Strategies for the one-step immobilization-purification of enzymes as industrial biocatalysts

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Abstract

Enzyme immobilization and purification are two steps that are usually required in the development of any industrial biocatalyst. In this review, we detail the efforts performed to couple the purification and the immobilization of industrial enzymes in a single step. The use of antibodies (versus the target enzyme or versus some domains), the development of specific domains with affinity for some specific supports or just to increase the affinity for standard ones (ionic exchangers, silicates) will be revised. We will show how the control of the immobilization conditions may convert some unspecific supports in largely specific ones. The development of tailor-made heterofunctional supports as a tool to immobilize-stabilize-purify some proteins will be discussed in deep, using low concentration of adsorbents groups and a dense layer of groups able to give an intense multipoint covalent attachment. The final coupling of mutagenesis and tailor made supports will be ~~a~~ the last part of the review.

Key words: controlled immobilization, enzyme stabilization, multimeric enzymes, chimeric proteins, covalent attachment, ionic exchange, IMAC.

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1. Introduction

Enzymes are biocatalysts with outstanding prospects as catalysts in industrial processes which include high activity under very mild environmental conditions, high selectivity, and high specificity (Gröger and Hummel, 2014, Reetz, 2013, Schrittwieser and Resch, 2013, Teixeira et al., 2014, Wells and Meyer, 2014). However, enzymes have also some limitations that may hinder their industrial implementation (Schoemaker et al., 2003).

Enzymes are water water-soluble molecules that need to be separated from the reaction media to be re-used. This is important for improving the economy of the process and also ~~to~~ for ~~facilitate~~ facilitating the control ~~of~~ in the reactor (Brady and Jordaan, 2009, Garcia-Galan et al., 2011, Sheldon, 2007). Furthermore, they may ~~not~~ neither be stable enough under industrially relevant conditions (presence of organic solvents, high temperatures to avoid contamination, etc.) nor ~~has~~ have high enough activity, selectivity or specificity towards the target industrial substrate (sometimes quite far from the physiological substrates). Moreover, they are produced in conjunction with many other similar proteins (some of them with undesired catalytic activity versus the substrates or even the products) and that decreases the final volumetric activity because some surface of the support will be occupied by other proteins. The activity of minority enzymes with opposite catalytic activity may also decrease the enantio or regioselectivity or specificity of the “biocatalyst” if it includes ~~some~~ any of these contaminant enzymes.

The latter issue is tackled using purification strategies, which in some cases may be a long and tedious process, while in other cases it includes just one chromatographic step (Clonis et al., 2000, Porath, 1992, Wilchek et al., 1984, Zeng

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and Ruckenstein, 1999). Nevertheless, even in the best case scenario this may have a negative economic impact in the final cost of the biocatalyst.

On the other hand, the most obvious solution to get a simpler recovery of the enzyme is its immobilization (Brady and Jordaan, 2009, Garcia-Galan et al., 2011, Sheldon, 2007). Moreover, considering that ~~the~~ immobilization is in most cases a requirement to use the enzyme as an industrial biocatalyst, many researchers have endeavored to couple immobilization with the improvement of other enzyme properties (Garcia-Galan et al., 2011, Guzik et al., 2014, Hernandez and Fernandez-Lafuente, 2011, Hwang and Gu, 2013, Rodrigues et al., 2013, Stepankova et al., 2013, Zucca and Sanjust, 2014). Multipoint (Mateo et al., 2007c) or multisubunit (in multimeric enzymes (Fernandez-Lafuente, 2009)) immobilization may improve enzyme rigidity and thus, improve enzyme stability (**Figure 1**). The rigidification of certain areas of the proteins surface and its controlled distortion resulting from the immobilization process have been shown to be able to ~~tuning~~ tune (in some instances significantly improving) enzyme activity, selectivity or specificity (Mateo et al., 2007c, Rodrigues et al., 2013).

Moreover, in certain cases, the immobilization protocol (including support, enzyme modification and immobilization conditions) has been designed to couple the immobilization of the enzyme and its purification in just one process preferably without sacrificing other potential enzyme improvements (Garcia-Galan et al., 2011). The present review will discuss the use of techniques that permit to join, in a single step, immobilization and purification. To this goal, it is very important to know if ~~-~~ the interaction of the enzyme molecule ~~interaction~~ with just one active group of the support is enough to keep the enzyme coupled to the support under the immobilization conditions, or, ~~by~~ on the contrary, only after several enzyme -support

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interactions the protein molecule remains attached to the support. These will be the key for the final adsorption selectivity ~~of the adsorption~~, even though the final objective will be a multipoint or multisubunit attachment to improve ~~the~~ enzyme stability (**Figure 2**) (Garcia-Galan et al., 2011).

First, a rapid view of different affinity immobilization strategies using supports bearing specific receptors to domains included in the target protein structure will be presented (Binz et al., 2005, Linder and Teeri, 1997, Ong et al., 1989, Saleemuddin, 1999). In general, these immobilizations will be just via one point (the domain) with scarce effect on enzyme stability (except those effects derived from the immobilization of enzymes inside a porous support), but in certain cases they may include several enzyme subunits of multimeric enzymes, with the positive effect on enzyme stability that this may have (Bolivar and Nidetzky, 2012b, Hernandez and Fernandez-Lafuente, 2011). A special case will be the immobilization of lipases on hydrophobic supports via interfacial activation, ~~and that way~~which produces some stabilization (Palomo et al., 2002). The use of tailor-~~-~~made supports to specifically immobilize proteins with certain structural particular features (large or small proteins, lipases via interfacial activation), with the final development of heterofunctional supports to achieve the specific enzyme immobilization followed by its stabilization via multipoint or multisubunit immobilization will be also an important part of this review (Barbosa et al., 2013). Finally, the coupling of site directed mutagenesis (to introduce specific domains in the desired areas of the protein) to these heterofunctional supports immobilization/stabilization of the protein will be discussed (Barbosa et al., 2013).

This strategy requires ~~to~~ having in mind that ~~the~~ immobilization involves different steps with different objectives. The first one is a somehow rapid

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immobilization, via the moieties that we have introduced which are able to recognize the protein. The second one, it is the promotion of covalent attachments (as many as possible to improve stability) between the enzyme and the support which may be quite a ~~quite~~ slow process and proceed at different conditions.

2. Coupled immobilization/purification of proteins via antibody specific adsorption.

One general strategy to couple immobilization ~~to~~ with purification with any protein is to immobilize it on a previously immobilized anti-target protein (Saleemuddin, 1999). This strategy may use monoclonal or polyclonal antibodies, and permits an extremely selective protein adsorption, only the target protein becomes immobilized (i) if the antibody is properly immobilized (Ahmed et al., 2006, Batalla et al., 2008, Cho et al., 2007, Iwata et al., 2008, Schmid et al., 2006) and (ii) if we can prevent any undesired adsorption (Fuentes et al., 2005a). Using polyclonal antibodies, the target protein will be immobilized following different orientations. This may be useful if it is going to be used in proteomics, if all the surface of the protein is available to interact with other proteins in one or another protein molecule, it may be used to detect any protein able to interact with any area of the immobilized protein (Fuentes et al., 2005a.) Using monoclonal antibodies, it is even possible to get a particular orientation of the enzyme regarding the support surface (**Figure 3**). That is interesting in Biocatalysis, as it may permit the selection of different protein orientations where the active center is fully exposed to the medium (Hernandez and Fernandez-Lafuente, 2011).

Moreover, if the selected epitope on the protein is a fragile region, the immobilization may improve enzyme stability to some degree, and the antibodies

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may also prevent the interaction of some areas of the protein with inactivating compounds (Fatima and Husain, 2007, Haider and Husain, 2009, Jan et al., 2001, Jan et al., 2006, Khan et al., 2005, Varshney et al., 2001, Younus et al., 2001, Younus et al., 2002, Younus et al., 2004). In general, immobilization yield is near ~~to~~ 100%, with almost complete pure immobilized enzyme, maximum stabilization achieved (in ~~adsencce~~absence of intermolecular inactivation causes) is under a factor of 20.

In other cases, the enzyme and the antibody ~~was~~ were exposed in free solution, and ~~them~~ then the antibody was captured using immobilized protein A or G (Kondo and Teshima, 1995, Wang et al., 2001a). This strategy, due to its complexity and cost (antibodies are expensive, their immobilization needs to be in a proper orientation, a final inert support is required, reuse is not that simple, and operation conditions should be compatible with antibody stability) has not been analyzed in detail in the literature.

In some instances, the antibody is not designed for the target protein, but for a specific domain, which is introduced in the target protein sequence using site-directed mutagenesis (Solomon et al., 1991, Vishwanath et al., 1997, Wang et al., 2001b, Witkowski et al., 1993). This permits to always have the desired orientation, but it will hardly have a very positive effect on enzyme properties. The advantage is that a single antibody-matrix may be used for the immobilization of any protein where this domain has been introduced. Immobilization (if using a *stoichiometric* amount of target protein and immobilized antibody) is near to 100%, purity of the immobilized enzyme is almost total, but stabilization in absence of intermolecular aggregation is not significant, since the domain prevents interactions between enzyme and support.

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These immobilization strategies, based on physical interactions, should produce reversible immobilization protocols, which is a requirement considering that the immobilization matrix may be far more expensive than the immobilized protein target. This becomes economically viable only if the resulting systems can be reused for many cycles. The stability of the antibodies is quite high, in fact some reports show that the recognition capacity is maintained after several weeks even at 50 ºC at neutral pH values (Batalla, 2010). However, it should be considered that high affinity antibodies may yield adsorptions under which only very drastic conditions will permit ~~the~~ enzyme desorption from the antibody~~-~~ matrix after the enzyme inactivation. These conditions also can inactivate the antibody, reducing the number of cycles during which they may reused to immobilize~~d~~ fresh proteins batches (Batalla, 2010). Thus, low affinity antibodies may be preferred ~~on~~ in this kind of processes.

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3. Coupled immobilization/purification of enzymes and proteins via specific domains.

There are many different peptides and proteins ~~having~~ which have a high affinity for different groups or structures, which may be added to the structure of the target protein by genetic routes, and thus, transfer this affinity property to the employed protein (**Figure 4**). These peptides may be very small (just a dozen of residues or even less), like in the poly-His tags, or domains with several kD (e.g. cellulose binding domain) (Linder and Teeri, 1997, Nordon et al., 2009, Ong et al., 1989). Perhaps the most popular domain is the poly-His tag, with high affinity for metal chelates (**Figure 5**) (e.g. for purification by IMAC) (Porath et al., 1975, Porath and Olin, 1983, Porath, 1992) but the range of these affinity peptides is huge and still growing: domains of affinity for cellulose, chitin binding domain, peptide tags, among

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others (Arroyo et al., 2011, Baumann et al., 1998, Bello-Gil et al., 2014, Bergeron et al., 2009, Bolivar and Nidetzky, 2012a, b, Cassimjee et al., 2011, Chern and Chao, 2005, Chiang et al., 2008, Chiang et al., 2009, Cho et al., 2007, Daunert et al., 2007, Fishman et al., 2002, Ha et al., 2013, Hernandez and Fernandez-Lafuente, 2011, Kondo and Teshima, 1995, Kowsari et al., 2014, Kumada et al., 2009, Kweon et al., 2005, Lee et al., 2005, Li et al., 2012, Li et al., 2009, Lin et al., 2011, Linder and Teeri, 1997, Linder et al., 1998, López-Gallego et al., 2012, Lu et al., 2012, Martinez et al., 2000, Mateo et al., 2001b, Moldes et al., 2004a, Moldes et al., 2004b, Richins et al., 2000, Scaramozzino et al., 2005, Seino et al., 2014, Shpigel et al., 1999, Simon et al., 2002, Tominaga et al., 2005, Vishwanath et al., 1995, Wang et al., 2013a, Wang et al., 1997, Wang et al., 2010, Wang et al., 2013b, Wiesbauer et al., 2011, Yang et al., 2013, Zhao et al., 2013). Table 1 shows a summary of the main domains used for this purpose while Table 2 shows some specific examples of uses of these domains.

It should be considered that the affinity ligand may have possibilities ~~of~~ for adsorbing natural proteins, because it will hardly ~~will~~ be fully physically inert (it may be hydrophobic, possess certain ionic character, or other physical properties). Thus, if many ligands are present on the enzyme support, they may be able to interact by multiple points with any biomacromolecule (Fuentes et al., 2007, Murza et al., 1999). Thus, the design of these matrices needs to be carefully performed to avoid unspecific adsorption of other proteins. In the case of multimeric enzymes, enzyme immobilization may involve all or at least several subunits, enabling the prevention of enzyme dissociation and thus increasing enzyme stability (Fernandez-Lafuente, 2009).

Another strategy is to use tags or domains that increase the tendency of the enzyme to become adsorbed on “standard” matrices, such as cation exchangers (using poly-Arg, poly-His, some cationic rich domains) (**Figure 6**) (Fuchs and Raines, 2005, Gräslund et al., 2000, Rodrigues et al., 2014) or anion exchangers (using poly-Glu or poly-Asp) (Hedhammar et al., 2004). Table 2 shows some examples of these uses. In this case, the conditions and support used by the researcher must be designed to favor the ion exchange of proteins of the tagged protein compared with standard proteins. These processes involve multipoint adsorption, and the anion tag permits to have a very intense one. Using cation exchangers, at pH 7 just a few proteins become adsorbed, thus a cationic domain may permit quite a selective adsorption of the tagged protein (**Figure 7**) (Bolivar and Nidetzky, 2012a, b, Gräslund et al., 2000, Gräslund et al., 2002a, Gräslund et al., 2002b, Hedhammar et al., 2006, Hedhammar and Hober, 2007, Nock et al., 1997, Sassenfeld and Brewer, 1984, Wiesbauer et al., 2011).

The affinity domains, in order to yield ~~a~~ very high purification factors, require to be properly designed to prevent unspecific adsorption of other proteins. ~~And a~~A ~~two~~ too low amount of groups in the support may produce a slow immobilization (taking many hours to have fully immobilized enzymes. Stabilization may be mainly found by preventing subunit dissociation of multimeric enzymes, the values found will depend on the free enzyme concentration ~~s~~used for comparison (ranging from factors of 10 to thousands) (Bolivar and Nidetzky, 2012a, b, Gräslund et al., 2000, Gräslund et al., 2002a, Gräslund et al., 2002b, Hedhammar et al., 2006, Hedhammar and Hober, 2007, Nock et al., 1997, Sassenfeld and Brewer, 1984, Wiesbauer et al., 2011). We have not found actual uses of these strategies at industrial level, perhaps the

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moderate stabilization achieved did not encourage this as a protocol ~~of~~ for industrial enzyme immobilization, even being quite a ~~quite~~ simple protocol.

4.- Coupled immobilization/purification of enzymes and proteins via control of the immobilization process.

4.1- The case of lipases immobilization via interfacial activation on hydrophobic supports

In some cases, it is possible to use some specific particularities of the catalytic mechanism of an enzyme to differentiate it from others. That is the case of lipases. These enzymes are capable of acting in the surface of drops of oils (Brzozowski et al., 1991, Van Tilbeurgh et al., 1993). To reach this goal, lipases have a mechanism of action called interfacial activation (Verger, 1997). In aqueous media, they usually have the hydrophobic catalytic center blocked by a polypeptide chain, called lid, with a very hydrophobic internal face and a hydrophilic external face. This is called the closed form of lipases. ~~in~~ In certain cases the active center is not fully secluded from the medium (e.g. lipase B from *Candida antarctica*) (Uppenberg et al., 1994), whereas in other cases the lid fully blocks the active center (e,g, lipases from *Thermomyces lanuginosus* (Brady et al., 1990) or *Rhizomur miehei* (Derewenda et al., 1992)), while in a few cases the enzyme even has a double lid that moves in a coordinated form (e.g., lipase from *Bacillus thermocatenulatus*) (Carrasco-López et al., 2009). This closed form is in equilibrium with an open form where this lid is displaced exposing a very large hydrophobic pocket to the medium (Brzozowski et al., 1991, Van Tilbeurgh et al., 1993). This form is unfavorable in aqueous homogenous medium and the equilibrium is shifted towards the closed form. In the presence of a hydrophobic

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surface, like that of an oil drop, the open form of the lipase becomes adsorbed via this very large hydrophobic pocket, shifting the equilibrium towards the open form (**Figure 8**) (Brzozowski et al., 1991, Van Tilbeurgh et al., 1993).

This oriented adsorption of lipases on hydrophobic surfaces is called interfacial activation of lipases and permits ~~the~~ lipases to act on insoluble substrates like triglycerides. However, this mechanism of action causes lipases to become an exception among water soluble proteins: while the surface of the closed form is fairly hydrophilic, the open form has a huge hydrophobic pocket. This permits that the lipases may suffer interfacial activation with different hydrophobic surfaces: a drop of oil (Verger, 1997) or a hydrophobic support (Bastida et al., 1998, Fernandez-Lafuente et al., 1998, Garcia-Galan et al., 2014, Hernandez et al., 2011), but also a hydrophobic protein or the open form of another lipase (Fernández-Lorente et al., 2003, Palomo et al., 2003, Palomo et al., 2005a). All of them have been used to purify and/or immobilize lipases. That way, immobilization of lipases on hydrophobic supports at low ionic strength is a much utilized protocol because it permits, in a single step, to immobilize and to stabilize the open form of the lipase (producing its hyperactivation in many cases and the tuning of the enzyme properties **(Figure 9)** (Fernandez-Lorente et al., 2008, Fernández-Lorente et al., 2007) and to purify it (Fernandez-Lafuente et al., 1998).

Lipase adsorption on hydrophobic supports at low ionic strength is not a conventional hydrophobic adsorption, but an affinity interfacial process (Fernandez-Lafuente et al., 1998). At low ionic strength (e.g. 5 mM of buffer, or even in the presence of glycerin or polyethyleneglycol), the only water soluble protein able to become adsorbed in a moderately hydrophobic support will be ~~the~~ lipases (**Figure 10**) (Fernandez-Lafuente et al., 1998). The influence of the experimental conditions

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~~neither~~ do not fit a conventional hydrophobic adsorption (Manoel et al., 2015). This process involves the open form of the lipase., ~~high~~ High ionic strength during adsorption has a double negative effect in immobilization/purification process: other proteins may become adsorbed on the hydrophobic support (reducing the purification impact of the strategy) and lipase adsorption is slower, because the closed form is favored and the lipase needs to be adsorbed via conventional hydrophobic adsorption, which is not as efficient a process ~~not as efficient~~ as interfacial activation (Fernandez-Lafuente et al., 1998, Manoel et al., 2015).

Irreversible inactivation of the lipases adsorbed on this kind of supports using chemical Ser-hydrolases inhibitors, that need to have access to the catalytic Ser and depend only in the exposition of this group to the medium, is much more rapid after the immobilization on hydrophobic supports than in the free enzyme or in other immobilized preparations (Carrasco-López et al., 2009, Manoel et al., 2015, Santos et al., 2014a, b). This confirms that this strategy keeps the active form of the lipase open.

On the other hand, as lipase adsorption is related to interfacial affinity, some lipases become adsorbed only on certain hydrophobic supports and not ~~in~~ on others even permitting ~~even~~ the separation of different lipases contained in a sample in certain cases. In some instances, this follows a hydrophobicity support criterion: some lipases are only ~~are~~ adsorbed on very hydrophobic supports while not ~~in~~ on other less hydrophobic ones (Cunha et al., 2009, Fernández-Lorente et al., 2005, Sabuquillo et al., 1998, Volpato et al., 2010, Volpato et al., 2011). In other cases, the nature of the group that is on the support is more relevant that the hydrophobicity: lipase from porcine pancreas immobilized very slowly on octyl supports but fairly rapidly on phenyl-ones (Palomo et al., 2005b, Segura et al., 2004, Segura et al.,

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2006). Thus, supports not specifically designed for specific adsorption ~~permit to have~~allow having selective immobilization of lipases due to this peculiarity of the lipase mechanism of action. The lipases thus immobilized may be used under a variety of conditions; even in relatively high concentrations of organic cosolvents the enzyme will remain attached to the support (Fernandez-Lafuente et al., 1998). After enzyme inactivation, ~~it~~ the enzyme may be desorbed using detergents, such as guanidine ~~etc.~~ and new enzyme loaded~~,~~ enabling the~~o~~ reuse of~~e~~ the support. Full lipase immobilization on these supports may proceed in few minutes (even using 1 g of support/50 ml of lipase solution), with increments on enzyme activity that may become even 100 fold for some substrates (Fernandez-Lafuente et al., 1998) while in other cases it may produce a decrease in activity (Garcia-Galan et al., 2014). Lipase stability ~~uses~~ tends to ~~be~~ increase for ~~thousand~~ thousand-fold factors using octyl agarose or Sepabeads decaoctyl (Palomo et al., 2002)~~.~~~~,~~ ~~this~~ This has been attributed to the importance of the stabilization of the open form of the lipases, that has a more compact and ordered structure (Peters et al., 1996).

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The protocol is so simple that it is simple to implement ~~it~~ at industrial level~~.~~~~,~~ ~~in~~ In fact the most popular immobilized preparation of a lipase, Novozym 435, is prepared using this technique, and Resindions Srl (Italy) commercialize Sepabeads octadecyl for this use, with several small companies using the support to prepare lipase biocatalysts.

4.2. Selective immobilization of large multimeric proteins in standard supports

4.2.1. One-step immobilization-purification of large proteins via physical adsorption on standard chromatographic matrices

In this point, it is critical to bear in mind that natural proteins are adsorbed on most ~~of the~~ matrices~~x~~ (ion exchanger, IMAC matrix) via interaction with several groups located ~~in~~ on the support (Kumar et al., 2000, Porath, 1992). Large proteins usually have two differences with small proteins: i-they can cover larger surfaces of the support, permitting the establishment of long distance multi-interactions and ii-they usually will have a higher number of groups on their surface able to interact with the support). This has been exemplified using multimeric proteins from 240 or 360 KDa (Fuentes et al., 2004, Fuentes et al., 2007, Pessela et al., 2004a).

The first fact has permitted to develop simple systems for the purification of large proteins using supports with very low activation degree (**Figure 11**) (Fuentes et al., 2004, Fuentes et al., 2007, Pessela et al., 2004a). These supports are designed by preparing decreasing superficial density of the groups on the supports (decreasing each by a half ~~each~~ step)~~.,~~ ~~the~~ The support that will have better performance will be the one with the lowest activation that is able to adsorb the desired amount of the target enzyme. ~~The purification~~ Purification depends on the distribution of charges and size of the target protein and the contaminant ones, having a protein of 200 KDa and contaminants not larger than 100 KDa purification may be almost total by this technique. Even stabilization of weakly associated proteins, shifting the equilibrium toward the associated form may be achieved using this strategy (Fuentes et al., 2005b, Fuentes et al., 2006, Fuentes et al., 2007). Unfortunately, this did not permit to use the enzyme in an immobilized form. The enzymes adsorbed on lowly activated supports are very easily desorbed from them. In the section below, this lowly activated adsorbent supports will be used for the implementation of heterofunctional supports (discussed later).

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The second peculiarity has permitted to use highly activated supports to selectively and very strongly immobilize large proteins. The number of enzyme-support interactions necessary to fix the enzyme to the support may be controlled by controlling the medium (Pohl et al., 1997). For example using an ion exchanger, the higher the ionic strength, the higher the number of enzyme-support interactions required to fix the enzyme to the support. Using IMAC matrices, a similar effect is achieved by adding the competitor imidazole during ~~the~~ protein adsorption (**Figure 12**). Thus, using a medium that hinders adsorption, only the proteins that can give many enzyme-support interactions may become adsorbed, favoring the larger proteins over the smaller ones.

Having a large multimeric enzyme (eg, over 200 KDa), this strategy may permit to discard around 80% of the monomeric proteins and the fraction of the multimeric ones that cannot become adsorbed on the support, which is an interesting purification factor associated to the immobilization (Pessela et al., 2004b). However, this may even have a higher interest if the target protein is the only large protein in the extract. This may be the case if the multimeric and large protein is from a thermophilic microorganism cloned in a mesophilic host; after a thermal treatment, all the multimers of the mesophilic host are destroyed, and the only large protein remaining in solution is the target termophilic enzyme (Pessela et al., 2004a, Pessela et al., 2004b).

Two papers may be found in the literature using anion exchangers or IMAC supports. In the first one, the authors showed that the immobilization of two large beta-galactosidases (from *Escherichia coli* and from *Thermus* sp.) on very highly activated anion exchangers supports (e.g., containing 40 μmol of ethylenediamine immobilized per wet gram of 4 BCL agarose) was so strong that the enzyme can

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hardly be released from the support (Pessela et al., 2006). Thus, these supports were not valid for protein purification, but the very high adsorption strength and activity retention suggested that they could be very suitable for immobilization of these large proteins. Using high ionic strength (e.g., 300 mM NaCl), both large target proteins may be still ~~be~~ fully adsorbed on these supports, while only around 20% of total proteins adsorb, permitting some purification of the large proteins but not a total one (Pessela et al., 2006). Moreover, adsorption under these conditions further increased the adsorption strength on the support (now there is no desorption of any enzyme molecule even using 800 mM NaCl) (Pessela et al., 2006). Thus, the partial purification and the strong reversible immobilization of both beta-galactosidases were performed in a very simple two-step process. On such a protocol, these large proteins were directly adsorbed on these supports after desorption (at 200 mM of NaCl) from poorly activated supports (Pessela et al., 2006). Furthermore, direct adsorption on very highly activated supports promotes a significant thermal stabilization of both enzymes, mainly under dissociation conditions, because the face of the protein that involves more enzyme subunits will be ~~those~~ that where it is easier to have a higher number of enzyme-support interactions.

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In a second example, IMAC supports were used to get the selective adsorption of the multimeric α- and β-galactosidases from *Thermus* sp. T2 (Pessela et al., 2007b). First, it was shown that both enzymes hardly desorbed from highly activated IMAC supports, even in the presence of 1 M imidazole. In the presence of 50 mM of imidazole, these very large proteins can be adsorbed on these supports while the medium-small proteins did not adsorb. In this way a very simple purification and reversible immobilization of these large proteins cloned on *Escherichia coli* can be performed by first heating of the crude preparation to leave as the only large

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protein the thermophilic one (Pessela et al., 2007b). Interestingly, immobilized β-galactosidase from *Thermus* sp. T2 was 10-fold more stable than the native enzyme when incubated at 70 °C and pH 7.0, very likely as in the other example; the involvement of the largest areas of these multimeric enzymes in the adsorption process may promote a multi-subunit adsorption with stabilizing effects (Pessela et al., 2007b). These immobilized-stabilized enzymes can be desorbed away from the support/the reactor after inactivation and a fresh solution of enzyme can be purified, immobilized and stabilized again for 3 cycles (Pessela et al., 2007b). The protocol has as main problem the use of drastic conditions, which in certain cases may produce enzyme precipitation, perhaps by this reason we have been unable to find any actual industrial biocatalyst using this technology.

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4.2.2. One-step immobilization-purification of multimeric proteins via covalent immobilization on glyoxyl supports

This strategy is based on the specific features of glyoxyl supports: the first immobilization must be via several enzyme-support weak imino bonds (Mateo et al., 2005). This produces a positive effect; the enzyme is oriented towards the area where there is the highest density of Lys residues, which is the area where there is the highest possibility of getting an intense multipoint covalent attachment. This has converted glyoxyl supports in very suitable ~~to~~ for obtaining important stabilization factors upon immobilization of many different enzymes via multipoint covalent attachment (Mateo et al., 2006). Moreover, the immobilization of enzymes on this support requires that it may have several reactive primary amino groups under the immobilization conditions. At pH 10, protein immobilization may be very rapid, because the ε-amino group of Lys has some reactivity (Mateo et al., 2005). However

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at pH 7, the immobilization of most proteins on glyoxyl agarose did not occur (**Figure 13**) (Mateo et al., 2005).

Multimeric proteins are the exception; they have several terminal amino groups, which may have a pK around 7-8, enabling ~~its~~ their selective immobilization on glyoxyl agarose if several terminal amino groups are in the same plane. One example of this strategy is the immobilization on glyoxyl agarose beads at pH 7 of a multimeric glutamate dehydrogenase from *Thermus thermophilus* cloned in *E. coli* (Bolivar et al., 2009c). This permitted to perform the immobilization, purification and stabilization of this enzyme in just one step. A first thermal shock of the crude preparation destroyed most mesophilic multimeric proteins. After this treatment, glutamate dehydrogenase became immobilized in less than 1 h on highly activated glyoxyl agarose beads at pH 7 with a 100% yield and a purity over 90% and its multimeric structure became stabilized. After immobilization, a further incubation at pH 10 improved enzyme stability under any inactivating conditions by increasing the number of enzyme-support bonds (**Figure 14**) (Bolivar et al., 2009c), becoming 3 times more stable than the glyoxyl biocatalyst prepared at pH 10. This enzyme could be even submitted to reactivation strategies following unfolding /refolding ~~strategies~~reactions, because subunits cannot be released from the support (Bolivar et al., 2010b).

Other multimeric proteins have been also immobilized on glyoxyl supports at pH 7, like β-galactosidase from *Escherichia coli* (Pessela et al., 2007a), catalase from bovine liver, and IgG from rabbit (Grazu et al., 2006). However, other multimeric proteins are not immobilized under these conditions, perhaps because several terminal amino groups cannot react simultaneously with the glyoxyl support at pH 7 (glucose oxidase from *Aspergillus niger* and *Penicillium vitale*; catalase from

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Micococcus sp., A. niger and bovine liver; alcohol oxidase from *Pichia pastoris, Hansenula sp*. and *Candida boidinii*, β-galactosidase from *Thermus sp*) (Grazu et al., 2006). The purification factor depended on the number of other multimeric proteins on the extract and the proteolysis of the proteins in the crude: each point of proteolysis produced a terminal amino bond. This means that in an extract with high proteolytic activity, purification of multimeric proteins by immobilization on glyoxyl agarose at pH 7 may be short (Grazu et al., 2006). This protocol has not been used at industrial level. ~~, together~~ Together ~~to~~ with the cited sample requirements, the glyoxyl supports require a final reduction with sodium borohydride as reaction end-point (Blanco and Guisán, 1989), and this may be a problem for many small biotechnological companies.

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4.2.3. One-step immobilization-purification of poly-Lys tagged proteins via covalent immobilization on glyoxyl supports

Terreni and coworkers have shown that the introduction of poly-Lys tags on the enzyme penicillin G acylase may permit the immobilization of the tagged protein even at pH 7 on glyoxyl supports (Scaramozzino et al., 2005). Although the objective of this tag was to get an oriented immobilization, this means that under these conditions only poly-Lys tagged proteins and multimeric proteins having the terminal amino groups on the same plane will be immobilized on the support, enabling a significant purification via a simple immobilization protocol (**Figure 15**). The immobilization gave 100% yield in less than 1 h, keeping the enzyme activity intact ~~the enzyme activity~~, and even more importantly keeping the properties of the free enzyme in kinetically controlled synthesis of antibiotics.

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5. Coupled immobilization, purification and multipoint or multisubunit immobilization of enzymes and proteins via covalent immobilization on heterofunctional supports.

Now, we will present the development of tailor-made heterofunctional supports to get the specific immobilization of target proteins. Heterofunctional supports ~~has~~ have been recently reviewed (Barbosa et al., 2013); here we will focus on the prospects to use them to perform one step immobilization-purification. Heterofunctional supports are defined as those matrices that present several functionalities on ~~its~~ their surface, with different physical or chemical properties, able to interact with a protein (Barbosa et al., 2013, Mateo et al., 2000, Mateo et al., 2003).

In this section, our interest lies in those heterofunctional supports bearing groups able to specifically adsorb a target protein, and other groups able to give a covalent attachment to make this enzyme-support interaction irreversible (**Figure 16**). A requirement for using this strategy to get the coupled immobilization-purification of proteins is that the covalently reactive groups on the support must be unable to immobilize the enzyme under the employed conditions., ~~that~~ Thus~~way~~ the selective adsorption of the protein produced by the other groups will permit the purification of the target protein. Most examples found in the literature involve multimeric large proteins. As chemically reactive groups, epoxydes (Mateo et al., 2007a, Mateo et al., 2007b) and glyoxyl groups (Mateo et al., 2006) have been utilized. Glyoxyl, as stated above, cannot immobilize monomeric proteins at neutral pH value (Mateo et al., 2005).

On the other hand epoxy supports react very slowly with free proteins., ~~in~~ In fact a previous adsorption of the proteins on the support is required to have

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covalently immobilized enzymes using epoxy supports (Melander et al., 1984, Smalla et al., 1988, Wheatley and Schmidt Jr, 1993, 1999), with the exception of ~~that~~ those proteins bearing an exposed Cys residue (Grazu et al., 2012). As selective adsorbents, cationic groups that require a multipoint ionic exchange to fix the protein on the support have been utilized (Kumar et al., 2000, Pohl et al., 1997, Porath, 1992).

As already explained above, large multimeric proteins will be able to interact with groups in the support quite far apart, enabling the selective adsorption of large versus small proteins (Pessela et al., 2004a). After this first adsorption, the very near proximity of the groups on the support and the groups on the enzyme may permit the establishment of some covalent bonds (Bolivar et al., 2009a). However, the increase of the pH value to increase the reactivity of the nucleophilic groups on the enzyme surface is recommended to achieve some additional enzyme-support bounds (Pedroche et al., 2007).

This strategy has been utilized to selectively immobilize, purify and stabilize a glutamate dehygrogenase from *Thermus thermophilus* cloned in *E. coli* after a thermal treatment using amino-epoxy supports (Bolivar et al., 2009b). In another paper, a tetrameric enzyme, β-galactosidase from *Thermus* sp., also cloned in E. coli and submitted to a thermal shock, was immobilized and purified by immobilization on amino-epoxy and amino-glyoxyl supports (Bolivar et al., 2010a). As this strategy permitted to ensure the identical orientation of the enzyme on the support, this paper showed that glyoxyl supports are more effective to stabilize proteins than epoxy supports. And this occurred even ~~al~~though epoxy groups can react with a handful of amino acids, including Cys, Tyr, Lys and even Asp and Glu (Turková et al., 1978) while glyoxyl only react with primary amino groups. These supports are not

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commercially available because their properties have been very recently described, thus, although the immobilization protocol is simple, they are not used at industrial level ~~but~~ although a gradual implementation in the middle term may be expected ~~a gradual implementation in the middle term~~.

Another application of the concept of heterofunctional supports is being applied to lipases. Interfacial activation of lipases on hydrophobic supports is a very useful reversible protocol to immobilize-purify lipases (see section 4.1 of this review) (Fernandez-Lafuente et al., 1998). However, it presents some limitations, as the enzymes may become desorbed from the support after inactivation or by cause of organic solvents or detergents, reducing the range of conditions where they may be applied. To solve this problem, the crosslinking of the lipase immobilized via interfacial activation using polymers (Fernandez-Lorente et al., 2010, Pizarro et al., 2012) or glutaraldehyde (Barbosa et al., 2012) has been proposed. A simpler solution may be forthcoming by achieving the covalent attachment of interfacially activated lipases. A recent paper shows the combination of ~~on~~ a siliceous support of hydrophobic moieties (e.g., octyl groups) and glyoxyl groups (Bernal et al., 2014). This way, at pH 7 and low ionic strength the lipase is selectively immobilized via interfacial activation, while after increasing the pH value to 10, some covalent attachments between enzyme and support are expected to be obtained (Bernal et al., 2014). Although the real establishment of the covalent attachments has not been studied, this new support permitted to improve the lipase stability in thermal and organic solvent induced inactivations (Bernal et al., 2014), keeping the hyperactivation achieved by the immobilization on hydrophobic supports. The problem is the necessity of having at least a primary amino group near the area involved in the immobilization., ~~in~~ In a recent paper it has been shown that this may

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not always occur, and some solution should be implemented to have 100% of the immobilized lipases covalently attached to the support (using octyl-glyoxyl agarose beads) (**Figure 17**) (Rueda et al., 2014). In other papers, amino-phenyl silicates were used to immobilize the lipases from *C. antarctica* (form B) (Boros et al., 2013) and *Burkholderia cepacia* (Abaházi et al., 2014). After adsorption, the immobilized enzymes were treated with glycerol diglycidyl ether to have some enzyme-support crosslinking. This strategy gave better results that the mono-derivatized supports. Immobilization was suggested to proceed via interfacial activation (activity improved after immobilization), and the final covalent attachments improve enzyme stability (Abaházi et al., 2014, Boros et al., 2013)

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This kind of heterofunctional hydrophobic supports may greatly improve the prospects of using interfacial activation of lipases as a tool to prepare biocatalysts useful under any experimental conditions, using the hydrophobic groups to have the a selective adsorption, and the other groups to transform this in an irreversible immobilization. The change of reversible to irreversible immobilization means that the support cannot be reused, therefore the strategy is only recommended when the improvement in enzyme stability or the prevention of enzyme release to the reaction medium are really relevant. This methodology of lipase immobilization is very simple, but the very recent description of the advantages and prospects (2014 was the first report) made that they are not implemented, neither as commercial supports or used to produce industrial lipase biocatalyst.

6. Coupled immobilization, purification and multipoint or multisubunit immobilization of domain tagged enzymes and proteins via covalent immobilization on heterofunctional supports

In some cases, the mere attachment of the enzyme and the support achieved by using some of the tags described in section 3 of this review is not desired by different reasons, for example by the risk of some desorption of the enzyme during operation, a necessity for improving enzyme stability, the intention of submitting the enzyme to processes of unfolding/refolding, etc. (Bolivar et al., 2010b). In these situations, the use of heterofunctional supports bearing a few groups able to give the specific interaction with the tag, and others able to give a further covalent attachment (multipoint if possible) may be a good solution for both problems. In the literature, it is possible to find two examples of this strategy, both using poly-His tagged proteins and IMAC-epoxy matrices. One involved a glutaryl acylase (Mateo et al., 2001a), while the other involved a β-galactosidase from *Thermus* sp. (Pessela et al., 2003). After the selective adsorption on the immobilized metal chelate groups via the His-tag (a very low activation was used to get this selective adsorption), the incubation at alkaline pH values was utilized to increase the number of enzyme support bonds (**Figure 18**). In both cases, the enzymes were stabilized via this protocol.

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This strategy could be extended to any other kind of tag or domain. However, this means that a reversible immobilization is transformed in irreversible., Thus, the convenience of this needs to be analyzed in each case. Even ~~al~~though the immobilization protocol is not complex, they are not implemented at industrial level, because the supports ae not commercially available and need to be prepared for each enzyme to maximize the purification.

7. Immobilization-purification based on different immobilization rates

In some cases, mainly using strategies based that require a multipoint enzyme-support interaction, the target enzyme may have a much faster

immobilization rate than the other contaminant proteins. This may not be enough for having a good purification if other proteins are also very rapidly immobilized because the difficulty in stopping the immobilization, and less at industrial level where the volumes that they manage may make it almost impossible to have a strict control of the immobilization process. However, this opens an opportunity for the purification of the enzyme. If the target enzyme is the one most rapidly immobilized on the support, using an amount of the target enzyme that oversaturates the loading capacity of the support, a very good purification could be achieved (Garcia-Galan et al., 2011).

The only example we have located in the literature has been the purification of a glutamate racemase by immobilization on glyoxyl agarose (Mateo et al., 2005). The enzyme was among the ones that more rapidly immobilized on the support. Using an excess of glutamate racemase enzyme, ultimate~~final~~ly most of the immobilized enzyme was this enzyme (Mateo et al., 2005). Obviously, this strategy implied that many of the target enzyme molecules did not immobilize and therefore the yield was not complete, but the purification factor was quite high (Mateo et al., 2005).

The immobilization rate can be modulated using genetic engineering to add an amino acid on the enzyme surface that facilitates its immobilization on the support. For example, using glyoxyl supports, this may be performed by adding poly-Lys tags or by adding new Lys on rich-Lys areas of proteins, this can be a simple way to get the one pot immobilization, orientation, purification and, at least in the second case, the hyper-stabilization of enzymes (Abian et al., 2004, Cecchini et al., 2007, Cecchini et al., 2012, Scaramozzino et al., 2005, Serra et al., 2009, Serra et al., 2013, Temporini et al., 2010).

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Conclusions

The coupling of immobilization to purification of enzymes and proteins has ~~an undoubtable~~ undoubted interest. The interest goes further if the final biocatalyst has an improved stability via multisubunit or multipoint covalent attachment, or we can get enzymes with a better orientation. The better understanding of the immobilization mechanism on the different supports may open new strategies to reach this objective. For example, glyoxyl supports have shown their real impact in this area only after recognizing the mechanism of the first immobilization of the enzyme on the support (via multiple enzyme supports interactions) (Mateo et al., 2005).

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In this context, heterofunctional supports may be a real alternative for the one step immobilization-purification (Barbosa et al., 2013). There are not many heterofunctional supports in the literature, but most of the chromatographic supports described to purify enzymes (affinity ligands, dyes, etc.) may be compatible with the introduction of other moieties that may permit a covalent immobilization, changing from a support useful to purify enzymes (that is, a support that can easily release the adsorbed proteins), to a support useful to immobilize proteins. Thus, other heterofunctional supports bearing groups different to amino or IMAC may open new opportunities to have different strategies for the one step immobilization-purification-stabilization and even orientation of the target enzyme.

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The industrial use of ~~the~~ heterofunctional supports technologies is still very poor~~,~~. ~~just~~ only lipase immobilization on hydrophobic supports ~~is~~ seems very popular at both~~,~~ academic and industrial level. This may be founded in the wide use of lipases, one of the most used enzymes at industrial level. For the other cases, the problem lies in the lack of commercial support suppliers, in most cases the supports need to be designed for each specific enzyme and still the demand will be too low to make this commercially available.

The integrated use of tailor made heterofunctional supports and tailor made enzymes (via site directed mutagenesis) may be a very useful and efficient strategy to develop new methodologies to reach the goals pursued in this review. The present time already offers a wide handful of possibilities, the future is full with unexpected promises in this very interesting area in the development of industrial biocatalysts.

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Figure legend

Figure 1. Improved properties of enzymes by immobilization on supports.

Figure 2. Strategies of enzyme immobilization in biocatalysts design.

Figure 3. Use of chimeric proteins with inserted tags and immobilized anti-tags to achieve site-directed immobilization of the target protein.

Figure 4. Site-directed immobilization of chimerical protein having biotin tags on avidin-coated-support.

Figure 5. Site-directed Immobilization of poly-His tagged proteins on IMAC support .

Figure 6. Improved ionic exchange of tagged proteins with cationic and anionic domains on ionic exchangers.

Figure 7. One step purification/immobilization of poly-Hys tagged proteins.

Figure 8. Interfacial activation of lipases on hydrophobic interface. The 3D structure of lipase RML was obtained from the Protein Data Bank (PDB) code 3TGL using Pymol vs. 0.99.

Figure 9. Interfacial activation of lipases on hydrophobic supports at low ionic strength.

Figure 10. One step purification-Immobilization of lipases on hydrophobic support at low ionic strength.

Figure 11. Purification of large proteins using ionic exchanger with very low activation degree.

Figure 12. Purification and selective immobilization of large proteins on highly activated heterofunctional amino supports and IMAC support by controlling the reaction medium.

Figure 13. Multipoint immobilization of proteins on glyoxyl-agarose supports.

Figure 14. Stabilization of multimeric enzymes by multisubunit immobilization on glyoxyl support.

Figure 15. Site directed immobilization and stabilization of poly-Lys tagged enzyme by multipoint attachment on glyoxyl support.

Figure 16. Enzyme immobilization on heterofunctional supports: amine-epoxy and amine-glyoxil supports are able to adsorb proteins first, and later generate a multipoint attachment with reactive groups of enzyme.

Figure 17. Immobilization of lipases on heterofunctional Octyl-glyoxyl supports. The lipase is immobilized by interfacial adsorption. Then, covalent attachment is generated by reaction between ε-amine groups of enzyme and glyoxyl groups of support.

Figure 18. Site directed immobilization and stabilization of poly-His tagged enzyme by multipoint attachment on heterofunctional IMAC support.

Table 1: Structural domains used for enzyme and protein immobilization/purification

Type of Protein Binding Domain	References
Cellulose Binding Domain (CBD)	Fishman et al. (2002), Linder and Teeri (1997), Linder et al. (1998), Lu et al. (2012), Richins et al. (2000), Zhao et al. (2013)
Chitin Binding Domain (ChBD)	Bergeron et al. (2009), Chern and Chao (2005), Chiang et al. (2008), Chiang et al. (2009)
Thermostable Chitin Binding Domain (TtChBD)	Wang et al. (2013b)
Substrate Binding Domain (SBD)	Lee et al. (2005)
Albumin Binding Domain (ABD)	Baumann et al. (1998)
Lectin Binding Domain (LecBD)	López-Gallego et al. (2012)
Biotin carboxyl carrier protein domain (BCCPD)	Cho et al. (2007), Wang et al. (1997)
Calmodulin Protein Domain (CaM-tag)	Daunert et al. (2007)
Ribosomal Protein L2 (203-273) domain (Si-tag)	Yang et al. (2013)
Polystyrene binding peptide domain (PS-tag)	Kumada et al. (2009)
Ig-G binding domain from Protein A and G (AG-tag)	Kondo and Teshima (1995)
Methionine tag domain (Met-tag)	Seino et al. (2014)
Aldehyde tag domain (Ald-tag)	Wang et al. (2013a)
Peptide tag domain (Pep-tag)	Simon et al. (2002), Tominaga et al. (2005)
PolyHis tag domain (PolyHis-tag)	Ha et al. (2013), Li et al. (2009), Lin et al. (2011), Wang et al. (2010)

Table 2: Structural domains used for enzyme and protein immobilization/ purification onto supports

Type of Protein Binding Domain	Type of Immobilization and/or purification	Example of protein used for enzyme Immobilization/purification	Biochemical properties improved by this methodology	Ref.
Cellulose Binding Domain (CBD)	Immobilization onto cellulose supports	Antibodies fused to CBD	Protein was immobilized with a yield over 99% in a column with cellulose as stationary phase	Linder et al. (1998)
		Organosphosphate hydrolase fused with CBD	The CBD-OPH fusion proteins could be immobilized onto a variety of cellulose ~~matrixes~~matrices, and retained up to 85% of their original activity for 30 days	(Richins et al., 2000)
		Horseradish peroxidase fused with CBD	A six-fold increase in the half-life of the enzyme in buffer resulted from immobilization onto cellulose via CBD. The immobilized enzyme was also more stable than the native enzyme in increasing concentrations of acetone (0-92%).	(Fishman et al., 2002)
		Heparinase fused with CBD	Enzyme was active against heparin for over 40 h and produced much lower molecular weight fragments of heparin at the same percentage of depolymerization.	(Shpigel et al., 1999)
		Beta-galactosidase fused with CBD	The immobilized enzyme reached a maximum yield of galactooligosaccharides (GOS) of 49% (w/w) from 400 g/L lactose (pH 7.6) at 45 °C for 75 min, with a high productivity of 156.8 g/L/h. Moreover, the immobilized enzyme could retain over 85% activity after twenty batches with the GOS yields all above 40%.	(Lu et al., 2012)

		Beta-glucosidase (BGL) fused with CBD by a peptide linker	A high immobilization efficiency of 90% was achieved. In addition, the fusion of CBD structure enhanced the hydrolytic efficiency of the BGL-CBD against cellobiose, which displayed a 6-fold increase in V_{max}/K_m in comparison with the BGL.	(Zhao et al., 2013)
Chitin Binding Domain (ChBD)	Immobilization onto chitin supports	Hydantoinase fused with ChBD	Immobilized d-hydantoinase exhibited higher thermostability with a half-life of 270 h at 45°C compared to soluble enzyme. Furthermore, d-hydantoinase immobilized on chitin could be reused for 15 times to achieve a conversion yield exceeding 90%.	(Chern and Chao, 2005)
		Carbamoylase fused with ChBD	Half-life of the immobilized carbamolyase CBL303 could reach 210 h at 45°C, whereas its free form had that of 17 h. D-p-hydroxyphenylglycine (D-HPG) production with immobilized enzyme could be carried out in 16 cycles with 100% conversion yield.	(Chiang et al., 2008)
		Penicillin amidase-Chaperone chimera fused with ChBD	During amoxicillin synthesis in aqueous-methanol mixtures, the total turnover number of immobilized chimera was 2.8 times higher after 95 h than the immobilized penicillin amidase lacking a chaperone domain.	(Bergeron et al., 2009)
		Levansucrase fused with ChBD	The chitin immobilization of hibrid *Z. mobilis* levansucrae (encoded by levU) was attempted for the repeated production of levan. Using the immobilized levansucrase with 20% sucrose, the production of levan was enhanced by 60% as compared to that by the free counterpart.	(Chiang et al., 2009)

		Chitinase Chit42 fused with ChBD	Chimeric chitinase was produced by fusing a ChBD from *T. atroviride* chitinase 18-10 to Chit42. The improved chitinase containing a ChBD displayed a 1.7-fold higher specific activity than chit42. This increase suggests that the ChBD provides a strong binding capacity to insoluble chitin.	(Kowsari et al., 2014)
Thermostable Chitin Binding Domain (TtChBD)	Immobilization onto chitin supports	Hemicellulase fused with ThChBD	Immobilized multifunctional hemicellulase exhibited high stability to producing xylose for at least 19 or 30 times in continuous operation with the achievement of 60% or 80% conversion yield at temperatures up to 65 ºC.	(Wang et al., 2013b)
Substrate Binding Domain (SBD)	Immobilization on poly-hydroxyl-alkanoate (PHA) beads	Different Fluorescent and virus proteins fused with SBD	Using this PHA microbead system combined with SBD fusion technology, immunoassays could be successfully carried out.	(Lee et al., 2005)
Albumin Binding Domain (ABD)	Immobilization on polystyrene coated with Rat Serum Albumin (RSA) microplates	Different protein fused to ABD	The ABD-RSA interaction remained stable after addition of different albumins. The use of RSA-microtiter plates for indirect immobilization of ABP fusion protein was shown to be superior to direct adsorption on plastic. The binding capacity of the RSA-microtiter plates was determined to be about 0.8 pmol of monomeric ABP protein.	(Baumann et al., 1998)
Lectin Binding Domain (LecBD)	Immobilization on Agarose beads	Lipase and green fluorescent protein fused with a LecBD	High immobilization rates on agarose are achieved. The strongest lectin-agarose interaction is also quite stable under a survey of different conditions such as high temperatures (up to 60 °C) or high organic solvent concentrations (up to 60% of acetonitrile).	(López-Gallego et al., 2012)
Biotin carboxyl carrier protein domain (BCCPD)	Immobilization on avidin -coated surfaces	Luciferase fused with BCCPD	Highly purified recombinant luciferase was obtained by a one-step purification protocol, utilizing immobilized metal affinity chromatography. The novel BCCP-luciferase had properties, stability, and activity similar to those of native luciferase.	(Wang et al., 1997)
		β-galactosidase fused with BCCPD	β-galactosidase was fused with BCCPD. The	(Vishwanath et al., 1995)

			immobilization of this biotinylated enzyme in aividin immobilized in poly(ether sulfone) membranes permitted a twenty-fold improvement in immobilized enzyme activity when compared to fully biotinylated enzyme.	
Calmodulin Protein Domain (CaM-tag)	Immobilization onto a silica surface and cellulose membrane modified by covalently attaching a phenothiazine ligand	Organophosphorus hydrolase (OPH) and beta-lactamase genetically modified with CM-tag	Immobilized OPH retained more than 80% of the activity of the free enzyme. The phenothiazine-modified silica particles are stable for long periods of time, i.e., up to 2 years when stored at 4 °C.	(Daunert et al., 2007)
Ribosomal Protein L2 (203-273) domain (Si-tag)	Immobilization on diatoms silica	Green fluorescent protein fused with Si-Tag	The results showed that the purification performance of this affinity purification method was good.	(Yang et al., 2013)
Polystyrene binding peptide domain (PS-tag)	Immobilization on polystyrene supports	Single-chain Fv antibodies (scFv) genetically fused with PS-tags	scFv-PS-tags immobilized on a hydrophilic PS (phi-PS) plate in the presence of Tween 20 showed high antigen-binding activity comparable to, or greater than, that of a whole monoclonal antibody (mAb) on a hydrophobic PS (pho-PS) plate.	(Kumada et al., 2009)
Ig-G binding domain from Protein A and G (AG-tag)	Immobilization on Poly(methylmethacrylate/N-isopropylacrylamide/methacrylic acid) [P(MMA/NIPAM/MAA)] and poly(styrene[N-isopropylacrylamide/methacrylic acid) [P(St/NIPAM/MAA)] latex particles	Beta-galactosidase fused with AG-tag (AGβgal)	Immobilized AG β-gal retained approximately 75% of its activity in solution and the binding is stable enough to allow repeated use.	(Kondo and Teshima, 1995)
Methionine tag domain (Met-tag)	Immobilization on Au/Fe-oxide composite nanoparticles	Subtilisin fused with Met-tag	Methionine-tagged immobilized enzymes showed 98% residual specific activity, while the untagged enzymes showed 78%. The methionine-tagged immobilized enzymes retained their activities in a wide temperature range of 30-70 °C.	(Seino et al., 2014)
Aldehyde tag domain (Ald-tag)	Immobilization on mesocellular siliceous foam (MCF) support activated with amino groups	Lipase fused with Ald-tag	The specific activity and the k_{cat}/K_m of the immobilized lipase using aldehyde tag (IL-AT) were 2.50 and 3.02 fold higher, respectively, than those of the traditionally immobilized	(Wang et al., 2013a)

			lipase using glutaraldehyde (IL-GA). The newly immobilized lipase also presented better thermo-stability than the traditionally immobilized one.	
Peptide tag domain (Pep-tag)	Immobilization on agarose gel beads chemically modified with β-casein to display reactive glutamine (Gln) residues	Alkaline phosphatase (AP) genetically modified with specific peptide tags containing a reactive lysine (Lys) residue with different length Gly-Ser linkers.	Enzymatically immobilized AP showed comparable catalytic turnover (k_{cat}) to the soluble counterpart and comparable operational stability with chemically immobilized AP.	(Tominaga et al., 2005)
PolyHis tag domain (PolyHis-tag)	Immobilization on nitrilotriacetic-acid-modified ordered mesoporous silicas (NTA-OMPSs)	Horseradish peroxidase genetically modified with histidine tags with different lengths (His6, His4, His3, and His2)	Immobilized enzymes exhibit good stability toward heat and pH changes. Negligible leakage of these enzymes over a wide range of acidic conditions was observed	(Lin et al., 2011)
	Immobilization on silica oxide beads activated with a chelating Co(II)	Different lipases genetically modified with His6 tags	Direct extraction of C. antarctica lipase B (CalB) from a periplasmic preparation with a minimum of 58% activity yield was produced in a one-step extraction-immobilization protocol. Immobilized CalB was effectively employed in organic solvent (cyclohexane and acetonitrile) in a transacylation reaction and in aqueous buffer for ester hydrolysis.	(Cassimjee et al., 2011)
	Immobilization onto single-walled carbon nanotubes functionalized with N_{α},N_{α}-bis(carboxymethyl)-l-lysine hydrate	NADH-oxidase genetically modified with His6 tags	Immobilization resulted in a good loading capacity and stability maintaining 92% maximum activity of the native enzyme. immobilization was reversible and can retain ca. 92% activity for a couple of loading cycles.	(Wang et al., 2010)

	Immobilization and purification on Ni^{2+}-Complexed poly(2-acetamidoacrylic acid) (PAAA) hydrogel support	Recombinant histidine-tagged glutamyl aminopeptidase (His-tagged GAP).	Catalytic activity of immobilized His-tagged GAP for the hydrolysis of alanyl-para-nitroanilide revealed 90% conversion after 30 min of incubation. The hydrogel-immobilized enzyme also exhibited enhanced thermal stability of sustained 70% activity after 1 h incubation at 60 °C, while the free enzyme activity was reduced to 50% at the same condition. After four cycles of hydrogel regeneration, the immobilized enzyme lost only 20% of its initial activity.	(Ha et al., 2013)
Cationic binding module	Immobilization on Sepabeads FP-SP 400 (Sepabeads) and Fractogel EMD SO3 (Fractogel) harboring sulfopropyl and sulfoisopropyl	Chimeras of d-amino acid oxidase (TvDAO) and sucrose phosphorylase (LmSPase) modified with Z basic2	70% of the LmSPase activity and 90% of TvDAO activity were bound to the carrier in each of the different conditions used. non-covalent attachment to anionic supports through a Zbasic2 module was a very useful approach for a reversible oriented immobilization of TvDAO, LmSPase.	(Wiesbauer et al., 2011)
	Immobilization on unmodified silica supports	Chimeras of d-amino acid oxidase and sucrose phosphorylase modified with Z basic2 functions as highly efficient silica binding module (SBM).	Immobilized enzymes displayed full biological activity, suggesting that their binding to the glass surface had occurred in a preferred orientation via the SBM. Z basic2 proteins were immobilized on porous glass in a loading of 30 mg protein/g support or higher, showing that attachment via the SBM combines excellent binding selectivity with a technically useful binding capacity.	(Bolivar and Nidetzky, 2012a)
	Immobilization on functionalized silica carrier with 3-(trihydroxysilyl)-1-propane sulfonic acid.	D-Amino acid oxidase from Trigonopsis variabilis (TvDAO) fused positively charged module Z basic2.	Immobilized TvDAO was not sensitive to bubble aeration and received substantial stabilization of the activity at 45°C as compared to free enzyme.	(Bolivar and Nidetzky, 2012b)
His-tagged polyhydroxyalkanoate (PhaZ) domine.	Immobilization on Ni^{2+} nitrilotriacetate-agarose matrix.	Recombinant depolymerase from Pseudomonas putida KT2442 fused with His-tagged polyhydroxyalkanoate (PhaZ)	The immobilized enzyme was more stable than its soluble counterpart and showed optimal hydrolytic activity on p-nitrophenylacetate at 37°C and 50 mM phosphate buffer pH 8.0.	(Arroyo et al., 2011)

poly-Lys tags domain		Penicillin G acylase genetically modified with poly-Lys tags	Enzyme keeps catalytic properties of the soluble enzyme on kinetically synthesis of cefamadole and cefonicid.	(Scaramozzino et al., 2005)
		Cyclodextrin glycosyltransferase of *Bacillus macerans* genetically modified with poly-Lys tags	Immobilized enzyme retained fully the CGTase activity. Furthermore, although the poly-lysine-mediated immobilization is reversible, the binding force is strong enough to block protein leakage from the solid support at neutral and basic pH.	(Kweon et al., 2005)
		Protease of *S. cerevisiae* fused with a poly-Lys tag	Enzyme is simply purified from cell extracts with very high purity in just one-step.	(Li et al., 2012)
Choline-binding domain (C-Lyt)	Immobilization on choline immobilized supports and DEAE	A fragment of a hepatitis virus protein was bound to C-Lyt.	The hydrophilic fragment of hepatitis C virus (HCV) E2 protein was expressed in Escherichia coli as a chimera, bound C-Lyt of the major autolysin of *Streptococcus pneumoniae*. The C- LytE2 chimera was purified by affinity chromatography using DEAE-Sepharose. The purified antigen was used to immunize rabbits and the specific humoral immune response to E2 protein was examined. Induction to high levels of antibodies against this HCV E2 protein was found.	(Martinez et al., 2000)
	Graphite electrode surfaces were modified with N,N-diethylethylenediamine groups, acting as choline analogs	C-LytA-β-galactosidase	The ability of the prepared electrodes to specifically bind C-LytA-tagged recombinant proteins was tested with a C-LytA-β-galactosidase fusion protein. The hybrid protein was immobilized in a specific and reversible way, while retaining the catalytic activity. Moreover, these functionalized electrodes were shown to be highly stable and reusable.	(Bello-Gil et al., 2014)