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Interrelations of Chemistry and Biotechnology—V†

# BIOCATALYSIS IN NON-CONVENTIONAL MEDIA: MEDIUM ENGINEERING ASPECTS

(Technical Report)

*Prepared for publication by*

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# Biocatalysis in non-conventional media: Medium engineering aspects (Technical Report)


## ABSTRACT

During the past decade much progress has been made in the fundamental understanding of the phenomena that govern biocatalysis in non-conventional media. The factors that affect biocatalytic reactions and the activity and stability of biocatalysts in these reaction media are generally associated with the crucial role of water and the need to keep biocatalysts in their active conformation. For the rational design of biocatalytic processes in the reaction media, discussed in this review, some basic rules have been formulated. These rules may serve as useful tools for future optimization of biocatalysis in non-conventional media and for engineering media for synthetic purposes.


## INTRODUCTION

Integration of biotransformation steps in synthetic chemistry is gaining much scientific as well as industrial interest (Laane *et al.*, 1987a, Tramper *et al.*, 1992). The specificity and selectivity of biocatalysts make them very attractive as possible catalysts for reactions which are difficult and/or expensive to carry out by chemical means. For a long time applications of biotransformations in synthetic routes have been hampered by the general idea that biocatalysts (enzymes and whole cells) are only active in aqueous solutions and under mild conditions. Recently, it has become clear that biocatalysts are not so sensitive as expected. They can function under harsh conditions, such as extreme pH, temperatures and pressures, high salt concentrations, or in the presence of other additives (Monsan and Combes, 1984). They are also found to be active in all sorts of non-conventional media, such as organic solvents, aqueous two-phase systems, solid media, gases and supercritical fluids (Tramper *et al.*, 1992). These findings drastically increase the applicability of biocatalysis in organic synthesis.

In this review we will focus on the latest progress made in medium engineering and its effect on both activity and stability of the biocatalyst. Medium engineering not only involves the substitution of aqueous reaction media by non-conventional media, it also implies the adaptation of the microenvironment of the biocatalyst by immobilization or by introduction of additives for stabilization of the biocatalyst.

The non-conventional media dealt with in this review are organic solvents and supercritical fluids. Other non-conventional media, which will not be discussed here, are aqueous two-phase systems and solid and gaseous media. Aqueous two-phase systems are formed, when two solutions of water-soluble polymers or a concentrated salt solution and a polymer solution are mixed. The physical characteristics and the effects of phase components, pH, temperature etc., as well as several biotechnological applications of these systems have recently been reviewed by Andersson and Hahn-Hägerdal (1990) and King (1992). The latest advancements in biocatalysis in gaseous and solid media have recently been discussed by Robert *et al.* (1992) and Lamare and Legoy (1993).

## POTENTIAL OF BIOCATALYSIS IN ORGANIC SOLVENTS

There are several potential advantages for the introduction of organic solvents in synthetic reactions. Organic solvents will increase the solubility of poorly water-soluble substrates, thereby improving the volumetric productivity of the reaction. The thermodynamic reaction equilibrium may be shifted to favor synthesis over hydrolysis, either by altering the partitioning of the substrate/product between the phases of interest, or by reducing the water activity. The latter can be achieved by replacing the water in the reaction mixture by a water-miscible organic solvent, or by introduction of polymers, sugars, or salts. Reduction of the water activity or the associated water content will also diminish water-dependent unwanted side reactions such as polymerization of oxidized phenols (Kazandjian and Klibanov, 1985), or hydrolysis during transesterification reactions (Dordick *et al.*, 1986). In addition, higher product yields will be achieved by reduction of substrate and/or product inhibition, either indirectly by maintaining a low concentration in the aqueous micro-environment of the biocatalyst (Schwartz and McCoy, 1977, Vermuë and Tramper, 1990), or directly by changing the interactions between the inhibitor and the active site of the enzyme (Zaks and Klibanov, 1988a). Application of low-boiling organic solvents will simplify recovery of the product and biocatalyst. The biocatalyst does not dissolve in the solvent and can thus easily be recovered from the reaction mixture, for instance by filtration, while the product can be obtained by evaporating the solvent, provided there is sufficient difference in boiling point. Other advantages of enzymatic catalysis in organic solvents are the improved thermostability of the enzyme, particularly when microaqueous reaction media are used (Zaks and Klibanov, 1984, Volkin *et al.*, 1991) and the possibility to manipulate the stereo- and regio-selectivity of the enzyme in such media (Sakurai *et al.*, 1988, Klibanov, 1990a).
Obviously not all these advantages are relevant to all the categories of organic-solvent reaction media and reactions, and of cource disadvantages of using organic solvents in biocatalysis also exist, e.g. the organic solvent may denaturate or inhibit the biocatalyst. In addition, introduction of an organic solvent into the reaction mixture increases its complexity.

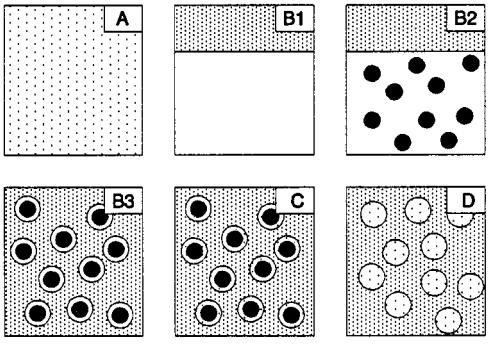


**Figure 1**: Schematic representation of the four categories of organic-solvent reaction media.
A : water-miscible solvent
B1: two-phase system, low volume organic solvent, solubilized biocatalyst
B2: two-phase system, low volume organic solvent, immobilized biocatalyst
B3: two-phase system, $a_w = 1$, high volume organic solvent, immobilized biocatalyst
C : micro-aqueous system, $a_w < 1$
D : reversed micelles

## ORGANIC-SOLVENT REACTION MEDIA

Four categories of organic solvent reaction media for biocatalysis can be distinguished (Figure 1). The water/organic-solvent mixtures may consist mainly of water with a relatively small amount of a water-miscible solvent (A). The mixture may consist of a two-phase system of a water-immiscible organic solvent and an aqueous buffer (B1, B2, B3), or it may be an organic solvent in which dry biocatalyst is suspended, so-called microaqueous organic-solvent mixtures (C). In the latter case the water present is located mostly on the solid enzyme particles. The fourth category of organic-solvent reaction media is the reversed micelles (D). Reversed micelles consist of tiny droplets of aqueous medium (radii in the range of 1-50 nm) stabilized by surfactant in a bulk of water-immiscible organic solvent. The preparation of reversed micelles and the properties of enzymes solubilized in the polar core of the reversed micelles have recently been discussed extensively in "Biomolecules in organic solvents" (Gómez-Puyou, 1992) and will therefore not be dealt with in this review.

## WATER-MISCIBLE ORGANIC SOLVENTS

*Introduction*

The addition of water-miscible solvents like acetone, ethanol, acetonitrile or dioxane has often been used to increase the solubility of apolar reactants. Usually, addition of small amounts of a water-miscible solvent has little effect on the biocatalyst activity and stability. In some cases, modest concentrations of these solvent even show an enhanched enzyme activity and stability (Butler, 1979, Guargliardi *et al.*, 1989, Vazquez-Duhalt *et al.*, 1993). However, when the concentration is increased, most water-miscible solvents have an inhibitory effect on the biocatalyst (Freeman and Lilly, 1987, Granot *et al.*, 1988, Blank-Koblenc *et al.*, 1988, O'Daly *et al.*, 1990, Guinn *et al.*, 1991, Fernandez *et al.*, 1991, Chatterjee and Russell, 1992, Wehtje, 1992, Vazquez-Duhalt *et al.*, 1993).

In reactions where hydrophobic interactions are involved in the complex formation between enzyme and substrate, the reduction of the activity of the biocatalyst at a high concentration of water-miscible organic solvent is mainly due to changes in the affinity of the enzyme for the substrate (Maurel, 1978). This is illustrated by the increased Michaelis Menten constant $K_m$ for the hydrolytic activity of trypsin (Maurel, 1978, Guinn *et al.*, 1991), papain (Fernandez *et al.*, 1991) and α-chymotrypsin (Maurel, 1978, Kise *et al.*, 1990). The $k_{cat}$ is much less affected than the apparent $K_m$ and a sharp decrease of the catalytic efficiency ($k_{cat}/K_m$) is often found when a water-miscible organic solvent is introduced in the reaction medium (Maurel, 1978, Kise *et al.*, 1990, Fernandez *et al.*, 1991). If electrostatic interactions play a dominant role, as is the case with ribonuclease (Maurel, 1978) and glucose oxidase (Blank-Koblenc, 1988), the addition of organic solvent can either increase, decrease or have no effect on the catalytic activity, depending on the solvent used.

*$LgP_{octanol}$*

Often the $lgP_{octanol}$ is used to predict the activity retention of a biocatalyst in organic media (Laane *et al.*, 1987b). The $lgP_{octanol}$ is defined as the logarithm of the partition coefficient of the solvent in a standard two-phase system of 1-octanol and water and is a measure of the hydrophobicity of the solvent. At low $lgP_{octanol}$ no activity is found, at intermediate $lgP_{octanol}$ the solvents show unpredictable toxic effects and at high $lgP_{octanol}$ no toxic effects are observed (Laane *et al.*, 1987b, Laane and Tramper, 1990). The correlation between

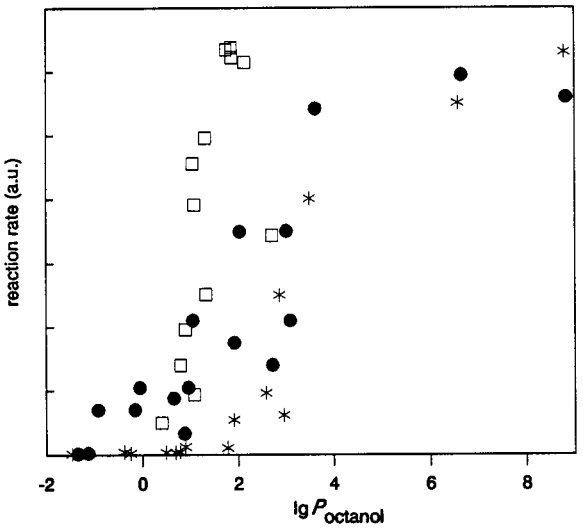


**Figure 2:** Activity of α-chymotrypsin (□), *Mucor* sp. lipase (●), and *Candida rugosa* lipase (*) as a function of $logP_{octanol}$ of the solvent, a.u. = arbitrary units (data from Reslow, 1989 and Zaks and Klibanov, 1985).

initial activity and/or stability and $lgP_{octanol}$ has been observed for several enzymes (Figure 2). However, examples of enzymic reactions exist for which no correlation between activity retention and $lgP_{octanol}$ is found (Figure 3, Estrada *et al.*, 1991, Kanerva *et al.*, 1990, Vazquez-Duhalt *et al.*, 1993). $LgP_{octanol}$ is thus not always a good parameter to predict the toxic effects of solvents in enzymic reactions and in some cases other parameters of solvents polarity, such as the Dimroth-Reichardt parameter $E_T(30)$ (Vazquez-Duhalt *et al.*, 1993), predict enzyme activity better.

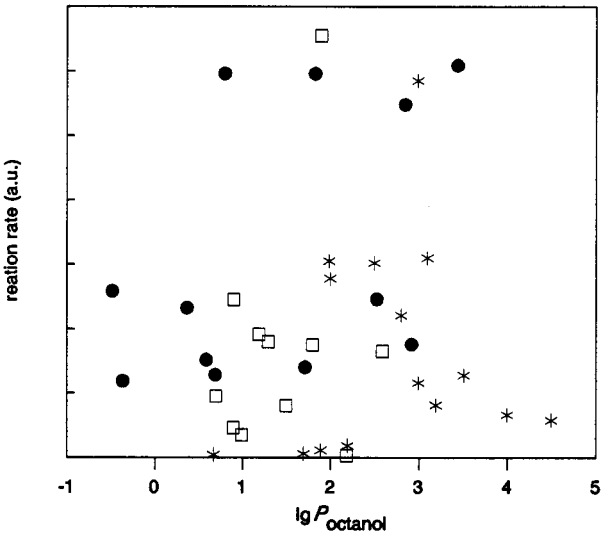


**Figure 3:** Activity of mandelonitril lyase (□), porcine pancreatic lipase (●), and polyphenol oxidase (*) as a function of $logP_{octanol}$ of the solvent, a.u. = arbitrary units (data from Wehtje, 1992, Zaks and Klibanov, 1985, and Yang *et al.*, 1992, respectively).

$Lg P_{octanol}$ in combination with either electron-pair-acceptance index or polarizability of the solvent has been used successfully to correlate the initial rate of a porcine pancreatic lipase-catalysed esterification reaction (Valivety *et al.*, 1991). In this approach direct polar interactions between the solvent and the enzyme (or the relatively polar phase around the enzyme) are accounted for. This approach has been extended by Schneider (1991), who suggests to use a three-dimensional solubility parameter to predict enzyme activity in all kind of solvents. Apart from the polar and dispersive interactions also hydrogen bonding is taken into account, but the usefulness of this approach is limited, due to lack of sufficient data.

*Denaturating capacity*

A more useful approach has been developed by Khmelnitsky *et al.* (1991). They have developed a thermodynamic model which describes the reversible denaturating effect of organic solvents, which is often observed after a certain treshold concentration of the organic solvent has been reached (Mozhaev *et al.*, 1989, Khmelnitsky *et al.*, 1991, Manjón *et al.*, 1992, Vazquez-Duhalt *et al.*, 1993). This inhibitory effect has been ascribed to reversible conformational changes (denaturation) of the enzymes as shown by fluorescence studies (Mozhaev *et al.*, 1989). Khmelnitsky *et al.* (1991) assume that reversible denaturation is primarily caused by the displacement of the essential hydration shell around the protein by the organic solvent. The model accounts for several physico-chemical characteristics, such as the hydrophobicity, solvating ability and molecular geometry of the organic solvent. Based on this model, the denaturating capacity (DC) of organic solvents can be quantified. When the denaturating capacity of only a few solvents is known for a given enzyme (protein), they form a DC-scale which permits the prediciton of the threshold concentration of any given organic solvent for the enzyme. The validity of the DC-scale for prediction of the threshold concentration of a solvent has been verified for different enzymes (proteins), such as α-chymotrypsin, laccase, trypsin, myoglobin, cytochrome c and chymotrypsinogen (Khmelnitsky *et al.*, 1991). Generally, predicted and experimental values for the threshold concentrations agree rather well, although one has to be careful in choosing the reference solvents for determination of the DC-scale, because some solvents show unpredictable toxic effects.

*Critical membrane concentration*

Toxic effects at threshold concentrations are also observed for cellular biocatalysts, exposed to subsaturating concentrations of an organic solvent in the aqueous phase. These so-called molecular-toxicity effects are ascribed to solvent effects on the cellular membrane (Osborne *et al.*, 1990, Vermuë *et al.*, 1993, Bassetti *et al.*, 1993). The threshold concentration evokes a critical concentration of the solvent in the membrane, which is hypothesized to be independent of the type of solvent (Osborne *et al.*, 1990).

The molecular toxicity of a solvent for cellular biocatalysts can be predicted by means of its $lg P_{octanol}$ value. The partition coefficient of the organic solvent in a membrane/aqueous buffer two-phase system can be related to $lg P_{octanol}$ using a Collander type of relationship (Collander, 1951):

$$P_{membrane} = R * P_{octanol}^{Y}$$

in which $P_{membrane}$ is the partition coefficient of the solvent in the membrane/aqueous buffer two-phase system, while $P_{octanol}$ refers to the octanol/aqueous two-phase system; $R$ and $Y$ are constants. The threshold concentration of organic solvent in the aqueous phase,

[$solvent_{aq,cr}$], is related to the critical membrane concentration *via* $P_{membrane}$ and the equation can thus be rearranged into

$$\lg [solvent_{aq,cr}] = \lg \frac{[solvent_{membrane,cr}]}{R} - Y * \lg P_{octanol}$$

If the logarithm of the threshold concentration of the solvent in the aqueous phase, lg[$solvent_{aq,cr}$], is plotted against $\lg P_{octanol}$, a straight line is obtained, as shown for the toxicity of alkanols and alkyl acetates for *Arthrobacter* and *Acinetobacter* cells in Figure 4 (Vermuë *et al.*, 1993). The intercept with the *y*-axis yields the logarithm of the ratio of the critical membrane concentration and *R*. A similar linear relation is also found for the hydroxylase activity of *Rhizopus nigricans* (Osborne *et al.*, 1990). These authors use a constant value of 0.19 for *R*, which has also been used for mammalian cells (Seeman, 1972) and they assume *R* to be independent of the solvent and the organism used. However, we doubt if *R* can be considered constant, because *R* represents the partition coefficient over a hypothetical membrane/octanol two-phase system and the solvent interactions with the membrane and with octanol will very likely be different for each type of solvent and for each type of membrane.

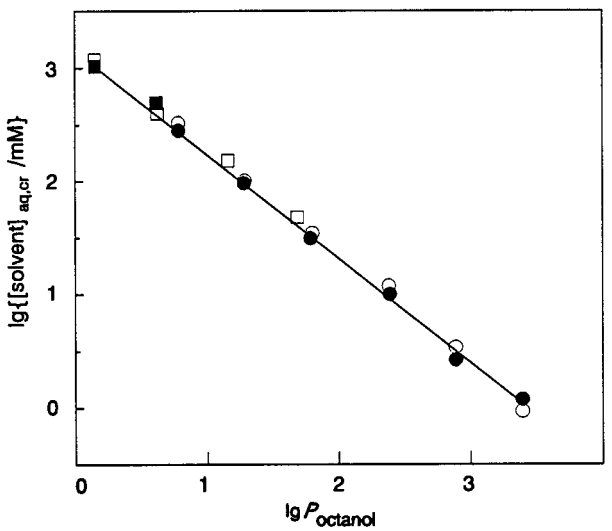


**Figure 4:** Relationship between the logarithm of the aqueous solvent concentration at which 50% of the initial oxygen consumption rate of *Arthrobacter* (closed symbols) and *Acinetobacter* (open symbols) is inhibited, and the $\log P_{octanol}$ of the solvents. The circles refer to alkanols, the squares to alkyl acetates (data from Vermuë *et al.*, 1993).

Despite our doubts about the constant value of *R* and thus the existence of *one* critical membrane concentration independent of the solvent, a linear relation between $\lg P_{octanol}$ and the log[$solvent_{aq,cr}$] has been observed (Figure 4). The value of log([$solvent_{membrane,cr}$]/R) thus has to be constant, which indicates that, if *R* varies with the solvent, the critical solvent concentration in the membrane has to vary in the same way (Vermuë *et al.*, 1993, Tramper and Vermuë, 1993). Although the existence of a constant critical membrane concentration is questionable, the plot can still be used to predict the molecular toxicity of any given solvent with known $\lg P_{octanol}$, since the threshold concentration in the aqueous phase can be estimated. It can be observed that the threshold concentration of polar solvents (low $\lg P_{octanol}$) is higher than the threshold concentration of more hydrophobic ones

and that the cellular biocatalyst is thus able to withstand higher concentrations of polar solvents. This seems to be in contrast with the general observation that solvents with high $\lg P_{octanol}$ are less toxic to biocatalysts (Figure 2). However, hydrophobic solvents generally exhibit such low maximum solubility in water, that the threshold concentration that would cause molecular toxicity effects cannot be reached.

*Effects on thermostability of the biocatalyst*

In general enzymes show enhanched thermostability when they are dispersed in pure organic solvents. This is observed for porcine pancreatic lipase and lipase from *Candida cylindracea* (Zaks and Klibanov, 1984), ribonuclease, chymotrypsin, lysozyme and several other enzymes (Volkin *et al.*, 1991 and references cited therein). The mechanisms of irreversible thermo-inactivation of enzymes in aqueous solutions at 90-100 °C have been indentified (Table 1). In most of these thermo-inactivation processes water is one of the reactants. Water also decreases the rigidity of the proteins involved, which will lead to reversible thermo-unfolding, heat-induced incorrect structure formation and aggregation of enzymes. In dry organic solvents the enzyme structure will maintain its rigidity and this explains the enhanced thermostability of enzymes in dry organic solvents, compared to aqueous solutions (Ahern and Klibanov, 1985, Zale and Klibanov, 1986, Volkin *et al.*, 1991).

**Table 1:** Mechanisms of irreversible thermo-inactivation of enzymes (Ahern and Klibanov, 1985, Zale and Klibanov, 1986, Volkin *et al.*, 1991).

| |
|---|
| - ß-elimination of cysteine residues |
| - thiol-catalysed disulfide interchange |
| - oxidation of cysteine residues |
| - deamination of asparagine and/or glutamine residues |
| - hydrolysis of peptide bonds at aspartic acid residues |

The extent to which enzymes show thermostability depends on the hydrophobicity of the organic solvent. For example, the thermostability of α-chymotrypsin (Reslow *et al.*, 1987), terpene cyclase (Wheeler and Croteau, 1986), ATP-ase and cytochrome oxidase (Ayala *et al.*, 1986) in hydrophobic solvents is better than in hydrophilic ones. This has been ascribed to the capacity of hydrophilic solvents to "strip" the essential water from the enzyme molecules, and thereby to diminish enzymatic activity (Zaks and Klibanov, 1988, Kanerva *et al.*, 1990). This capacity has recently been illustrated by Gorman and Dordick (1992). They have exchanged the enzyme-bound water of chymotrypsin, subtilisin Carlsberg and horseradish peroxidase by tritiated water ($T_2O$) prior to lyophilization of the enzyme preparation. After resuspending the enzyme in dry organic solvent, the highest degree of desorption of $T_2O$ has indeed been found after exposure to hydrophilic solvents. For example, methanol desorbs 56%-62% of bound $T_2O$, while hexane desorbs only 0.4%-2% (Gorman and Dordick, 1992).

*Effects on operational stability of biocatalysts*

In contrast to enzyme thermostability, not many reports deal with operational stability of enzymes in organic media. In general the water content of the reaction medium seems to be the most important parameter for both stability and activity because of its dual character. On the one hand, some water is essential for enzymatic activity, but on the other hand, the stability of the enzymes decreases with increasing water content. The operational stability of chymotrypsin adsorped on controlled-pore glass has been studied in diisopropyl

ether containing 0 - 0.75% v/v water. Very good stability is obtained in all reaction mixtures, except the one without extra water added. In the latter case, the solvent dehydrates the enzyme and decreases its activity (Reslow *et al.*, 1988a). Such good operational stability of chymotrypsin has also been observed in acetonitrile/water mixtures with moderate amounts of water. After 168 hours of reaction in media containing 2-4% v/v of water the residual activity is about 90% of the initial activity (Reslow *et al.*, 1988b).

The operational stability of mandelonitrile lyase in diisopropyl ether is poor if the substrate solution in solvent has not been presaturated with water (Wehtje *et al.*, 1990). This again indicates that essential water is extracted from the enzyme preparation by the solvent. The activity can almost be completely regained if extra water is added to the substrate solution, but some irreversible inactivation has also been observed (Wehtje *et al.*, 1990).

Irreversible inactivation is not necessarily due to interaction of the solvent with the hydration layer around the enzyme, but can be caused by direct interaction of the solvent with the enzyme. This has been illustrated by Van der Padt *et al.* (1992), who studied the inactivation of *Candida rugosa* lipase in glycerol/water mixtures. Glycerol concentrations up to 40% w/w ($a_w$ = 0.87) stabilize the lipase, but in glycerol solutions of 70 up to 95% w/w (in which the $a_w$ decreases from 0.63 to 0.11) inactivation is observed. If the lipase is stored for one week in vacuum desiccators above saturated salt solutions with known $a_w$, no inactivation occurs. This means that the inactivation in glycerol/water mixtures is caused by the interactions between the glycerol and the enzyme and not by the solvents' capacity to reduce the $a_w$. The latter is known to affect the hydration shell around the enzyme (Halling, 1990) and irreversible inactivation is thus not caused by solvent effects on this hydration shell in this particular case of glycerol.

## TWO-PHASE SYSTEMS OF WATER-IMMISCIBLE SOLVENTS AND WATER

*Introduction*

When a sufficient amount of a water-immiscible solvent is mixed with water, a two-phase system is generated. These systems are of particular interest for reactions with apolar substrates and products. Several examples in which the organic-solvent phase serves as a reservoir for apolar reactants and the biocatalyst is confined to the aqueous phase have been listed in Table 2. The two-phase reaction media are of particular interest when the reaction involves a toxic or inhibitory substrate and/or product. The inhibitory compound is not necessarily apolar. In extractive fermentations, water-immiscible solvents have successfully been applied to reduce the product inhibition by rather polar compounds, such as ethanol, butanol, acetone and butyric acid (Table 2). An example of the latter is the extractive ethanol fermentation using dodecanol. The distribution coefficient of ethanol over dodecanol and water is low (0.35 g/g) and high productivities can only be achieved when the ethanol is continuously removed from the organic-solvent phase e.g. by washout with hot water (Minier and Goma, 1982). In this way the ethanol concentration in the organic solvent is kept low and the driving force for mass transfer of the ethanol concentration from the aqueous phase in the organic-solvent phase remains as high as possible.

**Table 2:** Examples of biocatalytic reactions in two-phase reaction media which illustrate the advantages of these media.

| Examples of two-phase biocatalysis | References |
|---|---|
| **Organic solvent serves as reservoir for reactant** | |
| Stereospecific hydrolysis of *D,L*-menthyl acetate by *Bacillus subtilis* | Brookes *et al.*, 1986<br>Williams *et al.*, 1990 |
| Hydrolysis of *exo,exo*-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol diacetate ester by lipase P-30 from *Pseudomona* sp. | Patel *et al.*, 1992 |
| Bioconversion of naphthalene to 1,2-dihydronaphthalene-*cis*-1,2-diol | Harrop *et al.*, 1992 |
| Steroid bioconversion | Yamané *et al.*, 1979<br>Carrea *et al.*, 1988<br>Ceen *et al.*, 1988<br>Hocknull and Lilly, 1987, 1988, 1990 |
| **Reduction of substrate or product inhibition** | |
| Production of *L*-tryptophan | Ribeiro *et al.*, 1987 |
| Epoxidation of alkenes | Brink and Tramper, 1985<br>Harbron *et al.*, 1986 |
| Biodegradation of tetralin | Vermuë *et al.*, 1990 |
| Bioconversion of benzene to cyclohexa-3,5,diene-*cis*-1,2-diol | Van den Tweel *et al.*, 1987 |
| **Extractive fermentation** | |
| Extractive fermentation of ethanol | Daugulis and references cited therein, 1988 |
| Extractive fermentation of butanol and acetone | Roffler *et al.*, 1988 |
| Extractive fermentation of butyric acid | Evans and Wang, 1990 |
| Extractive fermentation of butanol | Barton and Daugulis, 1992 |
| **Equilibrium shift in synthetic reactions** | |
| Glycosidase-catalysed synthesis of alkyl ß-glucoside | Vulfson *et al.*, 1990 |
| Lipase-catalysed synthesis of acylglycerol and esters of decanoic acid and various alcohols | Janssen *et al.*, 1993a, 1993b, 1993c |
| Extractive fermentation of carboxylic acids | Aires-Barros *et al.*, 1989 |
| Peptide synthesis | Semenov and references cited therein, 1988<br>Kimura *et al.*, 1990<br>Clapés *et al.*, 1990 |

*Extractant screening*

Instead of dodecanol, more polar solvents can be applied, which show improved distribution coefficients for ethanol. However, better extractive solvents are in general more toxic to the biocatalyst. For the identification of organic solvents and solvent mixtures which are both biocompatible and effective extractant, Bruce and Daugulis (1991) have developed an extractant screening program (ESP) (Figure 5). This computer program first selects all solvents from a large database, that fulfil some specified requirements. The biocompatibility of the pre-selected solvents is predicted by using the correlation between biocatalytic activity and $\lg P_{octanol}$ (Laane *et al.*, 1987b) or the critical membrane concentration (Osborne *et al.*, 1990). The relation between $\lg P_{octanol}$ and the critical membrane concentration has been discussed above. For the estimation of the extractive power of the solvent and solvent mixtures, the computer program utilizes the UNIFAC group-contribution method for predicting liquid-liquid equilibrium data. The program calculates the distribution coefficients of solutes over two liquid phases and couples this information to the biocompatibility data. With this program Bruce and Daugulis (1992) have been able to identify solvents and solvent mixtures for effective extractive fermentation. For example, the solvent mixture of oleyl alcohol (octadec-9-enyl alcohol) with 5 % (v/v) 4-heptanone has a distribution coefficient for ethanol which is 12 % higher

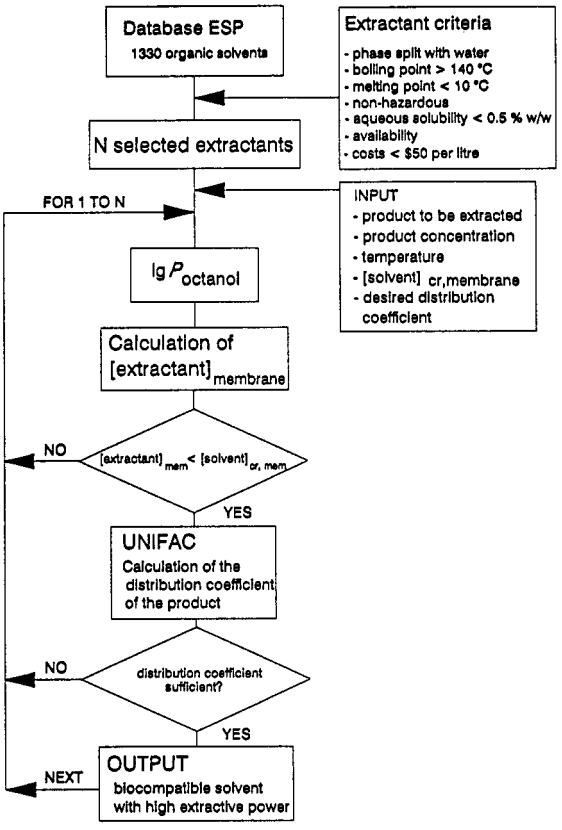


**Figure 5**: The extractant screening program (ESP) (adapted from Bruce and Daugulis, 1991).

than for pure oleyl alcohol and shows no significant inhibitory effect.

*Synthetic reactions*

In the above applications of two-phase reaction mixtures, relatively large amounts of aqueous phase have been applied. These reaction mixtures can also contain relatively little water, especially when used in synthetic reactions catalysed by hydrolytic enzymes (Table 2). The low water content is often credited for a shift in equilibirium towards synthesis, but this is often not justified, because the water activity ($a_w$) remains high. Even if the aqueous phase is restricted to the pores of the biocatalyst particles, the $a_w$ can still be close to 1, if water-immiscible solvents are used and the aqueous phase remains a dilute solution of reactants (Halling, 1984, Cassells and Halling, 1988).

Mass action of water only plays a role in shifting the equilibrium towards synthesis when highly concentrated solutions of substrates are used, as in the glycosidase-catalysed synthesis of alkyl ß-glucoside (Vulfson *et al.*, 1990) and in the lipase-catalysed acylglycerol synthesis and ester synthesis of decanoic acid and various alcohols (Janssen *et al.*, 1993a, 1993b, 1993c). A shift in the equilibrium position of hydrolytic reactions in two-phase reaction mixtures is often due to the partitioning behaviour of the reactants (Semenov *et al.*, 1988, Eggers *et al.*, 1989, Monot *et al.*, 1991). Several models which have been developed to predict the equilibrium position in two-phase systems have recently been reviewed by Janssen (1993d).

An interesting example where the partitioning behaviour has been exploited is reported by Aires-Barros *et al.* (1989). They introduce a novel means to extract carboxylic acids from aqueous solutions, by first converting the acids into a more hydrophobic ester *via* a lipase-

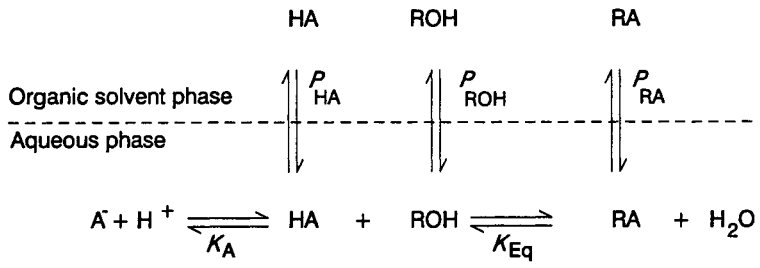


**Figure 6**: Equilibria involved in the esterification-coupled extraction of organic acids. $K_A$ is the dissociation constant of the acid/base equilibrium, $K_{Eq}$ is the equilibrium constant of the lipase-catalysed esterification, $P_{HA}$, $P_{ROH}$ and $P_{RA}$ are partition coefficients of the undissociated acid (HA), the alcohol (ROH) and the ester (RA), respectively (adapted from Aires-Barros *et al.*, 1989)

catalysed esterification (Figure 6). The high distribution coefficient of the ester outweighs the low equilibirum concentration of the ester in the presence of excessive water. The net result is a 4-15 fold increase in apparent distribution coefficient of the acid and a concentration of 80-95% (w/w) acid in the organic phase in the esterified form.

Partitioning behaviour may influence the equilibrium position in synthesis reactions in yet another way. For instance, peptide synthesis in buffer is often limited because the substrates predominantly exist in their ionogenic form. The $pK_a$ of the carboxylic acids usually range from 3 to 4 and the $pK_b$ of the amines from 8 to 10. In aqueous solutions one of the reactants will thus exist completely in the charged form, which makes peptide synthesis impossible over the whole range of pH. In water/organic-solvent two-phase reaction media, the apparent $pK_a$ of an acid usually increases, while the apparent $pK_b$ of the amines decrease, and in these media a pH range exists were both reactants are present in the uncharged form. This will result in a shift in the equilibrium of the reactions towards synthesis (Figure 7, Semenov, 1988, and references cited therein).

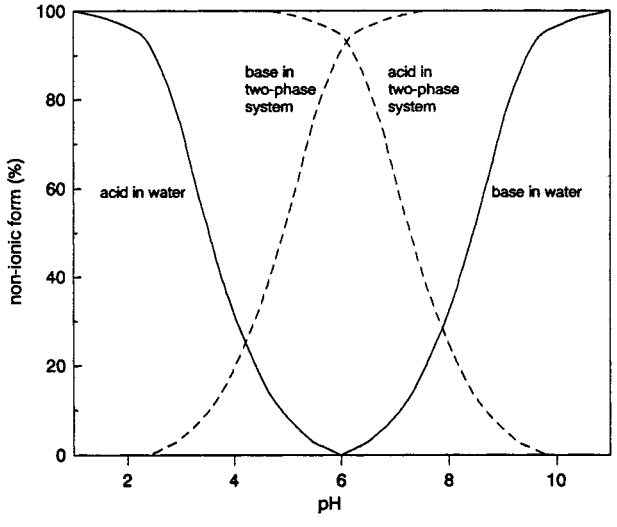


**Figure 7**: Theoretical titration curves for the carboxylic and amine components in water (____) and in a biphasic water-organic reaction mixture (----) (adapted from Semenov *et al.*, 1988).

In some cases, a high product yield in peptide synthesis has been achieved in aqueous buffer solutions by precipitation of the product in the aqueous buffer phase or by entrapment of the product inside the support of the immobilized enzyme used (Kimura *et al.*, 1990). Of cource, the latter can only be realised when the support shows affinity towards the product.

Peptide synthesis is not always thermodynamically controlled. It is possible to achieve high product yield in kinetically controlled reactions. The method is based on using carboxylic starting components whose hydrolysis energy is higher than that of the synthesized product. In kinetically controlled protein synthesis for example, an activated ester substrate is used (Figure 8). The substrate reacts with the enzyme, yielding an acyl enzyme intermediate. This intermediate can subsequently react with the amino group of an amino acid forming a peptide bond or with water, yielding a hydrolysis product. The maximum yield depends on the ratio between the transferase and the hydrolytic reaction rate. When the optimum peptide yield is reached, the reaction has to be stopped in order to avoid secondary hydrolysis of the synthesis product (Semenov *et al.*, 1988, Clapés *et al.*, 1992, Chatterjee and Russell, 1993).

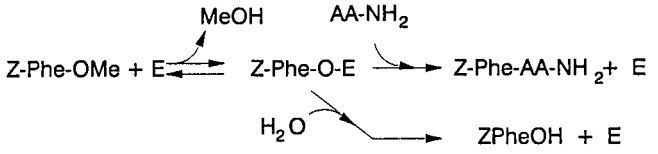


**Figure 8**: Reaction scheme for the kinetically controlled α-chymotrypsin catalyzed peptide synthesis.

*Toxic effects in two-phase reaction media*

The toxic effect on biocatalytic activity and stability in two-phase reaction media can be divided into two effects. The direct toxic effect of the solvent molecules, which are dissolved in the aqueous phase and interact with the biocatalyst, is called molecular-toxicity effect (Bar, 1987, 1988). This molecular effect has already been dealt with in the water-miscible organic-solvent reaction media. In two-phase reaction media an additional toxic effect is created by the presence of an interface between the aqueous and the organic solvent phase: the phase-toxicity effect. Several mechanisms to explain phase toxicity effects have been identified, such as nutrient extraction, disruption of cell membranes, limited access to nutrients due to emulsion formation, cell coating and attraction to the interface (Bar, 1987). Especially, disruption of the cellular membrane has been mentioned as the key toxic effect (Hocknull and Lilly, 1987, Hocknull and Lilly, 1988, Osborne *et al.*, 1990, Bruce and Daugulis, 1991, Vermuë *et al.*, 1993). Hocknull and Lilly (1988), for example, show that the toxic effect of solvents can partly be circumvented by the addition of the artificial electron acceptor, phenazine methosulphate (PMS), which replaces the need for a fully functioning cofactor-regeneration system, which is a typical membrane-associated enzyme complex.

Several parameters have been used to correlate solvent toxicity and cellular biocatalytic activity in two-phase reaction media. Apolar solvents having a low Hildebrandt solubility parameter ($\delta$) and high molecular weight show high biocatalytic activity retention for epoxidizing cells (Brink and Tramper, 1985). Better correlation between biocatalytic

activity and hydrophobicity is found when hydrophobicity is expressed by its $lgP_{octanol}$ (Laane, 1987, Laane *et al.*, 1987b). Generally, solvents having a relatively high $lgP_{octanol}$ value ($lgP_{octanol} > 5$) are biocompatible for cellular biocatalysts (Hocknull and Lilly, 1987, Buitelaar *et al.*, 1990, Hocknull and Lilly, 1990, Bruce and Daugulis, 1991, Vermuë *et al.*, 1993). The transition between toxic and non-toxic solvents depends on the type of organism used (Vermuë *et al.*, 1993) and the agitation rate (Habron *et al.*, 1986, Hocknull and Lilly, 1987). The latter, however, has a dual character. At low agitation rates, mass transfer of apolar reactants towards the aqueous phase may be rate limiting, because of the limited interfacial area for mass transfer. At higher agitation rates, the interfacial area is increased but this may also increase the amount of toxic interfacial effects, resulting in a decrease in activity. The rate of mass transfer from the organic solvent phase towards the aqueous phase can be measured by means of an apparatus called Lewis cell (Woodley *et al.*, 1991). Because this apparatus has a well-defined flat liquid/liquid interface, it is possible to expose biocatalyst to defined amounts of interface and to use the Lewis cell to study interfacial effects on biocatalytic activity and stability (Woodley and Lilly, 1992). Another technique for monitoring interfacial inactivation of enzymes has recently been reported by Ghatorae *et al.* (1993). They have used a liquid-liquid bubble column apparatus to study the inactivation of urease and chymotrypsin by 6 different solvents and have demonstrated that interfacial inactivation of these enzymes depends on the total area to which the enzyme solution is exposed, rather than the exposure time.
If predominantly phase-toxicity effects occur, immobilization of the biocatalyst in a hydrophilic gel can be a successful tool to protect the biocatalyst (Carrea *et al.*, 1988, Hocknull and Lilly, 1990, Harrop *et al.*, 1992).

## MICRO-AQUEOUS REACTION MEDIA

*Low water activity*
When the amount of water is reduced until no aqueous phase can be distinguished a microaqueous reaction medium is obtained (Yamane, 1988). The water activity ($a_w$) in these media, which are also called very low water systems, nonaqueous or anhydrous organic-solvent systems, may vary from close to one to very low values (Cassels and Halling, 1988). In this section only cases in which the $a_w$ is considerably lower than one will be discussed.
Strictly speaking, biocatalytic activity is not possible without water. Some water is required in all noncovalent interactions to maintain the native, catalytically active biocatalyst conformation. However, the minimal amount of water on the enzyme, which is required for biocatalytic activity, depends on the type of enzyme and may be restricted to less than a monolayer of water molecules around the biocatalyst (Zaks and Klibanov, 1988a, Zaks and Russell, 1988, Klibanov, 1989). For example, $\alpha$-chymotrypsin needs only 50 molecules of water per molecule of enzyme, while polyphenol oxidase requires about $3.5*10^7$ molecules per enzyme molecule (Dordick, 1989).
Due to the difference in distribution of water between the enzyme particles and the solvent, more water is needed for biocatalytic activity in hydrophilic solvents than in hydrophobic ones (Reslow *et al.*, 1987, Zaks and Klibanov, 1988b). If the amount of water required for biocatalysis is expressed as the amount of water bound to the enzyme particles, an optimum in activity is found independent of the solvent used (Zaks and Klibanov, 1988b). An alternative way to explain the difference in the amount of water required in hydrophobic and hydrophilic solvents is that hydrophilic solvents require more water to

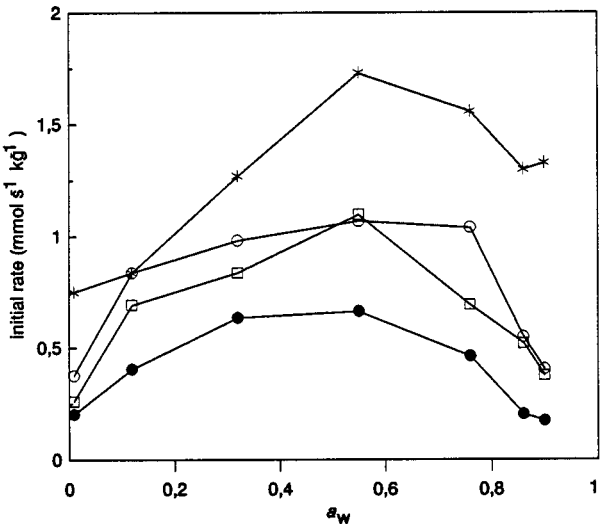


**Figure 9**: Activity of Lipozyme as a function of the water activity in hexane (*), toluene (○), trichloroethylene (□) and pentane-3-one (●) (data from Valivety *et al.*, 1992b).

reach the same water activity ($a_w$) (Valivety *et al.*, 1992a). The water bound by the enzyme particles is likely to be a function of the $a_w$ (Halling, 1990) and as a consequence the $a_w$ is likely to be a good predictor of the reaction rate. This has been shown by Valivety *et al.* (1992a, 1992b), who demonstrated that the reaction rate with suspended lipase shows similar dependence on water activity in different organic solvents (Figure 9). Water activity is a very suitable parameter for characterization of the reaction medium. It is equal in all phases at equilibrium and several methods for $a_w$-control are available (Halling and Valivety, 1992). Indirect effects of water partitioning between biocatalyst particles and the solvent on biocatalytic activity are circumvented at a constant $a_w$ and direct effects of several other parameters can therefore be revealed. For example, Yang *et al.* (1992) have studied the activity of polyphenol-oxidase in several organic solvents at constant water activity, controlled by 1) pre-equilibration of the enzyme preparation and the reaction mixture separately under constant humidity and 2) by direct addition of salt hydrates which act as water buffers to achieve a constant $a_w$ in the reaction mixture. The latter method is preferred because it is much simpler and both methods provide similar reaction rates. No obvious relationship has been found by these authors between the $\lg P_{octanol}$ of the solvent and the enzyme activity. Instead, the authors propose to use the ratio of the partition coefficients of product and substrate ($P_p/P_s$) to predict enzyme activity. Solvents showing a high $P_p/P_s$ ratio tend to extract the product from the microaqueous environment around the enzyme, while the substrate partitions out of the solvent into the microaqueous phase. However, this relationship will not be applicable if substrate inhibition occurs, or when the enzyme is not completely surrounded by water molecules but by less than a monolayer of water molecules. In that case part of the enzyme will exhibit direct interaction with solvent molecules and a correlation between $P_p$ or $P_s$ and enzyme activity does not necessarily exist. This has been observed for the α-chymotrypsin catalysed esterification in several solvents at $a_w = 1$ (Reslow *et al.*, 1987).

*Enantioselectivity*

The enantioselectivity of the enzyme can be manipulated by simply varying the organic solvent in which the reaction takes place (Sakurai *et al.*, 1988, Kitaguchi *et al.*, 1989, Fitzpatrick and Klibanov, 1991, Parida and Dordick, 1991, Tawaki and Klibanov, 1992). The enantioselectivity of an enzyme can even be completely reversed by a transition from water to organic solvents (Zaks and Klibanov, 1986, Tawaki and Klibanov, 1992). However, so far no explanation or generally valid correlation between the enantioselectivity and the physico-chemical properties of the solvent have been found for the change in stereoselectivity upon transition of a polar into an apolar solvent (Carrea *et al.*, 1992). Carrea *et al.* (1992) speculate that enantioselectivity is dictated by specific solvent-enzyme interactions rather than by physico-chemical properties of the solvent. They base their model on the observation that the enantiomeric solvents, *R*-carvone and *S*-carvone have different effects on the selectivity and transesterification rate of Lipase PS (Table 3).

**Table 3:** Effects of chiral solvents on enantioselectivity and transesterification rate of Lipase PS (data from Carrea *et al.*, 1992). The enantiomeric ratio (E) is used as an index of enantioselectivity for transesterification reactions as defined by Chen *et al.* (1987).

| | substrate | | | | | |
|---|---|---|---|---|---|---|
| | 2-cyclohexen-1-ol | | sulcatol | | *trans*-sobrerol | |
| solvent | E | rel. rate | E | rel. rate | E | rel. rate |
| *R*-carvone | 1.7 | 92 | 15.5 | 100 | >500 | 100 |
| *S*-carvone | 1.9 | 100 | 14.5 | 38 | >500 | 2 |

Because the physico-chemical properties of both solvents are identical, the effect on the enantioselectivity can only be attributed to the difference in structure of the solvents and therefore to the binding interactions with the enzyme. Although this model is likely to describe the solvent effect on the enantioselectivity of the enzymes sufficiently, it has little predictive value because of the large amount of possible interactions between solvents and enzymes.

*Preparation of the biocatalyst*

In microaqueous reaction mixtures it is possible to add enzymes "straight from the bottle" but the activity of the enzyme can be improved considerably by forcing the enzyme in its biocatalytically active conformation before addition to the reaction mixture. This can be achieved by lyophilizing or drying the enzyme from a buffer solution in which the pH is adjusted to the optimum pH for the enzyme activity in aqueous solutions (Zaks and Klibanov, 1985, Zaks and Klibanov, 1988a). Another way to lock the enzyme in its active conformation is by lyophilizing the enzyme from a solution to which ligands has been added, such as a competitive inhibitor (Zaks and Klibanov, 1988a, Russell and Klibanov, 1988, Zaks and Russell, 1988, Klibanov, 1989). This results in an enzyme with a very rigid structure which resembles the enzyme-substrate complex. As soon as small amounts of water are added to the enzyme preparation, the rigidity of the structure is lost and the enzyme memory is destroyed (Zaks and Klibanov, 1988a).

By using this "bioimprinting" method it is even possible to manipulate the enantio selectivity of enzymes. For example, α-chymotrypsin has been modified to accept the D-form of a derivative of tryptophan, phenylalanine and tyrosine, by precipitation of the enzyme-inhibitor complex between chymotrypsin and the *N*-acetylated amino acids in 1-

propanol. In a microaqueous reaction mixture the α-chymotrypsin prepared in this way exhibits high selectivity in the synthesis of the D-form of the ethyl ester of the *N*-acetylated amino ester present during precipitation. When precipitation of α-chymotrypsin is done in the presence of the L-form of the *N*-acetylated amino acid or in the absence of the D-isomer, no esterification of the *N*-acetylated amino acid occurs (Ståhl *et al.*, 1991, Månsson *et al.* 1992).

*Immobilization by deposition on a solid support*

It is often beneficial to deposit the enzyme on a support to avoid mass-transfer limitations, due to aggregation of the enzyme powder, and to facilitate separation of the biocatalyst from the reaction mixture. The support can indirectly influence the biocatalytic activity by affecting the partitioning of the reactants and water in the reaction mixture (Adlercreutz, 1991, 1992). It can also directly affect enzyme kinetics by inactivation during the immobilization procedure or by direct interactions between support and enzyme. The partitioning of water between the support and the solvent can be characterized by the aquaphilicity of the support (*Aq*), which is defined as the ratio of the amount of water on the support to the amount of water in the solvent under standard conditions and which is a practical way to quantify the water-adsorbing capacity of a support material.

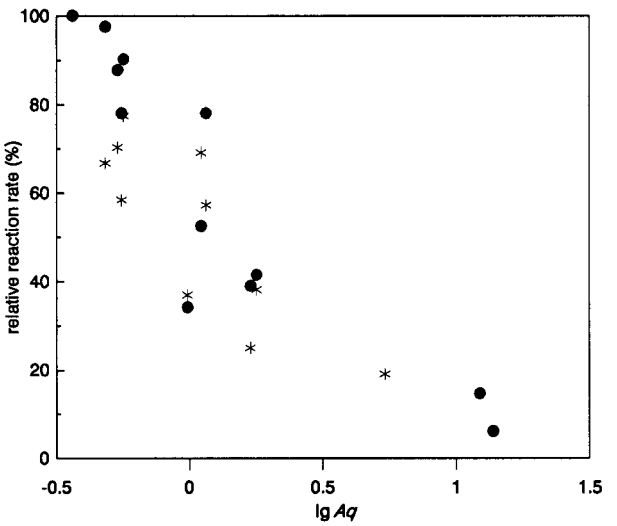


**Figure 10:** Relative reaction rate of α-chymotrypsin (*) and horse liver alcohol dehydrogenase (●) when deposited on support materials with different aquaphilicity (Aq). (data from Reslow *et al.*, 1988c).

For horse-liver dehydrogenase and for α-chymotrypsin deposited on a support the catalytic activity decreases with increasing aquaphilicity of the support material (Figure 10). In these cases a fixed amount of water has been added to each reaction mixture. This water partitions between the solvent, the support and the enzyme, and supports with a high aquaphilicity, such as the hydrophilic supports Sephadex and Biogel, will adsorb relatively large quantities of water compared to hydrophobic supports with low aquaphilicity, such as Celite and Bonopore. Enzyme immobilized on a support of high aquaphilicity will thus be less hydrated and subsequently show reduced biocatalytic activity, compared to the enzyme immobilized on a support of low aquaphilicity. An increase in enzyme activity has also been observed for the lipase catalysed esterification of heptanoic acid with 1-phenylethanol, when deposited on supports of increasing hydrophobicity (Norin *et al.*,

1988).
The direct effects of the support material on the enzyme activity can be studied separately at constant $a_w$, provided that supports are used which show a low tendency to adsorb substrate and products. At these conditions, the hydration of the enzyme will be fixed and indirect effects due to partitioning of water and reactants are minimized. This technique has been used by Adlercreutz (1991, 1992) who has studied the direct effects of the support on the activity of horse liver alcohol dehydrogenase (HLADH) and α-chymotrypsin (CT). For HLADH the reaction rate increases with increasing $a_w$ and the support with the lowest aquaphilicity, Celite, shows the highest activity among the support materials tested at fixed $a_w$. The highest catalytic activity is also observed in the CT-catalysed alcoholysis of *N*-acetyl-L-phenylalanine ethyl ester with 1-butanol, with the enzyme immobilized on Celite at high $a_w$. However, in the α-chymotrypsin catalysed reaction two competing reactions occur simultaneously, hydrolysis and alcoholysis and the ratio between these reactions varies with the $a_w$ and with the type of support. At high $a_w$ the enzyme shows high hydrolysis activity compared to alcoholysis when adsorbed to both hydrophilic and hydrophobic support. The reaction rate in the latter case is, however, much higher. At low $a_w$ almost no activity is found when the enzyme is adsorbed to the hydrophobic support but when adsorbed to the hydrophilic polyamide support, Accurel PA6, the enzyme shows considerable alcoholysis while no hydrolysis activity is detected. When alcoholysis is the preferred reaction, it will thus be more attractive to operate at low $a_w$ using the hydrophilic support material, Accurel PA6.

*Immobilization by covalent modification*
Although the simple deposit of enzyme on a solid support is often propagated as immobilization method for biocatalysis in microaqueous reaction mixtures, covalent attachment is also used to increase the stability and activity of the biocatalyst. Blanco *et al.* (1989) describe the use of chymotrypsin, multi-point attached to agarose, for peptide and amino-acid ester synthesis. During the immobilization procedure the active site of the enzyme can be protected by the addition of the competitive inhibitor, benzamidine (Blanco *et al.*, 1988), which blocks reactive groups in the active site of the enzyme, and prevents interaction of activated agarose with these groups. The result is a 10.000-fold more stable enzyme than the soluble one.
When chymotrypsin is immobilized through multi-point attachment, it can be used at lower $a_w$ than the free enzyme which is inactivated at $a_w < 0.4$ (Blanco *et al.*, 1992). This is of particular importance in ester and peptide synthesis, because at low $a_w$ the thermodynamic equilibria of these reactions will shift towards higher product yields. However, the reaction proceeds very slowly at these low $a_w$'s and expected high equilibrium yields of ester will not be reached within reasonable time (Blanco *et al.*, 1992).
Low reaction rates are often blamed to mass-transfer limitations. One of the methods to reduce mass-transfer limitations and to stabilize enzyme preparations is to modify the enzyme by covalent attachment to polyethylene glycol (PEG). The polyethylene glycol modified enzymes are soluble and active in water-immiscible organic solvents such as benzene, toluene and chlorinated hydrocarbons (Inada *et al.*, 1986, Inada *et al.*, 1990 and references cited therein).
In addition to immobilization, protein engineering has been propagated to improve the biocatalyst functioning and stability. The latest achievements with this technique have recently been reviewed by Arnold (1988, 1990) and Dordick (1992).

## SUPERCRITICAL FLUIDS

*Introduction*

Promising types of non-conventional medium for biocatalysis are the super- and near-critical fluids. Supercritical fluids are those compounds that exist at a temperature and a pressure above their corresponding critical value. Their physical properties make them very attractive for biocatalytic processes. They exhibit low surface tension and viscosity, and high diffusivity comparable with gases, all favouring efficient mass transfer. On the other hand they show liquid-like density, which promotes enhanced solubility of solutes compared to the solubility in gases. Probably the most important characteristic is that the solubility of solutes can be manipulated by changes in pressure and temperature, especially in the vicinity of the critical point. This makes product fractionation and purification possible directly from the reaction mixture without changing the solvent (McHugh and Krukonis, 1986).

**Table 4:** Critical temperature and pressure of possible compounds for biocatalysis in supercritical fluids

| Solvent | Critical temperature (°C) | Critical pressure (MPa) |
|---|---|---|
| Carbon dioxide | 31.1 | 7.3 |
| Ethane | 32.3 | 4.8 |
| Ethylene | 9.3 | 5.0 |
| Trifluoromethane | 25.9 | 4.6 |
| Nitrous oxide | 36.5 | 7.0 |
| Sulfur hexafluoride | N.A. | N.A. |

N.A.: Physical properties are not yet well characterized (Kamat *et al.*, 1992a)

For most biocatalytic reactions an operation temperature roughly below 60 °C is required for biocatalyst stability. The choice of supercritical fluids is thus limited to compounds having a critical temperature ($T_c$) between 0 and 60 °C. Table 4 shows a list of compounds that fulfil this requirement. Biocatalytic processes in supercritical fluids have been limited to carbon dioxide, except for only a few reports of bioprocesses executed in other supercritical fluids, such as the use of polyphenol oxidase for the oxidation of *p*-cresol and *p*-chlorophenol in supercritical trifluoromethane (Hammond *et al.*, 1985) and lipase catalysed transesterification of methyl methacrylate in supercritical ethane, ethylene, trifluoromethane and near-critical propane (Kamat *et al.*, 1992a). The latter also report for the first time on the use of an anhydrous, inorganic supercritical fluid, sulfur hexafluoride. This solvent shows the highest initial transesterification rate of all supercritical and conventional organic solvents tested. The improved activity is ascribed to its unusually high density compared to the other supercritical fluids (750 kg/m$^3$) and its high hydrophobicity compared to the conventional solvents used.

Carbon dioxide is the most popular among the supercritical fluids because it is nontoxic, nonflammable, not expensive and safe for human beings. Supercritical carbon dioxide ($SCCO_2$) has been used as a medium for reactions catalysed by several enzymes (Table 5). Most of the bioprocesses in supercritical fluids have been reviewed recently by Aaltonen and Rantakylä (1991) and Randolph *et al.* (1991). In this review, we will only focus on the effect of medium characterisitics of supercritical fluids on the stability and activity of enzymes.

**Table 5:** Examples of enzymatic reactions in supercritical carbon dioxide

| Enzyme | Reaction | Reference |
|---|---|---|
| - alkaline phosphatase | hydrolysis of *p*-nitrophenyl phosphate | Randolph *et al.*, 1985 |
| - polyphenol oxidase | oxidation of *p*-cresol and *p*-chlorophenol | Hammond *et al.*, 1985 |
| - thermolysin | synthesis of aspartame precursors | Kamihira *et al.*, 1987 |
| - cholesterol oxidase | oxidation of cholesterol | Randolph *et al.*, 1988 |
| - subtilisin | transesterification between *N*-acetyl-*L*-phenylalanine chloroethyl ester and ethanol | Pasta *et al.*, 1989 |
| - lipase | transesterification of triglycerides with fatty acids | Nakamura *et al.*, 1986<br>Chi *et al.*, 1988<br>Erickson *et al.*, 1990 |
| | transesterification of methyl methacrylate with 2-ethylhexanol | Kamat *et al.*, 1992a |
| | interesterification of trilaurin and myristic acid | Miller *et al.*, 1992 |
| | transesterification of ethyl acetate and nonanol | Vermuë *et al.*, 1992 |
| | esterification of oleic acid by ethanol | Marty *et al.*, 1992<br>Yu *et al.*, 1992 |
| | esterification of myristic acid and ethanol | Dumont *et al.*, 1992 |

*Stability*

Enzymes generally show enhanched stability in supercritical fluids. If stability losses are reported, they are ascribed to thermo-inactivation at the elevated temperatures used (Nakamura *et al.*, 1986, Randolph *et al.*, 1988) or to inactivation during depressurization, especially in case of enzymes, which have no stabilizing S-S bridges, like penicillum amidase. The degree of inactivation of monomeric enzymes with stabilizing S-S bridges, such as chymotrypsin and trypsin during the depressurization steps is much less pronounced (Kasche *et al.*, 1988). High moisture contents of the medium, like in organic solvents, decrease the operational stability of the enzymes. In humid $CO_2$ the enzyme tends to unfold more easily which further stimulates inactivation processes (Weder, 1984, Marty *et al.*, 1992).

*Activity*

Although improved activity of enzymes in supercritical carbon dioxide compared to conventional organic solvents has often been reported (Chi *et al.*, 1988, Randolph *et al.*, 1988, Pasta *et al.*, 1989), similar activity (Miller *et al.*, 1992) as well as decreased activity have also been found (Vermuë *et al.*, 1992, Kamat *et al.*, 1992a). In theory, supercritical fluids are expected to enhance the activity of enzymes in non-aqueous environments as a result of the high diffusivity of the bulk solvent, which diminishes external and internal mass-transfer limitations that occur in many conventional organic solvents (Kamat *et al.*, 1992b, Russell and Beckman, 1991). Supercritical fluids may, however, influence the reaction rate in several other ways. The reaction-rate constant itself may be influenced by the effect of the high pressures on the activation volume of the biocatalytic reaction (Nakamura, 1991, Randolph *et al.*, 1991). It is however, very difficult to predict this effect *a priori*.

Biocatalysis in supercritical fluids may also be promoted by effects on the solubility state of the reactants. For example, cholesterol oxidase shows an increase in activity at increased pressure. EPR investigations show that high pressures promote aggregation of the substrate molecules and the enzyme is surmised to be more active towards cholesterol aggregates than to cholesterol monomers (Randolph *et al.*, 1988).

Sometimes, it is questionable that the bioconversion actually took place in supercritical fluid and not in the aqueous microenvironment of the biocatalyst (Table 6). To be sure to operate at supercritical conditions, the Hildebrandt solubility parameter ($\delta$) can be helpful as a first estimate of the solubility of the substrates in the solvent at the reaction conditions

**Table 6:** Examples of biocatalytic reactions which have been claimed to be performed in supercritical fluid, but which were most likely performed in a two-phase system

| Biocatalytic reaction | Reference | Reasons to doubt supercritical conditions | Reference |
|---|---|---|---|
| Transesterifciation of triglycerides with stearic acid by lipase | Nakamura *et al.*, 1986 | the stearic acid concentration exceeds the maximum solubility at process conditions | Nakamura, 1991 |
| Oxidation of *p*-cresol and *p*-chlorophenol by polyphenol oxidase | Hammond *et al.*, 1985 | a microaqueous layer is observed during the reaction | Hammond *et al.*, 1985 |
| Hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase | Randolph *et al.*, 1985 | the substrate is not soluble at the process conditions | Krukonis *et al.*, 1988 |
| Transesterification of trilaurin and palmetic acid by lipase at pressures below 10 MPa | Erickson *et al.*, 1990 | the difference between the Hildebrandt solubility parameters of the solvent and the substrates is too high to expect sufficient solubilization of the substrates | Allada, 1984 Vermuë *et al.*, 1992 |

used (Allada, 1984, Vermuë *et al.*, 1992). If the difference in $\delta$ between supercritical solvent and an apolar compound becomes smaller than 9 $(MPa)^{0.5}$ a significant rise in solublity of the compound can be expected and reaction will take place at supercritical process conditions (Allada, 1984, Vermuë *et al.*, 1992).
The solubility parameter can also be used as a parameter to express the polarity of organic solvents. Brink and Tramper (1985) have found a correlation between the polarity of an organic solvent (expressed by $\delta$) in combination with a high molecular weight of the organic solvent and biocatalytic activity. Vermuë *et al.* (1992) have attempted to correlate the lipase-catalysed transesterification rate in supercritical $CO_2$ and the Hildebrandt solubility parameter of the medium in a similar way, but the biocatalytic activity seems to be hardly influenced by this parameter. The water content of the reaction mixture affects the activity much more. By increasing the water content from 0.05% to 0.2% (v/v) the product formation decreases considerably.

## CONCLUDING REMARKS

It is clear that during the past decade much progress has been made in the fundamental understanding of the phenomena that govern biocatalysis in non-conventional media. The factors that affect biocatalytic reactions and the activity and stability of biocatalysts in these reaction media are generally associated with the crucial role of water and the need to keep biocatalysts in their active conformation. For the rational design of biocatalytic processes in the reaction media, discussed in this review, some basic rules have been formulated. These rules may serve as useful tools for future optimization of biocatalysis in non-conventional media and for engineering media for synthetic purposes.

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