BIOCATALYSIS IN NON-CONVENTIONAL MEDIA: MEDIUM ENGINEERING ASPECTS

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ABSTRACT

The basic knowledge of the processes governing biocatalysis in non-conventional media has progressed significantly during the last decade. Biological catalyst activity and stability are influenced by a variety of circumstances, including the presence of water and the necessity to maintain biocatalysts in their active state. In order to rationally design biocatalytic processes in the reaction medium, certain fundamental guidelines have been developed and described in this review. Biocatalysis in non-conventional media and engineering media for synthetic purposes might benefit from these laws.

INTRODUCTION

Synthetic chemistry is becoming more interested in including biotransformation processes (Laane et al., 1987a, Tramper et al., 1992). Biocatalysts are interesting because of their specificity and selectivity, which makes them ideal catalysts for chemical processes that are difficult or costly to perform. For a long time, the prevalent belief that biocatalysts (enzymes and entire cells) are only active in aqueous solutions and at mild circumstances has impeded the use of biotransformations in synthetic methods. Biocatalysts aren't as sensitive as previously thought, according to recent research. Even under severe circumstances like high salt concentrations or dramatic pH and temperature and pressure changes they can still perform their essential functions (Monsan and Combes, 1984). Organic solvents, aqueous two-phase systems, solid media, gases, and supercritical fluids are all non-conventional media in which they are active (Tramper et al., 1992). Biocatalysis is now much more useful in organic synthesis as a result of these discoveries. In this study, we'll examine the most recent developments in medium engineering and how they affect the biocatalyst's activity and stability. Immobilization of the biocatalyst or the addition of stabilisers is only one aspect of medium engineering, which also includes replacing aqueous reaction media with non-conventional media. In this review, organic solvents and supercritical fluids are discussed as non-conventional media. Other non-conventional media, such as aqueous two-phase systems and solid and gaseous media, will not be treated here. Water-soluble polymers or a salt solution and a polymer solution may generate two-phase systems when they are combined together. There have been recent studies on the physical properties of these systems, as well as various biotechnological applications, by Andersson, Hahn-Hagerdal (1990), and King (1991). (1992). Recently, Robert et al. (1992) and Lamare and Legoy (1993) have explored the current developments in biocatalysis in gaseous and solid environments (1993).

POTENTIAL OF BIOCATALYSIS IN ORGANIC SOLVENTS

The use of organic solvents in synthetic processes has various potential benefits. The volumetric productivity of a process may be increased by using organic solvents, which improve the solubility of water-insoluble substrates. Changes in phase partitioning or reduction in water activity might change thermodynamic reaction equilibrium in favour of synthesis, rather than hydrolysis. A water-miscible organic solvent may be substituted for the water in the reaction mixture, or polymers, sugars, or salts can be added. Polymerization of oxidised phenols (Kazandjian and Klibanov, 1985) and hydrolysis during transesterification processes may be reduced by lowering the water activity or water concentration (Dordick et al., 1986). Increased product yields will also result from the reduction of inhibitory interactions between the enzyme and the substrate and/or product, either indirectly by keeping the inhibitor concentration low or directly by altering interactions between the inhibitor and the enzyme's active site (Schwartz and McCoy, 1977, Vermue and Tramper, 1990). (Zaks and Klibanov, 1988a). Recovery of the product and biocatalyst will be simplified by the use of low-boiling organic solvents. For example, filtering may be used to remove the biocatalyst from the reaction mixture, while evaporation of the solvent can be used to extract the product, provided there is a significant difference in

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boiling point between the two. Thermo- and regio-selectivity of the enzyme may also be controlled in organic solvents (Zaks and Klibanov, 1984, Volkin et al., 1991), as well as the increased thermostability of the enzyme when microaqueous reaction media are utilised (Sakurai et al., 1988, Klibanov, 1990a). Not every organic-solvent reaction media and reactions may benefit from all of these benefits. There are also downsides to employing organic solvents in biocatalysis such as the organic solvent might denaturate or inhibit the biocatalyst, to name just two. Adding an organic solvent to the reaction mixture further complicates the process.
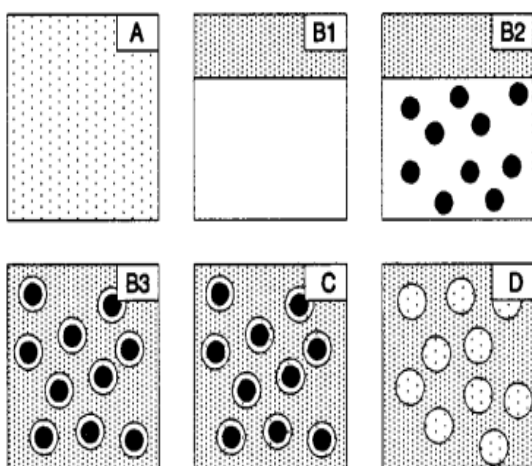

Figure 1: Schematic representation of the four categories of organic-solvent reaction media. A : water-miscible solvent B1: two-phase system, low volume organic solvent, solubilized biocatalyst B2: two-phase system, low volume organic solvent, immobilized biocatalyst B3: two-phase system, a, = 1, high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

ORGANIC-SOLVENT REACTION MEDIA

There are four distinct types of organic reaction medium for biocatalysis (Figure 1). Water and a water-miscible solvent may be the primary constituents of the water/organic-solvent combinations (A). Biocatalysts may be dissolved in an organic solvent or a water-immiscible buffer, or they can be dispersed in an organic solvent with a dry biocatalyst in suspension (C). This time, the water is primarily concentrated on the enzyme particles themselves. The reversed micelles are a fourth kind of organic-solvent reaction medium (D). Surfactant-

stabilized droplets of aqueous medium (ranging in diameter from a few nanometers to a few micrometres) in a bulk of water-insoluble organic solvent are known as reversed micelles. It is not necessary to go into detail on how reversed micelles are made or the features of enzymes solubilized in the polar core of reversed micelles, since this has recently been covered thoroughly in "Biomolecules in organic solvents" (G6mez-Puyou, 1992).

WATER-MISCIBLE ORGANIC SOLVENTS

Introduction These water-miscible solvents have frequently been employed to boost the solubility of non-polar reactants such water, ethanol, acetone, and dioxane. Adding tiny quantities of a water-miscible solvent to a biocatalyst often has no influence on its activity or stability. Small amounts of these solvents may potentially enhance enzyme activity and stability in some situations (Butler, 1979, Guargliardi et al., 1989, Vazquez-Duhalt et al., 1993). Even at relatively low dosages (Freeman and Lilly, 1987; Granot et al., 1988; BlankKoblenc et al., 1988; O'Daly et al., 1990; Guinn et al., 1991; Fernandez et al., 1991); at higher concentrations (Chatterjee and Russell, 1992; Wehtje, 1992; Vazquez-Duhalt et al., 1993); and Attributable to changes in the affinity of the enzyme for its substrate, a decrease in biocatalytic activity at a high concentration of a water-miscible organic solvent is mostly due to a decrease in enzyme activity (Maurel, 1978). The Michaelis Menten constant K, for the hydrolytic activity of trypsin (Maurel, 1978, Guinn et al., 1991), papain (Fernandez et al., 1991) and a-chymotrypsin, has been enhanced as a result (Maurel, 1978, Kise et al., 1990). An increase in the apparent catalytic efficiency (k,/K), on the other hand, has no effect on k, and typically results in a significant loss in catalytic efficiency (k,/K) (Maurel, 1978, Kise et al., 1990, Fernandez et al., 1991). In the case of ribonuclease and glucose oxidase, where electrostatic interactions play a prominent role, the addition of organic solvent may either enhance, reduce, or have no impact on the catalytic activity, depending on the solvent utilised. LgPoctan01 In organic media, the activity retention of a biocatalyst is often predicted using the lgPoctanol (Laane et al., 1987b). Logarithm of the partition coefficient in a conventional two-phase system of 1-octanol and water is defined as lgPoctanol and is a measure of the solvent's hydrophobicity. Low lgPoctanol is completely inactive, intermediate lgPoctanoI has variable harmful effects, and high lgPoctanoI has no toxic

effects at any level (Laane et al., 1987b, Laane and Tramper, 1990).
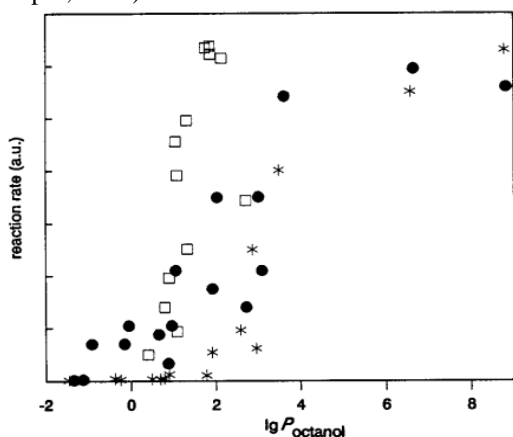

Figure 2: Activity of a-chymotrypsin (O), Mucor sp. lipase (O), and Candidurugosu lipase (*) as a function of IogP-, of the solvent, a.u. = arbitrary units (data from Reslow, 1989 and Zaks and Klibanov, 1985).nitial activity and/or stability and lgPoctanol has been observed for several enzymes (Figure 2). However, examples of enzymic reactions exist for which no correlation between activity retention and lgPoctano, isfound (Figure 3, Estrada et al., 1991, Kanerva et al., 1990, Vazquez-Duhalt et al., 1993). LgPoctanol 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 b(30) (VazquezDuhalt et al., 1993), predict enzyme activity better.

esterification process has been effectively correlated using LgPoctanolincombination with either an electron-pair-acceptance index or the solvent's polarizability (Valivety et al., 1991).
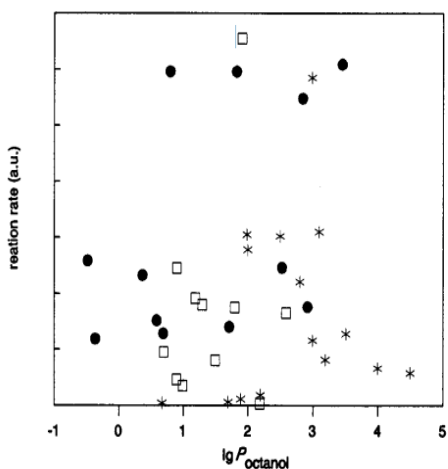
LogP-dependent activities of mandelonitril lyase (O), porcine pancreatic lipase (O), and polyphenol oxidase (*) (data from Wehtje, 1992, Zaks and Klibanov, 1985, and Yang et al., 1992, respectively). The initial rate of a porcine pancreatic lipase-catalyzed

These interactions are taken into account in this method, which takes into account the direct polar interactions between the enzyme and solvent. Using a three-dimensional solubility parameter, Schneider (1991) proposes to predict enzyme activity in all solvents. It is possible to take into consideration hydrogen bonding in addition to polar and dispersive interactions; however, the effectiveness of this technique is restricted because of insufficient data.

Denaturating capacity

Khmelnitsky et al. have come up with a better solution (1991). Organic solvents may undergo irreversible denaturation when their concentration rises over a particular threshold, as described by a thermodynamic model created by the researchers (Mozhaev et al., 1989, Khmelnitsky et al., 1991, Manj6n et al., 1992, Vazquez-Duhalt et al., 1993). As shown by fluorescence experiments, this inhibitory action may be attributed to the enzymes' reversible structural changes (denaturation) (Mozhaev et al., 1989). According to Khmelnitsky et al. (1991), organic solvent displacement of the protein's crucial hydration shell is the primary source of reversible denaturation. A variety of organic solvent properties, including hydrophobicity, solvating ability, and molecular shape, are taken into consideration in the model. The denaturing capacity (DC) of organic solvents may be measured using this approach. Denaturing capacities of enzyme (protein) may be predicted using the DC-scale when just a small number of organic solvents' denaturing capacities are known. Different enzymes (proteins) such as a-chymotrypsin, laccase, trypsin, myoglobin, cytochrome c, and chymotrypsinogen have been tested to see whether the DC-scale can accurately predict the threshold concentration of a solvent (Khmelnitsky et al., 1991). Predicted and experimental threshold concentrations are often in agreement, however one must be cautious when selecting the reference solvents for determining the DC-scale, since certain solvents have unanticipated hazardous effects.

Critical membrane Concentration

Subsaturating quantities of organic solvent in the aqueous phase may also have toxic effects on cellular biocatalysts. Solvent effects on the cellular membrane are blamed for these alleged molecular-toxicity consequences (Osborne et al., 1990, Vermue et al., 1993, Bassetti et al., 1993). For this reason, a critical concentration of solvent is thought to be independent of solvent type for determining a threshold concentration (Osborne et al., 1990). The toxicity of a solvent to cellular biocatalysts may be anticipated using its lgPoctanol value. A Collander relationship (Collander, 1951) may be used to connect the organic solvent partition coefficient in a membrane/aqueous buffer two-phase system:

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

Membrane-to-aqueous buffer partition coefficient is given by Pmembrane, whereas Poftanol refers to octane/aqueous buffer partition coefficient, with R and Y being constants. The critical membrane concentration, PmembrWe, is directly connected to the threshold concentration of organic solvent in the aqueous phase, [solvent,,,,].

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

Figure 4 shows the toxicity of alkanols and alkyl acetates to Arthrobacter and Acinetobacter cells when the logarithm of the threshold concentration of the solvent in the aqueous phase, lg[solvent,,,,], is plotted against lgPoctanol (Vermue et al., 1993). When the y-axis intersects with the x-axis, the logarithm of the ratio of the critical membrane concentration and R is obtained. The hydroxylase activity of Rhizopus nigricans is likewise observed to have a linear relationship (Osborne et al., 1990). A constant value of 0.19, which has been used for mammalian cells (Seeman, 1972), is assumed to be independent of the solvent and the organism utilised by these authors. The partition coefficient R in a hypothetical membrane/octanol two-phase system cannot be regarded constant, since the solvent interactions with the membrane and with octanol will be different for each kind of solvent and for each type of membrane, hence we doubt that R can be considered constant.
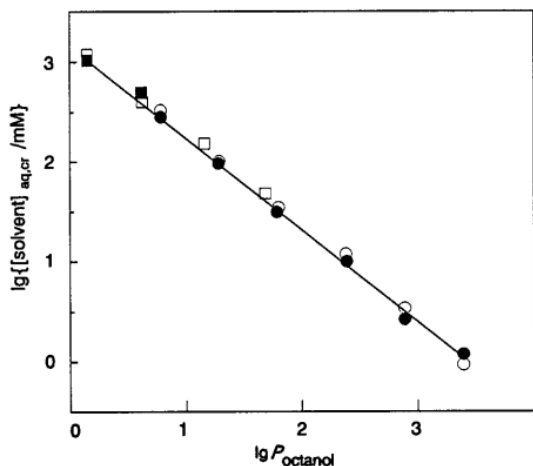
. Figure 4: Relationship between the logarithm of the aqueous solvent concentration at which 50% of the initial oxygen consumption rate of Arfhrobacfer (closed symbols) and Acinefobacfer (open symbols) is inhibited, and the IogP-,, of the solvents. The circles refer to alkanols, the squares to alkyl acetates (data from Vermut! 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 lgPoctanol and the log[solvent,,,,] has been observed (Figure 4). The value of log([solventmembrane,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 (Vermue et al., 1993, Tramper and Vermue, 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 lgPO,,,,,, since the threshold concentration in the aqueous phase can be estimated. It can be observed that the threshold concentration of polar solvents (low lgPocmnol) is higher than the threshold concentration of more hydrophobic onesand 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 lgPoctanol 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 a-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,O) prior to lyophilization of the enzyme preparation. After resuspending the enzyme in dry organic solvent, the highest degree of desorption of T20 has indeed been found after exposure to hydrophilic solvents. For example, methanol desorbs 56%-62% of bound T,O, while hexane desorbs only 0.4%- 2% (Gorman and Dordick, 1992).

Eflects 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 adsorbed on controlled-pore glass has been studied in diisopropyl Biotechnology in non-conventional media 353 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, = 0.87) stabilize the lipase, but in glycerol solutions of 70 up to 95% w/w (in which the a, 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,, 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,. 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.

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
	Williams et al., 1990
Hydrolysis of exo,exo-7-oxabicyclo[2.2.1]heptane-2,3-dimethanol diacetate ester by lipase P-30 from *Pseudomonas 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
	Carrea et al., 1988
	Cren et al., 1988
	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
	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
	Kimura et al., 1990
	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 preselected solvents is predicted by using the correlation between biocatalytic activity and lgPoctanol (Laane et al., 1987b) or the critical membrane concentration (Osborne et al., 1990). The relation between lgPoctmol 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 % (vh) 4-heptanone has a distribution coefficient for ethanol which is 12 % higher

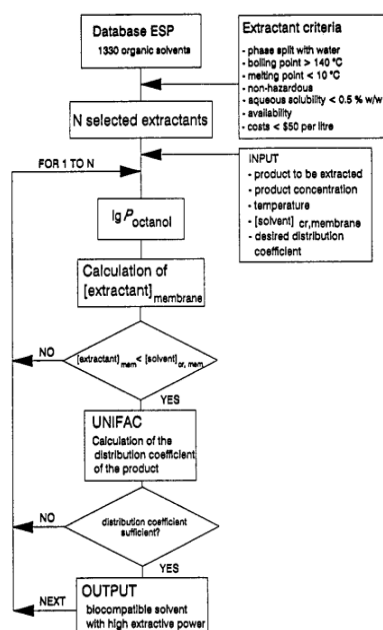*Biotechnology in non-conventional media*

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,) remains high. Even if the aqueous phase is restricted to the pores of the biocatalyst particles, the a, 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 D-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, 1993~). 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-
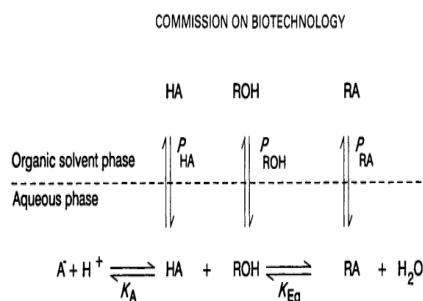

Figure 6: Equilibria involved in the esterification-coupled extraction of organic acids. KA is the dissociation constant of the acidhase equilibrium, KEq is the equilibrium constant of the lipase-

catalysed esterification, Pm, PRoH and PRA are partition coefficients of the undissociated acid (HA), the alcohol @OH) 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 pKa of the carboxylic acids usually range from 3 to 4 and the pK, 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 pKa of an acid usually increases, while the apparent pK, 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).
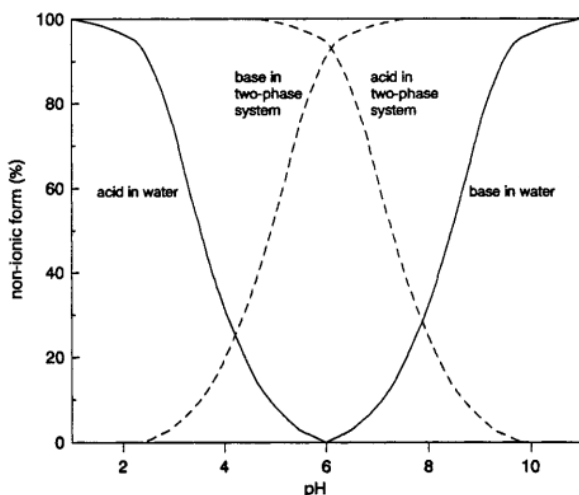

Figure 7: Theoretical titration curves for the carboxylic and amine components in water water-organic reaction mixture (----) (adapted from Semenov et al., 1988). and in a biphasicIn some cases, a high product yield in peptide synthesis has been achieved in aqueous buffer solutions by precipitation of 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, ClapCs et al., 1992, Chatterjee and Russell, 1993).
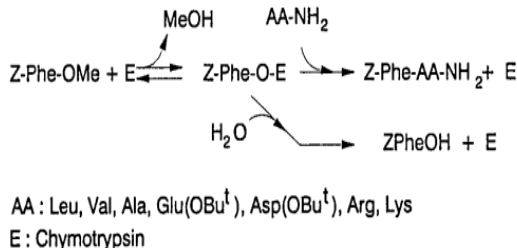

AA : Leu, Val, Ala, Glu(OBu^t), Asp(OBu^t), Arg, Lys

E : Chymotrypsin

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

Toxic eflects 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 watermiscible 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, Vermue 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 membraneassociated 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 (6) and high molecular weight show high biocatalytic activity retention for epoxidizing cells (Brink and Tramper, 1985). Better correlation between biocatalyticactivity and hydrophobicity is found when hydrophobicity is expressed by its lgPoCmol (Laane, 1987, Laane et al., 1987b). Generally, solvents having a relatively high lgPoctanol value (lgPocmol> 5) are biocompatible for cellular biocatalysts (Hocknull and Lilly, 1987, Buitelaar et al., 1990, Hocknull and Lilly, 1990, Bruce and Daugulis, 1991, Vermue et al., 1993). The transition between toxic and non-toxic solvents depends on the type of organism used (Vermue 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 liquidhquid 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 (G) 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, 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, a-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
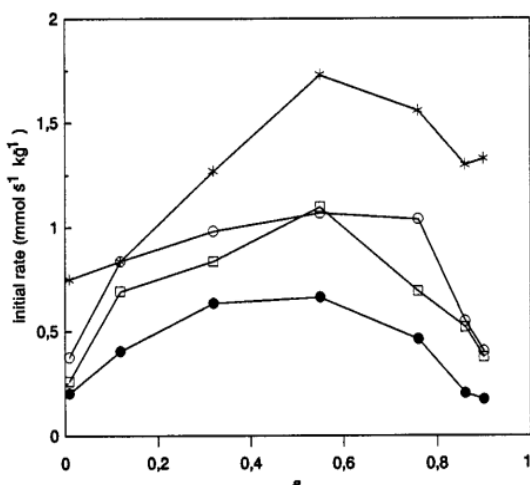
Figure 9: Activity of Lipozyme as a function of the water activity in hexane (*), toluene (0), trichloroethylene (0) and pentane-3-one (0) (data from Valivety et al., 1992b).

reach the same water activity (a,) (Valivety et al., 1992a). The water bound by the enzyme particles is likely to be a function of the a, (Halling, 1990) and as a consequence the a, 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,-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, 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, 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 lgPoctano,f the solvent and the enzyme activity. Instead, the authors propose to use the ratio of the partition coefficients of product and substrate (Pp/Ps) to predict enzyme activity. Solvents showing a high PplP, 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,, or P, and enzyme activity does not necessarily exist. This has been observed for the achymotrypsin catalysed esterification in several solvents at a, = 1 (Reslow et al., 19

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 Scarvone 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 Inmicroaqueous 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, a-chymotrypsin has been modified to accept the Dform 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 a-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 a-chymotrypsin is done in the presence of the L-form of the N-acetylated amino acid or in the absence of the Disomer, no esterification of the N-acetylated amino acid occurs (Stihl et al., 1991, Mansson 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.
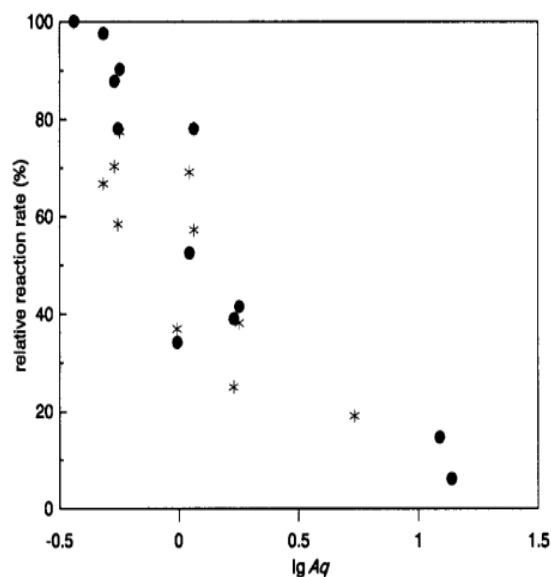
For horse-liver dehydrogenase and for a-chymotrypsin deposited on a support the catalytic activity decreases with increasing aquaphilicity of the support material (Figure 10). In these cases a fixed amount of water has been added to each reaction mixture. This water partitions between the solvent, the 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-

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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 G, 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 a-chymotrypsin (CT). For HLADH the reaction rate increases with increasing a, and the support with the lowest aquaphilicity, Celite, shows the highest activity among the support materials tested at fixed u,,,. The highest catalytic activity is also observed in the CT-catalysedalcoholysis of N-acetyl-L-phenylalanine ethyl ester with 1 -butanol, with the enzyme immobilized on Celite at high a,. However, in the a-chymotrypsin catalysed reaction two competing reactions occur simultaneously, hydrolysis and alcoholysis and the ratio between these reactions varies with the a, and with the type of support. At high a, 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, 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, using the hydrophilic support material, Accurel PA6.

Immobilization by covalent modijication

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, than the free enzyme which is inactivated at a, < 0.4 (Blanco et al., 1992). This is of particular importance in ester and peptide synthesis, because at low a, the thermodynamic equilibria of these reactions will shift towards higher product yields. However, the reaction proceeds very slowly at these low ~'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 nearcritical 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,) 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/m3) 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,) 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 Rantakyla (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 Chi et al., 1988 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 Yu et al., 1992
	esterification of myristic acid and ethanol	Dumont et al., 1992

Stability Enzymes generally show enhanced 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 C02 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 (Vermue 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 (6) 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
Transesterification 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 palmitic 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

CONCLUDING REMARKS

Research into the fundamentals of biocatalysis in non-conventional media has advanced significantly during the last decade. Biological catalyst activity and stability are influenced by a variety of circumstances, including the presence of water and the necessity to maintain biocatalysts in their active state. In order to rationally design biocatalytic processes in the reaction medium, certain fundamental guidelines have been developed and described in this review. Biocatalysis in non-conventional media and engineering media for synthetic purposes might benefit from these laws.

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