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Some Aspects of Biocatalysis in Organic Solvents

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1. INTRODUCTION

Enzyme catalysis in non-aqueous systems is now quite usual, although some decades ago it seemed exceptional. Considering the lipophilic environment in parts of the cell, e.g. membranes, it should not be so surprising that enzymes can work in non-aqueous media. Growth in fuels and oils is also well-known and costs society considerable amounts each year. These fuel systems can be characterized as two-liquid-phase systems and in many instances they approach low water systems.

Biocatalytic reactions have been performed by mankind in the fermentation of sugar to beer by the Sumerians from 7000 B.C.[1] Fermentation processes have been utilized in most civilizations since. As early as 1898 the reverse action of yeast extract was demonstrated in the synthesis of maltose from glucose.[2] Two years later the reversibility of lipase action on ethyl butanoate was demonstrated. The lipase was extracted from pig pancreas.[3] Around 1920, alcohols were used as media in the synthesis of alkyl glucosides from glucose and the corresponding alcohol. The reaction was catalysed by extracts from almonds.[4,5] Further, in the 50's even diethyl ether has been used as a reaction medium in enzymatic degradation of phosphatidyl cholin, catalysed by phospholipase A present in commercial pancreatin.[6]

In 1958 a continuous two-phase system based on partition chromatography was reported. The enzyme was dissolved in an aqueous phase retained by a hydrophilic support, cellulose. The immobilized enzymes were β-glucosidase and invertase catalysing hydrolysis of β-glucosides and sucrose, as well as transferring glycosyl groups to the mobile phase (alcohol). The system described was by no account inferior to many systems reported today.[7]

During the 60's and 70's several studies on two-liquid-phase systems appeared. Xanthine oxidase was used in non-polar media.[8,9] For steroid conversion both enzyme/cofactor[10,11] and whole cells (*Nocardia* sp.)[12] were utilized in combination with an organic solvent in order to increase the solubility of the substrates.

The importance of water was recognized at an early stage in biocatalysis in organic media, however **the appropriate amount** has been the object of numerous discussions and papers. Introduction of water activity, to correlate enzyme activity and content of water in biocatalysis in non-conventional media, has become accepted fairly recently. This, however, seems quite surprising considering that scientists in agriculture and food-technology have used the correlation between relative humidity (water activity) and enzymatic activity (deterioration of food) for almost 30 years.[13,14] The lack of a contagious effect on scientists studying biocatalysis probably reflects the limited interaction between the two disciplines.

Despite some disagreement on certain aspects, the following advantages in using biocatalysis in organic solvents are now generally accepted:

1) Increased solubility of hydrophobic substrates and products.
2) Enhanced stability of biocatalyst.
3) Synthetic reactions being possible.
4) Reduction of unwanted side reactions caused by water.
5) Easier integration into other chemical steps.

With the recognition of the above advantages there has recently been an exponential growth of interest in this field, as reflected by a parallel increase in review articles on this topic.[15]

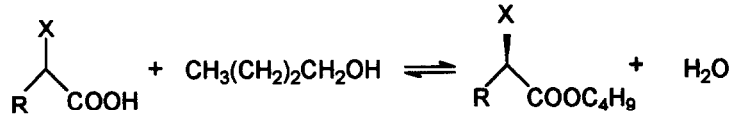
For the organic chemist it is probably the reverse action of the hydrolases that is the most attractive feature of biotransformations in organic media, e.g. synthesis of esters, lactones, amides, peptides, etc. in a chemo-, regio- and enantioselective manner. This aspect, as well as biotransformations in general, has been extensively covered recently.[16-18] This paper will only give a few examples of the most widespread biotransformations in organic solvents used by the organic chemist, namely reactions of hydrolases, otherwise the reader is referred to the references above.

Esterification/Hydrolysis

In ester synthesis an alcohol and a carboxylic acid are combined to form an ester and water catalysed by a hydrolase as shown in Scheme 1 (the genereal reaction is shown in 1a and a specific example in 1b). The opposite reaction, namely hydrolysis, is also often conducted in the presence of an organic solvent. With an immiscible solvent, a two phase system is created, which is beneficial to the lipases due to their interfacial activation. Addition of a miscible cosolvent is also quite common, however the amount is in this case more critical (Section 4).

Scheme 1 (Esterification)

1a $R^1COOH + R^2OH \rightleftharpoons R^1COOR^2 + H_2O$

1b 

(R)

X = Br, Cl, *p*-Cl-PhO

Several enzyme preparations are available from commercial suppliers[18] and new ones are regularly introduced to the market. The range of substrates (variation in R^1 or R^2) that has been successfully transformed in a chemo-, regio- or stereoselective way is very broad as can be verified by inspecting organic and bioorganic journals for the last 5-10 years. One example of this type of reaction is the kinetic resolution of racemic 2-halo carboxylic acids by esterification with 1-butanol in hexane and other organic solvents catalysed by lipase from *Candida rugosa* (Scheme 1b). This reaction afforded 95% ee for the *(R)*-ester at 42% conversion.[19] It is commonly stated that due to the production of water this process has limited application as high yields can never be achieved.

However it should be noted that 90-100% product was obtained in an esterification in a two-liquid-phase system consisting of 20% aqueous phase in hexane due to the extraction of the product into the non-polar phase.[20] This aspect is further discussed in the section considering the effect of water on the equilibrium position (Section 3.2).

Transesterification

In transesterification an ester and an acid, an alcohol, or another ester are combined. One possible way of dividing these reactions is into acidolysis, interesterification (Scheme 2) and alcoholysis (Scheme 3). Acidolysis and interesterification are often used in modification of triglycerides in order to obtain fat which resembles cocoa-butter (Scheme 2b).[21,22]

Scheme 2 (Acidolysis / Interesterification)

2a $$R^1COOR^2 + R^3COOR^5 \rightleftharpoons R^3COOR^2 + R^1COOR^5$$

2b $$\begin{array}{l} CH_2OCOR^1 \\ | \\ CHOCOR^2 \\ | \\ CH_2OCOR^3 \end{array} + R^4COOR^5 \rightleftharpoons \begin{array}{l} CH_2OCOR^4 \\ | \\ CHOCOR^2 \\ | \\ CH_2OCOR^3 \end{array} + R^1COOR^5$$

$R^{1,2,3,4}$= alkyl

R^5 = H (acidolysis)

R^5 = alkyl (interesterification)

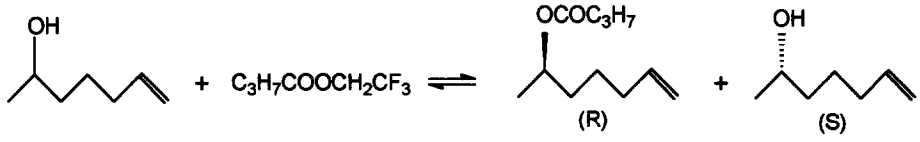
Alcoholysis (Scheme 3) is more frequently used by organic chemists when performing regio- and stereoselective biotransformations in organic solvents. Often no additional solvent is required as one of the substrates may also function as the reaction medium. In these reactions special acyldonors are often used to influence the reaction rate and/or the equilibrium position. Activated esters such as 2-haloethyl esters have been widely used.[19] In the resolution of 6-heptene-2-ol using trifluoroethyl butanoate an optical yield of 93% at 40% conversion was obtained (Scheme 3b).[23] The activated esters increase the reaction rate although their effect on the equilibrium position is not necessarily favourable.

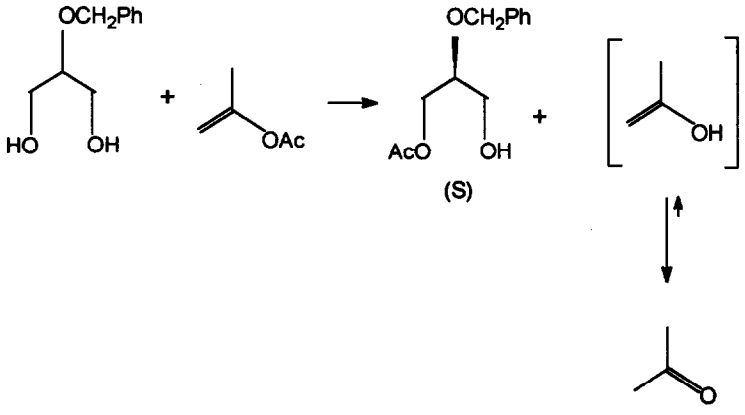
To manipulate the equilibrium position in order to maximize the yield, use of enol esters such as vinyl- or isopropenyl is popular. When the enols are liberated, they rapidly tautomerise to the corresponding volatile aldehyde or ketone. Besides the effect on the yield the reaction rate has

also been reported to increase.[24] In the reaction between 2-O-benzylglycerol and isopropenyl acetate catalysed by lipase from *Pseudomonas* sp., 96% ee for the *(S)*- ester was obtained at 72%

Scheme 3 (Alcoholysis)

3a $R^1COOR^2 + R^3OH \rightleftharpoons R^1COOR^3 + R^2OH$

3b 

3c 

conversion (Scheme 3c).[24] A drawback is that some enzymes seem to be sensitive to the acetaldehyde formed. However, means to avoid this problem have been addressed.[25]

The above examples are very few and the reader is referred to the previously cited reviews above for extensive examples. However, in order to draw full advantage of the potential of the field, biocatalysis in organic solvents, it is imperative to understand some of the basic features of these systems. It is the purpose of this paper to contribute to such an understanding.

2. GENERAL ASPECTS OF BIOCATALYSIS IN ORGANIC SOLVENTS

Most biocatalytic reactions in non-conventional media are multiphase systems with either an organic solvent, a gas or a supercritical fluid, in combination with an aqueous phase (Figure 1). The

biocatalyst will be confined to the aqueous phase. This phase can be of limited extent and sometimes only confined to the pores of the support material. Between the various phases are interfaces which can be of importance with respect to inactivation, mass-transfer and catalytic activity.[26]

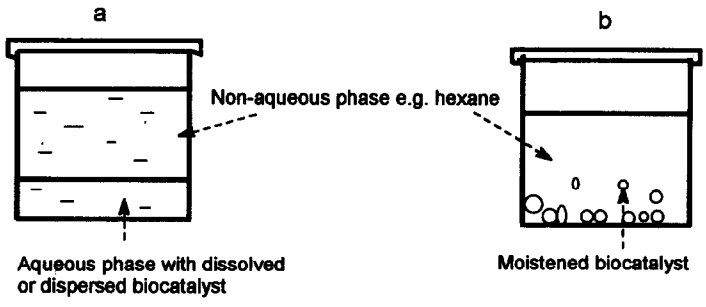


Figure 1

Two examples of multiphase systems with a) substantial aqueous phase b) aqueous phase confined to the pores of the biocatalyst

In these multiphase systems the distribution of species will normally be uneven between the different phases. To describe the amount of species in the various phases several approaches can be used. The use of concentrations is one way of treating this aspect. In a hexane/water-system it is clear that any polar species will have a high concentration in the aqueous phase and low concentration in the hexane phase, and vice versa for a very non-polar component. Any equation dealing with the total system will, in terms of biphasic concentrations, have a complex form and will vary with changes in the relative volume of the phases.[27]

For many purposes it seems reasonable to replace the use of concentrations with the activities. The activity (a_i) for a component (i) is related to the mole fraction (x_i) and the activity coefficient (γ_i) through the following equation:

$$a_i = x_i \gamma_i$$

Using pure water as the standard state implies that in aqueous solution the water activity, as well as the activity coefficient, is nearly unity. In general the value of the activity coefficients may range from 10^{-4} to 10^{4}.[28] A visual idea of water activity in organic solvents is that it indicates the intensity of interactions between water and solvent molecules.[29] A high interaction will be associated with low activity coefficients. Activities are, by definition, the same in all phases in equilibrium, assuming the same reference state is used. The partial vapour pressure can therefore

be used to calculate the activity for a component in the system, because in these systems ideal behaviour can be assumed in the gas phase.

Activity coefficients (and activities) can also be calculated from the partitioning (distribution) of a species between two liquid phases. Although in these phases non-ideality may be found, there exist data that make it possible to estimate the activity coefficients.[30] There are also models based on group contribution, such as UNIFAC[31] and ASOG[32], that make it possible to calculate the activity coefficients of the components in a liquid.

3. EFFECT OF WATER IN BIOCATALYSIS IN ORGANIC SOLVENTS

It has been shown that water is of special importance for biocatalytic reactions in non-conventional systems. A number of papers report reaction rate as a function of the amount of water added in biocatalytic reactions in non-conventional media.[33-42] etc. Recently, tritiated water was shown to be stripped from enzymes in organic solvents, the desorption appearing within a few minutes.[43] The following paragraphs discuss the effect of water on the biocatalyst, the hydrolytic equilibrium position and the kinetics.

3.1 Effect of Water on the Biocatalyst

Interactions between the enzyme molecules and the surrounding water are of crucial importance. Very often lowered reaction rate is caused by dehydration of the enzyme, however little is known about the process at the molecular level. It has been shown that the flexibility of the enzyme is related to the hydration state. Molecular events have been studied by gradual hydration of previously dried proteins. A stepwise hydration appeared with water first bound to charged and polar regions on the protein surface. Secondly, water condensed more generally and upon further addition of water, the mobility of the enzyme resembled that obtained in aqueous solution.[44]

Zaks and Klibanov[39] examined three unrelated enzymes, polyphenol oxidase, alcohol dehydrogenase and alcohol oxidase, in solvents of different hydrophobicity and water content. They showed that the activity of the enzymes was related to the amount of water bound to the protein, and not to the concentration of water in the solvents. These results have been replotted in terms of water activity in the reaction mixtures, confirming that optimum enzyme activity is related to water activity, independent of the solvent used.[45] Goderies *et al.*[46] showed that the relative humidity (i.e. water activity) was important for the orientation of the reaction process and for the yield. Recently there has been a growing acceptance for using either controlled or measured water activity.[47-53] The use of water activity as a means to describe the available water has been well known for many years in the food-industry.[14]

It is important to recognise that, even when optimum water content in terms of concentrations is achieved in a biocatalytic reaction, even small changes might change this optimum water content.[54] Some of the parameters that will influence this optimum are the solvent, reactants and support material:

Solvent

The solvent will compete with the biocatalyst for the water in the system and the ability of this is reflected in the solubility of water in the solvent. This aspect has been frequently reported.[39] The optimum water content in terms of water activity is, however, the same.

Reactants

When the nature or concentration of the reactants are changed, the requirements for water in the system will also change. An increase in concentration, or a change to a more polar component, will increase the water solubility in the bulk phase. This seems intuitively correct. However, if the changes in concentrations are conducted at the same water activity, a change in enzyme activity is not certain.[48] This aspect is probably reflected in the unchanged conversion rates, when increasing the substrate concentrations, in the presence of salt hydrates.[54] Salt hydrates may buffer water activity (see 3.4). The rate of transesterification of ethyl propionate catalysed by lipase from *Candida rugosa* was 48 times higher with nonanol than with heptanol in the case of dehydrated substrates, but only 2.2 times higher in the case of water saturated substrates (Figure 2).[48] This result demonstrates the necessity of considering the polarity of every substrate, because of its ability to modify the water partitioning between the solid phase (enzyme preparation) and the liquid phase.

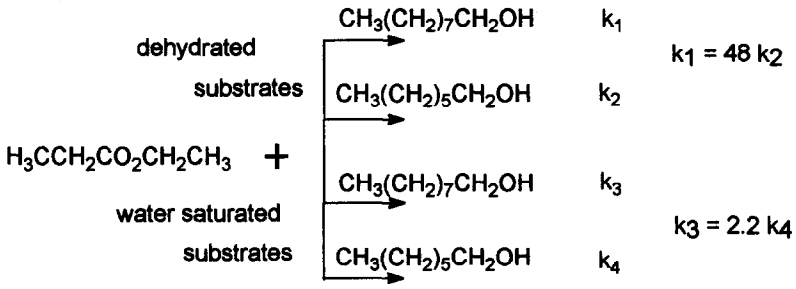


Figure 2

Effect of using water-saturated substrates on reaction rate in transesterification reaction catalysed by lipase from *Candida rugosa* [48]

Support materials

Support materials,[55-57] buffer salts[50] and additives to commercial enzyme preparations, such as sugar alcohols,[58] have also been shown to compete for the available water in the system.

Commercially available enzyme preparations as well as "home-made" preparations may vary in hydration state from batch to batch due to inconsistent drying procedures. During storage, change in hydration state may result from leaking seals and frequent opening of the biocatalyst containers. This will, at constant temperature, lead to different water adsorption.

3.2 Effect of Water on the Hydrolytic Equilibrium

In any reaction where water can take part, the water activity will predict the mass action of water on the equilibrium. This means that in a two-liquid-phase system, where the water activity is nearly unity, the equilibrium position in the organic phase will not change when the aqueous phase is reduced in volume (e.g. from 50% to 1%), as long as the activity of water and other species remains the same. However, if the content of water is reduced further, so that the water activity is reduced substantially, then an effect on the equilibrium position might be observed. The latter effect must not be confused with the equilibrium shift observed in two-liquid-phase systems due to favoured partitioning of the product into the organic phase (see 4.3).

A relevant examle has been reported in the transesterification of triglycerides with 1,3-specific lipases, where hydrolysis yielding diglycerides appeared always to be present. This hydrolysis was entirely dependent on the water activity, and it was not possible to create a water activity where enzymatic activity still persisted and at the same time avoiding the parasitic reaction producing diglycerides.[29]

3.3 Effect of Water on the Kinetics

Sometimes water may also affect the reaction rate by participating in the reaction, as well as through its effect on the hydration state of the biocatalyst.[59] Although unwanted, if water can take part in the reaction by e.g. attacking the acylenzyme in competition with another nucleophile (alcohol), it might be regarded as a competitive inhibitor. This has been reported in a chymotrypsin catalysed peptide reaction at known water activity.[60]

If the water level is very high undesirable hydrolysis of the product might be pronounced. However, this cannot account for reduced rate in initial rate measurements, but it will be of importance after some product has been formed, at which stage there may be a decrease in the overall net reaction rate.[26]

3.4 How to Control Water Activity (a_W)

There are several methods to set and control a fixed water activity in organic media and for further details the reader is referred to Ref. 61.

A common method for establishing a fixed water activity is by pre-equilibrating the biocatalyst, substrates, and solvent separately through vapour phase above saturated salt solutions (Figure 3a).[46] Different saturated salt solutions correspond to different water vapour pressures.[62] A limitation of this method is that only the initial water activity is set. If water is produced or consumed during the reaction or exchanged with air through leaking seals, the water activity will change. Even changes in the composition of the mixture as the reaction proceeds might change the water activity.

Another method to set the water activity is by the direct addition of salt hydrates into the reaction mixture (Figure 3b). Control of water activity by adding salt hydrates has been described for chymotrypsin catalysed reaction in the presence of $Na_2CO_3 \cdot 10H_2O$.[63] Later it was shown that salt hydrates were able to control water activity.[64] Studies on other enzymes, oxidase[65] and lipase,[54] have further confirmed the method. The background for using this method is discussed below.

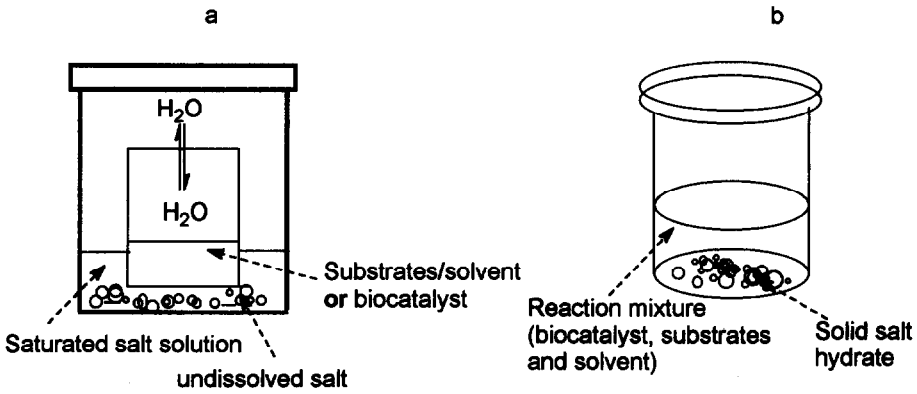


Figure 3

Examples of how to control water activity; a) separate pre-equilibration of substrates/solvent and biocatalyst b) direct addition of salt hydrate to reaction mixture

Ideally the chemical potential or activity of a solid phase is independent of the amount present. Thus a characteristic water vapour pressure will be found in equilibrium with the mixture of two hydrate forms of the same salt. Lists of salt hydrate pairs corresponding to different water activity have been reported.[66] It should be noted that the water activity obtained from the salt hydrate does not correspond to the water activity from the saturated solution of the same salt. During a reaction

where water is produced or consumed, the water activity will be maintained because the higher salt hydrate can release water whilst the lower can take up water. This means, for example, that when changing the amount of catalyst, which will change the requirement for water in the system, simply adding enough salt hydrates will ensure a constant value of water activity. Furthermore, when changing the solvent or increasing the concentrations of reactants, there is no need for separate experiments for water optimization, simply adding a "fair lump" of salt hydrates will satisfy the water requirements.[54] The accurate amount of salt hydrates needed can, however, be calculated from a simple water budget.[66]

Use of salt hydrates also provides a quick method to establish optimum water activity for an enzyme. This was performed for lipase P from *Pseudomonas fluorescens* and Lipozyme.[67] The results were in accordance with earlier work.[52]

3.5 Limitations to the Use of Salt Hydrates

There are some limitation to the use of salt hydrates:

* The salt hydrate must not react with any of the substrates. In the reaction between butanoic acid and butanol the use of $Na_2CO_3 \cdot 10H_2O$ resulted in a reaction between the salt and the acid, thereby proving that this salt was inappropriate in this particular reaction.[54]

* Temperature effects must also be considered. The water activity for a pair of salt hydrates normally increases with increasing temperature. Several salt hydrates also have relatively low melting points, e.g. $Na_2SO_4 \cdot 10H_2O$ and $Na_2CO_3 \cdot 10H_2O$ both have a melting point at 32°C. Above this temperature the salts will melt and form saturated salt solutions.

* Some salt hydrates show non-ideal behaviour and the following are effects that can be observed.[66]
  1) vapour pressure (e.g. water activity) above some salt hydrates varies smoothly with water content,
  2) significant effects of crystal size and/or shape can be observed,
  3) metastable intermediate hydrates or crystal forms may exist,
  4) final vapour pressure may depend on the direction from which it has been reached.

* For certain salt hydrates, even when the final equilibrium of the vapour pressure is correct, the rate at which it is reached may be too slow for the rate scale of the enzyme reaction (e.g. $CaSO_4 \cdot 2H_2O$).[54]

Despite these limitations, the use of salt hydrates to set and keep water activity is a very simple method and is regarded as a "real progress" in controlling the water activity.[68]

4. EFFECT OF ORGANIC SOLVENTS IN BIOCATALYSIS IN ORGANIC SOLVENTS

4.1 General

It is now generally accepted that enzyme catalysis can be performed in two-liquid-phase systems as well as in mainly organic solvents. Some empirical rules have emerged, e.g. that the more hydrophobic solvents are best due to their lack of water stripping from the enzyme.

There have been difficulties in finding a general solvent parameter that can be correlated to the enzyme activity. Laane *et al.*[69] have discussed various parameters such as Hildebrand solubility parameter (δ), dielectric constant (ε), and logarithm of partition coefficient of the solvent between octanol and water (log *P*). They concluded that the best parameter relating enzyme activity to solvent nature was log *P* and this parameter has been widely used since. Reslow *et al.*[70] were in general agreement, but found some discrepancies. Furthermore they showed that the inclusion of water solubility helped in their correlations. Valivety *et al.*[71] showed that the catalyst activity was best correlated as a function of both log *P* and either electron acceptance index or polarizability. Later no satisfactory correlation between the biocatalyst activity and log *P* was found.[72] The above results indicate that there is probably no single parameter for solvent polarity that will predict the enzyme activity in organic media.

4.2 Effect of Organic Solvent on the Biocatalyst

It seems reasonable that changes in the biocatalytic system will only affect the biocatalyst when they occur in the molecular vicinity of the enzyme. When dissolving an organic solvent in water, "immiscible" solvents will form a two-liquid-phase system at varying concentrations according to their solubility in water. This means that the biocatalyst in these systems will not experience a solvent concentration exceeding the solubility of the solvent in the aqueous phase. However, in these two-liquid-phase systems the biocatalyst will be exposed to the interface which may be harmful to it.[73]

For miscible solvents there is no limit to the solubility of solvent in the aqueous phase, and any concentration of solvent can be obtained in these systems. It has been shown that there is a threshold value of organic cosolvent addition where inactivation appears (Table 1).[74,75] It has been demonstrated quantitatively that non-polar solvents are not sufficiently soluble in water to reach the inactivation threshold concentration.[76] On the other hand a small addition of the miscible solvent to an aqueous phase does not harm the biocatalyst, indeed it may even increase the

activity.[15] In an esterification of aromatic amino acids catalysed by α-chymotrypsin in acetonitrile or 1,4-dioxane, it was observed that a small addition of water, sufficient to hydrate the enzyme, was beneficial to the activity. However, too much buffer resulted in decreased activity. Furthermore, it was observed that it was not the dissolved enzyme, but the *suspended* enzyme, that catalysed the reaction.[77] L-2-haloacid dehydrogenase has also been shown to be stable in anhydrous DMSO, but inactivated in a mixture of DMSO and water.[78] The recognition that a dry organic solvent is not so harmful to the enzyme stability is not of recent date. About one century ago dried enzyme extracts were shown to be stable in dry alcohol as opposed to alcohol in the presence of water.[2] Contrarily the stability of subtilisin BPN' was shown to be higher in 50% DMF in water than in anhydrous DMF.[79] However in the last example the enzyme reaction was stirred, and it is possible that the stirring could have influenced the result in the case of anhydrous DMF.[80] With the exception of the last example, the above results correspond to the two peak yield reported in peptide synthesis in a mixture of acetonitrile and water.[81] At low (about 10%) and high (more than 40%) water content the yield was high, however in between there was virtually no conversion.

Table 1

Effect of organic cosolvent (acetonitrile; trypsin and ethanol; α-chymotrypsin) addition on initial enzyme activity in miscible systems[74,75]

Enzyme	cosolvent (vol%)	relative activity (%)
trypsin	0	100
	45	100
	50	40
	60	10
α-chymotrypsin	0	100
	30	100
	40	.20
	>45	<10

It is more or less generally accepted that enzymes are more thermo-stable in low water media than in aqueous solutions. This has been explained by enhanced rigidity and that the covalent processes involved in irreversible inactivation, such as deamidation, peptide hydrolysis and cystine decomposition, require water. These processes are extremely slow in low water media.[82] Enhanced thermo-stability has been shown for porcine pancreas lipase,[83] ribonuclease, chymotrypsin and lysozyme[84] and tyrosinase.[65] For subtilisin BNP' in DMF the half-life depended very much on the pH from which the enzyme had been lyophilized.[85] For subtilisin Carlsberg the thermo-stability was less in ethanol with 4.8% water than in aqueous solution.[86] This result might be explained by the fact that subtilisin Carlsberg is soluble in high concentrations (up to 98%) of ethanol.

Very often a decreased activity in organic solvents is due to the water stripping effect of the solvent. When the effect of the organic solvent on the biocatalyst is considered the water stripping effect should be allowed for (see 3.1).

Kasche *et al.*[87] suggest that solvent effects on the reaction rate in enzyme catalysis in one- or two-liquid-phase systems can be of either *direct* or *indirect* nature (Figure 4). By *indirect* effects are meant partitioning of reactants and products, shift of equilibrium and mass transfer in two-phase systems. The *direct* effects are due to binding of organic (hydrophobic) molecules to the enzyme. It has been suggested that the only way to analyze direct solvent effects on the biocatalyst is by using saturated amounts of solvent in the buffer (one-phase system) or adsorbed enzyme on a hydrophobic surface in a two-phase system. Otherwise indirect effects will confuse the result. The effect of miscible solvents (DMF/DMSO) on the kinetics (amidase, esterase activity), the overall conformation (through fluorescence measurement) and the structure of the active site (through electron paramagnetic resonance spectroscopy, EPR) of trypsin has been studied.[88] It appeared that exceeding 10 % addition of solvent caused minor changes in the structure of the active site indicated by EPR spectroscopy, and the result correlated with a decline in K_{cat} of the amidase activity. It has been shown that at low phosphate concentrations in buffer systems saturated with solvents, soluble chymotrypsin is less affected by a polar solvent such as ethyl acetate, than a more hydrophobic one such as chloroform.[89]

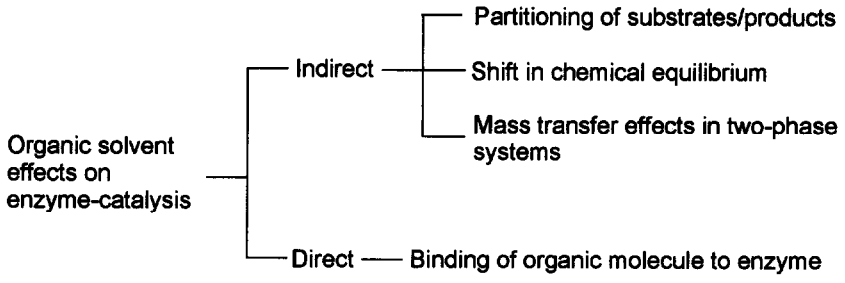


Figure 4

Examples of solvent effects on enzyme catalysis in one- or two-liquid-phase systems.[87]

The solvent effect on the biocatalyst is not independent of the nature of the enzyme. A study of the thermo-stability of invertase, glucose isomerase, α-amylase and lysozyme in H_2O compared to D_2O has been performed. The first three showed increased half-life in the deuterated water,

however lysozyme was not affected by the change in solvent.[90] Barbas *et al.*[91] reported that in the presence of a certain concentration of water-miscible organic solvent, the amidase activity of trypsin, chymotrypsin, papain and subtilisin was suppressed. However the esterase activity was not affected. These results may be partly explained in terms of K_m and partitioning.

4.3 Effect of Organic Solvent on the Equilibrium Position

The effect of the solvent on the equilibrium position of the biocatalytic reaction is independent of the biocatalyst present and should therefore be less complicated to predict. Estimations do not necessarily give a result that is obtainable within an acceptable amount of time, however they might be useful to avoid trying solvents that can never give a satisfactory yield.

For the reaction A+B $\rightleftharpoons$ C+D the yield can be found from K_x, an equilibrium constant in terms of mole fractions. This equilibrium constant, K_x, is related to an activity based equilibrium constant, K_a, which is independent of the solvent used. K_a is related to the activities (a_i), the activity coefficients (γ_i) and the mole fractions (x_i) through the following equation:

$$K_a = \frac{a_C\, a_D}{a_A\, a_B} = \frac{\gamma_C\, \gamma_D}{\gamma_A\, \gamma_B} \times \frac{x_C\, x_D}{x_A\, x_B} = K_\gamma\, K_x$$

In dilute solutions the mole fractions (x_i) become proportional to the concentrations and (as there is the same number of moles of reactants and products) the equation above can be rearranged and written as:

$$K_C = \frac{1}{K_\gamma}\, K_a = \frac{\gamma_A\, \gamma_B}{\gamma_C\, \gamma_D}\, K_a$$

where K_c is the equilibrium constant in terms of molar concentrations. This equation shows that K_c varies with the activity coefficients. As these are functions of the interaction between the solute and the solvent, they will vary in different solvents. The activity coefficients can be calculated using the UNIFAC group contribution method.[28]

It is also possible to estimate the change in solvent dependent equilibrium constants (K_{ci}) in terms of partition coefficients (P_i).[26] The equation relating this is:

$$K_{C1} = \frac{P_A P_B}{P_C P_D} K_{C2}$$

where the partition coefficients are defined for the concentrations in phase 2 divided by those in phase 1. The partition coefficients can be measured, calculated or found in the literature.[30]

An effect on the equilibrium position in peptide synthesis by using organic solvents as cosolvents has been reported.[92] Also in two-liquid-phase systems the equilibrium position is related to the reactants' partition coefficients.[93,94] This is an important point, as it is commonly and incorrectly believed that enzyme synthesis can only be performed in pure organic solvents. A two-liquid-phase system has been used in the synthesis of butyl butanoate, octyl 2-bromopropanoate and ethyl 2-bromopropanoate. Especially in the transesterification reactions between ethanol and octyl 2-bromopropanoate to octanol and ethyl 2-bromopropanoate, and vice versa, the effect of partitioning on the yield of ester in the organic phase is clear (Table 2).[95] Eggers *et al.*[96] have discussed the equilibrium position in terms of partition coefficients as well as activities. The effect of changing the organic solvent in some esterification reactions has been calculated theoretically to be of nearly 4 orders of magnitude.[97] Experimentally, an increase in equilibrium constant by a factor of 10 in the esterification of *N*-acetyl phenylalanine with ethanol, when the polarity of the organic media decreased (from ethyl acetate to heptane), has been reported.[72]

Table 2

Effect of partitioning on the yield of ester in a two-liquid-phase system consisting of 20 % aqueous phase in hexane [95]

Reactants	Ester product (%)
$H_3C(CH_2)_2CO_2H + H_3C(CH_2)CH_2OH$	~90
$H_3CCH(Br)CO_2(CH_2)_7CH_3 + CH_3CH_2OH$	~15
$H_3CCH(Br)CO_2CH_2CH_3 + CH_3(CH_2)_6CH_2OH$	~75

4.4 Effect of Organic Solvent on the Kinetics

Effect on Michaelis constant (K_m)

Generally there seems to be a change in K_m-values in both one- and two-liquid-phase systems in enzyme catalysis in organic media compared to an aqueous system. This change appears to be

related to the partitioning of the substrates. In a two-liquid-phase system the enzyme mainly experiences the substrate concentration in the aqueous phase, and the K_m (apparent) measured in the organic phase will be a function of the partitioning of the substrate between the two phases. This argument will be valid even if the aqueous phase decreases to a minimum, as well as in co-solvent systems. Several groups have reported changes in K_m-values that could be explained in terms of different partitioning of the substrates in the organic solvent. In the study of solvent effects on peroxidase catalysed conversion of phenolic substrates, the catalytic activity was reduced in organic solvents compared to water. This was manifested in increased K_m.[98] A 5000-fold increase of K_m in the fatty acid alcohol oxidase from *Candida tropicalis* was reported for the same substrate (dodecanol) when the reaction was carried out in octane compared to an aqueous system (Figure 5).[99] A decrease in K_m when changing from acetonitrile to toluene was reported in the papain-catalysed esterification of *N*-CBZ-glycine with methanol.[38] The results were explained in terms of better solubility of the substrate in hydrophilic solvents compared to hydrophobic ones. In the

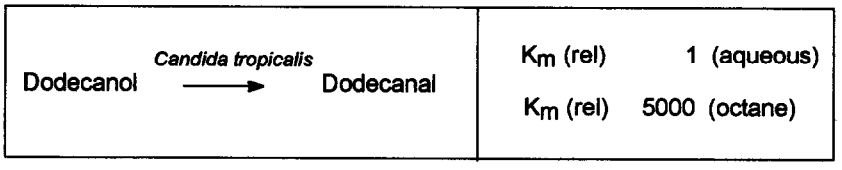


Figure 5

Effect of organic solvent on K_m in oxidation of dodecanol catalysed by fatty acid alcohol oxidase from *Candida tropicalis*[99]

esterification of *N*-acetyl phenylalanine with immobilized α-chymotrypsin, there was a trend in K_m value when changing from aqueous to organic media. Initially K_m increased, however, when the polarity of the media was further decreased, the opposite effect occurred, i.e. a decrease in K_m. The results were explained by the opposite partitioning of the substrate at the two extremes of the polarity scale.[72] No change in K_m was obtained for aminoacylases from porcine kidney or *Aspergillus* sp. in the deacetylation of *N*-acetyl-valine.[100] Finally, in a study of immobilized laccase suspended in water/organic mixtures of different compositions (low-water system), K_m was shown to exhibit a steep rise when water content increased. The same work also contained useful warnings in terms of misinterpretations of experimental data.[101]

From the above examples it appears that partitioning cannot account for all observed results. The remainder may therefore be due to *direct* effects on the biocatalyst.

Effect on activity, specificity and inhibition

The effect of solvent on activity and specificity is less clear and various explanations have been suggested, as may be demonstrated by the following examples:

Solvent effects on horseradish peroxidase had little influence on the catalytic turnover, in contrast to K_m, although changes in enzyme structure were observed through fluorescence spectra.[98] In the esterification of butyl stearate with laurinol catalysed by porcine pancreas lipase, the activity was halved in dodecane compared to that in benzene. This was explained by limited diffusion of the substrate into the enzyme surface due to too high hydrophobicity of the substrate.[102] In mushroom tyrosinase catalysed reaction the ratio of the partitioning of the product and substrate (P_p/P_s) was used to relate the organic solvent to the enzyme activity. This proved to be a better criterion than log *P*.[65] In papain catalysed reactions under a variety of conditions, substrate specificity was little changed from that in aqueous media.[38] In another protease catalysed reaction, the esterification of *N*-protected amino acids with methanol in acetonitrile and ethyl acetate catalysed by α-chymotrypsin, small variations in V_{max} were obtained when comparing the effect of two solvents.[103] Gololobov *et al.*[104] suggest that different factors are responsible for the specificity of enzymes in water and in mainly organic media. In the α-chymotrypsin catalysed acyl transfer in water, the specificity (nucleophile reactivity of amino acid amides) is correlated with the substrate specificity. In organic solvent (acetonitrile/DMF, 4% water) the specificity correlates with the size of the amino acid side chain.

For an organic chemist the enantioselectivity of the enzyme is probably one of the most interesting features. Several groups have reported variations in enantioselectivity when changing the solvent, as illustrated by the following examples.

In comparing hydrolysis (in buffer) of 2-chloroethyl esters of *N*-acetyl-L-and D-amino acids with the transesterification of the same substrate with propanol in butyl ether, a striking enantioselectivity was obtained in water and not in organic solvent.[105] Loss of enantioselectivity was observed in both hydrophilic solvents (dioxane and THF) and highly non-polar solvents (cyclohexane) compared to toluene, in the esterification of 2-hydroxy acids with primary alcohols.[106] In a study of the transesterification of 1-phenylethanol and vinyl butanoate catalysed by subtilisin Carlsberg in various solvents, enantioselectivity could be correlated with the dipole moment and dielectric constant of the solvent.[49] However, no correlation between enantioselectivity and physico-chemical characteristics of the solvent, such as hydrophobicity or dielectric constant, was found in the transesterifications between trifluoroethyl butanoate and (+/-)-sulcatol and (+/-)-3-bromo-5-hydroxymethyl isoxazoline catalysed by lipase from *Pseudomonas* sp. and porcine pancreas lipase in various organic solvents.[36] The above examples demonstrate that no clear relationship between solvents and enantioselectivity has yet been identified.

The solvent can also act as a specific inhibitor[74] as well as having an effect on the inhibition constants. In the production of cyclodextrines catalysed by cyclodextrin glycosyltransferase (CGTase) from unliquified corn starch in the presence of organic solvents, the yield was augmented 40% by addition of 5% of tertiary butanol. The increase in yield seemed to be due to a decrease of product inhibition of CGTase by forming an inclusion complex with cyclodextrins.[107]

5. CONCLUDING REMARKS

For the organic chemist enzyme catalysis in organic solvents has become an auxiliary tool in organic synthesis as discussed in the introduction. Results obtained are, however, sometimes inconsistent or negative. From the above examples it should be clear that there are many parameters that influence the observed results when performing enzyme reactions in organic solvents. The scientist often blames the catalyst, however the results may be due to small changes in the physico-chemical conditions of the reaction system. It is therefore essential to try to understand some of the basic features of these systems otherwise false conclusions may be drawn.

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