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INMOVILIZACIÓN DE AMINOACILASAS DE *STREPTOMYCES AMBOFACIENS*

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INDEX

1. INTRODUCTION 6

2. IMMOBILIZATION 6

2.1. WHAT IS IMMOBILIZATION ? 6

2.2. TYPES OF IMMOBILIZATION 6

2.3. MATERIAL FOR CATALYST CARRIERS 8

NANOTUBES 9

GRAPHENE 9

GRAPHITE NANOLAMINES 9

GRAPHENE OXIDE 10

MESOPOROUS SILICAS 10

2.4. IMMOBILIZATION OF ACYLASE IN GRAPHENE OXIDE 10

2.5. IMMOBILIZATION OF ACYLASES IN SBA-15 12

2.6. IMMOBILIZATION OF LIPASES IN SBA-15 12

3. MATERIAL AND METHODS 14

3.1. MATERIAL 14

3.1.1. THE ENZYMES 14

3.1.2. THE CARRIERS 14

3.2. METHODS 14

3.2.1. IMPACT OF IMMOBILIZATION PROCESS ON AMINOACYLASES PERFORMANCES14

3.2.2. IMPACT OF BUFFERS MOLARITY AND PH AMINOACYLASES IMMOBILIZATION . 15

3.2.3. IMPACT OF COBALT AND OF THE METHOD OF PREPARATION OF THE CARRIER16

3.2.4. DESORPTION OF ENZYMES 16

3.2.5. QUANTIFICATION OF ENZYMES 16

3.2.5.1. BCA METHOD 16

3.2.5.2. BRADFORD 17

3.2.6. ACTIVITY TEST 17

3.2.6.1. HYDROLYSIS 18

3.2.6.2. SYNTHESIS 18

3.2.7. ANALISIS OF THE PRODUCTION OF THE REACTION 19

3.2.7.1. THIN LAYER CROMATOGRAPHY (CROMATOGRAPHIE SUR COUCHE MINCE, CCM) 19

3.2.7.2. HIGH PERFORMANCE LIQUID CROMATOGRAPHY (HPLC) 19

4. RESULTS AND DISCUSSION 19
4.1. IMPACT OF IMMOBILIZATION PROCESS ON AMINOACYLASES PERFORMANCES 19
4.2. IMPACT OF BUFFERS MOLARITY AND PH AMINOACYLASES IMMOBILIZATION 21
4.2.1. SBA-15 21
4.2.2. GRAPHENE OXIDE (GO) 22
4.3. IMPACT OF COBALT AND OF THE METHOD OF PREPARATION OF THE CARRIER 24
4.4. DESORPTION OF ENZYMES 25
5. CONCLUSION 27
6. BIBLIOGRAPHY 28

LIST OF GRAPHS

Graph 1. Calibration line of the BCA method 17
Graph 2. Percentage of immobilization of the experiment that has been carried to study the impact of the immobilization method. 19
Graph 3. Loading enzyme from the experiment carried out with SBA-15 as support to study the impact of buffers molarity and pH aminoacylases in immobilization. 21
Graph 4. Percentage of immobilization from the experiment carried out with SBA-15 as support to study the impact of buffers molarity and pH aminoacylases in immobilization. 21
Graph 5. Loading enzyme from the experiment carried out with GO as support to study the impact of buffers molarity and pH aminoacylases in immobilization 22
Graph 6. Percentage of immobilization from the experiment carried out with GO as support to study the impact of buffers molarity and pH aminoacylases in immobilization 22
Graph 7. Loading enzyme (mg of enzyme/mg of support) of the immobilization in supports of SBA-15 without modification and SBA-15 modified with cobalt. 24
Graph 8. Percentage of immobilization of the immobilization in supports of SBA-15 without modification and SBA-15 modified with cobalt. 24
Graph 9. Specific activity (mg of product/(mg of enzyme · h)) presented by the free enzymes and the immobilized enzymes in supports of SBA-15 without modification and SBA-15 modified with cobalt., in a synthesis reaction. 25
Graph 10. Loading enzyme (mg of enzyme/mg of support) of the immobilization carried to check the enzymes desorption. 26
Graph 11. Concentration of C12K produced in the synthesis reaction carried to check the enzymes desorption. “1 3h”: 3 hours of synthesis reaction before centrifugation. “1 24h”: Synthesis reaction carried with the support after centrifugation. “1 24h liq”: Synthesis reaction carried with the supernatant after centrifugation. “2 24h”: Synthesis reaction carried without any centrifugation during 24 hours. 26
Graph 12. Relative activity 27

LIST OF TABLES

Table 1. Advantages and disadvantages of the different immobilization methods 7

LIST OF PICTURES

Picture 1. Four different modes of enzyme immobilization 8
Picture 2. Types of carriers for enzyme immobilization 9
Picture 3. Production of the graphene oxide from graphite.. 10
Picture 4. Graphic of the activity recovery and immobilization yield in function of the enzyme/support ratio. 11
Picture 5. Graphic of the activity recovery and immobilization yield in function of the time of immobilization.. 11
Picture 6. Graphic of the activity recovery and immobilization yield in function of the pH of the buffer used. 11
Picture 7. Graphic of the activity recovery and immobilization yield in function of the temperature of the immobilization. 12
Picture 8. Table that shows the immobilization efficiency and the activity of the immobilized enzymes varying the pore diameter and the concentration of the enzyme 13
Picture 9. Table that shows the immobilization efficiency and the activity of the immobilized enzymes varying the immobilization conditions... 13
Picture 10. Hydrolysis reaction, N-α-Acetyl-L-Lysine splits into Lysine and Acetic acid 18
Picture 11. Hydrolysis reaction, N-ε-Acetyl-L-Lysine splits into Lysine and Acetic acid.............. 18
Picture 12. Synthesis reaction, lauric acid and lysine produce α-lauroil-lysine as product........ 18
Picture 13. Results of the activity test from the experiment carried out to study the impact of the immobilization method.. 20
Picture 14. Results of the activity test of the hydrolysis reaction from the experiment carried out with SBA-15 and GO as supports to study the impact of buffers molarity and pH aminoacylases in immobilization. 23

1. INTRODUCTION

Enzymes are widely used as biocatalysts for different reactions. When operating with free-form enzymes they are in a liquid state so it will be difficult to separate them from the reaction product if complex methods are not used. If the enzyme is not fully recovered, the product will be contaminated. Besides, by using the enzyme in this way, it cannot be reused in other reactions and the stability time of the enzyme for the operation is low. These problems could be solved by immobilizing the enzymes since they are more stables when fixed to a carrier and since they are in a solid form they are better handled, easier to separate from the product and can be reused. Despite the advantages that the immobilization can have, when fixing the enzymes to a carrier they can lose activity. That is why is necessary to choose the support correctly and the immobilization method for the fixation to be effective.

In the laboratory aminoacylases are produced from *Streptomyces ambofaciens*; however, it is necessary to immobilize them because as it is been said, in their free form they are very unstable. The objectives of the present work is to be able to compare the immobilizations of these enzymes, the aminoacylases, in different supports, silicon or carbonated. In addition, optimal conditions have to be found to carry out the immobilization step, both in terms of temperature and pH, the buffer used and the support in which the immobilization is carried out.

2. IMMOBILIZATION

2.1. WHAT IS IMMOBILIZATION ?

It is difficult to define immobilization, so three terms are used to define it: effectiveness of immobilization, performance of immobilization and recovery of activity.

To define these terms it is first necessary to know these concepts:

- Initial activity: The activity that presents the enzyme before being immobilized.
- Immobilized activity: it is known by measuring the activity of the residual enzymatic solution and the water used to clean the support once the immobilization has been done.
- Observed activity: this is the activity of the enzyme that has been immobilized in the support.

In this way, the immobilisation efficiency can be defined as the relation between the activity observed and the immobilized activity, the immobilisation yield as the relation between the activity immobilized and the initial activity, finally, the recovery of activity as the relation between the activity observed and the initial activity. [1]

2.2. TYPES OF IMMOBILIZATION

The choice of the type of immobilization is very important in this process as it affects to the activity of the enzyme. The characteristics that must be taken into account when making the choice are several, physical properties such as the available material surface, size of the material pore, permeability, density... And chemical properties such as the possibility of modifying the surface of the support among other characteristics

Enzymes can be attached to the carrier by different interactions, from reversible physical adsorption or ionic bonding to stable covalent bonds. One way of classifying the different methods is into reversible and irreversible methods. Within the irreversible methods, three different methods can be found: binding to a carrier (attachment to a carrier), trapping (encapsulation) and cross-linking.

In the first case, the enzyme forms a bond with the carrier. This bond can be physical (such as hydrophobic and Van der Waals), covalent or ionic in nature. Generally, the physical link is very weak to keep the enzyme together in industrial conditions of high concentration of reagents and reactant products. The ionic bond is usually sufficient to hold the enzyme together. On the other hand, the covalent bond presents the problem that if the enzyme is irreversibly deactivated, both the enzyme and the carrier will be unusable.
In the entrapment the enzyme is physically retained within the pores of the support, in solution. The difference between entrapment and attachment to a carrier is that in the latter case the enzyme is attached to the external surface of prefabricated support while entrapment generally consists of the enzyme being present during the synthesis of the polymeric matrix.
The last case consists of immobilizing the enzymes without the need of a carrier. Since the use of a carrier always leads to a decrease in the activity. [1]

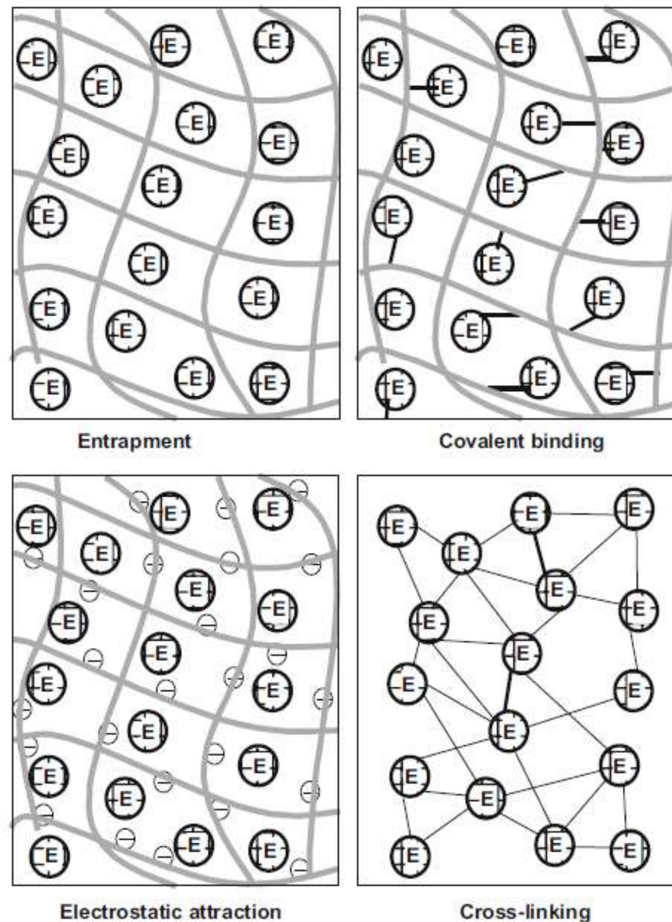
Moreover, within the reversible methods the most important is adsorption. This is the simplest method of immobilization and is based on physical adsorption where enzymes are attached to the support by hydrogen bonds, van der Waals forces or hydrophobic interactions. This type of immobilization is reversible since the conditions that influence the strength of the interaction such as pH, temperature, ionic strength, etc. can be changed. [2]

All methods have both advantages and disadvantages, here are some of them: [3]

Immobilization method	Advantages	Disadvantages
Adsorption	Its preparation is easy, the immobilization cost is low, it is a reversible method, it does not produce changes to the enzyme and the carrier can be regenerated after its use.	The binding force is weak which makes that the enzyme may get desorbed, the separation between the enzyme and the product is not easy.
Covalent binding	The binding force is strong so the enzyme will not get disunited, the enzyme activity once immobilized is still high	Its preparation is difficult, the cost of immobilization is high and the regeneration of the support is impossible.
Entrapment	The activity of the immobilized enzymes is high because as there are not chemicals interactions between the support and the enzyme, there are no loss of activity. In addition, the binding force is strong which means a good fixation and the immobilization cost is low.	The immobilization by this method is difficult to be done, the supports pore size affects in the reaction due to the diffusion of the substrate to arrive to the enzyme. Moreover, it is impossible to regenerate the support.
Cross-linking	The immobilized enzymes present a moderate activity, the binding force ins strong and the immobilization cost is moderate.	Its preparation is difficult. Moreover there is a risk of deactivation of the enzyme.

Table 1. Advantages and disadvantages of the different immobilization methods

Here is an image where some of the immobilization methods are represented:



Picture 1. Four different modes of enzyme immobilization

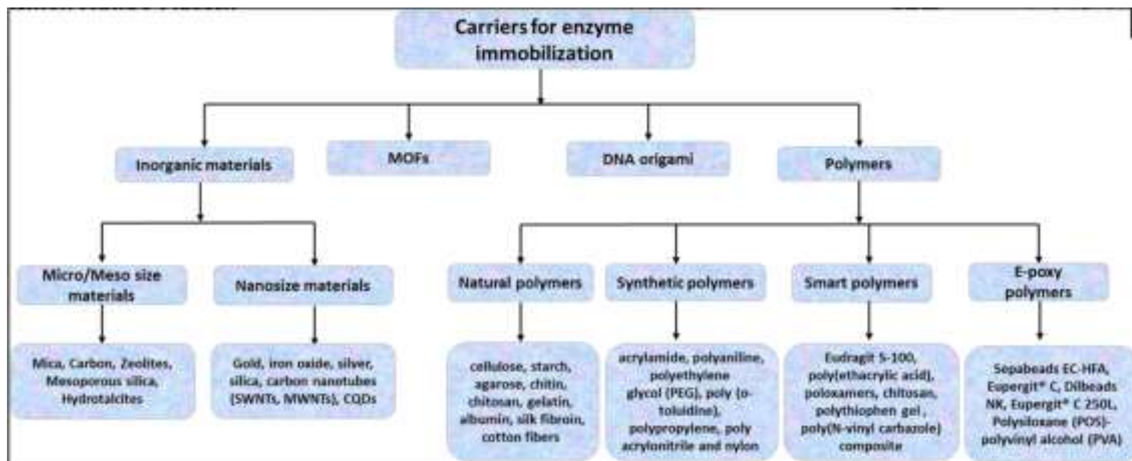
[4]

For example, Dong Yang et al. [5] perform an in-situ encapsulation immobilization of the enzyme PGA (Penicillin G Acylase) on an rGO/FeOOH/silica hydrogel carrier. Qingmei Yu et al. [6] immobilize PGA by covalent bonds on a GO-modified magnetic nanocomposite carrier. Ling Yang et al. [7] also immobilize PGA in a similar way, by means of covalent bonds, but on an epoxy-functionalized paramagnetic support. And Yung-Chin Yang et al. [8] carry out immobilization by absorption of an enzyme on a carboxylic acid functionalized SBA-15 carrier in order to enlarge its pores and thus facilitate the absorption of the enzyme.

2.3. MATERIAL FOR CATALYST CARRIERS

The choice of the carrier material on which the enzymes will be fixed is important, as the immobilisation will be better or worse, more or less stable and the enzyme will have a greater or lesser activity depending on the strength of the bond they form. The ideal properties of the support include physical resistance to compression, hydrophilicity, inertness towards enzymes, bio-compatibility, resistance to microbial attack, and availability at low cost. However, even though immobilization on solid supports is an established technology, there are still no general rules for selecting the best support for a given application. [2]

Many materials can be used as carriers, as shown in the following scheme:



Picture 2. Types of carriers for enzyme immobilization

[9]

Those materials that are going to be worked with are inorganic materials:

NANOTUBES

They are composed of rolled graphite sheets of cylindrical shapes whose length is of the order of micrometres and diameters of approximately 100 nanometres. In addition to their biological compatibility, these tubes have good thermal, mechanical and electrical properties.

There are two types of carbon nanotubes, those with a single wall and those with multiple walls. Those with multiple walls consist of a central tube that is surrounded by several layers of graphite while single-walled tubes have only the central tube, without the graphite layers.

Both covalent and non-covalent bonds can be formed to immobilize the top in the nanotubes. In the case of the non-covalent bond, both the properties of the enzyme and the nanotubes are preserved, but during the use of the enzyme-carrier complex, the enzyme may lose its immobilisation. On the other hand, in the covalent bond the enzyme-support bond, immobilisation will last longer but when fixing the protein it may be de-structured. [10]

For example, M.C. Bourkaib et al. [11] carried out an immobilization of Candida antarctica lipase B on chemically modified multiwalled carbon nanotubes. Also Wei Feng et al. [10] studied the different methods that can be used when immobilizing enzymes in carbon nanotubes.

GRAPHENE

It consists of a sheet of carbon atoms arranged in a hexagonal shape whose properties are very good as it has a high load bearing capacity as well as high thermal conductivity, great mechanical strength, inherent flexibility and a huge specific area. It is a hydrophobic material.

GRAPHITE NANOLAMINES

It is a nanomaterial, which is produced by oxidizing natural graphite and has numerous advantages because it is not a contaminant for the environment, is easily obtained, is not very expensive and has a good surface/volume ratio. [12]

GRAPHENE OXIDE

It is obtained through the oxidation and exfoliation of graphite and is an oxidized form of graphene. Both graphite and graphene oxide have the same structure, but GO includes functional groups, which contain oxygen. As a carrier, it has many functional groups that are necessary for the conjugation of biological macromolecules. In addition, it can be modified to obtain functional groups that conjugate with macromolecules such as enzymes.

Picture 3. Production of the graphene oxide from graphite

MESOPOROUS SILICAS

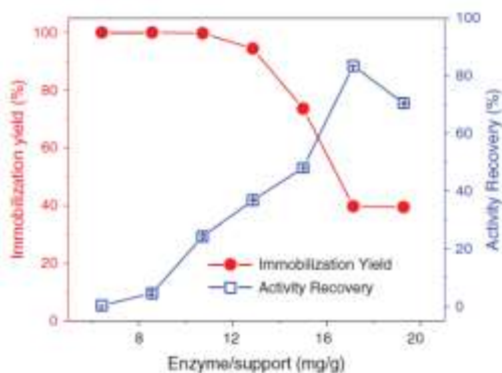
Mesoporous silica materials have been investigated as possible supports for enzyme immobilization due to their large and uniform pore size, open pore structure and good chemical and mechanical stability. In addition, they have a great potential to immobilize a high quantity of enzymes. [13]

For example, L. Dettori et al. [14] performed a physical adsorption immobilization of aminoacylases in unmodified SBA-15. In addition, they carried out an immobilization of these same enzymes by chemical adsorption on different modified SBA-15 carriers. Also Chengcheng et al. [13] performed an immobilization of penicillin G acylase in a support of mesoporous silica modified with lanthanum.

2.4. IMMOBILIZATION OF ACYLASE IN GRAPHENE OXIDE

Graphene oxide is prepared before the immobilization to obtain functionalized/modified graphene oxide. Covalent immobilization is used to optimize immobilization conditions to improve the stability of the immobilized enzyme. Immobilization is carried out with 50 mg of functionalized graphite oxide in an Eppendorf tube containing phosphate buffer with a concentration of 50 mM and the enzyme to be immobilized, glutaryl-7-aminocephalosporanic. Different immobilizations have been carried out varying the conditions, the enzyme/substrate ratio, the pH of the buffer, the temperature and the time of immobilization. In addition, once the immobilization has been done out the immobilization yield and the activity recovery has been calculated. [15]

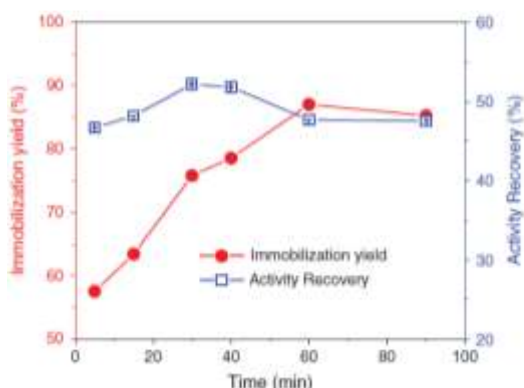
The results trying different ratios of enzyme/support are as follows:



Picture 4. Graphic of the activity recovery and immobilization yield in function of the enzyme/support ratio.

When increasing the enzyme/support ratio, the recovered activity also increases, although the maximum recovery of activity is obtained for a ratio of 17.13 mg/g. However, the amount of immobilised enzyme up to a ratio of 10.71 mg/g remains almost constant, but as it continues to increase, it decreases rapidly. This is why they choose the ratio of 15 mg/g for the following experiments.

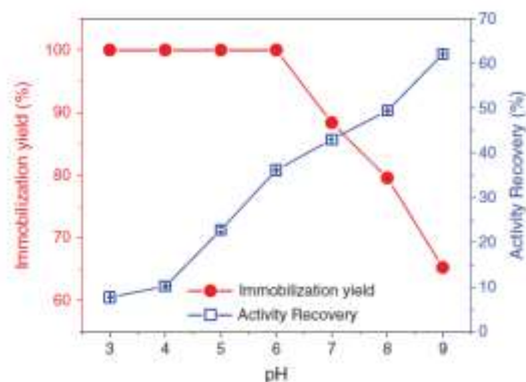
The results obtained by varying the time of immobilization are as follows:



Picture 5. Graphic of the activity recovery and immobilization yield in function of the time of immobilization.

It can be seen that there is not a significant change in the activity recovery with the time, while the amount of immobilized enzyme does increase significantly with the time. Finally, the time chosen for other immobilizations was 30 minutes.

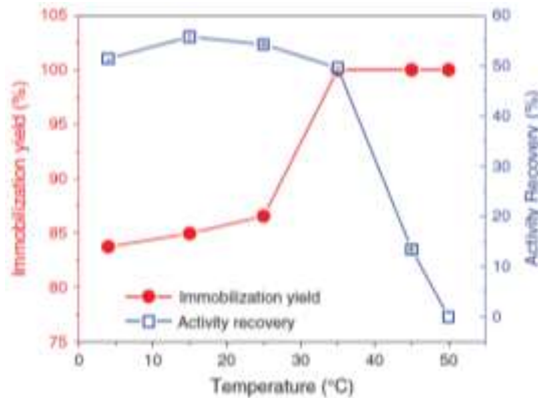
The results obtained by varying the pH of the buffer used are as follows:



Picture 6. Graphic of the activity recovery and immobilization yield in function of the pH of the buffer used.

The immobilization yield is at 100% from pH 3 to pH 6 and then decreases quickly. While the activity recovery at pH 3 is very low and increases up to 60% when the pH increases.

The results obtained by varying the temperature of the immobilization are as follows:



Picture 7. Graphic of the activity recovery and immobilization yield in function of the temperature of the immobilization.

The activity recovery remains almost constant from 4ºC to 30ºC, but when the temperature continues to rise, it decreases rapidly. The immobilization yield increases with the increase in temperature, reaching 100% when the temperature is 30ºC. [15]

2.5. IMMOBILIZATION OF ACYLASES IN SBA-15

Two immobilizations are carried out, both chemical and physical. The chemical adsorption immobilization is carried out in TRIS buffer at pH=8. 300 mg of support are taken and mixed with 54 ml of TRIS buffer solution. To this mixture, 2.3 ml of the amino-acylase solution with a concentration of 13 mg/ml is added. This solution with the enzymes is stirred for 3 hours at room temperature and 100 mbar pressure, and for 24 hours at room pressure. Once immobilized, the support with the immobilized enzyme is obtained by filtering it and washing it twice with TRIS buffer. It is left to dry overnight in the air. Immobilization by physical adsorption is done with 100 mg of SBA-15 suspended in 20 ml of TRIS buffer (25 mM in water, NaCl 50 mM) at pH=8, together with 4.6 mL of enzyme concentration 13 mg/mL. The mixture is shaken for 3 hours at room temperature and after the 3 hours of washing three times with TRIS buffer and left to air dry for one night. [14]

2.6. IMMOBILIZATION OF LIPASES IN SBA-15

SBA-15, with three different pore sizes, is used as a support where the enzyme, which is Antarctic Candida lipase B, is to be immobilized. The immobilization procedure is carried out with a phosphate buffer of 25 mM concentration, in a total volume of enzyme and buffer of 40 ml. To which 100 mg of SBA-15 is added and this mixture is stirred at room temperature. The samples are then filtered and washed with buffer. The immobilization conditions, time, pH and enzyme concentration were varied to study which are the best conditions. [16]

Table 1
Immobilization of CALB onto SBA-15 with pore diameters at 8.1 nm.[a]

Entry	pH	Time (min)	CALB concentration (μg/ml)	IE (%)[b]	Activity (U/g)[c]
1	6.0	15	40.6	86.08 ± 0.89	300.00 ± 33.33
2	6.0	30	40.6	87.84 ± 0.34	288.89 ± 38.49
3	6.0	60	40.6	89.71 ± 0.29	222.22 ± 38.49
4	7.0	15	40.6	82.36 ± 0.89	300.00 ± 33.33
5	7.0	30	40.6	85.85 ± 0.34	444.44 ± 38.49
6	7.0	60	40.6	89.15 ± 0.34	344.44 ± 19.25
7	7.0	90	40.6	89.39 ± 0.18	388.89 ± 19.25
8	7.0	120	40.6	93.07 ± 0.00	237.78 ± 26.94
9	7.0	30	20.3	92.17 ± 1.04	122.22 ± 19.25
10	7.0	30	40.6	85.85 ± 0.34	444.44 ± 38.49
11	7.0	30	60.9	74.24 ± 0.69	855.56 ± 69.39
12	7.0	30	80.12	69.35 ± 0.65	388.89 ± 19.25
13	8.0	15	40.6	63.90 ± 0.75	211.11 ± 19.25
14	8.0	30	40.6	66.08 ± 0.33	244.44 ± 38.49
15	8.0	60	40.6	68.00 ± 0.34	255.56 ± 19.25

[a] Immobilization conditions: Required amounts of the CALB solution were dissolved in 25 mM phosphate buffer, up to a total volume of 40 ml, contacted with 100 mg of SBA-15 at room temperatures. *Note:* Standard deviation values were calculated from triplicate experiments.
[b] Immobilization efficiency.
[c] Activity of the immobilized CALB.

Picture 9. Table that shows the immobilization efficiency and the activity of the immobilized enzymes varying the immobilization conditions.

Table 2
Immobilization of CALB onto SBA-15 with pore diameters at 6.6 nm and 12.5 nm.[a]

Entry	Supports	CALB concentration (μg/ml)	IE (%)[b]	Activity (U/g)[c]
1	SBA-15 (6.6)[d]	20.3	80.87 ± 0.26	200.04 ± 38.49
2		40.6	67.65 ± 0.55	322.22 ± 19.25
3		60.9	65.69 ± 0.27	944.44 ± 19.25
4		80.12	61.81 ± 0.63	822.22 ± 38.49
5	SBA-15 (12.5)[e]	20.3	52.41 ± 1.38	377.82 ± 38.49
6		40.6	53.13 ± 0.64	433.33 ± 19.25
7		60.9	58.15 ± 0.71	1188.89 ± 69.39
8		80.12	51.51 ± 0.86	755.56 ± 38.49

[a] Immobilization conditions: required amounts of the CALB solution were dissolved in 25 mM phosphate buffer, up to a total volume of 40 ml, pH 7.0, contacted with 100 mg of SBA-15 at room temperatures for 0.5 h. *Note:* Standard deviation values were calculated from triplicate experiments.
[b] Immobilization efficiency.
[c] Activity of the immobilized CALB.
[d] SBA-15 with pore diameters at 6.6 nm.
[e] SBA-15 with pore diameters at 12.5 nm.

Picture 8. Table that shows the immobilization efficiency and the activity of the immobilized enzymes varying the pore diameter and the concentration of the enzyme

The results obtained by varying the pH show that greater immobilization efficiency and greater activity is obtained at pH 6 or pH 7 than at pH 8. No great difference is observed between pH 6 and pH 7.

The results obtained by varying the immobilization time between 15 and 120 minutes indicate that between 15 and 60 minutes the immobilization efficiency increases proportionally with time. Between 60 and 90 minutes they did not observe a great difference in immobilization efficacy, this may be due to the formation of aggregations of lipase molecules that could block the support pores. And between 90 and 120 minutes an increase in immobilization effectiveness proportional to time is again observed. This could be due to the departure of some lipase molecules that were blocking the pores, again allowing immobilization in them.

In the results obtained by varying the concentration of the enzyme, it is observed that the immobilization efficiency decreases as the concentration of the enzyme increases. This may be due to the fact that the increase in enzyme concentration also generates a greater aggregation of enzymes in the support pores, preventing them from entering to be immobilized.

In the results obtained when varying the diameter of the pores of the SBA-15 it can be seen how an increase of the loading enzyme is produced when increasing the size of pore from 6.6 nm to 8.1 nm which is reasonable since the greater the pore the access for the enzyme is better. However, in the case of the 12.5 nm diameter there is a decrease in the loading enzyme with respect to the one of 8.1 nm. This can be explained by the fact that, due to the increase in the size of the pores, a change in the distribution of these is produced, causing the surface of the support to decrease and this is unfavourable to immobilisation. With regard to the activity, the enzymes immobilized in the support with the largest pores show a greater activity. This is explained by the fact that the larger diameter favours the diffusion of substrates and therefore improves the activity. [16]

3. MATERIAL AND METHODS

3.1. MATERIAL

3.1.1. THE ENZYMES

The enzymes used in the immobilization have been synthesized in the laboratory. These types of enzymes are produced by different microorganisms, including filamentous bacteria such as *streptomyces*. The enzymes used in this case are those produced by the *streptomyces ambofaciens*, which is why they are known as Sam. There are four types of Sam enzymes: Sam AA, Sam ELA, Sam PVA and Sam AA like.

The use of these enzymes is important because they are used in the N-acylation reaction. This reaction allows the synthesis of peptide derivatives, which can have various biological activities. In addition, this reaction is used in the production of amino acids based on biosufractants. Moreover, these molecules are widely used due to their great interest in the fields of cosmetics, cleaning, food and pharmacy. Nowadays it is very important to take into account the environmental impact that this process has, which is why new methods are being researched to obtain biosufractants. Besides the enzymes with high catalytic efficiency are very specific and eco-friendly so they are perfect to be used as green biocatalyst. [14]

3.1.2. THE CARRIERS

The carriers that has been used are graphene oxide (GO), SBA-15 and SBA-15 Co.

The preparation of the SBA-15 is carried out as follows: 5.3 g of surfactant P123 are weighed and mixed with 200 ml of hydrochloric acid (HCl), 1 M at pH=0. The mixture is taken to a water bath at 40°C under agitation to dissolve the surfactant. Once this is dissolved, 8.44 g of the precursor (TMOS) is added. This makes the surfactant in acid medium hydrolyze and begins polymerization. It is necessary to wait long enough for polymerization to take place and to precipitate the polymer. Once this has happened, the mixture is taken to the autoclave at 40ºC for 24 hours and at 100ºC for another 24 hours. Thus obtaining the SBA-15.

To obtain the SBA-15 Co it is necessary to wet impregnate the SBA-15 with cobalt nitrate hexahydrate (Co(NO3)2 - 6H2O), the amount of cobalt nitrate added depends on the desired cobalt concentration in the carrier at the end. Carriers have been prepared with 3 different amounts of cobalt 10%, 5% and 1%. Once impregnated the substrates can be used (SBA-25 Co impregnated) or they can be treated some more. In this case, they are calcined at 500ºC after impregnation to try to fix more cobalt in the support. After being calcined, the supports are used in the immobilization and are called SBA-15 Co calcined. Finally, the third method of preparing this type of support is calcination at 300ºC before impregnation with cobalt nitrate and another calcination at 500ºC after impregnation. This type of support used for immobilisation is called SBA-15 Co ClC.

3.2. METHODS

3.2.1. IMPACT OF IMMOBILIZATION PROCESS ON AMINOACYLASES PERFORMANCES

Three experiments are performed in parallel varying the conditions of immobilization in order to know which method gives the best results; this will be the one to be used in the following experiments. The enzymes that are used are amino-acylases and the carrier in this case is SBA-15.

First, 20 mg of enzyme (1 ml of enzyme whose concentration is 20 g/L) are mixed with 36 ml of buffer; in this case, tris HCl 25 mM with NaCl 50 mM was used. From this mixture, 0.5 ml is taken as a sample (t0) in order to measure the initial concentration of the enzyme. Then 200 mg of the SBA-15 carrier are added. As three experiments are to be performed, this procedure is repeated three times.

In the first case the mixture of buffer, enzyme and support is taken to the rotavapor at a pressure of 100 mbar, in order to extract the air from the pores of the support, during 3 hours. The other two samples are left during these three hours at room temperature and pressure. Then, the samples are taken to agitation, the first one, the one that was in the rotavapor, and the second one, one of the samples that were at room temperature and pressure, are taken to orbital agitation. While the third sample, the other of those that were at pressure and room temperature is taken to rotary agitation. The agitation takes place in the cold chamber, which is at 4ºC.

The samples are left in agitation for about 18 hours, after which the samples are collected to be filtered, thus separating the support with the enzymes that have been immobilised and the remaining liquid with the enzymes that have not been immobilised. From the filtration liquid, a sample (F1) is also taken in order to know the concentration of the remaining enzyme, which has not been immobilized, and compare it with the initial enzyme concentration so we can know the concentration of the immobilized enzyme. Finally, two washes are performed (L1) with 10 ml of buffer each and the support is left to dry with the immobilized enzymes.

3.2.2. IMPACT OF BUFFERS MOLARITY AND PH AMINOACYLASES IMMOBILIZATION

The impact of both the molarity of the buffer and its pH on enzyme immobilization is to be studied. For this purpose, several immobilizations are going to be carried out with Tris Buffer 50 mM HCl, NaCl, and Phosphate buffer of concentration 50 mM at pH 6.2, 7.2 and 8.2 and of concentration 100 mM at pH 6.2, 7.2 and 8.2. The immobilization will be done both in a silicon support, the SBA-15, and in graphene oxide (GO).

Dispersibility tests were carried out with both supports (SBA-15 and GO) and different buffers to check if any of the combinations between support and buffer presents any problem for sedimentation that could make difficult the separation of the support with the immobilized enzyme from the remaining free enzyme. In all cases, after centrifugation of the sample, the support has completely settled.

For the SBA-15, 20 mg of carrier were used, with 100 µL of enzyme at a concentration of 20 g/L and 900 µL of buffer. For GO the amount of buffer and enzyme are the same, however, 10 mg of support are used instead of 20.

The samples are taken to the cold chamber at 4ºC for 16 hours, leaving them in rotary agitation. After this time, the samples are removed from the agitation and the support with the immobilized enzyme is separated from the rest of the enzyme that has not been immobilized. To do so the samples are centrifuged to make the support with the immobilized enzyme settle. After recovering as much free enzyme as possible, the support is washed twice with 1 ml of the buffer that has been used in its immobilization, each with a subsequent centrifugation to finally obtain only the support with the immobilized enzyme.

3.2.3. IMPACT OF COBALT AND OF THE METHOD OF PREPARATION OF THE CARRIER

The aminoacylases used, get activated in presence of cobalt, so silicon carriers, SBA-15, with different percentages of cobalt have been used to compare them with the immobilization with the SBA-15 carrier without cobalt. In addition, the SBA-15 Cobalt carriers have been prepared in different ways in order to see what influence this may have on enzyme immobilization.

The method used in the immobilization is the same as the one used previously. 20 mg are taken from each carrier, to which 100 µL of enzyme at a concentration of 20 g/L and 900 µL of buffer are added. The buffer used in this case is phosphate buffer with a concentration of 100 mM and pH=7.2.

The samples are taken to the cold chamber at 4ºC for 24 hours, leaving them in rotary agitation as in the previous experiment. After this time, the samples are removed from the agitation and the support with the immobilized enzyme is separated from the rest of the enzyme that has not been immobilized. To do so the samples are centrifuged to make the support with the immobilized enzyme settle. After recovering as much free enzyme as possible, the support is washed twice with 1 ml of the buffer that has been used in its immobilization, each with a subsequent centrifugation to finally obtain only the support with the immobilized enzyme.

3.2.4. DESORPTION OF ENZYMES

To check whether the activity seen when carrying out the different reactions corresponds to the immobilized enzymes or to enzymes that have been desorbed, immobilization is carried out with the same process as previously using the unmodified SBA-15 carriers, SBA-15 Co 5% impregnated, SBA-15 Co 5% impregnated-calcinated and SBA-15 Co 5% calcinated-impregnated-calcinated: 20 mg are taken from each carrier, to which 100 µL of enzyme at a concentration of 20 g/L and 900 µL of buffer are added. The buffer it the phosphate buffer 100 mM, pH 7.2. The samples are taken to the cold chamber at 4ºC for 24 hours, leaving them in rotary agitation as in the previous experiment. After this time, the samples are removed from the agitation and the support with the immobilized enzyme is separated from the rest of the enzyme that has not been immobilized. To do so the samples are centrifuged to make the support with the immobilized enzyme settle. After recovering as much free enzyme as possible, the support is washed twice with 1 ml of the buffer that has been used in its immobilization, each with a subsequent centrifugation to finally obtain only the support with the immobilized enzyme.

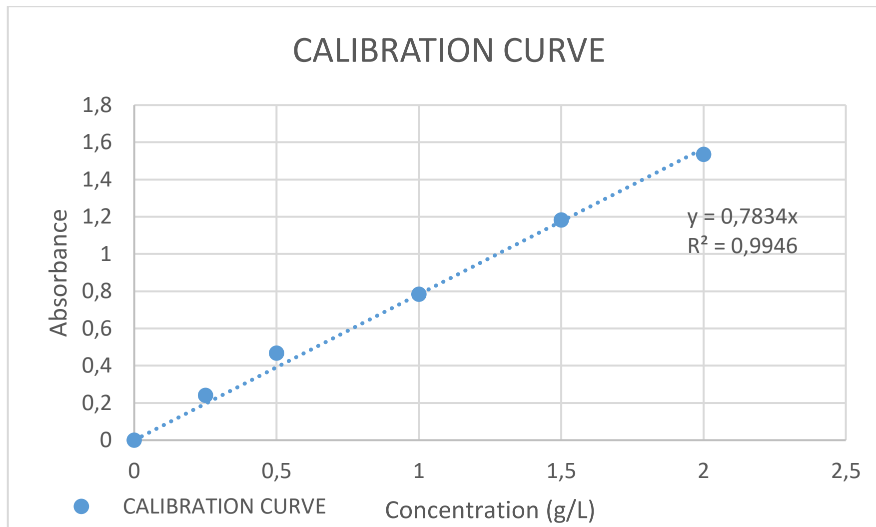
The synthesis reaction is launched with the different supports, these are left for 3 hours after which they are centrifuged to separate the reaction medium from the support. After centrifugation, the supernatant liquid is put back into agitation at 45ºC, and the reaction medium is added to the support and the reaction is launched again.

3.2.5. QUANTIFICATION OF ENZYMES

3.2.5.1. BCA METHOD

The BCA method has been used for the quantification of enzymes. The principle of this method consists of the formation of a Cu^{+2} - protein complex under alkaline conditions followed by the reduction of Cu^{+2} to Cu^{+1}. The rate of reduction is proportional to the amount of protein present. To carry out this method, a solution, known as Reagent A, of bicinchoninic acid, with sodium carbonate, sodium tartrate and sodium bicarbonate is used and mixed with a solution of copper sulfate pentahydrate known as Reagent B. The ratio shall be 50 units of Reagent A to each unit

of Reagent B. Of this mixture, 20 units shall be used for each sample unit to be quantified. Once everything has been mixed, it is left to incubate for 30 minutes at 37ºC (time which may vary depending on the temperature at which it is incubated). Finally, the absorbance is measured in the spectrophotometer. Samples of known concentration with BSA (Bovine serum albumin) are also measured to obtain a calibration line, which is used to know the concentration of the rest of the samples.



Graph 1. Calibration line of the BCA method

3.2.5.2. BRADFORD

The Bradford method is a spectroscopic analysis method used to measure the concentration of proteins in solution. A change in the colour of the sample occurs when a complex is formed with the basic amino acids present in the proteins. Moreover, it is this change in colour to a blue tone that is measured in the spectrophotometer.

To perform this method, the Bradford reagent is used, taking 20 µL of sample and mixing it with 980 µL of Bradford reagent. Once mixed, it is left for 15 minutes for the complexes to be formed and then the absorbance is measured. As in the BCA test, the measurement of samples of known concentration is performed in order to draw a calibration line and from it to know the concentration of the rest of the samples.

3.2.6. ACTIVITY TEST

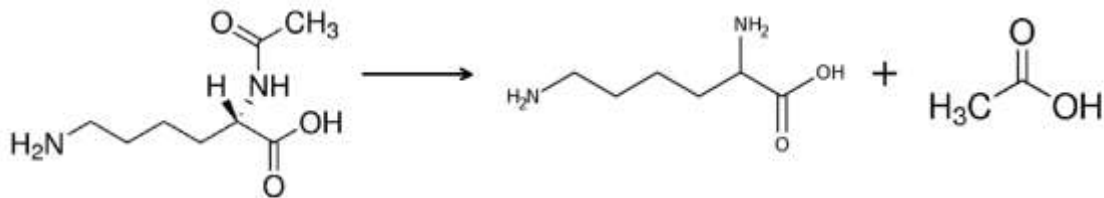
To check the activity of the immobilized enzyme, an activity test is performed, launching hydrolysis and synthesis reactions. In the enzyme used, as it is been previously said, there are four types of aminoacylases:

- Sam AA
- Sam ELA
- Sam PVA
- Sam AA-like

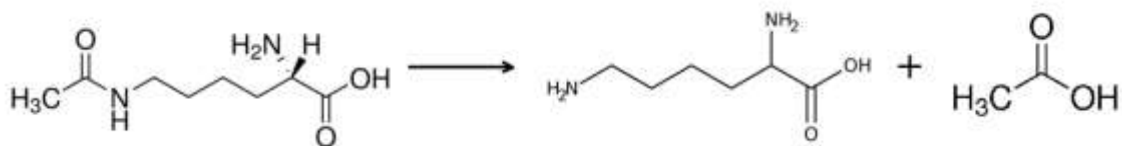
We only consider the first two; Sam AA is regioselective in alpha while Sam ELA is regioselective in epsilon. Therefore, we will do a hydrolysis or synthesis test on alpha and epsilon acetylisin. [17]

3.2.6.1. HYDROLYSIS

The hydrolysis reactions consists of Nα-acetyl-lysine (α-ACK) to obtain lysine. The reactions that takes place in presence of the free enzyme had 50 µL of the remaining free enzyme that has been taken after the centrifugation, 900 µL of buffer tris HCl 25 mM with NaCl 50 mM, at pH=8, and 100 µL of α-ACK. Moreover, for the reactions with the immobilized enzyme in the support we take all the support (10 mg in the case of GO and 20 mg in the case of SBA-15 and SBA-15 Co) and it is mixed with 100 µL of α-ACK and 900 µL of buffer tris HCl 25 mM with NaCl 50 mM, at pH=8. The mixture is left for 24 hours at 37ºC in agitation.



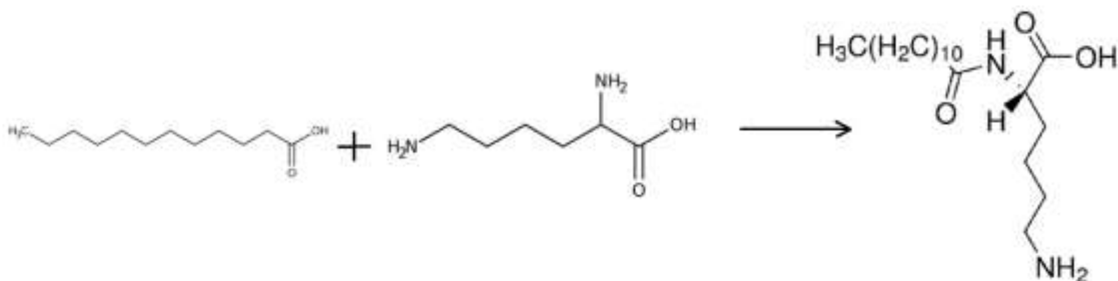
Picture 10. Hydrolysis reaction, N-α-Acetyl-L-Lysine splits into Lysine and Acetic acid



Picture 11. Hydrolysis reaction, N-ε-Acetyl-L-Lysine splits into Lysine and Acetic acid

3.2.6.2. SYNTHESIS

The synthesis reaction consists of the production of α-lauroyl-lysine from lauric acid and lysine. A 0.1 molar solution of lauric acid and 0.1 molar solution of lysine is prepared in buffer Tris HCl 25 mM with NaCl 50 mM, at pH 8. The reactions that takes place in presence of the free enzyme had 100 µL of the remaining free enzyme that has been taken after the centrifugation or 50 µL of the initial mixture of buffer and enzyme and 1 ml of the solution 0,1 molar of lauric acid and lysine. Moreover, for the reactions with the immobilized enzyme in the support we take all the support (20 mg) and it is mixed with 1 ml of the solution 0,1 molar of lauric acid and lysine. The mixture is left for 24 hours at 45ºC in agitation.



Picture 12. Synthesis reaction, lauric acid and lysine produce α-lauroil-lysine as product

3.2.7. ANALISIS OF THE PRODUCTION OF THE REACTION

After the reactions, hydrolysis or synthesis, have been carried out, the amount of product obtained must be quantified in order to know if the reaction has taken place as expected. Different methods can be used for this purpose.

3.2.7.1. THIN LAYER CROMATOGRAPHY (CROMATOGRAPHIE SUR COUCHE MINCE, CCM)

It is a chromatographic technique in which a chromatographic plate is used, immersed in a mobile phase (the eluent). This plate consists of a stationary polar phase of silica gel attached to a solid surface of aluminium. The eluent used is a mixture of acetic acid, butanol and water. A few drops of the samples which are to be quantified are applied along the length of the silica gel plate and this is introduced into a closed cell which contains the eluent. The eluent ascends through the plate due to capillarity and it will drag with it certain components, thus producing the separation of the products that will later allow their quantification. Once the eluent reaches a marked line at a fixed distance from the origin where the samples were, the plate is removed and allowed to dry. When it is dry, ninhydrin is applied, which reacts with the components that have been separated in such a way that when heat is applied they are revealed.

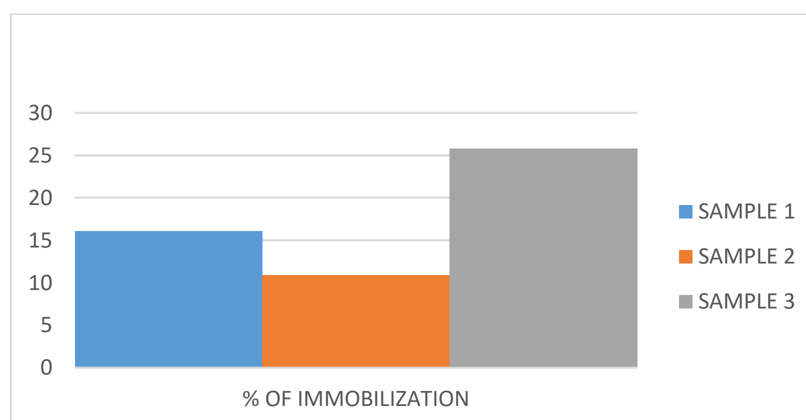
3.2.7.2. HIGH PERFORMANCE LIQUID CROMATOGRAPHY (HPLC)

It is a type of column chromatography widely used to separate the components of a mixture based on different types of chemical interactions between the substances analysed and the chromatographic column. In this case, HPLC is carried out by varying the composition of the mobile phase, also known as gradient elution. The two mobile phases used are solution A consisting of 60% methanol, 40% distilled water and 0.1% TFA, and solution B consisting of 100% methanol and 0.07% TFA. . Samples of known concentration with α.lauroil-lysine are also measured to obtain a calibration line, which is used to know the concentration of the rest of the samples.

4. RESULTS AND DISCUSSION

4.1. IMPACT OF IMMOBILIZATION PROCESS ON AMINOACYLASES PERFORMANCES

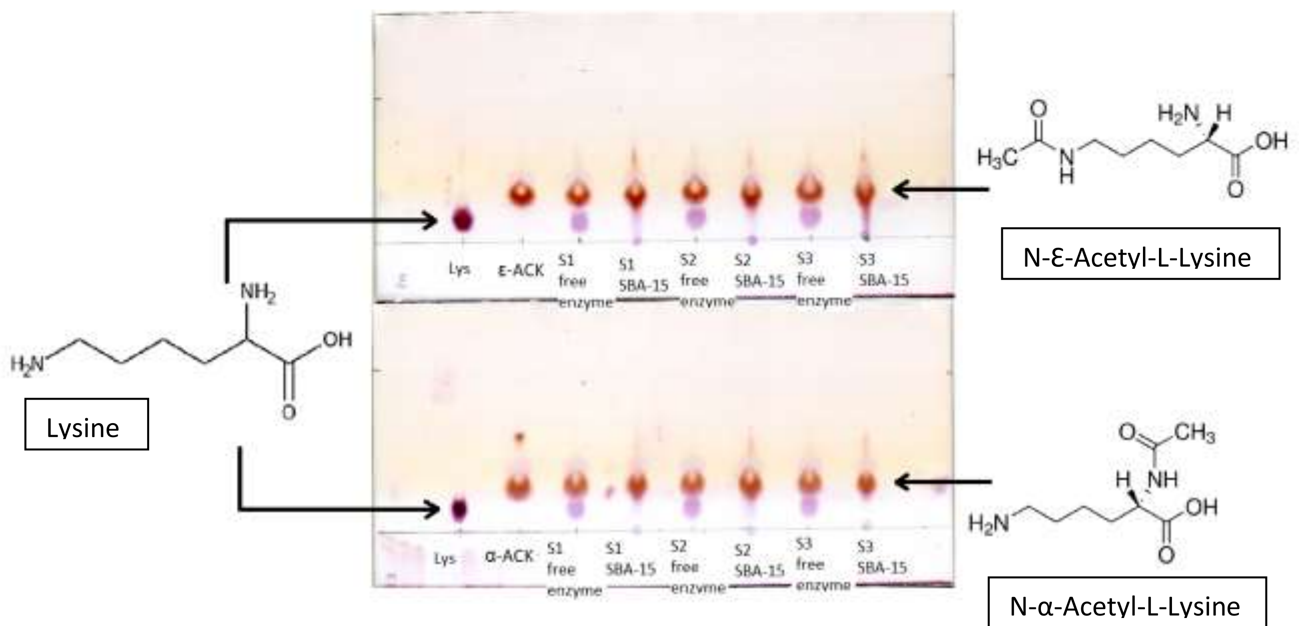
Among the three methods of immobilization that have been carried out, one has given notably better results than the other two.



Graph 2. Percentage of immobilization of the experiment that has been carried to study the impact of the immobilization method.

Sample one is that which during immobilization was at room temperature and pressure and in orbital agitation, sample two is that which was in the rotavapor and then in orbital agitation, and sample three is that which was at room pressure and temperature and then in rotary agitation.

When the activity test was performed on each of the samples, the following results were obtained:



Picture 13. Results of the activity test from the experiment carried out to study the impact of the immobilization method.

In the upper part of the picture, we have the epsilon activity test and in the lower part the alpha activity test. And, from left to right the samples are: lysine, Nε-Acetyl-L-lysine and Nα-Acetyl-L-lysine respectively; the remaining free enzyme from the immobilization with orbital agitation, the SBA-15 support with enzyme immobilized by orbital agitation, the remaining free enzyme from the immobilization with orbital agitation but which had previously been in the rotavapor, the SBA-15 carrier with enzyme immobilized by orbital agitation but which had previously been in the rotavapor, the free enzyme remaining from the immobilization by rotary agitation and the SBA-15 carrier with the enzyme immobilized by rotary agitation.

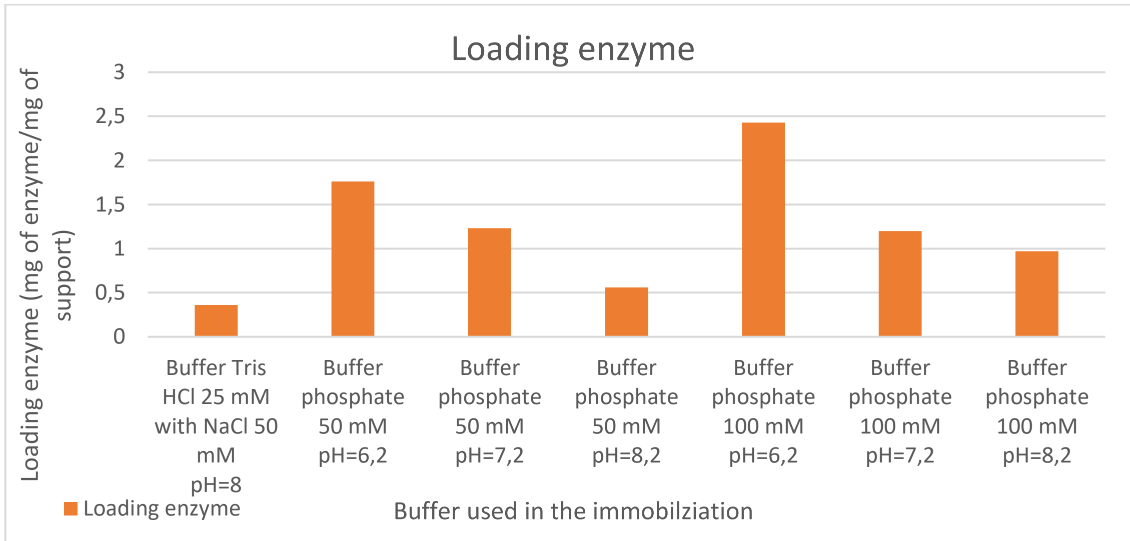
We can see that the free form enzymes do have activity, whereas when they are immobilized on the carrier they have lost the activity.

Therefore, the agitation that has worked best is rotary agitation, although the method used for immobilization should be changed to avoid loss of enzyme activity when immobilized.

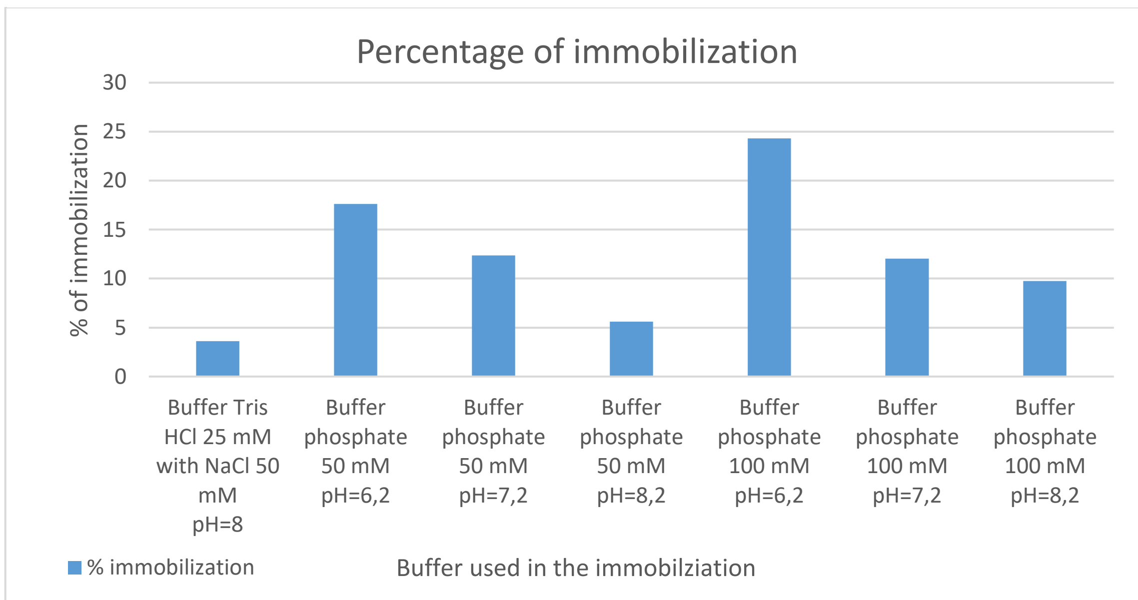
4.2. IMPACT OF BUFFERS MOLARITY AND PH AMINOACYLASES IMMOBILIZATION

4.2.1. SBA-15

The results obtained in the immobilization in the SBA-15 support with different buffers are the following:



Graph 3. Loading enzyme from the experiment carried out with SBA-15 as support to study the impact of buffers molarity and pH aminoacylases in immobilization.

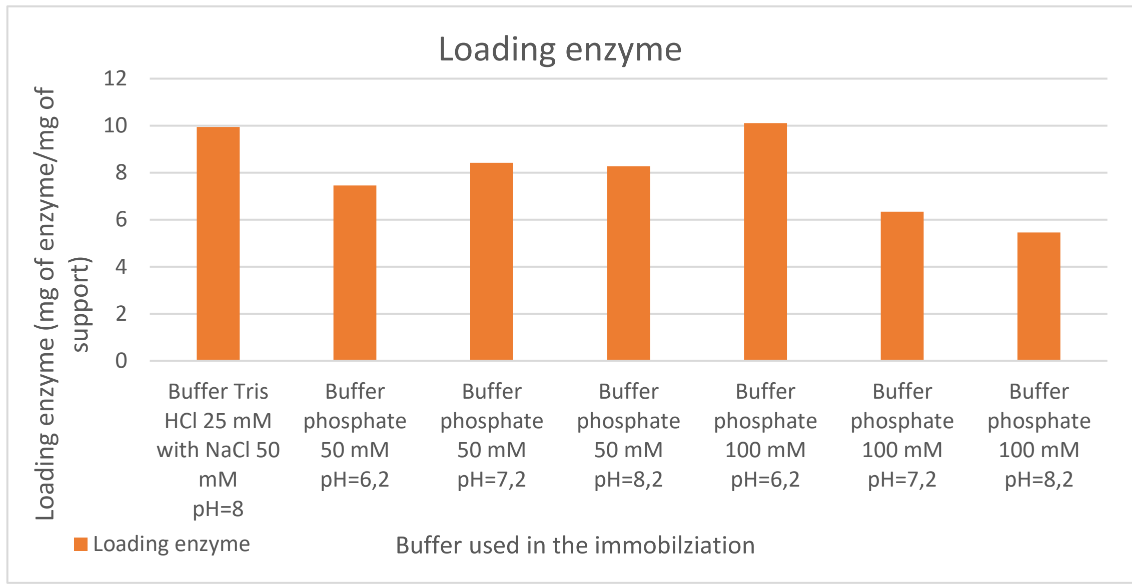


Graph 4. Percentage of immobilization from the experiment carried out with SBA-15 as support to study the impact of buffers molarity and pH aminoacylases in immobilization.

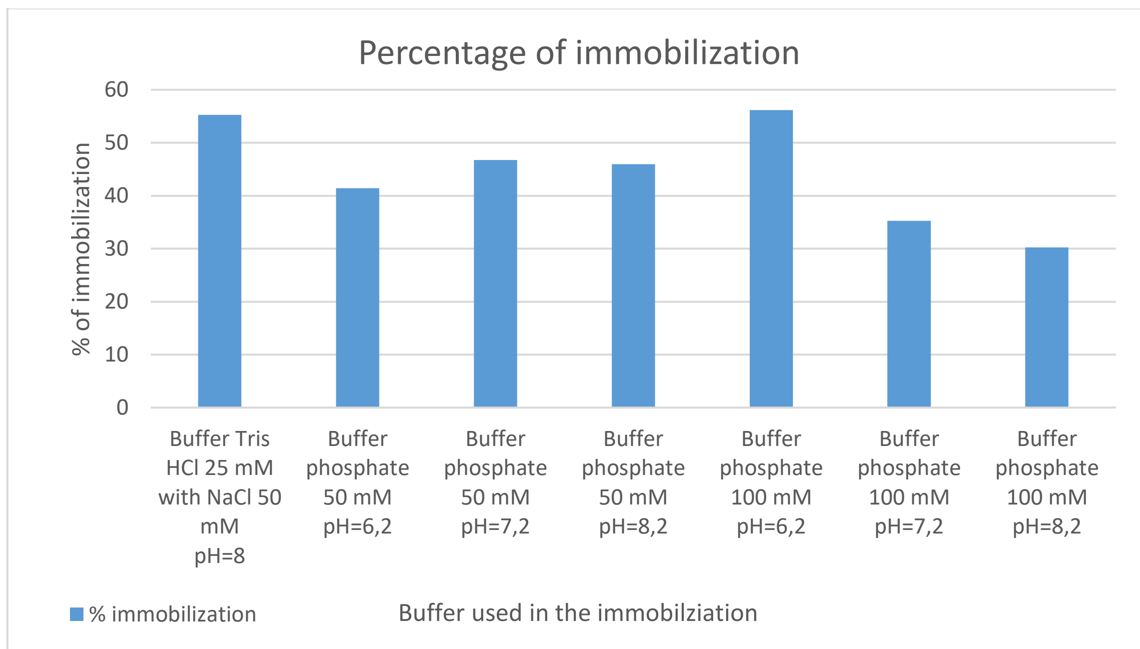
In this graph, it shows the percentage of immobilization obtained with each one of the buffers. It can be seen that a higher percentage of immobilization is obtained with the phosphate buffers than with the tris buffer. In addition, within the phosphate buffers a greater immobilization is achieved with a lower pH.

4.2.2. GRAPHENE OXIDE (GO)

The results obtained in immobilization with the support of graphene oxide with different buffers are as follows:

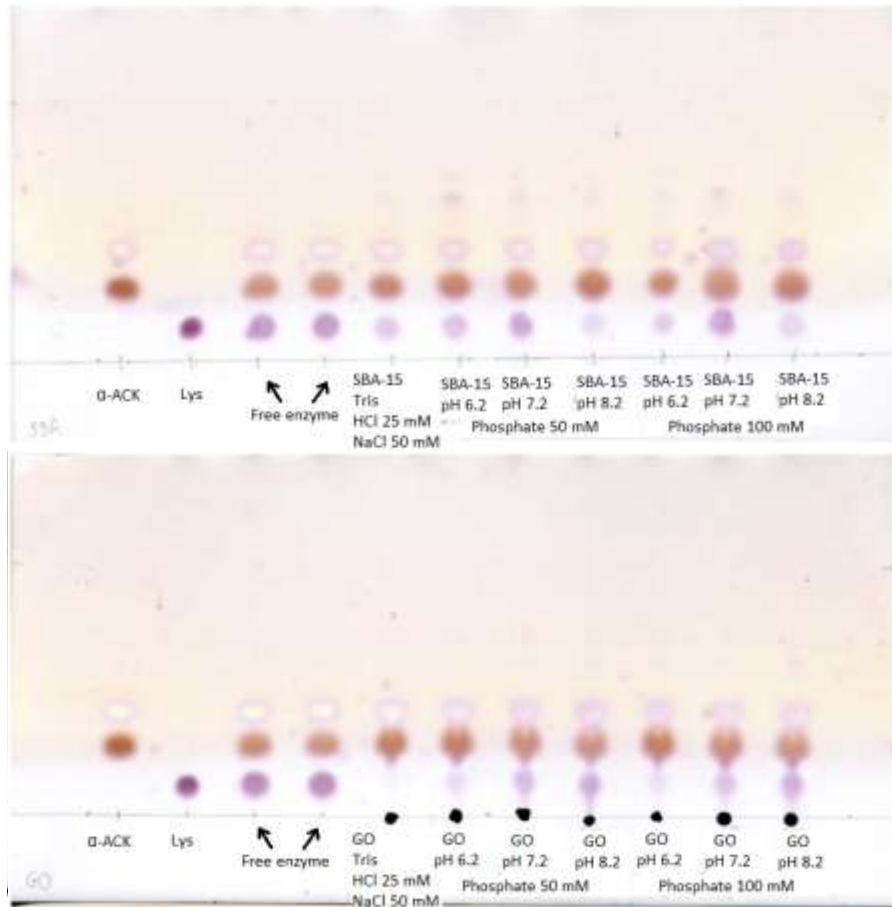


Graph 5. Loading enzyme from the experiment carried out with GO as support to study the impact of buffers molarity and pH aminoacylases in immobilization



Graph 6. Percentage of immobilization from the experiment carried out with GO as support to study the impact of buffers molarity and pH aminoacylases in immobilization

In the case of graphene oxide, it can be seen that the percentages of immobilization are more similar in all the buffers used. That is why it will be more important, to see which buffer is better, the activity that the enzymes present once immobilized.



Picture 14. Results of the activity test of the hydrolysis reaction from the experiment carried out with SBA-15 and GO as supports to study the impact of buffers molarity and pH aminoacylases in immobilization.

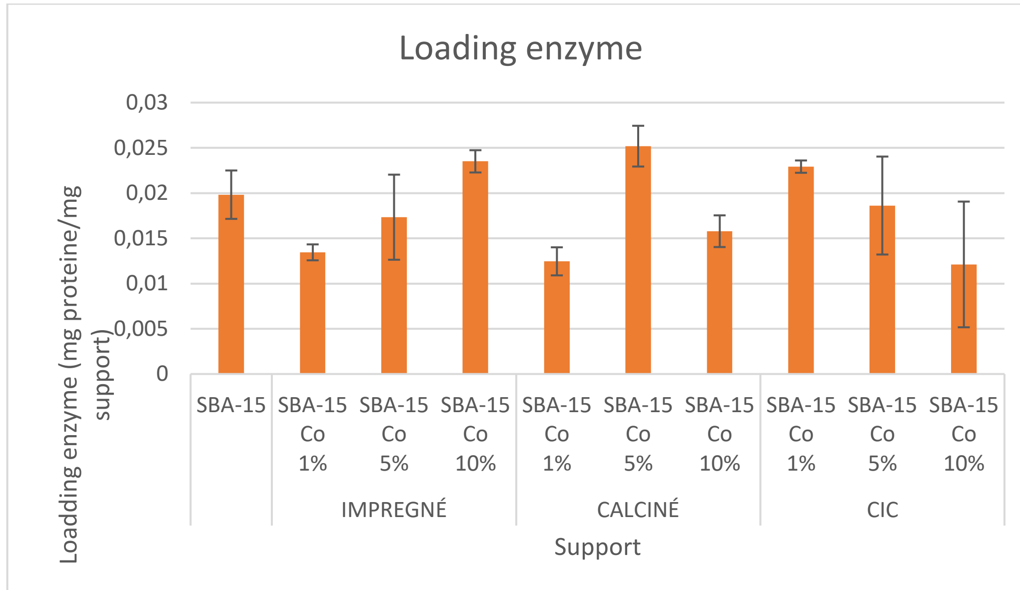
In the upper part of the picture, we have the alpha activity test of the hydrolysis reaction for the SBA-15 and in the lower part the alpha activity test of the hydrolysis reaction for the GO. From left to right we find the alpha-acetyl-lysine acid, the lysine, two samples of two reactions where there has been the free enzyme and then samples of the reaction where the enzyme has been immobilized with the tris buffer, with the phosphate buffer at a concentration of 50 mM and pH 6.2, 7.2 and 8.2 and at a concentration of 100 mM at pH 6.2, 7.2 and 8.2.

In the case of the enzymes immobilized in SBA-15, although all of them show some activity, those that present more activity are the ones that were immobilized in phosphate buffer at pH 7.2, with concentrations of 50 mM and 100 mM. In the case of GO, the ones that show more activity are those that have been immobilized in phosphate buffer at pH 7.2 and 8.2, at concentrations of 50 mM and 100 mM.

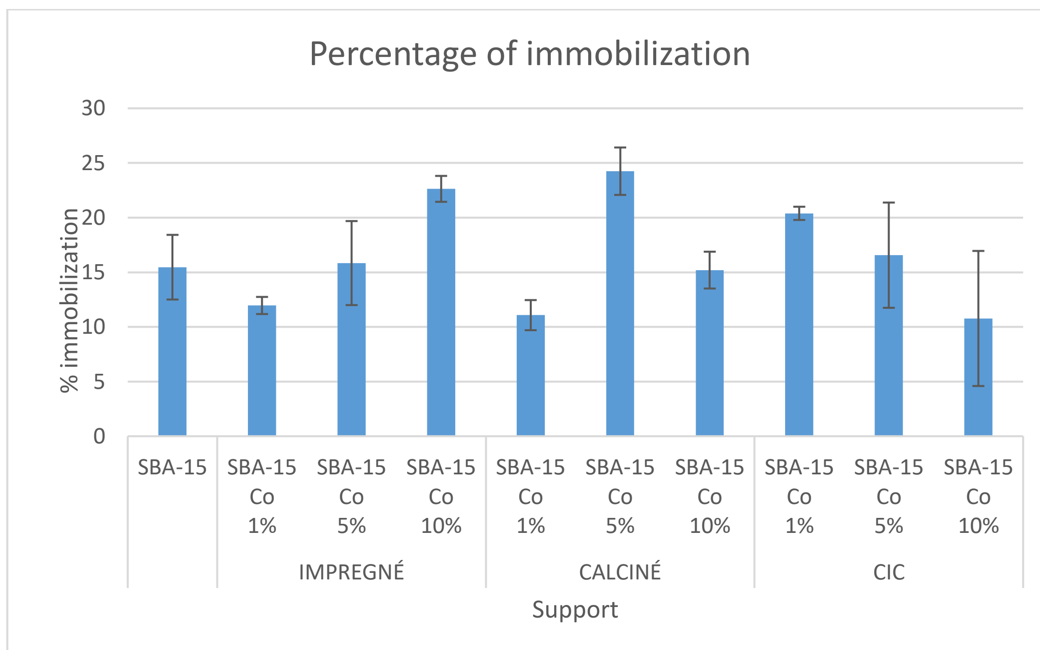
Therefore, it could be said that the best option combining both the percentage of immobilization and the activity presented by the enzymes is the use of phosphate buffer at pH=7.2.

4.3. IMPACT OF COBALT AND OF THE METHOD OF PREPARATION OF THE CARRIER

The results obtained by carrying out this experiment are as follows:



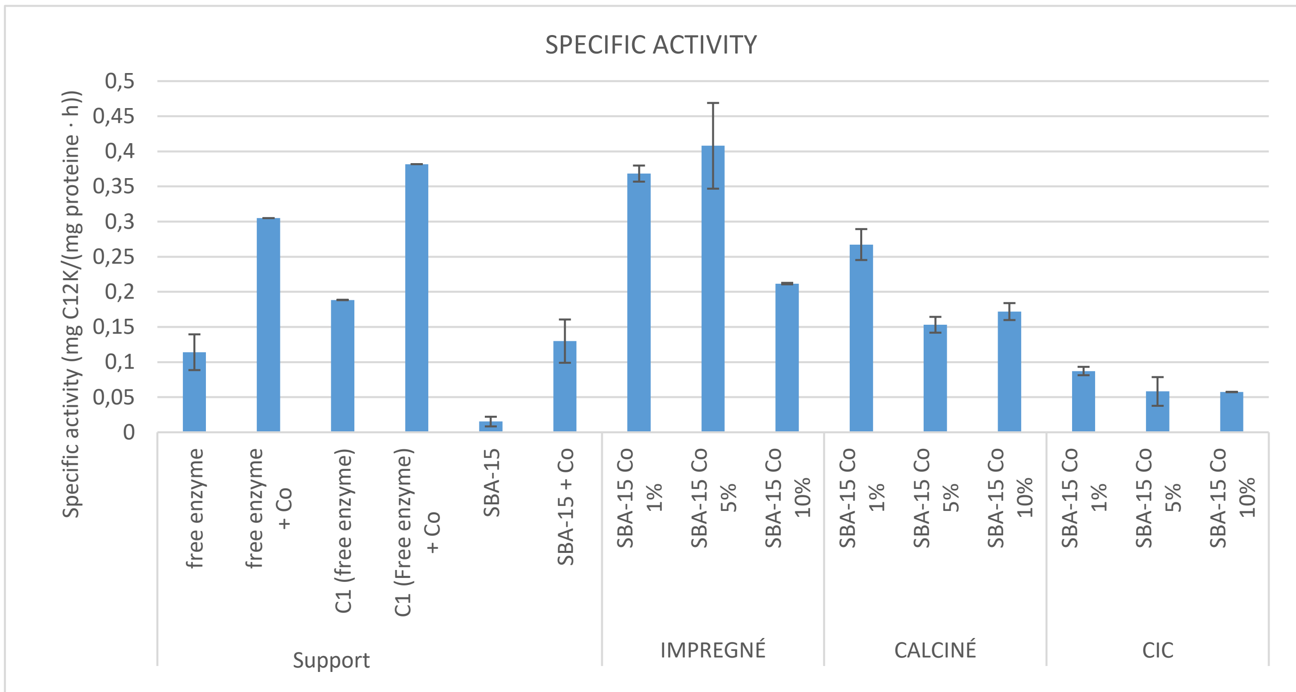
Graph 7. Loading enzyme (mg of enzyme/mg of support) of the immobilization in supports of SBA-15 without modification and SBA-15 modified with cobalt.



Graph 8. Percentage of immobilization of the immobilization in supports of SBA-15 without modification and SBA-15 modified with cobalt.

When the cobalt is impregnated, the immobilization increase with the percentage of cobalt. When the cobalt is impregnated and then calcined there is an increase and then a decrease. In addition, when the cobalt is calcined, impregnated and then calcined again the immobilization decrease when the percentage of cobalt increase.

Anyway, as it can be seen the amount of immobilized enzyme does not change much, in some cases the amount immobilized in SBA-15 Co is lower than in SBA-15 and in other cases it is higher. However, where a significant difference can be seen is in the activity presented by the enzymes in the presence of cobalt.

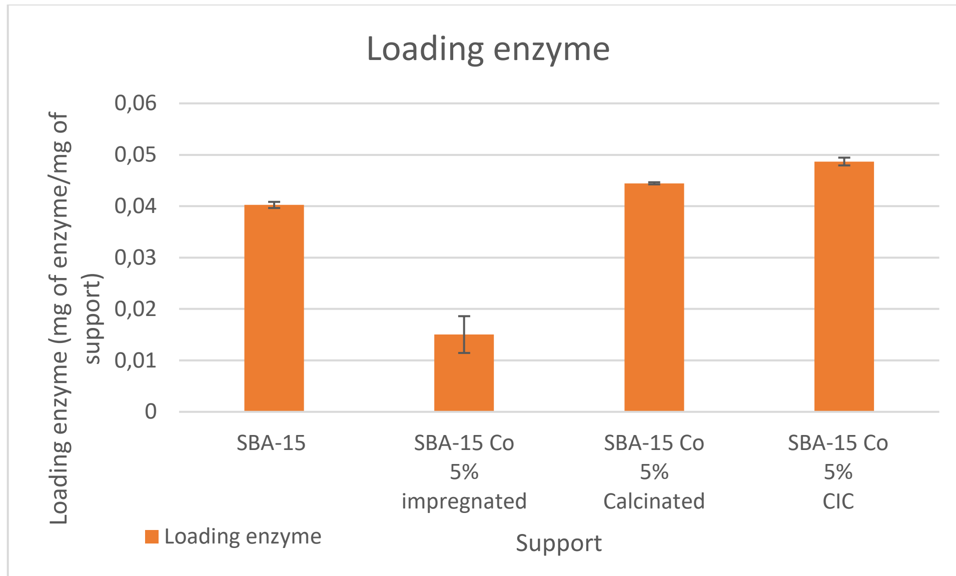


Graph 9. Specific activity (mg of product/(mg of enzyme · h)) presented by the free enzymes and the immobilized enzymes in supports of SBA-15 without modification and SBA-15 modified with cobalt., in a synthesis reaction.

In the reactions carried out in the presence of the free enzyme, a greater amount of product was obtained in the presence of cobalt than without it. The same happens with the reactions carried out with SBA-15, in one case cobalt was added in solution and in the other not and it can be seen how there is a higher production in those where there was cobalt in solution. However, in the reactions carried out in the presence of supports in which the cobalt has been previously impregnated, they give better results. That is, cobalt makes that enzyme present a higher activity.

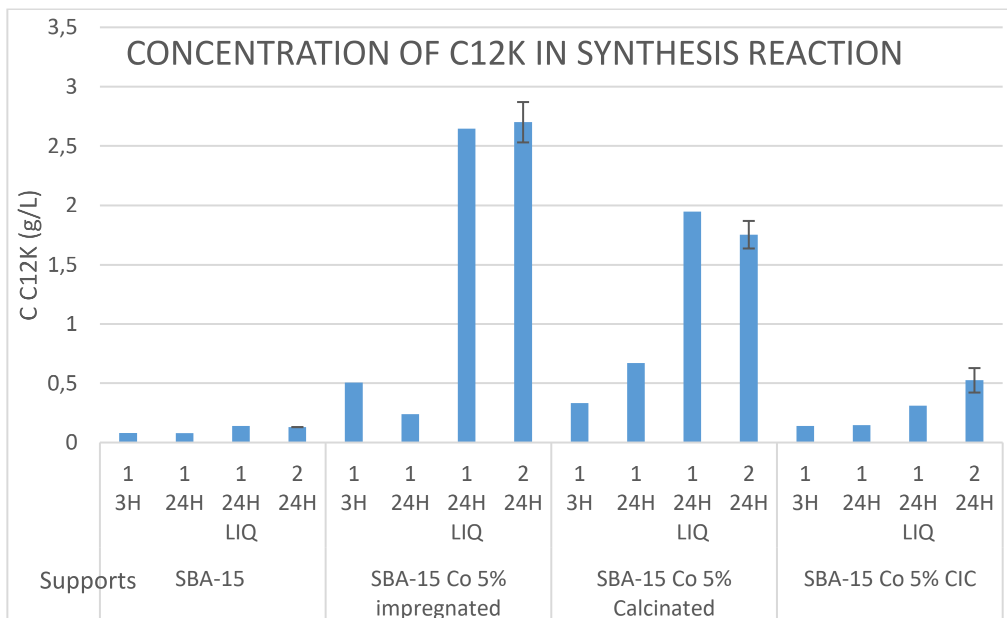
4.4. DESORPTION OF ENZYMES

The results obtained by carrying out this experiment are as follows:

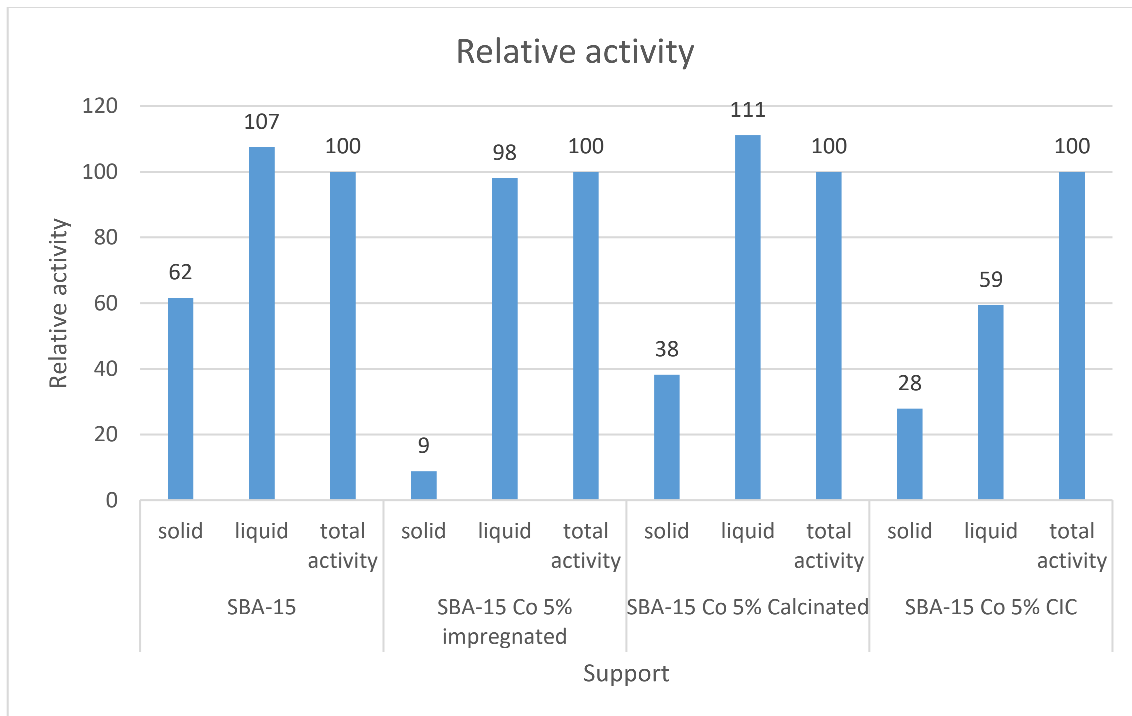


Graph 10. Loading enzyme (mg of enzyme/mg of support) of the immobilization carried to check the enzymes desorption.

After 24 hours from the launch of the reaction the samples, the production obtained 3 hours after the start of the reaction is measured and the production obtained after 24 hours of both the reaction containing the carrier and the supernatant liquid, which will contain the desorbed enzymes. The results obtained were as follows:



Graph 11. Concentration of C12K produced in the synthesis reaction carried to check the enzymes desorption. "1 3h": 3 hours of synthesis reaction before centrifugation. "1 24h": Synthesis reaction carried with the support after centrifugation. "1 24h liq": Synthesis reaction carried with the supernatant after centrifugation. "2 24h": Synthesis reaction carried without any centrifugation during 24 hours.



Graph 12. Relative activity

As can be seen, the amount of lauroyl lysine produced in a reaction where centrifugation is not performed is very similar to the amount produced by the supernatant liquid, which is due to the enzyme that has been desorbed. Therefore, it can be stated that the activity observed in the synthesis reactions corresponds for the most part to the enzyme that has been desorbed.

5. CONCLUSION

Several experiments have been carried out with the enzymes aminoacilases (from streptomyces ambofaciens) in graphite oxide supports, SBA-15 and cobalt modified SBA-15 (SBA-15 Co) and in the presence of different buffers at different concentrations and pH. Initially, the best method of agitation for immobilization was studied. As a result, rotary agitation gave a higher percentage of immobilization of the enzymes. It was also studied which buffer, Tris buffer or phosphate buffer, with which molarity and at which pH gave as a result a higher immobilization of the enzyme and a higher activity of it. We experimented with 25 mM Tris HCl buffer with 50 mM NaCl at pH 8 and phosphate buffer at 50 mM and 100 mM molarities and pH 6.2, 7.2 and 8.2. As a result, the phosphate buffer gave better results than Tris, and among the different phosphate buffers used, the one with the best results in terms of both immobilization and activity of the enzyme after being immobilized is the phosphate buffer with molarity 100 mM and pH 7,2. Next, the influence of cobalt on immobilization and the influence of the method by which cobalt has been fixed was studied using SBA-15 Co supports. With regard to immobilization, the presence of cobalt does not improve it notably, however, the activity of the enzymes improves very significantly, in the case of the cobalt being in solution, but especially when the cobalt is fixed in the support. Finally, a small experiment was carried out to see if the activity observed in the reactions is due to the immobilized enzymes or to the enzyme that has been desorbed. After the immobilization, the synthesis reactions were launched, and three hours later, the support was

centrifuged to separate it from the reaction liquid. The solution with the reagents is again added to the support and the supernatant liquid is put into agitation at 45ºC. Thus, the activity of the supernatant liquid after 24 hours is very similar to the activity presented by a reaction in which centrifugation has not been carried out. Therefore, it is possible to know that the enzyme is desorbed and the activity observed is due to the enzyme that has been desorbed. As a conclusion, it can be said that the best thing to do now would be to attempt immobilisation by covalent bonding to prevent the enzyme from dissociating from the support and to achieve greater activity.

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