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Enzymatic biosensors for anti-cancer drugs screening



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INTRODUCTION

01. LACTATE DEHYDROGENASE:

Lactate dehydrogenase EC 1.1.1.27. is a cytoplasmic enzyme [1] of the glycolytic pathway. It belongs to the oxidoreductases, and it intervenes by catalysing the reduction of pyruvate to lactate while oxidising NADH to NAD⁺. It allows the continuation of the process under anaerobic conditions [2] (figure 1).

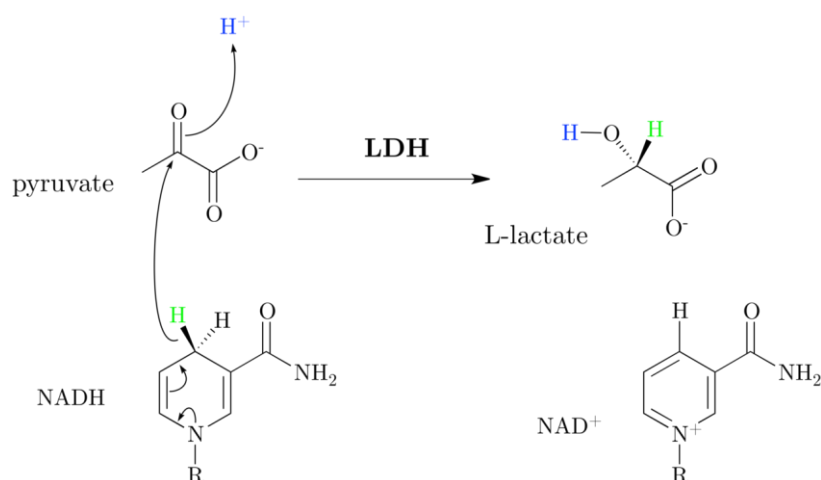


Figure 1: Lactate dehydrogenase catalyses the reversible reduction of pyruvate to lactate, coupled with the oxidation of NADH to NAD⁺ [3].

It is spread among various organisms with minor changes including plants and animals. Moreover, LDH is widely distributed throughout the body, with tissue-specific concentrations; the highest levels are found in muscles, liver and kidneys.

LDH has 5 different isomeric forms called LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5, each one composed of 4 subunits that can be of two different types, named muscle (M) and heart (H).

LDH-1 is also called LDH-B and it is formed by only H subunits while LDH-5 is also known as LDH-A and is composed by only M subunits. The former has a net charge of

–6 and is more affine to the transformation of pyruvate in lactate. On the contrary the latter has a net charge of +1 and favours the reverse reaction [1] [2].

The main focus of this thesis is LDH-A, as it has an important role in cancer metabolism (figure 2).

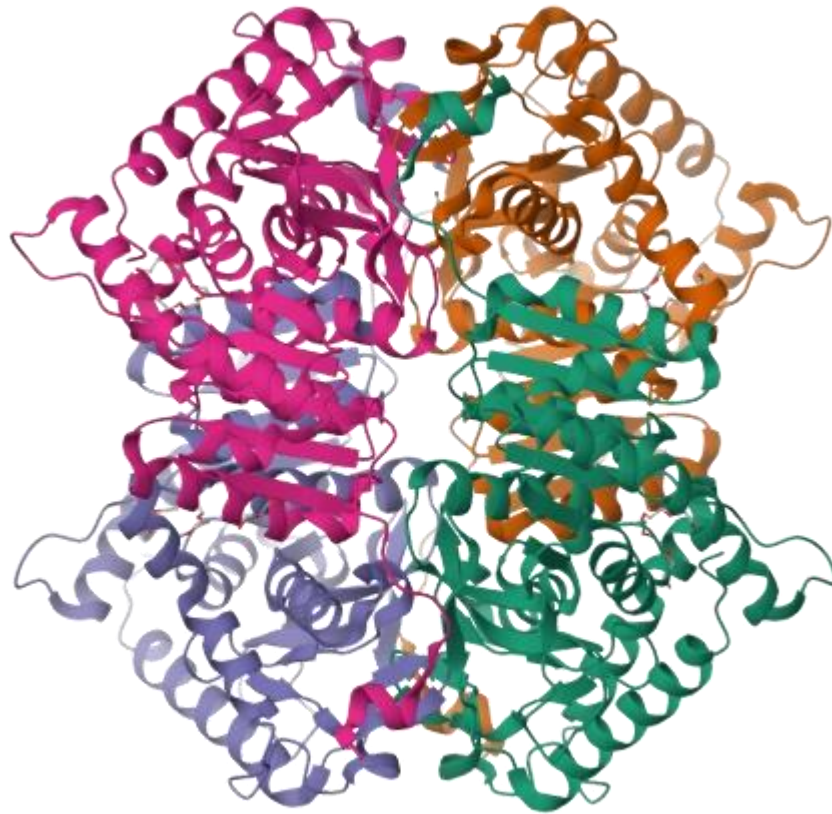


Figure 2: the 3D simulated structure of human-LDH-A from PDB - Protein Data Bank [4].

02. ROLE OF LDH-A IN CANCEROUS CELLS:

The energy required by human cells to sustain life largely comes from the oxidation of glucose. During glycolysis, one molecule of glucose is converted into 2 molecules of pyruvate, producing a net worth of 2 molecules of ATP. At this point, there are two possible pathways which the pyruvate could follow: oxidative phosphorylation and lactic fermentation. In the first one, which takes place in the presence of oxygen, pyruvate is transported in the mitochondria, and it allows a production of 32 molecules of ATP. The other one occurs under strong physical exercise that brings a lack of O_2 in the cytoplasm. In these conditions, pyruvate is reduced into lactate, thanks to LDH who a plus 2 net worth of ATP and regenerates NAD^+ , essential for glycolysis to continue. [5].

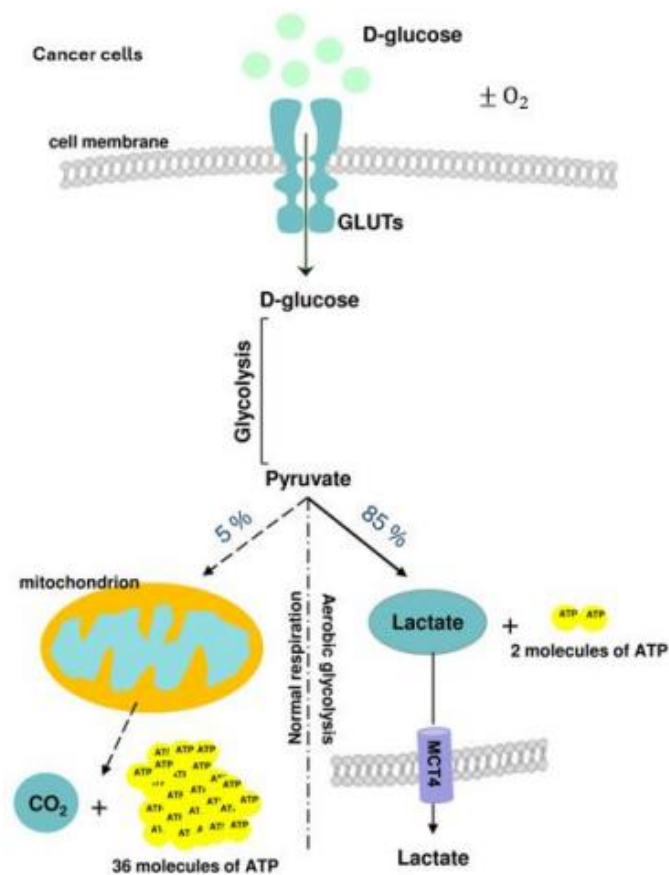


Figure 3: the Warburg effect, the shift in the metabolism of O_2 [6].

The latter is obviously less convenient for the cells but permits a faster production of energy under stress. This mechanism doesn't apply, though, to tumour cells where there

is a shift in the process that leads to the aerobic production of lactate by LDH-A [7]. This strategy, known as the Warburg effect, from Otto Warburg who was the first to study this effect in 1924, allows the tumour to grow and spread faster while amassing big quantities of lactate in the cytoplasm. At the time of this work, it is still not clear if the Warburg effect is a consequence of the tumour or the cause of it [8] [9].

Cancer is one of the most important causes of death in human life. According to a study from 2021, it was calculated at 15% the percentage of cancer deaths on the total yearly deaths worldwide [10].

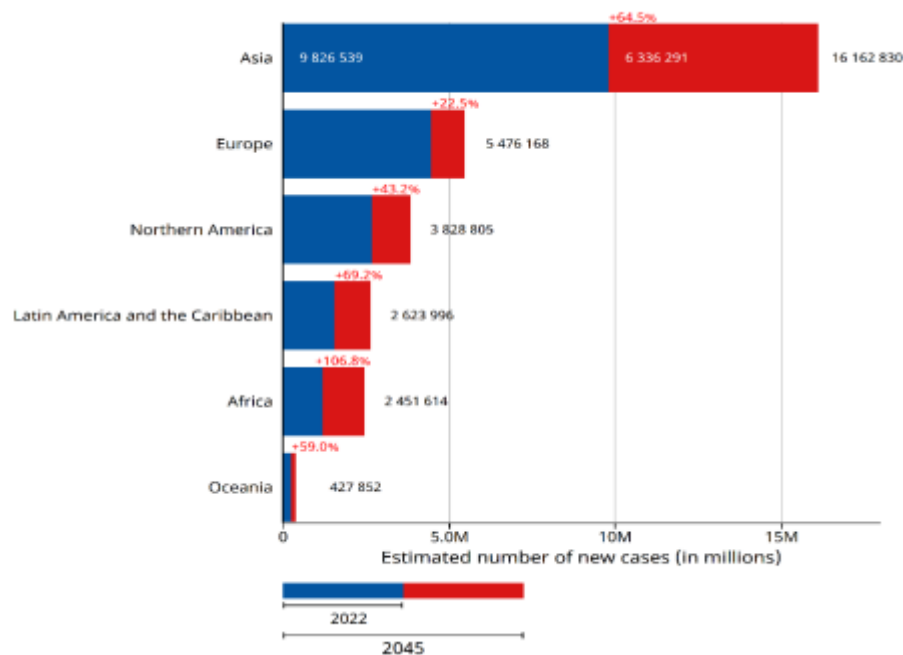


Figure 4: Number of new cases of cancer in 2022 (blue) and a projection towards the 2045 (red) [11].

The number of casualties attributed to this disease in 2024 is 9,7 million. The same study projected 1 out of 9 men and 1 out of 12 women to die of cancer in their lifespan. There are 5 types, in particular, responsible for near half of the deaths but confronting these data to the incidence, it is obvious that some cancers are more treatable than others. Breast cancer thanks to early diagnosis and an active screening is second at 2,308,897 new diagnoses in 2022 but in the same year the number of casualties is close at one quarter 665,684. The same can be said for prostate cancer which affected 1,466,680 new men but only 396,792 were the men who died of this disease.

On the other hand, some types like the liver one (865,269 new diagnoses and 757,948 deaths), the pancreas one (510,566 new diagnoses and 467,005 deaths) or even the lung one (2,480,301 new diagnoses and 1,817,172 deaths) have numbers close due to their aggressiveness and later diagnosis [12]. In figure 5 there are the complete data and the differences between sexes.

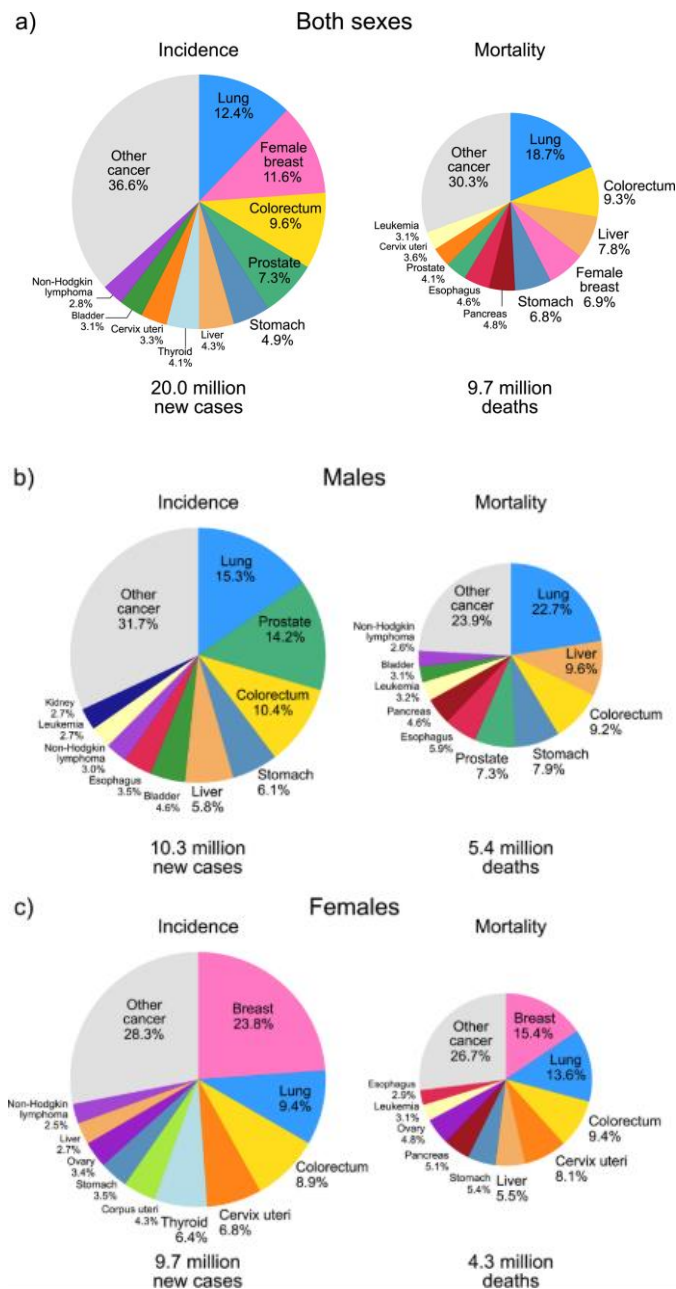


Figure 5: the percentual partition of new cases of cancer and deaths in 2022 divided by the type, a) in total, b) for only males and c) for females [12].

Some big differences can be found in the geographic incidence, as it is possible to see in figure \$. Approximately half of the new cases occur in Asia, largely due to its enormous population and the ageing population. In fact, older people are more exposed to age-based cancer type. The same can be said for Europe, in which the median age is 44 years old, moreover the 22,4% praises the incredible screening capabilities of the health system of the old continent. On the contrary, the young population of Africa has a low percentage of cancer incidence, but it is the only continent where the percentage of mortality is higher than incidence because of the low efficiency of the health system.

Another aspect of the different countries that influence the cancer data is the extent to which specific dietary and lifestyle habits are embedded in society, such as the consumption of red meat or alcohol. Moreover, the high incidence of lung cancer comes mainly from two central actors of the last century: smoking and air pollution. In fact, the lag period between the start of smoking or the exposure at massive air pollution and the development of cancer (20-30 years) drives the incidence of our times back to the 90s when the sensitivity on these subjects was way lower.

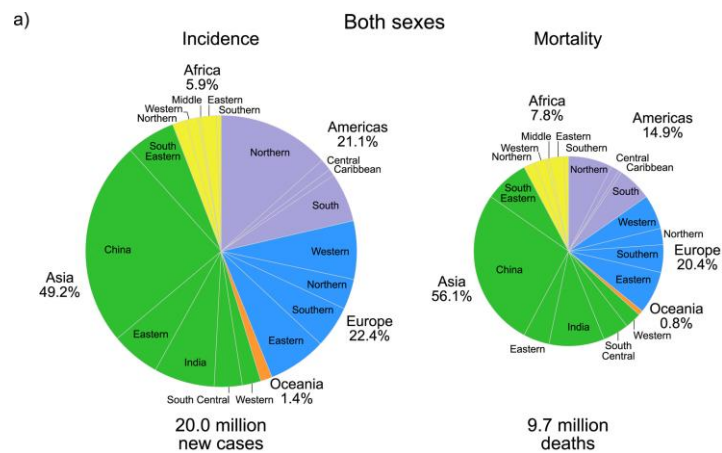


Figure 6: the percentual partition of new cases of cancer and deaths in 2022 divided by continents [12].

This study is not focused on finding a cure for it, but its main goal is about optimising the process of anti-cancer drug testing which as a matter of fact right now is very expensive and time requiring.

03. DRUG DEVELOPMENT PROCESS:

To introduce in the market a new drug, there are many phases that need to be passed. It can take more than 10 years from the beginning of the process, and the costs are close to billions. The first phase of this process is the discovery and development, where thousands of potential drugs are identified and studied to understand the potential effect on the target disease.

The second phase is called the preclinical research phase, and it examines the potential side effects of the drugs both *in vivo* and *in vitro*. If the results are promising, the clinical research can start. This stage is called Phase I and it is initially conducted on a small, targeted group of healthy humans, to see dosage range and other side effects. However, in oncology, Phase I trials are often performed directly in severe cancer patients due to the potential toxicity of anticancer drugs.

The process continues, then, with a bigger group of human patients (up to 300) who suffer from the targeted disease and ends with large-scale test on patients worldwide to compare with traditional treatments. If a drug passes all this phase, the regulatory review process begins. At this stage, all the data collected over the years are submitted to EMA for Europe or FDA for United States, through a formal application. Once the approval is granted and the drug enters the market, the producer continues to monitor the drug to look for rare side-effect [13].

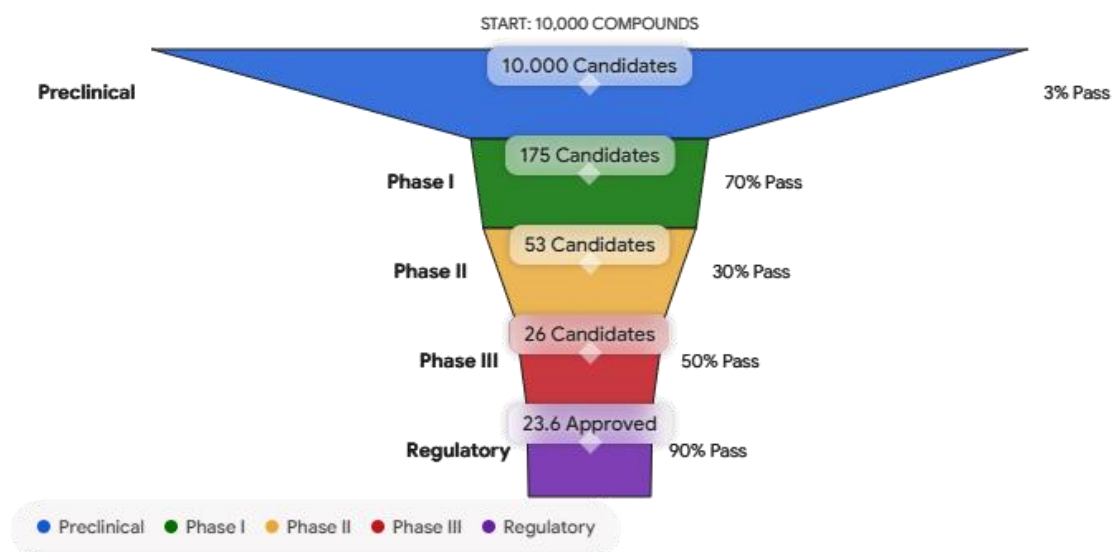


Figure 7: the funnelling effect of the drug development process.

04. ENZYMES IMMOBILIZATION:

Biocatalysis is the acceleration of a chemical reaction using enzymes instead of inorganic catalyst normally used in the past. Enzymes are composed by complex and heavy weight aminoacidic chains that have a very high specificity, allow an incredible velocity of reaction and last, they can even be regulated through inhibition and activation. The downside of biocatalysis is all really about the sensitivity to even a slight change of temperature or pH [14].

Table 1: comparison between enzymes and inorganic catalysis.

Feature	Enzymes (Biocatalysts)	Inorganic Catalysts
Composition	Proteins (complex organic molecules)	Metals, oxides, or small mineral ions
Reaction Rate	10^8 to 10^{17} times higher than uncatalyzed	Significant, but generally lower than enzymes
Specificity	Extreme (Lock-and-key or induced fit model)	Low (often catalyze multiple side reactions)
Regulation	Can be regulated (activated or inhibited)	Not adjustable once the reaction starts
Sensitivity	Highly sensitive to T and pH variations	More stable under extreme industrial conditions
Operating Conditions	Mild (ambient T, neutral pH)	Often require high T and high pressure

Enzymes immobilization was first introduced in 1916 and since then many different techniques were developed. It is a process where a bond between the enzyme and an organic or inorganic material support is created. It benefits various downsides of the lone

enzyme, such as the stability, allowing him to endure more extreme temperature and pH conditions and exposition to aggressive solvents. It can also decrease the cost of the process allowing the recycle of the catalyst and a more efficient separation of the final product [15] [16].

Enzymes immobilization strategies fall into two different categories based on the nature of the bond between the enzyme and the support material: chemical (in which it forms a covalent bond) and physical (where the bond is non-covalent).

- CHEMICAL STRATEGIES:

1. Covalent binding: functional groups often obtained with functionalizing techniques link enzyme residues to a support structure in an irreversible way, obtaining a very high efficiency in contrasting leakage and stabilizing the enzymes. The stabilized enzyme can easily resist high temperatures without denaturing, extreme pH values and harsh organic solvents. On the other hand, it is common a drop in the activity due to the many sites on the enzyme where the bond can be formed, in fact a simple miss-orientation can screen the active site [16].
2. Cross-linking: is a technique where an aggregate of enzymes is created through covalent bonds between the singularities. The CLEAs (cross-linked enzyme aggregates) is a famous variant in which a carrier is not required. However, when the aggregate is too voluminous there are some drawbacks, like the inactivation of the central part that is too difficult to reach for the substrate or even enzyme denaturation caused by the numerous covalent bonds [16].

- PHYSICAL STRATEGIES:

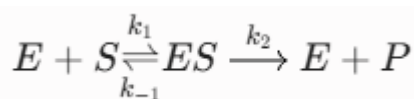
1. Adsorption: a weak binding between the support and the enzyme is created. These bindings are non-covalent, so reversible, like Van der Waals forces or hydrogen bonds. The main reasons why it is used are the very low cost.

Moreover, the nature of the bonds does not harm the enzyme; preserving is original structure and activity. On the contrary of covalent bonding a serious problem is the desorption that can bring to leaching [16].

2. Entrapment and encapsulation: these strategies consist of “entrapping” the enzymes in a complex structure, for the first it is caught in a porous network and for the second it is housed in a polymeric matrix. The confinement obtained avoids the problem of leaking because the pore entries are smaller than the enzyme itself while the substrate and the products can pass freely through. Another good point for these methods is the low probability of changing the enzyme structural integrity, enabling a high residual activity [16].

05. ENZYME KINETIC:

Enzyme kinetics is the study of rates of enzyme-catalysed chemical reactions. Enzymes change the process of a chemical reaction by lowering the initial energy activation needed. The standard model to understate the progression of a chemical reaction with a single substrate is the Michaelis-Menten model. It assumes that the enzyme E binds reversibly with the substrate S, generating the ES complex, which can convert into the product P.



Equation 1

k_1 : is the rate constant for the direct formation of the complex ES.

k_{-1} : is the rate constant for the dissociation of the complex ES back into E and S.

k_2 : is the rate constant for the irreversible formation of the product P from the ES complex.

The equation for the velocity of the process can be derived by making one principal assumption: the steady-state assumption. This principle states that, during the reaction

time, the concentration of the ES complex remains approximately constant during the initial phase of the reaction, because the formation rate of the complex is equal to its rate of dissociation.

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Equation 2

[S]: concentration of the substrate.

V_{max} : maximum velocity of the process which can be achieved when all the enzyme E is converted in the complex ES.

K_m : Michaelis constant, it is equal to the [S] when the velocity of the process is half V_{max} . It also serves as an inverse measure of the affinity of the enzyme to the substrate; a lower K_m refers to species more affine while a bigger K_m refers to species not much affine.

Considering the key reaction of this work, namely the reduction of pyruvate into lactate, it should be noted that is the reaction involves more than one substrate, in fact NADH is needed. NADH is an electron transporter and a co-enzyme.

To better describe the mechanism of the pyruvate to lactate reaction other models with two substrates need to be introduced. The most appropriate model is the ordered Bi-Bi sequential model, where the sequence of bindings is highly codified: first the NADH binds to the enzyme and create a complex ready to bind to the second substrate, the pyruvate. At this point also the release process must follow a strict order, first it is released the lactate and then the NAD^+ which re-generate the free enzyme [17] [18].

The steady-state velocity equation looks like:

$$v = \frac{V_{max}[A][B]}{K_i^A K_m^B + K_m^B[A] + K_m^A[B] + [A][B]}$$

Equation 3

[A] and [B]: concentrations of the substrates.

V_{max} : maximum velocity of the process.

K_m^A and K_m^B : Michaelis constant of the two substrates.

K^A_i : dissociation constant for the NADH-LDH complex.

Other models for two substrates are the random Bi-Bi sequential model, Ping-Pong Bi-Bi model and the Theorell-Chance model [17].

However, the Michaelis-Menten model can still be applied, under the condition of fixed concentration of NADH. More in detail, it should be in the medium reaction in strong excess so that the reaction behaves as a pseudo-first order with [S] equals to pyruvate, allowing the application of the Michaelis-Menten model [19].

06. SUPPORTS:

After the focus on the immobilization strategies, it is clear the importance of the selection of a suitable support material.

Firstly, it is fundamental the ratio between the costs and the effectiveness to ensure the feasibility of the solution not only in small-scale processes, but also for large-scale industrial applications where the required volumes are massive. This aspect is closely linked to the assessment of the material's environmental friendliness and overall sustainability.

Stability is another key factor to consider, including the chemical one, the thermal one and the mechanical one as well, in order to avoid the loss of enzyme due to the degradation or rupture of the support.

Furthermore, the material needs to be non-toxic to the enzyme and compatible with the medium used in the reaction.

In the end, it is important to consider specific requirements depending on the immobilization strategy. For instance, in the case of covalent immobilization, the support material should allow a wide range of surface modifiability through functionalization processes, thereby promoting high affinity towards the enzyme. In addition, a high surface area is desirable, as it enables a greater amount of immobilized enzyme per gram of support [20].

In contrast, an effective material for the encapsulation mainly depends on its porous structure. In particular, the pore volume should be sufficiently high to accommodate large amounts of enzymes, while the pore openings should be narrow enough to avoid the loss of enzyme, but sufficiently large to avoid mass transport limitations phenomena. Even the pore morphology is an important parameter, since an excessively branched pore network may hinder substrate diffusion toward the innermost regions of the material. The range of materials that could be used is wide and encompasses both inorganic and organic supports. However, considering the dimensions of the enzyme molecules, particular attention will be given to mesoporous or microporous structures. More in detail, mesoporous silica, in particular SBA-15, will be considered for covalent immobilization and metal-organic frameworks (MOFs), in particular ZIF-90 and MAF-7, for encapsulation [21] [22].

07. METAL ORGANIC FRAMEWORKS:

Metal Organic Frameworks are a very versatile class of crystalline porous materials with metal's nodes and organic linkers that mix and integrate properties of both components. The two most successful and stable sub-classes of MOFs are zeolitic imidazolate frameworks and zeolitic metal-azolate frameworks. These structures are topology isomorphous to inorganic zeolites substituting the silicon or aluminium tetrahedron with transitional metal cations and oxygen bridge with imidazole or azolate bridge [23]. For MOF MAF-7 zinc ions (Zn^{++}) are the nodes of the structure, while uses 3-methyl-1,2,4-triazole as ligands. The overall topology is isomorphic to the more well-known ZIF-8 framework.

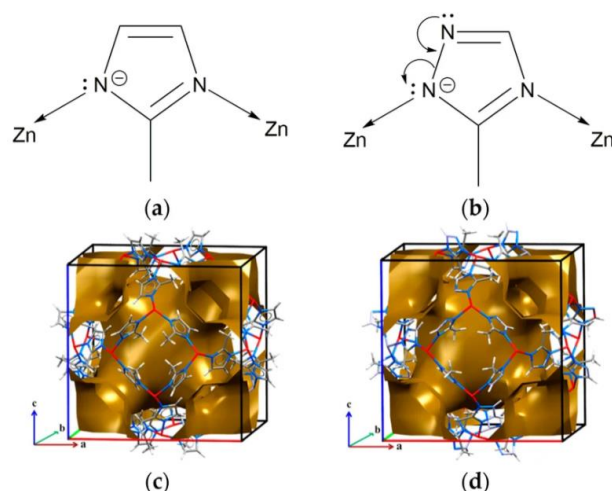


Figure 8: comparison between the ligand of ZIF-8 (a) and MAF-7 (b), and the SOD zeolitic topology of ZIF-8 (c) and MAF-7 (d) [24].

MAF-7 exhibits a typical sodalite (SOD) zeolitic architecture with a pore configuration that is simple to imagine as “the neck of a bottle” with small entries and large volume. Such a structural arrangement is particularly suitable for enzyme encapsulation, since the internal cavities can host biomolecules of appropriate size, while the narrow apertures help limit enzyme diffusion out of the framework as a result, MAF-7 may reduce leaching problems, making it an interesting candidate as a support material for immobilization through encapsulation. The ligands used change the structure, replacing the carbon atom at the 1-position of the ring of the ZIF-8 with an electronegative nitrogen atom. The nitrogen atoms used for coordinating to the metal ions are at 2- and 4-position this leaves the nitrogen atom at 1-position exposed to the pore surface. The presence of this uncoordinated nitrogen atom and the methyl groups at position 3 make the pore surface highly hydrophilic in contrast to the hydrophobic surface of ZIF-8 [24].

ZIF-90 instead uses imidazole-2-carboxaldehyde (ICA) as organic linker exposing to the framework cavity a highly polar and asymmetrical aldehyde which create a hydrophilic surface. However, its conventional structure is microporous, so it is generally unsuitable for the encapsulation of LDH. Nevertheless, one of his variants, the hierarchical ZIF-90, combines micropores with system of mesopores, perfect for trapping the enzyme [25].

08. SBA-15:

SBA-15 (Santa Barbara Amorphous no.15) first developed in 1998 at the University of California, Santa Barbara (the name comes from there) is a silica based amorphous material which can withstand very high temperature. It is characterized by straight cylindrical pores, whose size can reach up to 30 nm when an organic solvent is used during the synthesis. Another relevant feature is the thickness of the walls between the pores; thin walls are perfect for reducing the mass of the structure, while still preserving its ordered porous architecture.

The precursor used for the synthesis is typically TEOS (tetraethyl orthosilicate), which undergoes hydrolysis and condensation, forming the thin glassy inorganic walls around the micelle. The micelles are generated by the action of a structure-directing agent, which directs the formation of the structure.

There is more than one template which can be used, one is P123 (Pluronic) a non-ionic triblock copolymer made by two PEO (poly-ethylene oxide) chains at both ends and a PPO (poly-propylene oxide) chain in the middle, another template can be P104 which brings to the formation of smaller micelles or L121.

In general, the macromolecule has two hydrophilic tails and a hydrophobic core, and this is the reason why in aqueous solution it tends to form micelles trying to hide his PPO chain. Then, during calcination, the residues leave behind the pores needed.

If you want to obtain much larger pores a pore expanding agent can be used as TMB (1,3,5-trimethylbenzene). The TMB is highly hydrophobic when it is poured into the reaction medium which is aqueous it migrates in the space surrounded by the PPO chain expanding the micelle dimensions. Without the TMB the dimensions of the pores drop from 30 nm to 10 nm.

One of the reasons of the success of SBA-15 not yet discussed is the wide range of functionalising processes that it can undergo [26] [27].

Table 2: different templates for SBA-15 and the characteristics obtained from their use [27].

Block copolymer	Reaction temperature (°C)	d(100) (Å)	BET surface area (m ² /g)	Pore size (Å)	Pore volume (cm ³ /g)	Wall thickness (Å)
EO ₅ PO ₇₀ EO ₅	35	118 (117)	630	100	1.04	35
EO ₂₀ PO ₇₀ EO ₂₀	35	104 (95.7)	690	47	0.56	64
EO ₂₀ PO ₇₀ EO ₂₀	35, 80*	105 (97.5)	780	60	0.80	53
EO ₂₀ PO ₇₀ EO ₂₀	35, 80*	103 (99.5)	820	77	1.03	38
EO ₂₀ PO ₇₀ EO ₂₀	35, 90*	108 (105)	920	85	1.23	36
EO ₂₀ PO ₇₀ EO ₂₀	35, 100*	105 (104)	850	89	1.17	31
EO ₁₇ PO ₅₅ EO ₁₇	40	97.5 (80.6)	770	46	0.70	47
EO ₂₀ PO ₃₀ EO ₂₀	60	77.6 (77.6)	1000	51	1.26	39
EO ₂₆ PO ₃₉ EO ₂₆	40	92.6 (88.2)	960	60	1.08	42
EO ₁₃ PO ₇₀ EO ₁₃	60	80.6 (80.5)	950	59	1.19	34
PO ₁₉ EO ₃₃ PO ₁₉	60	74.5 (71.1)	1040	48	1.15	34

*Reaction at 35°C for 20 hours, then heating to the higher temperature for 24 hours, or for the second entry for 80°C, 48 hours.

09. SUPPORT FUNCTIONALIZATION:

Despite his many advantages, the SBA-15 is not equipped for an efficient covalent immobilization because, despite its large surface area, it possesses only silanol groups on the surface, and not the organic groups required for the creation of a stable bond with the enzyme. It is therefore fundamental to modify the surface to introduce these functional groups. However, the abundance of these anchors is not always a positive point. As previously discussed, it is possible to reach enzyme deactivation caused by an incorrect position of the enzyme or conformational distortion caused by the formation of multiple bonds [28] [29].

Functionalization is the process of chemically attaching specific organic molecules or metal complexes to the support walls. For silica-based materials, there are two principal functionalization strategies that are commonly used:

1. Post-synthesis: the first method highlighted is the simplest one; this process occurs after the complete synthesis and calcination of the SBA-15. It consists of using specific organosilanes in an organic solvent. Only the surface is modified in this case, unlike the other method that will be shown after this one. The downsides are basically all about the fact that the functionalization sometimes is

not homogeneous, which leads to the blocking of the pores altering the quantity of enzyme that is possible to immobilize. Moreover, it does not allow to have control on the final location of the attachment of the enzyme [28].

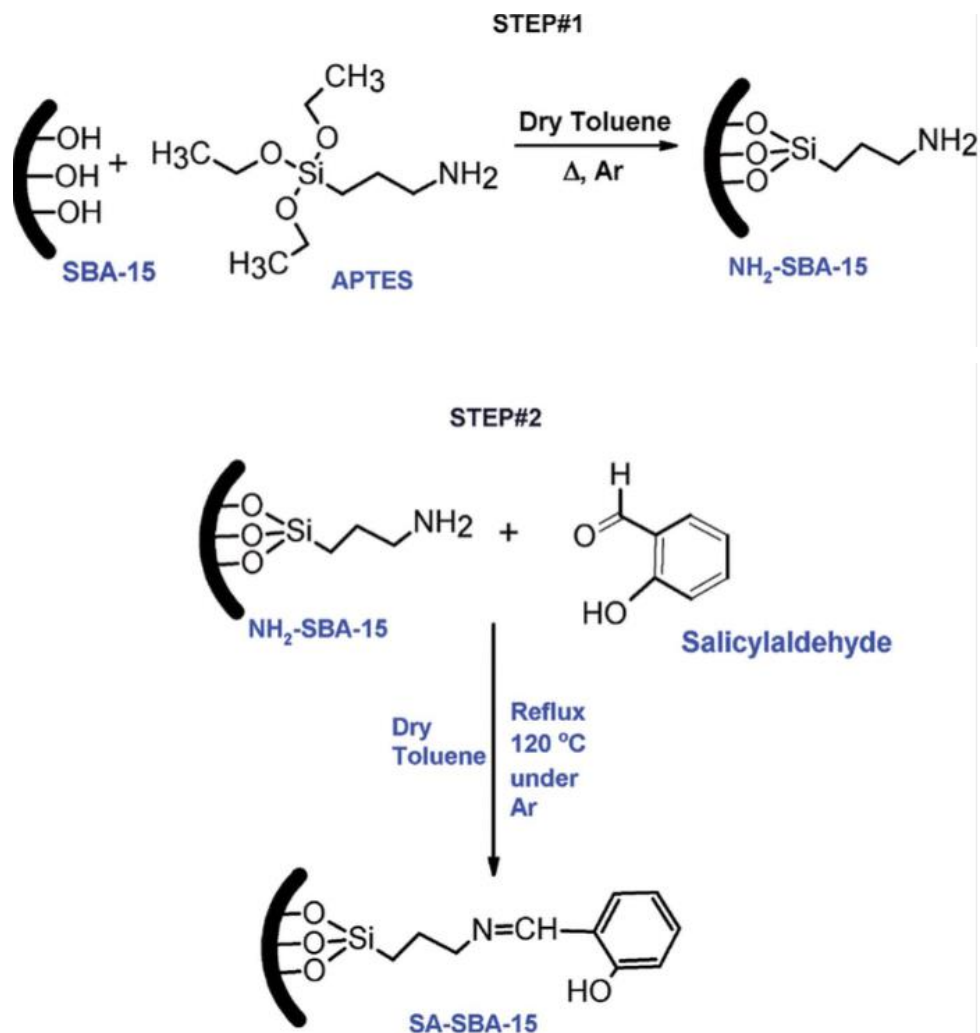


Figure 9: example of post-synthesis functionalisation [30]

2. Co-condensation: this technique takes place during the synthesis of the support. The functionalising agent is blended with other reagents and creates a mixed structure organic-inorganic. Unlike the post-synthesis method, here the result is a homogeneous distribution of the functional groups, although the problem of the limitation of the pores and their entries persists. Beyond that, it is very important to measure out precisely the reagents because an excessive amount of the functionalizing agent can lead to disorders in the crystallin structure of the material, turning it amorphous in the worst-case scenario [28].

10. LDH IMMOBILIZATION:

Measuring precisely the dimensions of a macromolecule is a difficult process but an appropriate approximation can be done through the weight and the molecular weight. A human LDH-A molecule can be contained in a cube of 8.5 nm of edge [31]. Even if the measure are not precise, it is still enough to define the dimensions of the pores required for the encapsulation of the h-LDH: the mesopores.

The two MOFs analysed have the windows of the structure around the dimension of 0,35 nm. This feature has both advantages and limitations. On the one hand, such small apertures reduce the probability of LDH leaking from the framework, thereby improving enzyme retention. On the other, efficient biocatalysis requires not only the preservation of enzyme integrity, but also the diffusion of substrates and cofactors toward the active site. The most critical substrate is obviously the bulkiest one: the NADH; it has a approximated diameter of 1,5 nm, circa [31].

The question therefore arises as to how the reaction can occur despite the apparent size mismatch between the MOF windows and NADH.

There is more than one solution to this problem. The first one derives from the action of the enzyme during the assembly of the structure: it disrupts the perfect lattice, creating defects or larger diffusion pathways that facilitate substrates' transport. Moreover, the enzyme is not always deeply embedded within the structure, but in some cases, it may be located close to the external surface, allowing the NADH to reach the active site from outside of the cage [32] [33].

In the end, it is important to understand that the structure is not always rigid and immobile. A key characteristic of the ZIF-90 is the flexibility of the gates, which moves like lungs during the inhaling and exhaling process, allowing the substrate to flow in. On the contrary the MAF-7 is famous for its rigid framework which suppresses the “breathing swing” [34] [24].

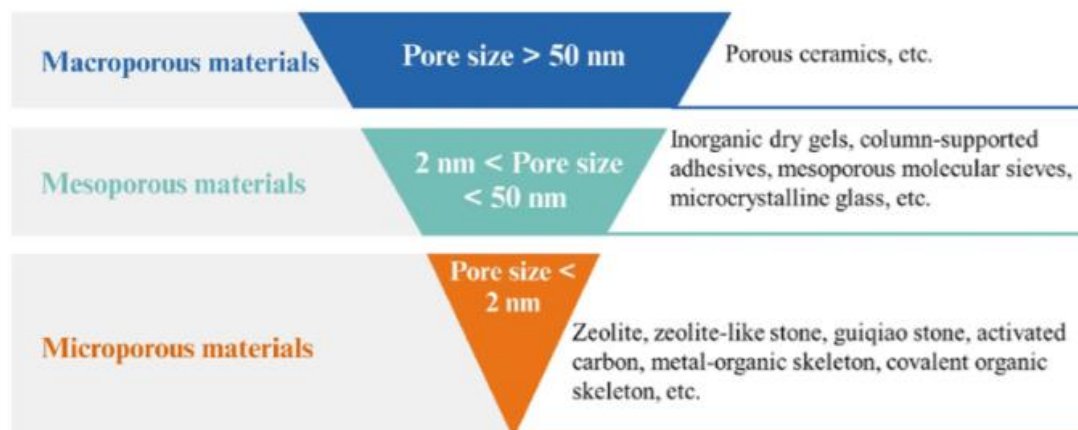


Figure 10: comparison between the different pore sizes.

Regarding the covalent immobilization, it is important to identify the amino acid residues that could be exploited to attach the enzyme to the support material. Lysine residues have many interesting features related to this purpose. First of all, they contain an extremely reactive amino group ($-NH_2$). Moreover, these groups are frequently exposed on the protein surface and are often not directly involved in the catalytic active site.

The immobilization process typically requires a basic environment, around pH 10, because the pKa of the amino group is 9.2, and in order to enable the reaction, it must be deprotonated.

However, these conditions are very harsh for the enzyme, and the risk of denaturation cannot be ignored: the alkaline bulk can strip the enzyme from some of its important bonds that create its 3D structure.

The solution of this problem comes from the use of protective additives, like trehalose. Trehalose is a molecule that creates an intense structure of water (the interaction trehalose-water is stronger than water-water) around itself and increases the surface tension of the solvent (kosmotrope). There is more than one theory on how the trehalose protect the enzyme like the formation of a thin pure-water shell around it which even compresses the structure disallowing an unfolding of the LDH or the creation of hydrogen bonds between the sugar and the enzyme, probably the correct behaviour is a synthesis of them [35] [16].

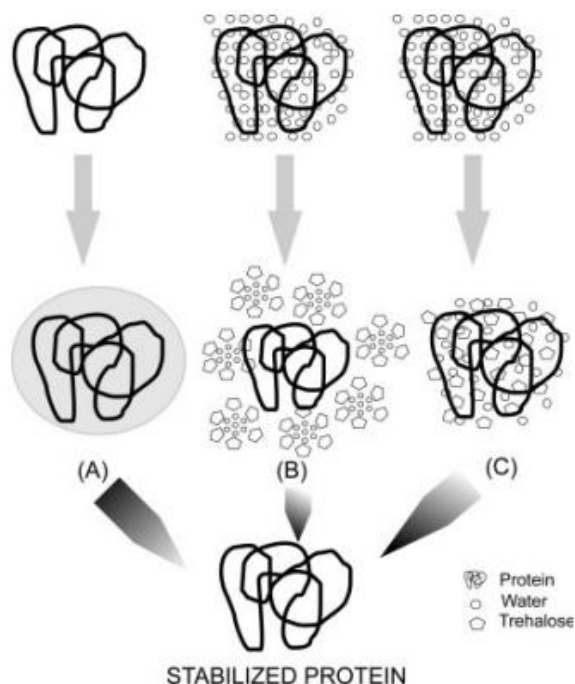


Figure 11: various theories on how the trehalose stabilize a protein [35]:

- a) through a vitrification shield,*
- b) through preferential exclusion and water entrapment,*
- c) by replacing the water into the structure*

The covalent immobilization between the enzyme and the functionalized support occurs through the reaction between the amino groups of lysine residues and the aldehyde groups present on the support surface. The Schiff's base is a reversible bond that can easily trigger hydrolysis. To avoid the leaching of the enzyme is important to reduce it to a more stable covalent bond using a reducing agent like Sodium Borohydride.

The Sodium Borohydride is a very strong reducing agent that not only attack the Schiff's base but also the LDH itself causing denaturation. For this reason, the amount of NaBH_4 , as well as the reaction time and conditions, must be optimized in order to stabilize the enzyme–support linkage while minimizing possible enzyme denaturation or loss of catalytic activity [36].

MATERIAL AND METHODS

11. SYNTHESIS OF SUPPORTS:

11.1. MOF MAF-7:

The following procedures are adapted from Wang Y, Luo X, Sun X, et al [22].

To obtain a crystalline structure of MAF-7, several attempts were carried out, modifying the synthesis procedure after each trial.

The source of the metal nodes used was zinc nitrate hexahydrate $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, which provided Zn^{++} ions to the structure. The organic bridge linker instead was 3-methyl-1,2,4-triazole. First of all, it is important to notice that the precursor of MOF MAF-7, 3-methyl-1,2,4-triazole, is an exceptional hygroscopic material. Therefore, in order to minimize moisture absorption and improve the reproducibility of the synthesis, it was dried overnight before use. Even though in MAF-7 synthesis, normally the reaction medium is made by DMF ($\text{HCOON}(\text{CH}_3)_2$), which is able to dissolve very quickly the reagents, in this study, the medium of reaction was made of milliQ water. In fact, DMF is toxic and cannot be used with the enzyme; therefore, it has been substituted by a “greener” solvent [37].

First try:

The synthesis of the material is based on mixing two distinct, separately prepared solutions, which we will refer to as Solution A and Solution B.

To begin, the solution A is prepared by weighing 50 mg of 3-methyl-1,2,4-triazole, which is then dissolved in 2.4 mL of MilliQ water. The mixture is kept under constant magnetic stirring until the solute is completely dissolved and the liquid appears perfectly clear.

While waiting for the first solution, the preparation of Solution B, dedicated to the metal precursor, is carried out in parallel. In this step, 60 mg of zinc nitrate hexahydrate are weighed and mixed with 2.5 mL of MilliQ water, again waiting for the complete dissolution of the salt under magnetic stirring. Once the solution A is ready, 100 μL of a 10% ammonia solution are slowly introduced under stirring. The ammonia plays a key

role here: it serves to deprotonate the organic linker, an essential chemical step that allows it to coordinate and bond effectively with the zinc ion.

At this point, the core reaction takes place: Solution B is added to Solution A drop by drop while maintaining gentle magnetic stirring. During this process, the interaction between the components generates an immediate cloudiness in the liquid, resulting in the appearance of a white powder in suspension.

Following this, the stabilization and crystal lattice growth phase begins; the suspension is transferred to a refrigerator at a controlled temperature of 4°C, where it is left to age for the next 24 hours.

The washing phase consists in a first centrifugation at 12000 rpm for 7 minutes in order to remove the reaction solvent. Then, the powder was redispersed in MilliQ water through the use of vortex and more importantly the ultrasonic bath. This step is necessary to remove the adsorbed species from the internal pores of MAF-7, which may not be completely eliminated by the first centrifugation step. The washing process was repeated two times and then the powder was let dry in a silica gel dryer.



Figure 12: Ultrasonic bath (from BioRender.com).

Second try:

The synthesis of the material is based on mixing two distinct, separately prepared solutions, which we will refer to as Solution A and Solution B.

To begin, the solution A is prepared by weighing 50 mg of 3-methyl-1,2,4-triazole, which is then dissolved in 2.4 mL of MilliQ water. The mixture is kept under constant magnetic stirring until the solute is completely dissolved and the liquid appears perfectly clear.

While waiting for the first solution, the preparation of Solution B, dedicated to the metal precursor, is carried out in parallel. In this step, 60 mg of zinc nitrate hexahydrate are

weighed and mixed with 2.5 mL of MilliQ water, again waiting for the complete dissolution of the salt under magnetic stirring. Once the solution A is ready, add a 10% ammonia solution slowly until the pH of the solution is 9, use a pHmeter to be precise and then bring the volume to 2,5 mL with milliQ water.

At this point, the core reaction takes place: Solution B is added to Solution A drop by drop while maintaining gentle magnetic stirring. During this process, the interaction between the components generates an immediate cloudiness in the liquid, resulting in the appearance of a white powder in suspension.

Following this, the stabilization and crystal lattice growth phase begins; the suspension is transferred to a refrigerator at a controlled temperature of 4°C, where it is left to age for the next 24 hours.

The washing phase consists in a first centrifugation at 12000 rpm for 7 minutes in order to remove the reaction solvent. Then, the powder was redispersed in MilliQ water through the use of vortex and more importantly the ultrasonic bath. The washing process was repeated two times and then the powder was let dry in a silica gel dryer.

Third try:

The synthesis of the material is based on mixing two distinct, separately prepared solutions, which we will refer to as Solution A and Solution B.

To begin, the solution A is prepared by weighing 50 mg of 3-methyl-1,2,4-triazole, which is then dissolved in 2.5 mL of MilliQ water. The mixture is kept under constant magnetic stirring until the solute is completely dissolved and the liquid appears perfectly clear.

While waiting for the first solution, the preparation of Solution B, dedicated to the metal precursor, is carried out in parallel. In this step, 60 mg of zinc nitrate hexahydrate are weighed and mixed with 2.4 mL of MilliQ water, again waiting for the complete dissolution of the salt under magnetic stirring. Once the solution B is ready, 100 µL of a 10% ammonia solution are slowly introduced under stirring. The ammonia plays a key role here: it serves to deprotonate the organic linker, an essential chemical step that allows it to coordinate and bond effectively with the zinc ion.

At this point, the core reaction takes place: Solution B is added to Solution A drop by drop while maintaining gentle magnetic stirring. During this process, the interaction between the components generates an immediate cloudiness in the liquid, resulting in the appearance of a white powder in suspension.

Following this, the stabilization and crystal lattice growth phase begins; the suspension is transferred to a refrigerator at a controlled temperature of 4°C, where it is left to age for the next 24 hours.

The washing phase consists in a first centrifugation at 12000 rpm for 7 minutes in order to remove the reaction solvent. Then, the powder was redispersed in MilliQ water through the use of vortex and more importantly the ultrasonic bath. The washing process was repeated two times and then the powder was let dry in a silica gel dryer.

Fourth try:

The synthesis of the material is based on mixing two distinct, separately prepared solutions, which we will refer to as Solution A and Solution B.

To begin, the solution A is prepared by weighing 50 mg of 3-methyl-1,2,4-triazole, which is then dissolved in 2.4 mL of MilliQ water. The mixture is kept under constant magnetic stirring until the solute is completely dissolved and the liquid appears perfectly clear.

While waiting for the first solution, the preparation of Solution B, dedicated to the metal precursor, is carried out in parallel. In this step, 60 mg of zinc nitrate hexahydrate are weighed and mixed with 2.5 mL of MilliQ water, again waiting for the complete dissolution of the salt under magnetic stirring. Once the solution A is ready, add a 10% ammonia solution slowly until the pH of the solution is 10.5, use a pHmeter to be precise and then bring the volume to 2.5 mL with milliQ water.

At this point, the core reaction takes place: Solution B is added to Solution A drop by drop while maintaining gentle magnetic stirring. During this process, the interaction between the components generates an immediate cloudiness in the liquid, resulting in the appearance of a white powder in suspension.

Following this, the stabilization and crystal lattice growth phase begins; the suspension is let rest for 30 minutes under gentle magnetic stirring.

The washing phase consists in a first centrifugation at 12000 rpm for 7 minutes in order to remove the reaction solvent. Then, the powder was redispersed in MilliQ water through the use of vortex and more importantly the ultrasonic bath. The washing process was repeated two times and then the powder was let dry in a silica gel dryer.

Fifth try:

The synthesis of the material is based on mixing two distinct, separately prepared solutions, which we will refer to as Solution A and Solution B.

To begin, the solution A is prepared by weighing 50 mg of 3-methyl-1,2,4-triazole, which is then dissolved in 2.4 mL of MilliQ water. The mixture is kept under constant magnetic stirring until the solute is completely dissolved and the liquid appears perfectly clear.

While waiting for the first solution, the preparation of Solution B, dedicated to the metal precursor, is carried out in parallel. In this step, 60 mg of zinc nitrate hexahydrate are weighed and mixed with 2.5 mL of MilliQ water, again waiting for the complete dissolution of the salt under magnetic stirring. Once the solution A is ready, add a 10% ammonia solution slowly until the pH of the solution is 10.5, use a pHmeter to be precise and then bring the volume to 2.5 mL with milliQ water.

At this point, the core reaction takes place: Solution B is added to Solution A drop by drop while maintaining gentle magnetic stirring. During this process, the interaction between the components generates an immediate cloudiness in the liquid, resulting in the appearance of a white powder in suspension.

Following this, the stabilization and crystal lattice growth phase begins; the suspension is transferred to a refrigerator at a controlled temperature of 4°C, where it is left to age for the next 24 hours.

The washing phase consists in a first centrifugation at 12000 rpm for 7 minutes in order to remove the reaction solvent. Then, the powder was redispersed in MilliQ water

through the use of vortex and more importantly the ultrasonic bath. The washing process was repeated two times and then the powder was let dry in a silica gel dryer.

11.2. MOF ZIF-90:

The procedure for the synthesis of ZIF-90 comes from Wang Y, Luo X, Sun X, et al [22]. It is based on the preparation of two solutions: A with the linker and B with the ion knots. The knots will be provided by zinc nitrate hexahydrate $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ while will be used imidazole-2-carboxaldehyde (ICA) as organic linker.

To prepare Solution A, 5 mL of Milli-Q water is pre-heated to approximately 50 °C in a beaker. Subsequently, 0.154 mg of imidazole-2-carboxaldehyde (ICA) is dissolved in the pre-heated water under magnetic stirring. The temperature is then increased to 80 °C, and the beaker is covered with aluminium foil to minimize evaporation-induced volume changes until the solution becomes completely clear. Simultaneously, Solution B is prepared by dissolving 0.12 mg of zinc nitrate hexahydrate in 5 mL of Milli-Q water, maintaining magnetic stirring until the solute is fully dissolved.

Once Solution A is ready, the heat source is turned off, allowing the mixture to cool to approximately 45 °C in order to not let too much of the solute to deposit while not creating a harsh environment for the enzyme. At this stage, a syringe filter is employed to remove any excess ICA that precipitates during the cooling process. Solution B is then introduced dropwise into Solution A under gentle stirring. The resulting suspension is stored in a refrigerator for 24 hours prior to the purification phase. To isolate the material, the mixture is centrifuged at 8000 rpm for 7 minutes to remove the solvent. The recovered precipitate is washed twice with Milli-Q water, utilizing vortex mixing and ultrasonication to ensure complete re-dispersion after each centrifugation cycle, before finally being left to dry.

11.3. SBA-15:

The template used in this case is P123 (Pluronic) a non-ionic triblock copolymer made by two PEO (poly-ethylene oxide) chains at both ends and a PPO (poly-propylene oxide) chain in the middle and TMB a micelle expander template.

The following procedure refers to the Piumetti et al [38].

160 mL of a 2 M HCl solution is prepared by mixing 31.53 mL of HCl with 128.47 mL of Milli-Q water inside a fume hood. To this acidic medium, 4 g of P123 is added under magnetic stirring at 40 °C and allowed to dissolve until the solution becomes completely clear. Subsequently, 1 g of TMB is introduced dropwise, and the mixture is stirred for 1 hour at 40 °C to optimize the pore size distribution and achieve a high surface area. The silica source is then added in the form of 8.5 g of TEOS, leaving the mixture under continuous stirring for the following 24 hours. This extended phase is critical to enable TEOS hydrolysis and condensation, which drives the formation of a rigid silica network around the hexagonally organized micelles of the templating agent.

The following day, the solution is transferred to an autoclave for hydrothermal treatment at 100 °C for 24 hours. The resulting precipitate is then isolated via centrifugation at 2000 rpm for 7 minutes and washed with Milli-Q water until the supernatant reaches a neutral pH, followed by two additional washes with ethanol. Finally, the synthesized silica is dried overnight at 60 °C and calcined at 550 °C for 5 hours to ensure the complete removal of the surfactant template.

The functionalization process will follow the post-synthesis method adapting the approach described by Saengdee et al [39].

- Generation of amino and epoxy groups:

The ratio between APTES e GPTMS (two organosilanes) will be 1:1 as was considered the best one following the results obtained by Chiara Vincenzi in her thesis. Add slowly to 20 mL of ethanol, 1 mL of APTES and 1 mL of GPTMS under magnetic stirring. When the solution is clear add 0,6667 g of m-SBA15, then let rest under stirring for 5 hours. Now recover the silica through centrifugation (5000 rpm, 10 minutes) and do two washes with milliQ water and

one with ethanol, using each time the ultrasonic bath for a better result. In the end filter it with Buchner funnel and let dry.

- Generations of diols:

Immerse the silica in 20 mL of a 0,1 M H_2SO_4 solution under hood, under magnetic stirring and at 85°C . Let rest at this temperature for 2 hours, then proceed to recover the silica through centrifugation (5000 rpm, 10 minutes) and do two washes with milliQ water and one with ethanol, using each time the ultrasonic bath for a better result. In the end filter it with Buchner funnel and let dry.

This phase helps to convert the epoxy groups generated before into diols while avoiding to denaturate the amino groups.

- Generation of glycoxylic groups:

Add the silica to 20,33 mL of a 0,1 M NaIO_4 and let rest for 2 hours under magnetic stirring. Now recover the silica through centrifugation (5000 rpm, 10 minutes) and do two washes with milliQ water and one with ethanol, using each time the ultrasonic bath for a better result. In the end filter it with Buchner funnel and let dry.

This phase facilitates the conversion of the diols in aldehydic groups through oxidation made by NaIO_4 .

12. MATERIAL ANALYSIS:

12.1. XRD:

The first step was of course made by Wilhelm Röntgen, who discovered the high-energy electromagnetic waves called x-radiation in 1895 [40].

In 1912 occurred the crystallographic breakthrough: it was demonstrated that x-rays could be diffracted by crystals [41].

The first successful analysis of an x-ray diffraction map is dated in June of 1913 (by William Henry Bragg and William Lawrence Bragg), where, through a mathematical model, the crystalline structure of sodium chloride was first described, laying the base of the modern structure analysis [42].

XRD is based on the scattering of electromagnetic waves by the electrons around the atoms of a lattice: more electrons are in the structure, the stronger is this scattering.

The electrons of a crystalline material irradiated by an X-ray beam become excited and start to oscillate at the same frequency of the incident radiation. In this way, they scatter electromagnetic waves in every direction. In amorphous structures, these scattered waves interfere destructively with each other in almost in every direction, producing no well-defined diffraction pattern. On the other hand, in case of a perfectly crystalline structure, where atoms are arranged periodically throughout the material, a constructive interference occurs along specific trajectories. When the path-length difference between waves scattered from adjacent parallel crystallographic planes is equal to an integer number of wavelengths, a strong signal (peak) is detected [43].

The phenomenon is governed by the Bragg's law (from the two scientists cited before):

$$n\lambda = 2d \sin(\theta)$$

Equation 4

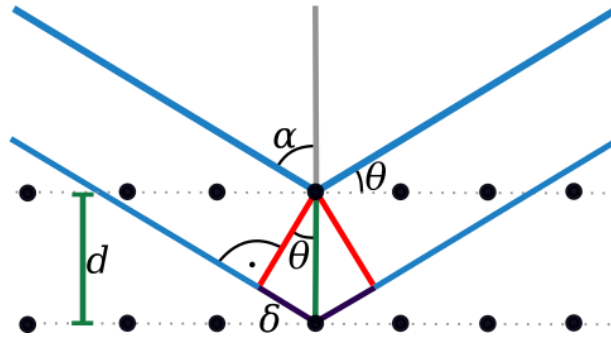


Figure 13: trajectories of the beam according to Bragg's law [44]

The Malvern Panalytical X'Pert Powder DY 3539 [45] is one of the most robust, flexible, and widely used powder diffractometers in materials science laboratories. One of its more useful features is that the sample holder remains perfectly horizontal and stationary, allowing the x-ray tube and the detector to move symmetrically, preventing slipping or shifting during the scan that would alter the analysis (θ - θ geometry). The goniometer radius is settled at 240 nm, offering a compromise between signal intensity and peak resolution. Moreover, it is equipped with the DOPS (direct optical encoding positioning system), a reading system on the goniometer circles that ensures highly reproducible step positioning down to micro-intervals. The x-ray tube is made of ceramic with a copper

(Cu) anode which generates a Cu-K α radiation with 0,15418 as average wavelength. It is equipped with CRISP technology to prevent air corrosion due to the ionized x-rays along the tube. The generator has 3 kW of power and operates at around 40-45 kV. To improve the efficiency of the analysis of powders a X'celerator detector is used, through the RTMS (real time multiple strip) technology it scans a wide angular range in just a few minutes with an excellent signal-to-noise ratio. Instead of capturing the signal point-by-point like older proportional detectors, the X'Celerator operates as an array of one hundred tiny detectors working in parallel [46].

12.2. FT-IR:

Fourier Transform Infrared (FT-IR) spectroscopy is a non-destructive analytical technique meant to probe the vibrational states of covalent chemical bonds. As a response, a fingerprint of the material is obtained. The phenomenon is based on the absorption of infrared photon, which induces transition between vibrational energy levels. These transitions are IR-active only when they are associated with a change in the molecular dipole moment. The intensity of the resulting absorption band is directly proportional to the square of this transition dipole moment.

Polar bonds undergo large dipole variations; on the contrary, non-polar bonds yield weak or entirely absent variation. Homonuclear diatomic gases like nitrogen (N₂) and oxygen (O₂) are entirely IR-inactive and invisible to the instrument due to their perfect symmetry. In fact, they are composed of two identical atoms, making their static dipole equal to zero. As a matter of fact, they can vibrate back and forth, but considering both ends are the same, this does not affect the symmetry. So, the two major components of air are invisible; the problem lies in CO₂ and H₂O, since they are both very evident in a FT-IR spectrum, exhibiting strong absorption bands that can interfere with the analysis. To minimize this contribution, the air is commonly removed from the sample cell through a vacuum pump [47].

There are two different types of vibrations of the bonds:

- Stretching vibration (changes the bond length):

The atoms move back and forth along the axis of the bond, changing the length of it; these movements can be both symmetrical and asymmetrical. In the former

case, both ends get closer and depart at the same time. In the latter instead, the atoms are not synchronized and an effect similar to a magnetic pulling is given [47].

- Bending vibration (changes the bond angle):

It alters the specific angle between two bonds. Because it takes less energy to bend a bond than to stretch it, these vibrations show up at lower frequencies on an FT-IR spectrum. There are two different categories of bending vibrations:

1. In-plane bending: divided in scissoring, where two atoms move toward and away from each other simultaneously, opening and closing the bond angle; and rocking where the angle between the two bonds does not change but it swings [47].
2. Out-of-plane bending: divided in twisting, where the atoms rotate out of the plane in different directions, and wagging, where both atoms bounce forward and backward together, breaking out of the molecule's structural plane [47].

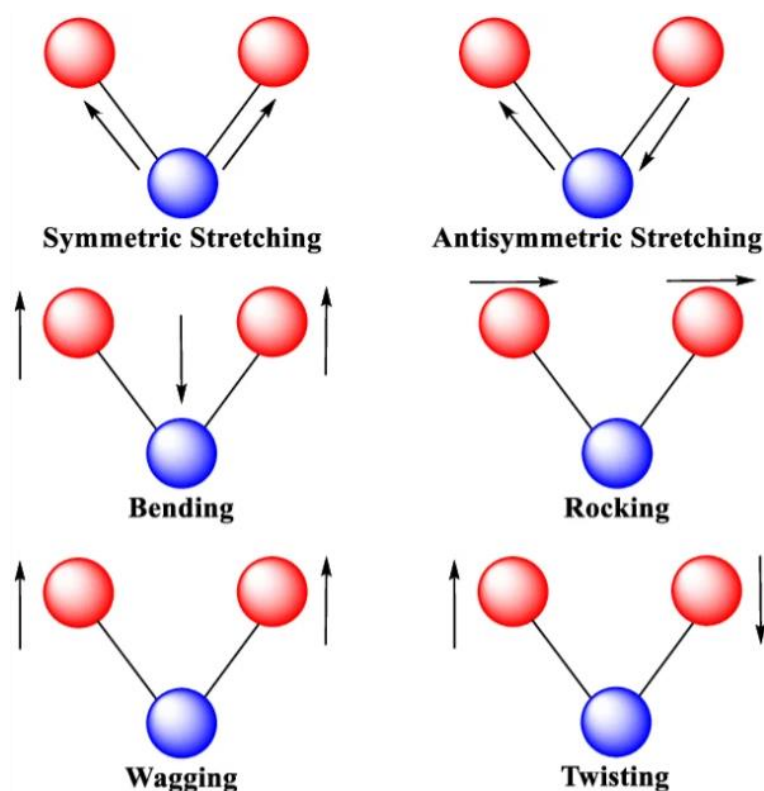


Figure 14: various type of bond's vibration [48]

12.2.1. PROCEDURE:

Performing an FT-IR analysis requires transforming the raw material into a solid, cohesive tablet. This process begins by thoroughly grinding the sample using a mortar and pestle to achieve a fine, uniform powder, which is then carefully transferred onto the disc of a press. To prevent any direct contact, and subsequent contamination, between the steel of the press and the sample, a layer of protective parafilm backing paper is placed on the disc beforehand. A razor blade is then used to distribute the material in a perfectly even layer across the surface before the entire press assembly is secured.

The material is compressed under a pressure of approximately 2 bar. Once the pressure is slowly released and the apparatus is dismantled, the newly formed tablet must be retrieved with extreme care to avoid cracking or structural failure.

Once the intact tablet is recovered, pincers are used to gently place it into a golden pellet-holder. After securing the holder, it is inserted into the detached glass cell of the FT-IR instrument. The cell is then remounted and meticulously aligned within the sample compartment to ensure that the infrared beam directly strikes the centre of the tablet. During the initial setup, the instrument's display screen allows the operator to monitor the system and distinguish the target material's spectrum from the ambient interference patterns typically generated by gaseous CO₂ and H₂O present in the atmosphere.

The analysis is conducted utilizing a Bruker Equinox 55 spectrometer (frequently designated as the IFS 55 Equinox). This specific instrument is highly regarded in research environments for its exceptional optical stability, modular architecture, and its capacity to interface seamlessly with specialized external components, such as custom high-vacuum lines.

To ensure data integrity, the system utilizes a high-precision, permanently aligned Michelson interferometer outfitted with advanced cube-corner mirrors. This specific optical configuration is crucial for laboratory environments, as it inherently prevents optical misalignments that might otherwise be triggered by ambient temperature fluctuations or routine mechanical vibrations, such as heavy footsteps and the operation of neighboring machinery.

His spectral range falls perfectly into the mid-infrared region (MIR) between 370 and 7500 cm^{-1} . The high resolution allows to distinguish sharp and close peaks. Moreover, the signal-to-noise ratio is extremely high allowing clean baselines even with a non-perfect pellet of the material. The detector used is a MCT (mercury cadmium telluride), a precise, quantum detector which needs cooling through liquid nitrogen (poured in the machine before the analysis). The platform is also equipped with a Bruker's OPUS software that can sustain more complex analysis through baseline correction and deconvolution algorithms.

To remove air from the cell a system of valves and glass tubes connected to a vacuum pump needs to be used, nitrogen liquid will also be used to help the pump to take out the air by refrigerating a glass condenser. The valves of the system must be opened slowly lowering the pressure one section at the time of the system to avoid damaging the pump.

12.3. NITROGEN PHYSISORPTION AT 77K:

As previously mentioned, for a support material suitable for enzyme immobilization the surface area or the dimension of the pores are fundamental characteristics. Characterizing the microstructure of a material is one of the cornerstones of modern material science. The texture of a solid surface is typically analysed via gas physisorption, which requires a clear distinction between physical adsorption and chemical adsorption. These processes represent different thermodynamic and kinetic regimes of gas-solid interactions.

The BET theory, first introduced in 1938 by Stephen Brunauer, Paul Hugh Emmett, and Edward Teller, represents a thermodynamic extension of Langmuir's monolayer adsorption model, which adapts the kinetic equilibrium of Langmuir's theory to describe infinite, multi-layered physical adsorption under the fundamental postulates:

- Homogeneous surface sites: adsorption occurs only on well-defined, energetically equivalent localized sites of the sample surface, with each site accommodating exactly one adsorbate molecule.

- Absence of lateral interactions: there are no lateral interactions or cooperative forces between adsorbed molecules within the same layer.
- Multilayer stack formation: gas molecules physically adsorb in infinite layers. A molecule in a layer serves as a localized adsorption site for a molecule in the next layer.
- Independent layer equilibrium: the Langmuir's model can be applied to each discrete layer. The uppermost layer is always in dynamic equilibrium with the gas phase, meaning the rate of molecular condensation equals the rate of evaporation.
- Differentiated layer energetics: the heat of adsorption for the first monolayer is constant and unique, reflecting the specific net interaction energy between the solid surface and the gas.
- Bulk liquefaction behaviour of higher layers: for the second and all subsequent layers, the heat of adsorption is assumed to be identical to the heat of condensation of the bulk adsorbate gas. Thus, the upper layers behave thermodynamically as a condensed liquid phase.
- Gas phase ideality: the gas phase behaves like an ideal gas.

The outcomes of gas physisorption are typically analysed through BET method to obtain the important parameters.

Before initiating the process, a pre-treatment is necessary to remove any adsorbed contaminants, like water or solvent, from the surface or the internal porosity of the material. It consists of a continuous flow of a slow, pure inert heated gas. In this way, the partial pressure of the desorbed contaminants is kept close to zero. The temperature for the degassing process should be chosen with extreme care to avoid structural modifications or degradation of the samples [49] [50].

The results of the analyses can be represented as graphs. One of the typical is an isotherm plot that reports the quantity of adsorbed gas (Y axis) as a function of relative pressure (X axis). To completely understand the informations provided by an adsorption isotherm, it is important to describe the different shapes that such curves can assume. According to IUPAC classification, there are 6 different types of standard isotherm.

Type I: characterized by a sharp, rapid rise at very low relative pressures, followed by a nearly horizontal plateau. This is typical for microporous materials. Moreover, it is split into two subcategories, “a” and “b” depending on whether the micropores are smaller than 1nm or are around 2nm. Type I(a) is associated with very narrow micropores, typically smaller than 1 nm, and displays a very steep uptake at extremely low relative pressures. Type I(b), instead, is generally observed for materials with wider micropores, with pore sizes closer to approximately 2 nm, and shows a less abrupt uptake shifted towards slightly higher relative pressures.

Type II: it is typical of the non-porous materials or with macropores so big that they act as a flat surface. It is characterized by a point of inflection called “B” which means the first layer of adsorbed gas has been completed and starts the multilayer phase, causing the curve to bend upward.

Type III: in this case the material has big macropores or no pores at all. Moreover, adsorbate–adsorbate interactions are stronger than adsorbent–adsorptive interactions, meaning that the molecules tend to cluster with each other rather than adsorb uniformly onto the surface. As a result, the isotherm displays a convex shape over the entire pressure range and does not present a clear point B.

Type IV: it is typical of mesoporous materials. This is characterized by an initial adsorption behaviour similar to that of Type II isotherms, followed by a sharp increase in the medium relative pressure range due to capillary condensation within the mesopores. At high relative pressures, the curve tends to level off, indicating the progressive saturation of the pore volume. There are 2 subcategories related to the dimensions of the mesopores: type IVa for pores wider than 4 nm and IVb for pores narrower than 4 nm. The IVa type moreover, shows a hysteresis loop since adsorption and desorption occur through different mechanisms during pore filling and emptying. In the IVb variations, the pores are too small to generate a hysteresis loop, so no pronounced hysteresis loop is present.

Type V: it is similar to the type III at low pressures, as they indicate weak interactions between the adsorbent surface and the adsorptive molecule. However, it is referred to

mesoporous or microporous materials. After an initial region with low adsorption uptake, a sharp increase occurs at intermediate relative pressures due to cooperative adsorption and pore filling. At higher relative pressures, the isotherm reaches a plateau corresponding to the saturation of the accessible pore volume. In many cases, Type V isotherms also display a hysteresis loop.

Type VI: it is the most particular one. It features a series of different steps due to the adsorption in different layers. Every step means a single distinct monolayer of the adsorbed specie. Therefore, this type of isotherm is generally observed only under very specific conditions, when the surface is sufficiently homogeneous and the adsorption process occurs layer by layer [51].

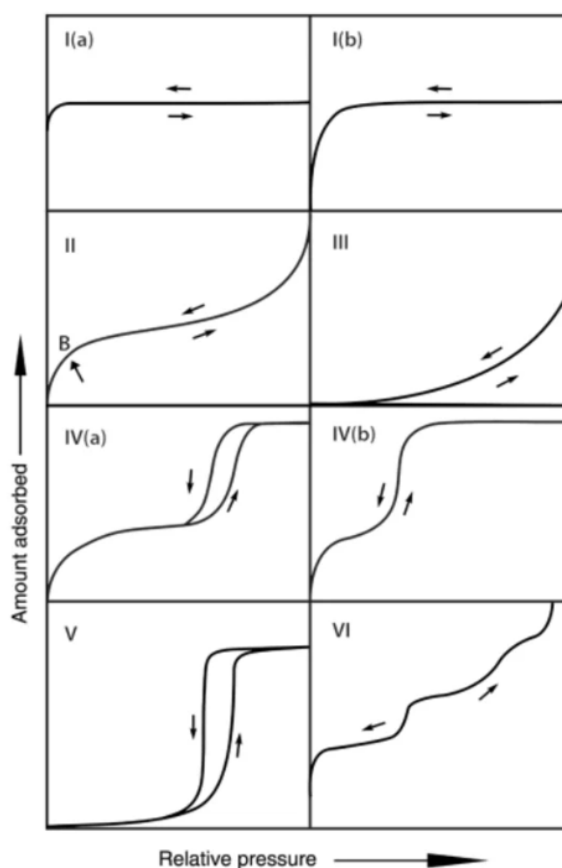


Figure 15: different types of isotherms according to IUPAC classification [51].

A hysteresis loop in the plot is essentially a "lag" between the path gas molecules take when accessing a pore versus when they leave it; this behaviour is generally associated with capillary condensation and evaporation phenomena occurring inside mesopores, and

it provides important information about pore geometry, pore connectivity, and pore-network effects. The hysteresis arises because the thermodynamic and kinetic conditions involved in pore filling during adsorption are different from those involved in pore emptying during desorption. There are 5 different types of hysteresis loops, according to IUPAC classification:

Type H1: the two branches are vertical and parallel; it normally means that the pores have a cylindrical shape.

Type H2: this type is driven by more complex network effects, often referred to as "inkbottle" pores (a wide pore cavity with a narrow neck). The desorption is delayed because the liquid gas is trapped inside the wide cavity until the narrow neck empties. It divides into two subcategories: H2a characterized by a steep desorption branch, which is caused by pore blocking or cavitation phenomenon; and H2b, which features a much more gradual desorption branch.

Type H3: the two branches are close to each other, and they do not reach a plateau at the end. It is typical for slit-shaped pores.

Type H4: it is similar to the H3 type, but the two branches have less slope and a high "uptake" at low relative pressure; it is typical of material with both micropores and mesopores like a hierarchical zeolite.

Type H5: a unique, uncommon shape that features a distinctive "step-down" on the desorption curve, creating an asymmetrical loop due to a structure with both open and partially blocked mesopores [52].

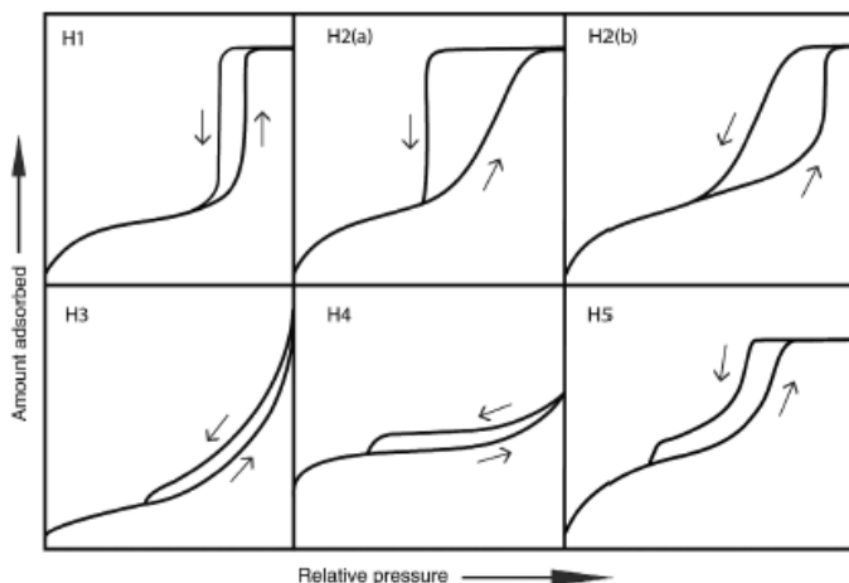


Figure 16: different types of hysteresis loops that is possible to find in an isotherm plot according to IUPAC [53].

12.3.1. PROCEDURE:

The procedure starts by taring the burette plus the funnel on the scale, adding between 50 and 100 mg of the material, marking this measure as measure 1, then re-weighting the burette with the cork and marking this as measure 2.

Prior to nitrogen physisorption analysis, the sample was subjected to a thermal pretreatment in order to remove water and other adsorbed species from the material surface and pore network. The burette containing the sample was equipped with the internal rod, taking care not to disturb the cork, and placed under a continuous nitrogen flow at 150 °C for 3 h.

After the pretreatment, the nitrogen flow was stopped and the burette was removed from the setup. The internal rod was carefully taken out while keeping the cork in place. The burette was then weighed, and this value was recorded as Weight 3. This measurement was used to determine the final dry mass of the sample after degassing, according to the following equation:

$$\text{Dry sample mass} = \text{Weight 3} - (\text{Weight 1} - \text{Weight 2})$$

where Weight 1 and Weight 2 refer to the previously recorded masses required for the tare correction of the burette and accessories.

After pretreatment, the burette was mounted on the analysis port of the instrument. Nitrogen adsorption–desorption measurements were performed at 77 K using an ASAP Accelerated Surface Area and Porosimetry System. The instrument consists of two main sections: a preparation/degas station, used for sample pretreatment, and an analysis station, where the adsorption–desorption experiment is carried out. During the analysis, the burette was immersed in liquid nitrogen to maintain the sample at 77 K throughout the measurement.

13. LDH ENCAPSULATION:

To maximize data collection and minimize experimental costs, initial LDH encapsulation trials are conducted using non-human LDH, specifically from a rat source. To ensure the stability and viability of the immobilized enzyme, a potassium phosphate buffer (pH 7.4) is prepared according to the following procedure.

First, 2.53 g of monobasic potassium phosphate (KH_2PO_4) and 14.18 g of anhydrous dibasic potassium phosphate (K_2HPO_4) are weighed and transferred into a 1 L beaker. Approximately 800 mL of Milli-Q water is then added under magnetic stirring. Once the salts are dissolved, a pH meter is immersed into the solution to monitor the pH value. If the pH is higher than 7.4, it is adjusted dropwise with diluted HCl; conversely, if the pH is lower, diluted KOH is used to correct it. Finally, the solution is brought to a total volume of 1 L with Milli-Q water.

13.1. LDH@MAF-7:

The procedure for the synthesis of LDH@MAF-7 comes from Wang Y, Luo X, Sun X, et al [22]. The synthesis of the material is based on mixing two distinct, separately prepared solutions, which we will refer to as Solution A and Solution B.

To begin, the solution A is prepared by weighing 50 mg of 3-methyl-1,2,4-triazole, which is then dissolved in 2.3 mL of MilliQ water. The mixture is kept under constant magnetic stirring until the solute is completely dissolved and the liquid appears perfectly clear,

then 0.04 mL of a 5 mg/mL solution of LDH is added (for the rat-LDH) or 0,096 mL of a 2,09 mg/mL of h-LDH.

While waiting for the first solution, the preparation of Solution B, dedicated to the metal precursor, is carried out in parallel. In this step, 60 mg of zinc nitrate hexahydrate are weighed and mixed with 2.5 mL of MilliQ water, again waiting for the complete dissolution of the salt under magnetic stirring. Once the solution A is ready, add a 10% ammonia solution slowly until the pH of the solution is 10.5, use a pHmeter to be precise and then bring the volume to 2.5 mL with milliQ water.

At this point, the core reaction takes place: Solution B is added to Solution A drop by drop while maintaining gentle magnetic stirring. During this process, the interaction between the components generates an immediate cloudiness in the liquid, resulting in the appearance of a white powder in suspension.

Following this, the stabilization and crystal lattice growth phase begins; the suspension is transferred to a refrigerator at a controlled temperature of 4°C, where it is left to age for the next 24 hours.

The washing phase consists in a first centrifugation at 12000 rpm for 7 minutes in order to remove the reaction solvent. Then add milliQ water in order to disperse the powder through the use of vortex and more importantly the ultrasonic bath. To obtain a better result repeat the washing process two time and then let dry the powder in a silica gel dryer.

13.2. LDH@ZIF-90:

The procedure for the synthesis of ZIF-90 comes from Wang Y, Luo X, Sun X, et al [22]. It is based on the preparation of two solutions: A with the linker and B with the ion knots. To prepare Solution A, 4.14 mL of pure Milli-Q water in a beaker is pre-heated to approximately 50°C. Once the temperature stabilizes, 0.154 mg of imidazole-2-carboxaldehyde (ICA) is added and dissolved under constant magnetic stirring. The mixture is then heated further to 80°C; during this stage, covering the beaker with tin foil

helps minimize any volume loss from evaporation. Stirring is maintained until the solution becomes completely clear.

In parallel, Solution B is prepared by dissolving 0.12 mg of zinc nitrate hexahydrate in 5 mL of Milli-Q water, allowing it to stir magnetically until the solute is fully dissolved.

Once Solution A is clear, the heat is turned off, allowing the mixture to cool down to roughly 45°C. At this temperature, the solution is passed through a syringe filter to remove any excess ICA that may have precipitated during the cooling process. Finally, the solution is blended with the enzyme, adding either 0.86 mL of a 5 mg/mL rat-LDH solution or 2 mL of a 2.09 mg/mL h-LDH solution.

Solution B is introduced dropwise into Solution A under gentle stirring, and the resulting suspension is stored in a refrigerator for 24 hours. Following this incubation period, the purification phase is initiated. The mixture is centrifuged at 8000 rpm for 7 minutes to remove the solvent, and the collected precipitate is washed twice with Milli-Q water, employing vortex mixing and ultrasonication to ensure complete re-dispersion after each cycle. The subsequent treatment depends on the intended downstream application: for structural characterization, the material is left to dry completely; conversely, for enzymatic activity assays, the support matrix containing the entrapped enzyme is directly re-dispersed in the previously prepared potassium phosphate buffer (pH 7.4).

14. LDH COVALENT BONDING:

The procedure followed for the immobilization was the one described by Cocuzza et al. [54]. Initially, a carbonate buffer (pH 9) is prepared by dissolving 1.91 g of NaHCO_3 and 0.24 g of Na_2CO_3 in 800 mL of Milli-Q water inside a 1 L beaker under magnetic stirring. The pH is monitored using a pH meter and adjusted dropwise with dilute solutions of HCl or KOH as required, before bringing the final volume to 1 L. A separate trehalose-containing buffer is then prepared by dissolving 3.40 g of trehalose in 30 mL of the newly prepared carbonate buffer within a Falcon tube.

The immobilization process is performed in parallel across three separate conditions to evaluate different reducing agent concentrations. For each condition, 0.048 mL of a 2,09

mg/mL enzyme solution is added to 4.5 mL of the trehalose-carbonate buffer under gentle magnetic stirring. A 0.5 mL aliquot is immediately withdrawn to assess the initial enzymatic activity. Subsequently, 100 mg of m-SBA15 is introduced to the mixture, which is then incubated at 4 °C for 3 hours under continuous gentle stirring. Shortly before the completion of this 3-hour incubation, a 0.7 mL aliquot is sampled and centrifuged at 500 rpm for 4 minutes to determine the activity of the supernatant. The analyzed supernatant volume is then replenished with fresh trehalose buffer to maintain the mixture volume.

Following the incubation phase, 6 mL of trehalose buffer is introduced into each vial, bringing the total volume of each reaction mixture to approximately 10 mL. Three distinct solutions of NaBH₄ in trehalose buffer are freshly prepared at concentrations of 10, 5, and 2.5 mg/mL. Because the reaction of NaBH₄ is highly rapid, resulting in immediate and visible hydrogen gas (H₂) evolution, these solutions are prepared immediately prior to use.

Next, 0.1 mL of each respective NaBH₄ solution is added dropwise to the three separate vials containing the immobilized enzyme, and the reaction is allowed to proceed for 18 minutes under gentle magnetic stirring. Finally, the mixtures are centrifuged at 2000 rpm for 7 minutes, and the resulting precipitates are washed twice with a potassium phosphate buffer (pH 7.4). The purified materials are then re-dispersed in the same phosphate buffer, with the final suspension volume calculated according to the required target concentration (C).

$$V_{buff} = \frac{m_{enz\ on\ sba}}{C} \quad m_{enz\ on\ sba} = \frac{m_{prot\ fin}}{1 - \frac{A_{3h}}{A_0}} \quad m_{prot\ fin} = C_{fin=in} V_{tot}$$

Equation 5, 6 and 7

15. BRADFORD PROTEIN ASSAY:

Bradford protein assay is a widely utilized colorimetric method to quantify total protein concentration in a sample solution. First introduced by Marion M. Bradford in 1976, this technique converts physical dye-protein binding into a measurable optical signal.

By utilizing the triphenylmethane dye Coomassie Brilliant Blue G-250 ($C_{47}H_{48}N_3NaO_7S_2$), this assay slashed sample processing times from hours to a 10-minute window [55].

Under the highly acidic conditions of the Bradford reagent, done in phosphoric acid, the dye is predominantly present in its doubly protonated, cationic state, presenting itself as a brown coloured liquid.

The presence of proteins alters the equilibrium of the solution; the binding between the dye and the protein shifts the equilibrium to the anionic form, with an instantaneous colour change to blue and a peak in absorbance at 595 nm. The intensity of this absorbance is proportional to protein concentration within the linear range of the assay.

The cationic brown dye donates its free electron to ionizable, positively charged groups on the protein. The redistribution of the charge causes a localized disruption of the protein's native tertiary fold, partially denaturing the macromolecule and exposing its hydrophobic internal part.

Once exposed, these hydrophobic pockets interact with the nonpolar, hydrophobic aromatic rings of the dye. These van der Waals forces form the initial non-covalent stabilizing anchors between the dye and the protein backbones.

This hydrophobic alignment places the positive amine sidechains of the protein close to the negatively charged sulfonic acid groups of the dye. Electrostatic salt bridges are then established, stabilizing the complex in its unprotonated blue anionic state [56].

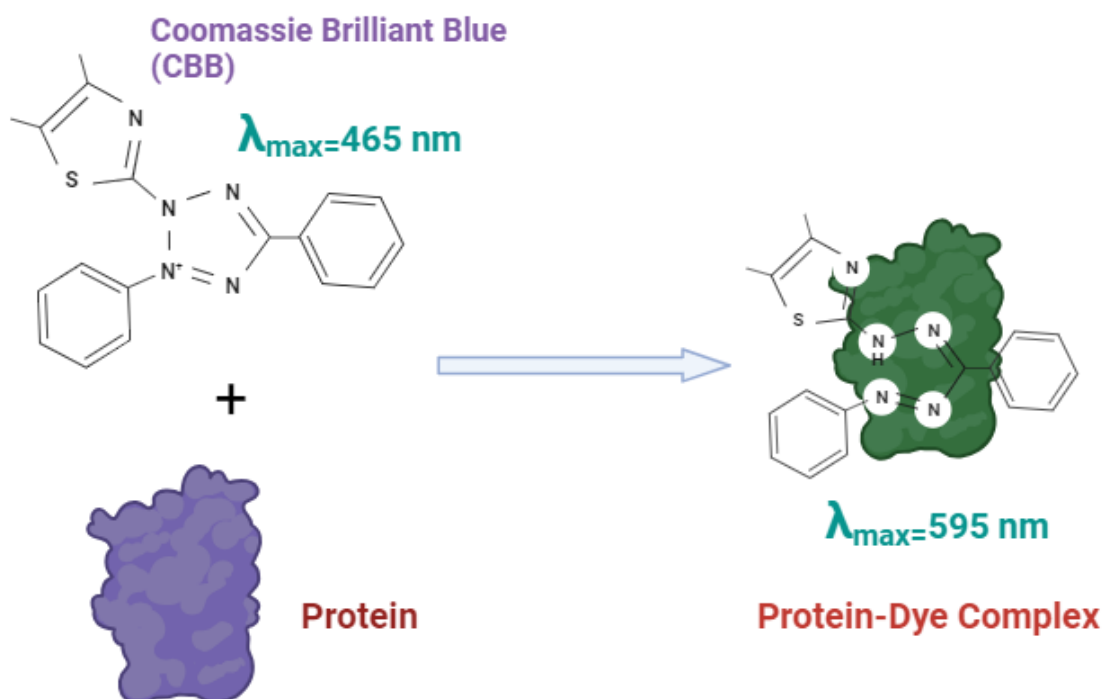


Figure 17: Reaction of the Bradford protein assay [57].

The accuracy of the test relies on the preparation of an appropriate protein standard curve. Ideally, the standard protein should be identical or closely similar to the protein present in the sample, since different proteins may produce different color responses in the assay. In the absence of a sample-specific standard, bovine gamma globulin (BGG) and bovine serum albumin (BSA) are commonly used as reference proteins. BGG is often considered suitable when a colour response representative of many proteins is required, whereas BSA can also be effectively used for the purposes of this thesis [54].

15.1. PROTEIN REPRESENTATIVE STANDARD CURVE:

The phosphate buffer (pH 7.0) was prepared by dissolving 0.093 g of K_2HPO_4 and 0.063 g of KH_2PO_4 in approximately 15 mL of Milli-Q water. After adjusting the pH to 7.0 using aqueous solutions of NaOH or HCl, the final volume was brought to 20 mL with Milli-Q water. Protein concentration was determined via the Bradford assay using a reagent composed of Coomassie Brilliant Blue G-250, phosphoric acid, and methanol. All spectrophotometric measurements were performed at 595 nm using cuvettes

compatible with a final assay volume of 2.2 mL, with bovine serum albumin (BSA) serving as the reference standard.

To establish the baseline, the spectrophotometer was blanked using a mixture of 2 mL of Bradford reagent and 0.2 mL of the prepared phosphate buffer. This blank solution was sealed with Parafilm, gently inverted to ensure homogeneity without spilling, and incubated for 10 minutes in the dark prior to measurement. A calibration curve was constructed using seven distinct BSA standard solutions prepared in the phosphate buffer, spanning a concentration range of 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL. Each calibration point was prepared and analysed in triplicate by combining 2 mL of the Bradford reagent with 0.2 mL of the respective BSA standard solution inside the cuvette. Following the same mixing and 10-minute dark incubation protocol used for the blank, the absorbance of each sample was recorded at 595 nm.

16. SPECTROPHOTOMETER ANALYSIS:

To analyse enzyme kinetics, ultraviolet-visible (UV-Vis) absorption spectrophotometry is still the standard method due to its exceptional reproducibility, low cost, and non-destructive nature. The spectrophotometry provides a real-time picture of the reaction, divided frame by frame in time, by monitoring changes in the optical properties of the sample.

The principle behind this analysis lies in the absorption of monochromatic light by the sample. When incident light of a specific intensity (I_0) encounters the sample, a portion is absorbed, and the remaining light is transmitted with a certain intensity (I). The ratio of transmitted light to incident light defines the transmittance (T):

$$T = \frac{I}{I_0}$$

I : intensity of transmitted light; I_0 : intensity of incidence.
Equation 8

The transmittance can be converted to absorbance through the following equation:

$$A = -\log_{10}(T)$$

Equation 9

In this assay, the variation in absorbance was monitored at 340 nm, corresponding to the absorption maximum of NADH. As highlighted at the start of this paper, during the enzyme reaction of LDH, NADH is consumed, producing NAD⁺, which, on the contrary, does not absorb at 340 nm. Therefore, following the evolution of the value of A₃₄₀ dependent on the concentration of NADH, the kinetics of the enzymatic reaction can be derived.

To calculate the enzyme activity, the time-dependent variation in absorbance was analysed from the graph $\Delta A / \Delta t$. At the beginning, for small concentrations of substrates, it is possible to have some disturbances. Once it settles, the line starts to drop, and it is fundamental to take the value of the slope, which represents the rate of NADH consumption. With the advance of time, the graph will flatten out [59].

The following formula was used to calculate the enzymatic activity:

$$\text{Activity (U/mL)} = \frac{\left(\frac{\Delta A}{\Delta t}\right) \times V_t \times \text{FD}}{\epsilon \times l \times V_s}$$

Equation 10

$\Delta A / \Delta t$: slope of the plot.

FD: dilution factor.

V_t: total volume of the cuvette.

V_s: volume of the enzymatic solution.

ϵ : molar extinction coefficient of the analyte (in this case, NADH), equal to 6.22 mM⁻¹ cm⁻¹.

l: optic length (1 cm for cuvettes).

1 U is defined by the conversion of 1 μ mol in 1 minute.

The spectrophotometer used is Jasco V-730 (figure &). It is a compact benchtop UV-Visible (UV-Vis) spectrophotometer designed for high-precision optical measurements. In this machine, the light beam is split into two different paths, one travels through the sample, while the other one goes simultaneously through the “blank” sample. This feature allows the analysis to be more stable and compensates for any fluctuations in the light source. The range of wavelength analysed is from 190 to 1100 nm, covering a very

large range of analysis, and will be put at 340 nm as was written before. Also, the light source is dual; there is a deuterium lamp (UV) and a halogen lamp (visible). The position designated to the cuvettes is equipped with an integrated stirring mechanism. By inserting a suitable magnetic anchor in the cuvette, it is possible to stir the sample, improving the mixing and therefore the reaction in case of a time analysis of an enzyme. Moreover, the station is supplied by a temperature-controlled bath which can maintain the buffer used at 37°C, a temperature that simulates the environment inside the human body [59] [60].

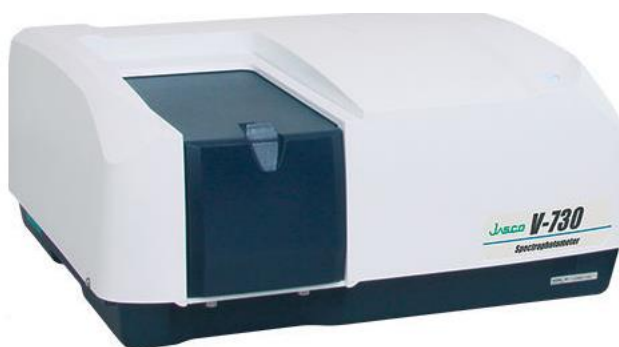


Figure 18: Jasco V-730 spectrophotometer used in FERMAN lab at PoliTO [59].

16.1. RESIDUAL ACTIVITY and IMMOBILIZATION YIELD:

In order to determine the immobilization yield, it is necessary to take a small amount of the supernatant after the immobilization process (around 0,6 mL). The supernatant has to be tested through the Bradford assay by mixing 0.2 mL of it with 2 mL of the Bradford reagent in a cuvette. The spectrophotometer was blanked as described before and also the preparation of the sample was analogous. To ensure reliability, the test has been run in duplicate. The protein concentration in the supernatant was calculated from the BSA calibration curve according to the following equation:

$$C_s = \frac{Abs_{media}}{K}$$

Equation 11 (K equals to the slope of the intercept of the protein standard curve).

$$C_{sup} = \frac{C_s V_{epp}}{V_{enz}}$$

Equation 12

$$I_{yield} = 100 - C_{sup} V_{enz}$$

Equation 13

To calculate the retained activity, the activity of the immobilized enzyme was compared with that of the free enzyme under the same assay conditions. First, an aliquote of the enzyme used for the immobilization process was diluted in pH 7.4 phosphate buffer in order to obtain a solution with a concentration of 0.01 mg/mL.

The enzymatic activity was measured by UV-Vis spectrophotometers at 340 nm, monitoring the consumption of NADH during the reaction, as was said before. The reaction mixture was prepared in a cuvette equipped with a suitable magnetic stir bar. For the blank cuvette, 2.71 mL of 0.1 M pH 7.4 phosphate buffer, previously equilibrated at 37°C, were mixed with 0,1 mL of a pre-made solution 7 mM of NADH and 0,1 mL of a pre-made solution 30.1 mM of pyruvate. The sample cuvette was prepared using the same reaction mixture of the blank. After blank correction using the instrument software, 0.1 mL of the enzyme solution were injected in the sample cuvette and the “absorbance in real time analysis” was run. From the graph obtained, the linear portion of the curve was selected, and the slope was extracted and then used in the equation & to obtain the activity.

The same procedure was then repeated using the immobilized enzyme, appropriately suspended in phosphate buffer to obtain an equivalent enzyme concentration of 0.01 mg/mL. The retained activity was calculated by comparing the activity of the immobilized enzyme with that of the free enzyme. To obtain the residual activity divide the activity of the immobilized enzyme by that of the free enzyme.

$$Residual\ Activity = \frac{Acitvity_{imm\ enz}}{Activity_{free\ enz}}$$

Equation 14

16.2. LEACHING TEST and KINETIC CURVE:

The leaching test will be run on the three different LDH@SBA-15 crafted using different concentrations of NaBH₄ of 0.025 mg/mL, 0,05 mg/mL and 0.1 mg/mL. In this phase the focus will be the LDH@SBA-15 because of the disappointing results of the LDH@MOFs that will be discussed in the Results and Conclusions sections.

The test will run for three hours, with 5 sampling times: 0, 30, 60, 90 and 180 minutes. It starts by centrifuging the suspension of LDH@SBA-15 at 2000 rpm for 7 minutes and removing all the supernatant, precisely measuring its volume. The supernatant is quickly tested at the spectrophotometer using it as the enzymatic solution in the same assay previously described.

After the removal of the supernatant, 3 mL of 0.1 M pH 7.4 phosphate buffer were added to the LDH@SBA-15 samples and they were put under refrigeration, through an ice-cold bath, and under magnetic stirring thanks to a little anchor. The same procedure was repeated at each selected time point for each LDH@SBA-15 sample prepared with the different NaBH₄ concentrations. Moreover, triplicates of each analysis were performed, to ensure better precision.

Through the equation &, the activity of each supernatant is calculated. The activity value was multiplied by the volume of the supernatant removed at each time point, then it is divided by the activity of the LDH@SBA-15 at the beginning of the process to obtain the leaching percentage.

$$Leaching\% = \frac{Activity_{sup}}{Activity_{imm\ enz}}$$

Equation 15

To define the kinetic curve, the activity has to be tested with different concentrations of pyruvate. For this purpose, 8 different pyruvate solutions were prepared with the following concentrations as it is written in the following table:

Table 3: various concentrations of pyruvate for a kinetic curve.

C of the solution (mM)	C in the cuvette (mM)
0,1505	0.005
0.7525	0.025
1.505	0.05
3.01	0.1
4.515	0.15
9.03	0.3
18.06	0.6
30.1	1

The spectrophotometric procedure was the same as that described for the previous activity assays, and the same reagent volumes were used in the cuvette. Each measurement was performed in triplicate for each pyruvate concentration. The initial linear portion of the absorbance decrease at 340 nm was used to calculate the initial reaction rate.

To have a further comparison, another kinetic curve will be made, in the presence of DMSO, which is a solvent used to insert inhibition agents in the cuvette. In this case, it will be only the curve with DMSO without inhibitors. In this case, the assay composition was slightly modified in order to keep the final volume constant while allowing the addition of DMSO. The amount of buffer will be reduced to 2.7 mL and 0.01 mL of DMSO will be added. The procedure, apart from this slight modification, is the same described before.

RESULTS

17. XRD of the MATERIALS:

17.1. MAF-7:

The procedures attempted did not grant a crystalline structure of MOF MAF-7, as it is possible to see from figure 20 to figure 24. the diffractograms of the synthesised materials show broad and poorly defined signals, rather than the sharp reflections expected for crystalline MAF-7. This indicates that the obtained materials are predominantly amorphous or poorly crystalline. In the crystalline reference pattern of MAF-7, well-defined diffraction peaks are observed, reflecting the periodic arrangement of the framework. In contrast, the synthesized samples show broad diffuse bands, commonly referred to as amorphous halos, which suggest the loss of long-range structural order. Therefore, although the structure maintains its core, formed by the tetrahedron of Zn^{++} linked by the azolate, the periodic organization required to generate a crystalline MOF lattice was not achieved under the tested synthetic conditions. The peaks of the XRD spectrum are replaced by a broad, smooth, diffuse “amorphous hump” or halo.

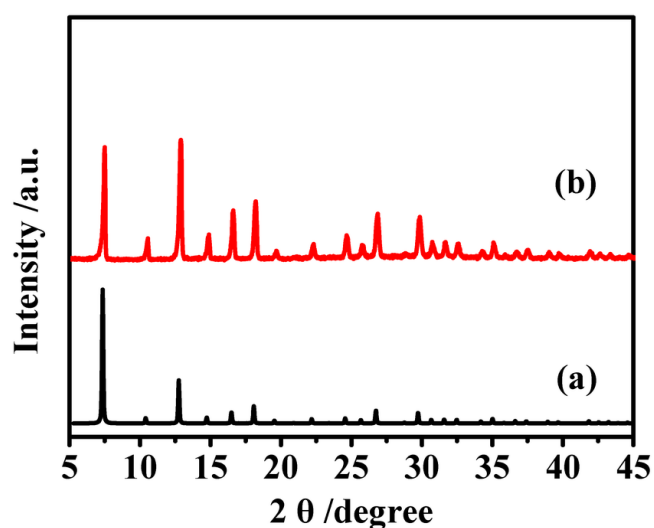


Figure 19: (a) PXRD of simulated crystalline MAF-7, (b) PXRD of synthesised crystalline MAF-7 [61].

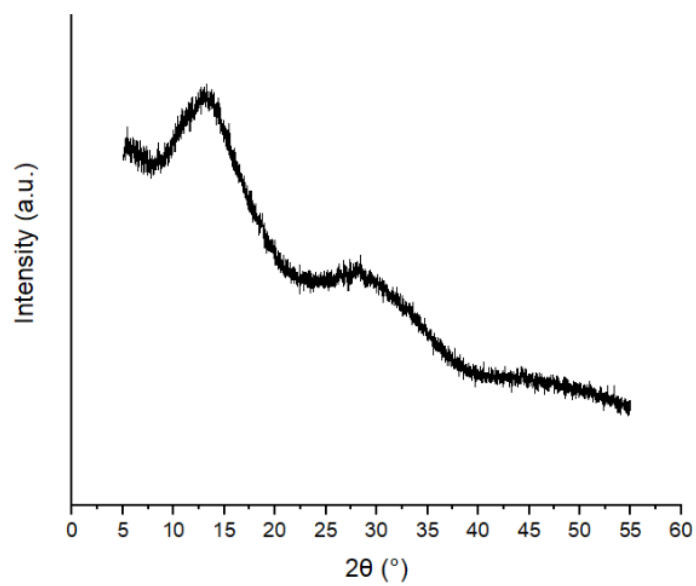


Figure 20: PXRD of synthesised amorphous MAF-7 through the first procedure.

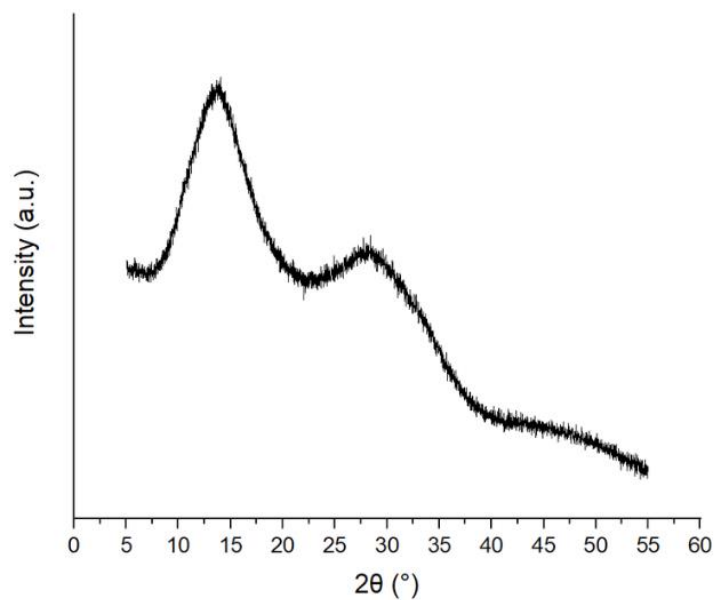


Figure 21: PXRD of synthesised amorphous MAF-7 through the second procedure.

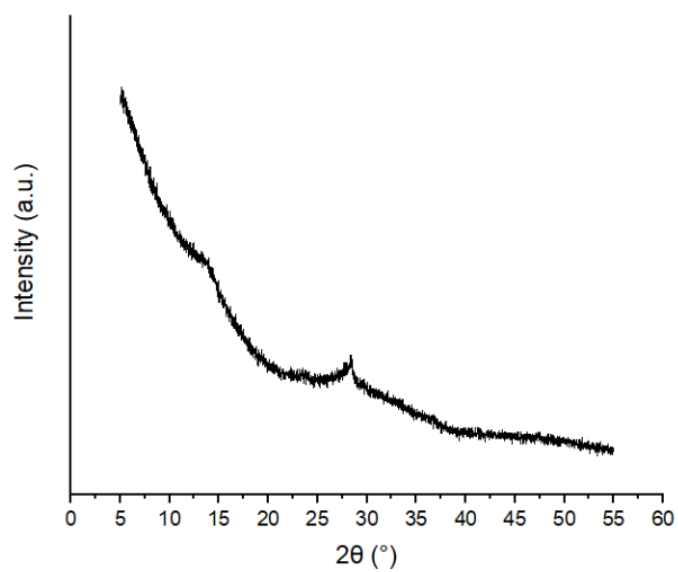


Figure 22: PXRD of synthesised amorphous MAF-7 through the third procedure.

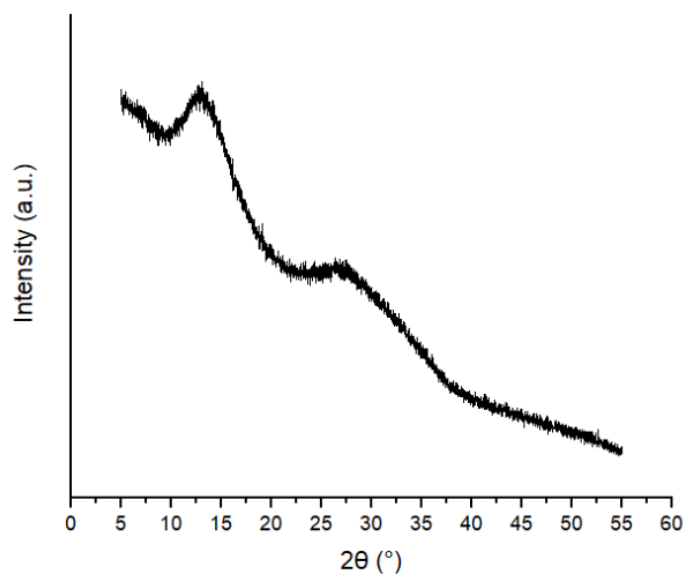


Figure 23: PXRD of synthesised amorphous MAF-7 through the fourth procedure.

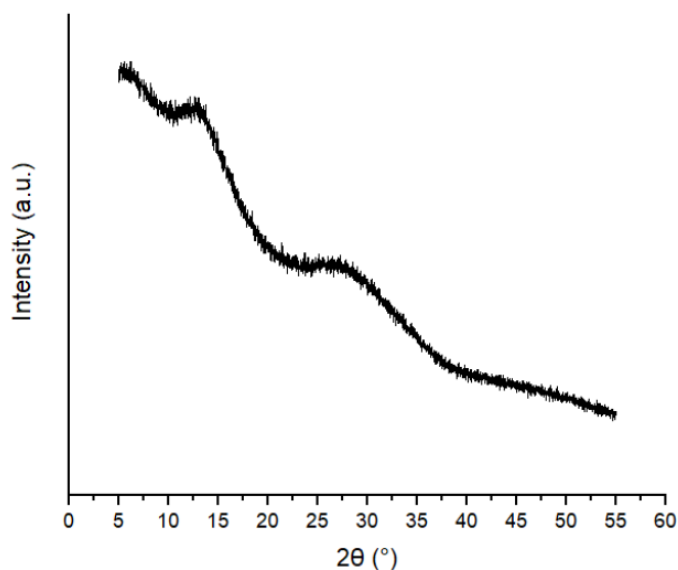


Figure 24: PXRD of synthesised amorphous MAF-7 through the fifth procedure.

The PXRD patterns obtained from the different synthetic procedures show comparable amorphous or poorly crystalline features, although some differences can be observed among the samples. Broad diffuse bands are observed, mainly in the low-angle region between approximately 10° and 18° 2θ and, in some cases, around 27° – 32° 2θ .

Among the tested procedures, the second sample shows the most defined diffraction features, with a relatively intense broad band around 14° 2θ and a second broad contribution around 28° – 31° 2θ . This suggests that this material may retain a higher degree of local structural organization compared with the other amorphous samples. The first and fourth procedures also show broad bands in similar angular regions, indicating partial short-range order, although the signals are less defined. In contrast, the third procedure gives the least structured pattern, dominated by a decreasing background and only weak, poorly resolved features, suggesting a higher degree of amorphization. The fifth sample shows an intermediate behaviour, with broad and diffuse signals similar to those observed for the fourth procedure but less clearly resolved.

Overall, the procedure chosen for the LDH@MAF-7 was the fifth one, because is the most similar to the article consulted and granted a better conversion than the others.

17.2. ZIF-90:

The synthesised ZIF-90 (figure 26) showed a highly crystalline structure, as evidenced by the presence of sharp and well-defined reflections in the XRD pattern. The peaks on the XRD diffractogram are in good agreement with the reference patterns from the bibliography (figure 25).

The first peak at circa $7,3^\circ$ is the signature peak of SOD-type ZIFs; it corresponds to the (011) crystal plane.

Table 4: peaks of XRD spectrum of ZIF-90 [62]

2 θ	Plane
$7,3^\circ$	011
$10,4^\circ$	002
$12,7^\circ$	112
$14,7^\circ$	022
$16,4^\circ$	013
$18,0^\circ$	222

The absence of any broad amorphous halo suggests that no significant amorphous fraction is present in the sample, together with high purity of the sample. Moreover, the narrow width of the diffraction peaks indicates a highly ordered crystalline lattice and relatively large, well-defined crystallite domain sizes.

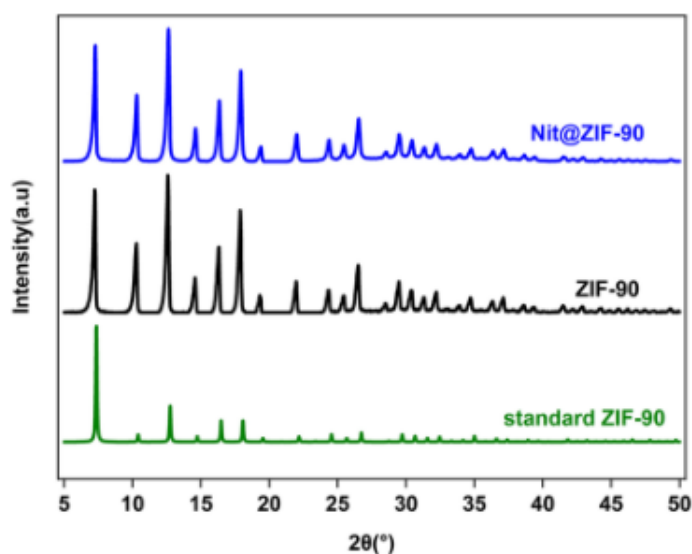


Figure 25: PXRD of crystalline ZIF-90 [62].

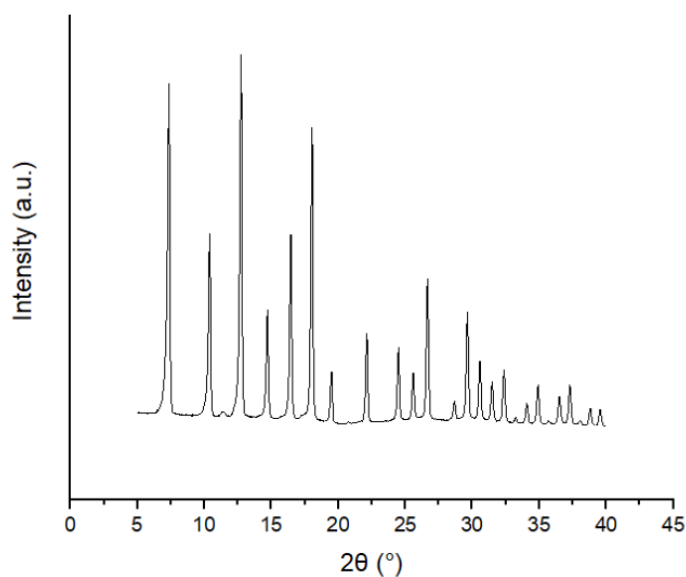


Figure 26: PXRD of synthesised crystalline ZIF-90.

17.3. SBA-15:

The structural fingerprint of SBA-15 is the intensive low-angle, sharp peak observed at around 1° ; it corresponds to the (100) reflection plane of a two-dimensional hexagonal lattice (figure 27, 28, 29). The presence of this peak in both the functionalized and non-functionalized SBA-15 means that the mesoporous structure does not change with the functionalization process. One sign of the successful functionalization is the drop of intensity of the main peak. This decrease can be attributed to the incorporation of organic groups inside the mesopores, which reduces the electron density contrast between the silica walls and the empty channels. Consequently, the diffraction signal becomes weaker. Therefore, the preservation of the low-angle peak, together with its reduced intensity, supports the successful functionalization of SBA-15 without collapse of the mesoporous structure.

The broad contribution observed between 2.5° and 5° (figure &) may be related to a combination of pore form factor (the scattering profile of individual cylindrical channels), partial loss of long-range mesoscopic order, and interparticle effects. In the functionalized sample (figure &), this feature appears less pronounced, likely due to the presence of organic groups inside the pores, which attenuate the scattering contrast.

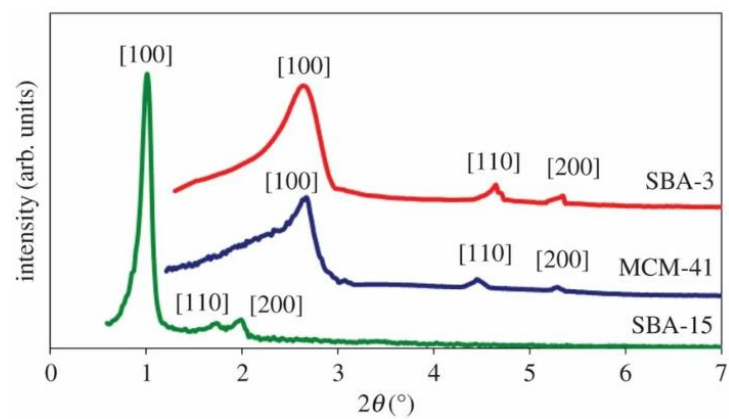


Figure 27: SAXRD (small angle XRD) of SBA-15 [63].

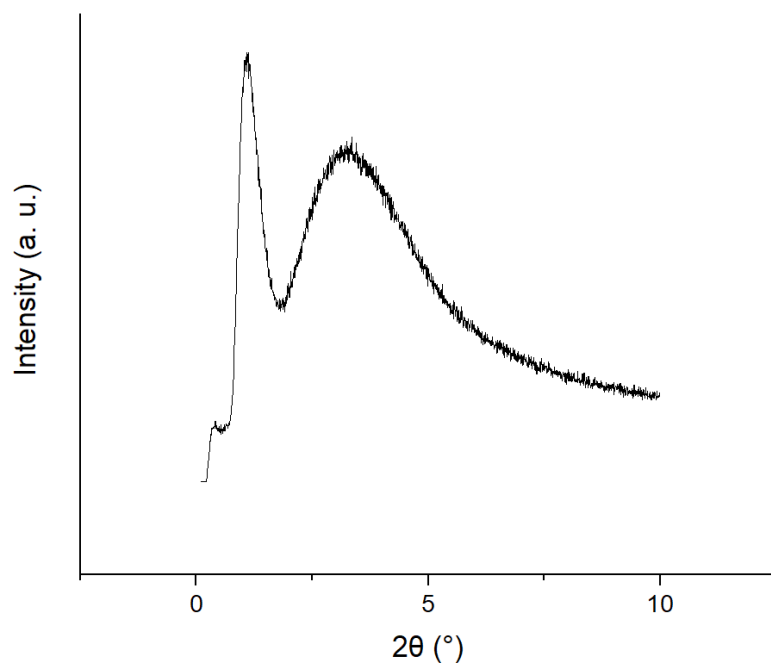


Figure 28: SAXRD of synthesised non-functionalised SBA-15.

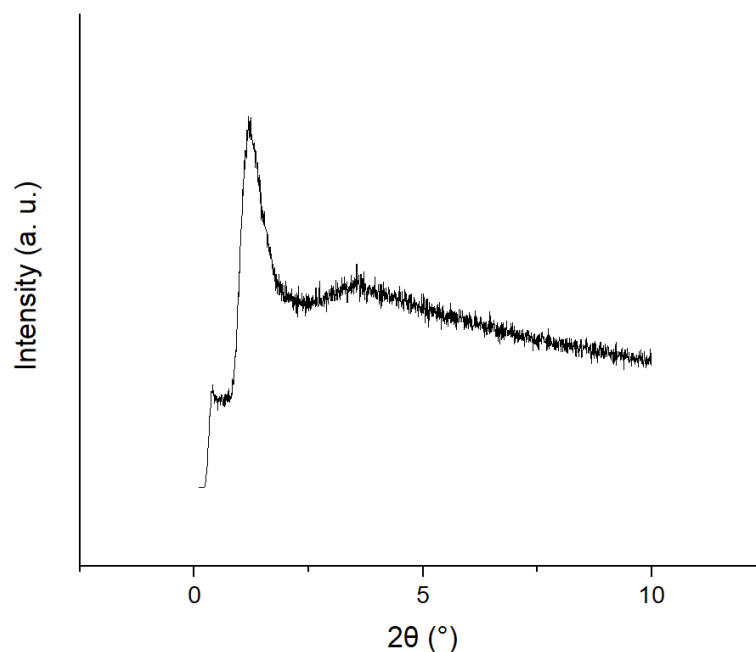


Figure 29: SAXRD of synthesised functionalised SBA-15.

18. FT-IR of the MATERIALS:

18.1. MAF-7:

FT-IR analysis was performed to further investigate the chemical composition of the powder obtained from the fifth synthesis procedure, which appeared amorphous according to PXRD analysis. Although FT-IR spectroscopy cannot confirm the presence of long-range crystalline order, it can provide information about the functional groups present in the material. It confirmed that the powder displays the main vibrational bands associated with the 3-methyl-1,2,4-triazole linker, supporting the formation of a MAF-7-like material (figure 30). In the table 5 the principal peaks of the spectra are described. The broad “hump” around 3400 derives from the water trapped in the structure, which is another clue for the amorphous structure of the MAF-7. The double peak at 2950-2850 corresponds to the symmetric and asymmetric stretching vibrations of the methyl group, $-\text{CH}_3$. The presence of these bands supports the incorporation of the 3-methyl-1,2,4-triazole linker in the material. The intense bands observed between 1500 and 1400 cm^{-1} are mainly associated with C–N and C=N stretching vibrations of the triazole ring. In

case of a crystalline material, they should be more defined. In this case, their broad and partially overlapping profile is consistent with the poorly ordered nature suggested by PXRD analysis. Finally, the band around 740 cm^{-1} can be assigned to out-of-plane bending vibrations of the triazole ring.

Table 5: analysis of the peaks of the FT-IR spectrum of MAF-7 [24] [64] [65].

Wavenumber (cm^{-1})	Peak
3100	stretching of the C-H bond of the triazole ring
2950-2850	symmetric and antisymmetric stretching of the C-H bond of the methyl group $-\text{CH}_3$
1500-1400	stretching of the C-N and C=N
740	bending out of plane of the triazole ring

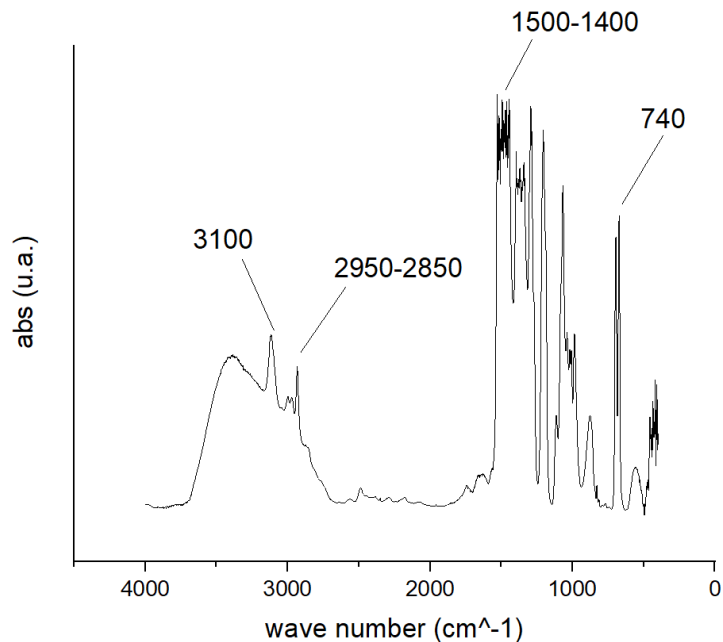


Figure 30: FT-IR spectrum of amorphous MAF-7.

Overall, the FT-IR spectrum confirms the presence of the characteristic functional groups of the organic linker, supporting the formation of an amorphous or poorly crystalline MAF-7-like material obtained through the fifth procedure.

18.2. ZIF-90:

The following table (6) analyses the peaks of the FT-IR spectrum of ZIF-90 (figure 31). The spectrum shows the characteristic vibrational bands of the imidazole-2-carboxaldehyde linker, supporting the successful formation of ZIF-90.

The sharp and clear peak of the aldehyde fingerprint at 1700 cm^{-1} shows that the aldehyde groups line the pores of the structure and they did not undergo an oxidation process, while the other peak traceable to the aldehyde groups is at 2850, and it distinguishes the ZIF-90 from ZIF-8. The sharpness and separation of the peaks between 1400-1450 show that the organic linker is well incorporated and chemically preserved within the material. Together with the PXRD results, the FT-IR spectrum supports the successful synthesis of crystalline ZIF-90. Finally, the band around 750 cm^{-1} can be assigned to out-of-plane bending vibrations of the imidazole ring.

Table 6: analysis of the peaks of the FT-IR spectrum of ZIF-90 [66] [67].

Wavenumber (cm^{-1})	Peak
3100	stretching of the aromatic C-H bond of the imidazole ring
2850	stretching of the C-H bond of the aldehyde group
1700	stretching of the double bond C=O of -CHO
1450-1400	stretching of the double bonds C=C and C=N
750	bending out of plane of the imidazole ring

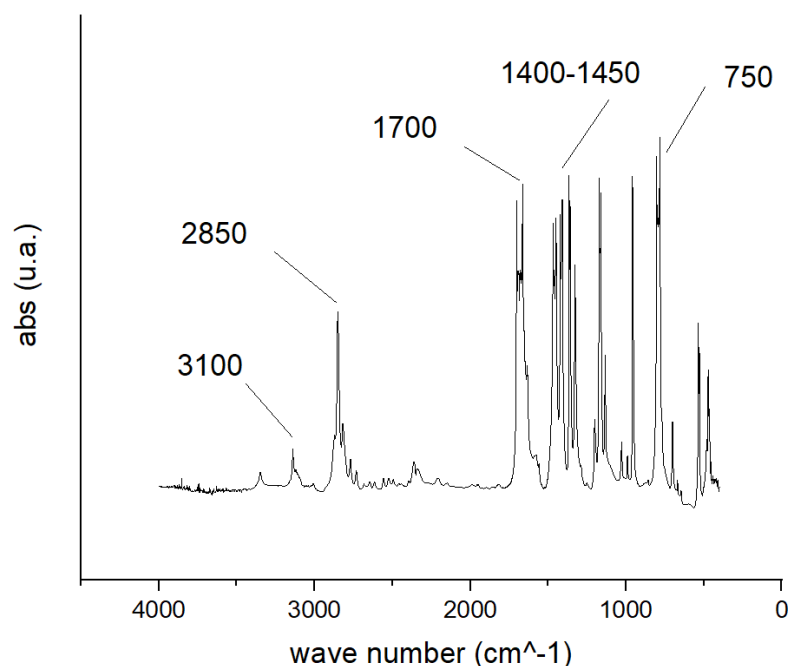


Figure 31: FT-IR spectrum of crystalline ZIF-90.

18.3. SBA-15:

The following table (7) analyses the peaks of the FT-IR spectrum of SBA-15 (figure 32). The broad band at 3400 is assigned to O–H stretching vibrations of surface silanol groups, Si–OH, with a possible contribution from adsorbed water, which is the main reason for the peak at 1630. However, from the shape of it is possible to say that there is only a small amount of water absorbed.

The peaks at 2930-2850 and at 1650 confirm the successful post-synthesis functionalisation process with APTES. In fact, they can be assigned to the symmetric and asymmetric stretching vibrations of aliphatic C–H bonds from the propyl chains of APTES. In general, it is safe to say it is a well-preserved, robust SBA-15 silica host (proven by the 1080, 800, and 460 cm⁻¹ bands) that successfully carries the organic/protein phase (proven by the aliphatic C-H stretch at 2930-2850 cm⁻¹ and the enzyme-related amide feature at 1650 cm⁻¹).

Table 7: analysis of the peaks of the FT-IR spectrum of SBA-15 [68] [69].

Wavenumber (cm ⁻¹)	Peak
3400	stretching of O-H bond of the Si-OH
2930-2850	symmetric and antisymmetric stretching of the C-H bond of the methyl group –CH ₃
1650	Asymmetric bending vibration of the primary ammonium ion
1630	H-O-H scissoring bending
1080	Si-O-Si asymmetric stretching
960	Si-OH stretching, vibration of surface silanol groups
800	Si-O-Si symmetric stretching
460	Si-O-Si bending out-of-plane rock/bend of the framework siloxane bonds

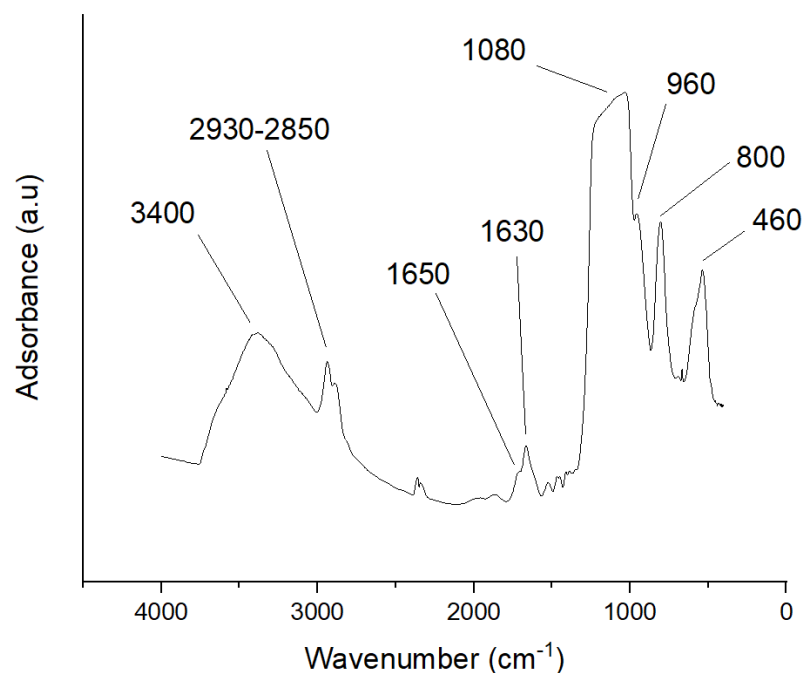


Figure 32: FT-IR spectrum of SBA-15.

19. PHYSISORPTION OF NITROGEN AT 77K:

19.1. ZIF-90:

The N₂ adsorption-desorption isotherm curve of ZIF-90 (figure 33) exhibits the classical features of a type Ia according to the IUPAC classification. The steep is nearly vertical, and it happens at a very low relative pressure, typical of a microporous material. The reason behind this trend is that the pores fill with gas rapidly. The hysteresis loop at around 0.35-0.45 does not meet the standard hysteresis loop of IUPAC because it is not due to the different conditions of emptying the pore instead of filling them. This loop is caused by the breathing swing of the gates of ZIF-90 that open its cage during the filling process; instead, during desorption, the cage does not snap back immediately but requires a slightly bigger drop of pressure to return to its original dimension. Moreover, the two branches do not completely overlap, probably because of a non-sufficiently long and inadequate pre-treatment degassing process.

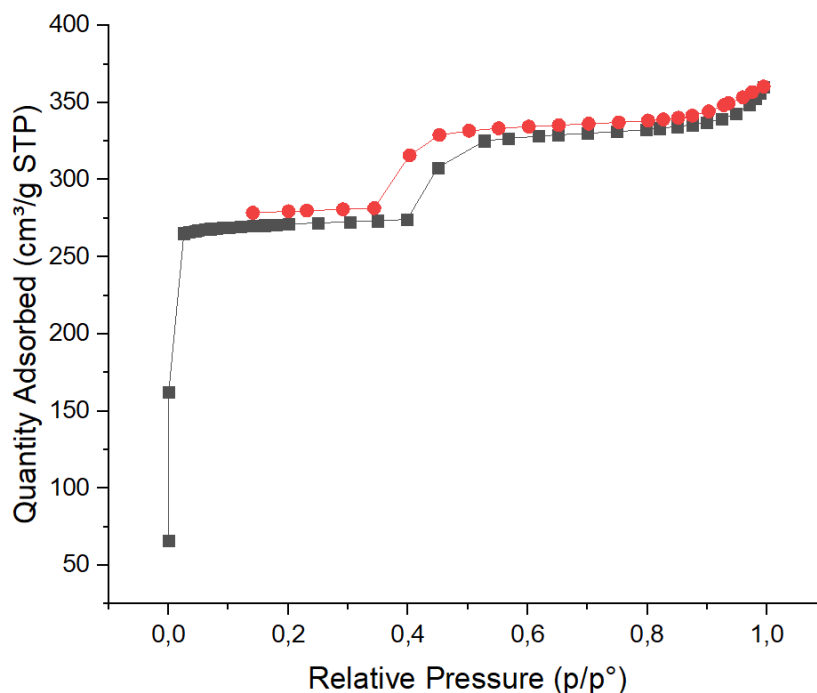


Figure 33: Isotherm plot of ZIF-90.

In the following table 7 it is possible to see the enormous value of the surface area of the ZIF-90, and its pore dimensions (even if, as it will be explained later, they are not precise because of the model of calculation).

Table 7: characteristics of ZIF-90 calculated through BJH method.

Surface area	$826 \pm 23 \text{ m}^2/\text{g}$
Pore volume	$0.54 \text{ cm}^3/\text{g}$
Pore size	2.6 nm

The pore distribution has been calculated through the BJH method, which is indeed not optimal, since it is mainly suitable for mesoporous materials and it is not so well reliable for pores smaller than 2 nm. Crystalline ZIF-90 is expected to possess microporous cavities of around 1 nm and smaller pore apertures of approximately 0.3 nm. Therefore, the pore size distribution obtained by BJH should not be interpreted as the intrinsic microporosity of the ZIF-90 framework. Instead, the peaks observed in the mesoporous range may be attributed to interparticle voids, external surface contributions, or limitations of the BJH model when applied to microporous materials. For this reason, the adsorption isotherm confirms the microporous nature of the synthesized ZIF-90, while the BJH pore size distribution should be considered only as a qualitative indication of

larger textural porosity rather than an accurate measurement of the intrinsic pore size of the framework.

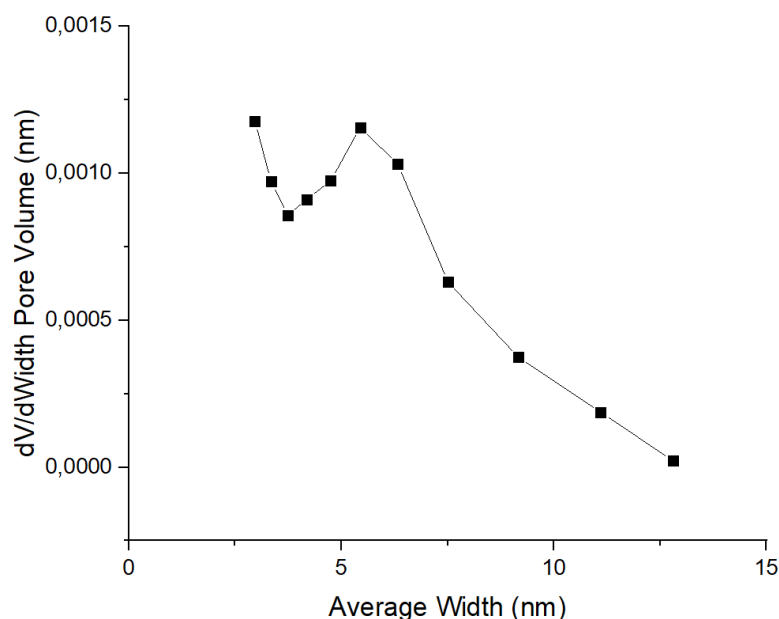


Figure 34: pore distribution plot of ZIF-90.

19.2. SBA-15:

The N₂ adsorption-desorption isotherm curve of Functionalised SBA-15 (figure 35) exhibits the classical features of a type IVa according to the IUPAC classification typical for mesoporous materials. The initial steep happens at a very low relative pressure, typical of monolayer-multilayer adsorption on silica walls. The reason behind the sharp steep is around 0.6-0.9 is the capillary condensation of the mesopores. The hysteresis loop at around 0.35-0.45 meets the type H2a of IUPAC, meaning the functionalization was successful and the pores assumed the shape of “inkbottle”. The reason behind this shape is the pore blocking effects, caused by the functionalisation molecules attached to the walls of the pores. Moreover, the two branches do not completely overlap, probably because of a non-sufficiently long and inadequate pre-treatment degassing process.

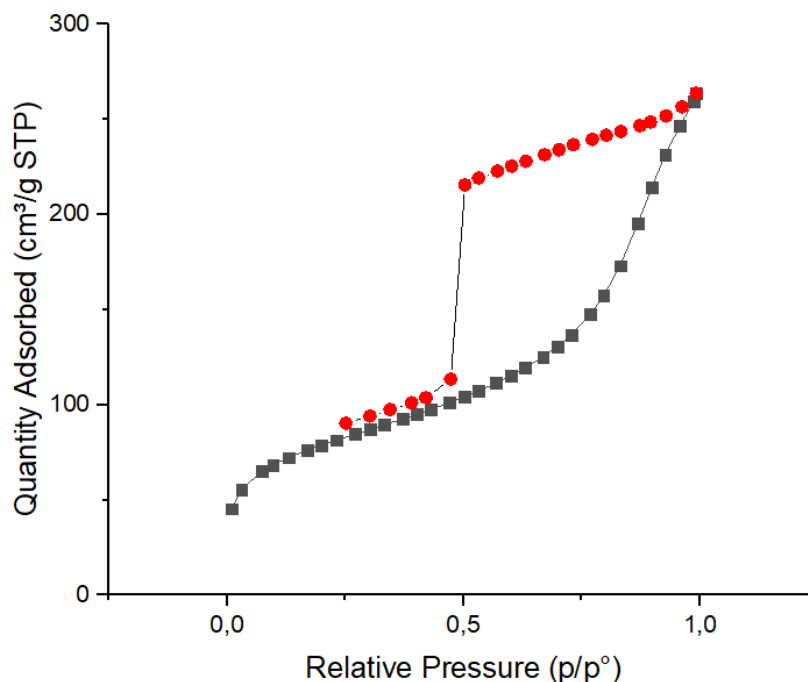


Figure 35: Isotherm plot of functionalised SBA-15.

The N₂ adsorption-desorption isotherm curve of Non-Functionalised SBA-15 (figure 36) exhibits the classical features of a type IVa according to the IUPAC classification typical for mesoporous materials too. It tops at much higher quantity adsorbed with respect to the functionalized sample (260 vs 460). The hysteresis loop at around 0.35-0.45 does not align perfectly with the type H2a of IUPAC, meaning the walls are smoother than the Functionalised SBA-15 and there is less roughness caused by the functionalisation molecules attached to the walls of the pores. Normally, Non-Functionalised SBA-15 should show a H1 type of hysteresis; this variation indicates a degree of network effects, surface roughness, or slight structural variations in the cylindrical channels (e.g., corrugated pore walls or minor internal blocking particles left over from synthesis).

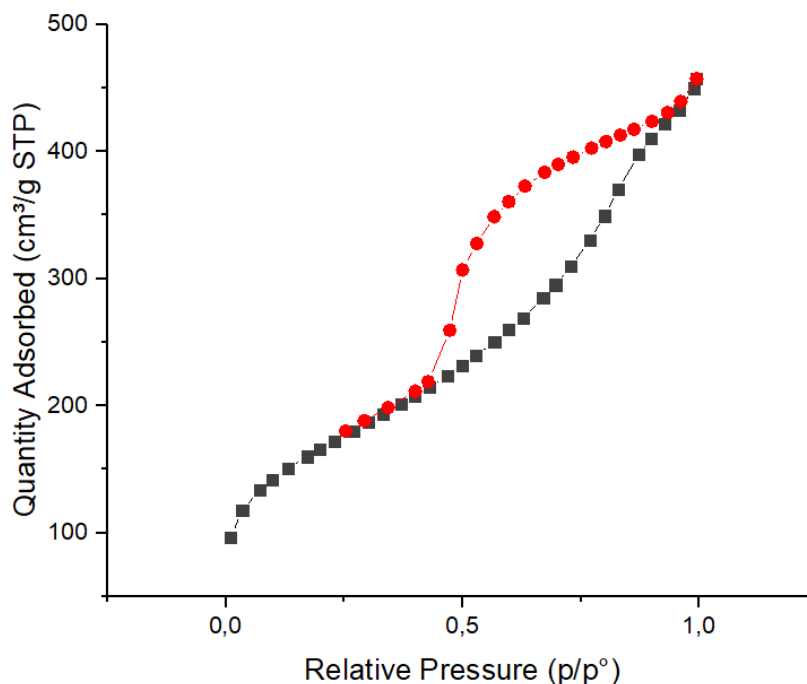


Figure 36: Isotherm plot of non-functionalised SBA-15.

The pore size distribution of the non-functionalized SBA-15 sample shows a sharp and intense peak centered at approximately 3.9–4 nm, indicating a highly ordered mesoporous structure with a narrow and homogeneous pore size distribution. This behavior is characteristic of SBA-15 materials and confirms the presence of well-defined cylindrical mesopores.

After functionalization with APTES and GPTMS, aimed at introducing amino and glyoxyl functional groups, the pore size distribution remains centered in the mesoporous range, with a maximum around 3.7–3.9 nm. However, compared with the pristine SBA-15, the peak intensity decreases markedly and the distribution becomes broader. This suggests a reduction in the accessible pore volume and an increase in pore size heterogeneity after surface modification.

Nevertheless, the persistence of a well-defined peak in the mesoporous region indicates that the ordered porous structure of SBA-15 is largely preserved after functionalization.

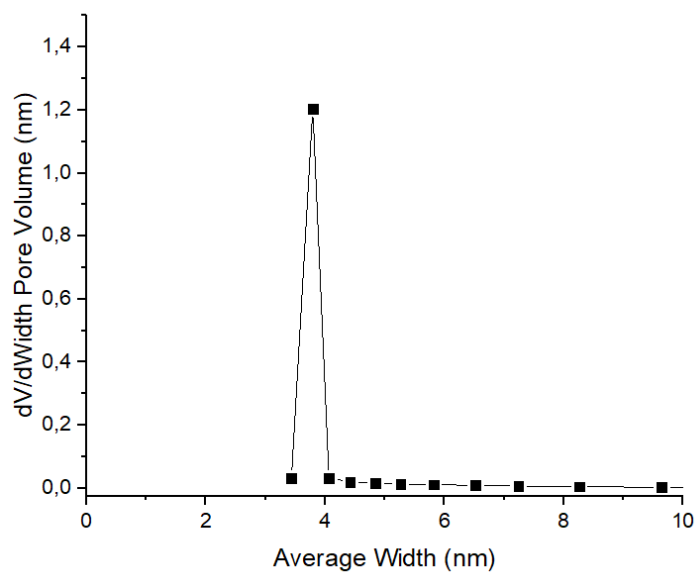


Figure 37: pore distribution plot of functionalised SBA-15.

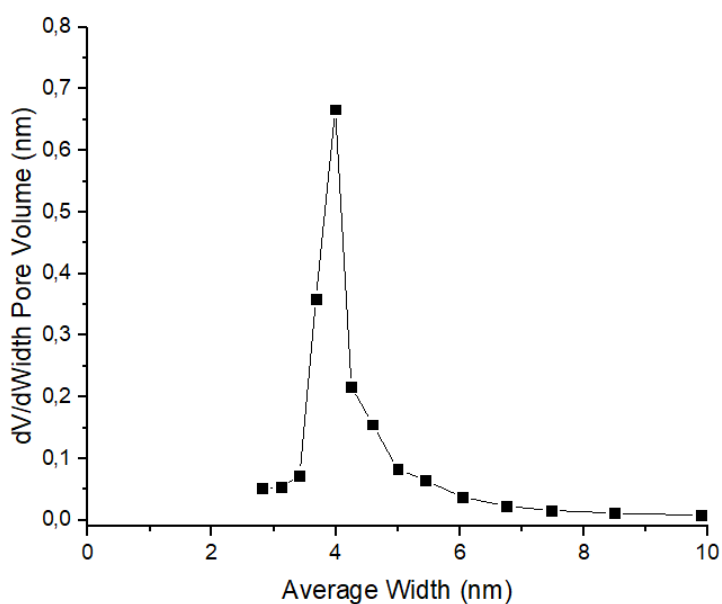


Figure 38: pore distribution plot of non-functionalised SBA-15.

From the following tables (8 and 9) the comparison appears more clearly, the surface area drops because of the attached species which block some pores, probably the smaller ones, this brings also to the increase in the pore size. The pore volume instead decreases always because of the obstruction of the pores.

Table 8: characteristics of functionalised SBA-15 calculated through BHJ method.

Surface area	$275 \pm 3 \text{ m}^2/\text{g}$
Pore volume	$0.4 \text{ cm}^3/\text{g}$
Pore size	6 nm

Table 9: characteristics of non-functionalised SBA-15 calculated through BHJ method.

Surface area	$591 \pm 4 \text{ m}^2/\text{g}$
Pore volume	$0.7 \text{ cm}^3/\text{g}$
Pore size	4.5 nm

20. XRD of LDH@MATERIALS:

20.1. LDH@MAF-7:

The PXRD pattern of LDH@MAF-7 shows a broad and diffuse profile without sharp Bragg reflections, indicating the absence of long-range crystalline order. Therefore, the synthesized LDH@MAF-7 sample cannot be considered crystalline. Instead, the diffractogram is consistent with the formation of an amorphous or poorly crystalline LDH@MAF-7-like material. The absence of the characteristic reflections of crystalline MAF-7 suggests that the presence of the enzyme, together with the synthetic conditions used for the encapsulation process, did not promote the formation of an ordered framework. However, PXRD alone cannot provide information about the local coordination environment or confirm the presence of LDH within the material.

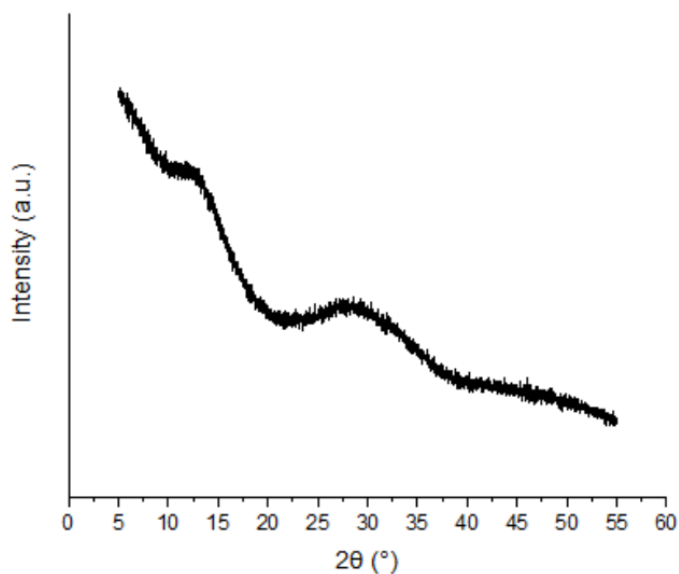


Figure 39: PXRD of synthesised amorphous LDH@MAF-7

20.2. LDH@ZIF-90:

The PXRD pattern reported in Figure 40 clearly shows that the crystalline structure of the synthesized ZIF-90 is not retained after enzyme encapsulation. In contrast to pristine ZIF-90, which is expected to display well-defined Bragg reflections, the LDH@ZIF-90 sample exhibits a broad and diffuse diffraction profile, commonly associated with amorphous or poorly crystalline materials. This evidence suggests that, under the selected synthetic conditions, the formation of a crystalline ZIF-90 framework was not achieved, leading instead to an amorphous LDH@ZIF-90-like hybrid material.

The loss of crystallinity may be attributed to several factors related to the encapsulation process. In particular, the addition of the enzyme-containing solution may have significantly altered the reaction environment, affecting parameters such as pH, ionic strength, ligand–metal coordination equilibrium, and nucleation kinetics. The presence of LDH during framework formation could interfere with the regular assembly of zinc ions and imidazolate-based linkers, preventing the development of the long-range order typically observed in crystalline ZIF-90. Moreover, the mild aqueous conditions required to preserve enzyme activity may further limit crystal growth and favor the formation of a disordered hybrid matrix.

Therefore, the PXRD results indicate that the obtained LDH@ZIF-90 material does not possess the long-range crystalline order characteristic of ZIF-90.

However, the absence of crystallinity does not necessarily exclude the successful incorporation of LDH within the material. PXRD only provides information about long-range structural organization and cannot directly confirm enzyme encapsulation or local coordination environments.

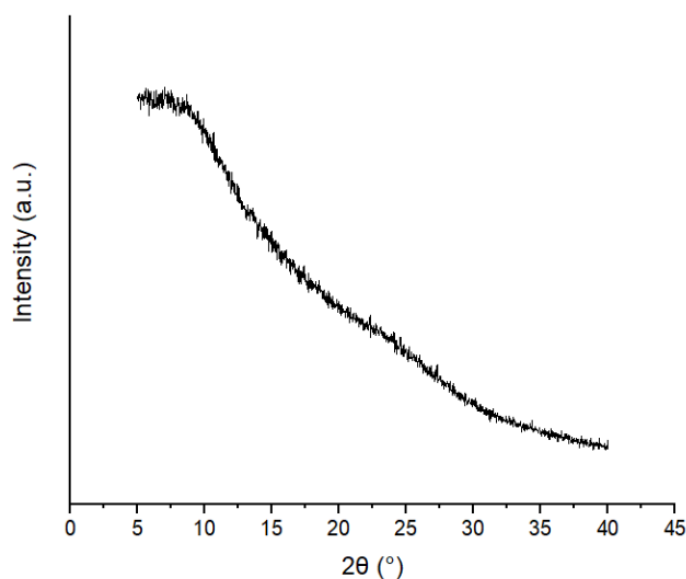


Figure 40: PXRD of synthesised amorphous LDH@ZIF-90

21. FT-IR of LDH@MATERIALS:

21.1. LDH@MAF-7:

FT-IR analysis was performed to investigate the chemical composition of the amorphous LDH@MAF-7-like material. The spectrum (figure 41) displays the characteristic vibrational bands associated with the 3-methyl-1,2,4-triazole linker, together with an additional contribution attributable to the protein component. In the table 10 are described the principal peaks of the spectrums. The broad “hump” around 3400 derives from the water trapped in the structure, with a possible contribution from N–H stretching vibrations of the enzyme.

The double peak at 2950-2850 is the ultimate confirmation that the 3-methyl-1,2,4-triazole linker is structurally intact within the material.

Moreover, the peaks at 1500-1400 are mainly assigned to C–N and C=N stretching vibrations of the triazole ring. Compared with a crystalline MAF-7 material, these signals appear less resolved, which is consistent with the amorphous or poorly crystalline nature indicated by PXRD analysis. The band around 740 cm⁻¹ can be assigned to out-of-plane bending vibrations of the triazole ring.

An additional band around 1650 cm⁻¹ can be attributed to the amide I band of the peptide backbone of LDH, mainly arising from C=O stretching vibration. The presence of this band, especially when compared with the FT-IR spectrum of MAF-7 without enzyme, supports the presence of LDH in the final material. However, FT-IR alone cannot distinguish whether the enzyme is fully encapsulated inside the framework or partially adsorbed on the external surface.

Table 10: analysis of the principal peaks of the FT-IR spectrum of LDH@MAF-7 [24] [64] [65].

Wavenumber (cm ⁻¹)	Peak
3100	stretching of the C-H bond of the triazole ring
2950-2850	symmetric and antisymmetric stretching of the C-H bond of the methyl group –CH ₃
1650	ammide I band of peptide backbone of LDH
1500-1400	stretching of the double bonds C=C and C=N
740	bending out of plane of the triazole ring

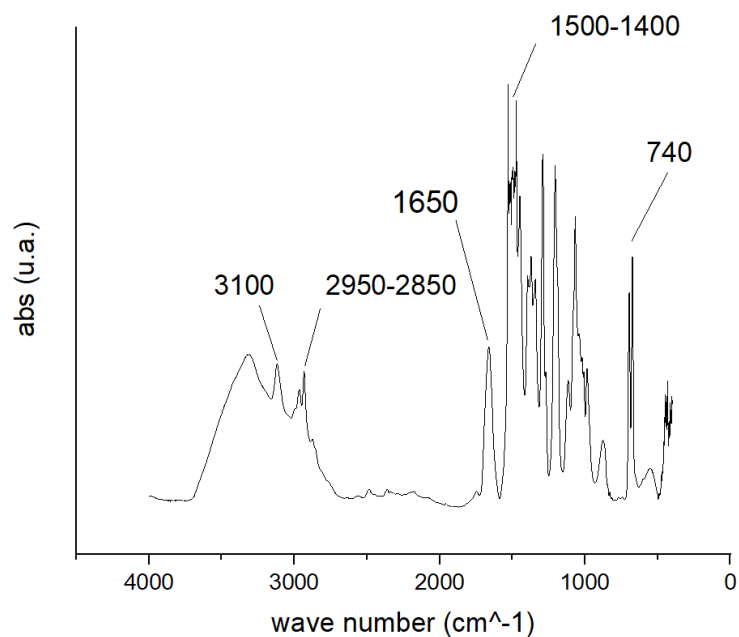


Figure 41: FT-IR spectrum of amorphous LDH@MAF-7.

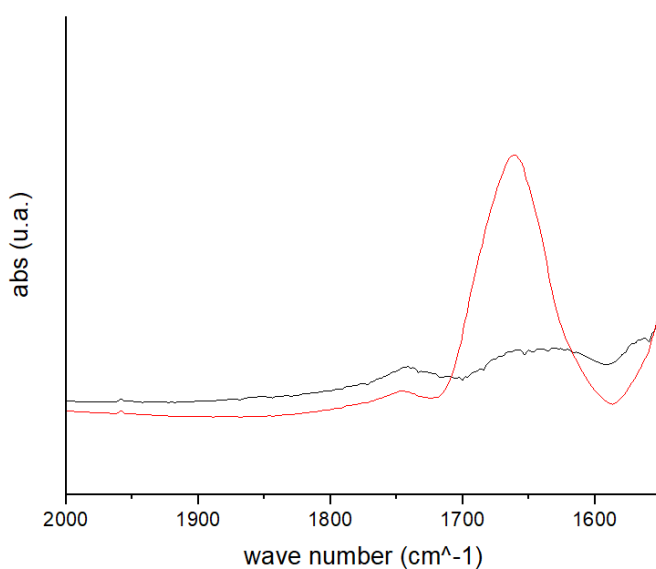


Figure 42: in red FT-IR spectrum of LDH@MAF-7,
in black FT-IR spectrum of MAF-7.

The comparison between FT-IR spectra of MAF-7 and LDH@MAF-7 (figure 42) shows that the main bands associated with the triazole linker are preserved after enzyme incorporation. This indicates that the organic component of the MAF-7-like material remains present in the LDH-containing sample. In addition, the LDH@MAF-7 spectrum

shows an extra contribution around 1650 cm^{-1} , not evident in the spectrum of MAF-7 without enzyme, supporting the presence of LDH in the final material.

Overall, the FT-IR results support the formation of an LDH-containing MAF-7-like material, while the PXRD pattern confirms that the material lacks long-range crystalline order.

21.2. LDH@SBA-15:

The following table (11) analyses the peaks of the FT-IR spectrum of LDH@SBA-15 (figure 43). The spectrum shows the characteristic vibrations of the SBA-15 silica framework, together with additional bands related to the APTES functionalization and to the presence of the immobilized enzyme. The broad band around 3400 cm^{-1} can be assigned to O-H stretching vibrations of surface silanol groups and adsorbed water, with a possible contribution of N-H stretching vibrations of APTES and the protein. The bands in the $2930\text{--}2850\text{ cm}^{-1}$ region are attributed to the symmetric and asymmetric stretching vibrations of aliphatic C-H bonds, mainly from the propyl chains introduced by APTES. Their presence supports the successful post-synthetic functionalization of SBA-15.

The band around 1630 cm^{-1} is mainly assigned to the H-O-H bending vibration of adsorbed water; however, in the LDH-containing sample, it may also partially overlap with the amide I band of the protein. Moreover, the small shoulder of the peak at 1630 cm^{-1} , around 1600 cm^{-1} , is the marker of a successful immobilization because of is the typical peak of the enzyme.

Overall, the FT-IR spectrum confirms the preservation of the SBA-15 silica framework, supports the presence of APTES organic groups, and provides qualitative evidence of LDH immobilization.

Table 11: analysis of the principal peaks of the FT-IR spectrum of LDH@SBA-15 [68] [69].

Wavenumber (cm ⁻¹)	Peak
3400	stretching of O-H bond of the Si-OH
2930-2850	symmetric and antisymmetric stretching of the C-H bond of the methyl group –CH ₃
1630	H-O-H scissoring bending
1600	ammide I band of peptide backbone of LDH
1080	Si-O-Si asymmetric stretching
960	Si-OH stretching, vibration of surface silanol groups
800	Si-O-Si symmetric stretching
460	Si-O-Si bending out-of-plane rock/bend of the framework siloxane bonds

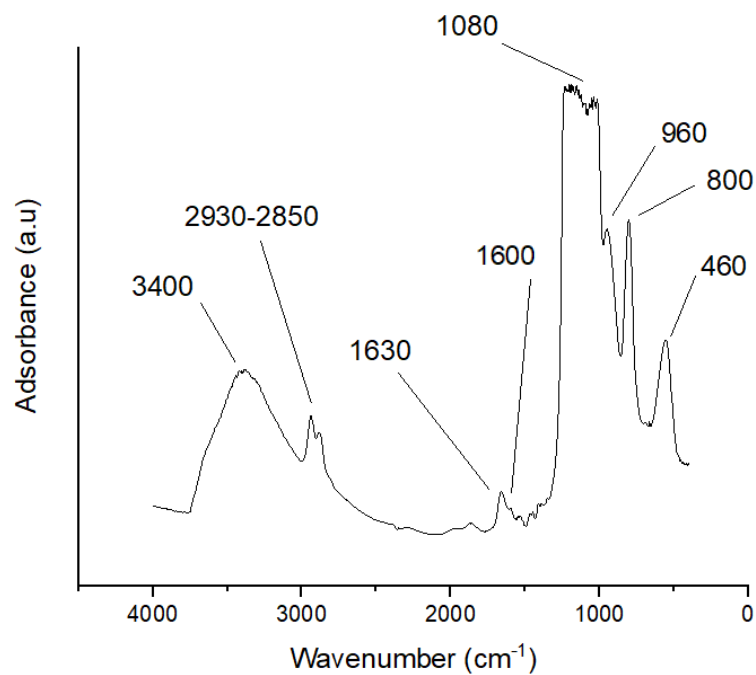


Figure 43: FT-IR spectrum of LDH@SBA-15.

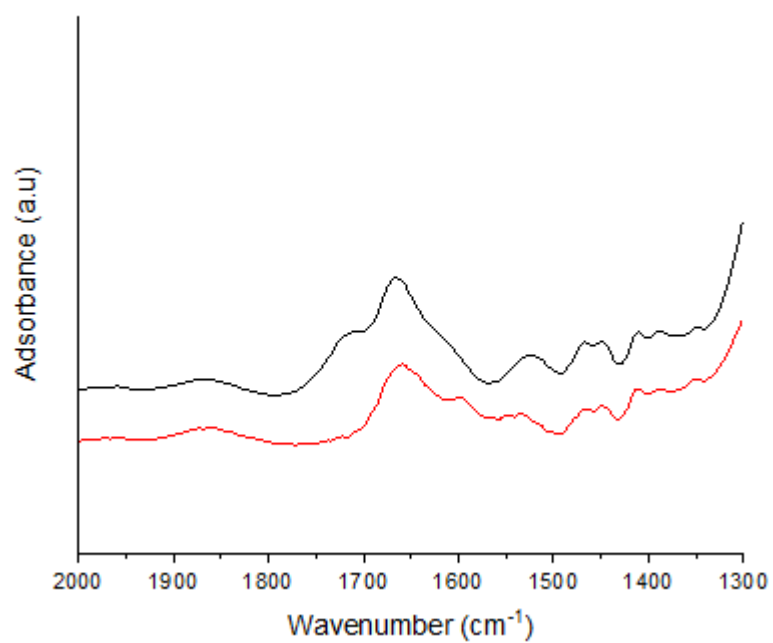


Figure 44: in red FT-IR spectrum of LDH@SBA-15,
in black FT-IR spectrum of SBA-15.

22. PROTEIN STANDARD REPRESENTATIVE CURVE:

The protein standard calibration curve was obtained by plotting the average absorbance values against the corresponding protein standard concentrations. All measurements were performed in triplicate in order to evaluate the reproducibility of the assay. The resulting calibration curve showed a linear trend over the selected concentration range, with a regression equation of $y = 2.38x$ and a coefficient of determination $R^2 = 0.996$.

This high R^2 value confirms the strong correlation between absorbance and protein concentration, indicating that the calibration curve is appropriate for protein quantification. The low variability among replicate measurements further supports the precision of the spectrophotometric analysis. Consequently, the obtained calibration equation was used to determine the protein concentration of the unknown samples.

Table 12: values of abs measured by the spectrophotometer.

[C]	Abs1	Abs2	Abs3	AbsMedia
0.01	0.038	0.038	0.040	0.039
0.05	0.141	0.156	0.147	0.148
0.1	0.251	0.264	0.268	0.261
0.2	0.514	0.54	0.535	0.530
0.3	0.742	0.749	0.792	0.761
0.4	0.949	0.969	0.985	0.968
0.5	1.113	1.124	1.129	1.122

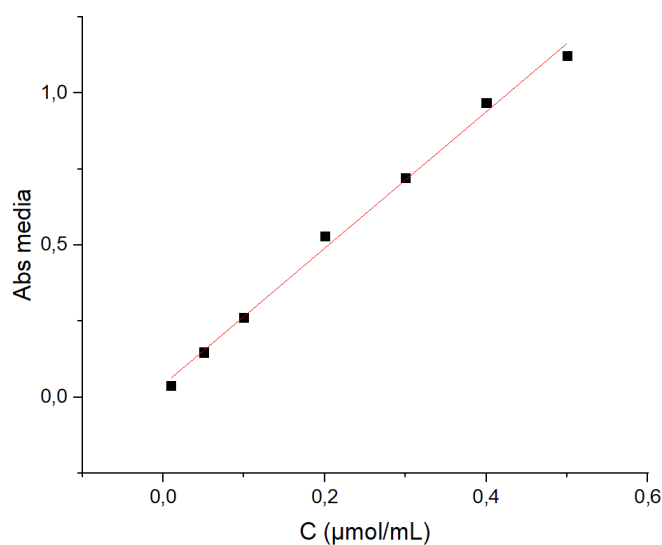


Figure 44: the protein representative standard curve.

$$R^2 = 0.996 \quad y = 2.38x$$

23. RESIDUAL ACTIVITY AND IMMOBILIZATION YIELD:

The immobilization yield and residual enzymatic activity of the different LDH-based hybrid systems are reported in Table &. These two parameters provide complementary information: the immobilization yield indicates the amount of enzyme retained within or onto the material, whereas the residual activity reflects the fraction of enzymatic activity preserved after the immobilization process.

The MOF-based systems, namely LDH@MAF-7 and LDH@ZIF-90, expressed very high immobilization yields, reaching 99% and 100%, respectively. This result suggests that almost all the enzyme initially added during the synthesis was retained within the final solid material, leading to negligible enzyme loss in the supernatant. However, the high immobilization yield was not accompanied by a comparable preservation of catalytic activity. In particular, LDH@MAF-7 retained only 33% residual activity when

rat-LDH was used and 5% in the case of h-LDH, while LDH@ZIF-90 showed an even lower residual activity of only 1%.

These results suggest that, although the MOF-based matrices are highly efficient in entrapping or retaining the enzyme, the immobilization conditions are likely not suitable for preserving its catalytic function. The loss of activity may be attributed to several factors, including enzyme denaturation during synthesis, unfavorable pH or ionic strength, interactions between the protein and the metal/linker components, restricted diffusion of substrate and cofactor, or conformational constraints imposed by the matrix. Therefore, in these systems, high enzyme loading does not necessarily correspond to high biocatalytic performance.

A different behavior was observed for the SBA-15. As expected, SBA-15 showed lower immobilization yields compared with the MOF systems, ranging from 35.4% to 52.5% depending on the NaBH₄ concentration used during the immobilization procedure. This lower yield can be explained by the fact that enzyme immobilization on functionalized SBA-15 relies on surface interactions and covalent binding, which may be less quantitative than enzyme entrapment during MOF formation. Nevertheless, SBA-15 displayed a significantly better ability to preserve LDH activity.

For the sample prepared using 0.025 mg/mL NaBH₄, the residual activity was calculated as 139%. Since residual activity values above 100% are generally not expected, this result should be interpreted with caution and is likely affected by experimental error, such as inaccuracies in protein quantification, pipetting errors, incomplete washing, or variability in the enzymatic assay. For this reason, this sample should not be considered representative for the evaluation of the optimal immobilization conditions.

Among the remaining SBA-15 samples, the material prepared with 0.05 mg/mL NaBH₄ showed the best compromise between immobilization yield and catalytic performance, with an immobilization yield of 47.7% and a residual activity of 85%. Increasing the NaBH₄ concentration to 0.1 mg/mL slightly improved the immobilization yield to 52.5%, but this increase was accompanied by a decrease in residual activity to 70%. This suggests that a higher degree of reduction or stronger immobilization may negatively

affect the enzyme, possibly by increasing structural rigidity, altering the enzyme conformation, or reducing the accessibility of the active site.

Table 13: comparison between the results of the different synthesised materials.

Material	Immobilization yield	Residual Activity
MAF-7	99%	33% (rat-LDH) 5% (h-LDH)
ZIF-90	100%	1% (rat-LDH)
SBA-15 (0.025 mg/mL NaBH ₄)	35,4%	139% (h-LDH)
SBA-15 (0.05 mg/mL NaBH ₄)	47,7%	85% (h-LDH)
SBA-15 (0.1 mg/mL NaBH ₄)	52,5%	70% (h-LDH)

24. LEACHING TEST:

The leaching test was performed to evaluate the stability of LDH immobilization on SBA-15 over time. The leaching percentage reported in Figure & is cumulative; therefore, each point represents the total amount of enzyme released into the supernatant up to that specific time. For this reason, the curves show an increasing trend over time, as the released LDH progressively accumulates in the liquid phase.

At the beginning of the experiment, a certain amount of LDH was already detected in the supernatant, suggesting that a fraction of the enzyme was weakly bound to the support or located in highly accessible regions of the material. During the first hours, the cumulative leaching increased almost linearly, indicating a progressive release of the less strongly immobilized enzyme fraction. After approximately three hours, the curves tended to reach a plateau, suggesting that most of the readily releasable LDH had already been removed from the material and that the remaining enzyme was more strongly retained within or onto the SBA-15 support.

Interestingly, the cumulative leaching percentage increased with increasing NaBH₄ concentration. This behavior indicates that a higher amount of reducing agent did not necessarily improve the stability of the immobilized system. A possible explanation is

that higher NaBH_4 concentrations may promote a less controlled immobilization process, favoring enzyme attachment on the external surface of SBA-15 or near the pore openings rather than inside the mesoporous channels. Enzyme molecules located in these more exposed regions are more easily released during incubation, leading to a higher cumulative leaching percentage.

In addition, the higher immobilization yield observed at increasing NaBH_4 concentration may include a larger fraction of enzyme that is retained only weakly or superficially. This would explain why the sample treated with 0.1 mg/mL NaBH_4 , despite showing the highest immobilization yield, also displayed greater enzyme release over time and lower residual activity. Overall, these results suggest that excessive NaBH_4 concentration may negatively affect the quality and stability of the immobilization.

Therefore, considering immobilization yield, residual activity, and cumulative leaching behavior, the SBA-15 sample treated with 0.05 mg/mL NaBH_4 appears to represent the best compromise among the tested conditions.

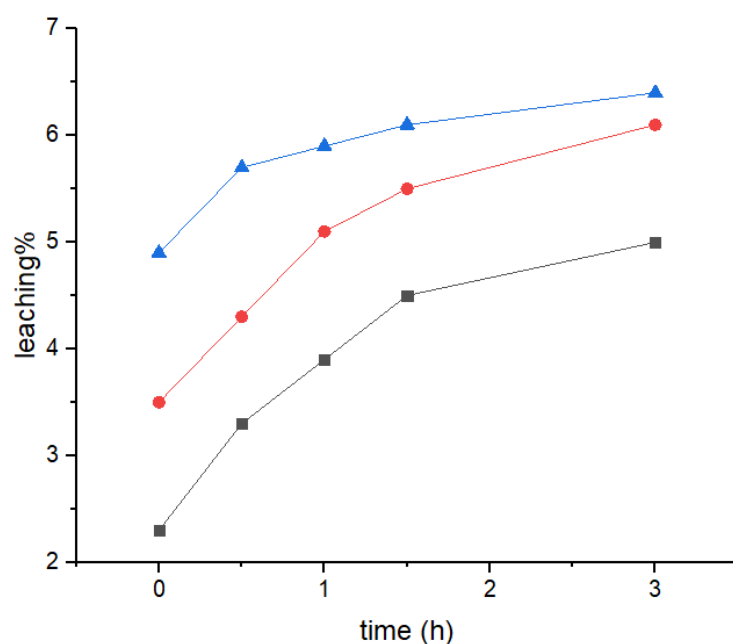


Figure 45: cumulative leaching % over time; in blue: SBA-15 (0.1 mg/mL NaBH_4), in red: SBA-15 (0.05 mg/mL NaBH_4), in grey: SBA-15 (0.025 mg/mL NaBH_4).

25. KINETIC CURVES:

The kinetic behaviour of the immobilized LDH was evaluated using the SBA-15 sample prepared with 0.05 mg/mL NaBH₄, which was selected as the most promising immobilization condition based on the balance between immobilization yield, residual activity, and leaching stability.

The kinetic curves reported in Figures 46 and 47 exhibit the typical hyperbolic profile associated with Michaelis–Menten behaviour. In both cases, the reaction rate increases rapidly at low substrate concentrations, where substrate availability is the limiting factor, and progressively approaches a plateau at higher concentrations, indicating the gradual saturation of the enzymatic active sites. This trend suggests that LDH immobilized on SBA-15 using 0.05 mg/mL NaBH₄ retains a Michaelis–Menten-like catalytic response after immobilization.

The kinetic parameters obtained from the fitting further support this interpretation. The LDH@SBA-15 sample analyzed in aqueous medium showed a K_M value of 0.083 mM and a V_{max} of 127.54 $\mu\text{mol min}^{-1}$, whereas in the presence of DMSO the K_M slightly increased to 0.1158 mM and the V_{max} increased to 166.63 $\mu\text{mol min}^{-1}$. The low K_M values obtained in both cases indicate a good apparent affinity between the immobilized enzyme and the substrate, suggesting that the immobilization process did not severely compromise substrate recognition. However, the slight increase in K_M observed in the presence of DMSO may indicate a modest change in the local reaction environment or in the apparent enzyme–substrate interaction.

At the same time, the higher V_{max} obtained with DMSO indicates an increase in the maximum apparent reaction rate. This suggests that DMSO improves the overall catalytic performance of the immobilized system. However, this enhancement should be interpreted with caution, since it may not necessarily correspond to an intrinsic increase in LDH activity, but rather to improved substrate accessibility and mass transport within the mesoporous support.

SBA-15 is characterized by ordered mesoporous channels, where diffusion limitations can strongly influence the apparent activity of immobilized enzymes. In aqueous buffer, incomplete wetting of the internal pore network, trapped air, or limited penetration of the reaction medium may restrict access of the substrate to enzyme molecules located inside the pores. The addition of DMSO may improve the wetting properties of the medium,

facilitating the penetration of the solution into the mesoporous channels and allowing a larger fraction of immobilized LDH to participate in the reaction.

Moreover, DMSO can modify the physicochemical properties of the reaction environment, including polarity, viscosity, and the hydration shell surrounding both the silica surface and the enzyme. These effects may reduce unfavorable interactions between the enzyme and the pore walls and improve the diffusion of substrates and products within the confined mesoporous space. As a result, the reaction rate measured in the presence of DMSO appears higher than that observed in aqueous medium.

Overall, the kinetic results indicate that the SBA-15 material immobilized using 0.05 mg/mL NaBH₄ preserves the catalytic behaviour of LDH and that the reaction medium plays an important role in determining the apparent enzymatic performance. The low K_m values suggest that the immobilized enzyme maintains good substrate affinity, while the increased V_{max} in the presence of DMSO supports the hypothesis of improved accessibility and reduced mass-transfer limitations within the SBA-15 pore network.

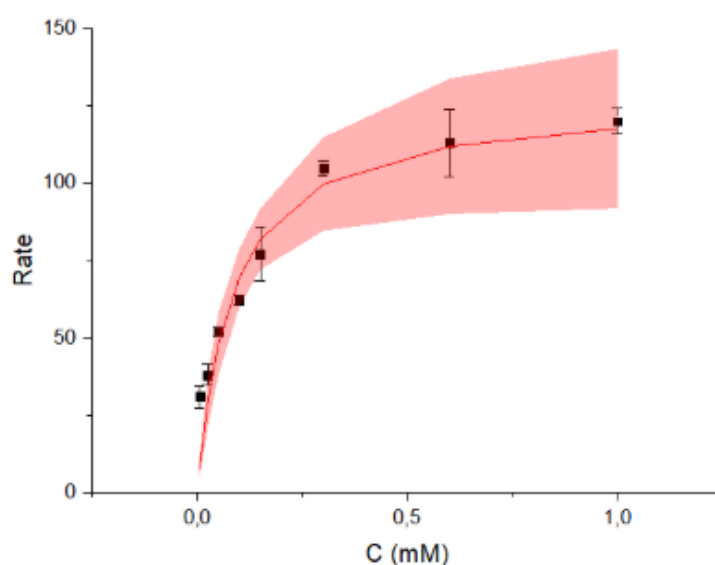


Figure 46: LDH@SBA-15 kinetic curve.

$$K_m = 0.083 \pm 0.006 \text{ mM}$$

$$V_{max} = 127.54 \pm 3.54 \mu\text{mol min}^{-1}$$

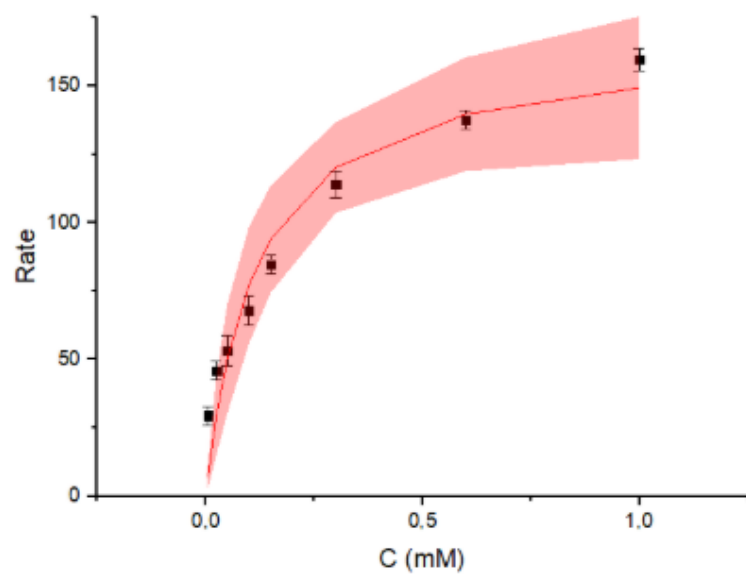


Figure 47: LDH@SBA-15 with DMSO kinetic curve.

$$K_m = 0.116 \pm 0.010 \text{ mM}$$

$$V_{max} = 166.63 \pm 4.28 \mu\text{mol min}^{-1}$$

CONCLUSIONS

The aim of this work was to contribute to the development of an enzymatic biosensor platform for the screening of anticancer drugs by investigating the behaviour of different enzyme-support materials for LDH immobilization. In particular, MOF-based systems, namely MAF-7 and ZIF-90, and functionalized SBA-15 mesoporous silica were evaluated in terms of structural properties, immobilization yield, residual enzymatic activity, and leaching behaviour.

Overall, the results indicate that SBA-15 is the most promising material among the investigated systems. Although SBA-15 did not provide quantitative enzyme immobilization, with a maximum immobilization yield of 52.5%, it allowed a significantly better preservation of LDH catalytic activity compared with the MOF-based materials. This aspect is particularly relevant for biosensor development, where enzyme retention alone is not sufficient: the immobilized enzyme must also remain catalytically active and accessible to the substrate.

The MOF-based systems showed almost complete enzyme retention, with immobilization yields close to 100%. However, this high immobilization efficiency was not accompanied by satisfactory residual activity. Both LDH@MAF-7 and LDH@ZIF-90 displayed a strong decrease in enzymatic activity after immobilization, reaching very low values, especially in the case of LDH@ZIF-90. This suggests that the synthesis conditions required for the formation of the MOF-based materials were not suitable for preserving LDH activity. Possible causes include enzyme denaturation, unfavorable pH or ionic strength, interactions with framework precursors, and diffusion limitations within the final material.

Moreover, the preparation of crystalline enzyme-containing MOFs appeared to be challenging. PXRD analysis showed that the presence of LDH during synthesis strongly affected the formation of ordered frameworks, leading to amorphous or poorly crystalline LDH@MOF-like materials. This suggests that the enzyme altered the reaction equilibrium, interfering with the nucleation and growth processes required for the

formation of crystalline MAF-7 and ZIF-90 structures. Among the two MOF systems, ZIF-90 showed better crystallinity in the absence of enzyme; however, after LDH encapsulation, it provided the lowest residual activity, making it less suitable under the conditions tested in this work.

In contrast, the LDH@SBA-15 systems showed a more favorable balance between immobilization yield and enzymatic activity. A general inverse relationship was observed between immobilization yield and residual activity as the NaBH₄ concentration increased. The sample prepared using 0.05 mg/mL NaBH₄ represented the best compromise, with an immobilization yield of 47.7% and a residual activity of 85%. Although the use of 0.1 mg/mL NaBH₄ slightly increased the immobilization yield to 52.5%, this improvement of only 4.8 percentage points was accompanied by a decrease in residual activity of 15 percentage points. Therefore, the higher enzyme loading obtained at 0.1 mg/mL NaBH₄ did not compensate for the loss in catalytic performance.

The leaching tests also provided important information on the stability of the immobilized systems. The cumulative leaching curves showed an initial release of enzyme, followed by a progressive increase and the formation of a plateau after approximately three hours. Interestingly, higher NaBH₄ concentrations resulted in higher cumulative leaching percentages. This suggests that excessive reducing agent may promote enzyme immobilization in more exposed or weakly stabilized regions of the SBA-15 support, such as the external surface or pore entrances, making the enzyme more prone to release over time. These results further support the selection of 0.05 mg/mL NaBH₄ as the most suitable immobilization condition.

In conclusion, functionalized SBA-15 appears to be the most suitable material for the intended biosensor application, as it provides the best balance between enzyme immobilization, catalytic activity, and stability. The MOF-based materials remain promising platforms due to their excellent enzyme retention capability, but their synthesis must be further optimized to preserve enzymatic activity and promote the formation of ordered enzyme-containing frameworks.

Future work should therefore focus on improving the MOF encapsulation strategy by optimizing parameters such as pH, precursor concentration, reaction time, enzyme

addition method, and solvent composition. In particular, the use of lyophilized LDH instead of frozen enzyme solutions is strongly recommended, as it may improve enzyme stability during synthesis and reduce the negative effects associated with dilution, storage, or freeze–thaw processes. Further optimization of these parameters could enhance the residual activity of LDH@MOF systems and allow a more reliable comparison with SBA-15-based materials. Additionally, future studies should include repeated-use tests, long-term storage stability, and biosensing performance toward anticancer drug screening in order to assess the real applicability of the most promising immobilized enzyme systems.

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