

TUTORIAL REVIEW

Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of organic polymeric carriers and immobilisation methods†

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Efficient immobilisation protocols are the result of perfect matching of factors depending on the enzyme, the process and the support for immobilisation. Physical–chemical phenomena, such as partition, solvation and diffusion, strongly affect the efficiency of the biocatalyst in each specific reaction system. Therefore, tailored solutions must be developed for each specific process of interest. Indeed, direct investigation of what occurs at the molecular level in a reaction catalysed by an immobilised enzyme is a quite formidable task and observed differences in the performance of immobilised biocatalysts must be interpreted very carefully. In any study dealing with enzyme immobilisation the prerequisite is the rigorous planning and reporting of experiments, being aware of the complexity of these multi-phase systems.

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Key learning points

- (1) The review would like to increase the independent thinking and the critical abilities of the reader when analyzing experimental data of processes carried out using immobilised biocatalysts. The reader is encouraged to use a rigorous scientific approach for the study of these complex-multi-phase systems, thus avoiding easy over-interpretations of experimental data.
- (2) The second aim is to provide a realistic overview of technical and economical constraints that determine the success of any immobilisation technology. This is of primary importance in a field of great industrial impact.
- (3) Thirdly, clear and schematic guidelines are presented for reporting minimal data of any immobilisation protocol. This is a pre-requisite for the development of successful and reproducible immobilisation methodologies.
- (4) Accordingly, schematic guidelines are provided for the reporting of the experiments needed for the accurate characterization of immobilised biocatalysts and their comparison in terms of efficiency.
- (5) Finally, the overall intent is to increase the awareness of the reader about the importance of partition and diffusion phenomena (often neglected) and in general of the physical–chemical variables involved in heterogeneous bio-catalyzed systems. That paves the way for the set-up of an appropriate experimental system, the selection of informative enzymatic assays and the production of useful experimental data.

1. Immobilising enzymes for industrial biocatalysis: technical and economic constraints

Immobilised enzymes are already used routinely in industry especially for synthesizing fine chemicals and pharmaceuticals.¹ Examples of bulk productions making use of immobilised enzymes can also be found in food and cosmetic sectors^{2–4} but there is an enormous catalytic potential waiting to be exploited for

different bulk production processes, going from biomass to oil and fat biotransformations. Like in a classical chemical process, working with heterogeneous catalysts enables their recovery and re-use, but the need for immobilising enzymes arises from several other different technical requirements. Firstly, the hydrophilic protein molecules of the free enzymes would aggregate when suspended in a hydrophobic environment that are used for many biotransformations, such as lipid transformations catalysed by lipases.² Secondly, applications of enzymes are often hampered by a lack of long-term operational stability, complex down-stream processing, low productivity and risk of contaminations. Ideally, optimal immobilisation technologies should provide solutions for all these problems. However, besides being highly efficient, immobilised enzymes for industrial applications must also be produced at affordable costs.

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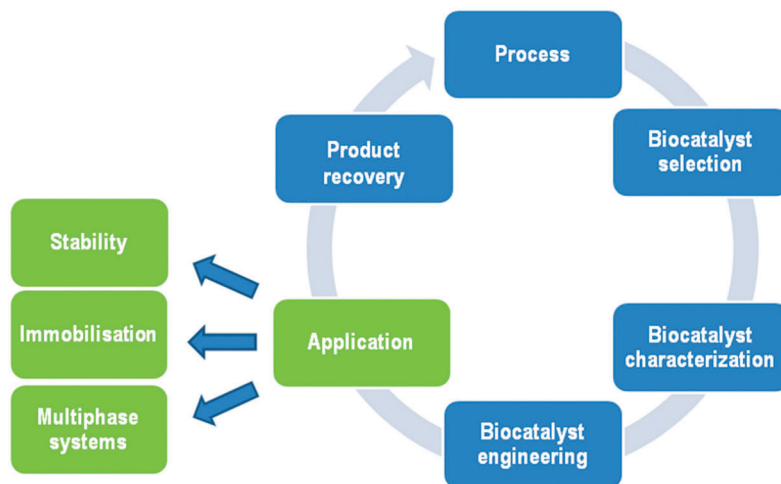


Fig. 1 Steps leading to the development of a biocatalysed process.

The steps leading to the development of a complete biocatalysed process are schematically reported in Fig. 1. As a rule of thumb, the cost of the catalyst should amount to a few per cent of the total production costs and this constraint, in many cases, represents a bottleneck for economic sustainability of biocatalysed synthesis at the industrial scale, especially in the case of production of commodities.⁵

It must be considered that only in a few cases, such as high-fructose syrup production by isomerisation of glucose, biocatalysis is a forced option, since no other technology is available. In the remaining situations as, for instance, in the production of biodiesel, biocatalysis is just one of the possible options. As a consequence, the transfer of biocatalytic technologies to the industrial scale is subjected to the demonstration that bio-conversions are not only environmentally sustainable and elegant but they are indeed competitive from all points of view. For example, in the production of fructose, a commodity, the isomerisation of glucose is catalysed by immobilised glucose isomerase having a productivity of 11 000 kg of the product per kg of enzyme.⁶

On the basis of these introductory concepts it becomes evident that solutions must be developed for each specific

process of interest. The ultimate goal is the achievement of highly productive catalysts, as this parameter determines also the impact of the cost of the biocatalyst per kg of the product.

The present review is not intended to provide a comprehensive analysis of the state of the art on enzyme immobilisation, but rather points out factors to be considered for the selection of appropriate methodologies for immobilisation of enzymes on solid organic carriers, focusing the attention on physical-chemical properties of carriers.

The attention will be focused mainly on commercially available supports and on the understanding of partition, solvation and diffusion phenomena which strongly affect the efficiency of the biocatalyst in each specific reaction system.

2. Immobilising enzymes: carrier bound technologies *versus* other methods

Carrier-bound immobilisation methods represent only one fraction of all available technologies, which are schematized in Fig. 2. In each case, immobilisation protocols try to find a



In the photo from the left: Dr Sara Cantone (COO of SPRIN), Dr Patrizia Spizzo, Prof. Lucia Gardossi, Dr Diana Fattor, Dr Valerio Ferrario, Dr Livia Corici, and Prof. Cynthia Ebert

The Laboratory of Applied and Computational Biocatalysis (LACB) is headed by Prof. Lucia Gardossi, who has been studying biocatalysis since the late 1980s. SPRIN s.p.a. was established in 2007 as a spin-off of LACB with the aim of providing immobilised enzymes and related services to industries. Experimental and computational strategies are employed synergistically to improve the understanding of enzymes' properties and to accelerate the development of industrial biocatalysts.

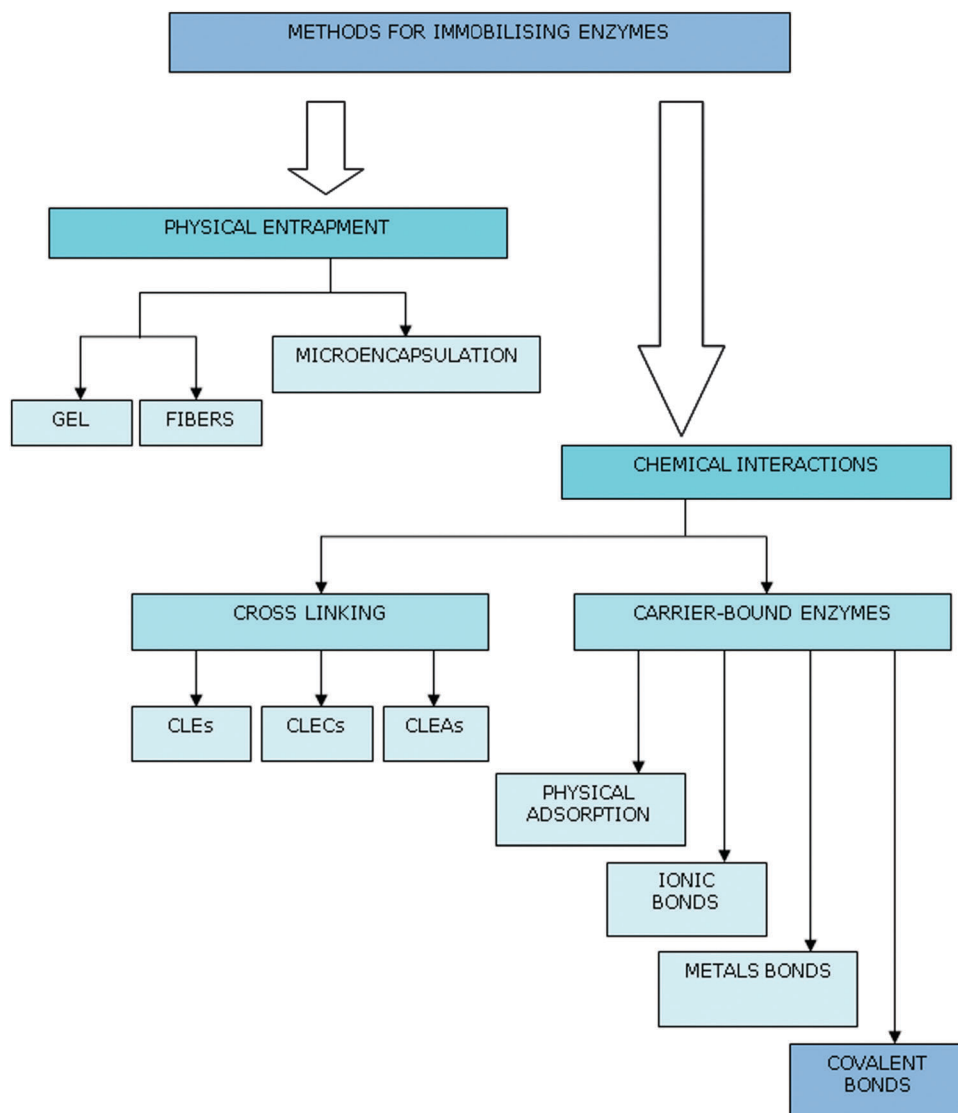


Fig. 2 Carrier bond immobilisation methods vs. other different immobilisation technologies. Abbreviations in the figure: CLEs – Cross-Linked Enzymes; CLECs – Cross-Linked Enzyme Crystals; CLEAs – Cross-Linked Enzyme Aggregates.

compromise between preservation of catalytic activity and the achievement of the expected technological advantages coming from immobilisation. As a matter of fact, immobilization procedures may infer different types of stress to the enzyme, thus inducing conformational modification and partial loss of activity.⁷

For the immobilisation of isolated enzymes the methods based on the establishment of chemical interactions are generally preferred. Physical entrapment is well suited for whole cell immobilisation and some remarkably efficient examples of industrial applications are described in the following section.

2.1 Entrapment

Physical entrapment involves encapsulation of cells or isolated enzymes into polymer networks of organic or inorganic (*e.g.* silica based) materials.⁸ Membrane devices such as hollow fibers or a microcapsule can also be used.⁹

The polymeric network must allow diffusion of reactants and products while avoiding the migration of the biocatalyst in the bulk medium. Because of that, entrapment is more suited for the immobilization of whole cells.¹⁰

It is noteworthy that Nitto Chemical (now Mitsubishi Rayon, Japan) has developed a process where the addition of water to acrylonitrile leading to acrylamide is catalysed by bacterial nitrile hydratases. The biotransformation is carried out using immobilised whole cells (cells entrapped in a cross-linked 10% (w/v) polyacrylamide/dimethylaminoethylmethacrylate gel) on 30 000 tons per year scale; fed-batch addition of the substrate allows for product concentrations of up to 20% (w/v), containing a negligible substrate and less than 0.02% (w/w) of side product (acrylic acid). Using 1% (w/v) immobilised-enzyme concentration the process takes about a day. A similar approach is used by DuPont for the regioselective hydration of adiponitrile to 5-cyanovaleramide, an early intermediate in the manufacture of

an herbicide. Cells containing nitrile hydratase are employed after entrapment in calcium alginate and are recycled multiple times, generating close to 3150 kg of product per kg of catalyst.⁵

Several specialty chemical companies also use living cells as catalysts for specific coenzyme-dependent reactions.¹¹

2.2 Cross-linked enzymes

Enzymes can be formulated into water-insoluble particles by means of extensive cross-linking. Enzyme crystals or aggregates are prepared in a spray-dried form. Cross-Linked Enzyme Aggregates (CLEAs) are prepared by firstly aggregating enzymes. The addition of precipitants such as acetone, ammonium sulfate, ethanol or 1,2-dimethoxyethane is followed by a cross-linker, commonly glutaraldehyde, thus forming diimine bonds. Often additives are included into the CLEAs. Cross-Linked Enzymes have the major

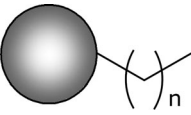
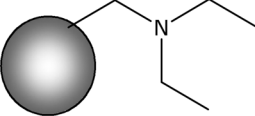
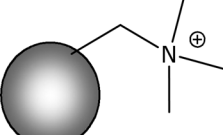
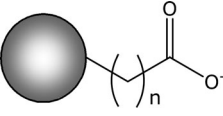
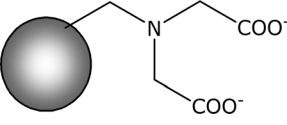
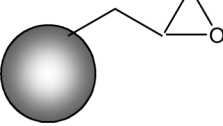
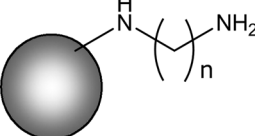
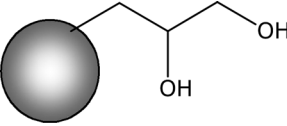
advantage that no support is required with a consequent important reduction of costs. They also combine high enzymatic activity with the ease of preparation from crude enzyme samples, although mechanical strength and filterability still have to be demonstrated on an industrially relevant scale. Cross-Linked Enzymes Aggregates (CLEAs) have been extensively reviewed by Roger Sheldon.¹²

2.3 Carrier-bound methods: general concepts

Classical methods of immobilisation of enzymes on solid carriers envisage the formation of chemical interactions or covalent bonds between a solid support and the enzyme.^{13,14}

Anchoring between the enzymatic protein and the support can take place either by adsorption or by formation of covalent bonds between functional groups present on the surface of the protein and appropriate reactive groups of the solid support (Table 1).¹³

Table 1 Most common functional groups present on organic polymeric carriers and used for establishing interactions and covalent bonds with enzymes

Method of immobilisation	Functional group	Structure	Binding	Reactive group on enzyme
van der Waals and hydrophobic interactions	Alkyl		The long alkyl chain enhances the hydrophobic nature of the carrier and maximizes hydrophobic interactions for physical immobilisation (adsorption)	Hydrophobic areas on the surface of lipases
Ionic interactions	Trialkyl ammine		Ionic adsorption	Negatively charged a.a.
	Tetra alkyl ammonium		Ionic adsorption	Negatively charged a.a.
	Carboxylate		Ionic adsorption	Positively charged a.a.
Metal affinity	Iminodiacetic		Loading metals such as Ni ²⁺ , Zn ²⁺ , Cu ²⁺	His-tag
Covalent bonds	Epoxy		Formation of covalent bonds <i>via</i> nucleophilic attack and opening of the epoxy ring	Nucleophilic groups (mainly -NH ₂ and -SH)
	Amino		Pre-activation with glutaraldehyde to introduce an aldehyde group that forms an imino bond <i>via</i> nucleophilic attack by a primary amine	Primary amines (terminal amines and Lys side chains)
	Diol		Activation with BrCN to give a reactive cyclic imido-carbonate Oxidation of adjacent <i>cis</i> -diols with NaIO ₄ to give dialdehydes	Primary amines (terminal amines and Lys side chains)

Physical forces, such as hydrophobic interactions, van der Waals binding or ionic interactions, are generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. In that respect, covalent binding of the enzyme to a support leads to more robust biocatalysts that can also be applied in aqueous, multi-phase or viscous mixtures.

The efficiency of supported enzyme preparations is governed by the properties of both the enzyme and the carrier material. The support (carrier) can be a synthetic organic polymer, a biopolymer or an inorganic solid. The interaction between the two provides an immobilised enzyme with specific chemical, biochemical, mechanical and kinetic properties (Fig. 3).

The immobilisation process generally occurs in aqueous solution containing the dissolved enzyme where the solid carrier is suspended. The ionic strength of the aqueous buffer can be adjusted to promote the partition of the protein onto the solid carrier. As a matter of fact, most enzymes have surfaces with predominant hydrophilic nature. The displacement of water molecules from the protein and the subsequent establishment of new polar interactions with the surface of hydrophilic carriers (*e.g.* a silicate or a carbohydrate) can occur at relatively low ionic strength, whereas a high salt concentration (up to 1 M phosphate) is recommended to promote the initial adsorption of the protein on polymeric organic resins (*e.g.* methacrylic or styrenic).¹⁴ Suitable incubation between the enzyme and the solid support is followed by recovery of the immobilised enzyme on the solid support, for example by filtration. In the case when the immobilised enzyme is to be used in organic systems (*i.e.* in low water media) it is necessary to remove the residual water, for example by washing with acetone or under reduced pressure.

The immobilisation of enzymes onto porous solid supports suspended in organic media has also been described.¹⁵ The method is intended for application of the enzyme in

non-aqueous media and it envisages the suspension of a porous silica support in a water-immiscible organic solvent, to which the aqueous solution containing the enzyme is then added. As a consequence, the aqueous solution is quickly absorbed into the hygroscopic solid support together with the enzyme, which thus remains in contact with an aqueous micro-phase inside the pores of the support. This enzymatic preparation, consisting of the hydrated support and the enzyme, can then be used directly for carrying out reactions in organic solvent while maintaining the enzyme hydrated inside the pores.

3. Which carrier for enzyme immobilisation?

Different immobilisation methodologies are continuously developed and published. The scientific literature of the last thirty years also reports the use of a considerable variety of materials, which, in many cases, are not only expensive but also hardly available in reasonable amounts. Moreover, complex protocols are often envisaged.^{13,14} Consequently, they are unlikely to be applied for the preparation of industrial biocatalysts although they bring fundamental knowledge of enzyme stabilisation and application and could be interesting for other advanced technological applications (*e.g.* in biosensors).¹⁶

The expertise accumulated by the chemical industry in decades of industrial production of chromatographic resins has been profitably transferred to the synthesis of an array of new families of resins. These polymeric products are generally in the form of beads, endowed with broad chemical and physical properties, with the attempt to match the technical needs of biocatalysed industrial processes. Immobilisation of enzymes on fully characterized and commercially available resins is still the easiest and quickest way to prepare an immobilised biocatalyst in any laboratory equipped with very basic chemical facilities, although the cost of polymeric organic

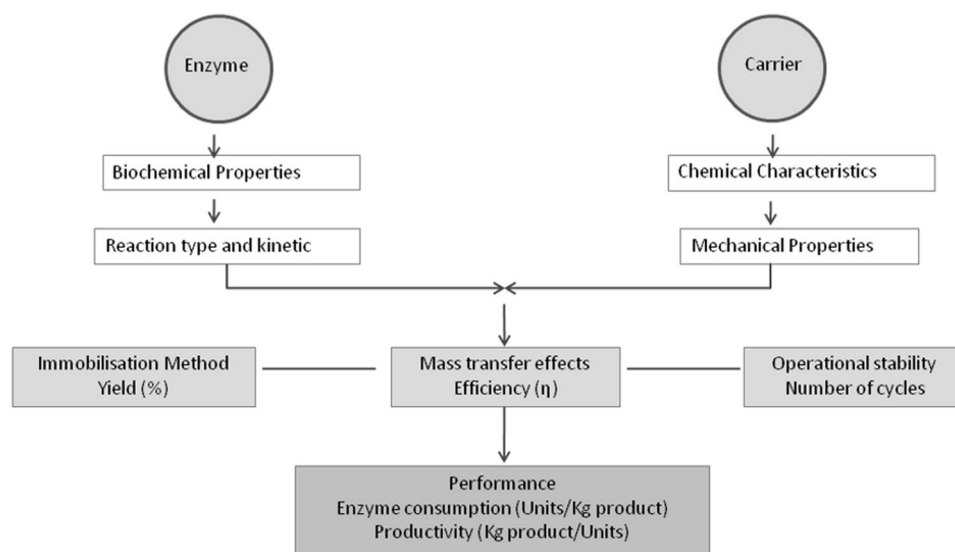


Fig. 3 Factors affecting the performance of enzymes immobilised on solid carriers.

carriers may represent an important issue in the selection of supports. It becomes obvious that the higher the cost of the carrier the higher should be the productivity of the immobilised biocatalyst.

Natural biopolymers may represent an attractive alternative from the economic point of view. Water-insoluble carbohydrates such as cellulose, starch, dextran, agarose and chitosan and proteins such as albumin and gelatin have been widely used as supports for immobilising enzymes.¹⁷ From this group, polysaccharides are of special interest, since they do not suffer from biological safety aspects like protein matrices isolated from animal sources and they are highly hydrophilic, which provides a desirable microenvironment for many enzymes.

The physical characteristics of the supports (such as particle diameter, swelling behavior, mechanical stability, and compression behavior) are of paramount importance for the performance of the immobilised systems and will determine the type of reactor used under technical conditions. In particular, pore volume, pore diameter and particle size determine the total surface area and thus critically affect the loading capacity of the resin.

In general, an essential requirement for any carrier is to have a large surface area ($>100 \text{ m}^2 \text{ g}^{-1}$) to promote the contact between the enzyme and the substrate.¹⁴ The pore size of the selected carriers should meet three requirements: (i) suitable to enable the adsorption of the enzyme molecule in the interior of the carriers; (ii) larger than the size of the enzyme molecules thus preventing the decrease of enzyme-conformation mobility; and (iii) diffusion constraints should be mitigated to ensure the accessibility of the substrate to the catalytic site of the enzyme. Very large substrates might require lower porosity to avoid the immobilisation of the enzyme in the inner pores that would be hardly accessible by bulky substrates. Maintaining the catalyst on the external layer of the support can be preferable when scarcely soluble substrates are used, which might precipitate inside narrow pores.

4. Chemical properties of organic carriers: polymeric composition and functionalization

The following section is intended to provide a schematic overview of the chemical features of the mostly largely employed organic carrier and the discussion will involve two different categories of chemical properties. The first one concerns the polymeric composition of the solid support, which identifies the class of biopolymers (*e.g.* carbohydrates such as cellulose, starch, dextran, agarose and chitosan or proteins such as albumin and gelatin) or synthetic polymers (*e.g.* acrylic, methacrylic and styrenic resins).¹⁴ The polymeric composition determines to a larger extent the hydrophobic/hydrophilic nature of the carrier and, consequently, its ability to establish polar or hydrophobic interactions with specific superficial areas of the enzymes. The chemical structure of the monomers will also strongly affect the ability of the carrier to be solvated by hydrophobic solvents/substrates as well as the water

retention capacity, although the latter property is also connected with porosity.

The second chemical feature is related to the presence of specific chemical functionalities on the surface of the carriers (Table 1).¹³ These functional groups can be part of the inherent structure of the monomers comprising the carrier (*e.g.* $-\text{OH}$ groups of carbohydrates) or can be deliberately introduced during the polymerization process (*e.g.* a certain percentage of the glycidyl methacrylate monomer was added in the synthesis of methacrylic polymers). Finally, they can also be introduced by chemical modification of the surface of the carrier through a “pre-activation” treatment. Some examples are reported in Table 1.

The most common functional groups available on organic supports are listed in Table 1, whereas examples of commercial products applicable for each different immobilisation technology can be found in ESI† (Tables S1–S3). Indeed, different organic resins are currently available on the market, suitable either for physical or covalent immobilisation. It must be underlined that a few companies commercialize both immobilisation supports and immobilised enzymes.

Regarding the effect of polymer–enzyme spacers, longer spacers are expected to allow a wider conformational flexibility to the protein. Shorter spacers can confer higher thermal stability since they restrict the enzyme mobility and prevent unfolding. Penicillin G acylase from *E. coli* was shown to be more stable when shorter spacers were selected. In the specific case, methacrylic polymers (Sepabeads[®], see Table S3 of ESI†) having amino groups preactivated with glutaraldehyde were used.¹⁸

4.1 Organic polymers for physical binding

van der Waals and hydrophobic interactions. Lipases are enzymes evolved to catalyze the hydrolysis of insoluble and hydrophobic substrates. Unlike the other hydrolase enzymes, their surface is characterized by significantly extended lipophilic areas that will interact well with a hydrophobic carrier and the immobilisation will take place thanks to van der Waals forces and entropy changes.¹⁹ The method is very mild and causes small or no conformational changes of the enzyme, thus maintaining its catalytic site in an active conformation.¹³

A range of hydrophobic carriers, such as polypropylene, acrylic or styrene, with different degrees of hydrophobicity and porosity are available on the market (Table S1 of ESI†).¹⁴ Indeed, styrenic polymers are widely used in refining of pharmaceuticals and natural extracts, since these are suitable for adsorbing large molecules because of their relatively large pore sizes and adsorption–desorption capacity.²⁰ As a consequence, a considerable number of acrylic or styrenic resins, with different degrees of hydrophobicity, are available and they usually have a surface area $>40 \text{ m}^2 \text{ g}^{-1}$ and a porosity $>400 \text{ Å}$. The data regarding the structural characterization of the solid supports showed that several materials used for enzyme adsorption can be considered macroporous, since pore diameters are higher than 50 Å .

Also porous acrylic resins, such as Amberlite XAD-7, are used to immobilise enzymes *via* adsorption and they can also be

functionalized with octadecyl groups to enhance the hydrophobic nature (Table 1, first entry). These highly hydrophobic supports allow very strong adsorption *via* hydrophobic interactions and their application is mainly restricted to lipases immobilisation. Lipases from *Humicola lanuginosa*, *Candida antarctica* and *Rhizomucor miehei* adsorbed on Sepabeads[®] containing octadecyl chains (see Table S1 of ESI[†]) showed the highest activity in the esterification of oleic acid with 1-butanol in isooctane.²¹ This was attributed to the hydrophobic nature of the support facilitating opening of the hydrophobic lid of the lipase, but the favorable partitioning effect of substrates cannot be excluded. Lipases, thanks to hydrophobic interactions, can be readily adsorbed on hydrophobic organic resins even at low ionic strength of the immobilisation buffer. This feature enables the exploitation of polymeric resins for achieving lipase purification and immobilisation in one single step starting from crude enzymatic solutions, where lipases represent the only hydrophobic component.¹⁶

One of the most successful examples of lipase adsorption on organic resins is the widely used enzyme *Candida antarctica* lipase B commercially available in the immobilised form as Novozym 435[®] which consists of the enzyme adsorbed on a macroporous (polymethyl/butylmethacrylate-divinylbenzene) resin.²²

SIRMS (synchrotron infrared microspectroscopy) imaging at 10 μm resolution of thin sections of this catalyst (Novozym 435[®]) revealed that the enzyme is localized in an external shell of the bead with a thickness of $\sim 100 \mu\text{m}$.²³ Furthermore, it was found that CALB secondary structure was not altered by immobilisation. Scanning electron micrograph (SEM) images of the Novozym 435[®] beads showed that the average pore size is 10 times larger than CALB molecules, implying that there is no physical barrier to enzyme or substrate diffusion throughout the bead. Protein–matrix and protein–protein interactions govern the distribution of the enzyme within the macroporous resin.

Among organic polymeric carriers, Accurel is also a hydrophobic polypropylene resin and its application to lipase immobilisation has been extensively investigated.²⁴

Ionic interactions. A number of reversible immobilisation methods have been inspired by principles on the basis of protein–ligand interactions commonly applied in chromatography. For example, one of the first applications of chromatographic principles in the reversible immobilisation of enzymes has been the use of ion-exchange resins. As can be observed in Table 1, positively (tetra alkyl ammonium) or negatively charged (carboxy) resins can be exploited for the scope.^{13,14} Polyethyleneimine has also been used to bind a variety of enzymes.²⁵ Depending on the pH of the solution and the isoelectric point, the surface of the enzyme may bear charges. Using widely available modelling systems, the surface charge and charge distribution of an enzyme can be readily calculated and displayed.⁹

The development of immobilisation protocols requires the identification of conditions under which the enzyme remains both strongly bound and fully active, which is not trivial.²⁶

A highly charged support can induce the distortion of kinetics when the substrates or products themselves are charged, as a result of partition or diffusion phenomena. Enzyme properties, such as pH optimum or pH stability, may also change, although this could be exploited to shift the optimal conditions of a certain enzyme towards more alkaline or acidic conditions, depending on the application.²⁷

A range of synthetic ion exchanger polymers, available for ionic immobilisation of enzymes, are presented in Table S2 of ESI[†]. However, in general, any ion exchanger can act as a support for enzyme immobilisation *via* ionic and strongly polar interactions.

The first full scale industrial use of an immobilised enzyme was the production of L-amino acids by resolution of racemic acylamino acids using an aminoacylase from *Aspergillus oryzae* immobilised by adsorption on DEAE-Sephadex, which consists of cross-linked dextran functionalized with diethylaminoethyl groups. The process was performed in continuous operation in a fixed-bed reactor (Tanabe process – 1960)²⁸ and also for the immobilisation of a recombinant epoxide hydrolase from *Aspergillus niger* for the resolution of various racemic epoxides by enantioselective hydrolysis.²⁹

Metal binding. Metal chelated supports are used extensively in protein chromatography (IMA – Immobilised Metal-Ion Affinity).³⁰ The DNA sequence specifying a string of six to nine histidine residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6xHis or poly-His tag fused to its N- or C-terminus. Expressed His-tagged proteins can be purified and detected easily because the string of histidine residues binds to several types of immobilised metal ions, including nickel, cobalt and copper, under specific buffer conditions. Chelator ligands can be immobilised on the solid supports by means of stable covalent bonds and the metal ions are then bound by coordination. The chelators most commonly used as ligands for IMAC are nitrilotriacetic acid (NTA) and iminodiacetic acid (IDA, see Table 1). The stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is subsequently regenerated by washing with a stronger chelator such as ethylene diamine tetraacetic acid (EDTA) when desired.

The concept at the basis of IMA has also been exploited for immobilising enzymes. A general immobilisation method for applications with enzymes suitable for His(6)-tagging was developed by Berglund and co-workers.³¹ The enzyme alanine racemase from *Geobacillus stearothermophilus* was cloned, over-expressed and fused to a His(6)-tag at its N-terminal. The enzyme was immobilised through cobalt ions. The metal binding technology remains confined on a laboratory scale.

4.2 Organic polymers for covalent immobilisation

Organic resins with epoxy functionalities. Covalent binding of enzymes on carriers provides the strongest linkages between enzyme and the carrier, enzyme leaching in aqueous media is minimized and no protein contamination of the product occurs.

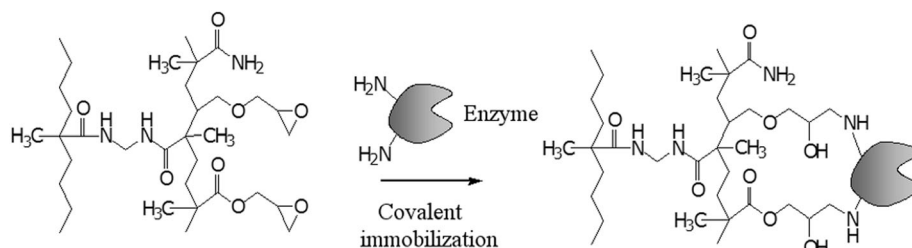


Fig. 4 Schematic representation of an epoxy functionalized methacrylic resin and the method for covalent enzyme immobilisation.

One very straightforward method implies the exploitation of epoxy groups of functionalised resins through the nucleophilic attack of groups available on the surface of the enzyme, mainly amino (see Table 1), to form stable secondary amino bonds (Fig. 4). The immobilisation procedure is carried out at neutral or alkaline pH and the resulting bonds are long-term stable within a pH range of 1 to 12.^{1,13,14}

Epoxy groups are stable at neutral pH values and react with different nucleophilic groups, besides primary amino, like thio and hydroxyl to form strong linkages (thioether and ether bonds) with minimal chemical modification of the protein.

Eupergit C has been one of the first epoxy-activated resins commercially available. It is a macroporous copolymer of methacrylamide, glycidyl methacrylate and allyl glycidyl ether, cross-linked with *N,N'*-methylene-bis(methacrylamide), with an average particle size of 170 μm and a pore diameter of 25 nm. Eupergit C is highly hydrophilic and absorbs about three times its weight of water.

Due to the high density of oxirane groups on the surface of the carrier beads (>0.6 mmol per g of dry Eupergit C), one enzyme molecule can form multiple bonds (multipoint attachment), which is considered as a major contributing factor to the high operational stability of enzymes bound to Eupergit C. Any remaining oxirane groups can be blocked (end capped) using a variety of reagents (mercaptoethanol, ethanolamine, glycine, etc.) to prevent any undesired support–protein reactions.¹⁴

Immobilisation by covalent attachment to Eupergit C has been successfully applied to a variety of enzymes for industrial application.³² Nowadays, different epoxy acrylate polymers are available (Table S3 of ESI†). Extended porosity of the resins can be achieved *via* cross-linking in the presence of a porogenic agent that allows the control of porosity. Only a few acrylic/styrene resins activated with epoxy groups are commercialized and they are particularly suitable for aqueous–hydrophobic biphasic systems and hydrophobic substrates.

One of the most remarkable examples of covalently immobilised enzyme is represented by penicillin G acylase immobilised on Eupergit C able to retain 60% of the initial activity over >800 cycles.^{32,33} The methacrylic resins are both chemically and mechanically stable over a pH range from 0 to 14 and they do not swell or shrink even upon drastic pH changes. The enzymatic cleavage of penicillin G is carried out in water and nowadays affords approximately 20 000 tons per year of 6-APA.

Resins and biopolymers with amino functionalities. A wide number of amino-functionalised acrylic resins are also available on the market (see Table S3 in ESI†). The amino functionality confers a higher hydrophilic character to the resins. The amino groups can be linked to the enzyme *via* pre-activation by glutaraldehyde or other bi-functional reagents (Fig. 5).¹⁴ Reaction of the aldehyde groups with amino groups of enzymes forms *Schiff's bases*, which are not stable at acidic pH.

The imine formed can subsequently be reduced (NaBH_4), ensuring irreversible immobilisation but with the risk of decreasing the activity of the biocatalyst.¹⁴

Among biopolymers, chitosan has been used for enzyme immobilisation because of its primary amino groups that were pre-activated with bi-functional reagents. Treatment with polyethyleneimine or hexamethylenediamine and glutaraldehyde can improve the mechanical characteristics of the enzyme on chitosan, although this causes some activity loss or an increase of diffusion limitations. The most relevant industrial application of chitosan as a biocatalyst carrier is related to the immobilisation of penicillin G acylase (PGA).³³

Aldehyde-agarose contains free aldehyde groups, to which enzymes can be covalently attached *via Schiff's base* formation with the free amino groups of lysine residues of proteins. The covalent immobilisation of penicillin G acylase, as well as various other enzymes, to cross-linked aldehyde-agarose has been widely studied, mainly with the objective of improving its thermal and operational stability.^{34,35}

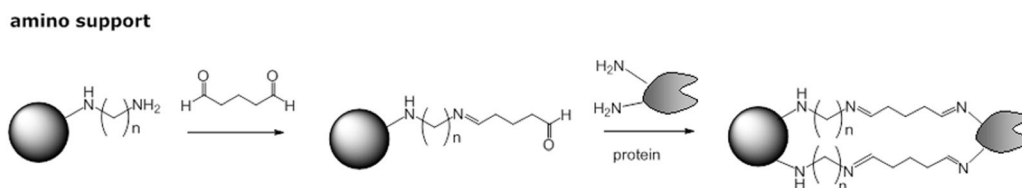


Fig. 5 Steps leading to immobilisation of enzymes on amino functionalised resins: (i) pre-activation of amino groups with a di-functional reagent (e.g. glutaraldehyde); (ii) nucleophilic attack of the amino group to the aldehyde and formation of the imine bond (*Schiff's base*).

Sugar based carriers. The hydroxyl groups of polysaccharides can be exploited for covalent immobilisation of proteins (Table 1). Cyanogen bromide reacts with vicinal diols to give the reactive cyclic imido-carbonate. This reacts with primary amino groups (*i.e.* mainly lysine residues) on the protein under mildly basic conditions (pH 9.0–11.5).³⁶ The most commonly used method for immobilising proteins on the research scale (*i.e.* using less than a gram of protein) involves Sepharose, activated by cyanogen bromide. Sepharose is a commercially available beaded polymer which is highly hydrophilic and generally inert to microbiological attack. Chemically it is an agarose (poly- $\{\beta\text{-}1,3\text{-D-galactose-}\alpha\text{-}1,4\text{-(3,6-anhydro)-L-galactose}\}$) gel. The hydroxyl groups of this polysaccharide combine with cyanogen bromide to give the reactive cyclic imido-carbonate. This reacts with primary amino groups (*i.e.* mainly lysine residues) on the enzyme under mildly basic conditions (pH 9–11.5). This is a simple, mild and often successful method but the high toxicity of cyanogen bromide confined its use to the laboratory scale.³⁷

Cellulose is also an acceptable support and can be activated in a similar way. The binding capacity for enzymes is generally lower as compared to agarose but it is inexpensive and commercially available in fibrous and granular forms. Some drawbacks are the low particle sizes, which affect their use in high pressure processes. Some immobilisation and engineering aspects have been reviewed.³⁸

5. The relevance of partition and diffusion phenomena in enzyme immobilisation

5.1 Preventing the partition of enzymes into the bulk medium (enzyme leaching)

Interactions at the basis of physical adsorption are not sufficiently strong to prevent the detachment of the protein from the support in the presence of an aqueous or hydrophilic phase in which it will tend to be distributed, also on account of mechanical stresses (*e.g.* viscosity of the reaction mixture).^{39,40}

As a general rule, the activity of an enzyme adsorbed on a solid support cannot be assessed in aqueous media, because one fraction of the protein might leach off the support.

Consequently, the reaction kinetics will reflect the contribution of both the immobilised and solubilised fractions, providing ambiguous information. Fig. 6 (diagram on the left) shows the tributyrin activity present in the supernatant in a classical hydrolytic assay for lipase enzymes. This is the result of the partition of the lipase (lipase B from *Candida antarctica*) adsorbed on a methacrylic carrier (Novozym 435[®]) into the assay medium (aqueous buffer with an emulsifier). In the case of the covalently immobilised lipase (diagram on the right), the supernatant displays negligible activity (different scale dimensions must be considered). Data demonstrate that the comparison of the hydrolytic activity of the two immobilised biocatalysts is not feasible on the basis of an assay carried out in an aqueous environment, since the solubilised free enzyme will strongly affect the observed kinetics.⁴⁰

Indeed, when the detachment of the protein from the carrier is a relevant issue for the final application the evaluation of the enzyme leached off the support under operational conditions is advisable.

This is particularly important for application of immobilised biocatalysts in food industry, where regulatory issues are particularly strict on the use of enzymes. As a matter of fact, the European Food Safety Authority (EFSA) clearly states that “information should be provided on the fate of the food enzyme during food processing and its behavior in the food matrix”. More precisely “in the case of immobilised food enzymes, information on the immobilisation procedure is required, *e.g.* enzyme support materials and immobilisation agents. Information on potential leakage of carriers, immobilisation agents and active enzymes into the food should be provided”.⁴¹

To overcome the problem of protein leaching, methods have also been developed that envisage cross-linking between the enzyme particles adsorbed on the support⁴² (Fig. 7), or the formation of a coating of silicone intended to hold the enzyme on the solid surface.⁴³

It must be underlined that the application of methods for covalent immobilisation of enzymes does not automatically assure that all protein molecules are tightly bound on the carrier. It is a rather common practice to load very high amount of protein on a carrier to achieve higher biocatalytic activity.

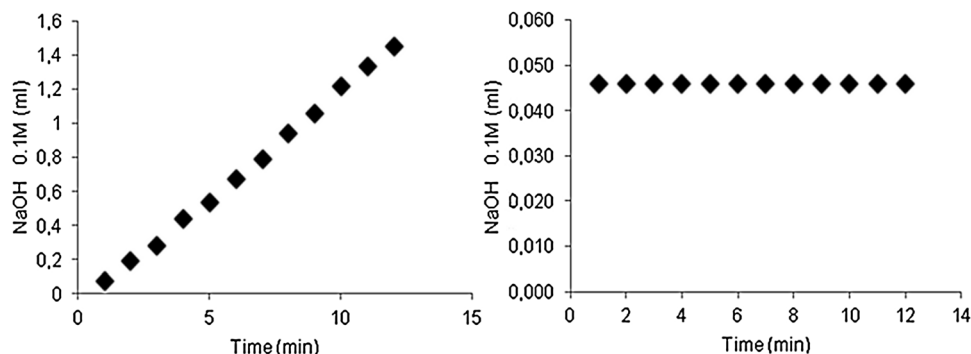


Fig. 6 Titration of the residual enzymatic activity present in the tributyrin emulsion after incubation of the immobilised enzymatic preparations for 15 min at 30 °C under stirring employing always the same units of immobilised enzyme. Adsorbed preparation on the left, covalent preparation on the right.⁴⁰

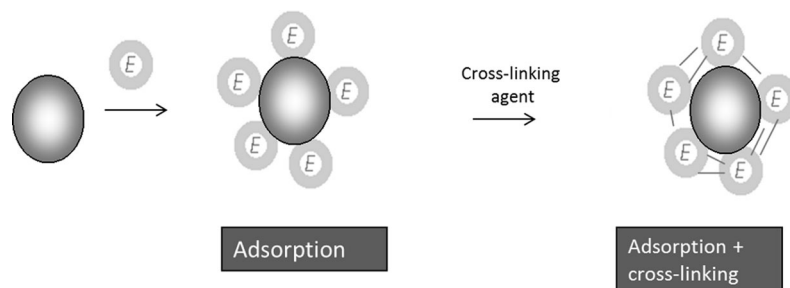


Fig. 7 Schematization of the immobilisation of enzymes on a solid support by the establishment of weak interactions (adsorption). The subsequent formation of cross-links is a strategy for preventing the detachment of the protein molecules from the carrier.

However the protein to be loaded on the support must not exceed the capacity of the functional groups to form covalent bonds.³⁹ Although in some cases the carrier producers state the concentration of functional groups on the support (generally in the range of 0.025 to 4.5 mmol per gram of dry polymer), it is quite difficult to determine *a priori* the optimum amount of protein to be loaded, since enzymes differ in terms of number of lysine residues on the surface and also molecular size. Washing steps after covalent immobilisation are advisable but they do not assure the complete removal of those protein molecules loaded on the carrier *via* simple adsorption,⁴⁴ so that the non-covalently bound enzyme molecules can leach off the support once applied in the biocatalytic process. Loading an excess of enzyme may cause not only a waste of biocatalyst³⁹ but also induce a decrease of the observed specific activity due to the crowding of enzyme molecules on the carrier surface and consequent diffusion limitations.

5.2 Diffusion and partition of solutes

Biocatalytic reactions that make use of immobilised enzymes are multiphase systems, where the direct investigation of partition, diffusion and solvation phenomena is quite complex.

The immobilisation of the biocatalyst introduces relevant variations in the mobility and partition of solutes and most often this translates into diffusion limitations.

As discussed in Section 4, the efficient immobilisation was one of the keys to the successful adoption of the enzymatic cleavage of penicillin G. Sepharose Gs (DSM Anti-infectives) consist of PGA immobilisation within a loose network of this highly hydrophilic polysaccharide that allows an efficient solvation of the enzyme and diffusion of reactants/products.³³ The biocatalyst is industrially applied to the enzymatic cleavage of penicillin G to 6-APA.

It has also been demonstrated that the reverse reaction, the PGA-catalysed synthesis of semi-synthetic β -lactam antibiotics, is adversely affected by diffusion limitations that occur after enzyme immobilisation.³³ Once the amide is formed and released from the active site of the enzyme, ideally the product (*e.g.* cephalixin or amoxicillin) should promptly diffuse out of the immobilisation carrier into the bulk medium (aqueous buffer). Accumulation of the product inside the carrier pores will favor the hydrolytic reaction, leading to poor yields and

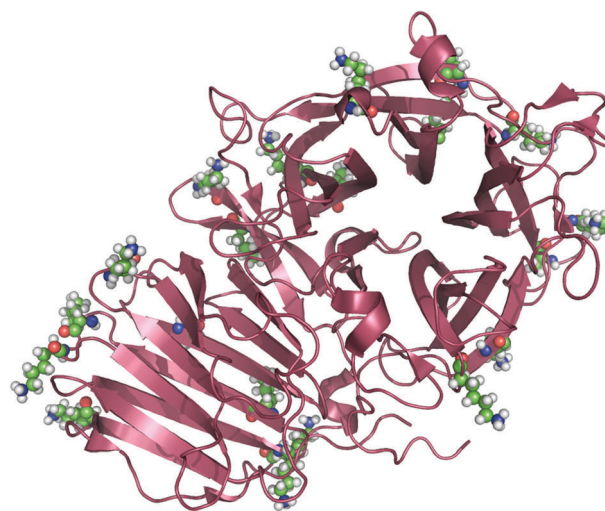


Fig. 8 3D structure of exoinulinase from *Bacillus stearothermophilus* obtained by homology modeling. The opening of the active site is evident on the upper right side, whereas superficial lysine residues are highlighted in the space-filling modality.

undesired side-products. It was claimed that the penicillin G acylase from *E. coli* performed much better in the synthesis of β -lactam antibiotics when immobilised in gelatin-chitosan than with other supports.²⁵ An industrial process is currently in use that is based on high concentration of reactants (0.2–0.8 M concentration in water) and an excess of an activated side-chain donor.⁴⁵

Not always immobilisation has a negative impact on diffusion of solutes. Fig. 8 shows the structure of exoinulinase from *Bacillus stearothermophilus* (EC 3.2.1.80).⁴⁶ Superficial features of the proteins were studied to reveal lysine residues potentially involved in the covalent anchorage of the enzyme to carriers and to acquire information useful for rational planning of an immobilisation protocol. Twenty six lysine residues were identified on the surface of exoinulinase, all positioned far from the opening of the active site and mostly on the backside of the protein. These structural features make the enzyme optimal for covalent immobilisation to a functionalized polymeric carrier since the anchorage of the protein on the support is expected to occur without occluding the access to the active site (Fig. 9).

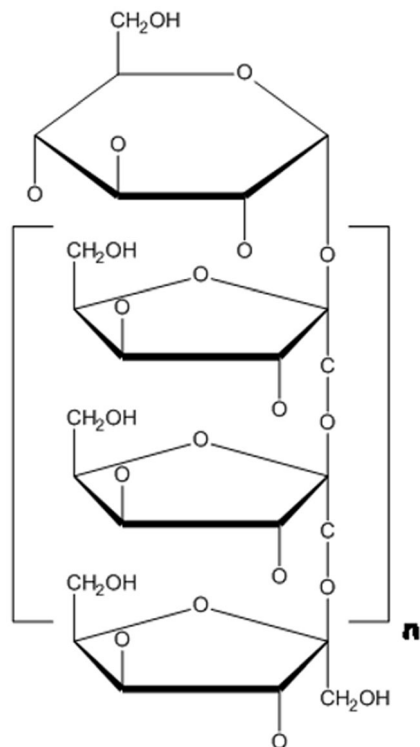


Fig. 9 Schematic representation of inulin, a polydisperse fructan with the degree of polymerization that typically ranges from 2 to 60. The fructosyl units in inulin are linked by β (2 \rightarrow 1) linkages and the resulting polymer chain terminates with a non-reducing glucose unit. Exoinulinase is a fructofuranosyl hydrolase (EC 3.2.1.80), which hydrolyses the terminal fructose from the inulin chain at the reducing end.

Experimentally it was evidenced that the exoinulinase covalently immobilised on acrylic amino resins led to an observed immobilisation yield >100%. This might be ascribed to a higher accessibility of the active site of the enzyme when employed in the immobilised form.

When the immobilised inulinase was marked with fluorescein and analyzed by fluorescent microscopy it was evident that the protein was present exclusively on the first external layer of the resin beads, where the bulky inulin can diffuse freely. Although no direct experimental evidence can be provided, the correct orientation of the enzyme upon binding on the support has also been suggested as a possible factor affecting positively the efficiency of the biocatalyst. The applicability of the immobilised inulinase was also demonstrated in batch and fluidized reactors.⁴⁷

Among biocatalysed reactions, processes catalysed by lipases involve complex partition phenomena that deserve special attention. Hydrolysis of oils and fats are carried out in the presence of an aqueous phase but on the hydrophobic and insoluble substrate. Table 2 shows how the substrate used in the activity determines wide variations in observed activity among three immobilised preparations of the same lipase enzyme, where the polymeric carriers differ in their hydrophobic nature (acrylic/styrenic-amino > methacrylic-epoxy > methacrylic-amino).⁴⁸

Table 2 Hydrolytic activities of three different immobilised preparation of lipase B from *Candida antarctica* determined using two different assays. The same amount of enzymatic units was loaded on each carrier

Immobilisation method	Carrier	Hydrolytic activity (U/g _{dry})	
		<i>p</i> -Nitrophenyl-palmitate	Tributyrin
Covalent	Methacrylic-epoxy	92	308
Covalent	Methacrylic-amino	25	40
Covalent	Acrylic/styrenic-amino	68	631

It appears clear that the efficiency of any immobilised enzyme should be evaluated using the specific reaction of interest as a reference activity assay, whereas the transfer of conclusions from one system to another can lead to misleading interpretation.

A further example is illustrated in Fig. 10 that reports the effect of different immobilisation protocols on the hydrolytic and synthetic activities of lipase from *Pseudomonas cepacia* covalently immobilised on a styrene-divinylbenzene resin functionalized with epoxy groups. The graphics show the effect of different experimental variables (*e.g.* loaded enzyme and temperature) on the observed hydrolytic activity (tributyrin hydrolysis) and synthetic activity (transesterification between 2-phenyl-2-ethanol and vinylacetate) (Fig. 11).

It is evident that the experimental variables exert opposite effects on the two observed activities, thus confirming that each biocatalytic application deserves an *ad hoc* method of immobilisation.⁴⁸

5.3 Partition of water

The chemical nature of the immobilisation carrier and partition phenomena can play a major role in the case of reactions carried out in low water media. It is largely recognized that water activity, namely the distribution of free water among the phases of a reaction system, greatly affects the enzymatic activity as well as the thermodynamics of the reaction.⁴⁹ Controlling the water activity becomes an important issue especially when reactions must be carried out using enzymes that retain their activity only when they are sufficiently hydrated. These enzymes, differently from lipases, lose most of their activity in nearly anhydrous organic solvent. In such cases, a straightforward solution can be the immobilisation of enzymes on highly porous supports having a high water retention capacity. The porosity of these carriers make possible the creation of a microenvironment that assures a sufficient hydration to the enzymes.⁵⁰ Although there are several methods for controlling the water activity⁵¹ their application to reaction systems catalysed by immobilised enzymes can be difficult. This is particularly true for biocatalysts bound on porous carriers. Water entrapped in the pores has a distinct behavior as compared to bulk water and it is by far less mobile. If we consider that even styrenic porous resins can contain 60–65% of water (w/w) it appears clear that the pre-equilibration of the biocatalyst immobilised on any porous support will take exceedingly long time if carried out in a vapor phase

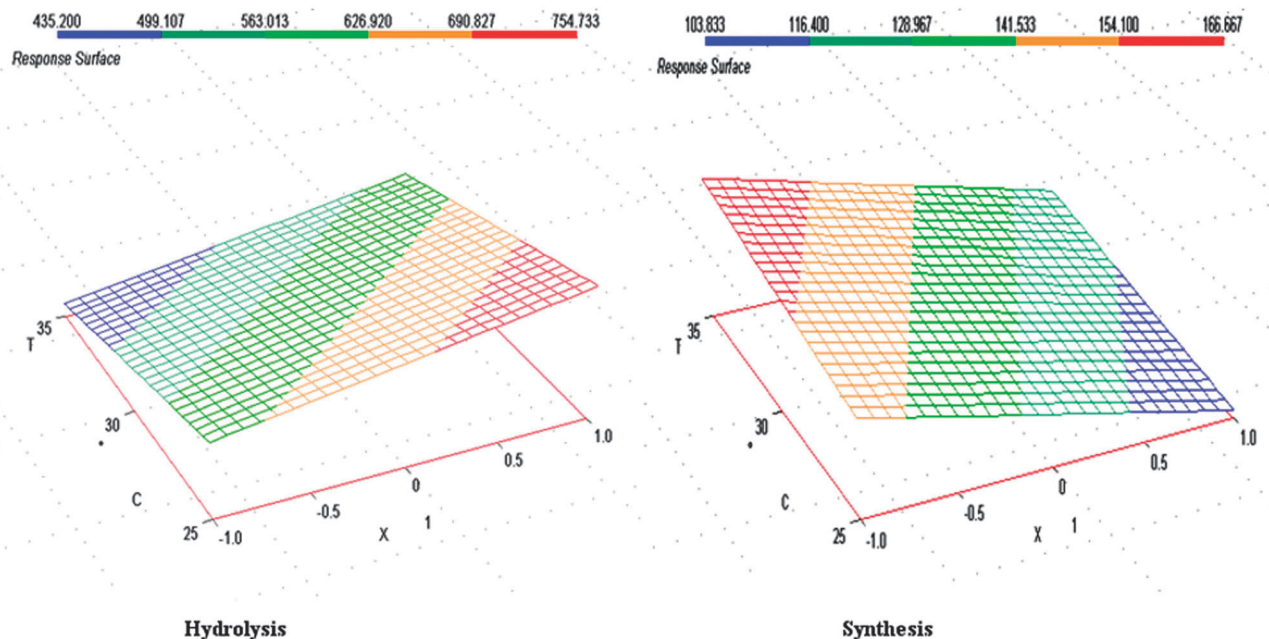


Fig. 10 Effect of experimental variables on the covalent immobilisation of lipase from *Pseudomonas cepacia* on a styrene–divinylbenzene resin functionalized with epoxy groups. Hydrolytic activity was assayed with the hydrolysis of tributyrin in aqueous media whereas the synthetic activity was measured by following the kinetics of the transesterification between 2-phenyl-2-ethanol and vinylacetate. X axis = enzyme loading; Y axis = temperature.

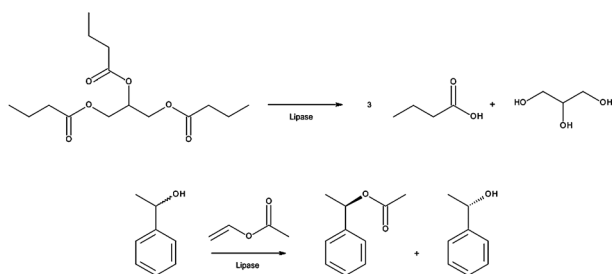


Fig. 11 Activity assays employed for the characterization of the efficiency lipase from *Pseudomonas cepacia* after immobilisation: tributyrin hydrolysis (on the top) and transesterification between 2-phenyl-2-ethanol and vinylacetate (synthetic activity).

(for instance a chamber containing hydrated salts is able to control the water activity of the closed system).⁵¹ More importantly, water molecules adsorbed on the external surface of the porous resins will change their distribution once the carrier is placed in hydrophobic media because the presence of the organic solvent (or any other hydrophobic phase) will promote the fast penetration of water into the pores.¹⁵ Here water will have restricted mobility and minimal tendency to partition into the bulk medium surrounding the resin. Therefore, the pre-equilibration of a porous carrier at a fixed water activity value might have no utility because the multi-phase system will reach the equilibrium only after the mixing of all the components. Moreover, when working in media of low water content (such as those based on organic solvents or ionic liquids) it is not sufficient to state the amount of added water to allow the reproducibility of experiments.⁵²

6. The importance of correct reporting of immobilisation protocols

The starting point for the development and optimisation of any immobilisation protocol is the rigorous reporting of essential experimental data, trying to account and control most of the experimental variables that might affect the final outcome. Recently, the scientific committee of the European Federation of Biotechnology Section on Applied Biocatalysis (ESAB) prepared and published guidelines for the correct reporting of experiments in biocatalysis.⁵²

Fig. 12 illustrates the fundamental information to be reported in order to assure reproducibility of protocols and actual comparison of different formulations of immobilised enzymes.

As a starting point, the enzymatic native preparation must be fully described taking into account also batch variability (see also <http://www.brenda-enzymes.info>: a comprehensive enzyme information system with structural and functional data). When relevant to the study, reference to information about glycosylation and other post-translational modifications of the enzyme used is advisable. For detailed guidelines when using immobilised biocatalysts, previously published recommendations should also be followed.⁵³

Enzymatic preparations of native lipases available on the market are not homogeneous in their components and protein content. As the general industrial aim is to minimize the enzyme purification and operate the process with the crudest form of catalyst possible (usually a lysate), many commercial enzymatic preparations contain a quite low percentage of

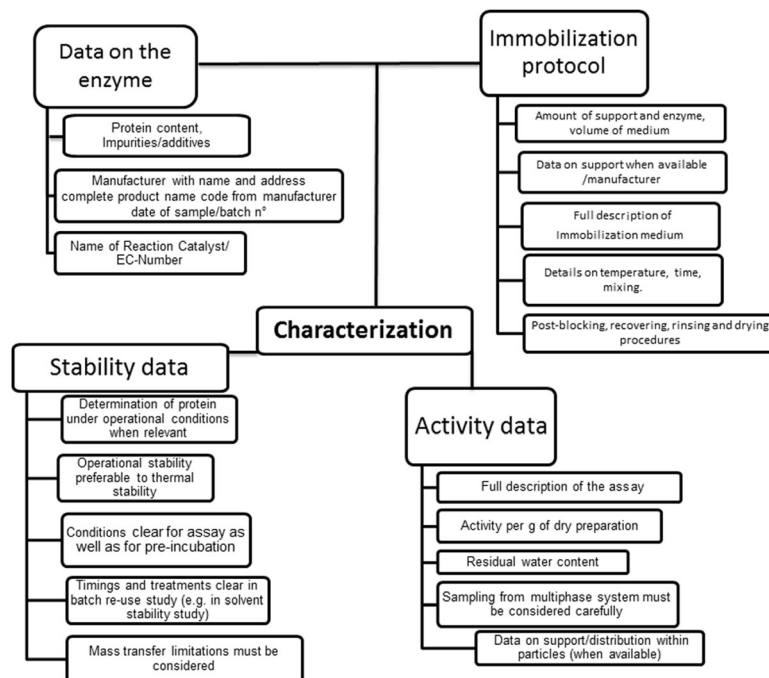


Fig. 12 Data to be reported for the description of immobilisation protocols and for the characterization of immobilised biocatalysts.

protein (sometimes <1%). In most cases, their production is directed to applications different than biotransformations, such as detergent formulations, so that a considerable amount of stabilizing agents, mostly polyols and salts, are present. Any additives included deliberately as part of the biocatalyst preparation should be specified fully. The interference of extra components might make difficult to apply structural and functional knowledge concerning the pure proteins to the rational planning of immobilisation protocols. Ultimately, the transfer of the immobilisation protocols might become impracticable between different commercial preparations of the same enzymatic protein.

Information on the protocol should include the amounts of support and enzyme employed for immobilisation and an estimation of the amount immobilised, usually calculated as the difference between amounts added and that remaining un-immobilised. This information is not to be considered as the immobilisation yield but rather as an indicator of the loading capacity of the carrier under a defined set of experimental conditions.¹⁴

Immobilised yield is calculated as the ratio between the enzymatic units expressed by the immobilised enzymes vs. the enzymatic units loaded on the carrier.

Counter-ions present in buffers must be specified, keeping in mind that calculation of the ionic strength of the buffer must be possible. The pH should normally be specified along with the temperature. An approximate range is preferable to room temperature.

If the polymer must be pre-activated by means of a bifunctional agent (e.g. glutaraldehyde) the composition of the glutaraldehyde solution must be fully described (concentration, buffer, pH)

as well as the time required for the pre-activation and any washing and/or dehydration steps.

Similarly, post-blocking procedures aiming at “capping” unreacted functional groups of the carrier must be specified.

When the immobilised enzyme must be applied in an anhydrous environment, procedures for the removal of the residual water present in the carrier should be described. The specification of the final residual water content (generally determined by measuring the weight loss upon heating the sample in an oven) is crucial for any immobilised preparation, because it allows determining the specific activity of the immobilised biocatalyst referring to its dry mass.

Reaction mixtures with immobilised enzymes are multi-phase systems that need to be agitated continuously and the method used can affect the observed reaction progress because agitation can affect mass transfer rates when they are limiting. Hence, it is desirable to describe the agitation conditions as clearly as possible. It must be kept in mind that some mixing methods can damage or grind the biocatalyst to fine particles,³⁹ leading to reaction kinetics that does not actually reflect the efficiency of the biocatalyst in its intact form.

Also the shape and size of the vessel greatly affect reaction kinetics and the shape and size of the vessel must be specified as well as the fraction filled with liquid. Furthermore, the total volume removed for analysis must account only for a small fraction of the total volume of this liquid phase, or the behavior of the reaction mixture as a whole will be perturbed by sample removal. Whichever analytical approach is adopted, the multi-phase reaction mixtures require careful description.

Stability studies on immobilised biocatalysts might also lead to misleading results, as activity is measured under conditions of

strong mass transfer limitation. The intrinsic activity of the catalyst can decrease significantly with little change in measured activity, because this is still largely limited by the same mass transfer rate.⁵⁴

Stability studies under operational conditions are more informative than general thermal stability data in aqueous media. Loss of activity upon recycling is also a very important parameter for determining the efficiency of the immobilised preparation. Whatever assay is used (continuous or batch system), any treatment (e.g. rinsing) of the biocatalyst after recovery can play a crucial role, therefore, these procedures should be specified. Presentation of results must clearly distinguish the total time spent under reaction conditions from elapsed time, particularly when the cycle includes an extended storage time between successive batch reactions. Consequently, the characterisation of any enzymatic preparation in terms of stability and activity should be preferably studied under the final operational conditions.⁵⁵

7. Conclusions

The considerable amount of scientific data published since the 1970s on enzyme immobilisation provides a wide array of technological options for developing optimal immobilised biocatalysts. However, the variables affecting the performance of immobilised biocatalysts are so many and so difficult to be measured directly that rational planning of the immobilisation protocol remains a complicated task. Most importantly, results obtained with one biocatalyst or reaction can be hardly transferred to different work cases. Planning any assay for determining the efficiency of an immobilisation strategy should take into account that performance of an immobilised biocatalyst can change from one reaction to another.

This vast variability in immobilisation studies can be faced and overcome thanks to new research approaches based on the analysis of structure–function relationships of enzymes along with statistical methodologies.^{46,56} A more rational approach towards the optimisation of immobilisation protocols is feasible through the understanding of the properties and requirements of biocatalysts but must be accompanied by adequate development of materials applicable as immobilisation carriers, which combine technological performance with renewability and economic sustainability. The parallel progress of these two research fields will enable the full exploitation of the catalytic potential of enzymes also in industry.⁵⁷

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References

- 1 R. A. Sheldon, *Adv. Synth. Catal.*, 2007, **349**, 1289–1307.
- 2 M. W. Christensen, L. Andersen, T. L. Husum and O. Kirk, *Eur. J. Lipid Sci. Technol.*, 2003, **105**, 318–321.
- 3 F. Hasan, A. A. Shan and A. Hameed, *Enzyme Microb. Technol.*, 2006, **39**, 235–251.
- 4 O. Kirk, T. V. Borchert and C. C. Fuglsang, *Curr. Opin. Biotechnol.*, 2002, **13**, 345–351.
- 5 S. M. Thomas, R. DiCosimo and V. Nagarajan, *Trends Biotechnol.*, 2002, **20**, 238–242.
- 6 W. Tischer and V. Kascher, *Trends Biotechnol.*, 1999, **17**, 326–335.
- 7 I. Petry, A. Ganesan, A. Pitt, B. D. Moore and P. J. Halling, *Biotechnol. Bioeng.*, 2006, **95**, 984–991.
- 8 A. C. Pierre, *Biocatal. Biotransform.*, 2004, **22**, 145–170.
- 9 L. Betancor and H. R. Luckarift, *Trends Biotechnol.*, 2008, **26**, 566–572.
- 10 P. Nikolova and O. P. Ward, *J. Ind. Microbiol.*, 1993, **12**, 76–86.
- 11 M. Fidaleo, S. Charaniya, C. Solheid, U. Diel, M. Laudon, H. Ge, L. E. Scriven and M. C. Flickinger, *Biotechnol. Bioeng.*, 2006, **95**, 446–458.
- 12 R. A. Sheldon, *Appl. Microbiol. Biotechnol.*, 2011, **92**, 467–477.
- 13 L. Cao, *Carrier-bound Immobilised Enzymes: Principle, Application and Design*, Wiley-VCH, Weinheim, 2005.
- 14 U. Hanefeld, L. Gardossi and E. Magner, *Chem. Soc. Rev.*, 2009, **38**, 453–468.
- 15 A. Basso, L. DeMartin, C. Ebert, L. Gardossi and P. Linda, *Chem. Commun.*, 2000, 467–468.
- 16 A. A. Khan and M. A. Alzohairy, Recent Advances and Applications of Immobilized Enzyme Technologies: A Review, *Res. J. Biol. Sci.*, 2010, **5**, 565–575.
- 17 M. M. M. Elnashar, The Art of Immobilization Using Biopolymers, Biomaterials and Nanobiotechnology, in *Biotechnology of Biopolymers*, ed. M. M. M. Elnashar, InTech, 2011.
- 18 A. Basso, P. Braiuca, S. Cantone, C. Ebert, P. Linda, P. Spizzo, P. Caimi, U. Hanefeld, G. Degrossi and L. Gardossi, *Adv. Synth. Catal.*, 2007, **349**, 877–886.
- 19 V. Ferrario, C. Ebert, L. Knapic, D. Fattor, A. Basso, P. Spizzo and L. Gardossi, *Adv. Synth. Catal.*, 2011, **353**, 2466–2480.
- 20 B. M. Brena and F. Batista-Viera, Immobilization of Enzymes, in *Immobilization of Enzymes and Cells*, ed. J. M. Guisan, Humana Press Inc., Totowa, New Jersey, 2nd edn, 2006, pp. 15–29.
- 21 M. Petkar, A. Lali, P. Caimi and M. Daminati, *J. Mol. Catal. B: Enzym.*, 2006, **39**, 83–90.
- 22 O. Kirk and M. W. Christensen, *Org. Process Res. Dev.*, 2002, **6**, 446–451.
- 23 Y. Mei, L. Miller, W. Gao and R. A. Gross, *Biomacromolecules*, 2003, **4**, 70–74.
- 24 A. Salis, E. Sanjust, V. Solinas and M. Monduzzi, *J. Mol. Catal. B: Enzym.*, 2003, **24–25**, 75–82.
- 25 C. Mateo, R. Torres, G. Fernandez-Lorente, C. Ortiz, M. Fuentes, A. Hidalgo, F. Lopez-Gallego, O. Abian, J. M. Palomo, L. Betancor, B. C. C. Pessela, J. M. Guisan and R. Fernandez-Lafuente, *Biomacromolecules*, 2003, **4**(3), 772–777.
- 26 T. Tosa, T. Mori, N. Fuse and I. Chibata, *Enzymologia*, 1966, **31**, 214–224.

- 27 L. Goldstein, *Biochemistry*, 1972, **11**, 4072–4084.
- 28 A. Liese, K. Seelbach and C. Wandrey, *Industrial Biotransformations*, Wiley-VCH, Weinheim, 2nd edn, 2006.
- 29 S. Karboune, A. Archelas, R. Furstoss and J. Barratti, *J. Mol. Catal. B: Enzym.*, 2005, **32**, 175–184.
- 30 J. Porath, *Protein Expression Purif.*, 1992, **3**, 263–281.
- 31 K. E. Cassimjee, M. Trummer, C. Branneby and P. Berglund, *Biotechnol. Bioeng.*, 2008, **99**(3), 712–716.
- 32 E. Katchalski-Katzir and D. M. Kraemer, *J. Mol. Catal. B: Enzym.*, 2000, **10**, 157–176.
- 33 A. I. Kallenberg, F. van Rantwijk and R. A. Sheldon, *Adv. Synth. Catal.*, 2005, **347**, 905–926.
- 34 I. Takata, T. Tosa and I. Chibata, *J. Solid-Phase Biochem.*, 1978, **2**, 225.
- 35 R. Fernandez-Lafuente, C. M. Rosell, G. Alvaro and J. M. Guisan, *Enzyme Microb. Technol.*, 1992, **14**, 489–495.
- 36 L. C. Katwa and M. R. Raghavendra Rao, *Biotechnol. Lett.*, 1983, **3**, 191–196.
- 37 M. T. Martin, M. Alcalde, F. J. Plou and A. Ballesteros, *Indian J. Biochem. Biophys.*, 2002, **39**(4), 229–234.
- 38 P. Gemeiner, V. Stefuca and V. Bales, *Enzyme Microb. Technol.*, 1993, **15**, 551.
- 39 L. Hilterhaus, B. Minow, J. Müller, M. Berheide, H. Quitmann, M. Katzer, O. Thum, G. Antranikian, A. P. Zeng and A. Liese, *Bioprocess Biosyst. Eng.*, 2008, **31**, 163–171.
- 40 L. Sinigoi, PhD thesis, Università degli Studi di Trieste, 2011, (<http://hdl.handle.net/10077/4568>).
- 41 European Food Safety Authority; Technical Report of EFSA: Explanatory Note on the Guidance of the Scientific Panel of Food Contact Material, Enzymes, Flavours and Processing Aids (CEF) on the Submission of a Dossier on Food Enzymes. Supporting Publication 2011, **177**, 1–18. Available online: www.efsa.europa.eu.
- 42 I. Mazeaud, P. Poulsen and M. Christensen, *PTC Int. Pat.*, W036235 A1, 2007.
- 43 L. O. Wiemann, P. Weisshaupt, R. Nieguth, O. Thum and M. B. Ansorge-Schumacher, *Org. Process Res. Dev.*, 2009, **13**, 617–620.
- 44 I. Petry, A. Ganesan, A. Pitt, B. D. Moore and P. J. Halling, *Biotechnol. Bioeng.*, 2006, **95**(5), 984–991.
- 45 E. de Vroom, *PCT Int. Pat.*, WO9704086, 1997.
- 46 A. Basso, P. Spizzo, V. Ferrario, L. Knapic, N. Savko, P. Braiuca, C. Ebert, E. Ricca, V. Calabrò and L. Gardossi, *Biotechnol. Prog.*, 2010, **26**, 397–405.
- 47 E. Ricca, V. Calabrò, S. Curcio, G. Iorio, L. Gardossi and A. Basso, *Int. J. Mol. Sci.*, 2010, **11**, 1180–1189.
- 48 D. Fattor, PhD thesis, Università degli Studi di Trieste, 2012, (<http://hdl.handle.net/10077/7778>).
- 49 P. J. Halling, *Enzyme Microb. Technol.*, 1994, **16**, 178–206.
- 50 A. Basso, L. De Martin, C. Ebert, L. Gardossi, P. Linda and V. Zlatev, *J. Mol. Catal. B: Enzym.*, 2001, **11**, 851–855.
- 51 G. Bell, P. J. Halling, L. May, B. D. Moore, D. A. Robb and R. Ulijn, Methods for Measurement and Control of Water in Non-aqueous Biocatalysis, in *Methods in Biotechnology: Enzymes in Nonaqueous Solvents*, ed. E. N. Vulfson, P. J. Halling and H. L. Holland, Humana Press Inc., Totowa, New Jersey, 2001, pp. 105–126.
- 52 L. Gardossi, P. B. Poulsen, A. Ballesteros, K. Hult, V. K. Švedas, D. Vasić-Rački, G. Carrea, A. Magnusson, A. Schmid, R. Wohlgemuth and P. J. Halling, *Trends Biotechnol.*, 2010, **28**, 171–180.
- 53 The EFB Working Party on Immobilised Biocatalysts, *Enzyme Microb. Technol.*, 1983, **5**, 304–307.
- 54 J. L. van Roon, M. M. Arntz, A. I. Kallenberg, M. A. Paasman, J. Tramper, C. G. Schroën and H. H. Beftink, *Appl. Microbiol. Biotechnol.*, 2006, **72**, 263–278.
- 55 A. S. Bommarius and B. Riebel, *Biocatalysis*, Wiley-VCH, Weinheim, 2004.
- 56 P. Braiuca, C. Ebert, A. Basso, P. Linda and L. Gardossi, *Trends Biotechnol.*, 2006, **24**, 419–425.
- 57 S.-T. Yang, *Bioprocessing for Value-Added Products from Renewable Resources: New Technologies and Applications*, Elsevier, Amsterdam, 2011.