# Semi-crystalline Fe-BTC MOF material as an efficient support for enzyme immobilization

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**ABSTRACT**. Metal-organic frameworks (MOFs) have revolutionized the potential applications of nanoporous materials. One of the most recent and promising applications of these materials is their use as supports for enzyme immobilization. In this context, the in-situ (one-step) methodologies, which do not require the use of MOFs with pores larger than the enzyme to be immobilized, seem to be particularly encouraging. This work presents a systematic study of the semi-crystalline Fe-BTC MOF material (commercialized as Basolite F300) employed as support of the enzymes laccase and lipase through either in-situ or post-synthesis methodology. The presence of the enzyme in the resultant solid biocatalysts was proved by CHNS chemical analysis, thermogravimetric analysis, Bradford assays and by SDS-PAGE electrophoresis. The enzymatic activity of the resultant Fe-BTC-based biocatalysts was also tested. The in-situ approach is particularly relevant due to various reasons: (*i*) the enzyme immobilization is given in one step; (*ii*) it is rapid (10 minutes); (*iii*) it is very efficient in terms of encapsulation capacity ($\geq$ 98 % for laccase and $\geq$ 87 % for lipase); (*iv*) the enzymes are fully retained and no leaching is observed after an initial release of only around 10% of the enzyme molecules weakly immobilized; and (*v*) the activity of the retained enzyme can be substantially maintained (97 % with respect to the free enzyme in the case of lipase). Any of these parameters systematically improves these given by the post-synthesis (two-step) approach. Moreover, Fe-BTC widely surpasses the benefits given by other MOF-based supports either by in-situ or post-synthesis approaches.

## 1. Introduction

Metal-organic frameworks (MOFs) are bi– or tridimensional nanoporous materials, generally crystalline, formed by either metal ions or metal clusters linked by multidentate (at least, bidentate) organic ligands [1]. Due to their extraordinary compositional and structural versatility, several thousands of MOF materials have been described since their permanent porosity was made clear [2]. Such emergence of nanoporous materials has entailed the reinforcement of the already known applications as well as their use in some other applications unexplored for other related porous materials [3-6].

One of the most recent applications of MOFs focuses on the immobilization of enzymes [7-13]. Enzymes are proteins with biocatalytic function, which are characterized by an extraordinary selectivity. Since the selectivity is a highly demanded property of a catalyst, enzymes have started to be used in industry [14, 15]. However, their industrial use is limited by their condition of homogeneous catalysts and their low stability and their relatively easy denaturalization. These drawbacks could be overcome by immobilizing them on solid supports, which ideally offers favorable interaction and high contact surface with the enzyme without altering their bioactivity [15-17]. In this sense, mesoporous supports, which are able to immobilize enzymes by encapsulating them within the pores through non-covalent bond, have been successfully used in an academic context [18-22]. In a similar way, the MOF materials that possess pores reaching the ‘meso’ range (diameter larger than 2.0 nm) could encapsulate certain small enzymes [12, 13, 23, 24]. Moreover, the hybrid organic-inorganic character of certain MOFs seems to favor the interaction of these materials with enzymes, since solid active biocatalysts can be formed by just contacting both species, even when the material does not contain enough large pores to host the enzyme [11].

Our group has patented [25] and published [26] a new methodology that enables to obtain enzyme@MOF composites in one step employing in-situ approach. Such method is environmentally sustainable (room temperature and water as unique solvent) [27], and in

principle it should help to preserve enzymatic activity (aqueous solution and moderate pH values). Indeed, it has been applied to different MOF supports, different enzymes and different synthesis media [26]. However, the first in-situ attempts gave rise to biocatalysts with relatively low catalytic activity despite their high enzyme immobilization capacity. Besides, their specific activities (*i.e.* activity per mg of immobilized enzyme) were quite lower than those given by the free enzyme and even lower than their counterpart biocatalyst prepared by post-synthesis strategy in which the enzyme is immobilized on a previous synthesized MOF [26]. The first successful approach to overcome this challenge was the use of the semi-crystalline Fe-BTC MOF material as support for in-situ immobilization of different enzymes [28], which led to solid biocatalysts with relatively high enzyme loadings and high specific activity.

Fe-BTC material is an unconventional MOF. Firstly, it presents a semi-crystalline/semi-amorphous nature. Despite it is not purely crystalline, its powder X-ray diffraction pattern (PXRD) suggests a close structural relationship with the crystalline mesocages-containing Fe-BTC MOF material known as MIL-100(Fe) [29-31]. Indeed, this material contains only one type of the two mesocavities found in MIL-100(Fe) [31, 32]. It has been widely demonstrated by means of several physicochemical characterization techniques that these two Fe-BTC materials are different [29-31]. Additionally, both materials, which are catalytically active in the same types of reactions, gave different order of activity [30, 33]. The semi-crystalline Fe-BTC is normally better catalysts for acid-required reactions, whereas MIL-100(Fe) becomes more active in redox reactions [30, 33]. Their photocatalytic role also resulted to be very different [32]. Secondly, this material is commercially available (its commercial name is Basolite F300). Maybe this fact has significantly contributed to make it one of the most widely studied MOFs as catalysts [30, 31, 32-37], despite its structure is still unknown and its direct preparation has been described only two years ago [31]. Finally, Fe-BTC offers two key advantages when used as potential support for enzymes: (*i*) Fe is a biocompatible and safe metal ion for human beings, so the resultant biocatalysts can be used in any application including food industry, biomedicine,

etc.; (*ii*) this material can be prepared instantaneously by sustainable and enzyme-compatible methods and, unlike other MOFs [27], is free of impurities from the very beginning [28, 31].

This work describes systematic studies regarding the immobilization of two enzymes, lipase and laccase, on Fe-BTC MOF material using both in-situ and post-synthesis methodologies. Its mesocages, similar to those found in MIL-100(Fe), are not large enough to host molecules as large as enzymes. Regardless of the particular enzyme, in-situ procedure resulted more efficient to instantly retain the enzymes and to fully preserve their respective catalytic activities while preventing enzyme leaching.

## 2. Experimental

### *2.1. Synthesis of enzyme-free Fe-BTC MOF material*

Enzyme-free MOF material was prepared according to the method described elsewhere [31]. Fe-BTC material was prepared in water and at room temperature starting from two solutions: The first one was colour–less, had a pH of *ca.* 8.0 and it was formed mixing 0.263 g of the organic linker trimesic acid ($H_3BTC$), 3.68 g of 0.1 M NaOH aqueous solution and 6.32 g of MilliQ water. The second solution was yellowish orange, had a pH value of *ca.* 1.0 and it was composed by 0.508 g of $FeCl_3 \cdot 6H_2O$ and 10 g of MilliQ water. Next, iron solution was added dropwise to the organic linker solution under permanent stirring. Instantaneously, a brownish orange precipitate was formed. After 10 minutes, the mixture was centrifuged, washed three times with MilliQ water and completely dried under a continuous nitrogen flow. The molar ratio of the mixture was 1.5 Fe: 1.0 $H_3BTC$: 3.0 NaOH: 880 $H_2O$. The resulting MOF material was named Fe-BTC-10min.

### *2.2. Post-synthesis immobilization of lipase or laccase on Fe-BTC MOF material*

For both enzymes 100 mg of the MOF material were suspended into 10 mL of an enzyme aqueous solution under slow and permanent agitation. In the case of lipase (Lip), the enzymatic solution was prepared in 50 mM solution of sodium phosphate/phosphoric acid buffer

($Na_2HPO_4 \cdot 2H_2O$ / $H_3PO_4$) at pH 7, whereas for the case of laccase (Lac), 50 mM solution of sodium acetate/acetic acid buffer ($CH_3COONa$ / $CH_3COOH$) at pH 5.0 was used. Next 50 mg of enzyme per g of support was added (Scheme S1). The suspension was kept under mild agitation for 10 minutes, 1 h and 3 h for Lac and for 10 min, 1 h and 4 h for Lip. Aliquots were withdrawn after such times, and the enzymatic activity of the blank, suspension and supernatant (after separating it by centrifugation) were also assayed spectrophotometrically, either by the p-NPA hydrolysis for the Lip or by oxidation of the ABTS substrate for Lac (see enzymatic assays for further details in SI). The end of the immobilization process was established by the detection of a decrease in activity of the supernatant to a minimum and constant value.

The percentage of enzyme immobilized and enzyme loading onto the support was calculated by the difference between the initial (or blank) enzyme concentration and one in the supernatant before and after the immobilization process, always using the Bradford assay described in SI. Afterwards, the suspension was filtered and the solid biocatalysts were washed with the same buffer used for each immobilization. No protein was detected in the washing residues using Bradford assay [38]. The solid samples were then filtered under vacuum and dried under a continuous nitrogen flow, weighted and stored at 4 ºC. In order to determine the activity of the immobilized enzyme, 10 mg of the corresponding biocatalysts were re-suspended in 1 mL of the same buffer used for immobilization. 100 µL of such solution were catalytically analysed following the processes described in enzymatic assays (that is, ABTS oxidation for Lac, and tributyrin hydrolysis for Lip). All measurements were performed at least three times and their averaged value was taken for plotting and discussion. The materials obtained with the post-synthesis procedure were named Lac#Fe-BTC and Lip#Fe-BTC respectively.

*2.3. In-situ immobilisation of lipase or laccase on Fe-BTC MOF material*

The in-situ preparation method of the biocatalysts Lac@Fe-BTC and Lip@Fe-BTC was similar to the above described for enzyme–free Fe-BTC MOF material employing water and room temperature [28, 31]. The only difference was that certain amount of water in the organic

linker solution was substituted by the same volume of enzyme extract solution (Scheme S2). The solid biocatalyst was prepared as follows: The organic linker solution (solution 1) was prepared dissolving 0.263 g of $H_3BTC$ in 3.68 g of NaOH 0.1 M. 29.5 mg of enzyme was added onto solution 1. The corresponding extract (5.90 mg/mL for Lip and 3.96 mg/mL for Lac) was previously dissolved in the needed amount of MilliQ water. Iron solution (solution 2) was formed by 0.508 g of $FeCl_3 \cdot 6H_2O$ in 10 g of Milli-Q water. The order of addition was chosen in order to prevent the enzymes exposure to the extreme acidic pH provided by solution 2. Solution 2 was gently added dropwise on the mixture under slow magnetic stirring. Like in the enzyme-free system, such mixture of solutions resulted in the instant appearance of a brownish orange precipitate. The suspension was maintained under permanent stirring at room temperature (25 °C) for different reaction times. The obtained solid was then recovered by vacuum filtration, washed with Milli-Q water and finally dried under a continuous nitrogen flow. The resultant biocatalysts were weighted, gently mashed to powder in a mortar and storage at 4 ºC. The solid biocatalysts were named as Lac@Fe-BTC-*x* and Lip@Fe-BTC-*x*, where *x* indicates the particular synthesis time (10 min., 1 h, 3 h, 22 h for Lac@Fe-BTC biocatalysts, and 10 min., 1 h, 4 h and 22 h for Lip@Fe-BTC, respectively).

## 3. Results and discussion

### *3.1. Post-synthesis immobilization of lipase and laccase on Fe-BTC material*

Fe-BTC material was prepared as described elsewhere [31]. Figure 1 shows the PXRD pattern and the $N_2$ adsorption/desorption isotherm of the so-obtained Fe-BTC sample compared with these of the commercial Fe-BTC sample, Basolite F300. Both techniques prove the successful preparation of the Fe-BTC sample, whose total porous surface was 740 $m^2/g$. From t-plot method it was estimated that only 43 $m^2/g$ belongs to the external superficial area, which is the only available to interact with enzymes.

**A**

**B**

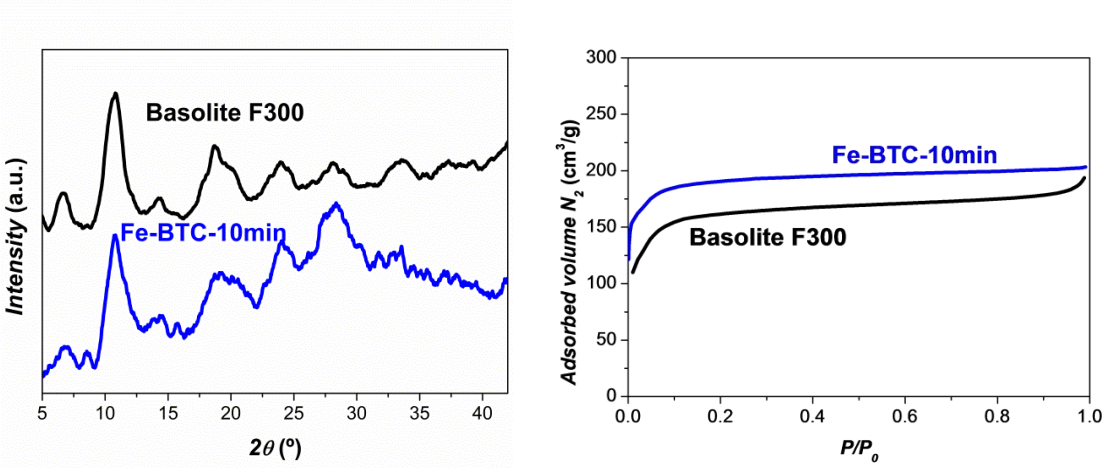


**Figure 1**. **A**) PXRD patterns belonging to commercial MOF Basolite F300 (black line) together with the lab synthesized material Fe-BTC (blue line) after 10 minutes of preparation. **B**) $N_2$ adsorption/desorption isotherms at –196 °C for commercial MOF Basolite F300 (black line) and lab synthesized material Fe-BTC after 10 minutes of preparation (blue line).

This sample was used as support of the enzymes laccase and lipase via post-synthesis immobilization strategy. It must be noted that the immobilization conditions were optimized for each enzyme. The results of the enzyme immobilization tests and the catalytic activity of the resultant post-synthesis biocatalysts (denoted as Lip#Fe-BTC and Lac#Fe-BTC) are given in Table 1. Only the results after 10 minutes of contact time between enzyme and support are shown, because the immobilization and catalytic efficiencies did not improve with longer contact. Despite the low surface area of the support significant amount of enzymes becomes retained (9 and 17 mg of laccase and lipase, respectively, per g of Fe-BTC), suggesting a relatively favorable interaction between enzyme and support.

The lack of accessibility problems for the reactants to reach enzyme active centers and the presumably non-covalent interaction with the MOF support may lead to expect a highly

preserved activity in both biocatalysts. However, the activity was only acceptable for Lip#Fe-BTC but negligible for Lac#Fe-BTC. It must be noted that the immobilization conditions were optimized for each enzyme.

The acceptable results of immobilization and catalytic activity of the lipase-containing biocatalyst Lip#Fe-BTC are in good agreement with the ones reported for post-synthesis immobilization of the same enzyme on other MOF material [11].

**Table 1.** Immobilization efficiency and catalytic performance of the post-synthesis biocatalysts Enzyme#Fe-BTC.

| Biocatalyst | Enzyme immobilized[a] / % | Enzyme loading[b] / mg·g$^{-1}$ | Activity[c] / U·g$^{-1}$ | Specific activity[d] / U·mg$^{-1}$ |
|---|---|---|---|---|
| **Lac#Fe-BTC[e]** | 18 | 9 | 2 | 0.2 (378) |
| **Lip#Fe-BTC[f]** | 29 | 17 | 1064 | 62 (371) |

[a]Percentage of retained enzyme relative to the total amount of enzyme per gram of support present in the immobilization media.
[b]Milligrams of enzyme per gram of support.
[c]Activity Units per gram of support.
[d]Activity Units per milligram of immobilized enzyme. The activity of the corresponding free enzymes is given in brackets.
[e]Tested reaction: Oxidation of the 2'azino-bis-(3-ethylbenzothiazoline-6-sulfonate diammonium salt (ABTS).
[f]Tested reaction: Hydrolysis of tributyrin (TB).

At present, it is not clear the reason why the behavior of both enzymes on the biocatalysts prepared via post-synthesis methodology is different. Probably, the different nature or different strength of the enzyme-MOF interaction is given by the differences in enzyme size and/or chemical surface composition of both enzymes.

Since the post-synthesis methodology generates biocatalysts clearly improvable in terms of both enzyme loading and catalytic activity of the retained enzyme at the same time, the in-situ immobilization approach was addressed.

*3.2. In-situ immobilization of lipase and laccase on Fe-BTC material*

As mentioned in the Introduction section, the in-situ immobilization of enzymes onto MOF materials is relatively recent [10, 25, 26, 28]. The in-situ approaches using other porous support materials showed some key advantages against the post-synthesis ones [18, 20, 39]. They entail a great challenge because they have to be carried out under the experimental conditions imposed by the formation of the MOF supports rather than under optimized conditions for maintaining the enzymatic activity. Therefore, previous effort trying to bring the experimental conditions closer to those required to avoid the enzyme denaturalization are essential to reach the desired goal. Fortunately, sustainable syntheses of MOF materials, which use conditions compatible with enzymatic activity, have been already developed in our group [27, 31, 32, 40].

The strategy to prepare in-situ biocatalysts was similar to that published elsewhere [26, 28]. Scheme S2 graphically summarizes the in-situ experimental procedure compared with the post-synthesis one (Scheme S1).

PXRD patterns of the enzyme-free Fe-BTC material and the Fe-BTC-based solid biocatalysts containing either lipase or laccase are shown in Figure 2. Although Fe-BTC cannot be strictly considered a crystalline material, the intensity and specially the position of their XRD bands (rather than peaks) can be almost used as fingerprint of this material [29, 31, 32]. From Figure 2, the presence of Fe-BTC can be inferred in all the enzyme@Fe-BTC samples. The decrease of the intensity of the XRD bands with respect to those belonging to the band of th pure Fe-BTC material can be attributed to a less ordered sample, suggesting that enzymes somehow alter the formation of the MOF material and that they are not mere spectators. The effect of a simple dilution due to the presence of certain amount of enzyme in the solid biocatalyst is hard to assume because of the low enzyme loadings (around 20 mg per g of MOF, Tables 2 and 3).

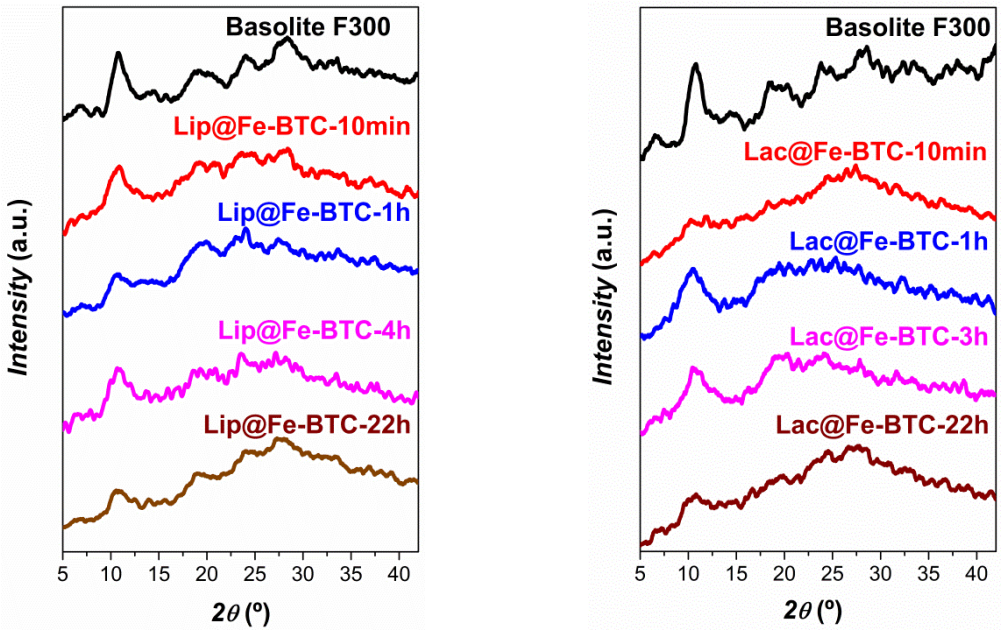


**Figure 2**. **A**) PXRD patterns of Basolite F300 (black line, at the top), and the in-situ biocatalysts Lip@Fe-BTC after 10 min (red line), 1 h (blue line), 4 h (pink line) and 22 h (brown line) of preparation. **B**) XRD patterns of the Basolite F300 (black line, at the top), and the in-situ biocatalysts Lac@Fe-BTC after 10 min (red line), 1 h (blue line), 3 h (pink line) and 22 h (brown line) of preparation.

**Table 2.** Immobilization efficiency and catalytic performance of the in-situ biocatalysts Lip@Fe-BTC-*x*, where *x* is the synthesis preparation time.

| Biocatalyst | Enzyme immobilized[a] / % | Activity [c] / U·g$^{-1}$ | Specific activity[d] / U·mg$^{-1}$ |
|---|---|---|---|
| **Lip@Fe-BTC-10 min** | 95 | 387 | 30 (371) |
| **Lip@Fe-BTC-1h** | 96 | 886 | 42 (371) |
| **Lip@Fe-BTC-4h** | 91 | 1278 | 142 (371) |
| **Lip@Fe-BTC-22h** | 87 | 3587 | 359 (371) |

[a]Percentage of retained enzyme relative to the total amount of enzyme per gram of support present in the immobilization media.
[b]Milligrams of enzyme per gram of support.
[c]Activity Units per gram of support.
[d]Activity Units per milligram of enzyme. The activity of the corresponding free enzymes is given in brackets.
Tested reaction: Hydrolysis of tributyrin (TB).

**Table 3.** Immobilization efficiency and catalytic performance of the in-situ biocatalysts Lac@Fe-BTC-*x*, where *x* is the synthesis preparation time.

| Biocatalyst | Enzyme immobilized[a] / % | Activity [c] / U·g$^{-1}$ | Specific activity[d] / U·mg$^{-1}$ |
|---|---|---|---|
| **Lac@Fe-BTC-10 min** | 98 | 102 | 4 (378) |
| **Lac@Fe-BTC-1h** | 99 | 751 | 39 (378) |
| **Lac@Fe-BTC-4h** | 100 | 237 | 11 (378) |
| **Lac@Fe-BTC-22h** | 100 | 54 | 2 (378) |

[a]Percentage of retained enzyme relative to the total amount of enzyme per gram of support present in the immobilization media.
[b]Milligrams of enzyme per gram of support.
[c]Activity Units per gram of support.
[d]Activity Units per milligram of enzyme. The activity of the corresponding free enzymes is given in brackets.
Tested reaction: Oxidation of the 2'azino-bis-(3-ethylbenzothiazoline-6-sulfonate diammonium salt (ABTS).

The enzymes present in the media were close to quantitatively entrapped from the first 10 minutes suspension in both cases. The activity of the lipase catalysts increases with interaction times, with maximum activity found at 22 hours. The corresponding catalytic efficiency at this time is very close to the activity of the free enzyme, as shown in Table 2. This is a noticeable result compared to the general behavior of immobilized enzymes, where (sometimes severe) loses of activity related to diffusional limitations and interaction with support are quite common. It is even more relevant considering that the conditions for in-situ immobilization have been designed for the right formation of the MOF support.

These good results were not reached with laccase. Although practically all the enzymes present in the synthesis media were entrapped, catalytic activity recovered was a small percentage of the initial one (39 units per milligram immobilized in the sample withdrawn after 1 h). Further in-depth studies must be performed in order to understand and improve this behavior. Due to the low catalytic activity of the laccase-containing solid biocatalysts, only the characterization and further studies of biocatalysts containing lipase is presented in this work.

*3.3. Presence of the enzyme in the biocatalysts*

Data from CHNS chemical analyses of the in-situ biocatalysts Lip@Fe-BTC-*x* are shown in Table 4. The MOF material without enzyme (sample Fe-BTC-10 min) does not contain sulphur, opposite to that corresponding to lipase extract and Lip@Fe-BTC-*x*. Sulphur content can be used as a proof of the presence of the enzyme in the biocatalysts due to the content of this element in the the cysteine or methionine amino acids in the protein primary structure. Nitrogen content is higher in the free enzyme and catalysts samples than in the enzyme-free MOF material, also suggesting the presence of the proteins in these samples.

**Table 4.** CHNS chemical analyses of the enzyme-free MOF material, lipase extract, and in-situ biocatalysts Lip@Fe-BTC-*x* synthetized after 10 min, 1 h, 4 h and 22 h of preparation.

| Sample | C (%) | H (%) | N (%) | S (%) |
|---|---|---|---|---|
| **Fe-BTC-10min** | 25.6 | 4.0 | 0.11 | 0.00 |
| **Lipase extract (5.9 mg/mL)** | 24.0 | 7.8 | 0.32 | 0.04 |
| **Lip@Fe-BTC-10 min** | 19.5 | 7.2 | 0.66 | 0.04 |
| **Lip@Fe-BTC-1h** | 24.8 | 3.7 | 1.03 | 0.09 |
| **Lip@Fe-BTC-4h** | 14.9 | 7.7 | 0.60 | 0.05 |
| **Lip@Fe-BTC-22h** | 10.8 | 6.9 | 0.45 | 0.03 |

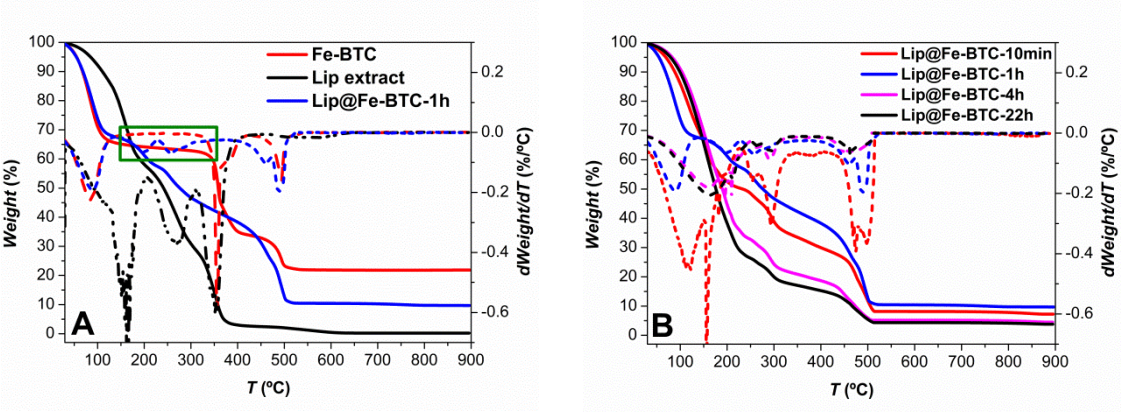


**Figure 3**. ***A***) TGA (left Y-axis) and DTG (right Y-axis) curves of the Fe-BTC material (red lines), the Lip extract (black lines) and biocatalysts Lip@Fe-BTC-1h after 1 h of preparation. **B**) TGA (left Y-Axis) and DTG (right Y-axis) curves of the biocatalysts Lip@Fe-BTC-*x* prepared with different synthesis times.

The thermogravimetric curve of the sample Fe-BTC (with no enzyme) underwent a small and lineal loss of weight in the interval between 150 ºC and 300 ºC, so its derivative (dotted red

line) does not show peaks (Figure 3A). Lip@Fe-BTC-1h shows two neat weight losses in the same interval with two peaks in its derivative (dotted blue line), attributed to the presence of the enzyme. The exact amount of enzyme lost in biocatalyst decomposition cannot be deduced due to the high complexity of these systems. Actually, TG curve of the enzyme extract is difficult to interpret considering the low enzyme concentration (3.59 mg/mL). However, thermogravimetric analysis coupled to mass spectrometry (Figure 4) demonstrates that the loss of water from the biocatalyst does not only occur at temperatures close to 100 ºC, but also between 200 ºC and 300 ºC (purple rectangle in the Figures). This finding suggests that the immobilized enzyme strongly retains certain amount of water up to temperatures much higher than its boiling point. Carbon dioxide mass curve (m/e 44, red line in Figure 4) displays a significant increase from 300 ºC to 500 ºC. Such temperatures correspond to the decomposition of the organic ligand (trimesic acid). $CO_2$ is also detected in the region between 150 ºC and 300 ºC, attributed to the presence of the enzyme.

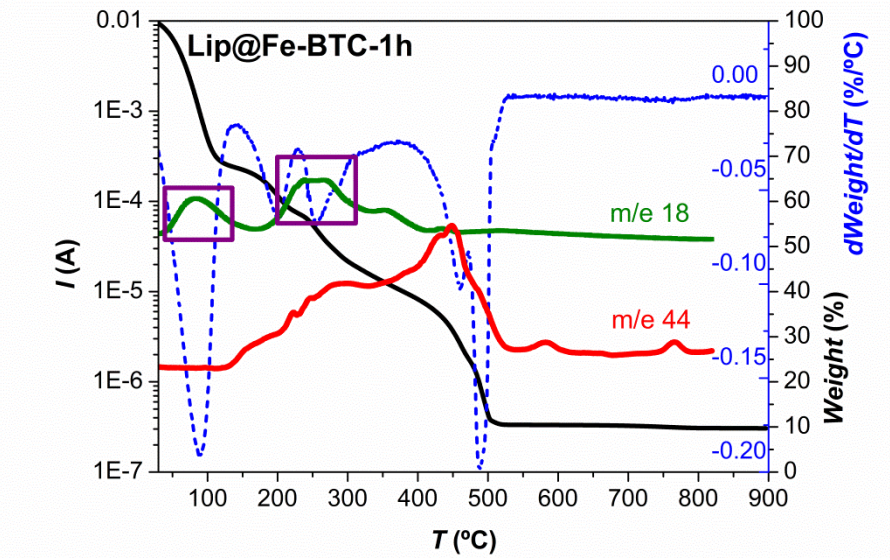


**Figure 4**. Variation of the m/e 18 (green line) and m/e 44 (red line) masses (left Y-axis) as a function of the temperature, and TGA and DTG (right Y-axis) curves of the biocatalysts Lip@Fe-BTC-1h after 1 h of preparation.

Thermograms of the Lip@Fe-BTC-*x* biocatalysts (Figure 3B) show that the water loss is around 30-35 % between 100 ºC and 150 ºC. The weight loss of organic ligand is around 50-60

% of the catalyst weight at temperatures close to 500 ºC, which again reinforces the hypothesis of the presence of lipase bound in the solid MOF Fe-BTC biocatalyst.

*3.4. Leaching tests*

Enzyme leaching tests are practically compulsory assuming the non-covalent nature of the enzyme-support bonding. The biocatalysts Lip#Fe-BTC-10min and Lip#Fe-BTC-1h were suspended in a solution particularly designed to extract the enzyme from the solids to the solution (*see* Supporting Information). The protein concentration of the supernatant was determined by Bradford assay at different times as displayed in Figure 5.

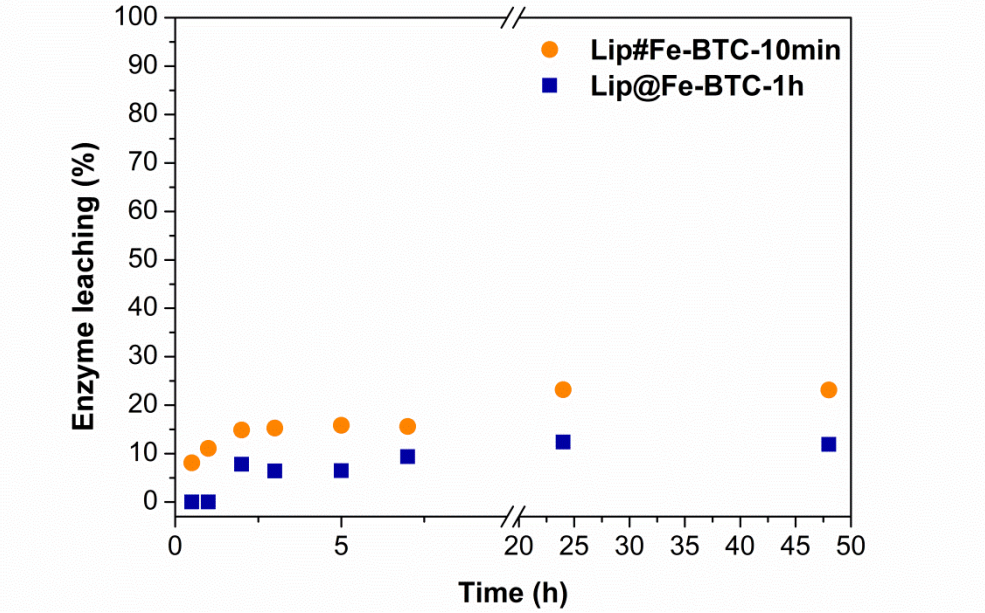


**Figure 5**. Percentage lipase leaching along the time for post-synthesis biocatalyst Lip#Fe-BTC-10min (orange circles), and in-situ biocatalyst Lip@Fe-BTC-1h (blue squares).

The leaching curves shown in Figure 5 present a plateau after around 3 h, suggesting a saturation profile. The same behavior was found in different solid biocatalysts prepared by immobilizing enzymes on either siliceous ordered mesoporous or MOFs materials [26, 41, 42]. The initial enzyme release could be attributed to weakly bound enzyme molecules like those immobilized on the external surface of the particles or within the external edges of the network.

The fact that the biocatalysts prepared by post-synthesis strategies have higher leaching somehow supports this interpretation. After a few minutes, no more enzyme molecules are significantly released (around 10 % for in-situ approach and 20 % for post-synthesis approach) even after incubation times longer than 48 h. It must be noted that the enzyme retention is comparable to permanent fixed covalent bonding systems. Once again, the relevance of support material design to improve the biocatalyst properties is illustrated.

*3.5. Electrophoresis of the Lip@Fe-BTC-x samples*

The biocatalysts were filtered and dried after 48 h of suspension for leaching studies. The solids were tested in SDS-PAGE electrophoresis experiment to check the presence of proteins inside the solid materials. Figure 6A shows lipase extract (lane 3) with a main band between 31 and 45 kDa, which is attributed to the lipase from *Candida antarctica* B. Whereas lane 2 represents the supernatant from denaturation of Lip#Fe-BTC-10min, showing a major band in the same position. In Figure 6B, supernatants of Lip@Fe-BTC-10min, Lip@Fe-BTC-1h and Lip@Fe-BTC-4h are displayed and compared to the lipase extract. In all cases the band corresponding to lipase can be clearly seen, which indicates that enzyme is still retained by the support after the leaching test.

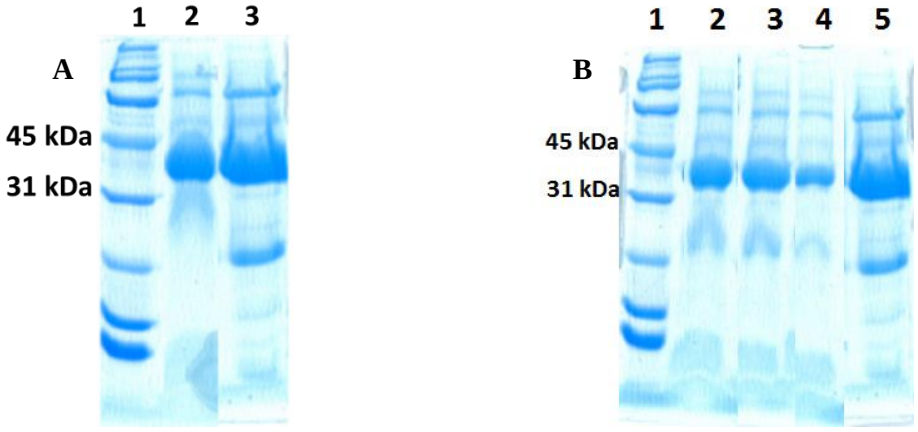


**Figure 6**. **A**) 10% SDS-PAGE electrophoresis gel of (from left to right): 1) a protein marker, 2) biocatalysts Lip#Fe-BTC-10min and 3) lipase extract. **B**) 10% SDS-PAGE electrophoresis gel of (from left to right): 1) a protein marker, 2) biocatalysts Lip@Fe-BTC-10min, 3) biocatalysts Lip@Fe-BTC-1h, 4) biocatalysts Lip@Fe-BTC-4h, and 5) lipase extract.

*3.7. Post-synthesis vs in-situ methodology*

Obtained biocatalysts through in-situ (or one-step) immobilization approach became highly efficient compared to the post-synthesis immobilization method. Almost 100 % of laccase was in-situ immobilized after 1 h (sample Lac@Fe-BTC-1h), on the contrary, 18 % was achieved with the biocatalysts prepared by post-synthesis immobilization after 10 min (sample Lac#Fe-BTC-10min). Additionally, it was found that lipase-containing biocatalysts do follow the same trend, since 87 % of enzyme was retained employing the in-situ approach after 22 h (Lip@Fe-BTC-22h), whereas less than 30 % of enzyme present in the immobilization media was finally incorporated into the biocatalyst after 10 min (Lip#Fe-BTC-10min). These results suggest that MOF material Fe-BTC is a suitable support for enzymes, especially when using the in-situ immobilization procedure.

Catalytic activity and catalytic efficiency of post-synthesis laccase biocatalysts are practically negligible, in spite of the conditions were specifically designed to both favor enzyme-support interactions and to preserve catalytic activity. In contrast, the use of the same support and same enzyme via in-situ approach yielded noticeably higher catalytic activity and efficiency values (Figure 7A). The results become even more relevant considering that this immobilization strategy focuses on the right formation of the material rather than on the optimal enzyme conditions. Previous data with laccase coming from other enzymatic source (Suberase from *Myceliophthora thermophila*) employed within the same post-synthesis immobilization procedure onto an amino-functionalized PMO material [43] displayed (after optimization) an enzyme loading of 119 mg/g, a catalytic activity of 19 U/g, and a catalytic efficiency of 0.16 U/mg. Data shown in this work (using laccase (Novozym 51003) from *Aspergillus oryzae*) gave a much higher catalytic activity of 751 U/g and a catalytic efficiency of 39 U/mg. In-situ immobilization of laccase on siliceous materials also yielded poor results, especially in terms of catalytic efficiency (*unpublished results*).

Similarly, lipase enzyme immobilization efficiency and enzymatic activity of the biocatalysts prepared by the in-situ immobilization procedure surpass those achieved with post-synthesis approach (Figure 7B). It is noteworthy that the in-situ biocatalyst was able to retain 97 % of the catalytic activity given by the free enzyme (Table 2). In particular, the catalytic efficiency of Lip@Fe-BTC-22h is 359 U/mg, pretty much the same as the free enzyme specific activity (371 U/mg), which means the enzyme does not lose practically any activity during the whole immobilization. As an example of the relevance of these results, the in-situ immobilization of the same lipase (Lipozyme CALB L from *Candida antarctica* B) on purely siliceous material [22] achieved a catalytic efficiency of 119 U/mg, far lower from the 359 U/mg given by Lip@Fe-BTC-22h.

It is clear from these data that in-situ immobilization on MOF Fe-BTC does not alter catalytic activity of enzymes, or at least it does in a much lesser degree than other immobilization systems. It means that the interaction of protein molecules with MOF does not involve conformational changes leading to loss of catalytic activity commonly found in most of the immobilized enzymatic systems. In addition, the fact of not being the enzymes encapsulated within the intrinsic mesocages of the Fe-BTC MOF system do contribute to the lack of diffusional restrictions that reactants may have in order to achieve the enzyme active centers.

In summary, Fe-BTC material has shown to be very efficient support for enzyme immobilization. The relevance of the results presented here is easier appreciated when compared with similar data published in the literature. The only MOF material studied in the same post-synthesis and in-situ immobilization procedures so far was $NH_2$-MIL-53(Al) [25, 26]. Post-synthesis immobilization of laccase in this material yielded a catalytic efficiency of 0.3 U/mg after 24 h of synthesis preparation. Post-synthesis immobilized laccase on Fe-BTC gave a catalytic efficiency two orders of magnitude higher in the same reaction (39 U/mg) after only 10 minutes. Such a short time of biocatalyst synthesis is an additional advantage for potential industrial exploitation. Regarding the in-situ immobilization of enzymes on the MOF material $NH_2$-MIL-53(Al), it was studied with another enzyme, β-glucosidase [25, 26]. In this case the

maximum catalytic activity retained (with respect that of the free enzyme) was 37 %, which is far lower than the retained activity of lipase on Fe-BTC, reaching 97 %.

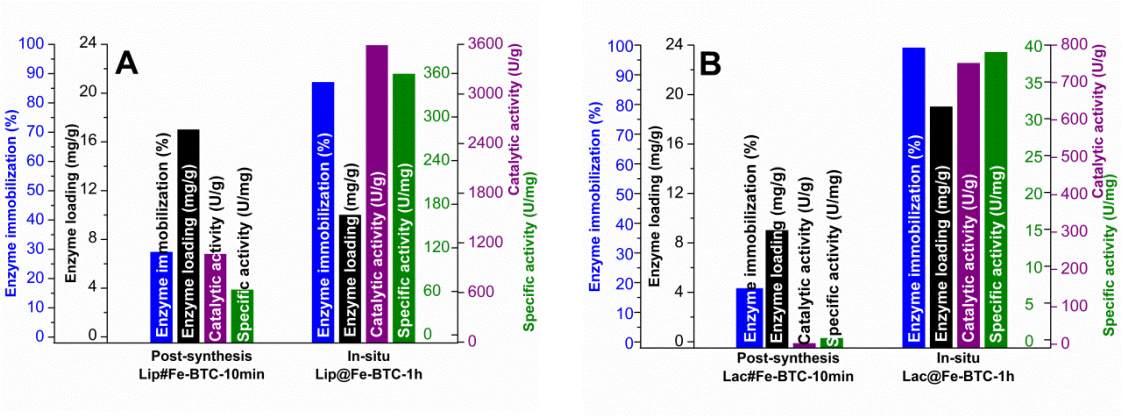


**Figure 7**. Percentage enzyme immobilization (blue columns, left Y-axis), enzyme loading (black columns, left Y-axis), catalytic activity (purple columns, right Y-axis), specific activity (green, right Y-axis) of: **A**) the biocatalyst Lip#Fe-BTC-10 min prepared by post-synthesis approach (left) and Lip@Fe-BTC-1h prepared by in-situ approach; **B**) the biocatalyst Lac#Fe-BTC-10 min prepared by post-synthesis approach (left) and Lac@Fe-BTC-1h (right) prepared by in-situ approach.

## 4. Conclusions

Fe-BTC MOF has proven to be an excellent material for enzyme immobilization. More specifically, the in-situ immobilization on such MOF material was more efficient than post-synthesis approach in terms of percent enzyme immobilization, catalytic activity, catalytic efficiency and leaching preventing for both studied enzymes: laccase and lipase. Additionally, in-situ immobilization of lipase in Fe-BTC MOF material has yielded to biocatalysts with the uncommon and desired property of being capable of fully preserving the same activity than the free enzyme. This is a clear milestone in enzyme immobilization field since neither diffusional restriction nor support interactions seem to affect the activity of this biocatalyst. Furthermore, the easy and strong enzyme-MOF interactions suggest an efficient affinity between lipase and Fe-BTC without altering catalytic properties of the enzyme. The potential of the semi-crystallin Fe-BTC as support for enzyme immobilization has been revealed even when compared with

some other MOF materials, all obtained through the same methodology under friendly conditions designed for enzymatic catalytic activity preservation.

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