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Lipases encapsulation onto ZIF-8. A comparison between biocatalysts obtained at low and high zinc:2-methylimidazole molar ratio in aqueous medium

Federica Pitzalis[†][a],Cristina Carucci[†][b], Maryam Naseri[a,c], Lida Fotouhi[c], Edmond Magner*[b], Andrea Salis*[a]

Abstract: Lipase AK from *Pseudomonas fluorescens*, and Lipase RM from *Rhizomucor miehei* were encapsulated into a zeolite imidazolate framework (ZIF-8) via "one-pot" synthesis to obtain AK@ZIF-8 and RM@ZIF-8 biocatalysts. The effect of a high (1:40) and low (1:4) Zn:2-methylimidazole molar ratio on the biocatalysts synthesis was investigated. The different Zn:L ratios affected both the surface area, the loading and the specific activity of the biocatalysts. Samples synthesised using high Zn:L ratio had high values of surface area whereas those obtained using low Zn:L ratio had higher loadings and specific activities. The decrease of pH (from 11.6 to 9.4) during the synthesis at high Zn:L ratio produced ZIF-8 samples with features similar to those observed for low Zn:L ratio samples. The low Zn:L (1:4) ratio AK@ZIF-8 biocatalyst retained 99% activity after storage for 15 days at 5°C and 40% activity after 5 reaction cycles.

1. Introduction

Enzymes are generally active under mild conditions, i.e. room temperature and atmospheric pressure,[1] conditions that enable the development of environmentally friendly 'green' industrial processes. However, in contrast to conventional chemical catalysts, enzymes are expensive and unstable in e.g. non aqueous solutions and at extremes of pH. Immobilisation can overcome these disadvantages.[2] When an enzyme is immobilised on a solid support it generally retains its catalytic activity, enhances its stability, and can be reused for several reaction cycles.[3, 4] In the past 15 years ordered mesoporous silicates have widely been used for enzyme immobilisation due to their textural and structural features.[5–9] More recently, metal organic frameworks (MOFs) have emerged as a possible new family of enzyme supports. MOFs, first synthesised by Yaghi et al.[10] consist of metal ions coordinated with organic ligands to form a porous 3D network. Zeolite imidazolate frameworks (ZIFs) are an important MOF subclass containing a zeolite-like crystalline structure where Zn^{2+} (or Co^{2+}) ions are coordinated by imidazole derivatives forming a tetrahedral network.[11–15] Systematic change of the organic ligand or the metal ion results in a variety of 3D structures, similar to those of natural zeolites.[16] ZIFs can be prepared in several ways, i.e. by solvothermal,[15–17] mechano-chemical,[18] sonochemical,[19] microwave-assisted,[20] or hydrothermal[12] synthetic methods. Among these, water synthesis is facile, rapid, and low cost.[12] Moreover, this is a very suitable way to obtain enzyme@ZIF conjugates.[21–23] ZIF-8 is a metal organic framework where Zn^{2+} ions are coordinated by 2-methylimidazole (2-MIM) to form rhombic dodecahedral structures.[15] These structures are often referred to as "SOD-like" due to their similarity to those of the natural zeolite "sodalite". Although water synthesis is predominantly used for the immobilisation of single enzymes[21–24] or to obtain bienzymatic systems,[25] some critical aspects of the mechanism of formation remain unknown. The mechanism of crystallization,[20] porosity,[12] and the surface area[26] of pure ZIFs prepared in aqueous media are strongly influenced by the molar ratio between Zn^{2+} ions and the alkyl-imidazole ligand, as well as by the water content.[26] Moreover, the addition of the enzyme to the reaction mixture during crystal nucleation can affect the crystal shape and morphology[22,24] and, consequently, the activity of the obtained immobilised biocatalyst.[22,24]

Lipases (E.C. 3.1.1.3 triacylglycerol acylhydrolases) are one of the most widely used enzymes for biotechnological applications,[27–29] such as the production of biodiesel,[28,30,31] as well as in the food,[32] and pharmaceutical[33,34] industries. To the best of our knowledge, only a limited number of works investigating the catalytic performance of lipases immobilised on MOFs in general[34–36] and on ZIFs in particular, have been reported. Very recently, Gascòn et al. immobilised lipase B from *Candida antarctica* on Basolite F-300 like materials.[37] Cao et al. measured the catalytic activity of *Bacillus subtilis* lipase immobilised on Cu-BTC MOF.[35] He et al. immobilised a thermophilic lipase from *Alcaligenes sp*. on ZIF-8, through a mechanochemical synthetic method.[38] Cheong et al. immobilised *Burkholderia cepacia* lipase in amino-functionalised ZIF-8 through physical adsorption, and measured both the hydrolytic and transesterification activity.[39] Shi et al. immobilised the lipase from *Candida rugosa* on ZIF-8 synthesised via a solvothermal method using methanol as the solvent.[40] Liang et al. immobilised several enzymes on ZIF-8, including a lipase (origin not specified).[24] Lyu et al. reported the in-situ immobilisation of lipase from *Thermomyces lanuginosus* on ZIF-8, using methanol as solvent and polyvinylpyrrolidone as a protective coating for the enzymes.[41] Finally, Huo et al. prepared a hierarchical magnetic ZIF-8/UiO-66/Fe_3O_4 support for the encapsulation of *Candida antarctica* lipase B.[42]

In this work the 'one-pot' water synthesis of ZIF-8 and the encapsulation of lipases from *Pseudomonas fluorescens* (lipase

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AK) and *Rhizomucor miehei* (lipase RM), to obtain AK@ZIF-8 and RM@ZIF-8 biocatalysts, is reported. These lipases are well characterized.[43–46] Lipase RM has a molecular mass of 32kDa, with an isoelectric point, pI, of 3.8.[47] Lipase AK has a molecular mass of 33kDa and a pI = 4.[48] These lipases are very promising enzymes due to their good activity toward triglyceride transesterification for biodiesel production.[47,49–51] While they have mainly been immobilized on polymers of silica supports, to the best of our knowledge, no data is available about the immobilization of AK and RM lipases on ZIF-8.Here, two stoichiometric ratios of Zn^{2+} and the 2-MIM ligand (L) i.e. Zn:L=1:40 [25] and Zn:L=1:4 [24] were used in previous reports. The Zn:L ratio affects both the texture of ZIF-8 and the activity of the obtained lipase@ZIF-8 biocatalyst. A similar effect on ZIF-8 structure and catalytic activity was obtained by changing the pH at which the biocatalysts were prepared. Finally, the storage stability and the ability to recycle of the most active lipase@ZIF-8 biocatalysts were examined.

2. Results and Discussion

2.1 Characterisation of ZIF-8 and lipase@ZIF-8 samples

ZIF-8 and lipase@ZIF-8 samples were synthesised by using two different Zn^{2+}:2-MIM (Zn:L) molar ratios. Samples named ZIF-8_{HR} and lipase@ZIF-8_{HR} were obtained by using a high Zn:L (1:40)[25] molar ratio, whereas samples ZIF-8_{LR} and lipase@ZIF-8_{LR} were obtained by using a low Zn:L (1:4)[24] molar ratio. All samples were characterised using a range of techniques i.e. X-ray diffraction (XRD), porosimetry, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR) and thermogravimetric analysis (TGA).

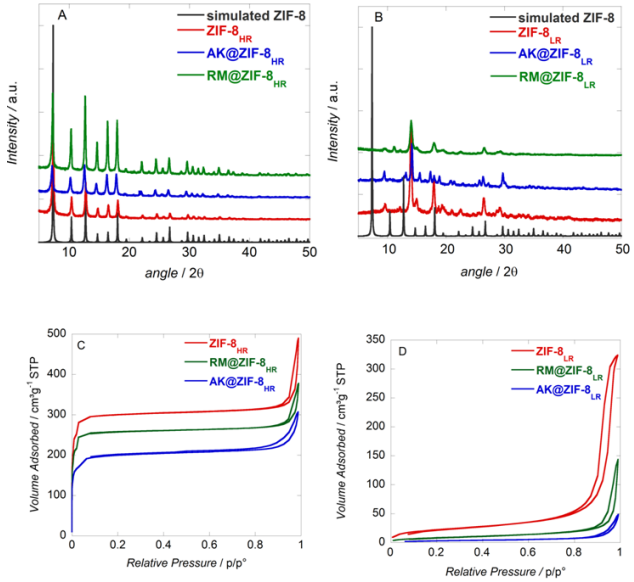

Figure 1. XRD patterns of ZIF-8 and lipase@ZIF-8 prepared using a Zn:L ratio of A) 1:40 and B) 1:4. N_2 adsorption and desorption isotherms of ZIF-8, AK@ZIF-8 and RM@ZIF-8 prepared using a Zn:L ratio of C) 1:40 and D) 1:4.

The XRD patterns of ZIF-8_{HR} and lipase@ZIF-8_{HR} (Figure 1A) are in good agreement with those of a ZIF-8 simulated pattern reported in the literature.[52,53] The diffractograms (Figure 1B) of ZIF-8_{LR} and lipase@ZIF-8_{LR} samples have different patterns. XRD patterns for ZIF-8 materials synthesised with various 2-MIM concentrations were previously observed in a variety of studies. Kida et al. observed the occurrence of a crystalline ZIF-8 SOD-like structure with high molar Zn:L ratios (1:40; 1:60; 1:80; 1:100), while, at lower ratios (Zn:L ratio 1:20) they observed the occurrence of mixed phases associated with the formation of $Zn(OH)_2$, $Zn(OH)NO_3$, and $Zn_5(OH)_8(NO_3)_2(H_2O)_2$.[26] Shi et al. synthesised a ZIF-8 sample (Zn:L ratio of 1:4) that had an XRD pattern which was assigned to an unknown mixed phase[54] while Liang et al. obtained a ZIF-8 sample with a typical SOD-like crystalline structure at a Zn:L ratio of 1:4.[24] The structure of ZIF-8 is affected by the presence of encapsulated protein. At a Zn:L ratio of 1:4 different–morphologies were observed for a wide range of proteins (i.e. nanoflowers for horseradish peroxidase, nanostars for trypsin etc.).[24] Similarly, Cui et al. observed a variety of XRD patterns and crystal morphologies for catalase@ZIF-8 samples obtained with Zn:L ratios in the range 1:1 - 1:20.[22] The concentration of protein concentration also affects the structure of ZIF-8. The peak intensity decreased with increasing concentration of BSA.[24] The XRD patterns of AK@ZIF-8 and RM@ZIF-8 samples showed different peak intensities (Figure 1A-B) suggesting an interaction between the enzyme molecules and the ZIF-8 material.[37,55] These findings demonstrate that the formation of pure ZIF-8 and lipase@ZIF-8 structures in aqueous solution is a complex process.

Table 1. Textural parameters obtained by the N_2 physisorption analysis of ZIF-8 and lipase@ZIF-8 samples

Sample	Zn:L Ratio	S_{BET} (m^2g^{-1})	Pore volume (cm^3g^{-1})
ZIF-8_{HR}	1:40	919	n.a.
AK@ZIF-8_{HR}	1:40	615	39
RM@ZIF-8_{HR}	1:40	790	n.a.
ZIF-8_{LR}	1:4	79	0.50
AK@ZIF-8_{LR}	1:4	4.4	0.049
RM@ZIF-8_{LR}	1:4	30	0.22

Figures 1C-D show the textural characterisation of ZIF-8 and lipase@ZIF-8 samples performed by nitrogen physisorption. A ZIF-8_{HR} sample displayed a typical type I isotherm associated with a microporous material (Figure 1 C) with a high surface area of 919 m^2/g (Table 1).The small hysteresis present at $p/p_0 > 0.8$ may be due to the presence of mesopores in ZIF-8_{HR} as well as in the lipase@ZIF-8_{HR} samples. This was previously observed for other enzyme@ZIF-8.[22] The decrease in surface area for lipase@ZIF-8 (Table 1) is likely due to enzyme encapsulation into the ZIF-8 structure.[22,24,37,38] The N_2 adsorption/desorption isotherms obtained for the samples synthesised with the low Zn:L (1:4) ratio (Figure 1D), are significantly different. Low ratios of Zn:2-MIM ligand result in low surface areas (Table 1). This is consistent with the work of Kida et al. who investigated the textural parameters of ZIF-8 samples at different Zn:L ratios over the range 1:100 - 1:20.[26] They observed surface areas of over 1500 m^2/g for SOD-type ZIF-8 at

high Zn:L ratios (from 1:40 to 1:100) and lower values of surface areas (from 1300 to 120 m^2/g) for the molar ratio 1:20.[26] Scanning electron microscopy (SEM) images of ZIF-8, AK@ZIF-8, and RM@ZIF-8 synthesised at a Zn:L=1:40 ratio and the corresponding samples synthesised at a Zn:L=1:4 ratio are shown in Figures S1A-C and Figure S1D-F (see Supporting Information file), respectively. Changes in particle size and morphology were observed in the presence and absence of enzyme. Figure 2 displays the FTIR spectra of the pure ZIF-8 and the lipase@ZIF-8 samples obtained at high (Figure 2A) and low (Figure 2B) Zn:L ratio. For ZIF-8, the C=N stretching mode shows a band at 1584 cm^{-1}. The band at 1420 cm^{-1} is associated with a stretching mode of imidazole ring.[14] A strong band at 760 cm^{-1} is due to out of plane bending of the imidazole aromatic ring, whereas the in-plane bending of the ring produces bands in the range 900 cm^{-1} - 1350 cm^{-1}.[14] Finally, the characteristic band at 421 cm^{-1} is associated with the Zn-N stretching mode.[14,56] For lipase@ZIF-8 samples the bands in the range 1644-1648 cm^{-1}, due to the C=O stretching of the amide I, confirm the successful encapsulation of lipase molecules in the ZIF-8.

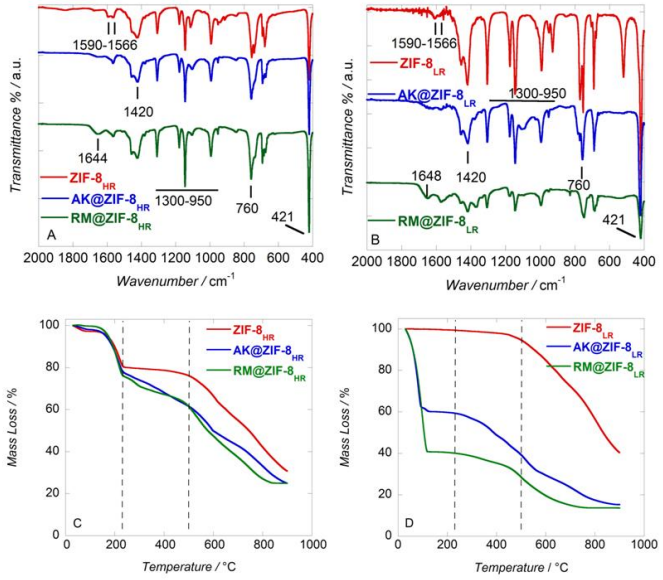

Figure 2. FTIR spectra of ZIF-8 and lipase@ZIF-8, A) high Zn:L (1:40) ratio; B) low Zn:L (1:4) ratio. TGA curves of ZIF-8 and lipase@ZIF-8 prepared by using the C) high Zn:L ratio, and D) low Zn:L ratio.

Thermogravimetric analysis (TGA) data show (Figure 2C-D) that ZIF-8_{HR} and lipase@ZIF-8_{HR} samples are stable up to 140°C. Both materials displayed a similar mass loss of ca. 20 % (see Table 2) in the temperature range140-230°C (Figure 2C), associated with the removal of water and unreacted reagents.[12] At higher temperatures, in the range 230°C - 500°C, a small mass loss was observed for pure ZIF-8_{HR} (5.1 %), while lipase@ZIF-8_{HR} biocomposites showed a gradual and continuous decomposition, with Δm of 17.0 % and 15.3 % for AK@ZIF-8_{HR} and RM@ZIF-8_{HR}, respectively (Table 2). This mass loss is probably due to the decomposition of enzymes encapsulated in the ZIF-8 network.[25,38] At temperature greater than 500°C the mass loss is likely due to the decomposition of the ZIF-8 structure.[12] In the case of the samples synthesised at a low (1:4) ratio of Zn:L, the curves in Figure 2D follow different trends. ZIF-8_{LR} sample has a small mass loss 4.7 % up to 500°C, similar to that of ZIF-8_{HR}. The steep mass loss above 500 °C is likely due to the thermal decomposition of the structure (Table 2). For the lipase@ZIF-8_{LR} samples the initial mass loss (T < 130°C) is associated with the loss of water. Between 230-500°C, the decomposition of the enzymes occurs with mass losses of 7.3% and 11.4% for AK@ZIF-8_{LR} and RM@ZIF-8_{LR}, respectively. The diversity in shape in Figures 2C and 2D are due to the different synthesis conditions.[22,26,37,57,58]

Table 2. Mass loss (Δm) of ZIF-8 and lipase@ZIF-8 samples at high (HR=1:40) and low (LR=1:4) Zn:L ratios.

Sample	Δm (%) T<230°C	Δm (%) 230°C <T<500°C	Δm (%) T>500°C
ZIF-8_{HR}	18.8	5.1	76.1
AK@ZIF-8_{HR}	21.5	17.0	61.6
RM@ZIF-8_{HR}	23.3	15.3	36.6
ZIF-8_{LR}	0.7	4.7	54.4
AK@ZIF-8_{LR}	29.3	7.3	63.4
RM@ZIF-8_{LR}	60.0	11.4	15.0

2.2 Loading and activity of AK@ZIF-8 and RM@ZIF-8 biocatalysts

Protein loading (Figure 3 A,B) and enzyme activity (Figure 3 C,D) of the lipase@ZIF-8 samples were quantified by the Bradford[59] and p-nitrophenyl butyrate assays,[60,61] respectively. Lipase@ZIF-8 samples obtained at a Zn:L ratio 1:4 had higher protein loadings than those obtained at a ratio of 1:40. The loadings were 138±13 mg/g (AK@ZIF-8_{LR}), 77±12 mg/g (RM@ZIF-8_{LR}), 40±10 mg/g (AK@ZIF-8_{HR}), and 12±6 mg/g (RM@ZIF-8_{HR}).

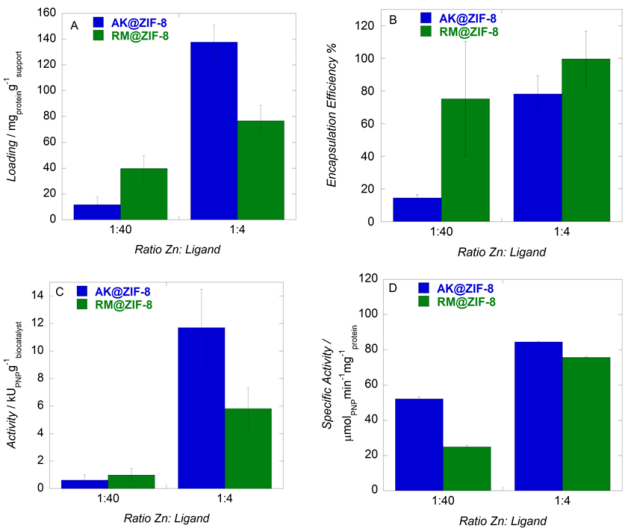

Figure 3. Comparison A) enzyme loading, B) encapsulation efficiency, C) enzymatic activity, and D) specific activity (PNP= p-nitrophenol) of lipase ZIF-8 prepared at Zn:L ratios of 1:40 and 1:4.

Moreover, the samples obtained using a ratio of 1:4 resulted in a very high encapsulation efficiency (EE%, the amount of immobilised protein relative to the total protein amount in the immobilising solution) (Figure 3B). Lipase RM in particular was completely immobilised in the ZIF-8_{LR} material, with an EE% of 99% while, with lipase AK, an EE% of 78% was observed. The encapsulation efficiency for samples obtained by Zn:L=1:40 ratios was 14% and 75% for AK@ZIF-8_{HR} and lipase RM@ZIF-8_{HR} respectively (Figure 3B). The catalytic activities of lipase@ZIF-8 samples are shown in Figures 3C-D. Specific activities ($\mu mol_{PNP}\ min^{-1}\ mg_{protein}^{-1}$ = U/mg) were 84.5±0.3 U/mg (AK@ZIF-8_{LR}), 75.8±0.4 U/mg (RM@ZIF-8_{LR}), 52.2±2 U/mg (AK@ZIF-8_{HR}) and 24.9±0.7 U/mg (RM@ZIF-8_{HR}). Although samples obtained at the low Zn:L (1:4) molar ratio had low surface areas (Table 1), they resulted in higher loadings, encapsulation efficiencies and specific activities than samples synthesised with a high (1:40) ratio. This means that lipases are better adapted to the ZIF-8_{LR} than to ZIF-8_{HR} structures.

2.3 Effect of pH on lipase@ZIF-8_{HR} samples

The two procedures used to synthesise ZIF-8 and lipase@ZIF-8 samples differ in terms of Zn:L ratio and in the pH of the synthesis solution. The pH was 11.6 at a Zn:L ratio of 1:40 and 10.6 for a ratio of 1:4. This pH difference might be responsible of at least part of the observed differences of surface area, loading and catalytic activity between the two types of samples. Thus, samples at a Zn:L=1:40 were prepared, but changing the pH of the synthesis solution (from 11.6 to 10.2, 9.8, 9.4) by adding HCl. The effect of the selected pH on surface area, protein loading, encapsulation efficiency and specific activity of the lipase@ZIF-8_{HR} biocatalysts was then examined. As shown in Figure 4, a change of pH of the synthesis solution (Zn:L=1:40) affects the surface area of lipase@ZIF-8_{HR} samples. Although the isotherms of the samples obtained at different pH values have similar shapes, the amount of adsorbed nitrogen decreases at decreasing pH for AK@ZIF-8_{HR} (Figure 4A) resulting in a decrease in the surface area (Figure 4C). On the contrary, for RM lipase the highest surface area was obtained with samples synthesised at pH 10.2. At pH 9.4 the surface area decreased dramatically for both AK@ZIF-8_{HR} and RM@ZIF-8_{HR}. At this pH the isotherms are similar to those obtained for the samples with Zn:L=1:4 ratio (Figure 1C), prepared at a pH of 10.6. These data clearly show the effect of pH on the surface area of lipase@ZIF-8_{HR} samples. The pK_a of 2-methylimidazole is 7.85.[62] As the pH of the ligand solution is lowered to values closer to the pK_a of 2-MIM, the concentration of the protonated form of the ligand increases. This may affect the formation of the structure of lipase@ZIF-8_{HR} samples. At pH 9 no solid sample could be collected by centrifugation after a synthesis time of 24 h. Moreover, the isotherms obtained at a ratio Zn:L=1:4 (Figure 1D) are similar to those obtained at a ratio of Zn:L=1:40 at pH 9.4 (Figures 4A and 4B) suggesting that a decrease of pH produces an effect similar to the decrease of the ratio of Zn:L. That hypothesis is supported by the data reported in Figure 4C, and in Table S1, where the comparison among surface areas obtained with the two synthesis conditions is shown. At ratio Zn:L=1:40, the highest surface area was achieved at pH 10.2 for AK@ZIF-8_{HR} (618 m^2/g) and RM@ZIF-8_{HR} (923 m^2/g) samples. Conversely, at pH 9.4 the surface areas of AK@ZIF-8_{HR} (33 m^2/g) and RM@ZIF-8_{HR} (43 m^2/g) were similar to those of the corresponding biocatalysts obtained at Zn:L=1:4 (4.4 m^2/g and 30 m^2/g). These data show that, in addition to pH, the type of enzyme influences the properties of lipase@ZIF-8.

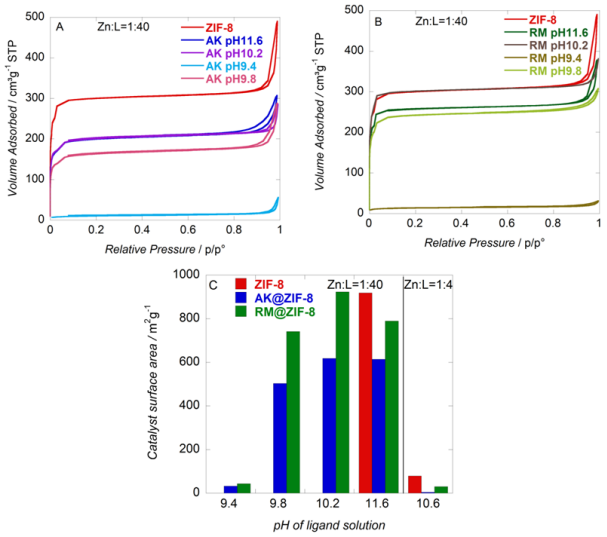

Figure 4. N_2 ads-desorption isotherms and comparison among surface areas obtained at different pH conditions of synthesis. A) AK@ZIF-8 and B) RM@ZIF-8, obtained by means of a Zn:L=1:40 controlling the pH during the synthesis. and; C) influence of pH on the surface area of all lipase@ZIF-8 samples at all synthesis conditions tested.

The effect of the synthesis pH on protein loading and catalytic activity was then investigated. Figure 5A shows that protein loading increases by decreasing the pH of the synthesis solution. This applies to both lipases, although the loading is always higher for lipase RM than for lipase AK. At pH 9.4 a loading of 102±10 mg/g and 55±6 mg/g was obtained for RM@ZIF-8_{HR} and AK@ZIF-8_{HR} samples, respectively. It should be noticed that the highest protein loading obtained at pH 9.4 is lower than that obtained with a Zn:L ratio of 1:4 (Figure 3A). Similarly, the highest encapsulation efficiency was reached for the samples obtained by the Zn:L=1:40 ratio at pH 9.4. EE% was 71% for RM@ZIF-8_{HR} and only 34% for AK@ZIF-8_{HR}. These values were, however, lower than those obtained for Zn:L= 1:4 ratio (Figure S3B).

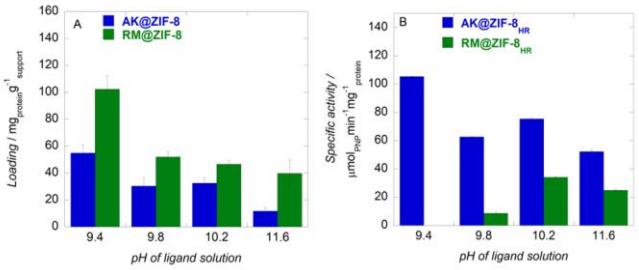

Figure 5. Lipase@ZIF-8 biocatalyst. A comparison among the samples obtained with Zn:L=1:40 ratio at different synthesis solution pH. A) Loading and B) catalytic activity.

The specific activities of the immobilised biocatalysts obtained by means of a Zn:L=1:40 ratio at different pH are shown in Figure 5B. AK@ZIF-8_{HR} samples increased their specific activity by decreasing the synthesis pH from 11.6 to pH 9.4. The specific activity measured for RM@ZIF-8_{HR} instead, was always lower than that of AK@ZIF-8_{HR} in the investigated pH range. Moreover the specific activity was about zero for RM@ZIF-8_{HR} samples synthesised at pH 9.4 (Figure 5B). It has been reported that *Mucor miehei* lipases display the maximal activity at pH 8.[47] Thus, we expect RM lipase to be less active in highly alkaline conditions (pH > 9.4) required for ZIF-8 formation. From these results we may argue that pH affects the synthesis of ZIF-8 and the consequent biocatalyst in a manner that cannot be ignored. A final comparison between the effect of pH and that of Zn:L ratio deserves notice. For the synthesis at Zn:L=1:4 ratio - whose initial pH of ligand solution was 10.6 - the biocatalysts are generally more active than those obtained at different pH tested at Zn:L=1:40 ratio (see par. 2.2). This suggests that the concentration of the 2-MIM ligand plays a more important role than pH on affecting the specific activity of the lipase@ZIF-8 biocatalysts.

2.4 Stability of lipase@ZIF-8_{LR} biocatalysts

The stability to store and to reuse an enzyme is a key issue for the development of a suitable immobilised biocatalyst. Free enzymes are generally quite expensive and unstable particularly in terms of long-term storage and harsh reaction conditions such as extremes of temperature or pH. Hence, a high storage stability allows the design of a large-scale biocatalyst production, while recycling allows for a reduction of operational costs. Figure 6 shows the storage stability and the recycling of lipase@ZIF-8_{LR} biocatalysts (Zn:L=1:4 ratio). These samples were chosen because, respect to those synthesised with the Zn:L=1:40 ratio, they reached the highest enzyme loading and specific activity. The biocatalysts were stored at 4°C for 15 days. An activity retention of 88% and 99% was obtained for RM@ZIF-8_{LR} and AK@ZIF-8_{LR}, respectively (Figure 6A). This demonstrates that, the investigated storage conditions do not significantly affect the relative activity of both biocatalysts. He et al. investigated the long-term stability and recycling of the thermophilic lipase from *Alcaligenes sp*. immobilised on ZIF-8.[38]

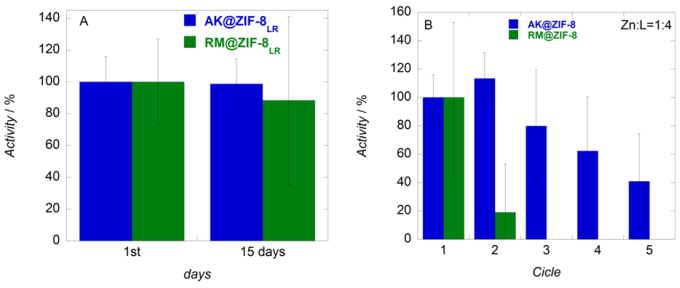

Figure 6. Stability and recycling of lipase ZIF-8_{LR} (Zn:L=1:4 ratio) biocatalyst. A) residual activity of the catalyst after 15 days and B) recycling.

After 5 days storage of the immobilised lipases at two different temperatures, they obtained an activity retention of 20.3% (37°C) and 6.0% (60°C). The operational stability to recycling of the two biocatalysts was then investigated (Figure 6B). RM@ZIF-8_{LR} biocatalyst retained only a 30% of its initial activity in the second reaction cycle, and became fully inactive in the third cycle. Instead, AK@ZIF-8_{LR} biocatalyst displayed a better performance, since it could be reused 5 times with activity retention of 40% at the 5th reaction cycle (Figure 6B). He et al. could recycle their biocatalyst 10 times with 75.7% retention of activity after the 10th reaction cycle.[38] Hence, although our and He et al. experimental conditions were not exactly the same, AK@ZIF-8_{LR} was less stable to recycling but more stable to storage than *Alcaligenes* lipase immobilised on ZIF-8. From these measurements it is difficult to discern the cause of the observed activity loss. Nonetheless, due to the fact that the enzyme is encapsulated within ZIF-8 structure it is more likely that deactivation rather than leaching is the cause of the observed trends.

3. Conclusions

Two synthetic methods differing for the ratio between zinc ion and 2-MIM ligand were compared for the preparation of lipase@ZIF-8 biocatalysts. A high Zn:L (1:40) ratio[25] and a low (1:4) ratio[24] gave lipase@ZIF-8_{HR} and lipase@ZIF-8_{LR} samples, respectively. The structural and textural features of ZIF-8 and lipase@ZIF-8 samples are both affected by Zn:L ratio. ZIF-8_{HR} samples had very high surface areas compared with ZIF-8_{LR} samples (Table 1). However, the latter samples had higher enzyme loadings and higher specific activities (Figure 3). The effect of decreasing the solution pH during the synthesis of lipase@ZIF-8_{HR} samples was then investigated. The obtained samples had lower surface areas (Figure 4) but higher loadings and specific activities (Figure 5), mimicking the behaviour of lipase@ZIF-8_{LR} samples. This observation, to the best of our knowledge, had not already been reported. AK@ZIF-8_{LR} and RM@ZIF-8_{LR} retained high levels of activity after 15 days storage at 4°C. Finally, AK@ZIF-8_{LR} could be recycled 5 times with activity retention of 40% at the 5th reaction cycle. This last biocatalyst might be a good candidate for further studies as, for example, the synthesis of biodiesel.[50]

Experimental Section

4.1 Chemicals

Lipase AK from *Pseudomonas fluorescens* (LAKGO751641) was purchased from Amano Enzymes, Japan. Lipase RM (2000 U g^{-1}) from *Rhizomucor Miehei* fungi was purchased from Sigma. Both lipases were used without any further purification. p-nitrophenyl butyrate, zinc nitrate hexahydrate (98%), 2-methylimidazole (99%), 2-propanol, bovine serum albumin (BSA, 98%), Bradford reagent, sodium phosphate dibasic (ACS Grade, ≥99%), magnesium nitrate (ACS Grade, ≥99%), were purchased

from Sigma-Aldrich. Sodium phosphate monobasic monohydrate (≥99%) was purchased from J.T. Backer.

4.2 Synthesis of ZIF-8 and lipase@ZIF-8 samples with a low Zn:2-MIM ratio (Zn:L=1:4).

For the preparation of ZIF-8,[24] a volume of 50 mL of an aqueous solution of zinc nitrate (40mM corresponding to 0.60 g and 2 mmol of $Zn(NO_3)_2 \times 6H_2O$) was quickly added under stirring to 50 mL of an aqueous solution of 2-methylimidazole (160 mM corresponding to 0.66 g and 8 mmol, pH 10.6). The reaction mixture was aged overnight at 25°C. Then the ZIF-8 sample was collected by centrifugation and washed three times with milliQ water. For the preparation of lipase@ZIF-8 biocatalyst, 0.4 g of lipase AK or 8mL (corresponding to 9.2 g of the commercial liquid preparation) of lipase RM were dissolved in the 2-methylimidaziole aqueous solution (160 mM). The resulting lipase/2-methylimidazole solution (pH=10.2) was quickly mixed under stirring to 50 mL the 40 mM zinc nitrate solution. Then the synthesis of Lipase@ZIF-8 was carried out with the same procedure followed for ZIF-8 sample (Zn:L=1:4).

4.3 Synthesis of ZIF-8 and lipase@ZIF-8 samples with a high Zn:2-MIM ratio (Zn:L=1:40).

For the preparation of ZIF-8,[12,25] 0.60 g of zinc nitrate (2mmol) were dissolved in 4 g of milliQ water and quickly added under stirring to 40 g of an aqueous solution containing 6.63 g (80 mmol) of 2-methylimidazole. The reaction mixture was left under continuous stirring for 10 minutes. Then the solution was aged overnight at 25°C. The ZIF-8 sample was recovered by centrifugation and washed three times with milliQ water. For the preparation of lipase@ZIF-8, 0.4 g of lipase AK or 8mL of lipase RM (corresponding to 9.2 g of the commercial liquid preparation) were dissolved into 40 g of an aqueous solution containing 6.63 g (80 mmol) of 2-methylimidazole aqueous solution. The resulting lipase/2-methylimidazole solution (pH=11.2) was quickly mixed under stirring to 4 g of the zinc nitrate solution. Then the synthesis of Lipase@ZIF-8 was carried out with the same procedure followed for ZIF-8 sample (Zn:L=1:40).The effect of pH on the synthesis of lipase@ZIF-8 samples was also investigated. To this purpose 4 additional lipase@ZIF-8 samples were prepared by decreasing the pH of 2-methylimidazole solution - before the addition of the lipases - from the initial value 11.6 to 10.2, 9.8, 9.4 and 9.0, by means of HCl addition. Then the synthesis proceeded as described above.

4.4 Characterization of ZIF-8 samples

A X'PERT Pro PANalytical diffractometer was used for X-ray diffraction (XRD) experiments with Cu-Kα radiation. The data were collected with a two theta step size of 0.02° from 5 to 50° and an accumulation time 20 s. The experimental XRD patterns were compared (Fig. 1A,B) with simulated patterns obtained using a deposited cif.file[53] and plotted with Mercury software. Thermogravimetric analysis (TGA) was carried out by means of a TGA 4000 Perkin Elmer. The temperature range was from 30 °C to 900 °C, the ramp rate selected was 10 °C per min, under nitrogen flow (flow rate=20 mL min^{-1}). Scanning electron microscopy (SEM) analysis was performed at various magnifications, by a HITACHI SU-70 equipment at 10 kV. Fourier transform infrared (FTIR) analysis was conducted with a Bruker Tensor 27 spectrophotometer, equipped with a diamond-ATR accessory and a DTGS detector. A number of 256 scans at a resolution of 2 cm^{-1} were averaged from wave number 4000 to 400 $cm^{-}1$. An ASAP 2020 (Micromeritics), was used to investigate the textural characteristics of all the samples. The N_2 adsorption/desorption isotherms were recorded at 77 K. Before the analysis, ZIF-8 (lipase@ZIF-8) samples were out-gassed under vacuum for 12 h at 200°C (120°C). The Brunauer-Emmett-Teller[63] (BET), and the Barret-Joyner-Halenda[64] (BJH) methods were used to determine specific surface area, the pore volume and the pore size distribution.

4.5 Determination of enzyme loading and encapsulation efficiency

Protein loading and the encapsulation efficiency of lipase@ZIF-8 samples were calculated by measuring the protein concentration in the initial and final immobilising solutions. The protein concentration was obtained spectrophotometrically (λ= 595 nm) through the Bradford Assay.[59] Encapsulation efficiency (EE%) is the percent ratio between the amount of immobilised protein and the amount of protein in the immobilising solution:

$$EE\% = (1 - [P]_f / [P]_0) \times 100\%$$

Where $[P]_0$ and $[P]_f$ are the initial and the final protein concentrations in the immobilising solution.[65]

Protein loading ($mg_{protein}/g_{ZIF-8}$) is the amount of immobilised protein per g of support:[66,67]

$$loading = \frac{[P]_0 V_0 - [P]_f V_f - [P]_w V_w}{m_{ZIF-8}}$$

Where, $[P]_0$, $[P]_f$ and $[P]_w$are the protein concentrations (mg/mL) in the initial, final and washing solutions; V_0 and V_f (mL) are the initial and final volumes of the immobilising solution; and V_w (mL) is the volume of washing solution; m_{ZIF-8} is the mass (g) of ZIF-8 support.

4.6 Determination of biocatalysts activity and stability

The activity of free and immobilised lipases was measured by means of a Varian Cary 60 UV-VIS spectrophotometer, equipped with an optical fibre probe with the p-nitro phenylbutyrate (PNPB)assay.[60,68,69] The activity of free lipases (AK and RM) was measured by adding 5 µL of the lipase aqueous solution to 2 mL of 0.1 M phosphate buffer pH 7, 200

μL of PNPB (0.02 M in 2-propanol) solution and 200 μL of 2-propanol. The activity of immobilised lipases was measured by adding 5 mg of lipase@ZIF-8 samples to 5 mL of 0.1 M phosphate buffer pH 7, 500 μL of PNPB solution and 500 μL of 2-propanol. The (free and immobilised) lipases catalyse the formation of p-nitrophenol which was quantified at λ=400 nm (molar extinction coefficient of p-nitrophenol, ε=9396.1 $M^{-1}cm^{-1}$) at room temperature. The lipase activity (μmol p-nitrophenol × min^{-1}) was corrected by subtracting the amount of p-nitrophenol formed in the absence of lipases. Storage and recycling stabilities were carried out by means of the PNPB assay, as described above. For storage tests, the wet biocatalyst was stored at 4°C and dried under vacuum the day before the activity measurements. For recycling tests, the used biocatalyst (5 mg) was recovered by centrifugation and washed with 1 mL of 0.1 M phosphate buffer pH 7 before the addition of the fresh substrate solution. All activity and stability measurements were carried out at least in triplicate.

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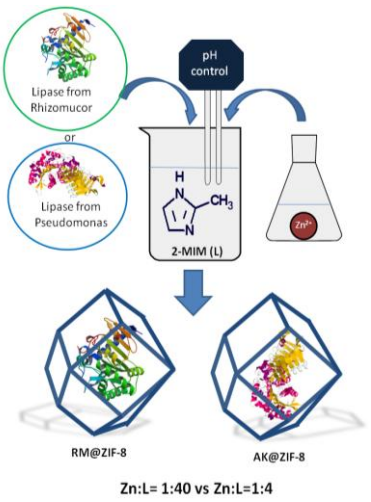
FULL PAPER

Rhizomucor miehei and *Pseudomonas fluorescens* lipases were encapsulated on ZIF-8 support via a “one-pot” aqueous synthesis. The effect of two different Zinc:Ligand molar ratios on enzyme loading and activity was investigated.



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Page No. – Page No.

Lipases encapsulation onto ZIF-8. A comparison between biocatalysts obtained at low and high zinc:2-methylimidazole molar ratio in aqueous medium