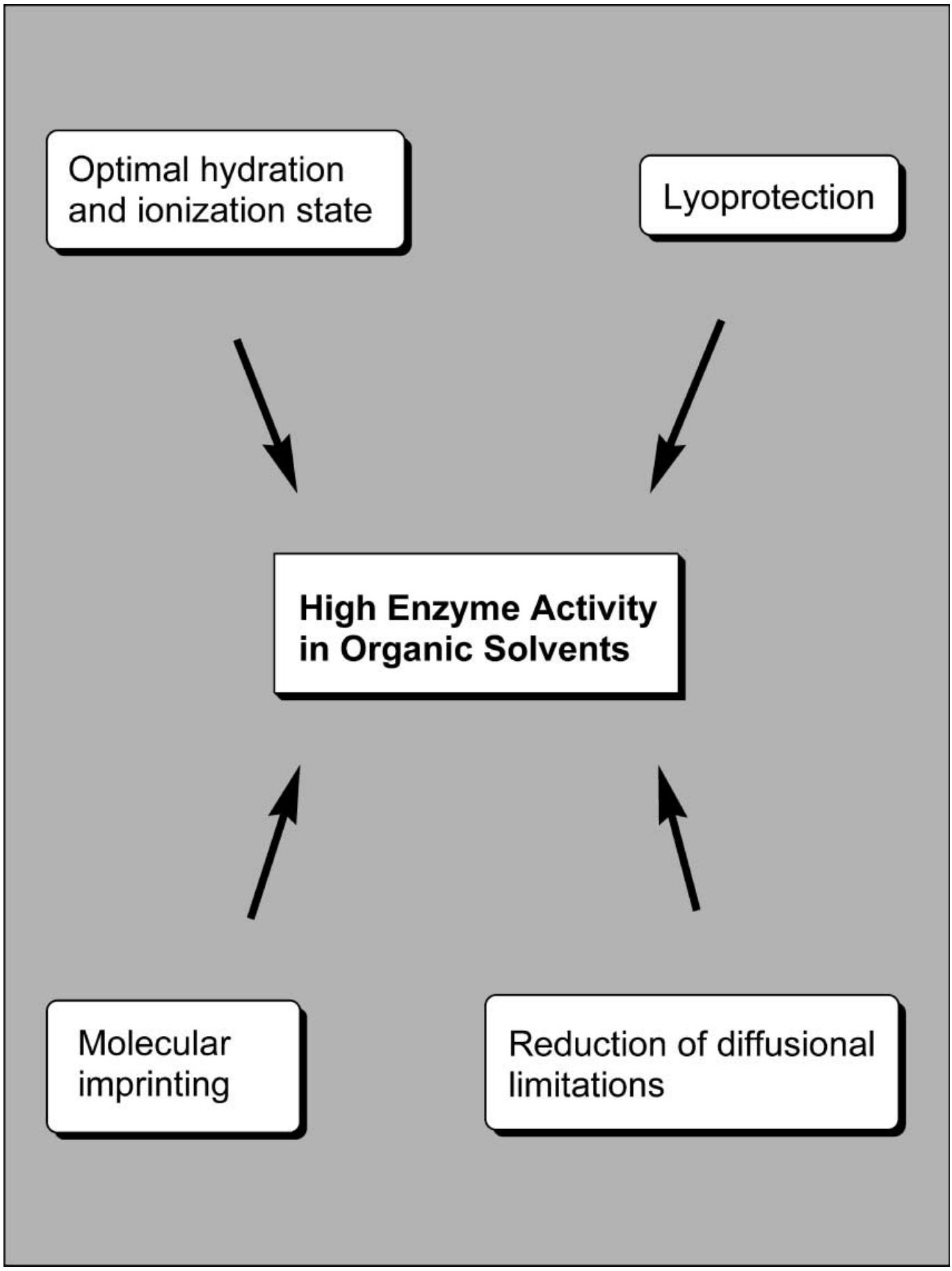




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# Optimization of Hydrolase Efficiency in Organic Solvents

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**Abstract:** The application of hydrolases in organic solvents for synthetic purposes is a procedure routinely adopted in organic chemistry, especially for the preparation of chiral building blocks. Numerous studies have shed light on several aspects of the mechanism of hydrolase action in low-water environments. Procedures suitable to improve the catalytic efficiency of enzymes and productivity of the synthetic processes have been reported. These fundamental and applied investigations have made hydrolase-catalyzed reactions in organic solvents of industrial interest. In this article we describe and discuss various approaches adopted to optimize the performance of hydrolases in organic media, with special emphasis on the formulation of the biocatalysts which, under proper conditions, can display an activity equal to that displayed in aqueous buffers.

**Keywords:** enzyme catalysis · immobilization · lipase · organic solvents · proteases

## Introduction

Although the coupling of enzymes and organic solvents seems quite unusual—biological systems are thought to be functional only in water—enzymes can be active also in non-aqueous media. This feature allows for expanding the number of biocatalytic applications, which make it possible to modify or synthesize innumerable compounds of interest in the pharmaceutical, agrochemical and fine chemical fields, exploiting the exquisite chemo-, regio- and enantioselectivity of enzymes.

The use of organic solvents in biocatalysis has been described in numerous studies.[1–3] For clarity, it should be emphasized that biocatalysis in organic solvents refers to those systems in which the enzymes (e.g. lyophilized powders or adsorbed onto inert supports) are suspended (or, sometimes, dissolved) in neat organic solvents in presence of enough aqueous buffer to ensure enzymatic activity. The amount of water present in the organic solvent may vary from any pre-saturation value (in general less than 5% *v*/*v*) to a very dry state. These reaction systems are clearly distinct from the aqueous ones where variable amounts of water-miscible solvents are added to favor the dissolution of insoluble reactants. Furthermore, they are also different from biphasic and reverse-micelles systems where, even if the organic solvent might constitute the most abundant part, the enzyme is dissolved in a discrete amount of water.

Enzyme applications in organic solvents have numerous advantages. For example, it is possible to transform substrates that are unstable or poorly soluble in water and prevent many side-reactions that are water dependent, including the denaturation of enzymes which, in several organic media, show higher thermal stability. Moreover, in absence of water, the synthesis by hydrolases (mainly lipases and proteases) of ester and amide bonds can be favored over hydrolysis. By varying the organic solvent it is also possible to modify the substrate specificity and the regio- and enantioselectivity of a given enzyme.[4] In spite of all these advantages, the use of this methodology it is not yet widely employed on an industrial level. One of the main reasons is certainly the lower catalytic activity (in most cases of several orders of magnitude) shown by enzymes in organic media compared with that in water. This behavior has been ascribed to different causes such as diffusional limitations, high saturating substrate concentrations, restricted protein flexibility, low stabilisation of the enzyme-substrate intermediate, partial enzyme denaturation by lyophilization that becomes irreversible in anhydrous organic media and, last but not least, non-optimal hydration of the biocatalyst.[5]

This concept article aims to present some of the more common and effective strategies, with emphasis in enzyme formulation, that have been adopted up to now to improve the activity of hydrolases in organic solvents and to discuss the

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**List of abbreviations**: $a_w$: water activity; CALB: lipase B from *Candida antarctica*; CALB+OA: CALB lyophilized with oleic acid; CALB + PEG: CALB lyophilized with PEG; Celite PC: lipase PC adsorbed onto Celite; CLEC-PC: cross-linked enzyme crystals (as commercialized by Altus); crude PC: lipase PC as commercialized by Amano; Novozym 435: immobilized CALB as commercialized by Novo-Nordisk (experimental product SP525); lipase PC: lipase from *Pseudomonas cepacia*; PC+PEG: lipase PC lyophilized with PEG; PC-PEG: lipase PC covalently linked to PEG; PEG: poly(ethylen glycol); Purified CALB: purified from crude CALB; sol–gel-AK-PC: lipase PC entrapped in sol–gel-AK (as commercialized by Fluka).

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possible mechanisms by which the enzyme activation is achieved.

## Enzyme Formulation

**Enzyme powders**: The simplest way by which enzymes can be utilized in organic solvents is to add them to the reaction media as dry powders, which are obtained by lyophilization or precipitation (for instance, by adding acetone) of crude or purified aqueous solutions of the biocatalyst. To enhance the catalytic activity, enzyme powders, which are not soluble in most organic solvents, are kept suspended in the medium by vigorous shacking or stirring. Because of its convenience, this method has been and still is widely employed, especially in preliminary and small-scale experiments. However, the activity of enzyme powders is generally rather low and their quantitative recovery from the medium is sometimes difficult because they tend to stick to the walls of the reaction vessels or to form sort of gels.

**Immobilization**: Better results in terms of catalytic efficiency, enzyme stability and catalyst reuse are obtained with immobilized hydrolases.

A largely employed immobilization method is based on the dispersion or deposition of the enzyme onto porous supports. To this end, numerous inorganic materials (Celite, silica gel, aluminas, zirconia, controlled-pore glasses)[6–8] and polymers (polyamides, polypropylene, polyacrylates),[9–11] with different porosity and bead size, have been used. Although this immobilization procedure is easy to use, particular care has to be dedicated to the optimization of the enzyme loading (weight of enzyme/weight of support).[7, 10, 12] In fact, if a low enzyme loading is deleterious for biocatalyst stability, because the interactions with the matrix can cause protein denaturation,[13, 14] a too high loading also leads to a decrease of the specific activity of the enzyme. This behaviour is exemplified in Figure 1 for lipase PC adsorbed onto Celite.[15] It can be seen that if the activity is referred to the whole biocatalyst (enzyme plus Celite), it increases as a function of the enzyme loading up to a maximum and then remains constant. Instead, if the relative activity is expressed as a function of the lipase protein, it increases up to a maximum and then decreases. These results can be explained when taking into consideration that a high enzyme loading causes the formation on the support of an enzyme multilayer. This hinders or prevents the accessibility of the substrate to the deeper buried enzyme molecules and, therefore, decreases the specific activity of the biocatalyst.

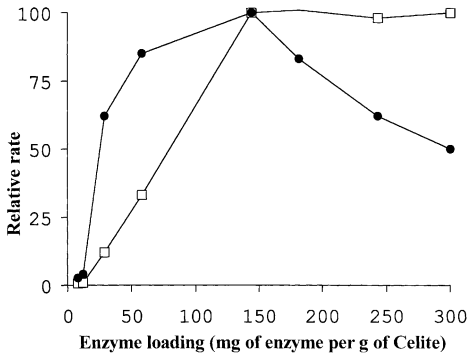


Figure 1. Relative transesterification rate of lipase PC, adsorbed onto Celite, as a function of enzyme loading. The relative rate is given per mg of whole material (lipase plus Celite, □) and per mg of lipase only (●). Transesterification was between (±)-sulcatol and vinyl acetate.

Several research groups have demonstrated that mass transfer limitations is a limiting factor for the catalytic activity in non-aqueous media.[10, 16, 17] Among these, Rees and Halling proved, by means of electrospray mass spectrometry and protein chemical modifications carried out with acyl chlorides in organic solvents, that the rate of modification was always much faster with smaller acyl chlorides and for protein adsorbed as monolayer onto a support than for lyophilized powders. In fact, in this latter case, only the enzyme molecules on the surface are accessible while the others, because of the extremely difficult penetration of the substrate (especially for bulky molecules) through the particles of the enzyme powders (solid-phase diffusion), behave as if they were inactive. This hypothesis is in agreement with the kinetic data obtained with different lipase PC formulations such as lipase powder, lipase adsorbed onto Celite and lipase co-lyophilized with PEG (Table 1).[7] It can be seen that, while the $V_{max}$ values change markedly as a function of enzyme dispersion ("soluble" enzyme-PEG complex > immobilized enzyme > enzyme powder), the $K_M$ values are very similar, if they are referred to the same $a_w$. Of course, the $K_M$ values increase as a function of water activity because of its competition with the substrate 1-octanol. This would indicate that with enzyme powders and immobilized enzymes only those enzyme molecules exposed at the surface are accessible to the substrate and, therefore, active.

Table 1. Apparent $K_m$[a] and $V_{max}$[b] values of different lipase PC formulations as a function of water activity ($a_w$) in toluene.

| | Crude PC[c] | | Celite PC[d] | | PEG+PC[e] | |
|---|---|---|---|---|---|---|
| $a_w$ | $V_{max}$ | $K_M$ [mM] | $V_{max}$ | $K_M$ [mM] | $V_{max}$ | $K_M$ [mM] |
| < 0.1 | 3 | 18 | 25 | 15 | 7 | 16 |
| 0.11 | 6 | 29 | 49 | 30 | 8 | 17 |
| 0.38 | 6 | 32 | 46 | 32 | 16 | 29 |
| 0.53 | 4 | 39 | 17 | 38 | 28 | 43 |
| 0.84 | 4 | 64 | 14 | 59 | 100 | 91 |

[a] $K_M$ values refer to the substrate 1-octanol in the transesterification reaction between vinyl butyrate and 1-octanol. [b] $V_{max}$ relative to PEG+PC at $a_w = 0.84$, taken as 100. [c] Lipase PC as commercialized by Amano. [d] Adsorbed onto Celite; or [e] co-lyophilized with PEG.

A different enzyme immobilization procedure is by entrapment in various types of materials.[2] Reetz and co-workers have entrapped lipases in different sol–gel compositions, by optimization of the nature of the silane monomers and of the water/silane stoichiometry, producing immobilized lipases with excellent specific activity and stability.[18] The activation mechanism of this approach, suitable also for proteases,[19] has been ascribed to the reduction of diffusional limitations. In fact, enzyme molecules distributed in the rigid tridimensional

net of the sol–gel material should be more accessible than those of the buried layers of the lyophilized powder. It is worth pointing out that also enzyme/support interactions might influence enzyme activity. In particular, in the case of lipases, in which the access of the substrate to the active site is regulated by a lid, a hydrophobic support resembling a lipid interface (e.g., some sol–gel matrices) could favour the open conformation of the enzyme and, therefore, activate it.[20, 21]

Peculiar immobilization procedures are represented by cross-linked enzyme crystals (CLECs)[22] and cross-linked enzyme aggregates (CLEAs).[23] These derivatives have shown high stability in organic solvents.

**Additives**: Enzyme activity in organic solvents can also be improved through the use of suitable additives.

Molecules such as substrate-resembling ligands, sugars, PEG, crown ethers, and inorganic salts, added to the aqueous enzyme solution before lyophilization, enhance the catalytic activity up to several orders of magnitude. The activation mechanism depends on the nature of the additive.

Competitive inhibitors or substrate analogues (removed from the enzyme, after lyophilization, by extraction with anhydrous solvents) may act through the so-called "imprinting" effect.[24, 25] In this case, the conformational changes induced in water by the binding of the ligand to the enzyme active site are maintained after ligand removal and suspension of the biocatalyst in organic solvents, because of the high enzyme rigidity in anhydrous media. When low water-soluble substrate analogues are used as ligands, their concentration in water can be increased by proper chemical modification or by adding organic cosolvents in the lyophilization medium.[26]

For other additives such as crown ethers,[27, 28] poly(ethylene glycol) (PEG)[29, 30] and sugars (sucrose, sorbitol, etc.),[31] it has been suggested that the increase of enzyme activity is due to lyoprotection. Conformational analyses carried out by several research groups on different proteins by means of FT/IR[32–35] have shown that lyophilization can affect protein secondary structure. The presence of lyoprotectants seems to prevent such conformational changes. For example, Griebenov and Klibanov[33] have demonstrated that bovine pancreatic trypsin inhibitor retains a more native-like secondary structure when co-lyophilized with sorbitol. The same phenomenon was observed by De Paz et al.[34] with subtilisin added with sucrose or trehalose and by Vecchio et al.[35] with lipase PC added with PEG. Moreover, the far UV/CD spectra of lipase PC, CALB, and subtilisin made soluble in dioxane by co-lyophylization with PEG or covalent linking to PEG,[37, 38] have shown that the enzymes have the same secondary structure in the organic solvent and in aqueous buffer.[36–39] Similarly, intrinsic protein fluorescence experiments have demonstrated that subtilisin and lipase PC, modified or complexed with PEG have native conformation in dioxane.[36, 38]

Interestingly, different mechanisms of activation have been hypothesized for different enzymes co-lyophilized with crown ethers. While van Unen et al.[40] suggested that the activation by crown ethers of $\alpha$-chymotrypsin and lipase from *Pseudomonas fluorescens* is mainly due to a lyoprotection effect, Santos et al.[28] proposed molecular imprinting as the primary cause for the activation of subtilisin by these additives.

Lyoprotection has also been invoked to explain the activation effects observed with subtilisin Carlsberg and lipase from *Mucor javanicus* lyophilized with potassium chloride (98% *w*/*w* KCl, 1% phosphate buffer, 1% enzyme).[41] The same effect is also exerted by other inorganic salts and activation seems to be proportional to the kosmotropicity (salting-out ability) of the salts.[42] Nevertheless, the increase of polarity of enzyme active site by salt ions and/or the water retained in its immediate surrounding, might contribute to activity increase by stabilization of the transition state.[41, 42]

## Water Activity

A crucial factor that has to be considered to optimize the efficiency of the biocatalyst and the yield of the product, is the $a_w$ of the medium.[43] In fact, besides the well known role of water as protein "lubricant", which influences the flexibility and, therefore, the activity of the enzyme, in the case of hydrolases-catalyzed synthetic reactions, the $a_w$ value directly modulates the balance (equilibrium) between the hydrolytic and the synthetic process. Several studies carried out with lipases[44–47] have demonstrated that the effect of $a_w$ on the catalytic activity depends on the nature of the enzyme. With lipase PC (Figure 2) and CALB[30] it has been shown that the optimal $a_w$ value depends also on the formulation.

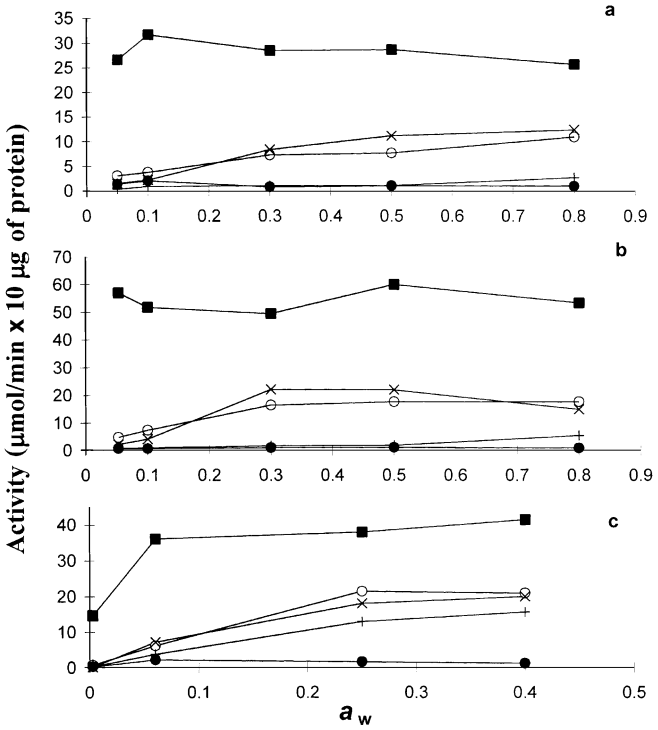


Figure 2. Total activity (transesterification plus hydrolysis) of sol–gel-AK-PC (■), PEG+PC (○), PEG-PC (×), crude PC (+) and CLEC-PC (●) in a) benzene, b) carbon tetrachloride, and c) 1,4-dioxane. Transesterification was between 1-octanol and vinyl butyrate.

The transesterification/hydrolytic activity ratio (and, therefore, product yield) is influenced both by enzyme formulation and $a_w$ (Table 2). It can be seen that at $a_w < 0.1$, this ratio is up

  

Table 2. Ratios of transesterification over hydrolytic activity for various lipase PC formulations in various organic solvents at different $a_w$ values.[a]

| Solvent | PC+PEG[b] | PC-PEG[c] | Crude PC[d] | CLEC-PC[e] | sol–gel-AK-PC[f] |
|---|---|---|---|---|---|
| $CCl_4$ ($a_w < 0.1$) | 4.4 | 3.9 | 1.5 | 8.4 | 2.0 |
| $CCl_4$ ($a_w = 0.84$) | 1.2 | 1.0 | 0.6 | 0.8 | 0.4 |
| benzene ($a_w < 0.1$) | 9.9 | 5.0 | 2.6 | 8.7 | 2.9 |
| benzene ($a_w = 0.84$) | 0.4 | 0.4 | 0.2 | 1.2 | 0.4 |
| 1,4-dioxane ($a_w = 0.003$) | 9.7 | 4.1 | 2.0 | 7.6 | 9.3 |
| 1,4-dioxane ($a_w = 0.4$) | 0.1 | 0.1 | 0.1 | 0.4 | 0.1 |

[a] The transesterification activity was estimated by measuring the amount of 1-octyl butyrate formed from the reaction of *n*-octanol (nucleophile) with vinyl butyrate (acyl donor). The concomitant hydrolytic activity was estimated by measuring the formation of butyric acid. [b] Lipase PC co-lyophilized or [c] covalently linked with PEG; [d] as commercialized by Amano; [e] as cross-linked enzyme crystals (as commercialized by Altus); [f] entrapped in sol–gel (as commercialized by Fluka).

to three times higher for CLEC-PC and PC+PEG than for crude PC. Analogously, Adlercreutz[11] has shown that, at the same $a_w$, $\alpha$-chymotrypsin immobilized onto the polyamide support Accurel PA6 has a alcoholysis/hydrolysis ratio higher than that obtained with the enzyme immobilized onto Celite. Moreover, Table 2 shows that at low $a_w$ values, the ratios between transesterification and hydrolysis rates are higher, which means that a lower amount of acylating agent is consumed per mole of ester product. Therefore, for synthetic applications the $a_w$ value will be a compromise assuring the highest catalytic activity of the enzyme and the lowest acylating ester consumption.

## Enzyme Ionization

Zaks and Klibanov first reported, in their pioneering studies on enzymatic catalysis in organic solvents,[48, 49] that enzymes have higher catalytic activity if they are recovered, by for example lyophilization or precipitation, from an aqueous buffer adjusted to the pH optimal for the given enzymes. They called this phenomenon "pH memory" and suggested that the protein retains in the organic solvent the same protonation state it had in the aqueous buffer. Nevertheless, if acidic or basic chemical species (reactants, products or impurities) are present in the reaction medium, a variation of the enzyme ionic state could occur, causing variations of catalytic activity. To overcome this drawback, buffers soluble in organic solvents (e.g. triisooctylamine with its hydrochloride and triphenylacetic acid with its sodium salt)[50] and even solid-state buffers (e.g., AMPSO, MOPS, PIPES, and their sodium salts)[51] can be added to the medium to keep optimal enzyme ionization.

## Conclusions and Perspectives

In spite of the wealth of literature information, it is hard to precisely state what are the best operational conditions and, even more so, the most appropriate enzyme formulations to utilize, both in general and for a given synthetic application. The reasons are to be ascribed, at least in part, to: a) a shortage of direct comparisons carried out among the various enzyme formulations and operational conditions; b) the almost complete lack of quantitative correlation conducted between the activity and performance of enzymes in organic media and the same properties in aqueous buffers. In most cases, in fact, dramatic or remarkable biocatalyst improvements are generically claimed.

When such comparisons have been done, for example with subtilisin (Table 3),[38] lipase PC (Figure 2 and Table 4),[29] and CALB (Table 4),[30] the results were quite informative. It was found that, besides solvent nature and $a_w$ of the medium, enzyme formulation has a profound effect on enzyme activity.

Table 3. Transesterification activity of subtilisin in dry dioxane.[a]

| Subtilisin formulation | Conversion ($\mu$mol $h^{-1}$ $mg^{-1}$ enzyme protein) |
|---|---|
| subtilisin powder[b] | 22 |
| subtilisin powder+PEG[c] | 89 |
| subtilisin powder+sorbitol[d] | 119 |
| PEG-subtilisin[e] | 83 |
| subtilisin adsorbed onto Celite[f] | 8 |
| subtilisin adsorbed onto Celite+sorbitol[g] | 98 |
| subtilisin adsorbed onto Celite+PEG[h] | 123 |

[a] Transesterification activity was measured using 1-hexanol as nucleophile and vinyl butyrate as acyl donor. Subtilisin, [b] as commercialized by Sigma; [c] lyophilized with PEG; or [d] sorbitol; [e] covalently linked to PEG; [f] adsorbed on Celite alone; or in presence of [g] sorbitol; or [h] PEG.

Table 4. Transesterification and total activity in organic solvent over hydrolytic activity in aqueous buffer of lipase PC and CALB formulations.[a, b]

| Enzyme form | Transesterification activity in organic solvent[c]/ hydrolytic activity in aqueous buffer[e] | Total activity in organic solvent[d]/ hydrolytic activity in aqueous buffer[e] |
|---|---|---|
| CALB+PEG[f] | 0.51 | 0.70 |
| Novozym 435[g] | 0.29 | 0.44 |
| CALB+OA[h] | 0.26 | 0.42 |
| purified CALB[i] | 0.16 | 0.40 |
| crude CALB[l] | 0.003 | 0.004 |
| sol–gel-AK-PC[m] | 0.83 | 1.3 |
| PEG+PC[m] | 0.26 | 0.39 |
| PEG-PC[m] | 0.32 | 0.48 |
| CLEC-PC[m] | 0.02 | 0.02 |
| crude PC[m] | 0.04 | 0.12 |

[a] The organic solvent was toluene in the case of the reaction catalyzed by CALB and carbon tetrachloride in the case of lipase PC. [b] The activities in organic solvent and aqueous buffer were referred to the same amount of lipase protein. [c] Transesterification activity was measured using 1-octanol as nucleophile and a vinyl ester (vinyl acetate in the case of CALB or vinyl butyrrate in the case of lipase PC) as acyl donor. [d] Total activity is the transesterification plus the hydrolytic activity occurring in organic solvent. [e] The hydrolytic activity in potassium phosphate buffer (0.05 M, pH 7) was determined using vinyl acetate as substrate in the case of CALB and tributyrin in the case of lipase PC. [f] CALB lyophilized with PEG; [g] as commercialized by Novo-Nordisk; [h] lyophilized with oleic acid; [i] purified from crude CALB; [l] as commercialized by Novo-Nordisk (experimental product SP 525). [m] For abbreviations of lipase PC see Table 2.

Thus, sol–gel-AK-PC, PC-PEG, PC+PEG, CALB+PEG and Novozym 435 showed transesterification and, even more so, total activity, which were of the same order of magnitude (in the case of total activity, 130, 48, 39, 70 and 44%, for the five formulations) of those displayed in aqueous buffer (Table 4). Instead, other formulations showed rather poor activity (Table 4). These data demonstrate that, with suitable enzyme formulations and proper $a_w$ values, the activity of hydrolases, specifically of lipases, in organic solvents is comparable to that displayed in aqueous buffer.

Of course, activity, though very important, is not the only parameter that has to be taken into consideration in choosing a catalyst. Stability, reusability, activity per catalyst weight (enzyme plus additives or supports), and cost have to be considered. Perhaps, immobilized hydrolases,[52] including several commercial preparations, are the preferable formulations for preparative purposes because of their satisfactory performance and handiness. However, for exploratory investigations or in the case of highly favorable product cost/catalyst cost ratio, also enzyme powders can be profitably used because of their readiness and convenience.

Further research aimed at a deeper understanding of the fundamental mechanisms that regulate enzyme activity and activation in low-water environments could also favor the development of new and better enzyme formulations. This, together with the discovery (also by molecular biology techniques[53–55]) of new hydrolases (and, more in general, new enzymes) with improved catalytic properties, should increase the number of efficient non-aqueous biocatalytic-systems suitable for a large variety of industrial applications.


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