maximum value at 4.8 nm, and another band (from 8 to 40 nm) with a maximum at 12.4 nm (Fig. S1, supplementary material). The adsorption isotherm for this material is type IV with a type H3 hysteresis loop, which corresponds to a disordered mesoporous system, with funnel shape pores, having pore diameters greater than 8 nm at one end and a lower value at the other end. Some effect on the specific activity of the immobilized CPO could be due to the particular shape of pores of this material as well as its lower total pore volume and specific area compared with the other supports.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.02.014.

Chloroperoxidase was immobilized by electrostatic interaction onto the three mesoporous materials, utilizing the difference in isoelectric points (IEP) between them and the enzyme. For CPO immobilization in silica-based materials the selected pH was 3, since the IEP of CPO is ~4.0, thus the overall net charge of the protein is positive and the silica-based materials are negatively charged at this pH (IEP of MSU-F and MCF is ~2.0). On the other hand, for TiO_2 the immobilization was performed at pH 5.0 in which the overall net charge of the enzyme is negative, while the TiO_2 is slightly cationic (IEP is ~5.8). The biocatalyst preparations were then assayed for activity and the specific activities are summarized in Table 3. The highest enzyme load was obtained with MSU-F support while the lowest was obtained with TiO_2. In the case of the mesoporous silicates no relationship was observed between the CPO load and some of their textural properties such as surface area and total pore volume. However, it seems that the enzyme load increases with a corresponding increase in the pore diameter between MCF and MSU-F. This fact could be attributed to the formation of CPO aggregates into the pores. According to Jung et al. [51], enzymes are naturally added in the cavities of siliceous mesocellular foams by the adsorption process and then the aggregates grow in a controlled manner according to the size of the cavities. On the other hand, the low enzyme load in TiO_2 could be explained because this material showed the lowest surface area, the lowest total pore volume and the lowest pore diameter of the three materials (Table 2). Furthermore, the TiO_2 showed a bimodal distribution of pore diameters, with a maximum at 4.8 nm. Considering that this diameter is smaller than the average dimension of the CPO (6.0 nm), and that the pores are funnel shaped, open at both ends, diffusion of the enzyme would be restricted only to entries that have diameters greater than 6.0 nm, without the possibility of aggregate formation inside due to the particular shape of the pores.

As expected, the specific activity of the preparations is correlated with the enzyme load (Table 3). However, the enzymatic activity is not only related with the amount of enzyme immobilized, also other factors may influencing the specific activity of a biocatalyst, such as an adequate orientation of the enzyme active site, the aggregation formation and mass transfer limitations, among others [54,58]. The immobilization yield (I_Y) indicates the percentage of immobilized enzyme, while the immobilization efficiency parameter (I_E) shows the fraction of immobilized enzyme that is active. As shown in Table 3, the I_E value is very small in all preparations because only a fraction of the immobilized CPO is accessible to the substrate, namely adequate orientation of active site. It is important to point out that there is a direct correlation between the I_E parameter and the pore diameter of materials, indicating that larger pore diameter greater is the fraction of enzyme exposed and accessible to the substrate. This statement would apply mainly to silicates type mesocellular foams, since their three-dimensional pores network allows easy substrate access inside of porous cavities [51]. The specific activity of the immobilized CPO obtained in this work is comparable or even higher than the activity reported in previous studies of CPO immobilized on mesoporous materials. Aburto et al. [54] obtained a maximum specific activity of 313 $nmol\ min^{-1}\ mg^{-1}{}_{SUP}$ in immobilized CPO on silicates type SBA-16, both by physical adsorption and covalent binding. On the other hand, Jung and co-workers [51] immobilized CPO on MCF, both by physisorption and by forming crosslinked enzyme aggregates (CLEA) inside the pores of materials, obtaining a specific activities of 1848 $nmol\ min^{-1}\ mg^{-1}{}_{SUP}$ ($U\,g^{-1}{}_{SUP}$) and 9073 $nmol\ min^{-1}\ mg^{-1}{}_{SUP}$ for CPO/MCF and CPO-CLEA/MCF, respectively.

The catalytic performance of the different biocatalysts was evaluated for styrene oxidation at two pH values, 3.0 and 6.0 (Fig. 3). These two pH were selected because previous studies reported an acidic pH optimum ($pH \approx 3.0$) for chloride-assisted peroxidation reactions and another pH optimum ($pH \approx 6$) for peroxidations carried out in the absence of chloride ion [59]. The specific activity was determined monitoring epoxides and diols under both assayed pH conditions. The produced epoxides may be partially (pH 6) or completely (pH 3) hydrolyzed to the corresponding diols. In most of cases, the kinetic data fitted a sigmoidal behavior, thus the Hill equation was used to determine the catalytic constants (Table 4). By far MCF showed the highest catalytic activity (k_{cat}) in both pH values, representing from 2.4 to 3.5 times these obtained with free enzyme. On the other hand, TiO_2 showed the poorest performance. In all preparations the catalytic activity (k_{cat}) at pH 6 was slightly higher that those found at pH 3. It is well known, that CPO is more active as halogenase at low pH, but the peroxidase activity is favored at higher pH [59]. Differences in catalytic constants from the different CPO preparations could be related with a defined orientation of the enzyme molecule that facilitates or hinders the substrate access to the CPO active-site. The enzyme orientation in the CPO/MSU-F and CPO/MCF biocatalysts seems to facilitate the styrene access to the active site. The immobilized enzyme onto mesoporous silicates should be differently oriented compared to the immobilized on TiO_2 due to the differences in net charge between enzyme and the support. In silicates, the enzyme is adsorbed to the support on the enzyme side where positive charges are more abundant, while the CPO on TiO_2 is mainly adsorbed on the enzyme side with a higher concentration of negative charges.

The substrate cooperativity to the enzyme (n) and the "substrate affinity" (K') are also shown in Table 4. In the cases where n was

Table 4
Catalytic parameters obtained for CPO biocatalysts from Hill's equation for two different pH values.

pH	Biocatalyst	k_{cat} (min^{-1})	K' ($mmol^n\ L^{-n}$)	n	Model adjustment (R^2)
3.0	Free CPO	99 ± 6	0.11 ± 0.04	1.7 ± 0.3	0.972
	CPO/TiO_2	50 ± 6	2.2 ± 0.1	2.5 ± 0.4	0.991
	CPO/MSU-F	646 ± 120	1.0 ± 0.6	1.5 ± 0.2	0.987
	CPO/MCF	2395 ± 240	0.8 ± 0.2	1.0 ± 0.3	0.966
6.0	Free CPO	90 ± 6	0.7 ± 0.2	1.1 ± 0.1	0.999
	CPO/TiO_2	87 ± 6	1.4 ± 0.1	1.7 ± 0.1	0.999
	CPO/MSU-F	590 ± 18	1.3 ± 0.1	2.0 ± 0.2	0.994
	CPO/MCF	3146 ± 180	4.3 ± 0.1	2.9 ± 0.7	0.982

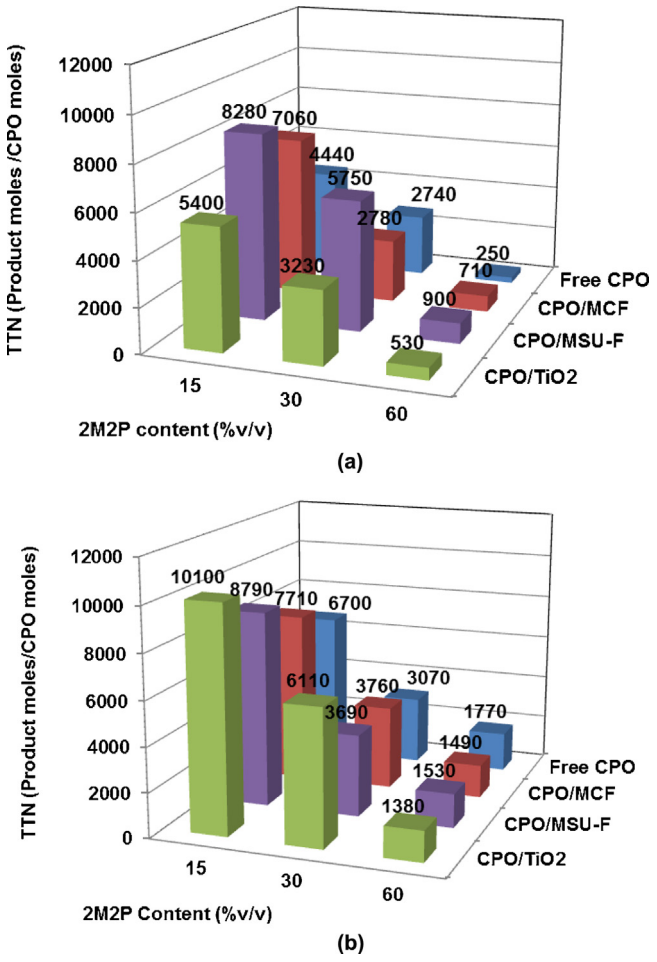

Fig. 4. Effect of 2M2P content as co-solvent on the operational stability of CPO in terms of TTN for the different biocatalysts in epoxidation reaction of styrene at (a) pH 3.0 and (b) pH 6.0.

equal to 1.0, the Hill equation becomes the rectangular hyperbolic equation and therefore K' equals to the Michaelis–Menten constant (K_m) corresponding to the substrate concentration that yields half-maximal velocity. This occurred only in two specific cases: the free CPO to pH 6 and the biocatalyst CPO/MCF to pH 3. Protein immobilization significantly increased de value of K' when compared with the free enzyme, suggesting mass transfer limitations for the substrate to reach the enzyme active site when the enzyme is immobilized.

The operational stability is the bottleneck for the industrial use of CPO. The total turnover number (TTN) of free and immobilized preparations for the styrene oxidation at pH 3.0 and 6.0 was determined using 2M2P as co-solvent (Fig. 4). In general, immobilized CPO showed higher TTN when compared to the free enzyme at both pH values. Surprisingly, at pH 6.0 the maximal TTN was obtained with CPO/TiO_2 biocatalyst (TTN = 10,100) representing an increase of operational stability of 51% with respect to the free enzyme. At pH 3.0 the best biocatalyst was CPO/MSU-F (TTN = 8280) with a TTN increase of 86% in relation to the free CPO. The interaction between the support and the protein surface could stabilizes the three dimensional structure of the enzyme, resulting in greater operational stability [60]. The values of TTN are slightly lower at pH 6 than at pH 3 when the solvent concentration is low, but a high organic solvent concentration the TTN values at pH 3 are higher that at pH 6. Interestingly, CPO/TiO_2 biocatalyst showed the highest operational stability when the TTN experiment was carried out at pH 6.0 in 15% 2M2P. On the opposite, in the experiments performed at pH 3.0 this preparation showed an operational stability closes to these from the free enzyme, and lower than the CPO/MCF and CPO/MSU-F. It is important to point out that CPO was immobilized on TiO_2 at pH 5, while on mesopourous silica at pH 3, thus, at pH 6 the electrostatic interactions on silica are disrupted, whereas in TiO_2 remain similar to those from immobilization.

The operational stability of CPO, measured as the total number of substrate molecules transformed by one molecule of enzyme in its lifetime or TTN, is the results of different factors, including the activity rate and the intrinsic stability of the enzyme. Peroxidase transformation mechanism is mediated by the production of free radicals and these produced free radicals are also the origin of its self-oxidative inactivation. As faster free radicals are produced faster the peroxidase inactivation occurs [10,61]. It is well known that CPO is more active at pH 3 than at pH 6, and thus the enzyme inactivation by hydrogen peroxide would be minimized at pH 6.0 [62]. This is consistent with the higher TTN at pH 6 (Fig. 4). On the other hand, the support material seems play an important role in the stability of the enzyme preparation. The high TTN values obtained with the CPO immobilized in TiO_2 could be due to many factors: The lower catalytic rate (Table 4) that slowly produces free radicals reducing the self-inactivation and increasing the enzyme lifetime, the pore size, which is comparable with the diameter of the enzyme (assuming that the pores are funnel-shaped) and thus the tight interactions between the support and the enzyme molecules stabilizing its structure [60], considering the immobilization pH. The importance of pore size has been demonstrated with immobilized catalase in MCF where higher thermal and storage stabilities were found when the pore diameters matched the diameter of the molecule [21].

On the other hand, at pH 3.0 the formation of enzyme aggregates inside the cavities of the mesocellular foams [51] could affect both activity and stability. On one hand, these aggregates play a role on the stabilization effect in CPO/MCF and CPO/MSU-F biocatalysts, which may reduce in some extent the inactivating action exerted by hydrogen peroxide on the CPO and lead to slightly higher TTN compared with the CPO/TiO_2 biocatalyst. On the other hand, the formation of enzyme aggregates increase the mass transfer limitations making it more difficult for substrate to access the active site in CPO. The shape of the pores in the TiO_2 does not allow the

Table 5
Catalytic parameters of epoxidation reactions of styrene and some derivatives catalyzed by free CPO, using 15% (v/v) 2M2P as co-solvent and 1250 peroxide/enzyme concentration ratio at the end of the reaction.

Substrate	k_{cat} (min^{-1})	K' ($mmol^n\ L^{-n}$)	k_{cat}/K'	n	Model adjustment (R^2)
Styrene	90 ± 6	0.73 ± 0.19	123	1.1 ± 0.1	0.999
3-Chlorostyrene	25 ± 1	0.39 ± 0.04	64	1.2 ± 0.2	0.980
3-Nitrostyrene	13 ± 2	6.7 ± 1.2	1.9	1.2 ± 0.2	0.994

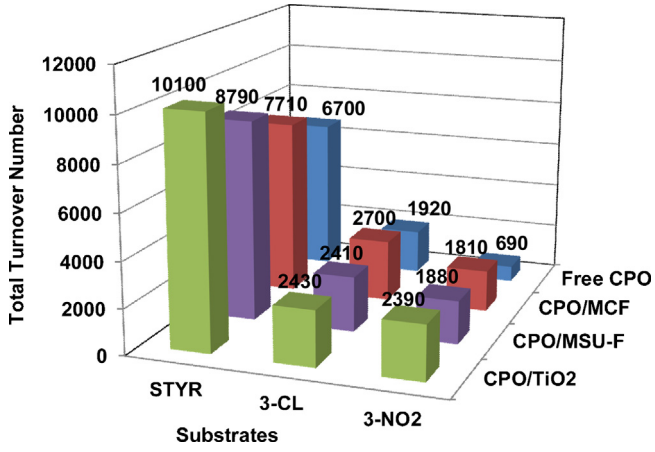

Fig. 5. Total turnover numbers for free and immobilized CPO in the epoxidation reaction of styrene (STYR), 3-chlorostyrene (3-Cl) and 3-nitrostyrene (3-NO_2), using 15% (v/v) 2M2P as co-solvent and 25 nmol s^{-1} H_2O_2 continuous flow, at 293 K.

formation of these aggregates leaving the entire enzyme exposed to the action of hydrogen peroxide. This fact could explain the drastic decrease in TTN values, mainly with CPO/TiO_2 system, going from pH 6.0 to pH 3.0 for the different co-solvents contents tested.

This behavior is directly related to the enzyme specificity toward each substrate (k_{cat}/K'), which follows the same trend observed for the TTN. These catalytic parameters were obtained for each of the substrates from the curves of specific activity of the free CPO in function of substrate concentration (Table 5). In this case, it was found that n value is close to 1 in the Hill equation and hence K' will be equal to K_m. The greater specificity of the CPO by styrene was established due to their high k_{cat} (90 min^{-1}) and low K' (0.73 mmol L^{-1}), while the low specificity for the 3-nitrostyrene was determined largely to high value of K' (6.7 mmol L^{-1}).

In the case of styrene, the highest TTN values were obtained at pH 6.0 and by using 15% (v/v) of 2M2P as co-solvent. Thus, the TTN under these conditions for two styrene derivatives; 3-chlorostyrene and 3-nitrostyrene, were determined (Fig. 5). The obtained TTN values follows the trend: styrene > 3-chlorostyrene > 3-nitrostyrene, for both free- and immobilized-CPO. Moreover, for all three substrates the TTN values are higher for the immobilized CPO than for free enzyme. The maximum TTN for 3-chlorostyrene was obtained with the CPO/MCF biocatalyst (TTN = 2700), achieving an increase of 41% compared to the free enzyme, whereas for 3-nitrostyrene, best biocatalyst was the CPO/TiO_2 (TTN = 2390) providing an increase of 246% relative to the free CPO. This behavior could be attributed to the stability that offers the supports to the enzyme, as above mentioned. Another interesting situation occurs with the styrene derivatives. The free enzyme showed a TTN of 1920 with 3-chlorostyrene and 690 with 3-nitrostyrene. The difference between their TTN is related to the greater specificity that presents the CPO for 3-chlorostyrene ($k_{cat}/K' = 64$) in comparison with 3-nitrostyrene ($k_{cat}/K' = 1.9$). Even more, the TTN values for each of the immobilized preparations with these two substrates, showed the same trend (Fig. 5). This could indicate that interactions between support and enzyme generate slight changes in the protein conformation. According the crystal structure of chloroperoxidase [63], the heme edge is not available for substrate interaction. There is a small opening above the heme which could allow access to the Fe^{4+}=O center of compound I. Thus, the active site (heme) in CPO is located deeply inside the protein molecule and the immobilization operation can only alter the conformation of the CPO surface. However, the interaction between the support and enzyme surface could modify affinity of the enzyme toward these two substrates and thereby change the catalytic activity of the immobilized CPO [64].

4. Conclusions

The CPO immobilization onto mesoporous silica and TiO_2 increased the enzyme operational stability up to 246% in the case 3-nitrostyrene biotransformation. This is the first report of CPO immobilization onto TiO_2 and MSU-F indicating that these materials are potential supports for CPO immobilization in view of a large-scale application. All immobilized preparations were successfully used in the epoxidation reaction of styrene, 3-chlorostyrene and 3-nitrostyrene.

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