Part One Biocatalysis in Neat Organic Solvents – Fundamentals

1
Fundamentals of Biocatalysis in Neat Organic Solvents

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1.1
Introduction

It is well established that enzymes can express catalytic activity in predominantly organic media [1–8]. In some cases the reported catalytic activities in organic media are several orders of magnitude lower than those in aqueous solution. However, by careful selection of the type of enzyme preparation to use and the reaction conditions, it is often possible to achieve catalytic activities in the same order of magnitude as in water. Types of enzyme formulations for catalysis in organic solvents are treated in a separate chapter, while this chapter is largely concerned with reaction conditions for enzymatic catalysis in organic media. The most important points to consider are the solvent, the water content, and enzyme ionization effects (corresponding to pH effects in aqueous media).

1.2
Effects of Water on Biocatalytic Reactions

This book concerns the use of enzymes in non-aqueous media, which means that the major part of the medium surrounding the enzyme is non-aqueous. However, it is extremely difficult to remove water completely from enzymes. Even after extensive drying, a few water molecules remain tightly bound to the enzyme [6], and in most cases enzyme activity can be increased considerably by supplying an optimal amount of water to the system. It is thus very important to control the amount of water in the reaction mixtures in a proper way.

1.2.1
Quantification of Water in Low-Water Systems

The most straightforward way to quantify the amount of water in a reaction mixture is to use the water concentration (in mol L^{-1} or % by weight or volume). However, the properties of the enzyme (catalytic activity, etc.) are much more

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influenced by the amount of water bound to the enzyme than by the total water concentration [9]. Unfortunately, it is difficult to measure the amount of water bound to the enzyme directly. On the other hand, it has been found that the amount of water bound to the enzyme is largely influenced by the thermodynamic water activity (relative humidity) [10], which can be measured by sensors and controlled in reaction mixtures using methods described below. The water activity has been widely accepted as the best way to quantify water in low-water systems for enzymatic synthesis [1, 5]. At equilibrium, the water activity is the same in all phases. This means that it can be measured wherever practical, which often means in the gas phase.

1.2.2 Water Activity Control

In order to study the effects of water activity on an enzymatic reaction, there is a need for practical methods to adjust the water activity in the reaction mixture. Likewise, it is highly desirable to keep this parameter close to the optimal value during large-scale conversions. A range of water activity control methods have been developed [11], and which one to choose depends on the scale of reaction, the quantities of water to be removed or added, and availability of equipment.

1.2.2.1 Water Activity Control Using Saturated Salt Solutions

A water activity control method that does not require special equipment and is useful on laboratory scale involves equilibration with saturated salt solutions via the gas phase [11]. Since the solubility of a salt in water has a fixed value (at a fixed temperature), the saturated solution has a fixed water activity (and water concentration). Small containers with enzyme preparation or substrate solution can be put into larger containers that are partially filled with saturated salt solutions. No salt solution should enter the small containers, but water in the form of vapor should be allowed to move between the saturated salt solutions and the material in the small containers. After the pre-equilibration period, both the enzyme preparation and the substrate solution will have the same water activity as the saturated salt solution. After mixing, the rate at this water activity can be measured. The saturated salt solutions are used as "buffers" of the water activity. There should be some solid salt present which can dissolve if water comes into the system (for example, as a product formed in an enzymatic condensation reaction). On the other hand, if water is consumed in the system (for example in a hydrolysis reaction) some salt will crystallize from the solution. As long as there is both a saturated solution and solid salt, the water activity will be kept constant provided that the mass transfer is fast enough in the system. By using different salts, a range of water activities can be obtained (Table 1.1). If the reaction is slow, the equilibration through the gas phase as described above can be used to maintain the water activity, but if large amounts of water must be removed or added to the reaction at fixed water activity, a more efficient system is needed. One way to achieve this is to pump the saturated salt solution through silicone tubing immersed in the

Table 1.1 Saturated salt solutions suitable for water activity control. Values are given for 25 °C [77].

Salt	*Water activity*
LiCl	0.113
$MgCl_2$	0.225
K-acetate	0.328
K_2CO_3	0.432
$Mg(NO_3)_2$	0.529
$SrCl_2$	0.708
KCl	0.843
KNO_3	0.936
K_2SO_4	0.973

reactor [12]. The surface area of the silicone tubing can be chosen to match the water transport capacity required.

1.2.2.2 Water Activity Control Using Sensors

When it is important to control the water activity in a reactor, a water activity sensor is quite useful. The sensor should ideally measure the water activity in the liquid reaction medium. However, the sensors available are designed for gas phase measurements, and, provided there is effective enough equilibration between the liquid and gaseous phases, they can be used to control the water activity in the reactor. If the measured water activity is above the set point, drying is initiated, for example, by passing dry air through the reactor. On the other hand, if the water activity is too low, water can be added, either as liquid water or as humid air. Automatically controlled systems of this kind have been successfully used to monitor and control enzymatic reactions in organic media [13, 14].

1.2.2.3 Water Activity Control Using Pairs of Salt Hydrates

An alternative method for water activity control is based on the fact that salt hydrates containing different numbers of water molecules are interconverted at fixed water activities [15]. The first salt hydrate used was $Na_2CO_3 \cdot 10H_2O$. This is converted to $Na_2CO_3 \cdot 7H_2O$ at a water activity of 0.74 at 24 °C. The salt hydrates act as a buffer of the water activity. As long as both salt hydrates are present, the water activity remains at 0.74. If another water activity is desired, another pair of salt hydrates should be chosen. The salt hydrates can be added directly to the organic reaction mixture. One should be careful that the salt hydrates do not interfere with the enzyme or the enzymatic reaction.

1.2.3 Distribution of Water

During biocatalysis in organic media, the small amounts of water present are associated with various components of the system: dissolved in the solvent, bound

to the enzyme and bound to support materials and other additives that might be present. Water is exchanged between these different components, and at equilibrium the water activity is the same throughout the reaction mixture and the gas phase above it. Data on the amount of water associated to the various components as a function of water activity therefore helps in understanding the behavior of enzymes in such environments. The amount of water bound to the enzyme is described by the water adsorption isotherm. The adsorption isotherms of different enzymes are often relatively similar: a typical one is shown in Figure 1.1.

Support materials used for enzymes in organic media bind various amounts of water. An example of a support material that binds quite low amounts of water is Celite (Figure 1.2). It should be noted that additives, such as buffer salts, present in the enzyme preparation can bind substantial amounts of water (Figure 1.2).

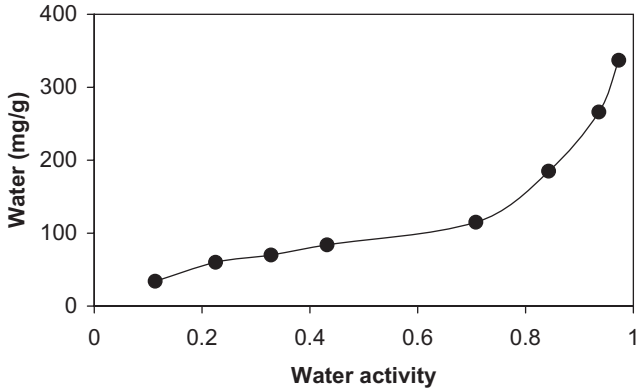

Figure 1.1 Water adsorption isotherm of α-chymotrypsin at 25 °C. Reprinted from Ref. [18].

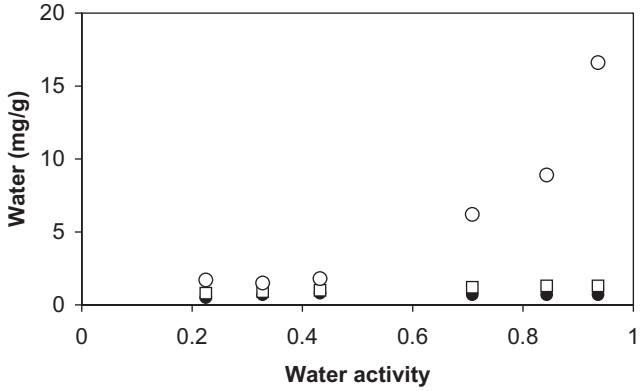

Figure 1.2 Water adsorption isotherms at 25 °C for Celite and preparations obtained by mixing Celite with different solutions (1.0 mL g^{-1} Celite) and drying. Pure Celite (●); Celite and α-chymotrypsin in water (4.0 mg mL^{-1}) (□); Celite and α-chymotrysin (4.0 mg mL^{-1}) in 50 mM sodium phosphate buffer, pH 7.8 (○). Reprinted from Ref. [18].

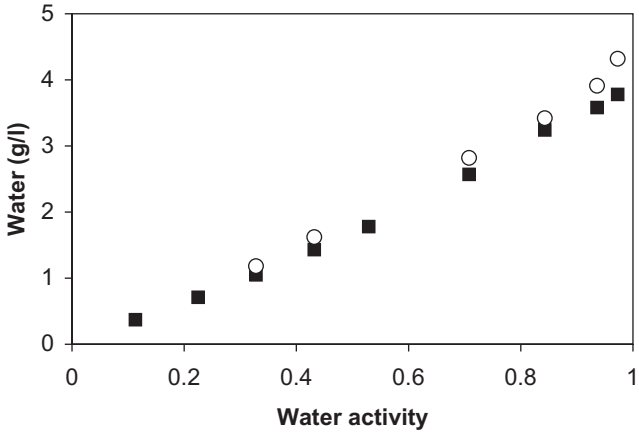

Figure 1.3 Solubility of water at different water activities in diisopropyl ether (■) and a substrate solution containing 10 mM Ac-Phe-OEt and 100 mM 1-butanol in diisopropyl ether (○). Reprinted from Ref. [18].

When working at fixed water concentration, the water-absorbing capacity of the support, called aquaphilicity [16], gives an indication of how well the support can compete with the enzyme for the water in the system. A high aquaphilicity means that the support absorbs a lot of water, leaving little for the enzyme, and this results in a low enzyme activity in most cases. When working at fixed water activity, more equal activities of enzymes on different supports are observed [17] although some differences still appear [18].

The solubility of water in a water-immiscible solvent at water activity 1 can be determined by equilibrating the solvent with pure water, followed by water analysis, for example, by Karl Fischer titration. At water activities lower than 1, lower amounts of water dissolve in the solvents, as shown in Figure 1.3. It should be noted that the solubility of water in the solvent changes when solvent composition is changed, for example, by dissolving substrates (Figure 1.3). In a hydrophobic solvent the increase in solubility of water can be substantial when substrates are dissolved in it.

The amounts of water associated with various components in a typical reaction mixture are shown in Table 1.2. Most of the water is dissolved in the reaction medium, and the amount of water bound to the enzyme is obviously just a minor fraction of the total amount of water. If the solvent was changed to one able to dissolve considerably more water and the same total amount of water was present in the system, the amount of water bound to the enzyme would decrease considerably and thereby its catalytic activity as well. Changing solvent at fixed water activity would just increase the concentration of water in the solvent and not the amount bound to the enzyme. Comparing enzyme activity at fixed enzyme hydration (fixed water activity) is thus the proper way of studying solvent effects on enzymatic reactions.

Table 1.2 Amounts of water associated with various components of a reaction mixture containing Celite-immobilized enzyme in diisopropyl ether at $a_w = 0.7$. Data from [18].

Component	*Amount of water (mg)*
1 mL diisopropyl ether with dissolved substrates	2.8
0.4 mg enzyme (α-chymotrypsin)	0.04
100 mg Celite	0.1
Buffer salts on the Celite	0.5
Total reaction mixture	3.44

1.2.3.1 Hysteresis Effects

Sometimes it can be difficult to know if the system has come to a true equilibrium concerning water distribution. It has been noted that water adsorption isotherms sometimes show hysteresis effects, which means that the water content, for example, that bound to the enzyme, depends not only on the water activity, but also on the hydration history [6]. More water is thus bound if a specified water activity is approached from a higher value (dehydration direction) than if the enzyme is hydrated from a drier state. The hysteresis effects might be due to slow conformational changes in the enzyme.

1.2.4 Water Effects on Activity

The catalytic activity of an enzyme in an organic medium often varies by several orders of magnitude depending on the degree of enzyme hydration [9, 19]. Control of enzyme hydration or water activity is thus a key issue when optimizing enzymatic conversions in organic solvents.

All enzymes to be used in organic media have at a previous stage been in an aqueous phase. They are then transferred to the organic medium, and this transfer process involves removal of water. This can be achieved by lyophilization or just drying of the enzyme solution, possibly in the presence of a support material or other additives. Another possibility is to dilute the aqueous enzyme solution with a water-miscible organic solvent which dissolves the water and causes the enzyme to precipitate. In one version, the enzyme solution also contains a crystal-forming solute such as an inorganic salt or an amino acid [20]. In this case, crystals are formed and the enzyme covers the crystals.

Lyophilization is a very common method to prepare enzymes for use in organic media, but the procedure often results in preparations having low catalytic activity. The method can cause inactivation of the enzyme both in the freezing step and in the drying step [7]. FT-IR spectroscopy has been used to study secondary structure in enzyme preparations, and lyophilization has been shown to decrease the α-helix content and increase the β-sheet content compared to native enzyme and

enzyme in aqueous solution [21]. The conformational changes during lyophilization can be prevented by lyoprotectants, such as substrate analogs or polyethylene glycol [22, 23]. Many other additives, including inorganic salts and crown ethers, have been used to improve enzyme activity after lyophilization, but methods to make enzyme preparations for use in organic media are treated in more detail in another chapter in this book and will not be discussed further here.

Rehydration of the enzyme preparation before use in organic media usually increases the catalytic activity considerably. Water is often called a molecular lubricant of enzymes in organic media [19]. Correlations between internal protein flexibility and degree of hydration have been shown using time-resolved fluorescence anisotropy studies [24] and electron paramagnetic resonance (EPR) spectroscopy [25]. A major effect of the increase in flexibility is probably that enzymes can reverse the conformational changes causing inactivation during lyophilization or other drying procedures for the preparation of the enzyme for use in organic media. In addition, increased flexibility can be beneficial for movements required in the catalytic process itself.

Relatively few detailed studies of enzyme kinetics in organic media have been carried out. Preferably, full kinetics should be studied, allowing the determination of K_m and k_{cat} values, but it is much more common to see just reports on the catalytic activity at fixed substrate concentrations as a function of water activity. That such studies can be misleading was shown in an investigation of lipase-catalyzed esterification [26]. When the reaction rate in the esterification reaction was plotted versus the water activity at three different substrate concentrations, maxima were obtained at three different water activities (Figure 1.4). Such maxima should not be used to claim that the optimal water activity of the enzyme was found. Detailed kinetic studies showed that both the k_{cat} and the K_m values (for the alcohol substrate) varied with the water activity. The K_m value of the alcohol increased with increasing water

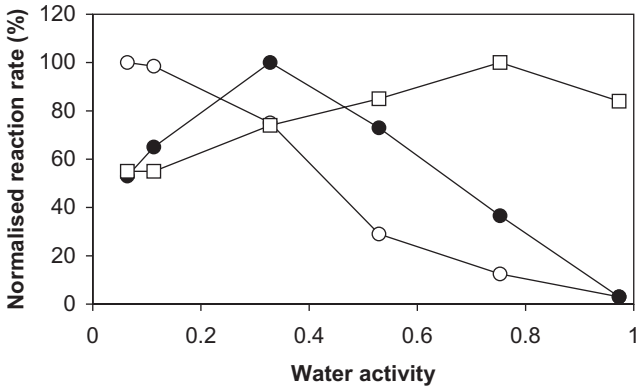

Figure 1.4 Normalized reaction rate as function of water activity for the esterification of dodecanol with decanoic acid catalyzed by *Rhizopus arrhizus* lipase at three different concentrations: 20 mM (○), 200 mM (●) and 800 mM (□) of each substrate. Data obtained from Ref. [26].

Table 1.3 Water activity at which the enzymes express 10% of their maximal activity.

Enzyme type	*Water activity*
Glycosidases	0.5–0.8
Lipases	0.0–0.2
Oxidoreductases	0.1–0.7

activity, mainly because water competed with the alcohol in the deacylation of the acyl enzyme. Variation of both substrate concentrations made it possible to estimate true k_{cat} values [26]. The maximal k_{cat} values were obtained at much higher water activities than the maxima observed at low substrate concentrations.

The response of enzymes to an increase in water activity varies considerably. There are examples of lipases that express considerable activity at water activities as low as 0.0001 [27], while many enzymes require considerably higher water activity. When comparing water activity dependence of enzymes it can be useful to compare water activities at which each enzyme expresses 10% of its maximal activity. Table 1.3 shows the results of a literature survey on this topic. It is clear that glycosidases require considerably higher water activity than lipases and that there is a large variation within the group oxidoreductases. In the majority of the experimental studies made, it has been found that enzymatic activity increases with increasing water activity. Maximal activity is often found quite close to a water activity of 1. As indicated above, this is the case also for most lipases, provided that measurements are made at high enough substrate concentrations.

It is not known which molecular features of lipases keep them active at low water activity. It is worth pointing out that when they are used to catalyze various reactions, such as hydrolysis, reversed hydrolysis, and transesterification reactions, the water activity dependence is similar in the different reactions [26, 28]. The same is true for phospholipase A_2 [29]. This shows that the effect of water on the enzyme is more important than the effect of water as a reactant when determining the reaction rate at different water activities.

1.2.5
Water Effects on Selectivity

When water molecules interact with an enzyme, it is natural that conformational changes can occur, which in turn can cause changes in the selectivity of the enzyme. Since enantioselectivity of enzymes is of major importance for many applications, it is a common task to investigate how to choose reaction conditions providing the maximal enantioselectivity. As might be expected, because water can interact with enzymes in many ways, it is difficult to generalize the effects. In some studies of lipase-catalyzed esterification reactions, no effects of water activity on enantioselectivity were observed [30]. In a similar study, no effects were observed in most cases, while the enantioselectivity of one lipase-catalyzed reaction decreased

with increasing water activity [31]. The enantioselectivity in reductions catalyzed by alcohol dehydrogenase from *Thermoanaerobium brockii* increased with increasing water activity in hexane [32]. The formation rate for both enantiomers of 2-pentanol increased with increasing water activity, but the formation of the S enantiomer was enhanced more, resulting in higher enantiomeric purity of the product. When even more water was added, forming a two-phase system with hexane, the enantioselectivity decreased [32]. A more detailed study showed that the formation of the S enantiomer was enthalpically favored in the whole range of water activities, while formation of the R enantiomer was entropically favored. In the competition between the two pathways leading to the R and S enantiomers, the enthalpy effect dominated, which resulted in the formation of an excess of the S enantiomer [33].

Another type of important selectivity is that between hydrolysis and transferase reactions (transesterification, transglycosylation, etc.) catalyzed by hydrolases. In this case, water can act both as a reactant and as a substance that modifies the properties of the enzyme. Effects of water as a reactant can be expected to be governed by the concentration or activity of water, as with other substrates. The effects of water as an enzyme modifier are considerably more difficult to predict.

The most straightforward way to quantify the competition between the transferase reaction and hydrolysis is to measure the initial ratio of these two reactions. Intuitively, one would assume the transferase/hydrolysis ratio to decrease with increasing water activity because of the effect of water as a reactant. This is often the case when lipases are used as catalysts [34–36]. However, in reactions catalyzed by glycosidases and proteases the transferase/hydrolysis ratio can either increase or decrease with increasing water activity [37, 38].

The competition between transferase and hydrolysis reactions can be described in terms of nucleophile (acceptor) selectivities of the enzymes, and selectivity constants can be defined. These constants are meant to quantify the intrinsic selectivity of the enzymes. Selectivity constants in combination with the concentrations (or thermodynamic activities) of the competing nucleophiles give the transferase/hydrolysis ratio. The selectivity constants are defined as follows [38, 39]:

$$\frac{r_s}{r_h} = S_c \frac{[\text{nucleophile}]}{[\text{water}]} \tag{1}$$

$$\frac{r_s}{r_h} = S \frac{a_{\text{nucleophile}}}{a_w} \tag{2}$$

where r_s and r_h are the rates of the transferase and hydrolysis reactions and S_c and S are the selectivity constants based on concentrations and activities, respectively.

The selectivity constants of glycosidases generally increase with increasing water activity [38], while those of lipases often decrease with increasing water activity [34]. The combination of high catalytic activity at low water activity and the good selectivity for transferase reactions at low water activity makes lipases very efficient transesterification catalysts [34]. When glycosidases are used for transglycosyl-

ation, it is surprisingly beneficial to use a water activity close to 1.0, which provides both high reaction rate and high selectivity for transglycosylation [38].

1.2.6 Water Effects on Stability

Water is often considered as the best solvent for enzymatic reactions. However, water is able to react in many ways with the enzyme, thereby causing its inactivation. Examples of such reactions include hydrolysis of peptide bonds in the enzyme, deamidation of amino acid side chains, and destruction of cystine residues [40]. Furthermore, the increased flexibility of the enzyme caused by water, which results in high catalytic activity, facilitates these inactivation reactions. It was thus realized that elimination of the major part of the water from the surroundings of the enzyme can cause pronounced stabilization compared to the situation in aqueous solution. It was shown that normal enzymes in organic media can express considerable activity even at 100 °C [41]. Several later studies have confirmed that enzyme stability generally decreases with increasing water activity [36, 42]. It should be pointed out that it is the absence of water that is important: other water-poor enzyme preparations, such as dry enzyme powders surrounded by gas, also exhibit high thermostability. When the *operational stability* of various protease preparations was studied, no conclusive effect of the water activity was observed when comparing water activities between 0.22 and 0.76 [43].

Since the catalytic activity of enzymes increases with increasing temperature, the possibility to use enzymes at high temperatures in low-water media might indicate that very high catalytic activities could be obtained. However, in one study of chymotrypsin-catalyzed reactions it was found that the reduction in catalytic activity due to the decrease in water activity was larger than the increase caused by the higher reaction temperature (Figure 1.5) [42]. Of course, there might be

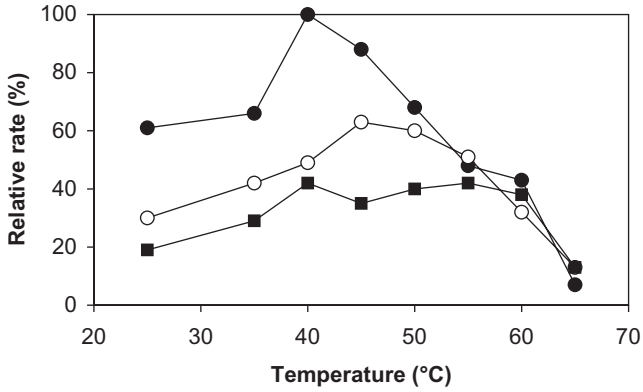

Figure 1.5 Relative activity of α-chymotrypsin in 5-methyl-2-hexanone at different temperatures and water contents: 100% water saturation (●), 75% water saturation (○), and 50% water saturation (■). Reprinted from Ref. [42] with kind permission of Springer Science and Business Media.

other cases where indeed higher overall catalytic activities can be obtained by a reduction in water activity combined with an increase in temperature.

1.3 Solvent Effects

Ever since it was discovered that enzymes can be catalytically active in neat organic solvents, the question of how to select the correct solvent for a specified enzymatic conversion has been of crucial importance. The solvent can influence an enzymatic reaction both by direct interaction with the enzyme and by influencing the solvation of the substrates and products in the reaction medium. An example of direct interaction between solvent and enzyme is when the solvent acts as an inhibitor of the enzyme. In other cases the solvent causes conformational changes in the enzyme, thereby changing its catalytic properties. The solvent can also influence the amount of water bound to the enzyme, but this effect can largely be avoided by the use of fixed water activity as described above. Direct interaction between solvent and enzyme can influence enzyme stability as well as activity.

A very common and important effect of the solvent on enzymatic reactions is that of affecting the solvation of the substrates and products of the reaction catalyzed. The solvation of the substrate influences its free energy and thereby its reactivity. Solvents which are able to dissolve a substrate very efficiently lower the free energy, and the rate of the catalyzed reaction is thereby decreased. The solvent also influences the equilibrium position of reactions, and here the solvation of both substrates and products must be considered.

Since there are so many solvents to choose from, it is natural that the search for guidelines for solvent selection has been intense. Researchers have tried to correlate enzyme activity, stability, and selectivity with different solvent descriptors, such as log P, dielectric constant, dipole moment, Hildebrand solubility parameters, and many others. When this approach is successful, the search for the optimal solvent can be limited to those having suitable values of the selected solvent descriptor(s). A list of solvent descriptors of a range of commonly used solvents is given in Table 1.4.

Below, solvent effects on activity and stability of enzymes will be discussed, while solvent effects on enzyme selectivity is a large topic which is treated in a separate chapter. Solvent effects on equilibria are treated in Section 1.4.

1.3.1 Solvent Effects on Enzyme Activity

It was early discovered that enzyme activity in organic solvents depends very much on the nature of the solvent. It was realized that the polarity or hydrophobicity of the solvent had a large influence, with non-polar hydrophobic solvents often providing higher reaction rates than more polar, hydrophilic solvents. When the kinetics of enzymatic reactions is studied, it is often found that K_m values in organic solvents are much higher than those in water for the corresponding

Table 1.4 Solvent descriptors of organic solvents commonly used for biocatalysis. Sw/o (solubility of water in solvent, wt%) So/w (solubility of solvent in water, wt%) and ε (dielectric constant) values from [78], log P (P = partition coefficient between octanol and water), ET (empirical polarity parameter by Reichardt-Dimroth) and HS (Hildebrand solubility parameter, $J^{1/2}cm^{-3/2}$) from [79].

Solvent	*log P*	*ε*	*ET*	*HS*	*Sw/o*	*So/w*
DMF	–1.01	36.71	0.404	20.3	100	
Methanol	–0.77	32.66	0.762	29.7	100	100
Ethanol	–0.31	24.55	0.654	26.1	100	100
1,4-Dioxane	–0.27	2.21	0.164	20.7	100	100
Acetone	–0.24	20.56	0.355	20.5	100	100
2-Butanone	0.29	18.51	0.327	19	10	24
Pyridine	0.65	12.91	0.302	21.7	100	100
Ethyl acetate	0.73	6.02	0.228	18.6	2.94	8.08
1-Butanol	0.88	17.51	0.506	23.7	20.5	7.45
Diethyl ether	0.89	4.2	0.117	15.1	1.47	6.04
Diisopropyl ether	1.52	3.88	0.102	14.4	0.57	1.2
Butyl acetate	1.7	5.01		17.4	1.2	0.68
Benzene	2.13	2.27	0.111	18.7	0.0635	0.179
1,1,1-Trichlorethane	2.49	7.25	0.17	17.4	0.034	0.132
Toluene	2.73	2.38	0.099	18.2	0.0334	0.0515
Hexane	3.98	1.88	0.009	14.9	0.0111	0.00123
Heptane	4.57	1.92	0.012	15.2	0.0091	0.000357

reactions. These effects are due to the effective solvation of the substrate in the organic solvent [44], reducing its free energy, so that the free energy of activation of the enzymatic reaction increases, resulting in a lower reaction rate. The reaction rate can as usual be increased by increasing the substrate concentration. However, if the substrate concentration is fixed at a moderate level, one should avoid solvents able to dissolve much higher substrate concentrations or else there is a severe risk that the reaction rate will be low due to the increase in apparent K_m value.

One way of measuring the solvation of a substance in a range of solvents is to study the partitioning of the substance between those solvents and a standard solvent which is immiscible with the others. The partitioning of two different substrates between water and a range of water-immiscible solvents was thus studied and was correlated with the relative rate of enzymatic conversion of these two substrates [45]. In this case, solvent effects on enzyme *specificity* were studied, but the same principles apply to rates of enzymatic conversions of single substrates. It is useful to contemplate the situation when an enzyme is acting in a two-phase system consisting of water and the organic solvent under study. The partitioning of the substrate between the phases will influence the substrate concentration in the aqueous phase, which in turn determines the reaction rate. A

solvent that very efficiently extracts the substrate from the aqueous phase will thus lower the aqueous substrate concentration and thereby lower the reaction rate (unless the aqueous substrate concentration is much higher than the K_m value). The same kind of reasoning can be used even though the aqueous phase is small or even non-existent. What really matters is the partitioning of the substrate between the bulk solvent and the active site of the enzyme, and this is not influenced by the introduction of an aqueous phase in between.

Partitioning between water and water-immiscible organic solvents is thus a straightforward way to get quantitative data for predicting solvent effects on the conversion of a certain substrate. However, the method is not applicable to water-miscible solvents. An alternative way to quantify solvation is to carry out theoretical calculation of interactions between the various components in the reaction mixture. The most frequently used method to do this is the UNIFAC group contribution model which can be used to calculate activity coefficients of all components in mixtures [46]. In this model, a molecule is treated as the sum of its different building blocks, such as methyl groups, carbonyl groups, etc. To calculate the difference in solvation between the two substrates N-Ac-L-Phe-OEt and N-Ac-L-Ser-OEt, activity coefficients of methanol and toluene in a range of organic solvents were calculated. Methanol and toluene represented the amino acid side chains of the substrate molecules, which otherwise were identical. There was a good correlation between the ratios of reactions rates and the ratios of the partition coefficients calculated from activity coefficients in the different solvents [47] (Figure 1.6). The UNIFAC model is under constant development to increase the range of compounds that can be handled and to improve the accuracy [48]. Some version of it is included in several calculation software packages.

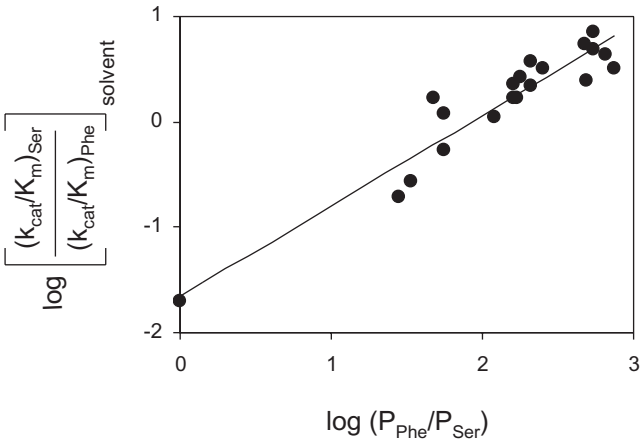

Figure 1.6 Dependence of the substrate specificity of subtilisin Carlsberg on the calculated ratio of the solvent-to-water partition coefficients of N-Ac-Phe-OEt and N-Ac-Ser-OEt in various solvents. Reprinted with permission from [47]. Copyright (1993) American Chemical Society.

When substrate activities are used instead of substrate concentrations in studies of enzyme kinetics in organic media, solvent effects due to substrate solvation disappear. Remaining solvent effects should be due to direct interactions between the enzyme and the solvent. In a study of lipase-catalyzed esterification reactions, it was found that K_m values based on activities were indeed more similar than those based on concentrations in different solvents, but still some differences remained [49].

As discussed in Section 1.2.3, it is crucial that the effects of solvents are studied at fixed water activity, or else indirect effects due to competition for water between enzyme and solvent will cause strong effects and mask the true solvent effects. In general, when correcting for substrate solvation, hydrophobic solvents seem to give higher rates than other solvents [5].

1.3.2
Solvent Effects on Stability

Enzyme stability in organic solvents depends on the direct interactions between enzyme and solvent. In addition to water, solvents like DMSO, formamide, and ethyleneglycol are able to dissolve proteins [50, 51]. A thorough study on the solubility of lysozyme revealed several other polar solvents able to dissolve more than $10\,g\,L^{-1}$ of the enzyme, including glycerol, 2,2,2-trifluoroethanol, methanol, and phenol [51]. Recent studies have shown that enzymes can also be dissolved in ionic liquids that are good H-bond acceptors, such as those containing acetate, lactate, or nitrate as anions [52]. In the majority of all these cases, dissolution results in inactivation of the enzyme due to disruption of its tertiary and sometimes also secondary structure. However, there are interesting exceptions. Lysozyme can fold correctly in glycerol [53]. Furthermore, morphine dehydrogenase was found to be catalytically active when dissolved in the ionic liquid 1-(3-hydroxypropyl)-3-methylimidazolium glycolate [54]. In organic solvents other than those mentioned, enzymes are practically insoluble. However, these undissolved enzyme preparations are often catalytically active and very useful for synthesis. The fact that the enzyme is present in a solid phase makes it easy to separate the reaction product from the enzyme, which simplifies both product purification and enzyme re-use.

Organic solvents can interact with enzymes in several ways. There can be specific interactions between isolated solvent molecules and enzyme molecules. This kind of interaction also occurs in water containing dissolved solvent molecules. When a separate organic solvent phase is present, interfacial inactivation can also occur. This is sometimes called phase toxicity [55] to distinguish it from the molecular toxicity of isolated solvent molecules. Interfacial inactivation can be studied in detail by bubbling solvent through an aqueous enzyme solution under controlled conditions [56].

In most experimental studies of the influence of organic solvents on enzyme stability, the remaining catalytic activity after exposure to different solvents has been measured. In such a study it was found that the remaining activity of a

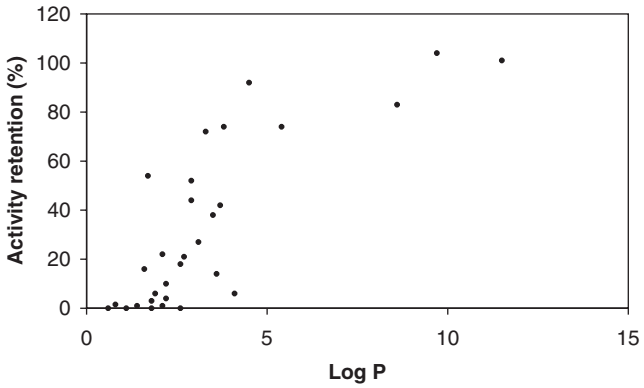

Figure 1.7 Activity retention of immobilized cells catalyzing an epoxidation reaction after exposure to organic solvents with different log P values. Data from Brink and Tramper [76] plotted according to Laane et al. [57]. Reprinted with permission of Wiley-Liss, a subsidiary of John Wiley and Sons, Inc.

whole-cell biocatalyst catalyzing epoxidation reactions correlated well with the log P value of the solvent (Figure 1.7) [57]. Solvents having log P values above 4 caused negligible inactivation, while those with log P values below 2 were highly inactivating. In the intermediate range of log P values a large scatter in the results was observed, making generalizations difficult. Similar results have been obtained in several subsequent studies, and log P is the most frequently used solvent parameter in this kind of investigations. Among studies giving contradictory results, one investigation of two glycosyl hydrolases and one phosphatase can be mentioned [58]. In this study, solvents with high log P values, like n-alkanes between n-octane (log P = 4.5) and n-hexadecane (log P = 8.8), were highly inactivating, while good stability was observed with butyl acetate having a log P value of 1.7. It is not surprising that a single solvent descriptor, such as log P, cannot give good predictions for all combinations of solvents and enzymes. In an attempt to create a more accurate model for predicting the inactivation of enzymes by solvents, principal component analysis was applied and 14 solvent descriptors were taken into account, resulting in good correlation between predicted and observed remaining activity of free and Celite-immobilized horse liver alcohol dehydrogenase [59]. A slightly worse model was obtained using only three solvent descriptors (log P, dielectric constant, and melting point), but a correlation coefficient of 0.957 between predicted and observed remaining activity was still obtained [59].

Large differences in sensitivity toward interfacial inactivation were observed between α-chymotrypsin and *Candida rugosa* lipase [56]. The lipase was most rapidly inactivated by 1-butanol and tolerated the hydrophobic hydrocarbons quite well, while the opposite was true for α-chymotrypsin. A detailed study of interfacial inactivation by 12 different solvents, all having log P values around 4, revealed

large differences between the solvents and also between the different enzymes studied [60]. The previously mentioned tendency of hydrocarbons to cause interfacial inactivation of α-chymotrypsin was confirmed. For both α- and β-chymotrypsin the interfacial inactivation increased with increasing interfacial tension of the solvents [60]. Proteins which are rigid and less prone to structural changes are less sensitive to interfacial inactivation, ribonuclease and papain being typical examples [60].

In conclusion, most protein-dissolving solvents cause enzyme inactivation, and, among the other solvents, those having high log P values are less inactivating than others in many cases. However, the chemical nature of the solvents is certainly also of importance, and there are clearly large individual variations between different enzymes.

1.4 Effects on Equilibria

When choosing reaction conditions, such as water activity and solvent, for an enzymatic reaction, possible effects on the equilibrium position of the reaction should be considered. When the aim is to produce an equilibrium mixture as the final product, the position of this equilibrium is of course of vital importance. It is, however, also important that the biocatalyst expresses sufficient catalytic activity under the conditions used, so that equilibrium is reached within a reasonable time. In practice, it often happens that a compromise must be made between high reaction rate and high equilibrium conversion.

The thermodynamic equilibrium constant of reaction A + B ⇆ C + D is defined as follows:

$$K = \frac{a_C \cdot a_D}{a_A \cdot a_B} \tag{3}$$

The value of K does not depend on the medium composition, and in principle this is the only equilibrium constant needed. However, since activities of substrates and products are often hard to get at, concentration-based equilibrium constants are often used instead. Concentrations can, for example, be expressed as molar ratios (x_A, etc.). For each substrate or product, mole ratio and activity can be interconverted using activity coefficients (γ_A, etc), where $a_A = \gamma_A \cdot x_A$.

The different equilibrium constants are thus related as follows:

$$K = K_\gamma \cdot K_x = \frac{\gamma_C \cdot \gamma_D}{\gamma_A \cdot \gamma_B} \cdot \frac{x_C \cdot x_D}{x_A \cdot x_B} \tag{4}$$

where K_x is the molar ratio-based equilibrium constant and Kγ is the combination of activity coefficients shown in the formula. The effects of the medium on the concentration-based equilibrium constant are described by the activity coefficients.

For reactions of the type acid + alcohol ⇆ ester + water, catalyzed by hydrolases, an equilibrium constant involving both concentrations and the water activity has been suggested [61]:

$$K_0 = \frac{[\text{ester}] \cdot a_w}{[\text{acid}] \cdot [\text{alcohol}]} \quad (5)$$

The reason for using an equilibrium constant like this is that the water activity is easily measured and/or controlled in the reaction mixtures and is often fixed to provide good conditions for the enzymatic reaction. Concentrations are more practical to use than activities for the other reactants.

1.4.1 Water Effects on Equilibria

One of the most common reasons for using low-water media for enzymatic reactions is that one wants to use a hydrolase for catalyzing reactions other than hydrolysis. In low-water media these enzymes can be efficiently used to catalyze reversed hydrolytic reactions and various types of tranferase reactions.

It is clear that the water activity is of crucial importance for the equilibrium yield in a reversed hydrolysis reaction. As expected, the equilibrium yield increases with decreasing water activity. This has been shown, for example, for the condensation of glucose and octanol [62], esterification of lysophospholipids with fatty acids [29, 63], and in normal lipase-catalyzed esterification reactions [64, 65]. The same situation is observed in ionic liquids [66].

In addition to its direct influence via the water activity in the system, the amount of water also influences the activity coefficients of the other components in the mixture, and therefore equilibrium constants like K_0 can vary with the water activity in the system (Table 1.5) [29, 63, 64]. This can be seen as a solvent effect on the equilibrium constant. The tendency in esterification reactions is that K_0 increases with decreasing water activity, which means that it is favorable to use low water activity because of both the direct effect of water activity on the equilibrium and the influence of water on K_0.

Table 1.5 Equilibrium yield and equilibrium constants (K_0) in the synthesis of phosphatidylcholine from lysophosphatidylcholine and oleic acid (800 mM) at different water activities. Data from Ref. [29].

Water activity	*Equilibrium yield (%)*	*K_0 (M^{-1})*
0.22	37.2	0.163
0.33	29.9	0.176
0.43	20.8	0.141
0.53	13.4	0.102

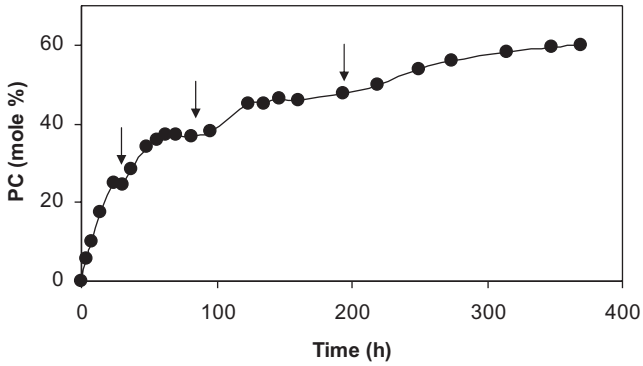

Figure 1.8 Time course of the synthesis of phosphatidylcholine from lysophosphatidylcholine and oleic acid catalyzed by phospholipase A_2. The water activity was initially 0.43 and was decreased stepwise to 0.33, 0.22 and finally to 0.11 (at times indicated by arrows). Reprinted with permission from Elsevier [29].

Reversed hydrolysis reactions should ideally be carried out at a water activity close to 0. One reason for not obeying this general rule is that many enzymes require a water activity considerably above 0 to express reasonable catalytic activity (see Section 1.2.4). Therefore compromises are often made between high activity and high yield. In order to optimize the synthesis reaction, it can be worthwhile to start the reaction at a relatively high water activity to achieve a high reaction rate and then stepwise or continuously decrease the water activity to obtain a high final yield (Figure 1.8) [29].

It is worth pointing out that the equilibrium positions in many esterification reactions in hydrophobic solvents are quite favourable, so that high yields can be obtained even if the water activity is close to 1. In these cases, it is the effective solvation of the ester product in the medium that is a main driving force the reaction. This is further discussed below.

1.4.2 Solvent Effects on Equilibria

Solvent effects on enzymatic reactions have been most thoroughly studied for esterification reactions. It has been observed that those reactions are favorably carried out in relatively hydrophobic solvents, while the equilibrium position is less favorable for esterification in more hydrophilic solvents. Correlations between equilibrium constants and solvent parameters have been evaluated. It was shown that the solubility of water in the solvent ($S_{w/o}$) gave better correlation with esterification equilibrium constants than log P and other simple solvent descriptors [61].

Solvent effects on esterification equilibria have also been described using UNIFAC calculations of activity coefficients. This method was claimed to give

somewhat better correlation than the one with solubility of water ($S_{w/o}$) [67]. A drawback with the UNIFAC method is that it does not consider the location of the various groups in the molecules and therefore it cannot account for the influence from neighboring groups. An alternative method to determine activity coefficients is the COSMO-RS method. This is based on the approximated continuum description of a solvent [68]. It was shown to give somewhat better correlation with experimental results for esterification reactions than the UNIFAC method [68].

1.5 Effects of pH in Organic Solvents

When enzymatic reactions are carried out in aqueous solutions, it is common practice to incorporate buffering species that keep pH constant. The reason is that the pH of the solution influences the ionization of important functional groups in the enzyme and thereby has a direct impact on the rate of the enzymatic reaction. Adding or removing a proton can turn the enzyme on or off or at least change the activity drastically. In a similar way, the ionization of these groups is important when enzymes are used in organic solvents. The buffers that are used in aqueous media are insoluble in most organic solvents, so there is a need for other methods to control the "pH" in such reaction media. The questions concerning "pH" control in organic media have been investigated by Halling and coworkers, and useful guidelines have been presented [69]. When ions cannot move freely in the medium, counter ions will also have an influence on the protonation of ionizable groups. Two separate equilibria are of importance:

1. Exchange of hydrogen ions and cations, such as sodium ions, with acidic groups of the enzyme
2. Transfer of both hydrogen ions and anions, such as chloride, onto basic groups of the enzyme.

In many cases, an enzyme in an organic solvent keeps the ionization state from the aqueous solution in which it was present before removal of water and transfer to organic medium. This is sometimes called the "pH memory" of enzymes in organic media [41]. If the reactions catalyzed do not involve the formation or consumption of acidic or basic substances, it can be sufficient to use a suitable pH value in the aqueous solution used to prepare the enzyme. In most of the procedures used, the enzyme will also keep some counter ions and other ions around it, and some of those will act as pH buffers to some extent. In cases when more pH buffering capacity is needed, it is worthwhile to consider the use of buffers suitable for organic media. Some buffers are soluble in organic media, triphenylacetic acid and its sodium salt being a typical example of a pair controlling the exchange of hydrogen ions and cations [70, 71]. A corresponding pair controlling exchange of hydrogen ions and anions is tri-*iso*-octylamine and its hydrochloride [70]. These buffers are soluble mainly in relatively polar organic solvents, like

pentanone and acetonitrile. Dendritic polybenzyl ether derivatives have been developed as alternatives suitable for more hydrophobic media [72].

A drawback of using organo soluble buffers for pH control is that in order to obtain the reaction product in a pure form after the enzymatic reaction, the buffer substances must be removed, which complicates the procedure. The use of solid-state buffers for organic media has thus been proposed, lysine and its hydrochloride being a typical example [73]. In addition, a wide range of "biological buffers" such as PIPES, MOPS, TES, HEPES, HEPPSO, TAPS, and AMPSO have been used in combination with their sodium or potassium salts [74]. Transfer of ions between the solid-state buffer and the enzyme can be slow in hydrophobic solvents, resulting in lag phases of up to 30 min [69].

1.6 Concluding Remarks

In many cases it is easy to detect some catalytic activity of an enzyme in an organic solvent. However, in order to get practically useful reaction rates there is normally a need to design the enzyme preparation and the reaction conditions with much care and thought. Hopefully, this chapter and its references can help in this task. A more extensive collection of practical advice on this topic can be found in [75].

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