Immobilized enzymes: crystals or carriers?

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The advantages of immobilized over soluble enzymes arise from their enhanced stability and ease of separation from the reaction media, leading to significant savings in enzyme consumption. Immobilization methods range from binding to prefabricated carrier materials to packaging in enzyme crystals or powders. During their use, mass-transfer effects can produce substrate or pH gradients, which reduce the reaction rates and product yields. The costs of immobilized enzymes must be minimized in order to increase their competitiveness for technical applications.

Enzymes are normally tightly packed in cellular organelles or in enzyme cascades, such as the fatty-acid-synthetase complex, thus enabling catalytic processes to take place precisely when and where they are needed. Artificial applications of such compartmentation or packing go back to the 1950s, when immobilized enzymes (enzymes with restricted mobility) were first prepared intentionally[1] by inclusion in polymeric matrices or binding onto carrier materials. Considerable effort was also put into the cross-linking of enzymes, by either protein cross-linking or the addition of inert materials[2]. Since then, numerous methods of immobilization on different materials have been developed. Binding to prefabricated carrier materials appears to have been preferred but, recently, cross-linking enzyme crystals has been reported to be an interesting alternative[3].

Immobilized enzymes are currently the subject of considerable interest because of their advantages over soluble enzymes or alternative technologies, and the steadily increasing number of applications for immobilized enzymes[4]. However, experimental investigations have produced unexpected results, such as a significant reduction or even an increase in enzyme activity compared with soluble enzymes. For example, cross-linked crystals of subtilisin hydrolysed an amino acid ester with a 27-times lower activity than the soluble enzyme[5]. However, the use of lipoprotein lipase in the solvent-mediated synthesis of esters gave a 40-fold increase in activity using immobilized enzymes or enzyme preparations with grafted polymers compared with enzyme powders[6].

The reasons for these observations will be discussed in this article, together with immobilization methods that would enable us to circumvent such peculiarities and achieve the desired benefits. Emphasis is placed on porous diffusion of substrates, reaction-generated protons and the dispersion of enzymes in solvents.

Desired benefits and characteristics

There are several reasons to use immobilized enzymes. In addition to the convenient handling of enzyme preparations, the two main targeted benefits are: (1) easy separation of enzyme from the product; and (2) reuse of the enzyme. Easy separation of the enzyme from the product simplifies enzyme applications and permits reliable and efficient reaction technology. Enzyme reuse provides a number of cost advantages, which are often an essential prerequisite for establishing an economically viable enzyme-catalysed process.

The properties of immobilized enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties (Fig. 1). Of the numerous parameters that have to be taken into account[7–9], the most important are outlined in Box 1.

As far as manufacturing costs are concerned, the yield of immobilized enzyme activity is determined by the immobilization method in relation to the amount of soluble enzyme used. Under process conditions, the resulting activity can be further reduced by mass-transfer effects. That is, the yield of enzyme activity following immobilization does not only depend on losses caused by the binding procedure but can be further reduced as a result of the diminished availability of enzyme molecules within pores or by slowly diffusing substrate molecules. Such limitations lead to lowered efficiency. However, improved stability under working conditions can compensate for such drawbacks, resulting in an overall benefit. Altogether, these interactions are a measure of productivity or enzyme consumption, expressed as, for example, enzyme units per kilogram of product. If we replace 'enzyme units' with 'enzyme costs' we obtain the essential product-related costs.

In order to estimate the cost advantages of immobilized enzymes, it is necessary to look at the individual manufacturing steps and their contribution to the overall costs. These include, first, the cost of biomass from plant or animal sources, or from microbial fermentations. In the latter case, the costs are determined mainly by the fermentation scale and the expression rate of the enzymes. Downstream processing is needed to achieve the required purity but is accompanied by loss in activity. The use of larger fermentation scales might be necessary in order to compensate for loss of activity and also for some increase in cost (Fig. 2). The costs of the immobilization procedure further increase the manufacturing costs. Thus, leaving aside the potential advantage of easier removal of the enzyme from the product, immobilized enzymes so far provide no cost benefit.

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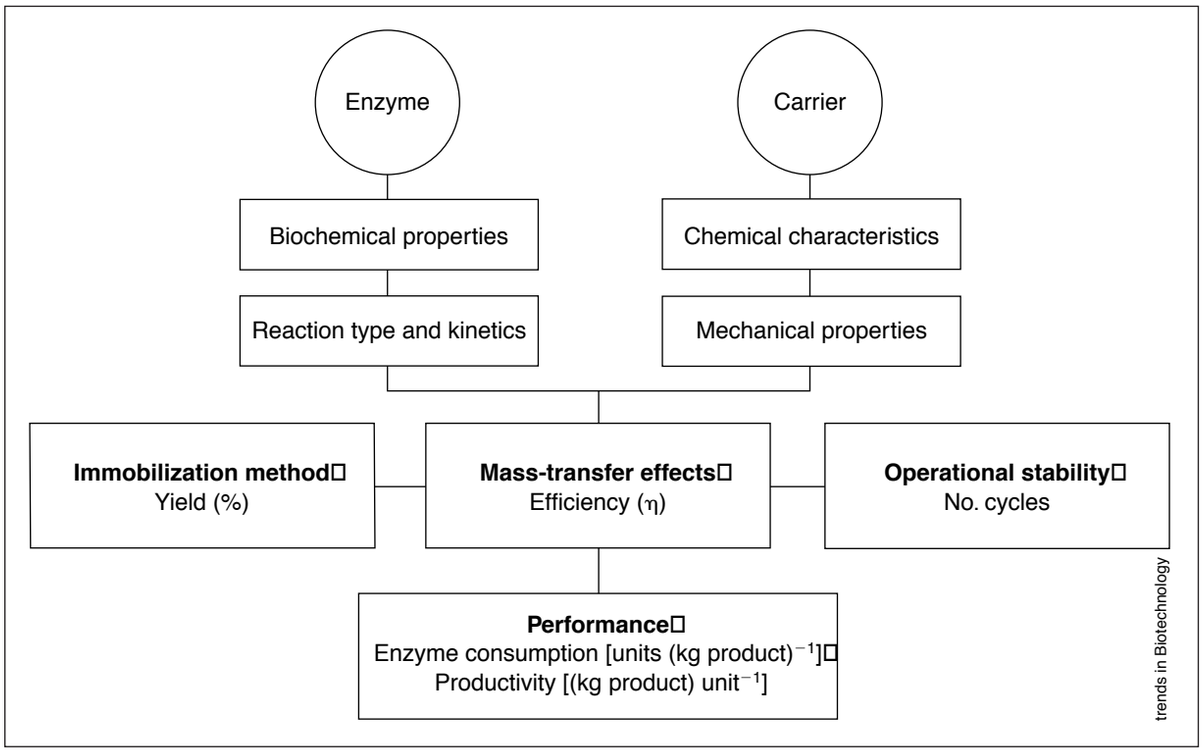

Figure 1

The properties of immobilized enzymes are governed by the interactions of the properties of the enzyme and the carrier material. Measurements are made of the yield of enzyme activity or bound enzyme protein as a result of the immobilization method, its efficiency as a result of physicochemical interactions (mass-transfer effects) and its operational stability (in stirred tanks, as number of cycles). These provide performance data, which can be expressed as productivity (product produced per unit enzyme) or enzyme consumption (enzyme consumed per unit product produced) until, for example, the residual enzyme activity has halved.

However, cost savings will be achieved by the repeated reuse of the immobilized enzyme. Prolonged use also means downscaling of the unit operation and thus increased costs for manufacture of the enzyme itself, which must also be taken into account. In conclusion, only repeated use will lead to dramatic cost reductions, and this can be easily tracked by monitoring the amount of enzyme required per kilogram of product formed. The costs of the enzyme must not be higher than a few percent of the production costs for

Box 1. Selected characteristic parameters of immobilized enzymes

Enzyme	**Biochemical properties** Molecular mass, prosthetic groups, functional groups on protein surface, purity (inactivating or protective function of impurities) **Kinetic parameters** Specific activity, pH and temperature profiles, kinetic parameters for activity and inhibition, stability against pH and temperature, solvents, contaminants, impurities
Carrier	**Chemical characteristics** Chemical basis and composition, functional groups, swelling behaviour, accessible volume of matrix, pore size, chemical stability of carrier **Mechanical properties** Particle diameter, single-particle compression behaviour, flow resistance (for fixed-bed applications), sedimentation velocity (for fluidized beds), abrasion (for stirred tanks)
Immobilized enzyme	**Immobilization method** Bound protein, yield of active enzyme, intrinsic kinetic parameters (i.e. properties free of mass-transfer effects) **Mass-transfer effects** Partitioning (different concentrations of solutes inside and outside the catalyst particles), external and internal (porous) diffusion; this gives the effectiveness in relation to free enzyme determined under appropriate reaction conditions **Stability** Operational stability (expressed as activity decay under working conditions), storage stability **'Performance'** Productivity (amount of formed product per unit activity or mass of enzyme) Enzyme consumption (e.g. units per kg product)

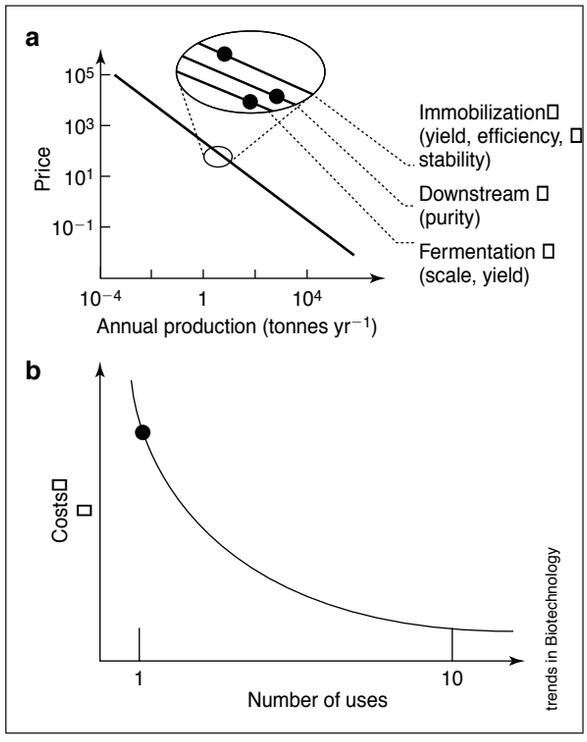

Figure 2

Manufacturing costs for immobilized enzymes (price per gram enzyme) slow down as their annual production increases, roughly along a straight line when plotted on a logarithmic scale (**a**). Individual costs are a matter of scale and enzyme yields in fermentation, of downstream steps to achieve the required purity, and of performance achievements by immobilization. Each contributes additively to the manufacturing costs (the magnified lines). In general, immobilization increases the manufacturing costs of enzymes. Their overall benefit in terms of often-dramatic cost savings arises by repeated or prolonged use in their applications; here, the cost of synthesizing the desired product is given per unit product formed against the number of times the immobilzed enzyme is used (**b**).

the desired product. Productivities range from 600 (kg product) (kg immobilized enzyme)$^{-1}$ for fine chemicals [e.g. the production of 6-aminopenicillanic acid by the hydrolysis of penicillin G with penicillin-G amidase, according to the manufacturer's description (Roche Molecular Biochemicals)] to 11 000 (kg product) (kg immobilized enzyme)$^{-1}$ for food products (e.g. the production of fructose by isomerizing glucose with glucose isomerase)[10].

Box 2. Methods used to immobilize enzymes

Covalent binding
Cross-linking
Binding onto prefabricated carrier materials
Non-covalent binding
Enzyme crystallization
Adsorptive or ionic binding onto carrier materials
Dispersing enzymes (e.g. dried enzyme powders in organic solvents)
Inclusion
Inclusion into membrane device
Incorporation into polymeric networks
Phase separation

Immobilization methods

Immobilization methods are often classified by the type of chemical reaction used for binding[11,12] (Box 2). Enzyme cross-linking was investigated thoroughly during the pioneer phase of enzyme immobilization. However, because of the low mechanical and hydrodynamic stability of the proteinaceous materials obtained by this method, interest turned to other methods. Dedicated carrier materials with functional groups for covalent binding were made commercially available and these met the chemical and mechanical requirements for industrial processes, either in stirred tanks or plug-flow reactors.

The use of enzyme crystals is one example of a non-chemical binding method in which the crystallized protein is both the carrier and catalyst. Multiple non-covalent forces form and stabilize the crystals and make them more rigid than cross-linked amorphous protein assemblies. Additional chemical bonding via cross-linkers is also required to stabilize the crystals against dissolution in an aqueous environment[13].

Immobilization by non-covalent adsorption has been shown to be very useful in non-aqueous systems, in which desorption can be neglected owing to the low solubility of enzymes in these solvents (in aqueous systems, desorption cannot be neglected). This method is widely used for reactions of lipases in water-immiscible solvents[14,15]. The use of adsorbed enzymes[10] and proteins[16] is widespread in commercial applications because of its simplicity.

Immobilization is also achieved by the simple dispersion of a dried, water-soluble enzyme preparation in a water-immiscible organic solvent. Lipases, in particular, can be successfully immobilized by this means. In organic solvents, they catalyse otherwise hydrolytic reactions in the reverse direction, if the water content is carefully controlled[17].

Immobilization by inclusion involves the enzyme being retained within a membrane device such as a hollow fibre, polymeric network or microcapsule. In simple inclusion, there is no need for derivatization of the enzyme or contact with another surface and so none of the detrimental effects of binding forces have to be taken into account. However, there is no additional stabilization as a result of protein–carrier interactions. Incorporation into polymeric networks is most efficient when combined with additional binding methods by, for example, the attachment of vinyl groups to the enzyme surface and covalent integration into a growing polymer chain. Such chemically modified enzymes can be incorporated into 'biocatalytic plastics' and are active in aqueous and organic media[18].

As for the derived enzyme particles, there are essential differences between binding on carriers and cross-linking or crystallization. The latter procedures lead to particles of high enzyme density, usually with a fairly broad size distribution from which unwanted sizes have to be eliminated. However, binding onto prefabricated carrier materials enables binding onto materials with preselected properties, with comparatively low enzyme density but with preferential binding on the outer shell of the carrier material[19,20].

Mass-transfer effects

Enzyme immobilization means the deliberate restriction of the mobility of the enzyme, which can also

affect the mobility of the solutes. These phenomena, referred to as mass-transfer effects, can lead to a reduced reaction rate and consequently to decreased efficiency compared with soluble enzymes. A reduced reaction rate can result from external diffusional restrictions on the surface of carrier materials. Furthermore, partition effects can lead to different concentrations inside and outside the carriers and this has to be taken into account for solutes that might interact with carrier materials by ionic or adsorptive forces[21,22]. Additional effects are observed in porous particles because of internal or porous diffusion, as outlined below.

The rates of reactions catalysed by cross-linked enzyme crystals are occasionally reported to be free of diffusional limitations[3,23,24]. All reactions of immobilized enzymes must obey the laws of mass transfer and their interactions with enzyme catalysis. The question is, therefore, what are the reasons for the restrictions caused by mass transfer and can they be avoided with cross-linked enzyme crystals? The mathematical description of the diffusional limitations of enzyme kinetics in the combined action with mass transfer is well established[1]. The presentation of those interactions is very complex, however, especially when, in addition to the comparatively simple Michaelis–Menten kinetics, terms for product inhibition, proton generation or enzyme deactivation are also incorporated.

For Michaelis–Menten enzyme kinetics, the extent of mass-transfer control is usually expressed by the efficiency coefficient or effectiveness factor η (Eqn 1),

$$\eta = \frac{\nu_{imm}}{\nu_{free}} \quad (1)$$

where ν_{imm} and ν_{free} are the rates of the reaction catalysed by the same enzyme concentrations with immobilized and free enzyme under otherwise-identical conditions.

Numerical values of η can be calculated when substrate diffusion is considered in Michaelis–Menten-type kinetics. They can be presented in graphical form, expressing η as a function of the Thiele modulus (Φ_R), the Sherwood number (Sh) and a dimensionless substrate content $[S] \div K_m$. The square of the Thiele modulus is the ratio between the maximum rate of the enzyme-catalysed reaction and the maximum rate of substrate transport to the carrier (Eqn 2),

$$\Phi_R = R \times \sqrt{\frac{V_{max}}{D_{eff} \times K_m}} \quad (2)$$

where R is the carrier radius, V_{max} the maximum rate, K_m the Michaelis–Menten constant of the immobilized enzyme and D_{eff} the effective diffusion coefficient of the substrate in the carrier.

The Sherwood number (Sh), a dimensionless quantity that gives the ratio of the convective to the diffusive mass-transfer rate outside the carriers, is defined in Eqn 3,

$$Sh = 2R \div \delta \quad (3)$$

where δ is the thickness of the unstirred diffusion layer outside the carrier that can be reduced by stirring (batch reactor) or increasing the flow rate (fixed-bed reactor); in an unstirred system, it has a value of 2. These quantities must be known to estimate the effectiveness factors for different immobilized enzyme preparations from graphs for η as a function of Φ_R (Ref. 25).

This can be illustrated from the estimation of the effectiveness factors of different immobilized preparations of penicillin-G amidase from *Escherichia coli* (Box 3). R and V_{max} in Eqn 2 can be easily determined and, for K_m, the value for the free enzyme can be used for estimations. The effective diffusion coefficient D_{eff} can then be calculated from Eqn 4[26],

$$\frac{D_{eff}}{D_0} = \frac{\epsilon_p}{\tau}\left(1 - \frac{r_{substrate}}{r_{pore}}\right)^4 \quad (4)$$

which is based on D_0, the diffusion coefficient in free solution, ϵ_p, the particle porosity, τ, the tortuosity factor (a measure of the 'straightness' of the pores), $r_{substrate}$, the substrate radius and r_{pore}, the pore radius. The Sherwood number for both batch and fixed-bed

Box 3. Effectiveness factors (η) for immobilized penicillin-G amidases

To determine the data in Table I, penicillin-G amidase was immobilized on prefabricated carriers or insolubilized as cross-linked crystals. The mean particle radius of swelled carrier (R) was 80 μm for Eupergit® (Ref. 27). For cross-linked crystals, a value of 7.5 μm was used as R, which corresponds to half the lowest diameter of needle- or plate-like crystals[5].

Table I. Effectiveness factors (η) for immobilized penicillin-G amidases

Carrier	S (mM)	Φ_R	η
Eupergit® C[a]	268	68	1.0
	10	68	0.995
	0.013	68	0.023
Eupergit® 250L	268	69	1.0
	10	69	0.995
	0.013	69	0.023
CLEC™	268	141	1.0
	10	141	0.273
	0.013	141	0.011

[a]Eupergit is a porous, spherical enzyme carrier (Röhm, Darmstadt).
Abbreviations: CLEC, crosslinked enzyme crystals (Altus Biologics); S, substrate concentration; η, effectiveness factor; Φ_R, Thiele modules.

The value of V_{max} was determined to be 90 U cm^{-3} for Eupergit C and 170 U cm^{-3} Eupergit 250L (1 unit = 1 μmol min^{-1} at 28°C) (Ref. 27). The V_{max} of cross-linked crystals of penicillin-G amidase was 16 000 U cm^{-3} (Altus Biologics).

The calculated diffusion coefficient of penicillin G (based on the molecular weight) in free solution (D_0) was found to be 4.0×10^{-6} cm^2 s^{-1} (Ref. 48). For the Eupergit carriers, the effective diffusion coefficient D_{eff} was determined by curve fitting based on measured and calculated progress curves[49]. For Eupergit C and Eupergit 250L, they were 40% and 70% of the free-solution values, respectively; the higher value in the latter is due to the larger pores in this carrier. For the cross-linked crystals, D_{eff} was calculated from Eqn 4 using the following values: $r_{substrate}$, 0.5 nm; r_{pore}, 1.25 nm; ϵ_p, 1; τ, 1). For K_m, the value for the free enzyme (13 μM) was used[27]. A substrate concentration [S] of 268 mM corresponds to the substrate concentration of a 10% solution of penicillin-G salt, which is what is normally used in large-scale penicillin hydrolysis. η was determined from calculated curves of effectiveness factors with Sh = 16 and Eqns 2–4 (Refs 1, 27).

reactors has been found to be in the range 10–20 (R. C. Schlothauer, PhD thesis, Technical Universität Hamburg–Harburg, Germany, 1996). Higher values of Sh give marginal changes in η (Ref. 25).

The data in Box 3 show that there are no diffusional limitations as long as the substrate concentration remains much higher than K_m, which is the case under the starting conditions of large-scale penicillin hydrolysis. At lower substrate concentrations, mass-transfer effects lead to reduced effectiveness for both carrier-fixed enzyme and cross-linked crystals. Limitations of crystals, indicated by the high Φ_R, are caused by their high enzyme loading despite their small size. From Box 3, it follows that crystals with a tenth of the dimension of carriers can have a hundred times the activity without suffering from increased diffusional limitations.

Low substrate concentrations occur in hydrolytic reactions such as penicillin splitting when substrate conversion is near the end of the reaction. Under these conditions, however, other factors, such as product inhibition[27] (R. C. Schlothauer, PhD thesis), will govern the reaction rate. An additional effect, which can overshadow all other factors at any substrate concentration, is the formation of reaction-generated proton gradients, discussed below.

In practice it is desirable to be able to detect such mass-transfer effects reliably and simply[2,9]. To investigate whether the immobilized enzyme is mass-transfer limited, it is advisable to assay the enzyme activity under more drastic conditions. These can include increasing the stirrer speed or flow rate to minimize external diffusion, crushing the particles to reduce the Φ_R, or adding buffer to avoid pH shifts. If the reaction rate is increased by any of these means, it is likely that mass-transfer effects control the reaction to some extent. Additional methods to do this are given in published recommendations on the characterization of immobilized biocatalysts[7,8].

Appropriate means that can be used to increase the reaction efficiency include the following.

- Decreasing the particle size of the carriers. In technical applications the lower limit for the diameter of spherical particles is 100 μm, which enables them to be retained on common sieve plates even in large enzyme reactors. For smaller enzyme crystals, other retention techniques have to be used.
- Reducing the enzyme loading is recommended for enzymes with a high specific activity and is easily achieved by common fixation methods. In crystals, enzyme activity can be diluted by cocrystallization with inactivated enzyme[5]. However, for enzymes with low specific activities, tight packing can be a useful fixation method when excessive inert carrier material would not favour reasonable reaction conditions.
- Preferential binding at the outer shell of carrier materials will enable increased efficiencies[20]. An efficiency increased by a factor of about two can be expected when only the outer shell (a tenth of the radius) is occupied by enzyme[19].

Thus, given these considerations, enzyme crystals and carrier-fixed enzymes do not differ substantially in their mass-transfer effects. The main difference is a result of their enzyme loadings and particle sizes. Equal restrictions for crystals can be expected when their particle-size ratio approaches 1:10 and for carrier-fixed enzymes when their enzyme-loading ratio approaches 100.

Support-generated (static) and reaction-generated (dynamic) proton gradients

Compared with free enzymes, the pH–activity profiles of immobilized enzymes can be shifted by 3–4 pH units. This has been observed for N-protected amino acid esters in solutions with a low buffer capacity and a low ionic strength that are hydrolysed using trypsin immobilized in cation-exchange carriers[21] or cross-linked subtilisin crystals[5].

For immobilized trypsin, these shifts were found to be due partly to static proton and substrate gradients. These originate from interactions (partition) between charged groups of solute molecules with stationary charges on the carrier. The pH in the carrier is then much lower than the bulk pH, causing the observed pH shifts[28,29]. In this case, the observed pH shift can be reduced simply by using a solution with a high ionic strength that minimizes such interactions. These static gradients are of little technical relevance to the reactions of charged substrates that provide high ionic strengths.

Such static pH gradients can also be formed in enzyme crystals at low ionic strengths. At pH values greater than the isolelectric point, a negatively charged crystal can act as a cation exchanger and partitioning of protons can give rise to a static pH gradient. However, this has not yet been studied.

In addition, dynamic proton gradients have frequently been observed when immobilized enzymes liberate protons in hydrolytic reactions[9,30–32]. They can overshadow any other effects, even when substrate- or product-related mass-transfer limitations are without any significance[30]. The reason is that, in enzyme-catalysed reactions such as the hydrolysis of esters (Eqn 5) or amides (Eqn 6), the formation of even small amounts of protons contributes significantly to pH shifts and hence to changes in reaction rates.

$$R_1COOR_2 + H_2O \leftrightarrow R_1COOH + R_2OH \leftrightarrow R_1COO^- + H^+ + R_2OH \quad (5)$$

$$R_1CONHR_2 + H_2O \leftrightarrow R_1COOH + H_2NR \leftrightarrow R_1COO^- + R_2NH_3^+ \quad (6)$$

The weight-related maximum activities of immobilized penicillin amidases (Box 3) enable 0.3 M and 0.0015 M acid and base to be formed per second in the pores of enzyme crystals and Eupergit® C carriers, respectively. In unbuffered or slightly buffered systems, even small amounts of acids (or bases) may give a pH in the carrier that differs significantly from the external pH. This is given by the dissociation constant, the pK_a value for the dissociation of acids and pK_b value for the deprotonation of bases. When penicillin is hydrolysed (Eqn 6), the formed acid ($pK_a \approx 3$) and base ($pK_b \approx 5$) give an intermediate pH value $\{pH = [(pK_a + pK_b) \div 2] \approx 4\}$ in the pores of immobilized enzyme that is much lower than the bulk pH of 8 that is required for optimal product yield.

In the course of a hydrolytic reaction, a pH shift can be reduced by the continuous addition of a base, which has to diffuse into the carrier. By these means, a

dynamic pH gradient is formed along the particle radius and this indicates diffusional control. In the same manner, ester hydrolysis (Eqn 5) creates a lower pH value ($pH \leqslant pK_a$) than the targeted pH optimum. Such dynamic pH gradients are important in the hydrolytic reactions of hydrolases such as lipases, esterases and amidases. These include penicillin amidases (synonymous with penicillin acylases) and cephalosporin acylases, which are used for the industrial scale hydrolytic splitting of penicillins and cephalosporins[33]. This hydrolytic splitting has to be performed at a pH of about 8, which is close to the optimum pH of the enzyme. Lower pH values lead to lower reaction rates and reversibility of the reaction, and hence to a significant loss in product formation. Higher pH values are not advisable because of the instability of the reaction partners. Moreover, the addition of buffers is not acceptable because of the expense of removing the buffer components.

To gain a better understanding, several mathematical models have been created that extend Michaelis–Menten-like kinetics by adding proton-generating terms[34] and consider product inhibition and pH values above the optimum pH of the enzyme[35]. In addition, they include facilitated transport arising from the buffering capabilities of substrates and products or added buffers[31,34,36]. In view of the complexities of the calculations, only a basic outline can be presented here.

Enzyme reactions are often characterized by a bell-shaped pH–activity profile (Eqn 7),

$$V_{max} = \frac{V_{max}}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \quad (7)$$

where K_1 and K_2 are pH-profile-related constants, giving a profile such as that shown in Fig. 3a. Accumulated protons generated within the pores of a catalyst cause the formation of a proton gradient, the extent of which is predominantly a function of the proton-formation rate. This is dependent on the immobilized enzyme's activity, the mass-transfer-driven transport of protons to the outside of the catalyst particles and the bases used to neutralize them. At steady state, a mass balance occurs.

Ruckenstein[31] calculated the resulting diffusion-restricted enzyme activities at high substrate concentrations. Shifts in pH and the resultant reductions in activity occur even at substrate concentrations high enough to exclude any substrate-related diffusional restrictions (i.e. at effectiveness factors close to 1). Incidentally, this is the situation under the conditions at the start of the penicillin-G-amidase reaction outlined in Box 3. In his calculations, Ruckenstein introduced a modified, proton-related Thiele modulus, replaced the substrate-related K_m with the pH-related K_1 (Eqn 7) and used the effective diffusion coefficient of protons instead of substrates. Thus, without buffer, the reaction rates decrease significantly even at comparably low values of the modified Thiele modulus and, instead of a bell-shaped profile, a curve is obtained that becomes flatter towards alkaline pH values (Fig. 3b). In the presence of buffers, facilitated transport of protons is assumed. Consequently, significantly increased proton transport can be achieved, so that the activity–pH profile is shifted to more-alkaline pH values (Fig. 3c).

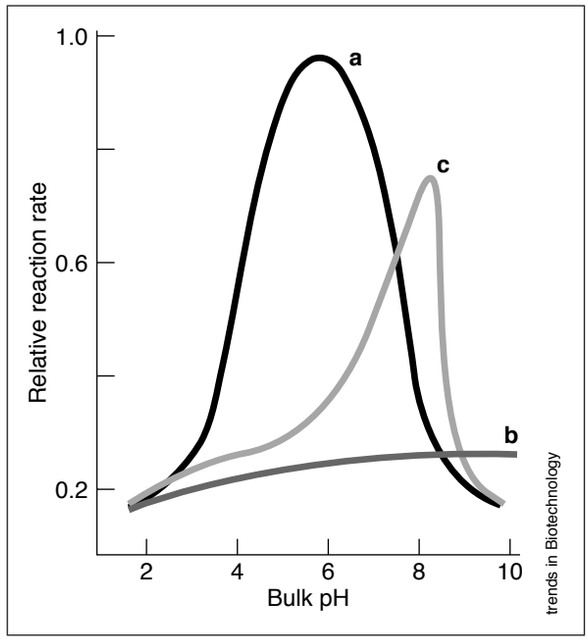

Figure 3

The efficiency of proton-generating enzyme reactions. The graph shows the influence of acid generation on enzyme activity as a function of the external (bulk) pH of free and immobilized enzymes. (**a**) Soluble-enzyme reaction with a bell-shaped activity–pH profile. $K_1 = 10^{-4}$ M, $K_2 = 10^{-8}$ M. (**b**) Severe diffusional control of an immobilized enzyme when the proton-related Thiele modulus $\Phi_R \approx 12$ (Ref. 36), bulk substrate concentration [S] = 145 mM and $K_m = 14.5$ mM. (**c**) Moderate internal diffusional control of an immobilized enzyme in the presence of buffer [using the same conditions as (**b**) and 10 mM buffer; simplified graphical presentation, derived from Ref. 36.

Direct experimental proof of such dynamic pH gradients has been obtained in porous carriers for immobilized penicillin amidase and glutaryl amidase. Direct fluorescence measurements of coimmobilized fluorochromes with pH-dependent fluorescence intensity revealed pH gradients of 2–3 pH units during hydrolysis in unbuffered systems (R. C. Schlothauer, PhD thesis). Additional evidence is provided by activity assays of carrier-fixed α-chymotrypsin[25,32]. In this case, the rate of the enzyme-catalysed reaction on the substrate content, buffer capacity and particle size was studied (Fig. 4). At high buffer capacity [ionic strength (I) > 0.05 M], the reaction rate indicates substrate-related diffusional control at low substrate concentration (Fig. 4a). At low buffer capacity ($I > 0.001$ M), the enzyme activity at high substrate concentrations is significantly lower and indicates control by pH shifts within in the carrier (Fig. 4a). Even with crushed particles, the activity is to some extent dependent on the buffer capacity (Fig. 4b). Low buffer capacities were also used to assay the pH-dependent activity of subtilisin crystals[5], as mentioned above. Thus, the formation of a dynamic pH gradient during hydrolysis could explain the observed pH shift in activity and the 27-times reduction in activity under these assay conditions.

In conclusion, differences between the pH dependence of the free enzyme and enzyme crystals seem to

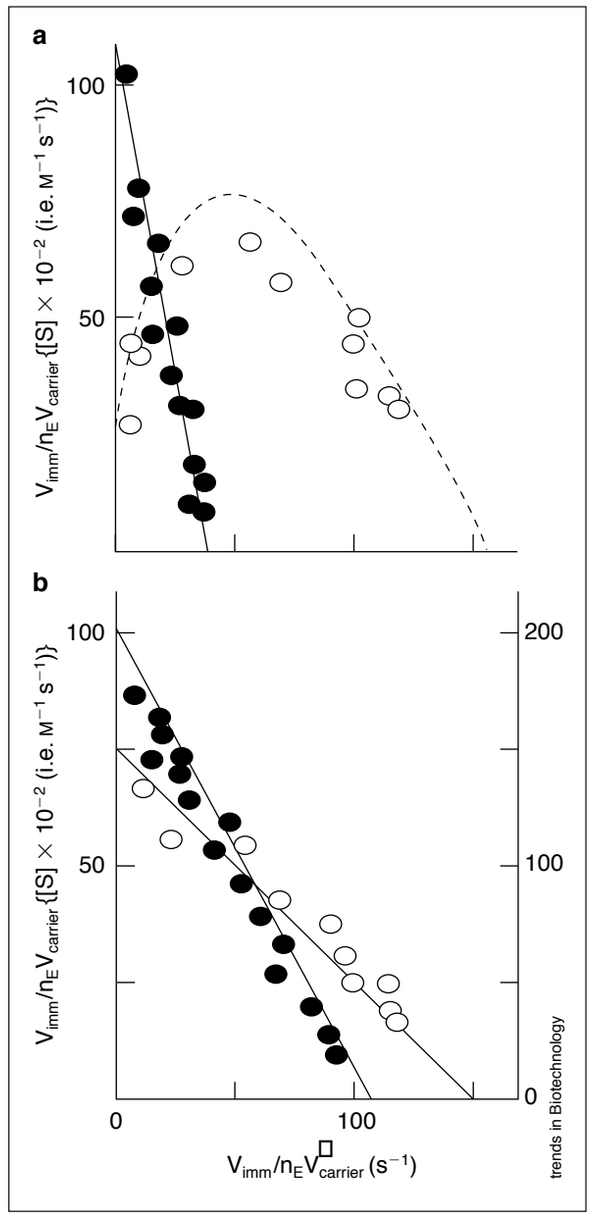

Figure 4

Porous diffusion by substrate- and reaction-generated protons. Effects of porous diffusion by substrate- and reaction-generated protons on the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester at a bulk pH of 8.0 and 25°C. α-Chymotrypsin was bound onto spherical Sepharose® particles with an average radius of 60 μm (**a**). The particles were then crushed to an average radius of 3 μm (**b**). The ionic strength of the buffer components was either 0.001 (filled circles) or 0.05 (open circles); the bulk pH was controlled by titration; n_E is the enzyme content in the carrier of volume V_{imm}, determined by active-site titration. Adapted from Ref. 32.

be caused by the same factors that cause the differences between free and otherwise-immobilized enzymes. In order to optimize productivity and reduce the loss of catalyst or products, it is advisable to minimize the effects of reaction-generated dynamic pH gradients. This can be achieved in several ways:

- by reducing the enzyme density or particle size, or both, for substrate-mediated diffusional control;
- by using buffers with sufficient capacity (>0.05 M) to minimize the dynamic pH gradients, with a p*K* value greater than the optimum pH for the enzyme-catalysed process[31] (occasionally, the substrates or products themselves provide such properties so that only the optimal external pH value has to be adapted);
- by operating at a higher external pH than the optimum pH of the enzyme;
- by coimmobilizing a proton-consuming enzyme (e.g urease, which forms ammonia *in situ* and neutralizes the generated protons[34]; however, ammonia can form a byproduct when it acts as a nucleophile during deacylation of the acyl-enzyme and this has to be taken into consideration).

Immobilization by dispersion in water-immiscible solvents

For reactions carried out in water-immiscible organic solvents the simplest method of immobilization is to suspend dried enzyme powders in the solvents[37]. The enzymes can be removed by filtration or centrifugation and reused. However, even this simple method can cause mass-transfer problems if the enzyme is poorly dispersed. Improper storage conditions and formulations of powders that have not been specially developed are also likely to be responsible for low activities, for example when humidity or reaction-generated water make hygroscopic lyophilisates sticky and increase the particle size of the enzyme powder. Moreover, in organic solvents, water activity and enzyme conformation and stability can affect the assayed activity[5,17,38–42].

The effects of formulation on lipoprotein lipase (LPL)-catalysed acetylations are shown in Table 1. The acetylation activity of lyophilized LPL in toluene is low despite its high activity in aqueous environments, which rules out inactivation during the lyophilization procedure. The activity was significantly increased by adding polyethylene glycol (PEG) during the lyophilization procedure, providing a highly dispersed LPL preparation in the organic medium. Covalent binding to PEG gave the best results and high activities could also be obtained following adsorption to a carrier material[6] (e.g. Celite®).

In contrast to the poor reaction rates reported elsewhere[43], crude lipase powder from *Pseudomonas cepacia* acylated secondary phenylethyl alcohol at reasonably high reaction rates (Roche Molecular Biochemicals) (Table 1). Based on active enzyme protein, the highest reaction rates were observed for carrier-fixed enzymes; this is also true for lipases from *Candida antarctica*. *P. cepacia* lipase crystals catalysed the reaction with a comparable activity when they had been pretreated with surfactants[43].

There are several means of enhancing the reaction rates of immobilized enzymes for use in water-immiscible solvents.

- In the case of dispersed enzyme powders, correct formulation and storage conditions will ensure reasonable activities. This means the addition of various compounds during the drying process to improve dispersion. Such compounds can also be quite useful as stabilizers and as protective agents. Suitable preparations are usually provided by enzyme manufacturers for dedicated enzymes.
- Solubilization of the enzyme in the organic solvent by covalent coupling of lipophilic compounds.

Table 1. Acetylation activities of dispersed lipases

Enzyme	Formulation	Activity	
		Units (mg dried preparation)$^{-1}$	Units (mg enzyme protein)$^{-1}$
Acetylation of (±)-sulcatol in toluene			
LPL[a]	Powder	<0.06	
	Powder + PEG	<0.06	
	Lyophilized together with PEG	1.8	
	With covalently bound PEG	3.5	
	Carrier fixed	2.3	
Acetylation of secondary phenylethyl alcohol			
PSL	Powder[a]	1.5	15
	Carrier-fixed enzyme[a]	0.08	8
CAL-B			
	Powder[a]	1.7	17
	Carrier-fixed enzyme[a]	0.4	40

[a]In water-saturated *n*-hexane at 25°C in units mg^{-1}; either with lyophilized preparations (powders) containing ~10% protein or with lyophilized carrier-fixed preparations [~1% protein by weight (Roche Molecular Biochemicals)].
Abbreviations: CAL-B, lipase from *Candida antarctica*, fraction B; LPL, lipoprotein lipase; PSL, lipase from *Pseudomonas cepacia*.

Immobilization must then be achieved by inclusion into membrane devices or by multiphase reactions[44–46].

- Immobilization of the enzyme. Even simple adsorption onto porous carriers can significantly increase the availability of single catalytic centres and also ensure easy separation from the product. Cross-linked crystals appear to require surfactants to compensate for their low activity in water-immiscible organic solvents[43].

Inert carrier or enzyme protein only?

With the exception of cross-linked enzymes, crystals and enzymes included within membranes, the inert carrier material is usually present in excess of the active enzyme protein. The range of active enzyme on carriers can be fairly broad, determined by bound enzyme protein (usually 0.1%–10% of the weight of the carrier material) and by active enzyme activity [0.1–500 units (mg protein)$^{-1}$]. It is therefore possible to establish a well-balanced relationship between reaction volume and carrier by adjusting the amount of bound enzyme on selected carrier materials. Nevertheless, the pores of inert carriers can retain significant amounts of product and thus require additional washing steps, leading to dilution and higher manufacturing costs. However, carriers can endow novel properties on enzyme preparations, such as pressure stability in plug-flow reactors.

In crystals, the carrier is the enzyme protein itself, whose specific activity strictly determines the weight-related activity of the crystal and hence provides an extremely high enzyme density, a particular advantage for low-activity enzymes. However, limitations caused by low activity are less a question of immobilization technology than of cost targets, because enzymes are usually expensive when their specific activity is low. This is not the case for high-enzyme-density preparations, which enable high activity per unit volume and hence short reaction times. Here, limitations occur when low concentrations of immobilized enzyme have to be handled without losses in the course of frequently repeated recycling.

Stability and productivity of immobilized enzymes

Even when manufacturing costs and mass-transfer effects have been minimized, the essential cost contributions of immobilized enzymes are determined by the time over which they can be used (i.e. their stability). Increased operational stability of immobilized enzymes is essential in order to achieve the highest cost benefits.

Enzyme stability can be determined by assaying the activity decay over time under operational conditions. The residual activity can even be predicted when the activity decay proceeds according to simple kinetic rules, as in thermal inactivation, which obeys first-order kinetics. Complications occur when mixtures of different enzyme species have differences in their binding or intrinsic stabilities, or when mass-transfer effects lead to low efficiencies. In the latter case, lowered activity as a result of low efficiency indicates that only a proportion of the enzyme is active. The unused portion might, in simple terms, replace the enzyme as it is inactivated. In other words, mass-transfer-controlled reactions appear to be much less sensitive to the decay of enzyme activity, thus falsely creating an impression of stabilization. In practice, it is advisable not only to determine the operational stability by tracing the time course of activity but also to follow its productivity or, alternatively, its consumption in relation to the product formed (Fig. 1).

A useful example to illustrate enzyme consumption is carrier-fixed penicillin-G. The enzyme is applied in stirred tanks with sieve plates in order to retain the enzyme particles when the product solution is drained off. The stirred tank is emptied and refilled repeatedly until the activity has dropped to ~50% of the original level. Until then, the consumption of enzyme in such a process is <10 (mg enzyme protein) (kg 6-amino penicillanic acid)$^{-1}$ or <0.2 kU kg^{-1}. The product-related enzyme costs can be calculated from these data.

When cross-linked crystals of thermolysin are used for peptide synthesis in ethyl acetate, stability is maintained

Table 2. Selected characteristic features of immobilization methods

Aspect	Prefabricated carrier	Cross-linked or crystallized enzyme
Enzyme source and quality	Enzymes of any purity Multienzyme-catalysed conversions (preferentially by whole-cell immobilization) also feasible	Pure enzymes only, which is advantageous when impurities are the reason for drawbacks
Specific activity and enzyme density on carrier	Activity loading adjustable on carrier (usually ~0.1%–10% w/w) Carrier may be preselected to fit to the intended binding and reaction technologies	High activity density enables low carrier volume (specific activity of enzyme determines volumetric activity of crystal) No excess of inert carrier (pure enzyme is the carrier)
Operational stability	High operational stability demonstrated in aqueous environment and water-immiscible organic solvents	Allow reactions at higher temperatures and in aqueous–organic-solvent mixtures

for several hundred hours with a very low enzyme-consumption rate, whereas a soluble enzyme preparation becomes inactive within a short period of time. When soluble thermolysin is stored in a mixed aqueous–organic solution, ~50% of its activity is lost within the first day of incubation and it then remains relatively stable for the next 15 days. It is possible that the initial inactivation is caused by an unstable fraction of thermolysin and that the thermolysin crystals no longer contain this unstable fraction[13]. Comparable productivity to that of crystals was achieved with thermolysin adsorbed on Amberlite® XAD-7 resins in continuous plug-flow reactors[47].

Some distinguishing characteristics of enzymes bound onto prefabricated carriers and enzymes immobilized as cross-linked enzymes or enzyme crystals are summarized in Table 2 but which is the best will ultimately be decided by both the specific technical requirements and financial considerations. With regard to purity, quality and formulation, it can be useful to define 'productivity' as the fermentation volume required to prepare the immobilized enzyme activity needed to synthesize a given amount of product. This is also of use when the overall performance of an immobilized-enzyme-catalysed process has to be competitive with other technologies, such as fermentation or whole-cell biotransformations.

Conclusions

The immobilization of enzymes is a useful tool to meet cost targets and has a number of technological advantages; for example, it enables the repeated use of enzymes and hence produces significant cost savings, and immobilized enzymes can be easily separated from the reaction liquid and thereby reduce laborious separation steps. Additional benefits arise from stabilization against harsh reaction conditions, which are deleterious to soluble enzyme preparations. Owing to the wide variety of properties of individual enzyme species and the varying requirements of reaction technology for the target compounds, it is necessary to exploit the wealth of methods and techniques of immobilization.

The physicochemical description of the kinetics (mass-transfer effects and the formation of substrate and pH gradients) of immobilized-enzyme systems is independent of whether the enzyme is immobilized on carriers, cross-linked in crystals or dispersed as aggregates (powders), but depends mainly on particle size, pore dimensions and enzyme density.

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‘Smart’ polymers and what they could do in biotechnology and medicine

Igor Y. Galaev and Bo Mattiasson

Stimulus-responsive or ‘smart’ polymers undergo strong conformational changes when only small changes in the environment (e.g. pH, temperature, ionic strength) occur. These changes result in phase separation from aqueous solution or order-of-magnitude changes in hydrogel size. Smart polymers are used in bioseparation and drug delivery, for the development of new biocatalysts, as biomimetic actuators, and as surfaces with switchable hydrophobic–hydrophilic properties.

Life is polymeric in its essence: the most important components of living cell (proteins, carbohydrates and nucleic acids) are all polymers. Nature uses polymers both for construction and as part of the complicated cell machinery. The salient feature of functional biopolymers is their all-or-nothing, or at least highly nonlinear, response to external stimuli – small changes happen in response to a varying parameter until a critical point is reached, when a large change occurs over a narrow range of the varying parameter; after the transition is completed, there is no significant further response of the system.

These nonlinear responses by biopolymers are caused by highly cooperative interactions. Despite the weakness of each particular interaction taking place in a separate monomer unit, when summed over hundreds and thousands of monomer units, these interactions can provide significant driving forces for the processes occurring in the whole system.

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Not surprisingly, an understanding of the mechanism of cooperative interactions in biopolymers has opened the floodgates to attempts to mimic this cooperative behavior in synthetic systems. Recent decades have witnessed the appearance of synthetic functional polymers that respond in some desired way to a change in temperature, pH, electric or magnetic field, or some other parameter. These polymers were originally called ‘stimulus responsive’ but the name ‘smart’ polymers was coined based on their similarity to biopolymers[1].

Smart polymers and hydrogels undergo fast, reversible changes in microstructure from a hydrophilic to a hydrophobic state. These changes are triggered by small changes in the environment but are apparent at the macroscopic level as precipitate formation from a solution or order-of-magnitude changes in the size and water content of hydrogels. These macroscopic changes are also reversible, the system returning to its initial state when the trigger is removed[2]. The driving force behind these transitions varies, with common stimuli including the neutralization of charged groups by either a pH shift[3] or the addition of an oppositely charged polymer[4], changes in the efficiency of hydrogen bonding with an increase in temperature or ionic strength[5], and

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