Importance of the support properties for immobilization or purification of enzymes

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Abstract

Immobilization and purification of enzymes are usual requirements for their industrial use. Both purification and immobilization have a common factor: they use a solid activated support. Using a support for enzyme purification means having mild conditions for enzyme release and a selective enzyme-support interaction is interesting. Using a support for immobilization, enzyme desorption is a problem. The improvement of enzyme features via immobilization is a usual objective (e.g., stability, selectivity) Thus, a support designed for enzyme purification and a support designed for enzyme immobilization may differ significantly. In this review, we will try to focus the attention on the requirements of a support surface to produce the desired objectives. The ideal physical properties of the matrix, the properties of the introduced reactive groups, the best surface activation degree to reach the desired objective, and the properties of the reactive groups will be discussed.

Key words: Enzyme purification, enzyme immobilization, enzyme stabilization, superficial density of reactive groups, multipoint attachment, one point attachment.

1- Introduction

The chemical industry of the 21st century intends to produce very complex products in a sustainable way to give solutions to the public demands concerning pharmaceutical, food and fine chemistry companies.[1] It is in this sense where enzymes have found their natural niche as industrial biocatalysts. They are extremely selective and specific, able to catalyze reactions at low temperature and pressure, even in aqueous media.[2] However, these natural biocatalysts have been designed by nature during evolution to fulfill some physiological requirements, and some of their properties are quite far from the industrial requirements.[3] Thus, enzymes are water soluble, prone to suffer inhibitions, and with low stability. Their exceptional activity, specificity and selectivity are found within physiological substrates and reactions. Then again, in industry the researcher intends to use the enzyme with a different substrate and, in many instances, in a very different reaction.[4] Moreover, enzymes are accompanied by many other enzymes in microorganisms, sometimes with activities against the same substrate. A particular enzyme may be specific and selective, but if the contaminant enzymes have opposite (or just different) properties, this may reduce the apparent performance of the prepared biocatalyst.[5] Even in the best scenario, the contaminant proteins will reduce the volumetric activity of our biocatalyst.[6]

Fortunately, researchers have many tools to improve enzyme performance that have experienced a very rapid development in the last years, for example, microbiology and genetics have promoted a revolution in biocatalysis.[7] Enzyme performance has been improved via site-directed mutagenesis.[8] Moreover, it is now possible to mimic the natural evolution targeting the desired enzyme property via directed evolution.[3c] Metagenomic tools permit to produce enzymes whose origin may be fully unknown,[9] e.g., extremophiles is nowadays a source of thermostable enzymes with novel properties

just by using DNA from thermophilic environments.[9a] However, these improvements of enzyme properties have not avoided the fact that enzymes still need to be purified and their recovery after the reaction may be complex. Activated supports are a key to solve both problems, easing the industrial implementation of enzymes; enzyme purification (to eliminate all enzymes able to catalyze any unwanted modification of the substrates or products of the reaction)[10] and immobilization (to simplify the enzyme reuse) [11] may require the use of activated supports. The rapid development on materials science has permitted to increase the availability of new activated supports and to better understand the behavior of others: nanomaterials,[12] hybrid materials,[13] tailor made silicates,[14], etc.

Immobilization, being almost compulsory, has been used by many researchers as a tool to improve various enzyme features, such as activity, selectivity, specificity, resistance to inhibitors, etc.[15] Rocha-Martin, J., Acosta, A., Berenguer, J., Guisan, J.M., Lopez-Gallego, F.Selective oxidation of glycerol to 1,3-dihydroxyacetone by covalently immobilized glycerol dehydrogenases with higher stability and lower product inhibition (2014) Bioresource Technology, 170, pp. 445-453 In some cases, some immobilization protocols have permitted the one-step immobilization and purification by a careful control of the support and/or immobilization conditions (Figure 1).[16] Nowadays, an immobilization protocol that does not permit the solution to some other enzyme limitations is not considered a good immobilization protocol.

Both for immobilization and purification, an adequate selection of the support and of the protocol are critical. There are many parameters that define a support: internal geometry (e.g., flat surfaces or thin fibers), specific surface area, superficial activation degree, mechanical resistance, pore diameter, etc.[11b,17] In some instances, some positive properties of a support for enzyme immobilization may be also positive

for enzyme purification, but in general they have a critical difference in the objective that may cause the optimal properties for each support to be different. While for enzyme purification, having an easy release of the enzyme from the support is mandatory to avoid enzyme inactivation, enzyme immobilization requires a strong enzyme-support attachment. Thus, the undesired release of the enzyme during operation needs to be avoided for enzyme immobilization while a too strong enzyme-support interaction may be unsuitable for enzyme purification (Figure 2).[15]

One critical key parameter when using a support for purification or immobilization is the identification of the first cause for enzyme interaction with it. In some instances, the interaction of one group in the support with the enzyme is enough to fix the enzyme to the support, while in many other cases, it is necessary to establish the interaction of several groups of the support with several groups of the enzyme.[18] After immobilization, other phenomena (desired or undesired) may occur, but the researcher must detect these phenomena and develop tools to control them. That is, the first immobilization may be via a one point or a multi-interaction, and after this immobilization, the support may continue increasing the number (or even the quality) of the interactions,[19] involving new groups as it is the case when using heterofunctional supports (Figure 3).[20]

Next, we will discuss how the properties of the support and the reactive groups placed on its surface may determine its suitability for being used for enzyme immobilization or purification, mainly at industrial level. In most cases, the same parameter may have some positive and some negative effects, compromise solutions use to be required to get optimal results, and each of this may depend on the particular enzyme, reactor and even final application desired.

2- Mechanical properties of the support for immobilization or purification

In this case, immobilization and purification have similar demands. The selection of the mechanical properties will depend on the final configuration of the reactor more than in the application for purification or immobilization .[6] If the reactor is a fixed bed reactor, it should possess very high rigidity to withstand high pressures without pressure problems.[6] Silica-based materials,[14b,21] carbon materials,[22] inorganic oxides,[23] porous glass and other mineral materials as copper phosphate or cobalt phosphate (by mineralizing cobalt-phosphate in the presence of His-tagged enzymes) Selective biomineralization of $Co_3(PO_4)_2$-sponges triggered by His-tagged proteins: efficient heterogeneous biocatalysts for redox processes Fernando López-Gallego and Luis Yate *Chem. Commun.*, 2015, **DOI:** 10.1039/C5CC00318K, montmorillonite and bentonite[24] may be a very good selection for a column. The situation is different if a stirred tank reactor is used, the mineral materials will be broken in these systems, while other flexible materials may be more adequate, such as agarose beads (the bad mechanical resistance to magnetic stirring has led to consider these material inadequate for stirred tank configurations, but using mechanical stirring the resistance is very high),[25] cellulose beads,[26] Lentikats (polyvinyl alcohol polymers shaped like a lens),[27] etc. Agarose beads are usually used in enzyme purification using a column configuration, but this requires using a very low flow rate, because the low rigidity produces pressure problems at high column height and rapid flows.[25a,25c] Some new more rigid agarose beads permit to stand higher pressures.

Some commercial materials, mostly from organic polymers, are more or less adequate for both reactor configurations. Examples are epoxy acrylic beads (e.g., Sepabeads, Eupergit),[28] styrene,[29] styrene-divinylbenzene beads,[30] Lewatit,[31] etc. Other reactor configurations, like fluidized-bed,[32] vortex reactors,[33] etc. may be less

demanding for the support mechanical resistance. Although agarose beads are traditionally used in purification due to their good inertness properties, over 100 papers and patents have been published on the use of organic polymers in enzyme purification.

The purification in a stirred tank is only possible if the support is able to produce a selective adsorption of the target enzyme, as the selective desorption using a gradient is far less efficient using a stirred system than using a column.[34] In biocatalysis, the selection of one reactor configuration may depend on many different factors (enzyme kinetics, control of pH, supply of oxygen, and other features of the reaction may make a stirred tank or a bed reactor more or less suitable), sometimes even on the available facilities on a specific factory or the volume of substrate to be processed.[35] That way, the properties of the support need to be chosen considering the final reactor to be utilized, but a handful of supports with different mechanical properties are available at similar prices.

3. The role of the particle size in a support for enzyme immobilization or purification.

The particle size plays a very important role in the handling of the particles, both for purification and biocatalysts preparation. Large particles may be retained more easily than small ones, but they may have some problems that we will detail below. Too small particles may become almost impossible to handle under industrial conditions. Production of large particles may be a problem in some instances, and the use of the support may generate fine particles that can produce problems after several cycles, thus this may be to a certain degree related to the mechanical resistance of the support. Thus, a compromise solution needs to be reached.[6]

In a column (for enzyme purification or biocatalysis), very large particles may favor the occurrence of preferential ways in the column decreasing the resolution of the column or the observed catalytic activity. Moreover, some diffusional problems of the enzyme molecules in very long pores may decrease the rate of enzyme adsorption, slowing down the full coating of the support surface by the enzyme (Figure 4).[36] Also, as these particles may have an ionic nature and thus they may behave as a "solid buffer", the change in the pH in enzyme purification using ionic exchange may not be as immediate as desired, producing also a decrease in the final resolution of the column.

If the purification is performed in batch via selective adsorption, just a delay in the adsorption rate may be found using a large particle, in many instances the advantages of large particle handling may make up for this delay on the enzyme adsorption.

In biocatalysis (under any reactor configuration), large particles may give rise to larger diffusional problems. The diffusional problems are not always negative for the enzyme performance, but in general they may produce a decrease in enzyme activity.[37] For example, if the substrate diffusion rate is slower than the substrate consumption rate, it is possible that the enzyme molecules located in the core of the support will not receive any substrate, thus being apparently fully inactive and decreasing the observed activity of the biocatalysts (Figure 5).[37b,37e] Other phenomena favored by the size of the particle are the promotion of pH gradients if the product has an ionizable group (e.g., in hydrolysis of an ester releasing a carboxylic acid).[38] This produces a decrease in the pH along the pore of the biocatalysts particle, and makes it impossible to use the enzyme under optimal pH conditions (because each enzyme molecule may be at a different pH value depending on their position on the pore) (Figure 6). This is not always negative. In some instances, this may be used to improve enzyme stability, for

example if the reaction is performed at alkaline pH value (e.g., due to product solubility or stability) and the enzyme stability/activity is higher at acidic pH value. An example of this is the hydrolysis of penicillin G catalyzed by penicillin G acylase.[39] The reaction is performed at pH 8 due to the adequate enzyme activity and substrate/product stability under these conditions, but the enzyme is more stable at acidic pH values. If a pH gradient is permitted, the initial activity decreased by almost a 50%, but the time to get full hydrolysis only increased by 20% and the stability of the enzyme increased several times, making it interesting to explore this pH gradient as a way to improve enzyme productivity.

Other problem related to the promotion of gradients of substrates, products, pH, etc. inside the pores of the particle is the apparent changes in enantio-specificity of an enzyme used in a kinetic resolution of racemates.[40]

The existence of substrate limitations for the biocatalyst in the inner part of the particle may make that these enzyme molecules may only act versus the "worst" substrate because the "good" one has been fully modified by the enzymes nearer to the particle surface, worsening the final results. On the other hand, the change on the internal pH, by pH gradients, may alter the behavior of the enzyme, improving or decreasing the final enantiomeric excess of the product; this fact needs to be considered in the studies.[15a] In these cases, the particle size is very important to determine the relevance of the diffusion limitations. Boniello, C., Mayr, T., Bolivar, J.M., Nidetzky, B. Dual-lifetime referencing (DLR): A powerful method for on-line measurement of internal pH in carrier-bound immobilized biocatalysts (2012) BMC Biotechnology, 12, art. no. 11.

If the particle is coated with some ionic group or an ionic polymer (e.g., polyethylenimine), this particle will behave as a "solid buffer".[15a]. This may decrease

the existence of pH gradient produced by the catalytic enzyme activity. Moreover, this may permit to keep the enzyme inside the particle protected from external changes in the pH value caused by the addition of titrating reagents (e.g., used in many reactions where a carboxylic acid is released) (Figure 7), this protection will increase when the size the particle does.

Thus, the size of the particle, and if this particle is loaded to the maximum or an intermediate enzyme load is recommended, depend on many points. For example, the reactor configuration, if the enzyme activity versus the target substrate is very high or it is very low, the real effect of the changes in medium on enzyme properties, the kind of reaction that we are performing. In some cases, only a decrease in activity is observed and this slower reaction time may be assumed by the easing of the biocatalyst handling (that also may save time). However, in a kinetic resolution a decrease in the final product quality cannot be assumed in any case.[40] Obviously, the handling of the particle will be kept in mind during all the optimization process of the biocatalyst.

4- Relevance of the support pores size and specific area for enzyme immobilization or purification

Pore size and specific surface area of a porous particulate support are related parameters: in general, the larger the pores, the smaller the specific area.

The specific area, if the enzyme can penetrate the pores of the particle, determines the loading capacity of the support.[11b,17a,41] At first glance, the best support is that having the highest loading capacity, because that way it is possible to purify/immobilize a larger amount of enzyme using a lower volume of support. It is possible to decrease the loading of the support if any kind of problem arises due to a very high enzyme activity (see section 3 of this review), but we cannot load more

enzyme than that determined by the specific area of the matrix if the specific activity of the enzyme is low and diffusional problems are not expected. Thus, in general a large specific area is recommended from an economical point of view.

The pore diameter determines the size of the protein that can be immobilized on that support.[42] It should be considered that the pore must permit the entry of new enzyme molecules once there are confronted enzyme molecules already immobilized to avoid the closing of the pore. Thus, the diameter of the pore needs to be 4-5 folds larger than the enzyme larger diameter if we intend to ensure the full coating of the support surface with the enzyme in a reasonable time.[43] It should be considered that the required pore diameter will be determined by the size of the larger protein able to become adsorbed on it, both in purification or immobilization, and the use of pores based just on the size of the target protein may generate problems to have a full loading of the support (Figure 8).[6] Even if a selective adsorption is intended, the immobilization of traces of very large proteins (that on the other hand are those that can more easily become immobilized due to their higher possibilities of establishing multi-interaction with the support surface) may produce the closing of the pores, drastically reducing the loading capacity of the supports.[6]

Some other factors may be modulated by the pore diameter size. For example, this parameter may affect the diffusion of the substrates. The use of supports bearing very small diameter pores may increase the diffusional limitations and this may have special relevance if the substrates are very large macromolecules, such as proteins or other polymers (Figure 9).[43a] Moreover, very large pores produce a lower mechanical resistance of the support particle.

Thus, it is again necessary to reach a compromise solution that may permit sufficiently good results in the different parameters, considering loading capacity and size of protein/substrates.

5- Influence of the internal morphology of the support on the intensity of the protein/support interactions

Previous points affected mainly the biocatalysts performance at "macroscopic" level. From this point, the interaction between enzyme molecules and the supports will be analyzed at "molecular level".

After the enzyme penetrates the particle, the internal morphology of the support will determine the possibilities of getting a very intense (ideally involving all likely groups of the protein)[44] or a very limited enzyme molecule-support interaction (ideally just one point).[45]

If the support is composed of macromolecular fibers, having a diameter size smaller than that of the enzyme, it is hard to get an intense enzyme-support multi-interaction[44b] (e.g., Toyopearl, Sephacel). If the support has large internal surfaces, enough to resemble that the enzyme molecule is interacting with a flat surface, it is possible to get an intense multi-interaction involving over 30% of the enzyme surface (e.g., agarose beads, porous glass or silicates, Sepabeads).[6] Moreover, these supports may have a higher mechanical resistance. Some supports are in between (e.g., Eupergit),[28c,46] those permit only a moderate enzyme-support multi-interaction.[28a,47]

The enzyme support-multi-interaction has different relevance for enzyme purification or immobilization.

Most of the chromatographic matrices for protein purification base their enzyme adsorption capacity on the establishment of many weak enzyme-support interactions,

290 that is, the enzyme is only incorporated to the support if many enzyme-support
291 interactions are achieved.[48] Ionic exchangers, hydrophobic, immobilized metal chelates
292 (IMAC) (for native proteins) matrices are examples of this kind of supports, all of them
293 widely used in protein purification.[49] These supports usually are intended to adsorb
294 most of the proteins on the crude extract and the purification is obtained via selective
295 desorption (Figure 10). Therefore, a support that did not permit an intense enzyme-
296 support multi-interaction does not seem very adequate; e.g., many proteins can be
297 unable to become adsorbed on thin fibers. However, if enzyme adsorption is based on a
298 single and strong point interaction with the support (e.g., affinity chromatography,
299 IMAC for poly His tagged proteins),[50] the possibilities of a multi-interaction far for
300 being an advantage becomes a problem.[45,51] It should be considered that many of the
301 ligands used in affinity chromatography and the IMAC matrices are not physically inert
302 (they are ionic, hydrophobic, able to give hydrogen bridges, etc.), but may interact with
303 different moieties of the enzyme molecules.[52] If a multi-interaction is favored,
304 undesired protein molecules may become adsorbed on the support via this alternative
305 mechanism, reducing the purification factor achieved (Figure 11). Moreover, even if the
306 adsorption of the protein remains selective, the affinity adsorbed protein molecules may
307 unspecifically interact with these groups, leading to enzyme inactivation or to
308 difficulties to get the enzyme desorption. In the case of large proteins, able to establish a
309 very intense enzyme-support multi-interaction, this may produce the impossibility of
310 getting enzyme desorption in an active form.[53] The purification via selective adsorption
311 of the target protein will save support surface and may permit the purification using a
312 stirred tank configuration and not columns, because it is no longer necessary to have a
313 sophisticated gradient to have the selective desorption of the enzyme from the support.

In protein immobilization, the control of the number of enzyme-support interactions is even more relevant. In the case of a physical adsorption, the number of enzyme-support interactions determines the range of conditions where the biocatalysts may be used: a higher number of interactions will permit a stronger adsorption, and thus, a biocatalyst that may be used in a broader range of conditions and for more enzymes.[6] In some instances, the support surfaces are even coated with ionic polymers to permit a tridimensional enzyme-support interaction, and that way, a very strong enzyme adsorption (Figure 12).[54]

In the case of covalent attachment, the enzyme multipoint covalent immobilization has been described as a powerful tool to improve enzyme rigidity, and that way, enzyme stability.[15b,17a,55] This is based on the fact that the relative positions of all groups involved in the immobilization cannot alter their relative positions under any circumstance (no more than the size of the spacer arm).[44,56] Thus, for this goal, a support permitting an intense multipoint interaction with the enzyme is preferred: this stabilization strategy may be only obtained using supports bearing large flat surfaces.[44b] However, if the support is not fully inert after immobilization, the possibility of suffering an intense uncontrolled interaction with the support may have negative effects on enzyme stability (Figure 3). For example, the blocking of the support with hydrophilic compounds is critical using Sepabeads (having large flat surfaces) while it is not so relevant using Eupergit (presenting not too thick fibers).[28a,47] Without the proper blocking, Sepabeads –penicillin acylase was less stable than Eupergit-penicillin acylase, while with the proper treatment, the Sepabeads biocatalyst was significantly more stable due to the higher multipoint attachment.[28a] Thus, the negative effects of the hydrophobicity of the support reverted the positive effects of a more intense multipoint covalent attachment.

Another point to be considered is if the stabilization of a multimeric enzyme is intended via immobilization by involving all enzyme subunits.[57] A thin fiber may involve both subunits of dimeric proteins (not as easily as a flat surface), but will be unable to involve the four subunits of a tetrameric planar enzyme. Thus, in this case a support having large flat surfaces seems to be recommended.

If the immobilization of the enzyme tries to keep the enzyme properties intact, and it is performed just to have a model enzyme where aggregation or other intermolecular processes are no longer possible, a low geometrical congruence of the support surface with the enzyme seems recommended. In this case thin fibers may be a more suitable solution than large flat surfaces.[6]

Thus, the internal morphology of the support internal structure is a key point to understand the interaction between enzyme and support at a molecular level.

6- Effect of the activation degree of the support on the interaction between protein and support

The number of active groups on a support surface is another key factor to control the enzyme-support multi-interaction.[6,15b] The effects are more relevant if a support having flat surfaces is used, with a lower incidence if the support is formed by thin fibers is employed.[28a,47] Only if there are several active groups under the enzyme molecule in the support, is a multi-interaction likely. This multi-interaction may be more intense when the number of reactive groups in the support increases. In a similar way, to ensure that just one interaction between enzyme and support is established, just one group should be under each enzyme molecule.[6] Considering that the surface density of reactive groups and adsorption rate are related terms (sometimes even in an exponential way), a very lowly activated support may offer an extremely low

immobilization rate.[25b] In some instances in order to get a reasonable immobilization rate a higher number of active groups in the supports is required, even although that way a just one-point attachment may be hard to ensure.[25b]

If the enzyme-support interaction is via too many points and this produce some undesired effect on the enzyme properties, it is always possible to perform an activation of the support under the maximum activation level, thus in general it is desirable that the support may offer a very high maximum number of active groups.

In some instances, for example if the support will be used for enzyme purification via affinity chromatography, a low activation of the support is preferred (Figure 13).[48,58] This is for two reasons. First, as stated before, the groups will be not physically inert and can produce the adsorption of the target protein via another mechanism not related to the affinity, or adsorb other proteins.[52,59] Second, and even more important, if there are many ligands under the enzyme molecules, and considering that the recognition pocket in the enzyme may be more or less an internal one, we are creating a “wall” where these pockets may have serious steric problems to access the ligand (and that is not solved by using large spacer arms). This way, it is possible that at a higher ligand loading, a lower affinity adsorption is achieved.[52,59]

The use of a highly activated support in a flat surface is necessary when a very intense multipoint covalent attachment is desired.[6,15b] The surfaces of a protein and a support are not complementary and only if the distance between groups fits the distances between reactive groups in the protein (inside the range of mobility of the structures), the multipoint covalent attachment may be achieved.[44a,55c,60] The possibility of this happening obviously increases if the number of groups in the support increases.[61]

Recently it has been shown that this may be achieved also by increasing the number of reactive groups in the protein via genetic or chemical tools.[62] Nowadays, it is possible to play simultaneously with the enzyme and the support number of reactive groups.[63]

The use of supports for physical unspecific adsorption may differ if they are used for immobilization or purification. To purify enzymes (via selective desorption), the activation degree needs to be enough to adsorb many of the proteins in a crude, but not too high, to prevent problems during the desorption step. Recently, it has been proposed that a control of the activation degree may be used to purify large proteins from small ones.[51c,53] Due to the multipoint adsorption requirements, supports with the lowest degree of groups able to adsorb the target large enzyme molecule, that due to their size are able to establish long distance interactions, will only immobilize these large molecules.[51c,53] The smaller ones will be unable to become adsorbed because they cannot give a multipoint adsorption. If the target enzyme is the only large one, a full purification may be obtained just by this adsorption step (e.g., multimeric thermophilic enzymes cloned in a mesophilic host and submitted to a thermal shock).[51c,53] This strategy may be also used to selectively adsorb and even to stabilize weak multi-protein complexes, using supports able to absorb the protein complex but not the individual components.[64]

To immobilize enzymes via physical adsorption, the enzyme adsorption should be as strong as possible to prevent enzyme desorption.[54a,54b] After enzyme inactivation, the enzyme will be desorbed under drastic conditions and the support may be reused. The use of very highly activated supports has proved to be useful to get the one step immobilization-purification of large proteins if the experimental conditions are properly selected: immobilization in the presence of an adsorption competitor causes

only large proteins to become adsorbed.[16b,65] This is due to the fact that these large proteins are able to give more enzyme-support interactions per enzyme molecule than small monomeric enzymes. These large enzymes tend to be multimeric, and this adsorption involves the face of the enzyme that implies more enzyme subunits, thus the dissociation of the enzyme may be prevented (fully if the enzyme is planar) or at least decreased after immobilization.[16b,65] These strategies enable the one step purification/immobilization/stabilization and present obvious economic advantages.

7- Relevance of the support active groups on the performance of a matrix for enzyme purification or immobilization.

The relevance of the nature of the active groups on the support is obvious for enzyme purification; the nature of the support must be able to permit enzyme adsorption in a selective way (e.g., affinity chromatography) or in an unspecific way (most of the used chromatographic matrices).[58d,66] Most of the active support groups are moderately stable and do not require special precautions. Other considerations have been treated in a previous point of this review; here we will only refer to the design of supports for ion exchange.

The adsorption of an enzyme molecule on ion-exchangers is based on the generation of several enzyme-support ionic interactions, exchanging the counter-ions of the enzyme and support (Figure 14).[49d,67] That means that the net charge of a protein is not enough to define the possibility of the protein to become adsorbed onto ion exchangers, but their capacity to establish an ionic net with the support is the key to permit this adsorption.[64b] This has been clearly exemplified when mixed cation-anion supports have been designed, having a “null net charge” in the surface and have been

able to adsorb many proteins, some of them are unable to become adsorbed on similar fully cationic or fully anionic supports under similar conditions (Figure 15).[68]

On the other hand, the reactive group properties are critical if the support is going to be used in enzyme covalent immobilization. The properties of the support group will determine if it may be considered a good or a bad immobilization methodology to get a very intense multipoint covalent attachment.[6] The properties of the ideal group to get an intense multipoint covalent attachment may be resumed in the following points:

- The reactive group should react with groups of the enzyme frequently placed on the enzyme surface (one of the target groups is the ε-amino group of Lys). It is interesting to involve the maximum number of enzyme groups on the immobilization.
- They should be able to immobilize enzymes in a broad range of conditions, to be used with a wide range of enzymes, or alter the enzyme orientation (Figure 16).[18,20] In many instances, after a first enzyme immobilization, the proximity of the support groups to the enzyme may permit to increase the enzyme reactivity with the support, and that way some attachments may be established,[19] and may stabilize the enzyme and permit the incubation under harsher conditions, conditions that can permit a more intense multipoint covalent attachment.
- They should preferably react with the enzyme without any kind of activation step (avoiding complex and dangerous activation of the protein before its immobilization).[6]
- They should be stable under a wide range of experimental conditions, the half live under storage conditions should be measured in months-years, under the immobilization conditions should be measured at least in weeks. Multipoint covalent attachment is a slow process that requires the correct alignment of the enzyme and support groups, depending on the support reactivity and the enzyme an optimal immobilization can take

between 3 h and one week.[61] Moreover, in order to favor the reactivity of the nucleophilic groups presented in the enzyme, alkaline pH are usually the most adequate to produce an intense multipoint covalent attachment.[61]

- They should permit the enzyme-support reaction with very low steric hindrances. The multipoint covalent attachment is a quite complex process that requires the alignment of groups placed in rigid structures. This process is difficult enough even if the reaction has no steric hindrances to the reaction.[6]

- They should permit some simple end point to the enzyme-support reaction, to prevent undesired enzyme-support reactions and to produce a final inert surface (see in point 9 of this review the relevance of this).[6]

-The changes in physical properties of the enzyme should be kept to a minimum (e.g., changing primary to secondary amino groups may be preferred to changes to amide groups).[6]

-They should permit simple immobilization protocols. At industrial level, where perhaps hundreds or even thousands of kg of support may be used, the use of complex support treatments may become a serious drawback.[6]

Following literature, there are a handful of reactive groups that may be adequate, although all of them have certain drawbacks.

Glutaraldehyde chemistry is perhaps the most widely used protein covalent immobilization methodology in the literature.[69] Many of its characteristics are far from the ideal support. For example, the glutaraldehyde activated support is not very stable even at low temperature even at neutral pH value.[69b] At alkaline pH values the stability is really very short. Moreover, there are some steric hindrances to the enzyme-support reaction. However, it has some good properties: primary amino groups will be involved in the reaction and they are transformed to secondary amino bonds. Moreover, the

chemical reactivity of the support may be suppressed by reduction with sodium borohydride but it is not strictly required due to chemical changes of the glutaraldehyde molecules along time.[69b] Thus, it did not seem the best option to get an intense multipoint covalent attachment. However, the full potential of this reagent for enzyme immobilization has been recently reported, showing its large versatility in enzyme immobilization. Glutaraldehyde activated supports are normally prepared from supports bearing primary amino groups, and under standard conditions, they mainly react with primary amino groups of the protein. That way, a glutaraldehyde activated support is really a heterofunctional support:[69b] they have a chemical reactive group, but also an anion exchanger, and certain hydrophobicity (Figure 17). This support has been recently reviewed, and here we will rapidly resume some of its characteristics.[69b] The activation may involve one or two glutaraldehyde molecules per amino group on the support, being the activation with two glutaraldehyde molecules which gives the highest reactivity versus amino groups.[70] Using very high ionic strength, the first enzyme insolubilization may be via hydrophobic adsorption.[71] Using a low ionic strength, the first enzyme immobilization will be via anion exchange.[71] If a moderate ionic strength is used (except in the case of lipases), the enzyme will be immobilized via a first covalent attachment.[71] In the case of lipases, the immobilization under these conditions is produced via interfacial activation versus the hydrophobic layer of glutaraldehyde dimers. To get an immobilization via covalent attachment, detergents also need to be added.[71b] This way, the enzyme may become immobilized on the support following different mechanisms, involving different areas and producing different results in terms of activity, stability, selectivity or specificity. As all this needs to be performed at a maximum pH of 8, the enzyme-support multipoint covalent attachment may be not very intense.

To solve this, it is possible to adsorb the enzyme molecules in a support containing primary amino groups and later treat the composite with glutaraldehyde.[72] This strategy has been recommended as the most suitable to get an intense multipoint covalent attachment using glutaraldehyde chemistry, as it involves the reaction between two amino-glutaraldehyde groups, quite reactive even at neutral pH values. However, it implies the global modification of the enzyme surface and this may be a problem in certain cases.[72] The existence of a surface under the enzyme with cationic and hydrophobic groups may also generate some problems on enzyme stability,[6] and also make the development of methodologies of enzyme reactivation via unfolding-refolding strategies complex.[73] Thus, this reagent has some potential to stabilize enzymes, but also some drawbacks.

One of the support reactive groups that has afforded the highest enzyme stabilization factors after immobilization is the glyoxyl.[25c] Glyoxyl (usually agarose) has given the highest values of stabilization via immobilization of many enzymes.

Glyoxyl group is very stable, does not present steric hindrances for the reaction with the enzyme and can react with the primary amino groups of enzymes. The mild reduction with sodium borohydride converts the aldehyde groups in inert hydroxyl groups and the reversible imino bonds formed with the enzyme in very stable secondary amino bonds.[74] They have peculiar features; under standard conditions they can only fix enzyme molecules to the support via a multipoint covalent attachment.[25b] This makes that the immobilization is directed via the area where there are most Lys residues, that is the one where the highest prospects of getting a high multipoint covalent attachment exist. The main limitations of these groups is the necessity of performing a reduction as reaction end-point (some enzymes cannot withstand even very mild reductions), and the necessity for immobilizing the enzyme at alkaline pH,

around 10, to permit the simultaneous establishment of several enzyme-support linkages that will permit the enzyme fixation to the support.[25c] This necessity has been converted in an advantage. First, these supports may permit the one-step purification, immobilization and stabilization of multimeric enzymes having the terminal amino groups (with a pK between 7 and 8.5) in the same plane by performing the immobilization at neutral pH value.[25a,75] Second, they are very suitable supports to prepare heterofunctional supports (see [20]). Glyoxyl heterofunctional supports bear groups able to fix the enzyme to the support via a desired mechanism and glyoxyl groups to have a former intense multipoint covalent attachment.[76] As glyoxyl supports cannot immobilize most enzymes at pH 7, at this pH the orientation of the enzyme will determined by the other groups (ionic groups, IMAC, thiol, etc.), altering the enzyme orientation regarding the support surface. The immobilization via different orientations may alter the enzyme stability, because not all areas have the same relevance for enzyme stability. Moreover, this strategy has been revealed as a simple way to tune enzyme selectivity, specificity or resistance to inhibitors.[20] Rocha-Martin, J., Acosta, A., Berenguer, J., Guisan, J.M., Lopez-Gallego, F. Selective oxidation of glycerol to 1,3-dihydroxyacetone by covalently immobilized glycerol dehydrogenases with higher stability and lower product inhibition (2014) Bioresource Technology, 170, pp. 445-453.

Moreover, immobilization on glyoxyl supports may be performed using neutral pH values if an imino bond stabilizer is added to the immobilization medium: tri-methyl-aminoborane, cyano-borohydride or thiol groups.[77] In this case, enzyme immobilization will proceed via the most reactive group (that tends to be the terminal amino group), that may be near to other primary amino groups (or not, avoiding an intense multipoint covalent attachment).

Epoxy activated supports groups are very popular immobilization matrices.[28c,46,78] Different epoxy supports have been commercialized by several companies and some industrial biocatalysts are prepared on them.[28c,78] They can react not only with amino groups, but also with thiol, hydroxyl and even carboxylic acids,[79] have no steric hindrances towards the reaction and present a good stability under a broad range of conditions. After immobilization, the remaining epoxy groups may be blocked by any compound having amino or thiol groups, leaving a chemically inert surface, but it is more difficult to have a fully physically inert support surface (the blocking reagents are not physically inert).[28a] However, they can immobilize enzyme molecules in a very slow fashion, because their reactivity is not very high.[80] In fact, commercially available epoxy-supports have some hydrophobicity and the companies recommend the use of high salt concentrations during enzyme immobilization. This is to force the hydrophobic adsorption of the enzymes to these supports, after this the enzyme-support reactions become intramolecular and the enzyme may become covalently attached to the support on a reasonable time. Based on this idea, a battery of different epoxy-heterofunctional supports have been developed, now the first adsorption may be via ionic exchange, thiol exchange, adsorption on immobilized borane or immobilized metal chelates, or even more specific (dyes) introduced in the support via different techniques.[20,28b,81] However, the low reactivity of the epoxy groups becomes a problem and stabilizations using epoxy activated supports are lower than that obtained using glyoxyl supports under comparable conditions (support activation degree and nature, enzyme orientation, etc.).[76b]

Activation of the supports with di-vinyl-sulfone (DVS) has been recently proposed as an alternative to the epoxy supports to get an intense multipoint covalent attachment (Figure 18).[82] These supports have been used for a long time for enzyme

immobilization,[83] but only recently their features for producing intense multipoint covalent attachment of enzymes have been analyzed. The enzyme groups that are involved in the immobilization and other properties (stability, steric hindrances, end point of the reaction) are similar to epoxy supports (except reactivity versus carboxylic acids), but they are far more reactive. In fact, they immobilize proteins from pH 5 to 10, and produce very intense multipoint covalent attachments after adequate incubations.[82] The results show that they can give even more intense multipoint covalent attachment than glyoxyl supports because the longer spacer arm (see section 9 of this review) and the implication of groups different to primary amino groups.[82] Stabilization is in some instances also higher than using glyoxyl supports, although in certain cases the activity recovery is too low or the stabilization did not reach the values obtained using glyoxyl supports, even after having more enzyme-support attachments.[84] The fairly hydrophobic nature of the group has been used to explain these results. These supports have been used to modulate lipase properties by immobilizing the enzymes under different conditions, favoring the first immobilization by one or another type of amino acid[85] However, they cannot be used in heterofunctional supports due to their moderate-high reactivity in a very wide range of conditions. It would be very hard to discard the DVS covalent immobilization and ensure the implication of the secondary groups in the first enzyme immobilization.

The protocols to get multipoint to covalent attachment involve some steps and long immobilization periods. Economic balance will depend on the final stabilization achieved (that should increase the operational life of the immobilized enzyme). Epoxy activated matrices are the only ones that are already produced in an activated form, glyoxyl and DVS activated supports may be produced by a support-producing company (require activation steps that not all biotechnological companies can perform) and

Comentario [AB1]: Queremos decir "to get from multipoint to covalent" u otra cosa? No queda claro

supplied to the final user. Glutaraldehyde, due to the low stability of the active group, needs to be prepared at the moment of use. These facts may also condition the final selection by a company of the immobilization protocol.

That way, the search for new support activation methods for enzyme immobilization via multipoint covalent attachment still remains as one of the more relevant topics of research in enzyme technology.

8- Effect of the spacer arm length on the intensity of the interaction between protein and support

The nature and length of the spacer arm has a great relevance on the behavior of a support for enzyme purification and immobilization. The longer the spacer arm, the higher mobility will have the support reactive group and the higher area of the protein may become involved in the enzyme support multi-interaction (Figure 19).[6] Moreover, the spacer arm may not be fully inert (e.g., an arm of CH_2 groups will become quite hydrophobic if it is too long), producing undesired interactions with the enzyme.

Thus, the length of the spacer arm will need to consider the exact purpose of the support.

In the purification of proteins via affinity chromatography, the ideal spacer arm should be quite long, to prevent any steric hindrance to the entry of the ligand to the enzyme recognition site.[52] The nature of the spacer arm, to avoid risks of hydrophobic interactions, should be a hydrophilic and flexible non-ionic polymer, such as dextran,[86] or even the sugar chains if the enzyme is glycosylated.[87] However, the activation of the dextran should be very low; the introduction of several ligands on the polymer may produce unspecific adsorptions of the enzyme. Moreover, the number of groups should be related to the length of the dextran to prevent that ligands placed in different dextran

molecules may simultaneously interact with protein molecules (that is, enabling again a certain undesired multi-interaction).

The use of IMAC columns to purify poly-His-tagged proteins is different. Native proteins require the interaction with several metal ligands; His tagged enzymes may become adsorbed in just one chelate.[49h,50a,88] A long spacer arm will increase the possibilities of multi-interaction; therefore, the selectivity of the adsorption will decrease.[45] In fact, it has been shown that the promotion of a dextran over the IMAC groups favored the selectivity of the adsorption (that is, an even "negative" spacer arm seems to be preferred).[51d]

In order to get a massive physical adsorption of proteins (both for purification or immobilization), a long spacer arm seems to be preferred, to favor the multi-interaction. In fact, polyethylenimine (PEI) or dextran sulfate coated supports have been described as optimal cationic supports to strongly but reversibly immobilize proteins, even more than 90% of proteins contained in a crude may be immobilized on each of both supports at pH 7 (that means that a large percentage of proteins immobilize on both supports under identical experimental conditions).[54a,54b,89] Moreover, as this coating forms a polymeric bed, PEI coated supports permit a tridimensional chromatography, useful to purify proteins that cannot become immobilized on planar surfaces.[90]

In covalent immobilization, the spacer arm plays different roles. A long spacer arm may reduce steric hindrances for the enzyme-support reaction, but reduces the rigidity conferred via multipoint covalent attachment (Figure 19).[6] On the other hand, a long spacer arm may involve more percentage of the surface of the protein in the immobilization, increasing the number of groups involved in the multipoint covalent attachment. If the arm is hydrophobic, this may have some negative effects on enzyme thermal stability. Next, we will show some examples of these facts.

Lowly activated dextran is an ideal spacer arm to have an immobilized enzyme with properties fairly similar to the free enzyme.[86] The enzyme is able to have freedom of movement, but may be recovered, which may be the best way to evaluate a protein in absence of any possibility of aggregation or other intermolecular artifacts. Enzymes will almost keep their properties unaltered after one-point immobilization (for example stability). Immobilization of renin or protein A using dextran activated supports has permitted to keep the activity of the enzyme and the recognition capacity almost intact, because of the decrease in the steric hindrances for the contact with the very large substrates.[86]

Glyoxyl groups have a very short spacer arm (O-CH_2-CHO), permitting to transmit the rigidification achieved by the multipoint covalent attachment, and being long enough to avoid that the support surface may generate some steric hindrances to the reaction[25] . Moreover it is so short that it does not produce any physical interaction with the enzyme. DVS activated supports (O-CH_2-CH_2-S-CH=CH) is longer, and permits a more intense multipoint covalent attachment.[82] However, the effects on enzyme stability are not always improved accordingly, because the rigidity conferred for the bonds is not so high, and they have a certain hydrophobicity, that may have negative effects for enzyme properties.[82]

Thus, a long enough spacer arm is preferred to a group directly on the surface, but if the spacer is too long, the rigidity transmitted may be decreased. Epoxy heterofunctional supports are a clear example that shows that the promotion of any problem to the enzyme-support reaction has a negative effect on the number of enzyme support bonds achieved.[18,81b,91] The modification of the epoxides with different moieties to get the adsorption of the enzymes produced the existence of a layer of these groups over the epoxide layer, and that makes it very difficult to get an intense

multipoint covalent attachment. This occurs even if just a SH group was the one used to modify the epoxy layer, and increases when the length of the ligand group increases.[92]

9- Relevance of the inertness of the support surface of the support for enzyme immobilization or purification

In this point, it is convenient to consider that the inertness of the support will affect both the matrix itself and the groups that the researcher introduces to perform the enzyme incorporation to the support. At first glance, the final inertness of the support core is in most cases advantageous, both in immobilization and purification.[6] It should be considered that polysaccharides matrices like agarose and cellulose beads are among the most inert ones, very similar to water (water may be considered the material showing maximum inertness). In general, the researcher should look for matrices as similar to these as possible.

The final inertness of the active groups placed in the support surface is a more complex target. In purification or in immobilization of enzymes via physical adsorption, the groups placed on the support will keep their capacity for physical interactions over time, and that is inevitable.[6] However, if a covalent immobilization is performed, it is possible and recommendable to have a final surface as inert as possible, both chemical and physically.[6]

In purification, an inert surface is critical to avoid enzyme adsorption via another mechanism different to that designed by the researcher. If an uncontrolled mixed adsorption is produced, desorption of the enzyme may become quite difficult, and the results obtained may be difficult to understand. Moreover, this secondary interaction of the enzyme and the support may be responsible for enzyme inactivation and other undesired effects. Obviously, the active group in the support surface will remain being

712 able to interact with the protein continuously. This may cause a not very frequently
713 studied phenomenon: if the adsorbed enzyme is desorbed immediately after adsorption,
714 this may be achieved under much milder conditions that when the adsorbed enzyme is
715 left to interact with the support for several hours).[51d] This is caused by the increase in
716 the enzyme-support bonds number; to reach the maximum level of enzyme-support
717 interactions a certain time is required after the enzyme is already incorporated to the
718 support, as explained for the multipoint covalent attachment, but now becoming an
719 undesired effect.

720 For enzyme immobilization via physical adsorption, a controlled mixed
721 adsorption may be interesting to prevent enzyme desorption during operation. However,
722 it is more suitable to get this if the researcher introduces both kinds of groups on the
723 support, because that way the relevance of each phenomenon may be controlled. An
724 example of this may be the interfacial activation of lipases versus hydrophobic supports
725 (Figure 20).[93] This lipase immobilization method is very useful and has a handful of
726 advantages (e.g., involve the open form of the lipase),[94] but the enzyme may be
727 released to the medium in the presence of organic solvents.[95] If the support also has
728 some ionic groups, able to form some ionic bridges after enzyme interfacial activation,
729 this may improve the usefulness of these preparations.[96] The idea has been already
730 used, even transformed to produce covalently forms of lipases interfacially activated
731 versus hydrophobic supports.[97]

732 If the enzyme is covalently attached to the support, it is possible to design
733 strategies to have a surface as inert as possible. For example, reduction of glyoxyl-
734 agarose supports produces a support having just inert hydroxyl groups.[74] The blocking
735 of epoxy or DVS activated supports may also get surfaces moderately inert.[28a,82] Using
736 glutaraldehyde, an inert surface is not possible; the cationic group will remain there,

737 together with the hydrophobicity of the glutaraldehyde groups.[69b,71] The possibility of
738 physical enzyme-support interactions may have different effects on enzyme features,
739 from stability to activity or selectivity, usually the effects are difficult to predict and in
740 most cases negatives. However, in certain cases, the change of the support surface
741 physical properties during blocking has been used to modulate the enzyme
742 properties.[98] Results using this technique may be positive in certain reactions and
743 conditions and very negative in other ones.

744 If the enzyme is going to be submitted to strategies of unfolding/refolding to
745 recover their activity after inactivation, the inertness of the support has a markedly
746 special relevance This strategy has been employed with some success with different
747 immobilized enzymes, even it has been reported than a multipoint covalent attachment
748 improves the rate and yield of recovered enzyme activity after unfolding/refolding.[99]
749 Most results have been reported using enzymes immobilized on inert glyoxyl-enzyme
750 biocatalysts, thanks to their inertness. However, if the support is able to interact with
751 groups of the enzyme, partially unfolded structures may become stabilized and the
752 correct enzyme refolding may become not possible.[73,100]

753 In other cases, some positive effects of immobilization derive directly for the
754 physical properties of the support surface. This is the case of the partition of some
755 deleterious compounds away from the enzyme molecules by immobilization on
756 polymeric beds. Thus, enzymes stability has been improved versus organic solvents or
757 oxygen by using supports coated with ionic polymers,[101] while the coating with
758 hydrophobic polymers has improved the stability versus hydrogen peroxide.[102]
759 Immobilization of lipase B from *Candida antarctica* on hydrophobic supports permitted
760 to prevent enzyme inactivation by hydrogen peroxide (Figure 21).[30d,103]
761

10- Conclusions

The design of supports to be used in immobilization or purification requires a deep knowledge of the phenomena that can occur between a support surface and a biomacromolecule. Many macroscopic and molecular level features need to be considered in the design of the support, with different relevance depending on the final use of the support and of the biocatalyst and most of them are interacting some way each other and in many instances a compromise solution need to be taken to have an overall good matrix. This review has tried to point some of the most relevant features of a support, and shown the many possibilities that the research has and the many decisions that may be taken before selecting a determined support. Immobilization and purification of enzymes using activated supports have very different objectives, but in fact are quite related topics, the researcher needs to determine the cause for the first enzyme insolubilization (one strong interaction or weak multipoint interactions) and avoid or at least, control any other likely support-enzyme interactions. The potential of a proper support used under proper conditions may be impressive and with many applications. A proper design of a support may permit to shift equilibrium of very weak protein complexes permitting their accumulation on the support, or improve the stability of an enzyme stability thousands folds, or its activity or their selectivity. However, to fully reach these goals, it is necessary to improve the knowledge and control of the interactions between support and enzyme, and even to design new reactive groups with even better properties than the currently available ones. These apparently old fashioned techniques remain as necessary as ever in the era of the biotechnology revolution and may be quite far from their limits to improve the performance of industrial enzymes.

Acknowledgements

We gratefully recognize the support from the Spanish Government, CTQ2013-41507-R, Colciencia (Colombia) and CNPq (Brazil). The predoctoral fellowships for Mr dos Santos (CNPq, Brazil) are also recognized. Á. Berenguer-Murcia thanks the Spanish Ministerio de Ciencia e Innovación for a Ramón y Cajal fellowship (RyC-2009-03813). The authors wish to thank Mr. Ramiro Martínez (Novozymes, Spain) for his continuous support to our research.

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Figure Legends

Figure Legends

Figure 1. Immobilization and purification of enzymes by control of the support and/or immobilization conditions.

Figure 2. Supports for immobilization or for enzyme purification.

Figure 3. Possibility of enzyme-support uncontrolled interactions.

Figure 4. Effect of large support particles on the adsorption rate of the enzyme-support.

Figure 5. Diffusional limitations by effect of a large support particle. Effect on enzyme activity.

Figure 6. pH gradients promotion in the production of an ionized product (e.g., production of a carboxylic acid by hydrolysis of an ester) favored by the particle size of the support.

Figure 7. Immobilization support coating an ionic polymer as a solid ‘‘buffer’. Protective effect on the enzyme.

Figure 8. Effect of pore size on the loading capacity of the support for immobilization and purification of enzymes.

Figure 9. Controlling the diffusional limitations of substrates by using supports bearing large pore diameter.

Figure 10. Different types of standard supports for protein purification.

Figure 11. Effect of internal geometry of the support on the possibilities of producing one-point interactions or multipoint interactions: large surfaces versus thin fibers.

Figure 12. Protein immobilization by physical adsorption on supports activated with polymers or standard groups: volume versus planar adsorption.

Figure 13. Effect of support activation degree on the effectiveness in affinity chromatography.

Figure 14. Immobilization and purification of enzymes on ion exchanger supports: a multipoint interaction is required.

Figure 15. Immobilization and purification of enzymes on mixed cation-anion supports.

Figure 16. Altering the enzyme orientation by controlling the immobilization conditions.

Figure 17. Glutaraldehyde supports as multifunctional supports for enzyme immobilization.

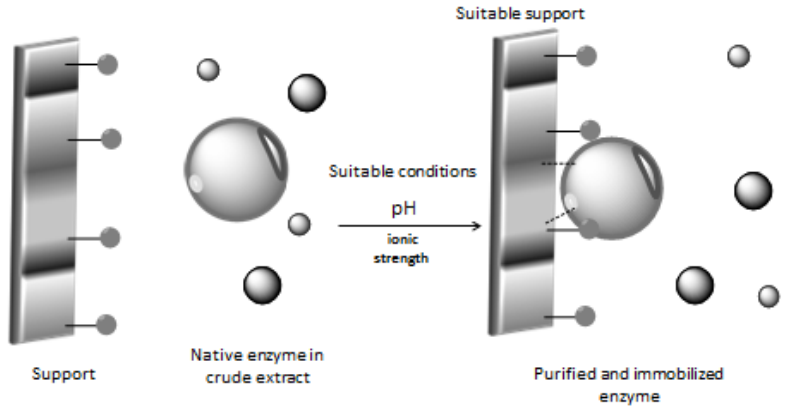
Figure 18. Di-vinyl-sulfone (DVS) activated supports for enzyme immobilization via multipoint covalent attachment

Figure 19. Relevance of the spacer arm in enzyme stabilization via multipoint covalent attachment.

Figure 20. Immobilization of lipases via interfacial activation on hydrophobic supports.

Figure 21. Stabilization of lipases versus hydrogen peroxide by generation of a partition effect using a hydrophobic support.

Figure 1



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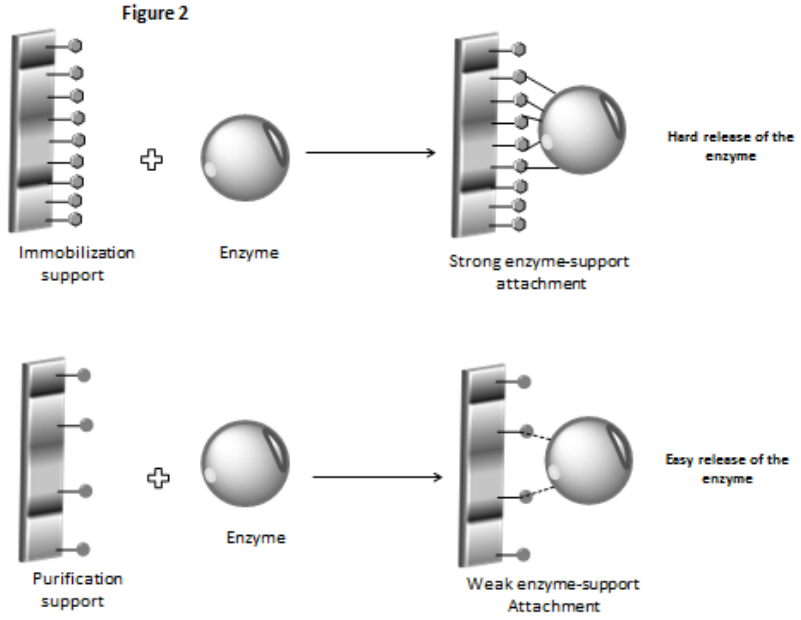

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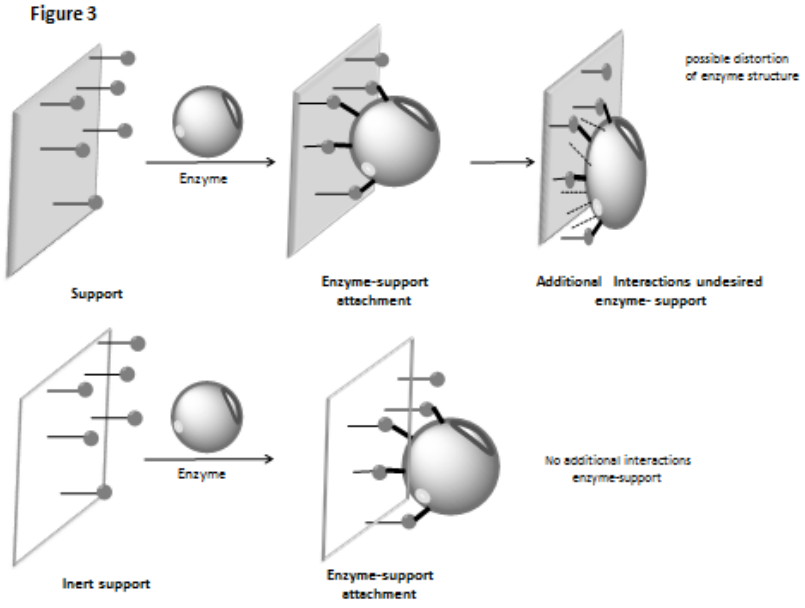

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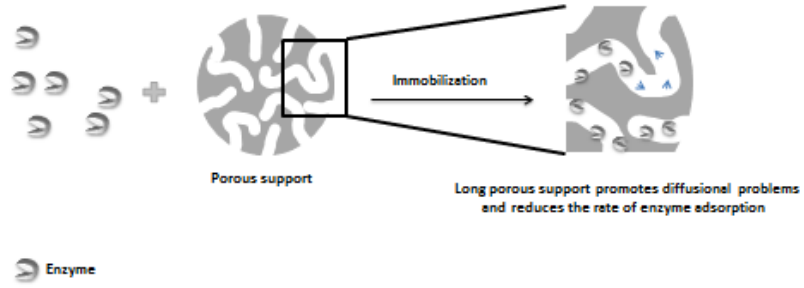

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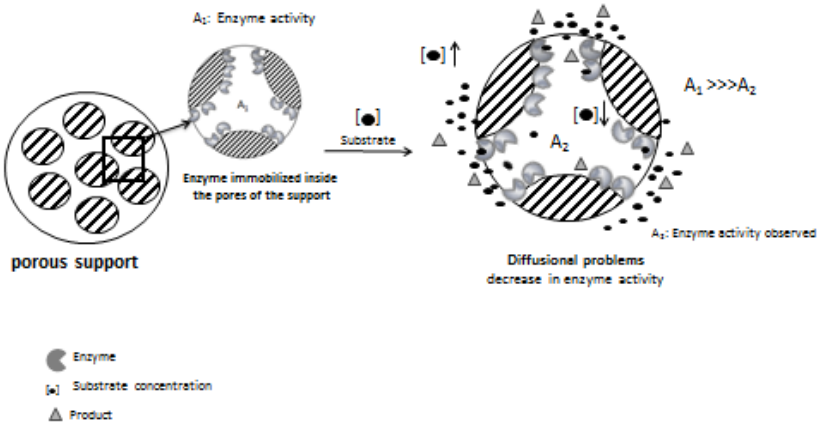

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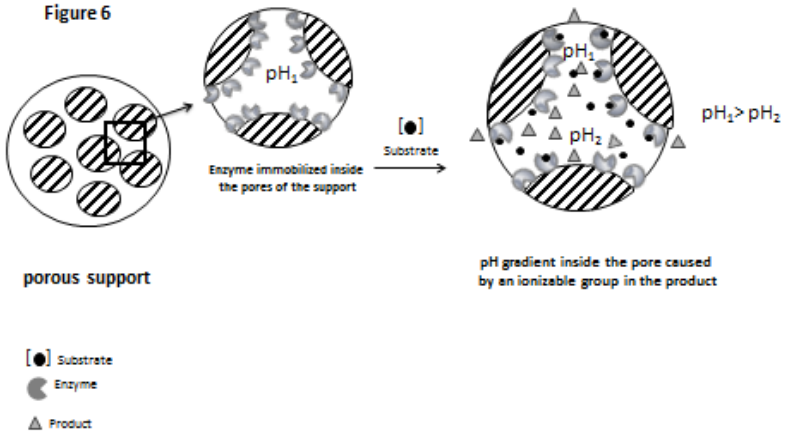

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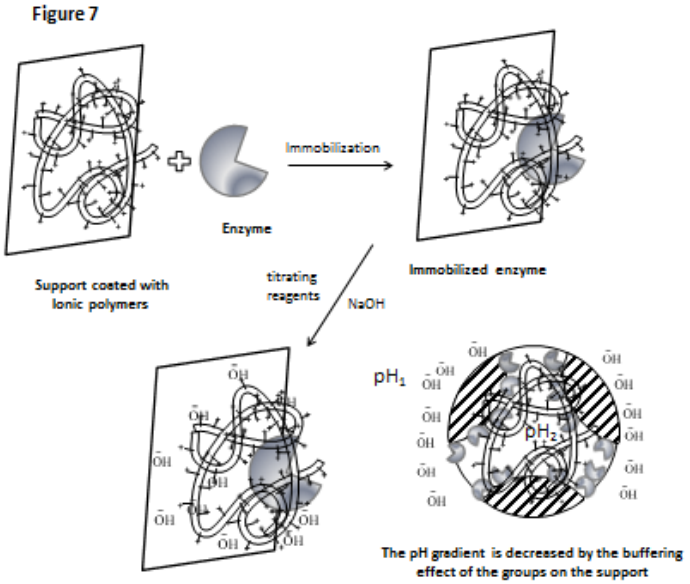

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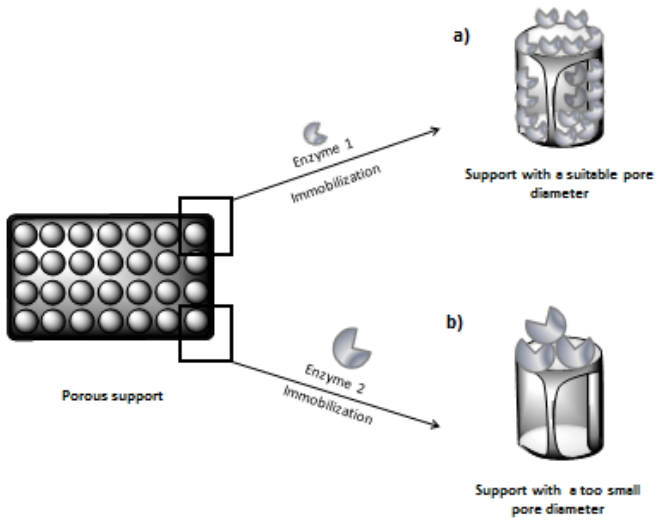

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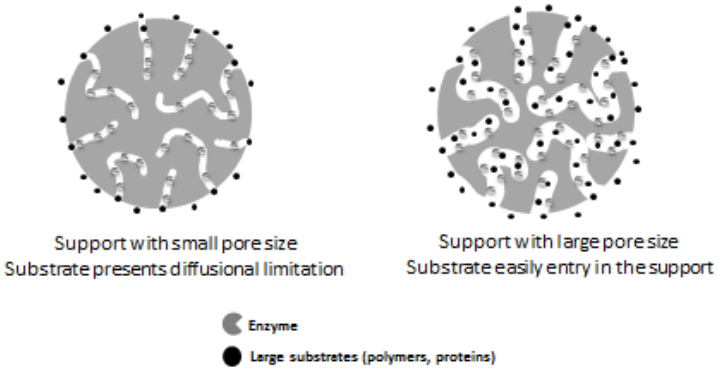

Figure 9

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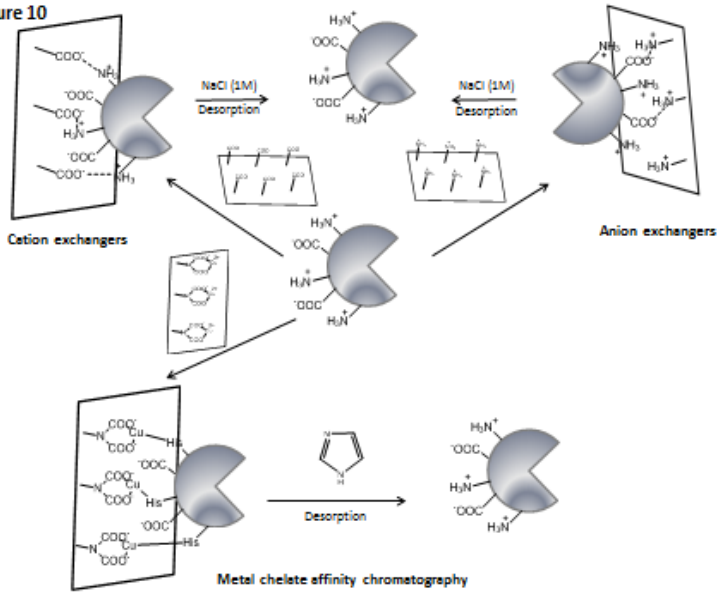

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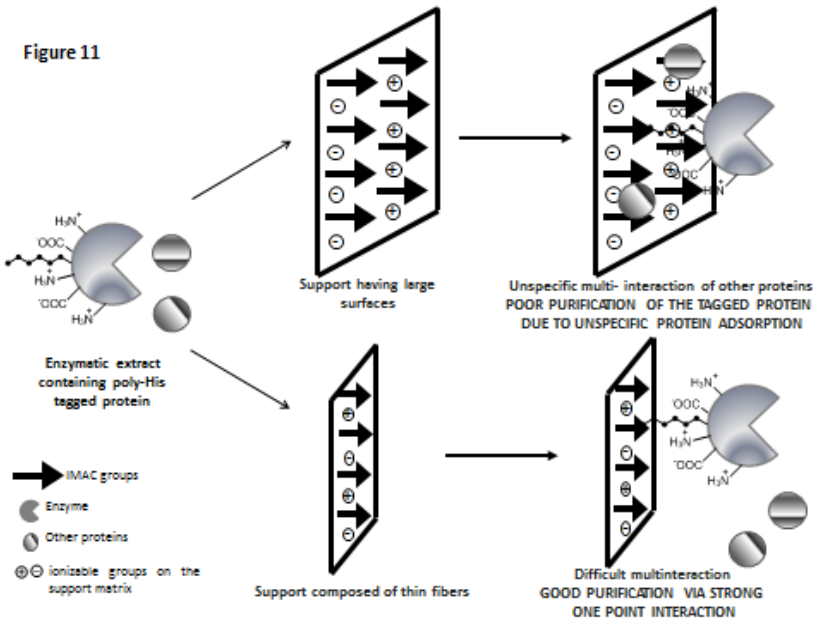

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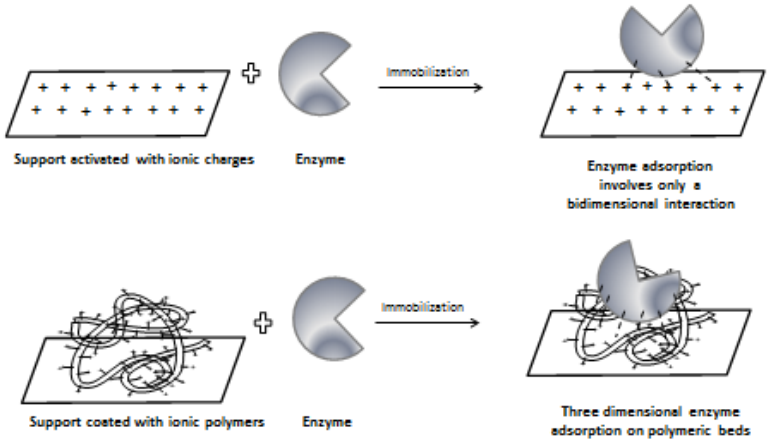

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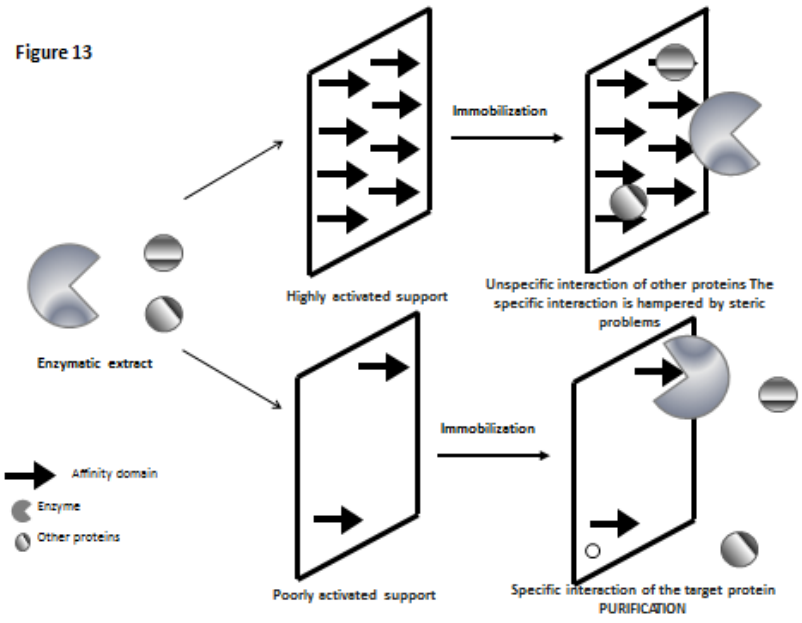

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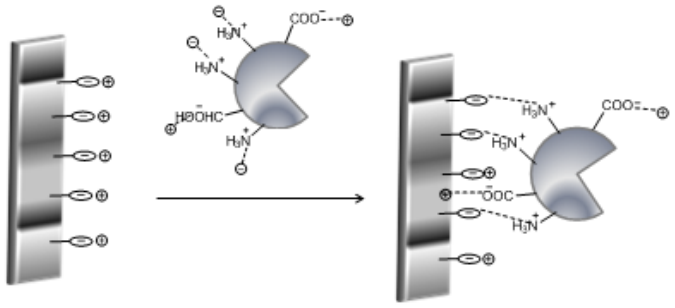

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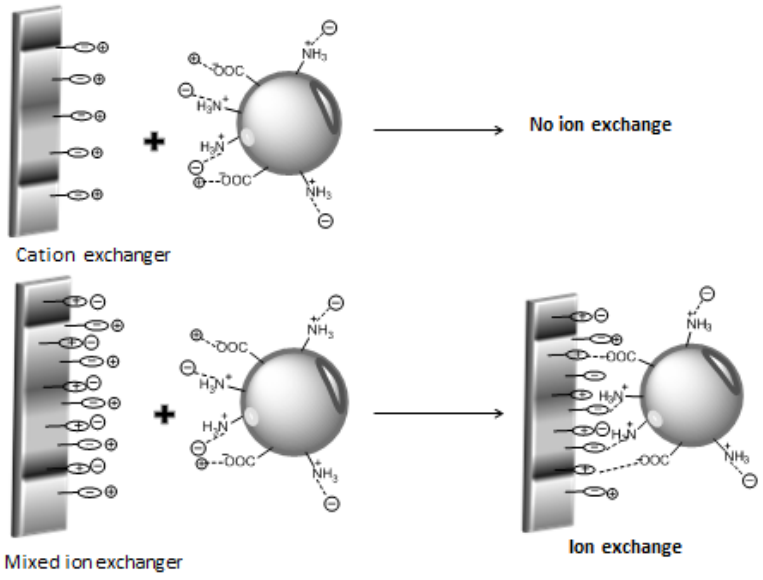

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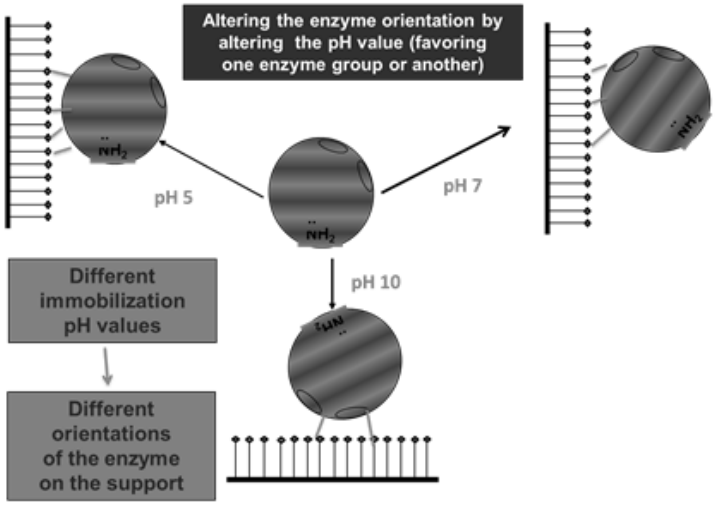

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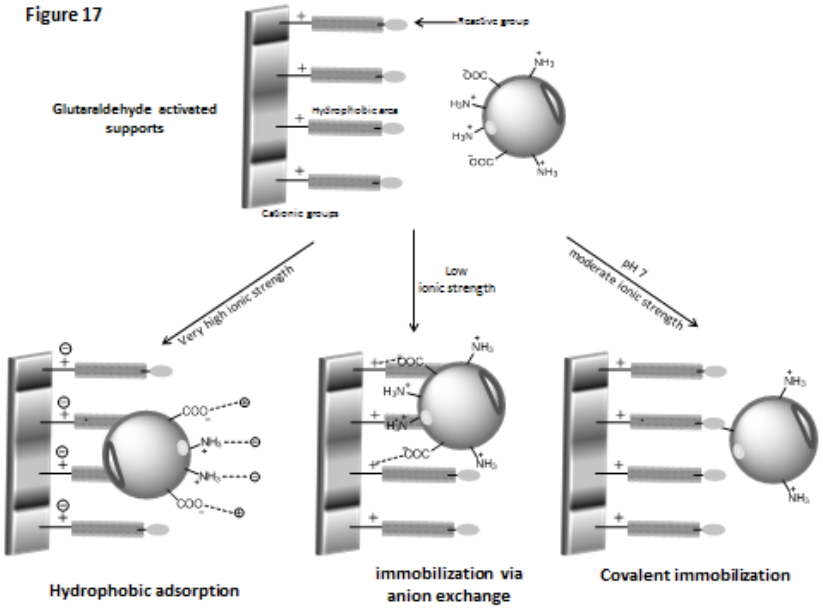

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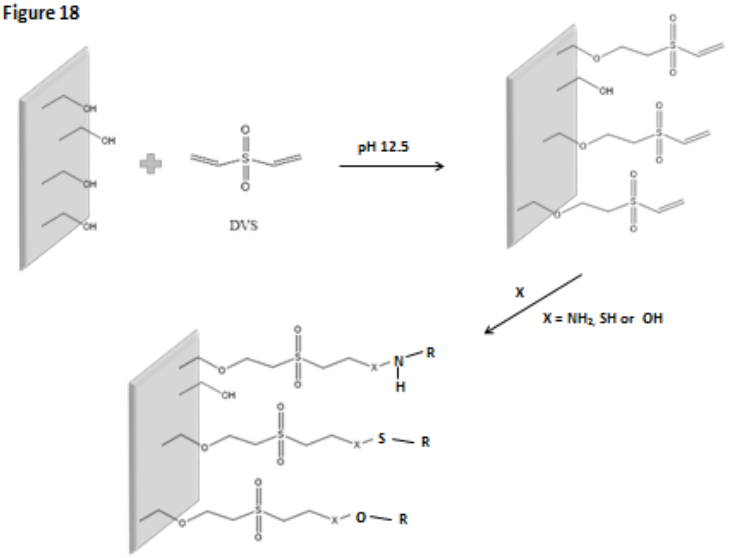

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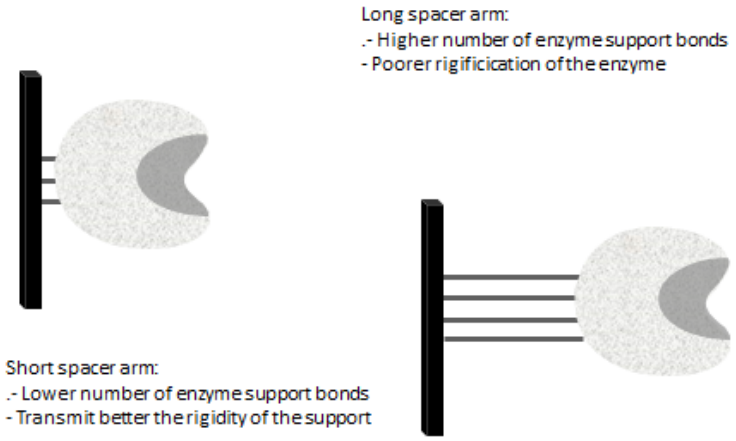

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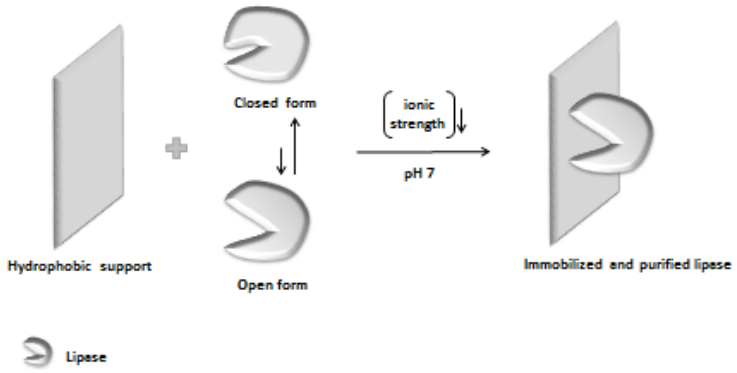

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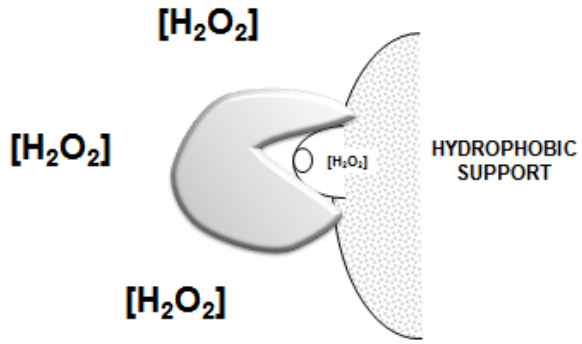
Figure 21

HYDROPHOBIC SUPPORT
LIPASE IMMOBILIZED VIA INTERFACIAL ACTIVATION



HYDROGEN PEROXIDE IS
PARTITIONED AWAY FROM THE
ARE INVOLVED IN THE IMMOBILIZATION

1246