BIOCATALYSIS IN NON-CONVENTIONAL MEDIA: KINETIC AND THERMODYNAMIC ASPECTS

Promotor: dr.ir. J. Tramper
hoogleraar in de bioprocestechnologie

Marian Vermuë

BIOCATALYSIS IN NON-CONVENTIONAL MEDIA: KINETIC AND THERMODYNAMIC ASPECTS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 1 december 1995
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

ISBN 90-5485-462-6

Ontwerp kaft:
Joanneke Marchal.

Stellingen

1. Yang *et al.* suggereren ten onrechte dat het mechanisme dat ten grondslag ligt aan de toxiciteit van organische oplosmiddelen voor cellulaire biokatalysatoren te vergelijken is met het mechanisme van toxiciteit voor enzymen.

   Yang, B., Kuo, S.-J., Hariyadi, P., Parkin, K.L. (1994) *Solvent suitability for lipase-mediated acyl-transfer and esterification reactions in microaqueous milieu is related to substrate and product polarities.* Enzyme Microb. technol. **16**: 577-583.

2. In de vakliteratuur over biokatalyse in ongebruikelijke media wordt de polariteit van het medium vaak aangeduid met de term "hydrofobiciteit" terwijl "hydrofobie" wordt bedoeld.

   Laane, C., Boeren, S., Vos, K., Veeger, C. (1987) *Rules for optimazation of biocatalysis in organic solvents.* Biotechnol. Bioeng. **30**: 81-87.

3. De eigenschappen van organsich oplosmiddel "niet met water mengbaar" en "apolair" zijn niet relevant voor de efficiëntie van een enzym, zolang er geen polaire interacties optreden bij de vorming van het enzym-substraat complex.

   Narayan, V.S., Klibanov, A.M. (1993) *Are water-immiscibility and apolarity of the solvent relevant to enzyme efficiency?* Biotechnol. Bioeng. **41**: 390-393.

4. Uitspraken over de enzymactiviteit in superkritische vloeistoffen moeten kritisch bekeken worden, omdat er veelal sprake is van niet-superkritische proces-omstandigheden.

   Hoofdstuk 2 van dit proefschrift

5. Het milieubewustzijn van individuële automobilisten zou groter zijn als de knalpijp van de auto in plaats van aan de achterzijde, op de moterkap wordt gemonteerd.

6. De door genetici gehanteerde gedragsregel om mogelijke dragers van een heterozygoot overerfelijke ziekte pas op 18-jarige leeftijd uitsluitsel te geven over hun dragerschap, getuigt van hun beperkte kijk op het proces van relatievorming.

7. Zolang de overheid geen maatregelen neemt om het collectieve motor- en autorijden in stiltegebieden te verhinderen, heeft het geen zin landelijke gebieden als stiltegebied aan te wijzen.

8. Je kunt wachten tot je een ons weegt, voordat het SI-eenhedenstelsel algemeen aanvaard en gebruikt wordt.

9. De gemiddelde huizenprijs wordt systematisch omhoog gedreven doordat makelaars hun courtage baseren op de waarde van het pand in plaats van op het aantal bestede bemiddelingsuren.

10. Bij privatisering van onderdelen van een overheidsinstelling is een gegarandeerde afname van diensten door de overheidsinstelling alleen gerechtvaardigd, wanneer de diensten die het geprivatiseerde onderdeel levert, zowel kwalitatief als prijstechnisch te vergelijken zijn met die van reeds gevestigde commerciële bedrijven.

11. Gezien het toenemende aantal vrouwelijke wetenschappers zou het tijdens internationale congressen gebruikelijke "ladies' programme" moeten worden vervangen door een "partners' programme".

    Enzyme Engineering Conference XIII, San Diego, oktober 1995.

12. Creativiteit is deadline-gebonden, al kunnen zwangere vrouwen die deadline beter niet zetten op de uitgerekende datum van de geboorte van hun kind.

Stellingen behorende bij het proefschrift "Biocatalysis in non-conventional media; thermodynamic and kinetic aspects".

Marian Vermuë
Wageningen, 1 december 1995.

VOORWOORD

Meestal wordt het voorwoord van een proefschrift als een van de laatste stukken tekst op het papier gezet en daarom zou je het ook een nawoord kunnen noemen. Het voorwoord geeft me echter de gelegenheid om de mensen met wie ik al die jaren zo plezierig heb mogen samenwerken, persoonlijk te bedanken. Zonder deze mensen was dit boekje er waarschijnlijk niet gekomen en zij verdienen daarom een plaats voorin mijn proefschrift.

Allereerst wil ik mijn promotor Hans Tramper bedanken. Hij heeft me alle vrijheid gegeven in het onderzoek en stuurde bij als dat nodig was. Ook kreeg ik van hem de kans het internationale congres "Biocatalysis in Non-Conventional Media" mee te organiseren, een onvergetelijke ervaring. Zijn niet-aflatende vertrouwen in het gereedkomen van het boekje heeft mij enerzijds wel eens verbaasd, maar anderzijds heeft het mij ook gestimuleerd. Zo'n vertrouwen kon ik eenvoudigweg niet beschamen.

Met Wim Beverloo heb ik menig keertje rond de tafel gezeten. In feite is hij naast Hans mijn copromotor geweest. Als thermodynamisch geweten was hij steeds betrokken bij de verschillende projecten die in dit proefschift staan beschreven, maar ook als kritisch lezer en rekenaar kon ik steeds een beroep op hem doen. Wim, veel dank.

Jan Sikkema wil ik bedanken voor de jarenlange plezierige samenwerking in het kader van ons gezamelijke P.C.I.B. project "Hydoxylering van aromaten tot geur- en smaakstoffen". Zijn microbiologische inbreng in met name het onderzoek naar tetraline afbraak in de liquid-impelled loop reaktor, is bijzonder geweest en heeft geresulteerd in een aantal gezamelijke publicaties.

Het werk met superkritische oplosmiddelen is uitgevoerd op TNO-voeding te Zeist. Jeroen de Jong en Wilbert Oostrom wil ik bedanken voor hun aktieve

begeleiding van de studenten die in het kader van een afstudeeronderwerp of een stage een aantal maanden bij hen mochten vertoeven. Hennie Haman wil ik bedanken voor de hulp die ik van hem kreeg bij het verzamelen van de uiteindelijke data.

I want to thank Dr. Dieter Kirstein for the opportunity to work with him in the field of immobilization of enzymes on magnetic carriers. His hospitality during my stay at the "Akademie der Wissenschaften der DDR" in Berlin has been tremendous.

Binnen de vakgroep zijn een aantal mensen van dichtbij betrokken geweest bij mijn onderzoek. Bij Henk van Sonsbeek kon ik steeds terecht met problemen met de "liquid-impelled loop reactor". Anja Janssen en Albert van der Padt waren steeds te vinden voor discussies over de onderwerpen evenwichten en a_w en Rik Beeftink heeft veel van mijn concept artikelen van kritische opmerkingen voorzien. Henk, Anja, Albert en Rik, bedankt voor jullie inbreng.

Aan dit onderzoek hebben veel studenten, stagaires en buitenlandse gasten meegewerkt. Kirsten Zagt, Petra Koenraad, Rudolf Bakker, Tiny Lootsma, Nuria Xaus, Margot Bergmans, Annette Verheul, Peter van der Laar, Emerence Veldkamp, Paul Tap, Mark Resink, André van der Schaaf, Gisela Janssen, Margot Tacken en André Meyerink, ik wil jullie bedanken voor jullie enthousiastme en inzet voor mijn onderzoek. Zonder jullie was mijn boekje niet zo dik geworden.

Op deze plek wil ik ook Dhr. Wegh van de vakgroep Luchtverontreiniging bedanken voor zijn hulp bij de gaschromatografische analyse van tetraline en de vakgroep Organische Chemie voor hun hulp bij de estersyntheses.

Speciaal ook wil ik de diverse afdelingen van de technische dienst bedanken voor het vakwerk wat ze steeds afleverden. Met name de glasblazerij die menig maal technische hoogstandjes heeft moeten uitvoeren om de liquid-impelled loop reactor aan te passen en Jan Teunisse en Evert Janssen van de technische dienst die steeds met kreatieve oplossingen kwamen voor de reaktoropstellingen.

Tijdens al die jaren op Proceskunde heb ik genoten van de kameraadschappelijke sfeer. Naast de gezamenlijke reizen, uitstapjes en avonden was het ook tijdens de normale werkdagen altijd gezellig. Voor die prettige werksfeer wil ik al de proceskundigen bedanken en mijn kamergenoten in het bijzonder.

Pa en ma wil ik bedanken voor de mogelijkheden die ze mij steeds hebben

geboden en hun belangstelling, ondanks dat het voor hen niet altijd gemakkelijk zal zijn geweest om te volgen waar ik nou eigenlijk mee bezig was.

Rob, de kombinatie van ons beider werk, het proefschrift schrijven, en kinderen krijgen en opvoeden is niet altijd eenvoudig geweest. Gelukkig weet jij de zaken altijd heerlijk voor mij te relativeren waardoor we beiden plezier in ons werk houden en genieten van de vele leuke momenten die we samen en met Steven en Joris meemaken.

voor Steven en Joris

CONTENTS

INTRODUCTION

One of the most challenging subjects of study in applied biocatalysis is the use of reaction media which are significantly less polar than water. The interest in the use of such non-conventional media has grown immensely after the recognition that biocatalysts, such as enzymes, cell organelles and whole cells, are active in all kind of non-conventional media, such as organic solvents, gases and even supercritical fluids.

The application of biocatalysts in these non-conventional media is very attractive in organic-synthesis routes which desire the advantageous characteristics of biocatalysts such as a high substrate specificity and which involve substrates and products which are poorly soluble in water. When water is one of the products of the synthesis reaction, application of these media is even more attractive, since the equilibrium may be shifted in favour of the synthesis by lowering the water activity or by altering the partitioning behaviour of the substrates and products between the relevant phases. Other reasons to justify the use of non-polar media have been listed in Table 1.

Table 1 Potential advantages of the use of non-polar media in biocatalysis

- increased solubility of non-polar substrates and products
- shift in thermodynamic equilibria to favour synthetic reactions
- reduction of water-dependent side reactions by decresed water activity
- reduction of substrate and/or product inhibition
- possibility to manipulate the stereo- and regioselectivity of an enzymic biocatalyst
- simple recovery of the insoluble enzyme from the non-aqueous medium
- facilitated product recovery from volatile organic solvents or from supercritical fluids
- possible stabilization of the biocatalyst

Obviously disadvantages also exist, *e.g.* upon introduction of a solvent phase, the biocatalyst may be denaturated or inhibited by the solvent and the complexity of the reaction system increases. It is clear, however, that situations exist where the advantages of using non-conventional media outweigh the disadvantages.

The potentials of the use of non-conventional media in biocatalysis have initially led to a boom of publications in which promising applications in this field are presented (see reviews of Carrea, 1984, and Dordick, 1989). After this exploring period, the urge for a more fundamental understanding of the involved phenomena has arisen. This became particularly clear at the international conference "Biocatalysis in Non-Conventional Media" where the fundamentals of biocatalysis in non-conventional media have been addressed extensively (Tramper *et al.*, 1992, Halling, 1994).

OUTLINE OF THIS THESIS

The progress made in medium engineering presented during this conference, are reviewed in Chapter 2. In this chapter also some basic design rules are formulated which serve as a tool for optimization of biocatalytic processes in non-conventional media.

A typical example of a non-conventional medium is a two-phase mixture of water and water-immiscible organic solvent. Especially for this type of reaction medium a new bioreactor has been developed, the so-called liquid-impelled loop reactor (Van Sonsbeek, 1992). This bioreactor has been used for the bioconversion of tetralin, a toxic apolar substrate. Chapter 3 describes the general strategy for selection of a suitable solvent for the bioconversion of apolar compounds in the liquid-impelled loop reactor, using the tetralin conversion as a typical example. In the selection procedure the basic design rules as presented in Chapter 2 are applied.

In order to find the rate-limiting substrate for the tetralin conversion in the liquid-impelled loop reactor the mass transfer of oxygen and tetralin has been investigated (Chapter 4).

One of the possible disadvantages of the use of non-conventional media is their toxicity towards microbial cells (Sikkema *et al.*, 1995). Toxicity in a two-liquid phase

reaction medium can be caused by phase-boundary effects (phase toxicity) and by the solvent molecules that are dissolved in the aqueous phase (molecular toxicity) (Bar, 1988). In Chapter 5 these effects have been described and on the basis of the presented theory of the critical-concentration in the membrane (Osborne *et al.*, 1990), prediction of the toxicity of a solvent with a given $\log P_{octanol}$ is feasible. $\text{Log}P_{octanol}$ 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 for the hydrophobicity of the solvent.

Another hydrophobicity indicator that has been used successfully to predict the toxicity of organic solvents to cellular biocatalysts is the Hildebrand solubility parameter (Brink and Tramper, 1985). In Chapter 6 this parameter is used to predict the solubility of substrates in near-critical carbon dioxide and to investigate its effect on the activity of an immobilized lipase in this micro-aqueous medium. In addition, the crucial role of water for the enzyme activity in this medium has been investigated.

Water is not only crucial for enzyme activity, but its presence also reduces the theoretical product yield in synthetic reactions. Reduction of the water activity diminishes water-dependent side-reactions in transesterification reactions (Chapter 6) and shifts the thermodynamic reaction equilibrium towards synthesis in esterification reactions (Chapter 7). In Chapter 7 a new method to control the water activity (a_w) during an esterification reaction in organic solvent is presented. The a_w is kept at an optimum value at which the enzyme is sufficiently active and yet high product yields are achieved.

Throughout this thesis, several thermodynamic quantities are used. Chapter 8 shows how thermodynamics provides us with the tools to predict the values of the parameters such as $\log P_{octanol}$, the Hildebrand solubility parameter and a_w and what the challenges of applying thermodynamics as a basic tool can be in medium engineering.

REFERENCES

Bar, R. (1988) Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. *J. Chem. Technol. Biotechnol.* **43**: 49-62.

Brink, L.E.S., Tramper, J. (1985) Optimization of organic solvent in multiphase biocatalysis. *Biotechnol. Bioeng.* **27**: 1258-1269.

Carrea, G. (1984) Biocatalysis in water-organic solvent two-phase systems. *Trends Biotechnol.* **2**: 102-106.

Dordick, J.S. (1989) Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.* **11**: 194-211.

Halling, P.J. (1994) Thermodynamic predictions for biocatalysis in nonconventional media: theory, tests, and recommendations for experimental design and analysis. *Enzyme Microb. Technol.* **16**: 178-206.

Osborne, S.J., Leaver, J., Turner, M.K., Dunnill, P. (1990) Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.* **12**: 281-291.

Sikkema, J., De Bont, J.A.M., Poolman, B. (1995) Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**: 201-222.

Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) (1992) Biocatalysis in non-conventional media, Elsevier Science Publ., Amsterdam, The Netherlands.

Van Sonsbeek, H.M. (1992) Physical aspects of liquid-impelled loop reactors. PhD-thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Chapter 2

BIOCATALYSIS IN NON-CONVENTIONAL MEDIA; MEDIUM ENGINEERING ASPECTS - A REVIEW

SUMMARY

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.

Biocatalysis in non-conventional media: medium engineering aspects.
Vermuë, M.H., Tramper, J. (1995) *Pure & Appl. Chem.* **67**: 345-373.

Chapter 2

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 relatively harsh conditions, such as extreme pH, elevated 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 favour 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.

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-

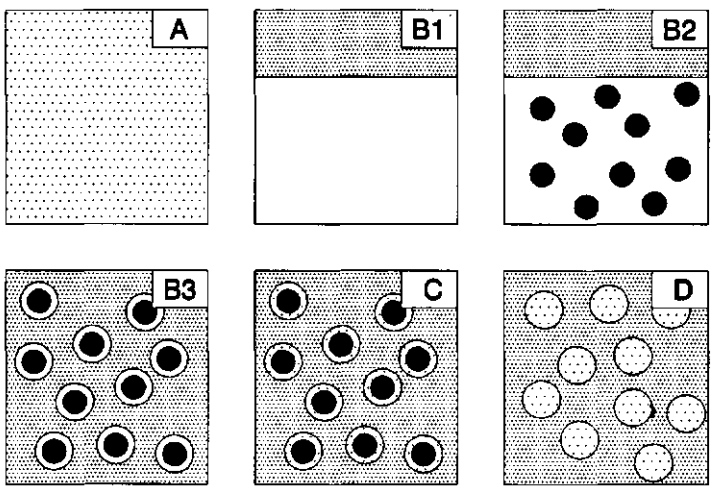


Figure 1: Schematic representation of the four categories of organic-solvent reaction media.
A : water-miscible solvent
B1: two-phase system, low volume organic solvent, solubilized biocatalyst
B2: two-phase system, low volume organic solvent, immobilized biocatalyst
B3: two-phase system, $a_w = 1$,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

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 solvents even show an enhanced 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 maintain the catalytic activity,

depending on the solvent used.

Log$P_{octanol}$

Often the log$P_{octanol}$ is used to predict the activity retention of a biocatalyst in organic media (Laane *et al.*, 1987b). The log$P_{octanol}$ 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 log$P_{octanol}$ no activity is found, at intermediate log$P_{octanol}$ the solvents show unpredictable toxic effects and at high log$P_{octanol}$ no toxic effects are observed (Laane *et al.*, 1987b, Laane and Tramper, 1990). The correlation between initial activity and/or stability and log$P_{octanol}$ has been observed for several enzymes (Figure 2). However, examples of enzymic reactions exist for which no correlation between activity retention and log$P_{octanol}$ is found (Figure 3, Estrada *et al.*, 1991, Kanerva *et al.*, 1990, Vazquez-Duhalt *et al.*, 1993). Log$P_{octanol}$ is thus not always a good parameter to predict the toxic effects of solvents in enzymic reactions and in some cases other polarity indicators, such as the Dimroth-Reichardt parameter $E_T(30)$ (Vazquez-Duhalt *et al.*, 1993), predict enzyme activity better.

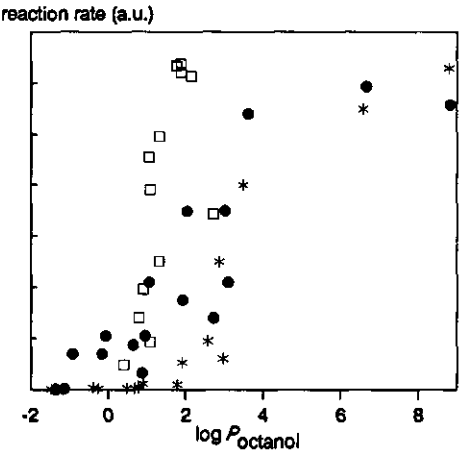


Figure 2: Activity of α-chymotrypsin (□), *Mucor* sp. lipase (●), and *Candida rugosa* lipase (*) as a function of log$P_{octanol}$ of the solvent, a.u. = arbitrary units (data from Reslow, 1989 and Zaks and Klibanov, 1985).

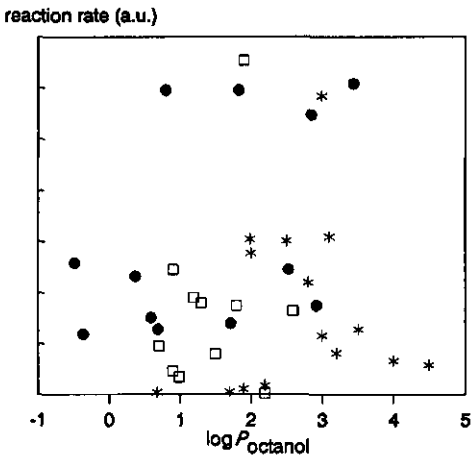


Figure 3: Activity of mandelonitril lyase (□), porcine pancreatic lipase (●), and polyphenol oxidase (*) as a function of $\log P_{octanol}$ of the solvent, a.u. = arbitrary units (data from Wehtje, 1992, Zaks and Klibanov, 1985, and Yang *et al.*, 1992, respectively).

$\text{Log}P_{octanol}$ 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 subsaturated 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 $\log P_{octanol}$ value. The partition coefficient of the organic solvent in a membrane/aqueous buffer two-phase system can be related to $\log P_{octanol}$ 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

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

If the logarithm of the threshold concentration of the solvent in the aqueous phase, $\log[solvent_{aq,cr}]$, is plotted against $\log P_{octanol}$, 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

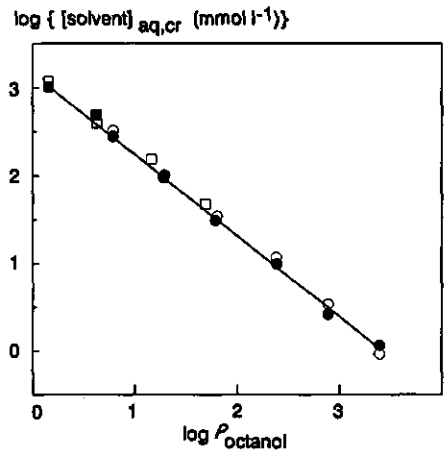


Figure 4: Relationship between the logarithm of the aqueous solvent concentration at which 50% of the initial oxygen consumption rate of *Arthrobacter* (closed symbols) and *Acinetobacter* (open symbols) is inhibited, and the $\log P_{octanol}$ of the solvents. The circles refer to alkanols, the squares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

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 (Sikkema *et al.*, 1994).

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 $\log P_{octanol}$ 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 $\log P_{octanol}$, since the threshold concentration in the aqueous phase can be estimated. It can be observed that the threshold concentration of polar solvents (low $\log P_{octanol}$) 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 $\log P_{octanol}$ 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 enhanced 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).

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-catalyzed disulfide interchange
- oxidation of cysteine residues
- deamination of asparagine and/or glutamine residues
- hydrolysis of peptide bonds at aspartic acid residues

The mechanisms of irreversible thermo-inactivation of enzymes in aqueous solutions at 363-373 K 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).

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 operational stability of enzymes in organic media is not often investigated. 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 water/g dodecanol) 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.

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

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 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 naphthalene-*cis*-glycol	Harrop *et al.*, 1992
Steroid bioconversion	Yamané *et al.*, 1979 Carrea *et al.*, 1988 Ceen *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 and Tramper, 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 aceton	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-catalyzed synthesis of alkyl-ß-glucoside	Vulfson *et al.*, 1990
Lipase-catalyzed 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

mixtures which are both biocompatible and an 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

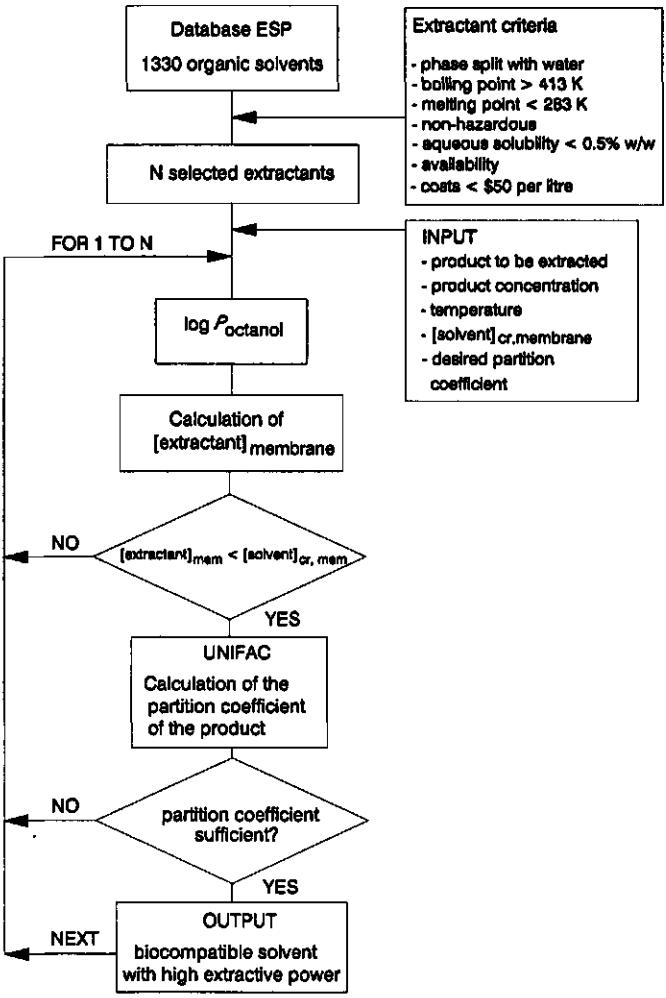


Figure 5: The extractant screening program (ESP) (adapted from Bruce and Daugulis, 1991).

correlation between biocatalytic activity and $\log P_{octanol}$ (Laane *et al.*, 1987b) or the critical membrane concentration (Osborne *et al.*, 1990). The realtion between $\log P_{octanol}$ 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 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

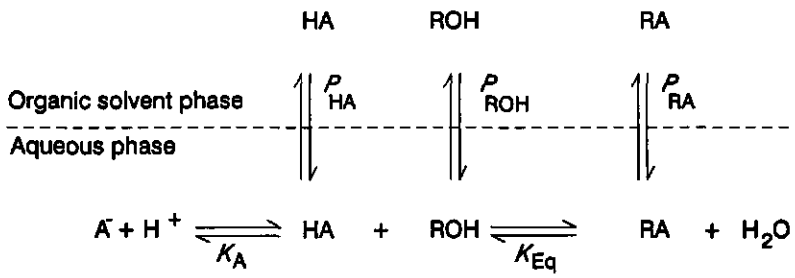


Figure 6: Equilibria involved in the esterification-coupled extraction of organic acids. K_A is the dissociation constant of the acid/base equilibrium, K_{Eq} is the equilibrium constant of the lipase-catalyzed esterification, P_{HA}, P_{ROH} and P_{RA} are partition coefficients of the undissociated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

synthesis when highly concentrated solutions of substrates are used, as in the glycosidase-catalyzed synthesis of alkyl ß-glucoside (Vulfson *et al.*, 1990) and in the lipase-catalyzed 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-catalyzed esterification (Figure 6). The high distribution coefficient of the ester outweighs the low equilibrium 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 at least one of the reactants will thus exist fairly 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 an increase of the equilibrium concentration of the ester product (Figure 7, Semenov, 1988, and references cited therein).

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

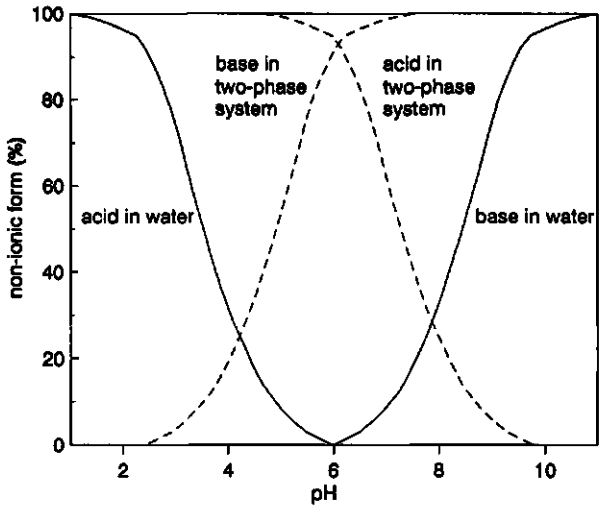
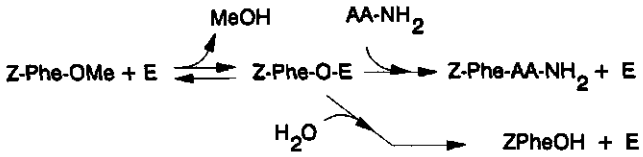


Figure 7: Theoretical titration curves for the carboxylic and amine components in water (——) and in the biphasic water-organic solvent reaction mixture (---) (adapted from Semenov *et al.*, 1988).

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



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

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

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 acylenzyme 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).

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 chapter on 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 interface-toxicity effect. Several mechanisms to explain interface-toxicity effects have been identified, such as nutrient extraction and adsorption, disruption of cell membranes by interfacial forces, limited access to nutrients due to emulsion formation, cell coating and attraction to the interface (Bar, 1987). In addition, several researchers have demonstrated that disruption of the cellular membrane is 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 avoided by the addition of an alternative artificial electron acceptor such as 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 (δ) 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 $\log P_{octanol}$ (Laane, 1987, Laane *et al.*, 1987b). Generally, solvents having a relatively high $\log P_{octanol}$ value ($\log P_{octanol} > 5$) 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 combined with lower turbulence intensity. 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 reproducible 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 on the exposure time.

If predominantly interface-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).

Chapter 2

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, α-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 reach the same water activity (a_w) (Valivety *et al.*, 1992a). The amount of water bound by the enzyme particles is likely to be a function of the a_w (Halling, 1990) and as a consequencethe 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

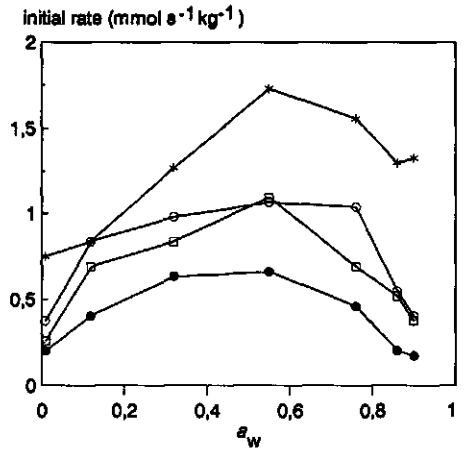


Figure 9: Activity of Lipozyme as a function of the water activity in hexane (*), toluene (○), trichloroethylene (□) and pentane-3-one (●) (data from Valivety *et al.*, 1992b).

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 in excess 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 $\log P_{octanol}$ 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 catalyzed 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). 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.

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

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 at all (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.

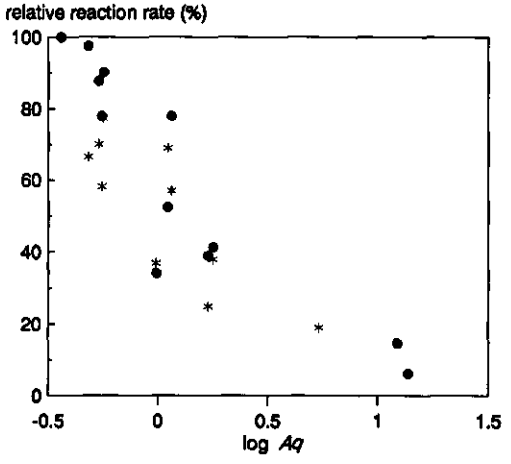


Figure 10: Relative reaction rate of α-chymotrypsin (*) and horse liver alcohol dehydrogenase (●) when deposited on support materials with different aquaphilicity (*Aq*) (data from Reslow *et al.*, 1988c).

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 catalyzed 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-catalyzed 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 catalyzed 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 attributed 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).

Chapter 2

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 compared to liquids, and high diffusivity comparable with subcritical 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). For most biocatalytic reactions an operation temperature roughly below 333 K is required for biocatalyst stability. The choice of supercritical fluids is thus limited to compounds having a critical temperature (T_c) between 0 and 333 K. For practical reasons the critical pressure of the compound should not be too extreme. Table 4 shows a list of compounds that fulfil this requirement.

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

Solvent	Critical temperature (K)	Critical pressure (MPa)
Carbon dioxide	304.1	7.3
Ethane	305.3	4.8
Ethylene	282.3	5.0
Trifluoromethane	298.9	4.6
Nitrous oxide	309.5	7.0
Sulfur hexafluoride	N.A.	N.A.

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

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 catalyzed 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 g l^{-1}) 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 catalyzed 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

Table 5: Examples of enzymatic reactions in supercritical carbon dioxide

Enzyme	Reaction	Reference
- alkaline phosphatase	hydrolysis of *p*-nitro-phenyl 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 methylmethacrylate with 2-ethylhexanol	Kamat *et al*, 1992a
	interesterification of trilaurin and myristic acid	Miller *et al*, 1992
	transesterification of ethylacetate 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

only focus on the effect of medium characteristics of supercritical fluids on the stability and activity of enzymes.

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 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 whether 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 (δ) can be helpful as a first estimate of the solubility of the substrates in the solvent at the reaction conditions used (Allada, 1984, Vermuë *et al.*, 1992). If the difference in δ 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 δ) in combination with a high molecular weight of the organic solvent and biocatalytic activity. Vermuë *et al.* (1992) have

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*-nitro-phenyl 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

attempted to correlate the lipase-catalyzed 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.

REFERENCES

Aaltonen, O., Rantakylä, M. (1991) Biocatalysis in supercritical CO_2. *CHEMTECH* **April 1991**: 240-248.

Adlercreutz, P. (1991) On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* **199**: 609-614.

Adlercreutz, P. (1992) On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. In: *Biocatalysis in Non-Conventional Media*, 55-61. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Ahern, T.J., Klibanov, A.M. (1985) The mechanism of irreversible enzyme inactivation at 100 °C. *Science* 228: 1280-1284.

Aires-Barros, M.R., Cabral, J.M.S., Willson, R.C., Hamel, J.-F.P., Cooney, C.L. (1989) Esterification-coupled extraction of organic acids: partition enhancement and underlying reaction and distribution equilibria. *Biotechnol. Bioeng.* **34**: 909-915.

Allada, S.R. (1984) Solubility parameters of supercritical fluids. *Ind. Eng. Chem. Process Des. Dev.* **23**: 344-48.

Andersson, E., Hahn-Hägerdal B., (1990) Bioconversions in aqueous two-phase systems. *Enzyme Microb. Technol.* **12**: 242-254.

Arnold, F.H. (1988) Protein design for nonaqueous solvents. *Protein Eng.* **2**: 21-25.

Arnold, F.H. (1990) Engineering enzymes for nonaqueous solvents. *Trends Biotechnol.* **8**: 244-249.

Ayala, G., Tuena de Gomez-Puyou, M., Gomez-Puyou, A., Darzon, A., (1986) Thermostability of membrane enzymes in organic solvents. *FEBS Lett.* **203**: 41-43.

Bar, R. (1987) Phase toxicity in a water-solvent two-liquid phase microbial system. In: *Biocatalysis in organic solvents*, 147-153. Laane, C., Tramper, J., Lilly, M.D. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Bar, R. (1988) Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. *J. Chem. Tech. Biotechnol.* **43**: 49-62.

Barton, W.E., Daugulis, A.J. (1992) Evaluation of solvents for extractive butanol fermentation with *Clostridium acetobutylicum* and the use of poly(propylene glycol) 1200. *Appl. Microbiol. Biotechnol.* **36**: 632-639.

Bassetti, L., Tramper, J. (1994) Organic-solvent toxicity in *Morinda citrifolia* cell suspensions. *Enzyme Microb. Technol.* **16**: 642-648.

Blanco, R.M., Guisan, J.M. (1988) Protecting effect of competitive inhibitors during very intense insolubilized enzyme-activated support multi-point attachments: trypsin (amine)-agarose (aldehyde) system. *Enzyme Microb. Technol.* **10**: 227-232.

Blanco, R.M., Guisan, J.M., Halling, P.J. (1989) Agarose-chymotrypsin as a catalyst for peptide and amino acid ester synthesis in organic media. *Biotechnol. Letters* **11**: 811-816.

Blanco, R.M., Rakels, J.L.L., Guisan, J.M., Halling, P.J. (1992) Effect of thermodynamic water activity on amino-acid ester synthesis catalyzed by agarose-chymotrypsin in 3-pentanone. *Biochim. Biophys. Acta* **1156**: 67-70.

Blank-Koblenc, T., Tor, R., Freeman, A. (1988) Cosolvent effects on gel-entrapped oxidoreductase: the glucose oxidase model. Biotechnol. *Appl. Biochem.* **10**: 32-41.

Brink, L.E.S., Tramper, J. (1985) Optimization of organic solvent in multiphase biocatalysis. *Biotechnol. Bioeng.* **27**: 1258-1269.

Brookes, I.K., Lilly, M.D., Drozd, J.W. (1986) Stereospecific hydrolysis of *d,l*-menthyl acetate by *Bacillus subtilis*: mass transfer-reaction interactions in a liquid-liquid system. *Enzyme Microb. Technol.* **8**: 53-57.

Bruce, L.J., Daugulis, A.J. (1991) Solvent selection strategies for extractive biocatalysis. *Biotechnol. Prog.* **7**: 116- 124.

Bruce, L.J., Daugulis, A.J. (1992) Extractive fermentation by *Zymomonas mobilis* and the use of solvent mixtures. *Biotechnol. Letters* **14**: 71-76.

Buitelaar, R.M., Vermuë, M.H., Schlatmann, J.E., Tramper, J. (1990) The influence of various organic solvents on the respiration of free and immobilized cells of *Tagetes minuta*. *Biotechnol. Techniques* **4**, 415-418.

Butler, L.G. (1979) Enzymes in non-aqueous solvents. *Enzyme Microb. Technol.* **1**: 253-259.

Carrea, G., Riva, S., Bovara, R., Pasta, P. (1988) Enzymatic oxidoreduction of steroids in two-phase systems: effects of organic solvent on enzyme kinetics and evaluation of the performance of different reactors. *Enzyme Microb. Technol.* **10**: 333-340.

Carrea, G., Ottolina, G., Riva, S., Secundo, F. (1992) Effect of reaction conditions on the activity and enantioselectivity of lipases in organic solvents. In: *Biocatalysis in Non-Conventional Media*, 111-119. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Cassells, J.M., Halling, P.J. (1988) Effect of thermodynamic water activity on thermolysin-catalyzed peptide synthesis in organic two-phase systems. *Enzyme Microb. Technol.* **10**: 486-491.

Ceen, E.G., Dunnill, P., Herrmann. J.P.R. (1988) Two-liquid phase reactor studies of 11α-hydroxylation of progesterone by *Aspergillus ochraceus*. *Biotechnol. Bioeng.* **31**: 743-746.

Chatterjee, S., Russell, A.J. (1992) Activity of thiosubtilisin in organic solvents. *Biotechnol. Prog.* **8**: 256-258.

Chatterjee S., Russell, A.J. (1993) Kinetic analysis of the mechanism for subtilisin in essentially anhydrous organic solvents. *Enzyme Microb. Technol.* **15**: 1022-1029.

Chen, C.-S., Wu, S.-H., Girdaukas, G., Sih, C.J. (1987) Quantitative analysis of biochemical kinetic resolution of enantiomers. 2. Enzyme-catalyzed esterification in water-organic solvent biphasic systems. *J. Am. Chem. Soc.* **109**: 2812-2817.

Chi, Y.M., Nakamura, K., Yano, T. (1988) Enzymatic interesterification in supercritical carbon dioxide. *Agric. Biol. Chem.* **52**: 1541-1550.

Clapés, P., Adlercreutz, P., Mattiasson, B. (1990) Enzymatic peptide synthesis in organic media. A comparative study of water miscible and water immiscible solvent systems. *J. Biotechnol.* **15**: 323-338.

Collander, R. (1951) The partition of organic compounds between higher alcohols and water. *Acta Chem. Scand.* **5**: 774-780.

Daugulis, A.J. (1988) Integrated reaction and product recovery in bioreactor systems. *Biotechnol. Prog.* **4**: 113-122.

Dordick, J.S. (1989) Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.* **11**: 194-211.

Dordick, J.S. (1992) Designing enzymes for use in organic solvents. *Biotechnol. Prog.* **8**: 259-267.

Dordick, J.S., Marletta, M.A., Klibanov, A.M. (1986) Peroxidase depolymerize lignin in organic media,but not in water. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 6255.

Dumont, T., Barth, D., Corbier, C., Branlant, G., Perrut, M. (1992) Enzymatic reaction kinetic: comparison in an organic solvent and in supercritical carbon dioxide. *Biotechnol. Bioeng.* **39**: 329-333.

Eggers, D.K., Blanch, H.W., Prausnitz, J.M. (1989) Extractive catalysis: solvent effects on equilibria of enzymatic reactions in two-phase systems. *Enzyme Microb. Technol.* **11**: 84-89.

Erickson, J.C., Schyns, P., Cooney, C.L. (1990) Effect of pressure on an enzymatic reaction in a supercritical fluid. *AIChE Journal* **36**: 299-301.

Estrada, P., Sanchez-Muñiz, R., Acebal, C., Arche, R., Castillón, P.M. (1991) Characterization and optimization of immobilized polyphenol oxidase in low-water organic solvents. *Biotechnol. Appl. Biochem.* **14**: 12-20.

Evans, P.J., Wang, H.Y. (1990) Effects of extractive fermentation on butyric acid production by *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.* **32**: 393-397.

Fernandez, M.M., Clark, D.S., Blanch, H.W. (1991) Papain kinetics in the presence of a water-miscible organic solvent. *Biotechn. Bioeng.* **37**: 967-972.

Fitzpatrick, P.A., Klibanov, A.M. (1991) How can the solvent affect enzyme enantioselectivity? *J. Am. Chem. Soc.* **113**: 3166-3171.

Freeman, A., Lilly, M.D. (1987) The effect of water-miscible solvents on the Δ^1-dehydrogenase activity of free and PAAH-entrapped *Arthrobacter simplex*. *Appl. Microbiol. Biotechnol.* **25**: 495-501.

Ghatorae, A.S., Guerra, M.J., Bell, G., Halling, P.J. (1993) New technique for monitoring interfacial inactivation of enzymes by organic solvents. In: *Stability and Stabilization of Enzymes*, 329-336. Van den Tweel, W.J.J., Harder, A., Buitelaar, R.M. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Gómez-Puyou, A.M.D. (Ed.) (1992) *Biomolecules in organic solvents*. CRC Press, Boca Raton, Florida, USA.

Gorman, L.A.S., Dordick, J. (1992) Organic solvents strip water off enzymes. *Biotechnol. Bioeng.* **39**: 392-397.

Granot, I., Aharonowitz, Y., Freeman, A. (1988) Cosolvent effect on Δ^1-steroid-reductase activity of free and PAAH-entrapped *Mycobacterium* Sp. NRRL B-3805 cells. *Appl. Microbiol. Biotechnol.* **27**: 457-463.

Guagliardi, A., Manco, G., Rossi, M., Bartolucci, S. (1989) Stability and activity of a thermostable malic enzyme in denaturants and water-miscible solvents. *Eur. J. Biochem.* **183**: 25-30.

Guinn, R.M., Blanch, H.W., Clark, D.S. (1991) Effect of a water-miscible organic solvent on the kinetic and structural properties of trypsin. *Enzyme Microb. Technol.* **13**: 320-326.

Halling, P.J. (1984) Effects of water on equilibria catalyzed by hydrolytic enzymes in biphasic reaction systems. *Enzyme Microbiol. Technol.* **6**: 513-516.

Halling, P.J. (1990) High-affinity binding of water by proteins is similar in air and in organic solvents. *Biochim. Biophys. Acta* **1040**: 225-228.

Halling, P.J., Valivety, R.H. (1992) Physical-chemical nature of low water systems for biocatalysis: especially phase behaviour, water activity and pH. In: *Biocatalysis in Non-Conventional Media*, 13-21. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Hammond, D.A., Karel, M., Klibanov, A.M., Krukonis, V.J. (1985) Enzymatic reactions in supercritical gases. *Appl. Biochem. Biotechnol.* **11**: 393-400.

Harbron, S., Smith, B.W., Lilly, M.D. (1986) Two-liquid phase biocatalysis: epoxidation of 1,7-octadiene by *Pseudomonas putida*. *Enzyme Microb. Technol.* **8**: 85-88.

Harrop, A.J., Woodley, J.M., Lilly, M.D. (1992) Production of naphthalene-*cis*-glycol by *Pseudomonas putida* in the presence of organic solvents. *Enzyme Microb. Technol.* **14**: 725-730.

Hocknull, M.D., Lilly, M.D. (1987) The Δ^1-dehydrogenation of hydrocortisone by *Arthrobacter simplex* in organic- aqueous two-liquid phase environments. In: *Biocatalysis in Organic Media*, 393-398. Laane, C., Tramper, J., Lilly, M.D. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Hocknull, M.D., Lilly, M.D. (1988) Stability of the steroid Δ^1-dehydrogenation system of *Arthrobacter simplex* in organic solvent-water two-liquid phase environments. *Enzyme Microbiol. Technol.* **10**: 669-674.

Hocknull, M.D., Lilly, M.D. (1990) The use of free and immobilised *Arthrobacter simplex* in organic solvent/aqueous two-liquid-phase reactors. *Appl. Microb. Biotechnol.* **33**: 148-153.

Inada, Y., Takahashi, K., Yoshimoto, T., Ajima, A., Matsushima, A., Saito, Y. (1986) Application of polyethylene glycol-modified enzymes in biotechnological processes: organic solvent-soluble enzymes. *TIBTECH* July: 190-194.

Inada, Y., Matsushima, A., Takahashi, K., Saito, Y. (1990) Polyethylene glycol-modified lipase soluble and active in organic solvents. *Biocatalysis* **3**: 317-328.

Janssen, A.E.M., Van der Padt, A., Van Sonsbeek, H.M., Van 't Riet (1993a) The effect of organic solvents on the equilibrium position of enzymatic acylglycerol synthesis. *Biotechnol. Bioeng.* **41**: 95-103.

Janssen, A.E.M., Van der Padt, A., Van 't Riet, K. (1993b) Solvent effects on lipase-catalyzed esterification of glycerol and fatty acids. *Biotechnol. Bioeng.* **42**: 953-962.

Janssen, A.E.M., Wessels Boer, N., Van 't Riet, K. (1993c) Solvent effects on the equilibrium position of lipase- catalyzed esterification of decanoic acid and various alcohols. *Biocatalysis* **8**: 133-135.

Janssen, A.E.M. (1993d) *Enzymatic synthesis of polyol esters in aqueous-organic two-phase systems*. PhD-Thesis, Wageningen Agricultural University, Wageningen, NL.

Kamat, S., Barrera, J., Beckman, E.J., Russell, A.J. (1992a) Biocatalytic synthesis of acrylates in organic solvents and supercritical fluids: 1.Optimization of enzyme environment. *Biotechnol. Bioeng.* **40**: 158-166.

Kamat, S., Beckman, E.J., Russell, A.J. (1992b) Role of diffusion in non-aqueous enzymology. 1. Theory. *Enzyme, Microb. Technol.* **14**: 265-271.

Kamihira, M., Taniguchi, M., Kobayashi, T. (1987) Synthesis of aspartame precursors by enzymatic reaction in supercritical carbon dioxide. *Agric. Biol. Chem.* **51**: 3427-3428.

Kanerva, L.T., Vihanto, J., Halme, M.H., Loponen, J.M., Euranto, E.K. (1990) Solvent effects in lipase-catalyzed transesterification reactions. *Acta Chem. Scand.* **44**: 1032-1035.

Kasche, V., Schlothauer, R., Brunner, G. (1988) Enzyme denaturation in supercritical CO_2: stabilizing effect of S-S bonds during the depressurization step. *Biotechnol. Lett.* **10**, 569-574.

Kazandjian, R.Z., Klibanov, A.M. (1985) Regioselective oxidation of phenol catalyzed by polyphenol oxidase in chloroform. *J. Am. Chem. Soc.* **107**: 5448-5450.

Khmelnitsky, Y.L., Mozhaev, V.V., Belova, A.B., Sergeeva, M.V., Martinek, K. (1991) Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis. *Eur. J. Biochem.* **198**, 31-41.

Kimura, Y., Nakanishi, K., Matsuno, R. (1990) Enzymatic synthesis of the precursor of Leu-enkephalin in water- immiscible organic systems. *Enzyme Microb. Technol.* **12**: 272-280.

King, R.S., (1992) Aqueous two-phase partitioning in biotechnology. In: *Polym. Appl. Biotechnol.*, 204-243 Soane, D.S. (Ed) Prentice Hall: Englewood Cliffs, N.J.

Kise, H., Hayakawa, A., Noritomi, H. (1990) Protease-catalyzed synthetic reactions and immobilization-activation of the enzymes in hydrophilic organic solvents. *J. Biotechnol.* **14**: 239-254.

Kitaguchi, H., Fitzpatrick, P.A., Huber, J.E., Klibanov. A.M. (1989) Enzymatic resolution of racemic amines: crucial role of the solvent. *J. Am. Chem. Soc.* **111**: 3094-3095.

Klibanov, A.M. (1989) Enzymatic catalysis in anhydrous organic solvents. *TIBS* April: 141-144.

Klibanov, A.M. (1990a) Assymmetric transformations catalyzed by enzymes in organic solvents. *Acc. Chem. Res.* **23**: 114-120.

Krukonis, V.J., Hammond, D.A. (1988) On the solubility of disodium p-nitrophenyl phosphate in supercritical carbon dioxide. *Biotechnol. Lett.* **10**: 837-841.

aane, C. (1987) Medium-engineering for bio-organic synthesis. *Biocatalysis* **1**: 17-22.

Laane, C., Tramper, J., Lilly, M.D. (Ed.) (1987a) *Biocatalysis in organic media*. Elsevier Science Publ., Amsterdam, The Netherlands.

Laane, C., Boeren, S., Vos, K., Veeger, C. (1987b) Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **30**: 81-87.

Laane, C., Tramper, J. (1990) Tailoring the medium and reactor for biocatalysis. *Chemtech* August: 502-506.

Lamare, S., Legoy, M.D. (1993) Biocatalysis in the gas phase. *Tibtech* **11**: 413-418.

Manjón, A., Obón, J.M., Cánovas, M., Iborra, J.L. (1992) Correlations between enzyme activity, water activity, and logP in one-liquid-phase systems. In: *Biocatalysis in non-conventional media*, 121-128. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Månsson, M.-O., Ståhl, M., Mosbach, K. (1992) Induced stereo and substrate selectivity of bio-imprinted α-chymotrypsin in anhydrous organic media. In: *Biocatalysis in Non-Conventional Media*, 321-327. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Marty, A., Chulalaksananukul, W., Willemot, R.M., Condoret, J.S. (1992) Kinetics of lipase-catalyzed esterification in supercritical CO_2. *Biotechnol. Bioeng.* **39**: 273-280.

Maurel, P. (1978) Relevance of dielectric constant and solvent hydrophobicity to the organic solvent effect in enzymology. *J. Biol. Chem.* **253**: 1677-1683.

McHugh, M., Krukonis, V. (1986) *Supercritical fluid extraction*. Butterworth Publ., Boston, USA.

Miller, D.A., Blanch, H.W., Prausnitz, J.M. (1991) Enzyme-catalyzed ineresterification of triglycerides in supercritical carbon dioxide. *Ind. Eng. Chem. Res.* **30**: 939-946.

Minier, M., Goma, G. (1982) Ethanol production by extractive fermentation. *Biotechnol. Bioeng.* **24**: 1565-1579.

Monot, F., Borzeix, F., Bardin, M., Vandecasteele, J.-P. (1991) Enzymatic esterification in organic media: role of water and organic solvent in kinetics and yield of butyl butyrate synthesis. *Appl. Micribiol. Biotechnol.* **35**: 759-765.

Monsan P., Combes D. (1984) Effect of water activity on enzyme action and stability. *Ann. N.Y. Acad. Sci.* **12**: 4860.

Mozhaev, V.V., Khmelnitsky, Y.L., Sergeeva, M.V., Belova, A.B., Klyachko, N.L., Levashov, A.V., Martinek, K. (1989) Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures. *Eur. J. Biochem.* **184**: 597-602.

Nakamura, K. (1990) Biochemical reactions in supercritical fluids. *TIBTECH* **8**: 288-292.

Nakamura, K., Chi, Y.M., Yamada, Y., Yano, T. (1986) Lipase activity and stability in supercritical carbon dioxide. *Chem. Eng. Commun.* **45**: 207-212.

Norin, M., Boutelje, J., Holmberg, E., Hult, K. (1988) Lipase immobilized by adsorption. Effect of support hydrophobicity on the reaction rate of ester synthesis in cyclohexane. *Appl. Microbiol. Biotechnol.* **28**: 527-530.

O'Daly, J.P., Crumbliss, A.L., Henkens, R.W. (1990) Activity of carbonic anhydrase immobilized on porous silica beads in organic media. *Biotechnol. Appl. Biochem.* **12**: 11-19.

Osborne, S.J., Leaver, J., Turner, M.K., Dunnill, P. (1990) Correlation of biocatalytic activity in an organic- aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.* **12**: 281-291.

Parida, S., Dordick, J.S. (1991) Substrate stucture and solvent hydrophobicity control lipase catalysis and enantioselectivity in organic media. *J. Am. Chem. Soc.* **113**: 2253-2259.

Pasta, P., Mazzola, G., Carrea, G., Riv, S. (1989) Subtilisin-catalyzed transesterification in supercritical carbon dioxide. *Biotechnol. Lett.* **2**: 643-648.

Patel, R.N., Liu, M., Banerjee, A., Szarka, L.J. (1992) Stereoselective enzymatic hydrolysis of (*exo,exo*)-7- oxabicyclo[2.2.1]heptane-2,3-dimethanol diacetate ester in a biphasic system. *Appl. Microbiol. Biotechnol.* **37**: 180-183.

Randolph, T.W., Blanch, H.W., Prausnitz, J.M., Wilke, C.R. (1985) Enzymatic catalysis in a supercritical fluid. *Biotechnol. Lett.* 7: 325-328.

Randolph, T.W., Blanch, H.W., Prausnitz, J.M. (1988) Enzyme-catalyzed oxidation of cholesterol in supercritical carbon dioxide. *AIChE Journal* **34**: 1354-1360.

Randolph, T.W., Blanch, H.W., Clark, D.S. (1991) Biocatalysis in supercritical fluids. In: *Biocatalysts for industry*, p219-237. Dordick, J.S. (Ed.) Plenum Press, New York.

Reslow, M. (1989) *Enzymes as catalysts in organic media.* PhD-Thesis, University of Lund, Lund, Sweden.

Reslow, M., Adlercreutz, P., Mattiasson B. (1987) Organic solvents for bioorganic synthesis 1. Optimization of parameters for a chymotrypsin catalyzed process. *Appl. Microbiol. Biotechnol.* **26**: 1-8.

Reslow, M., Adlercreutz, P., Mattiasson, B. (1988a) Selection of solvents for bioorganic synthesis. *Ann. N.Y. Acad. Sci.* **542**: 250-253.

Reslow, M., Adlercreutz, P., Mattiasson, B. (1988b) The influence of water on protease-catalyzed peptide synthesis in acetonitrile/water mixtures. *Eur. J. Biochem.* **177**: 313-318.

Reslow, M., Adlercreutz, P., Mattiasson, B. (1988c) On the importance of the support material for bioorganic synthesis. Influence of water partition between solvent, enzyme and solid support in water-poor reaction media. *Eur. J. Biochem.* **172**: 537-578.

Ribeiro, M.H., Cabral, J.M.S., Fonseca, M.M.R. (1987) Production of L-tryptophan in a two-phase system. In: *Biocatalysis in organic media*, 323-329. Laane, C., Tramper, J., Lilly, M.D. (Ed.), Elsevier Science Publ. Amsterdam, The Netherlands.

Robert, H., Lamare, F., Parvaresh, F., Legoy, M.D. (1992) The role of water in gaseous biocatalysis. In: *Biocatalysis in non-conventional media*, 85-92 Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ.: Amsterdam, The Netherlands.

Roffler, S.R., Blanch, H.W., Wilke, C.R. (1988) In situ extractive fermentation of acetone and butanol. *Biotechnol. Bioeng.* **31**: 135-143.

Russell, A.J., Klibanov, A.M. (1988) Inhibitor-induced enzyme activation in organic solvents. *J. Biol. Chem.* **263**: 11624-11626.

Russell, A.J., Beckman, E.J. (1991) Should the high diffusivity of a supercritical fluid increase the rate of an enzyme-catalyzed reaction? *Enzyme Microb. Technol.* **13**: 1007.

Sakurai, T., Margolin, A.L., Russell, A.J., Klibanov, A.M. (1988) Control of enzyme enantioselectivity by the reaction medium. *J. Am. Chem. Soc.* **110**: 7236-7237.

Schneider, L.V. (1991) A three-dimensional solubility parameter approach to nonaqueous enzymology. *Biotechnol. Bioeng.* **37**: 627-638.

Schwartz, R.D., McCoy, C.J. (1977) Epoxidation of 1,7-octadiene by *Pseudomonas oleovorans*: fermentation in the presence of cyclohexane. *J. Appl. Environ. Microbiol.* **34**: 47.

Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. *Pharm. Rev.* **24**: 583-655.

Semenov, A.N., Cerovski, V., Titov, M.I., Martinek, K. (1988) Enzymes in preparative organic synthesis: the problem of increasing the yield of product. *Collection Czechoslovak Chem. Commun.* **53**: 2963-2985.

Sikkema, J., De Bont, J.A.M. (1994) Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **269**: 8022-8028.

Ståhl, M., Jeppsson-Wistrand, U., Månsson, M.-O., Mosbach, K. (1991) Induced stereoselectivity and substrate selectivity of bio-imprinted α-chymotrypsin in anhydrous organic media. *J. Am. Chem. Soc.* **113**: 9366-9368.

Tawaki, S., Klibanov, A.M. (1992) Inversion of enzyme enantioselectivity mediated by the solvent. *J. Am. Chem. Soc.* **114**: 1882-1884.

Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) (1992) *Biocatalysis in non-conventional media*. Elsevier Science Publ., Amsterdam, The Netherlands.

Tramper, J., Vermuë, M.H. (1993) Tailoring the medium and bioreactor for biocatalysis. *Chimica* **47**: 110-115.

Valivety, R.H., Johnston, G.A., Suckling, C.J., Halling, P.J. (1991) Solvent effects on biocatalysis in organic systems: equilibrium position and rates of lipase catalyzed esterification. *Biotechnol. Bioeng.* **38**: 1137-1143.

Valivety, R.H., Halling, P.J., Macrae, A.R. (1992a) Reaction rate with suspended lipase catalyst shows similar dependence on water activity in different organic solvents. *Biochim. Biophys. Acta* **1118**: 218-222.

Valivety, R.H., Halling, P.J., Macrae, A.R. (1992b) Effect of water activity on rate of lipase catalyzed esterification. In: *Biocatalysis in Non-Conventional Media*, 549-555. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Van den Tweel, W.J.J., Marsman, E.H., Vorage, M.J.A.W., Tramper, J., De Bont, J.A.M. (1987) The application of organic solvents for the bioconversion of benzene to cis-benzeneglycol. In: *Bioreactors and biotransformations*. 231-241, Moody, G.W., Baker, P.B. (Ed.), Elsevier Science Publ. London, GB.

Van der Padt, A., Sewalt, J.J.W., Agoston, S.M.I. Van 't Riet, K. (1992) *Candida rugosa* lipase stability during acylglycerol synthesis. *Enzyme Microb. Technol.* **14**: 805-812.

Vazquez-Duhalt, R., Semple K.M., Westlake, D.W.S., Fedorak, P.M. (1993) Effect of water-miscible organic solvents on the catalytic activity of cytochrome *c*. *Enzyme Microb. Technol.* **15**: 936-943.

Vermuë, M.H., Tramper, J. (1990) Extractive biocatalysis in a liquid-impelled loop reactor. In: *Proceedings of the 5th European congress on biotechnology*, 243-246 Christiansen, C., Munck, L., Villadsen, J. (Ed.) Munksgaard Int. Publ.: Copenhagen, Denmark.

Vermuë, M.H., Tramper, J., De Jong, J.P.J., Oostrom, W.H.M. (1992) Enzymic transesterification in near-critical carbon dioxide: effect of pressure, Hildebrand solubility parameter and water content. *Enzyme Microb. Technol.* **14**: 649-655.

Vermuë, M.H., Sikkema, J., Verheul, A., Bakker, R., Tramper, J. (1993) Toxicity of homologous series of organic solvents for the gram-positive bacteria *Nocardia* sp. and *Arthrobacter* sp. and the gram-negative bacteria *Pseudomonas* sp. and *Acinetobacter* sp. *Biotechnol. Bioeng.* **42**: 747-758.

Volkin, D.B., Staubli, A., Langer, R., Klibanov, A.M. (1991) Enzyme thermoinactivation in anhydrous organic solvents. *Biotechnol. Bioeng.* **37**: 843-853.

Vulfson, E.N., Patel, R., Beecher, J.E., Andrews, A.T., Law, B.A. (1990) Glycosidases in organic solvents: 1. Alkyl-ß-glucoside synthesis in a water-organic two-phase system. *Enzyme Microb. Technol.* **12**: 950-954.

Weder, J.K.P. (1984) Studies on proteins and amino acids exposed to supercritical carbon dioxide extraction conditions. *Food Chem.* **15**: 175-190.

Wehtje, E., Adlercreutz, P., Mattiasson, B. (1990) Formation of C-C bonds by mandelonitrile lyase in organic solvents. *Biotechnol. Bioeng.* **36**: 39-46.

Wehtje, E. (1992) *Activity of mandelonitrile lyase in different reaction media - from aqueous solutions to nonpolar organic solvents.* PhD Thesis, Chapter 7, University of Lund, Sweden.

Wheeler, C.J., Croteau R. (1986) Terpene cyclase in organic solvent/minimal water media: demonstration and optimization of (+)-α-pinene cyclase activity. *Arch. Biochem. Biophys.* **248**: 429-434.

Williams, A.C., Woodley, J.M., Ellis, P.A., Narendranathan, T.J., Lilly, M.D. (1990) A comparison of pig liver esterase and *Bacillus subtilis* as catalysts for the hydrolyis of methyl acetate in stirred two-liquid phase reactors. *Enzyme Microb. Technol.* **12**: 260-265.

Woodley, J.M., Brazier, A.J., Lilly, M.D. (1991) Lewis cell studies to determine reactor design data for two- liquid-phase bacterial and enzymic reactions. *Biotechnol. Bioeng.* **37**: 133-140.

Woodley, J.M., Lilly, M.D. (1992) Process engineering of two-liquid phase biocatalysis. In: *Biocatalysis in non-conventional media*, 147-154. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Yamané, T. (1988) Importance of moisture content control for enzymatic reactions in organic solvents: a novel concept of "microaqueous". *Biocatalysis* **2**: 1-9.

Yamané, T., Nakatani, H., Sada, E., Tanaka, A., Fukui, S. (1979) Steroid bioconversion in water-insoluble organic solvents: Δ^1-dehydrogenation by free microbial cells and by cells entrapped in hydrophilic or lipophilic gels. *Biotechnol. Bioeng.* **21**: 2133-2145.

Yang, Z., Robb, D.A., Halling, P.J. (1992) Variation of tyrosinase activity with solvent at a constant water activity. In: *Biocatalysis in Non-Conventional Media*, 585-592. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Yu, Z.-R., Rizvi, S.S.H., Zollweg, J.A. (1992) Enzymatic esterification of fatty acid mixtures from milk fat and anhydrous milk fat with canola oil in supercritical carbon dioxide. *Biotechnol. Prog.* **8**: 508-513.

Zaks, A., Klibanov, A.M. (1984) Enzymatic catalysis in organic media at 100 °C. *Science* **224**: 1249-1251.

Zaks, A., Klibanov, A.M. (1985) Enzyme-catalyzed processes in organic solvents. *Proc. Natl. Acad. Sci. USA* **82**: 3192-3196.

Zaks, A., Klibanov, A.M. (1988a) Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.* **263**: 3194-3201.

Zaks, A., Klibanov, A.M. (1988b) The effect of water on enzyme action in organic media. *J. Biol. Chem.* **263**: 8017-8021.

Zaks, A., Russell, A.J. (1988) Enzymes in oganic solvents: properties ad applications. *J. Biotechnol.* **8**: 259-270.

Zale, S.E., Klibanov, A.M. (1986) Why does ribonuclease irreversibly inactivate at high temperatures? *Biochemistry* **25**: 5432-5444.

Chapter 3

STRATEGY FOR SELECTION OF A SUITABLE SOLVENT FOR EXTRACTIVE BIOCATALYSIS IN A LIQUID-IMPELLED LOOP REACTOR

SUMMARY

The liquid-impelled loop reactor (LLR) has been developed for biocatalysis in two-phase reaction mixtures of water and a water-immiscible organic solvent. Here, the steps are introduced which are essential in the selection procedure for a suitable solvent for application in the LLR. As a model reaction the biodegradation of the apolar toxic compound, tetralin, by *Arthrobacter* T2 and *Corynebacterium* C125 has been chosen to illustrate the necessity of each step.

Strategy for selection of a suitable solvent for extractive biocatalysis in a liquid-impelled loop reactor
Vermuë, M.H., Sikkema, J., Bakker, R., Janssen, G., Tramper, J.
Submitted for publication in the *Journal of Biotechnology*.

Chapter 3

INTRODUCTION

An important area in biotechnology is biocatalysis in media containing water-immiscible organic solvents (Laane *et al.*, 1987b, Tramper *et al.*, 1992). The main reason to use these solvents is to increase the solubility of poorly water-soluble substrates and/or products. They are also applied to regulate the concentration of the reactants in the micro-environment of the biocatalyst, thereby reducing substrate and/or product inhibition (Brink and Tramper, 1985, Harbron *et al.*, 1986, Van den Tweel *et al.*, 1987, Ribeiro *et al.*, 1987, Daugulis, 1988, Vermuë and Tramper, 1990). In addition, water-immiscible organic solvents facilitate the separation of the biocatalyst and the reactants, because the former generally does not dissolve in organic solvents.

For the use of these media a new type of bioreactor has been developed in our laboratory, the liquid-impelled loop reactor (LLR) (Tramper *et al.*, 1987). This reactor consists of two vertical parts which have an open connection at the bottom and the top (Figure 1). In the LLR a water-immiscible organic solvent with a lowere or a higher density than water is continuously injected in one of the tubes.

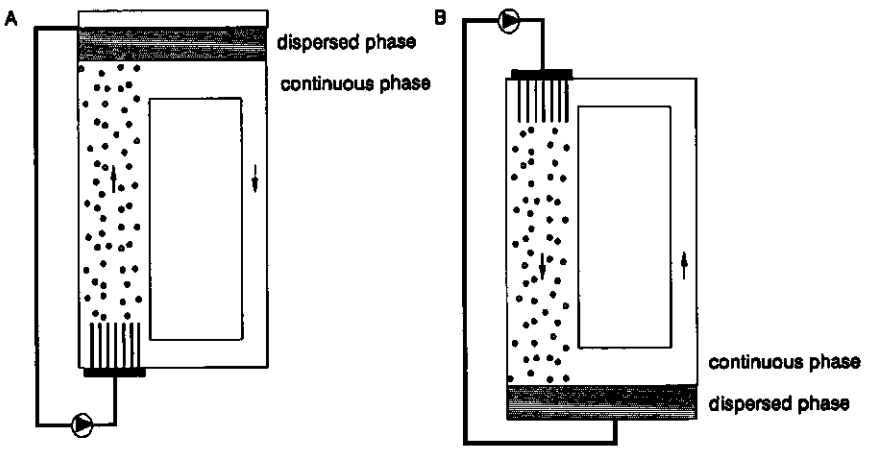


Figure 1: The liquid-impelled loop reactor in an upflow (A) and in a downflow (B) configuration.

The presence of the dispersed phase in this tube creates a density difference between the two tubes of the reactor and this causes circulation of the continuous phase. In this way, efficient mixing and subsequent mass transfer is achieved in the reactor. The solvent may have a lower density than water resulting in a reactor with an upflow configuration (Figure 1A). It may also have a density higher than water, in which case the LLR has a downflow configuration (Figure 1B). The physical aspects of liquid-impelled loop reactors, such as mixing properties, hydrodynamics and mass transfer characteristics have been studied extensively (Van Sonsbeek *et al.*, 1990, 1992a, b).

In the work described here, the strategy for the selection of a suitable solvent for the biodegradation of a toxic and apolar compound in the LLR is introduced (Table 1). To illustrate the necessary steps in the selection strategy the bioconversion of the highly apolar compound 1,2,3,4-tetrahydro-naphthalene (tetralin) has been chosen as a model reaction.

Several bacteria are able to degrade tetralin (Sikkema and De Bont, 1991a). Among them is *Arthrobacter* T2, which degrades tetralin (I) by initial oxidation to 1,2,3,4-tetrahydro-1-naphthol (II) which is further oxidized into 2,3,4-trihydro-1-naphthalone (III). *Corynebacterium* C125 degrades tetralin by initial oxidation by a dioxygenase, yielding the 1,2,5,6,7,8-hexahydro-*cis*-1,2-naphthalene diol (IV) (Sikkema and De Bont, 1991b). Both intermediates will be further metabolized yielding energy and biomass (Sikkema and De Bont, 1991b, 1993, Figure 2).

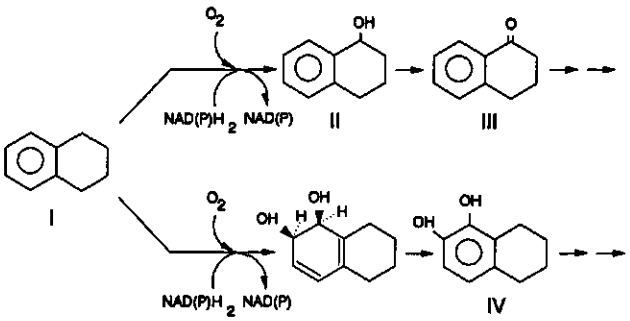


Figure 2 Schematic presentation of the bioconversion of tetralin by *Arthrobacter* T2 (upper reaction scheme) and *Corynebacterium* C125 (lower reaction scheme) (Adapted from Sikkema and De Bont, 1991a).

Tetralin has a very low solubility in water (about 125 μmol l^{-1}), but is already toxic for the bacteria at concentrations above 75 μmol l^{-1} in aqueous medium (Sikkema *et al.*, 1992). Therefore, water-immiscible organic solvents are introduced in the reaction mixture to improve the overall solubility of the substrate and simultaneously to prevent the toxicity of tetralin to the bacteria present in the aqueous phase.

SELECTION PROCEDURE

The first step in the selection of a suitable organic solvent is to investigate the biocompatibility of the solvent. The biocompatibility of a solvent can be predicted by the $\log P_{octanol}$ (Laane *et al.*, 1987a) which is defined as the logarithm of the partition coefficient in a standard octanol/water two-phase system and is a measure of the hydrophobicity of the solvent. Laane *et al.* (1987a) have observed that biocatalyst-activity retention in organic solvents is low in solvents having a $\log P_{octanol} < 2$, can not be predicted in solvents having a $\log P_{octanol}$ between 2 and 4, and is high in apolar solvents having a $\log P_{octanol} > 4$. A similar relation between $\log P_{octanol}$ and activity retention has been found for several other cellular biocatalysts, among which are bacteria, yeasts, fungi and plant cells, but the inflection point between toxic and non-toxic solvent seems to depend on the type of cells (Table 2). Generally, water-immiscible solvents with a

Table 1 Strategy for the selection of a suitable solvent for the biodegradation of a toxic apolar substrate in the Liquid-impelled Loop Reactor (LLR).

1	Test biocompatibility of water-immiscible organic solvents
2	Determine biodegradability of the biocompatible solvents by the biocatalyst
3	Assess toxicity of the substrate for the biocatalyst
4	Determine detoxifying effect of the selected water-immiscible organic solvents
5	Measure metabolic activity of the biocatalyst in the LLR with the selected solvent
6	Analyze effects of immobilization of the biocatalyst on the process operation
7	Determine bioconversion of the toxic apolar substrate in the LLR in the presence of the selected solvent

Table 2 The sensitivity of cellular biocatalysts towards organic solvents, as expressed by the $\log P_{octanol}$ at which inflection between toxic and non-toxic organic solvents occurs

Cellular biocatalyst	$\text{Log}P_{octanol}$-inflection region between toxic and non-toxic solvents	Reference
Mycobacterium sp.	$2 < \log P_{octanol} < 4$	Laane *et al.*, 1987a
Saccharomyces cerevisiae	$4 < \log P_{octanol} < 6$	Bruce and Daugulis, 1991
Clostridium acetobutylicum	$4 < \log P_{octanol} < 6$	Bruce and daugulis, 1991
Zymomonas mobilis	$\log P_{octanol} = 4$	Bruce and Daugulis, 1991
Rhizopus nigricans	$3 < \log P_{octanol} < 4$	Osborne *et al.*, 1990
Tagetes minuta	$\log P_{octanol} = 5$	Buitelaar *et al.*, 1990
Morinda citrifolia	$\log P_{octanol} = 5$	Bassetti and Tramper, 1994

$\log P_{octanol} < 2$ are toxic and can therefore be excluded from the list of potential suitable solvents, while solvents with a $\log P_{octanol} > 5$ are surely biocompatible. However, the latter are often poor extractants especially for moderately polar product such as the lower alcohols (Bruce and Daugulis, 1991). In order to find solvents that combine optimal product extraction capacity with high biocompatibility, it is thus essential to test the biocompatibility of solvents having a $\log P_{octanol}$ between 2 and 5. Among the biocompatible solvents, some can be used as sole energy and carbon source by the organisms under study. These solvents must be excluded from the list of applicable solvents, because they may lead to unwanted by-products.

The next step in the selection strategy is to study the toxicity of the substrate for the biocatalyst and to find out if addition of the selected biocompatible and non-biodegradable organic solvents have an apparent 'detoxifying' effect. After a suitable solvent is selected, it can be used in the LLR to study the metabolic activity of the bacteria within this type of reactor.

The following step in our strategy is to study the effect of immobilization of the biocatalyst. Immobilization helps to maintain a high cell load in the bioreactor in case of continuous operation at relatively high flow rates and it may prevent aggregation and clotting of the cells at the liquid-liquid interface (Hocknull and Lilly, 1990, Buitelaar *et al.*, 1990). The final step in the selection strategy is the study of the bioconversion of

the substrate within the LLR in the presence of the most suitable selected solvent.

MATERIALS AND METHODS

Microorganisms

The microorganisms, *Arthrobacter* T2 and *Corynebacterium* C125 are obtained from the Division of Industrial Microbiology of the Wageningen Agricultural University. *Arthrobacter* T2 has been isolated on tetralin (Sikkema and De Bont, 1991b), and *Corynebacterium* C125 on *o*-xylene (Schraa *et al.*, 1987). Tetralin can be used by these organisms as sole energy and carbon source, when this substrate is added via the vapour phase (Sikkema and De Bont, 1991b). The cells are pre-cultured in 3 l Erlenmeyer flasks, containing 1 l of growth medium (mineral medium supplied with 5 g l^{-1} sodium succinate and 0.5 g l^{-1} of yeast extract (Oxoid)). The composition of mineral medium in g l^{-1} is: K_2HPO_4, 1.55; $NaH_2PO_4.2H_2O$, 0.85; NH_4Cl, 2.0; $(NH_4)_2.SO_4$, 0.1; $MgCl_2.6H_2O$, 0.075 and 0.2 10^{-3} ml m^{-3} of a trace-elements solution (Vishniac and Santer, 1957).

Samples of the cells (100 ml) for the incubation experiments have been taken during the late-exponential growth phase of the cells. Before the cells are used, they are washed twice in mineral medium, resuspended in 100 ml mineral medium and starved for 2 days in mineral medium, in order to minimize endogenic respiration during the experiments. The suspension contains about 7.5 g protein per litre, as measured with the method of Bradford (1976), using Bovine Serum Albumin as standard. All experiments have been performed under sterile conditions at 303 K. For incubation of the cell suspension, a rotary shaker has been used.

Chemicals

Solvent source, purity, density and $\log P_{octanol}$ value are presented in Table 3.

Immobilization of the biocatalyst

The pre-cultured cells (200 ml) are centrifuged for 600 s at 10.000 rpm and the pellet is resuspended in 30 ml mineral medium. The suspension is mixed with 270 ml κ-carrageenan solution at 318 K. The final concentration of κ-carrageenan is 26 g l^{-1}. The solution is pressed through a needle having a diameter of 1.5 mm and the drops are collected in an ice-cold 0.75 mol l^{-1} KCl solution with a layer of ice-cold decane on top. This layer is added to assure the fast formation of equal round-shaped beads (Buitelaar *et al.*, 1988). The beads are left to harden for 1 hour and then washed with mineral medium.

Table 3 Physical properties of the organic solvents used.

Solvent	Source[a]	Purity (%)	$logP_{octanol}$[b]	Density ($g\ l^{-1}$)
Saturated aliphatic hydrocarbons				
pentane	D	~99	3.0	621
hexane	D	>99	3.5	654
heptane	D	>99	4.0	684
octane	D	99.5	4.5	699
iso-octane	D	98	4.5	688
nonane	D	98	5.1	714
decane	D	>95	5.6	726
undecane	A	99	6.1	742
dodecane	D	>99	6.6	745
tridecane	A	>99	7.1	756
tetradecane	A	99	7.6	760
pentadecane	A	>99	8.2	770
hexadecane	D	>99	8.8	770
heptadecane	A	99	9.3	780
octadecane	A	99	9.8	770
Aliphatic alkanols				
1-butanol	D	99.5	0.8	806
1-pentanol	A	>99	1.3	811
1-hexanol	D	>98	1.8	815
1-heptanol	C	98	2.4	820
1-octanol	D	>99	2.9	822
1-nonanol	A	99	3.4	830
1-decanol	C	99	4.0	830
1-undecanol	A	99	4.5	830
1-dodecanol	A	99	5.0	830
Fluorinated hydrocarbons				
perfluor hexane	B	~85	2.5	1684
perfluor decalin	A	95	6.5	1908
FC-40	C	NA[c]	11.4	1870
Esters of dicarboxylic acids				
dimethyl phthalate	D	99	2.3	1188
diethyl phthalate	D	99	3.3	1120
dibutyl phthalate	C	99	5.4	1047
dioctyl phthalate	C	98	9.6	979
didecyl phthalate	B	NA[c]	11.7	966

Table 3 *continued*

Aliphatic ethers				
diethyl ether	D	>99	0.9	708
dipropyl ether	A	>99	1.9	742
dibutyl ether	D	>99	2.9	764
dipentyl ether	A	99	3.9	779
dihexyl ether	D	>97	5.0	790
diphenyl ether	B	>98	4.3	1070
Aromatic hydrocarbons				
toluene	D	99.5	2.9	862
ethyl benzene	C	99	3.1	863
propyl benzene	A	98	3.6	857
butyl benzene	A	>99	4.1	856
tetralin	C	99	3.9	966
Unsaturated hydrocarbons				
1-hexene	D	99	3.1	668
1-heptene	C	>98	3.6	693
1-octene	D	>99	4.2	711
1-nonene	A	97	4.7	725
1-decene	D	96	5.2	741
1-undecene	A	99	5.7	751
1-dodecene	A	95	6.2	755
1-tetradecene	A	NA[c]	6.7	770
1-hexadecene	A	94	7.3	784
Esters of saturated aliphatic monocarboxylic acids				
methyl acetate	C	99.4	0.16	928
ethyl acetate	D	99.5	0.64	895
propyl acetate	D	>99	1.17	883
butyl acetate	D	NA[c]	1.7	876
pentyl acetate	C	>99	2.23	872

a) A = Aldrich-Chemie; B = Fluka; C = Janssen Chimica; D = Merck
b) Log$P_{octanol}$ values have been calculated according to Rekker and De Kort (1979)
c) NA = not available

Biocompatibility

1 ml of the solvent to be tested, is added to 9 ml of growth medium in a 100 ml serum bottle. To the reference bottle 1 ml of mineral medium is added instead of the solvent. The bottles are incubated for 3 days to check the sterility of the added solvent. After this pre-incubation period 0.1 ml of the bacterium suspension is added to the

mixture. The carbon-dioxide production is determined after 1, 3 and 7 days of incubation.

Biodegradability

The biodegradability of the biocompatible solvents has been tested to find out if they can be used by the microorganisms as sole source of energy and carbon. The same procedure as described for the toxicity test is followed, except that mineral medium is supplied instead of growth medium. The bio-degradability of the solvents is determined by measuring the carbon-dioxide production after 1, 3, 7 days of incubation.

Toxicity of tetralin

The toxicity of tetralin is tested by the addition of various amounts of tetralin to 250 ml serum bottles containing 50 ml of a starved-cell suspension in mineral medium, using a 5 mm^3 Hamilton syringe. The detoxifying effect of organic solvent has been studied by replacing 5 ml of the mineral medium by 5 ml of the selected biocompatible and non-biodegradable organic solvent. The bottles are sealed with Teflon Mininert valves (Pierce Europe).

The metabolic activity of the cells is determined by measuring the carbon-dioxide production and the tetralin content in the aqueous phase after 1, 3, 7 and 12 days of incubation and in the organic solvent phase after 7 days of incubation.

Partitioning of tetralin

The partition coefficient of tetralin has been determined for a FC-40/mineral medium mixture and for a dodecene/mineral medium mixture. 1 ml of a tetralin solution in the organic solvent is mixed well with 100 ml mineral medium. The mixture is allowed to separate into two phases overnight and samples of the aqueous phase have been analyzed. Mass balance equations give the concentration in the organic-solvent phase and the partition coefficient over both phases.

The Liquid-impelled Loop Reactor (LLR)

A schematic presentation of the LLR set-up in downflow configuration is given in Figure 3. This configuration is used for the bioconversion of tetralin by *Arthrobacter* T2 and *Corynebacterium* C125. The total reactor volume is 3 l and it contains 2.7 l aqueous medium and 300 ml of the perfluoro compound, FC-40 (gross formula $N(C_4F_9)_3$). The solvent is aerated outside the LLR by surface aeration in the aeration vessel, which contains about 700 ml solvent. The outlet air has been led through octanol to check the possible loss of FC-40 and tetralin by evaporation. No FC-40 and tetralin could be detected gas chromatographically in the octanol over 14 days of operation. From the aeration vessel the solvent is pumped into the reactor with a Watson-Marlow 5003U pump equipped with a Micropump pumphead (Model 200). The organic solvent

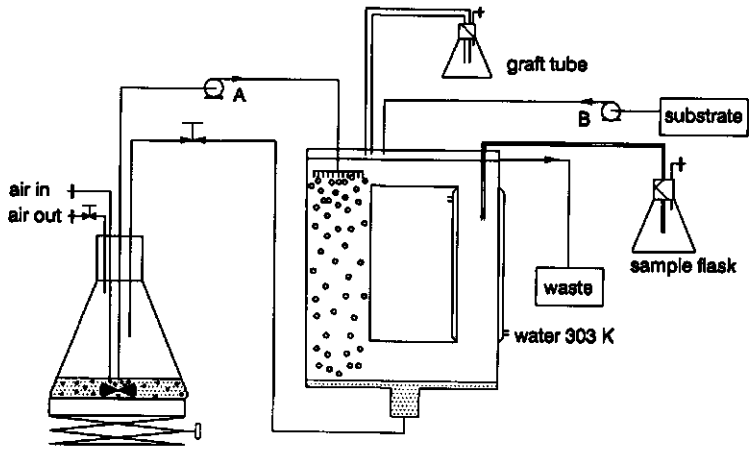


Figure 3 Reactor set-up for the bioconversion of tetralin.

is dispersed with a sparger, which has 41 hollow needles of 10 mm length and an internal diameter of about 0.5 mm. At the flowrate of 5 10^{-3} l s^{-1} jetting occurs, which results in the formation of small, equal, round-shaped, organic-solvent drops. At the bottom part of the reactor, where phase separation takes place, the organic solvent flows back into the aeration vessel. The liquid level inside the LLR is controlled by adjustment of the height of the screw table underneath the aeration vessel, making use of the law of communicating vessels. After 2 days pre-equilibration of the medium inside the LLR, 100 ml of the free cell suspension is added via the graft tube. In case of immobilized cells, 300 g beads are added via the same graft tube. Samples of the medium (~ 70 ml), and the immobilized cells (~ 4 g) can be taken aseptically via the sample flask.

Growth of the cells in the LLR

The growth of the free cells is measured by determination of the optical density at 660 nm and the protein content of the samples using the method of Bradford (1976). The tetralin concentration in the aqueous phase is determined daily and besides this the remaining tetralin concentration of the FC-40 is determined twice a week and adjusted, if neccessary, to 600 μmol l^{-1}, resulting in a tetralin concentration in the aqueous phase of approximately 5 μmol l^{-1}. Occasionally, the number of living cells has been determined by plate counting (5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract in mineral medium to which Oxoid no.3 agar (15 g l^{-1}) has been added).

When immobilized cells are used, the growth of cells is determined by measuring the protein content of the beads according to the method of Smith *et al.* (1992). The beads (3 g) are lyophilized and resuspended in 3 ml demi-water. From this suspension

0.5 g is heated for 120 s in 0.5 ml NaOH solution (1 mol l^{-1}) and 0.1 ml of the solution is thoroughly mixed with 5 ml Bradford reagents (1976). The adsorption at 595 nm is measured spectrophotometrically, using Bovine Serum Albumin as a standard protein solution.

Analytical techniques

Carbon-dioxide production is determined by injecting head-space samples (0.1 ml) into a Packard 427 gas chromatograph (Packard/Becker, Delft, The Netherlands) fitted with a Porapack Q column (Chrompack B.V., Middelburg, The Netherlands). The temperature of the T.C.D. detector is 413 K.

The tetralin biodegradation is determined by injecting 5 μl samples from the aqueous phase into a Hewlett Packard 7510A gas chromatograph, equipped with a 3 m packed PAW-DMCS column (Chrompack B.V.). The temperature of the F.I.D. detector is 523 K. The tetralin concentration in FC-40 is determined spectrophotometrically (Perkin Elmer) at 274 nm.

RESULTS AND DISCUSSION

Biocompatibility

The first step in the selection of a suitable solvent for the biodegradation of tetralin in the LLR, involves testing of the biocompatibility of several organic solvents (Table 1). A solvent is considered to be biocompatible, if the carbon-dioxide production of the cells after 7 days of incubation with 100 ml l^{-1} solvent is > 50% of the metabolic activity in the absence of organic solvent. Figure 4 shows the relation between the metabolic activity of the tested microorganisms and the $\log P_{octanol}$ of the solvents. It can be observed that the $\log P_{octanol}$ is indeed a good measure to predict the biocompatibility of organic solvents. Solvents having a $\log P_{octanol} < 3$ are toxic for both *Arthrobacter* T2 and *Corynebacterium* C125. Solvents having a $\log P_{octanol}$ between 3 and 5 are either toxic or biocompatible, while those having a $\log P_{octanol} > 5$ are biocompatible.

For whole cell biocatalysts, the mechanism for the toxicity of two-phase reaction mixtures is proposed to be caused by direct effects of the organic-solvent molecules dissolved in the aqueous phase (molecular toxicity) or by indirect effects which are due to the presence of the organic-solvent phase and the phase boundary between the aqueous and the organic-solvent phase (phase toxicity) (Bar, 1988, Vermuë *et al.*, 1993).

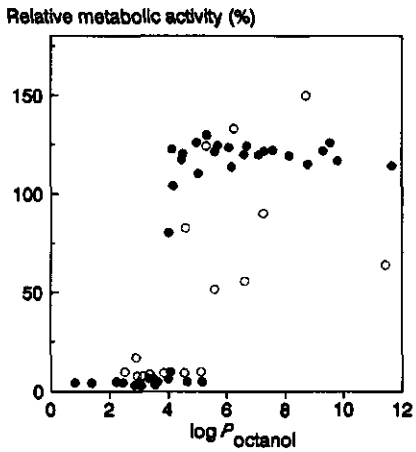


Figure 4 Relationship between the relative metabolic activity of *Arthrobacter* T2 (●) and *Corynebacterium* C125 (○), exposed to 100 ml l^{-1} organic solvent, and $\log P_{octanol}$.

Generally, molecular-toxicity effects are ascribed to direct interactions of the dissolved solvent molecules with the cell membrane of the organisms used (Osborne *et al.*, 1990, Bassetti and Tramper, 1993, Vermuë *et al.*, 1993, Sikkema *et al.*, 1992, 1994). These specific solvent/membrane interactions will determine the sensitivity of cells towards organic solvents, and thus the position of the inflection point between toxic and non-toxic solvents. The position of the inflection point may also be partly determined by phase-toxicity effects, such as extraction of membrane components, extraction of essential nutrients or limited access of the cells to nutrients (Bar, 1988). If the agitation rate increases, the interfacial area will increase and this will cause extra phase-toxicity effects (Hocknull and Lilly, 1988). It is thus possible that non-toxic organic solvents with intermediate $\log P_{octanol}$ become toxic, when higher agitation speeds are applied.

Many of the biocompatible solvents show an increase in carbon-dioxide production relative to the carbon-dioxide production in the absence of organic solvent (Figure 4). A likely explanation for the increased metabolic activity of some of the biocompatible solvents is that the organisms may be able to use the organic solvent as an extra carbon and energy source. Therfore the next step in the selection procedure

(Table 1) is to test the biodegradability of the biocompatible solvents. It has also been suggested that organic solvents have a direct stimulating effect on the membrane fluidity and the mobility of membrane-bound metabolic enzymes (Vermuë *et al.*, 1993, Sikkema *et al.*, 1992). This effect is also observed at low concentrations of toxic organic solvents,

Table 4 Biodegradability of organic solvents by *Arthrobacter* T2 and *Corynebacterium* C125.

Organic solvent	Metabolic activity measured by the CO_2 production in 7 days[a]	
	Arthrobacter T2	*Corynebacterium* C125
hexane	+	NT
heptane	+	NT
octane	+	-
iso-octane	+	NT
nonane	+/-	NT
decane	++	+
dodecane	++	++
hexadecane	+	++
1-decanol	-	NT
1-undecanol	-	NT
perfluor hexane	-	NT
perfluor decalin	-	NT
FC-40	-	-
dibutyl phthalate	+	NT
dioctyl phthalate	+	NT
didecyl phthalate	+	NT
dipentyl ether	-	NT
dihexyl ether	-	+/-
diphenyl ether	-	NT
1-octene	-	NT
1-nonene	-	NT
1-decene	-	NT
1-dodecene	-	++
1-tetradecene	++	++
1-hexadecene	++	++

a) - no carbon-dioxide production is observed
+ some carbon-dioxide production is observed but no increase in biomass can be detected
+ carbon-dioxide production, associated with an increase in biomass is observed
NT the solvent has not been tested

but when the concentration of these toxic solvents reaches a treshold concentration in the cell-membrane, the so-called critical membrane concentration, the organic solvent becomes inhibitory (Osborne *et al.*, 1990, Bassetti and Tramper, 1994, Vermuë *et al.*, 1993).

Biodegradability

Table 4 shows that among the 25 biocompatible solvents tested, 13 solvents are used as sole energy and carbon source by *Arthrobacter* T2. These solvents cannot be applied in the LLR, since their use may result in formation of unwanted by-products. Some of the remaining solvents might be converted by the organisms if a co-substrate such as succinic acid or tetralin is available. These solvents should also be excluded. Solvents which show a relative metabolic activity > 110% in the biocompatibility test (Figure 4), have therefore been omitted from the list of suitable solvents. The perfluoro-compounds, the alkylethers, and some of the alkenes (C10-C12) have been selected as potential suitable solvents for bioconversion of tetralin by *Arthrobacter* T2 (Table 4). The alkyl ethers have been excluded because of their high volatility while some of the perfluorocompounds and alkenes have been omitted because they are not readily available in large quantities for a reasonable price. Eventually, from the original list of 57 solvents, only FC-40 and dodecene remain as potentially suitable solvents for the biodegradation of tetralin by *Arthrobacter* T2. In case *Corynebacterium* C125 is used, only 18 solvents with a $\log P_{octanol} > 2$, a density difference with water of at least 100 g l^{-1} and with a boiling point at atmospheric pressure above 373 K have been tested for biocompatibility and biodegradability. FC-40 proves to be the only non-toxic and non-biodegradable solvent for *Corynebacterium* C125 (Figure 4 and Table 4).

Toxicity of tetralin

The toxicity of tetralin to *Arthrobacter* T2 and *Corynebacterium* C125 has been investigated and Figure 5 shows that the metabolic activity (expressed as the amount of carbon dioxide formed during 3 days of incubation) of the cells, drops at tetralin concentrations above 74 μmol l^{-1} and about 115 μmol l^{-1} for *Arthrobacter* T2 and *Corynebacterium* C125, respectively. The toxic concentrations are lower than the

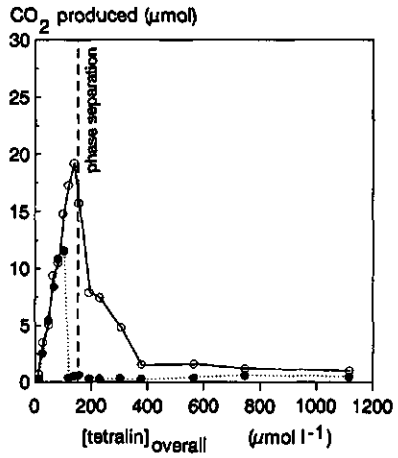


Figure 5 Carbon-dioxide production in 3 d; incubation in mineral media with various overall tetralin concentrations by *Arthrobacter* T2 (●) and *Corynebacterium* C125 (○).

aqueous solubility of tetralin and therefore molecular toxicity effects prevail.

Recently Osborne *et al.* (1992) have suggested that the actual concentration of apolar compounds in the aqueous phase may drop in the presence of cells, due to partitioning of the apolar compound in the cellular membrane. In these experiments, however, the membrane-lipid concentration is very low (approximately 4 10^{-3} g l^{-1}). The partition coefficient of tetralin has been determined to be 1100 on a weight basis in an artifical membrane/buffer mixture (Sikkema *et al.*, 1992). It can thus be calculated that the presence of cells in the aqueous phase hardly affects the actual aqueous tetralin concentration.

Introduction of a water-immiscible solvent

Because tetralin shows molecular toxicity, the next step in the selection strategy is to investigate the 'detoxifying' effect of addition of a biocompatible and non-biodegradable solvent (Table 1). If FC-40 is added to the reaction medium the toxicity of tetralin to *Arthrobacter* T2 is indeed reduced (Figure 6). However, the 'detoxifying' effect is much less than expected. On the basis of the partition coefficient of 120 as

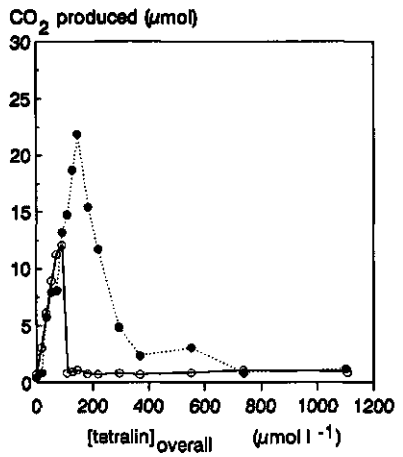


Figure 6 Effect of the addition of organic solvent on the carbon-dioxide production by free cells of *Arthrobacter* T2 after 3 d incubation with various concentrations of tetralin. Incubation with 10% v/v FC-40 (●) and without FC-40 (○).

found for tetralin in a mixture of FC-40 and mineral medium, the overall toxic concentration of tetralin should have increased from 74 μmol l^{-1} to 955 μmol l^{-1} upon addition of 100 ml FC-40 per liter mineral medium. As can be observed in Figure 6 the overall toxic concentration only increases about two-fold.

Addition of 100 ml l^{-1} dodecene has a similar 'detoxifying' effect. The partition coefficient of tetralin over dodecene and mineral medium is at least 5000 (the minimum value of 4 independent measurements) and based on this value, the overall toxic concentration should be at least 500 times the toxic concentration in mineral medium. However, the toxic concentration increased only 190 fold (Figure 7).

This indicates that toxicity in the two-phase reaction mixture is not only caused by the molecular toxicity of tetralin but that an additional toxic effect exists, which is probably caused by the presence of the interface between the aqueous phase and the FC-40/tetralin layer.

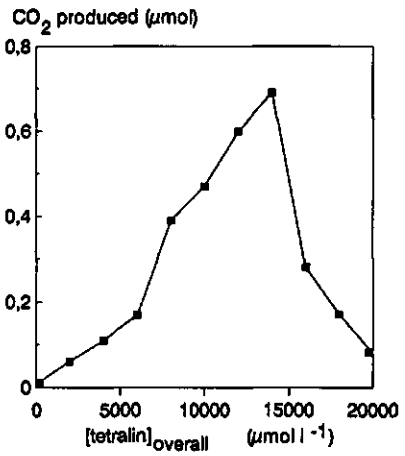


Figure 7 Effect of the addition of organic solvent on the carbon-dioxide production by free cells of *Arthrobacter* T2 after 3 d incubation with various concentrations of tetralin. Incubation with 10% v/v dodecene (■).

Metabolic activity of free *Arthrobacter* T2 and *Corynebacterium* C125 in the LLR

The next step in the selection strategy is to investigate if the biocompatible and non-biodegradable solvents, dodecene and FC-40 can be used in the LLR. As soon as dodecene is introduced in the LLR filled with a free cell suspension, a stable emulsion is formed. Due to the absence of a density difference between the two tubes of the reactor, circulation of the continuous phase stops and the solvent is therefore not suitable for application in the bioreactor.

In the presence of FC-40 free cells of both *Arthrobacter* T2 and *Corynebacterium* C125 are able to grow on sodium succinate, both in conical flasks and in the LLR (Table 5). The doubling time of the individual organisms is the same and does not depend on the type of bioreactor used. However, for both bacteria the maximum optical density (OD_{660}) reached is much higher in conical flasks than in the LLR. Furthermore, the formation of large aggregates of cell material at the liquid-liquid interface in the LLR can be observed during the stationary-growth phase. The maximum cell density as observed in the gently shaked, conical flasks containing 10% v/v FC-40 can not be

Table 5 Growth of *Arthrobacter* T2 and *Corynebacterium* C125 on growth medium (succinic acid and yeast extract) and 100 ml l^{-1} FC-40 in conical flasks and in the LLR.

Organism	Bioreactor	Doubling time (hours)	Maximum OD_{660} observed (-)
Arthrobacter T2	Conical flask	2.4	3.04
	LLR	2.4	0.35
Corynebacterium C125	Conical flask	2.6	4.02
	LLR	2.6	0.42

reached. Probably, this inhibitory effect is caused by additional phase-toxicity effects, which the cells experience in the LLR. The total interfacial area created inside the LLR (estimated to be about 50 10^{-3} m^2) (Vermuë *et al.*, 1994) is about 2 times higher than the interfacial area inside the conical flasks. In addition, the total volume ratio between organic solvent and aqueous medium in the LLR and the aeration bottle is higher than in the conical flask (100 ml l^{-1} in the conical flasks and 270 ml l^{-1} in the LLR), and this increases the total extractive capability in the LLR. However, the latter effect is negligible, since increasing the amount of organic solvent in the conical flasks has no significant additional toxic effect.

Bioconversion of tetralin by free cells in the LLR

At this stage of the selection procedure only one solvent of the original list of 57 potential solvents remains, FC-40. This solvent is now used to test the bioconversion of tetralin in the LLR.

Figure 8 shows the growth of *Corynebacterium* C125 on tetralin in the LLR. The tetralin concentration in the aqueous phase has been controlled at 5 μmol l^{-1} by addition of extra tetralin in the FC-40 phase, if neccessary. During the first 50 hours the optical density, as well as the protein concentration in the aqueous medium, hardly change. At 50 hours after inoculation the cells start to grow, however, severe aggregation of cells at the interphase is observed. Although tetralin is constantly available for the microorganism, the OD_{660} is significantly lower ($\sim$ 30%) than observed when succinate

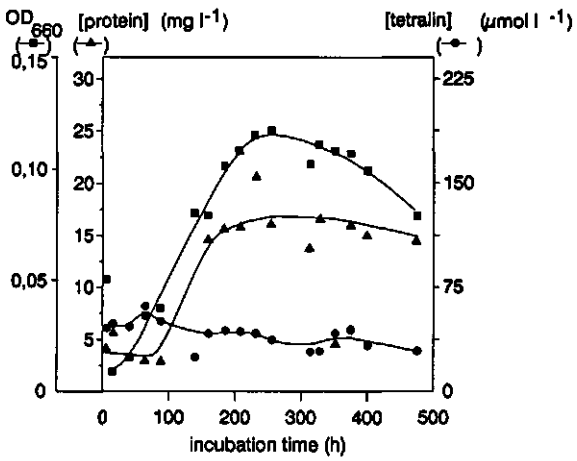


Figure 8 Growth of free *Corynebacterium* C125 cells on tetralin in the LLR with FC-40 as organic solvent.

is used (Table 5). After 150 hours the cells reach the stationary phase; no addition of tetralin is neccessary and the cells arenot active any more, as checked by plate counting. Obviously the free cells of *Corynebacterium* C125 experience a dramatic toxic effect in the LLR, which must be caused by increased interface, in comparison with the liquid-liquid interface in conical flasks.

When free *Arthrobacter* T2 cells are used in the LLR some tetralin conversion can be observed, but the cells aggregate and a stable emulsion is formed in the LLR. For that reason, the experiment has been stopped within 2 days.

Bioconversion of tetralin by immobilized cells in the LLR

The problems caused by emulsion formation and cell aggregation can be reduced by immobilization of the biocatalyst. Immobilization may also protect the cells against phase-toxicity effects (Hocknull and Lilly, 1990). Therefore, cells of *Corynebacterium* C125 and *Arthrobacter* T2 have been immobilized in κ-carrageenan. Both cell types are able to grow on succinate and within 10 hours the protein content in the gel beads increases from 0.1 mg g^{-1} to 0.8 mg g^{-1}. Besides growth inside the beads, cells start to

grow in suspension. These free cells tend to aggregate and emulsion formation is observed again. The latter problem is partly solved by the gel beads, which tend to break the emulsion.

The immobilized cells of *Arthrobacter* T2 and *Corynebacterium* C125 have been used for bioconversion of tetralin in the LLR. When *Corynebacterium* C125 is immobilized, however, the cells loose the ability to degrade tetralin. No increase in biomass inside the beads has been detected. Several attempts have been made to initiate tetralin conversion by these immobilized cells, for example by increasing the total initial biomass content inside the beads or by changing the tetralin concentration in the aqueous phase, but without success. The inactivation must be caused by a combination of the relative high temperature (318 K) during the immobilization and the exposure to the toxic substrate afterwards, but no sound explanation can be given.

When immobilized cells of *Arthrobacter* T2 are used, tetralin conversion proceeds successfully in the LLR (Figure 9). After 1 day of incubation in the presence of tetralin ($\sim$ 50 μmol l^{-1} in the aqueous phase), the protein content of the beads starts to increase and when the beads are filled, an increase in the optical density indicates growth of free cells in the medium (Figure 9). The maximum protein content of the beads is approximately the same as observed with succinate as the substrate ($\sim$ 0.9 mg g^{-1}, data not shown), which indicates that the additional phase-toxicity effects as observed with free cells, have been suppressed. Also, the formation of an emulsion is no longer observed, although some aggregation of free cell material still occurs at the interface between the aqueous and the organic solvent phase. Obviously, the gel beads are able to break the emulsion and prevent subsequent blocking of the bioreactor. After two weeks of semi-continuous operation, (tetralin is added three times a week), the experiment has been stopped.

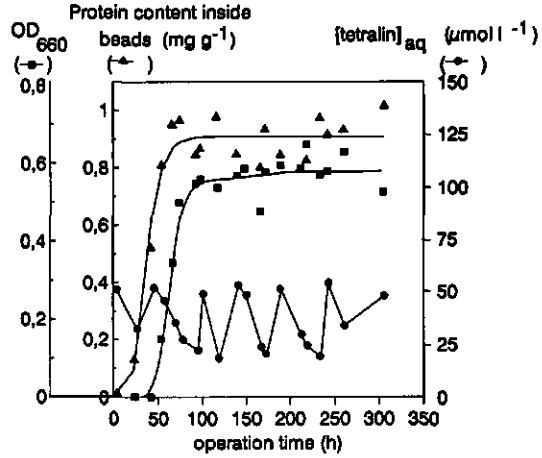


Figure 9 Growth of immobilized *Arthrobacter* T2 cells on tetralin in the LLR with FC-40 as organic solvent.

CONCLUSIONS

For the selection of a suitable solvent for the bioconversion of tetralin in the LLR, all steps in the selection strategy as presented in Table 1 are essential. $\text{Log}P_{\text{octanol}}$ is a helpful tool to predict the toxicity of organic solvents for cellular biocatalysts. Solvents having a $\text{log}P_{\text{octanol}} > 5$ are non-toxic for *Arthrobacter* T2 and *Corynebacterium* C125, but many of the biocompatible solvents are also biodegradable. Tetralin is a toxic substrate for both bacteria. Addition of the biocompatible and non-biodegradable solvent FC-40 has a 'detoxifying' effect, but the protective effect is limited, because additional phase-toxicity effects are observed in the LLR, as a result of the good mixing properties of this reactor. The phase toxicity can partly be avoided by immobilization of the cells. Immobilization of the cells is also advantageous when emulsion formation takes place. The gel beads are able to break the emulsion and prevent subsequent blocking of the reactor. Immobilized cells of *Corynebacterium* C125 are no longer able to convert tetralin. Successful bioconversion of tetralin in the LLR has been achieved with immobilized *Arthrobacter* T2.

ACKNOWLEDGEMENTS

Part of the work was financially supported by the Dutch Committee on Industrial Biotechnology. The authors thank Annette Verheul, Paul Tap, Mark Resink and Margot Tacken for their contribution to this work.

REFERENCES

Bar, R. (1988) Effect of interphase mixing on a water-organic solvent two-phase microbial system: ethanol fermentation. *J. Chem. Technol. Biotechnol.* **43**: 49-62.

Bassetti, L. and Tramper, J. (1994) Organic solvent toxicity in *Morinda citrifolia* cell suspensions. *Enzyme Microb. Technol.* **16**: 642-648.

Bradford, M.M. (1976) A rapid and sensitive method for quantification of microgram quantities of protein, utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Brink, L.E.S. and Tramper. J. (1985) Optimization of organic solvent in multiphase biocatalysis. *Biotechnol. Bioeng.* **27**: 1258-1269.

Bruce, L.J. and Daugulis, A.J. (1991) Solvent selection strategies for extractive biocatalysis. *Biotechnol. Prog.* **7**: 116-124.

Buitelaar, R.M., Hulst, A.C. and Tramper, J. (1988) Immobilization of biocatalysts in thermogels using the resonance nozzle for rapid drop formation and an organic solvent for gelling. *Biotechnology Techniques* **2**: 109-114.

Buitelaar, R.M., Vermuë, M.H., Schlatmann, J.E. and Tramper, J. (1990) The Influence of various organic solvents on the respiration of free and immobilized cells of *Tagetes minuta*. *Biotechnol. Techniques* **4**: 415-418.

Daugulis, A.J. (1988) Integrated reaction and product recovery in bioreactor systems. *Biotechnol. Prog.* **4**: 113-122.

Harbron, S., Smith, B.W. and Lilly, M.D. (1986) Two-liquid phase biocatalysis: epoxidation of 1,7-octadiene by *Pseudomonas putida*. *Enzyme Microb. Technol.* **8**: 85-88.

Hocknull, M.D. and Lilly, M.D. (1988) Stability of the steroid Δ^1-dehydrogenation system of *Arthrobacter simplex* in organic solvent-water two-liquid phase environments. *Enzyme Microb. Technol.* **10**: 669-674.

Hocknull, M.D. and Lilly, M.D. (1990) The use of free and immobilised *Arthrobacter simplex* in organic solvent/aqueous two-liquid-phase reactors. *Appl. Microbiol. Biotechnol.* **33**: 148-153.

Laane, C., Boeren, S., Vos, K. and Veeger, C. (1987a) Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **30**: 81-87.

Laane, C., Tramper, J. and Lilly, M.D. (Ed.) (1987b) *Biocatalysis in organic media*, Elsevier Science Publ., Amsterdam.

Osborne, S.J., Leaver, J., Turner, M.K. and Dunnill, P. (1990) Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.* **12**: 281-291.

Osborne, S.J., Leaver, J. and Turner, M.K. (1992) Membrane concentrations of primary alcohols which inhibit progesterone 11α-hydroxylase in *Rhizopus nigricans*. In: *Biocatalysis in Non-Conventional Media*, pp. 31-36, Tramper, J., Vermuë, M.H., Beeftink, H.H., and Von Stockar, U. (Eds.), Elsevier Science Publ., Amsterdam.

Rekker, R.F. and De Kort, H.M. (1979) The hydrophobic fragmental constant; an extension to a 1000 data point set. *Eur. J. Med. Chem.* **14**: 479-488.

Ribeiro, M.H., Cabral, J.M.S. and Fonseca, M.M.R. (1987) Production of L-tryptophan in a two-phase system. In: *Biocatalysis in organic media*, pp. 323-329, Laane, C., Tramper, J. and Lilly, M.D. (Eds.), Elsevier Science Publ., Amsterdam.

Schraa, G., Bethe, B.M., Van Neerven, A.R.W., Van den Tweel, W.J.J., Van der Wende, E. and Zehnder, A.J.B. (1987) Degradation of 1,2-dimethylbenzene by *Corynebacterium* strain 125. *Antonie van Leeuwenhoek* **53**: 159-170.

Sikkema, J. and De Bont, J.A.M. (1991a) Isolation and initial characterization of bacteria growing on tetralin. *Biodegradation* **2**: 15-23.

Sikkema, J. and De Bont, J.A.M. (1991b) Biocatalytic production of hydroxylated aromatic and alicyclic compounds: products derived from tetralin. *Recueil des Travaux Chimique des Pays-Bas* **110**: 189-194.

Sikkema, J., Poolman, B., Konings, W.N. and De Bont, J.A.M. (1992) Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. *J. Bacteriol.* **174**: 2986-2992.

Sikkema, J. and De Bont, J.A.M. (1993) Metabolism of tetralin (1,2,3,4-tetrahydro- naphthalene) in *Corynebacterium* sp. strain C125. *Appl. Environ. Microbiol.* **59**: 567-572.

Sikkema, J., De Bont, J.A.M. and Poolman B. (1994) Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **269**: 8022-8028.

Smith M.R., De Haan, A., Wijffels, R.H., Beuling E.E., Vilchez, C. and De Bont, J.A.M. (1992) Analysis of growth and specific activities of immobilized microbial cells. *Biotechnology techniques* **5**: 323-326.

Tramper, J., Wolters, I. and Verlaan, P. (1987) The liquid-impelled loop reactor*: a new type of density-difference-mixed bioreactor. In: *Biocatalysis in organic media*, pp. 311-316, Laane, C., Tramper, J. and Lilly, M.D. (Eds.), Elsevier Science Publ., Amsterdam.

Tramper, J., Vermuë, M.H., Beeftink, H.H. and Von Stockar, U. (Eds.) (1992) *Biocatalysis in non-conventional media*, Elsevier Science Publ., Amsterdam.

Van Sonsbeek, H.M., Verdurmen, R.E.M., Verlaan, P. and Tramper, J. (1990) Hydrodynamic model for liquid-impelled loop reactors. *Biotechnol. Bioeng.* **36**: 940-946.

Van Sonsbeek, H.M., Van der Tuin, S.P. and Tramper, J. (1992a) Mixing in liquid- impelled loop reactors. *Biotechnol. Bioeng.* **39**: 707-716.

Van Sonsbeek, H.M., De Blank, H. and Tramper, J. (1992b) Oxygen transfer in liquid- impelled loop reactors using perfluorocarbon liquids. *Biotechnol. Bioeng.* **40**, 713- 718.

Van den Tweel, W.J.J. Marsman, E.H., Vorage, M.J.A.W., Tramper. J. and De Bont, J.A.M. (1987) The application of organic solvents for the bioconversion of benzene to *cis*-benzene glycol. In: *Bioreactors and biotransformations*, pp. 231-241, Moody, G.W. and Baker, P.B. (Eds.), Elsevier Science Publ., London.

Vermuë, M.H. and Tramper, J. (1990) Extractive biocatalysis in a liquid-impelled loop reactor. In: *Proceedings of the 5th European congress on biotechnology*, pp. 243-246, Christiansen, C., Munck, L., Villadsen, J. (Eds.), Munksgaard Int. Publ., Copenhagen.

Vermuë, M.H., Sikkema, J., Verheul, A., Bakker, R. and Tramper, J. (1993) Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp.. *Biotechnol. Bioeng.* **42**: 747-758.

Vermuë, M., Tacken, M. and Tramper, J. (1994) Tetralin and oxygen transfer in the liquid-impelled loop reactor. *Bioprocess Eng.* **11**: 224-228.

Vishniac, W. and Santer, M. (1957) The thiobacilli. *Bacteriol. Rev.* **21**: 195-213.

TETRALIN AND OXYGEN TRANSFER IN THE LIQUID-IMPELLED LOOP REACTOR

SUMMARY

During the bioconversion of tetralin in the liquid-impelled loop reactor, oxygen and tetralin are transferred from the organic-solvent phase to the aqueous phase. Mass transfer of either tetralin or oxygen is likely to be the rate-limiting step in this bioconversion. In order to establish which of the two is limiting, the overall mass-transfer coefficients ($K_{i,L}A$) of both substrates were determined. Theoretical calculations did not reveal large differences. Therefore both $K_{i,L}A$'s were experimentally determined as well. From the results it is concluded that neither tetralin nor oxygen can be assigned to be the mass-transfer-limiting factor if tetralin is completely converted into CO_2 and H_2O. On the other hand, if tetralin is oxidized only partly (the aim of our synthetic studies), it very likely is the limiting substrate and process control can thus be achieved by controlling the supply of this toxic compound.

Tetralin and oxygen transfer in the liquid-impelled loop reactor.
Vermuë, M.H., Tacken, M., Tramper, J. (1994) *Bioprocess Eng.* **11**: 224-228.

INTRODUCTION

The application of two-phase systems consisting of an aqueous and a water-immiscible organic-solvent phase in biocatalysis has gained much interest over the past decade [Laane *et al.*, 1987, Tramper *et al.*, 1992). The potential advantages of using these two-phase systems are numerous. It is possible to eliminate limitations caused by low solubility of apolar substrates and products. Product and/or substrate inhibition can be reduced and in case such toxic compounds are used, their concentrations in the aqueous phase can be controlled. Furthermore, product recovery is facilitated. On the other hand, the use of these media is restricted to a relatively small set of suitable solvents, which are non-toxic, non-biodegradable and which have a favourable partition coefficient for the substrates/products. In addition, their use should not result in emulsion formation (Bruce and Daugulis, 1991, Vermuë and Tramper, 1990).

Especially for biocatalysis in two-phase systems the liquid-impelled loop reactor (LLR) has been designed (Tramper *et al.*, 1987). In this reactor the advantages of an air-lift loop reactor (good mixing properties, low shear forces, controlled oxygen supply) are combined with the advantages of using water-immiscible organic solvents. The hydrodynamics (Van Sonsbeek *et al.*, 1990) and mixing properties (Van Sonsbeek *et al.*, 1992a) of this bioreactor have recently been characterized as well as the mass transfer of oxygen from the organic-solvent phase into the aqueous phase (Van Sonsbeek *et al.*, 1991, 1992b, 1992c).

In our laboratory the bioconversion of tetralin in the LLR is studied using a perfluoro compound (FC-40) as a suitable organic solvent (Vermuë and Tramper, 1990). Tetralin (1,2,3,4-tetrahydro-naphthalene) is an apolar bicyclic molecule with an aromatic and a saturated ring. It can be converted by several microorganisms through oxidation of its saturated ring or *via* a dioxygenase-catalyzed oxidation of the aromatic ring (Sikkema and De Bont, 1991). The tetralin solubility in aqueous solutions is very low (ca. 150 μmol l^{-1}) and the toxic concentration of this substrate for the biocatalysts is even lower which makes the use of a two-phase bioreactor attractive (Sikkema, 1993).

Apart from tetralin, oxygen is needed for this bioconversion and in the liquid-impelled loop reactor both substrates can be supplied via the organic-solvent phase. In order to be able to distinguish which of the two substrates will be rate limiting in case of mass-transfer limitation, both the tetralin and oxygen supply were studied in the liquid-impelled loop reactor.

THEORY

The rate of mass transfer, *MTR* (mol s^{-1}) of a solute *i* from the organic-solvent phase (S) to the aqueous phase (L) is defined by the overall mass-transfer coefficient, $K_{i,L}$ (m s^{-1}) and the concentration difference, $C^*_{i,L}$-$C_{i,L}$ (mol m^{-3}) over the exchange area, *A* (m^2 of drop-surface area) (Van 't Riet and Tramper, 1991):

$$MTR = K_{i,L}A\ (C^*_{i,L} - C_{i,L}) \qquad (1)$$

in which

$$C^*_{i,L} = \frac{C_{i,S}}{m_i} \qquad (2)$$

where m_i (mol m^{-3}/mol m^{-3}) is the partition coefficient of the solute over the organic solvent and the aqueous phase and $C_{i,S}$ is its concentration in the organic-solvent phase.

In a two-phase system of FC-40 and aqueous buffer the partition coefficients of tetralin and oxygen are 120 and 12 (Van Sonsbeek *et al.*, 1992b), respectively. However, in our investigations, the organic-solvent phase was constantly sparged with oxygen and mass transfer through the organic-solvent phase was not rate limiting (as shown in results and discussion). The $C^*_{ox,L}$ was thus approximately equal to the maximum solubility of oxygen from ambient air in the aqueous buffer (270 mmol m^{-3}).

In case of complete bioconversion of tetralin ($C_{10}H_{12}$) into CO_2 and H_2O, theoretically 13 moles of oxygen are needed for 1 mole of tetralin. Therefore, the mass-transfer rate of oxygen (*OTR*) is maximally 13 times the mass-transfer rate of tetralin (*TTR*).

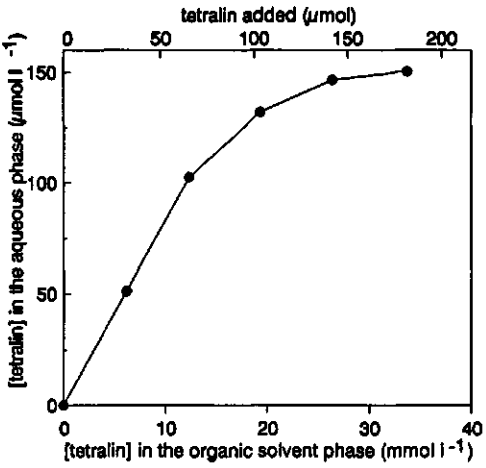


Figure 1 Determination of the partitioning of tetralin over aqueous buffer (100 ml) and FC-40 (5 ml) at 303 K to illustrate the non-ideal behaviour at high inlet concentrations of solute.

An increase of the amount of tetralin added to the organic-solvent phase will result in a proportional increase of the equilibrium concentration in the aqueous phase ($C^*_{tet,L}$). Eventually, however, the increase will no longer be proportional when the equilibrium concentration in the aqueous phase reaches the maximum solubility (150 μmol l^{-1}) (Figure 1). If this is the case and if the oxygen and tetralin concentrations in the aqueous phase ($C_{i,L}$) are zero, due to a relatively fast bioconversion, the maximal ratio between the mass-transfer coefficient of oxygen ($K_{ox,L}$) and tetralin ($K_{tet,L}$) can be estimated (equation 3).

$$\frac{K_{ox,L}}{K_{tet,L}} = \frac{OTR}{TTR} * \frac{C^*_{tet,L}}{C_{ox,L}} = 13 * \frac{150}{270} \approx 7 \qquad (3)$$

If the ratio, $K_{ox,L}/K_{tet,L}$, calculated from the separately determined K_i's, is higher than the estimated value (equation 3), tetralin transfer is rate limiting and process control can be achieved by controlling tetralin transfer rather than oxygen transfer.

Chapter 4

MATERIALS AND METHODS

The partition coefficient of tetralin in FC-40 and aqueous buffer

Six amounts of tetralin (1,2,3,4-tetrahydro-naftalene) (Merck) in the range of 0 - 185 μmol were added to 100 ml mineral medium (Sikkema and De Bont, 1991) and 5 ml perfluor compound, FC-40 (3M) in 100 ml sealed flasks. After 14 hours shaking at 303 K on an orbital shaker, the concentration of tetralin in the aqueous phase was determined. The concentration in the organic phase was calculated from the mass balance.

Dynamic $K_{i,L}A$ determination in the LLR

The overall mass-transfer coefficients $K_{i,L}$ for oxygen and tetralin were determined using the dynamic method (Van Sonsbeek *et al.*, 1991). A 3.5 l LLR was filled with 2.7 l mineral medium (Sikkema and De Bont, 1991) and 0.3 l FC-40 (3M) (Figure 2). Two buffer vessels with 0.5 l organic solvent were used; vessel A was sparged with nitrogen and contained no tetralin, vessel B was aerated and supplied with tetralin (Merck). To de-aerate the reactor, vessel A was connected to the reactor setup during 50 minutes, and solvent recycled. When all oxygen was removed from the reactor, a step change in inlet oxygen and tetralin concentration was obtained by switching the feed tap from vessel A to vessel B. The response of the concentration in the aqueous phase to this step-wise change in the oxygen and tetralin concentrations in the organic-solvent phase was measured as a function of time.

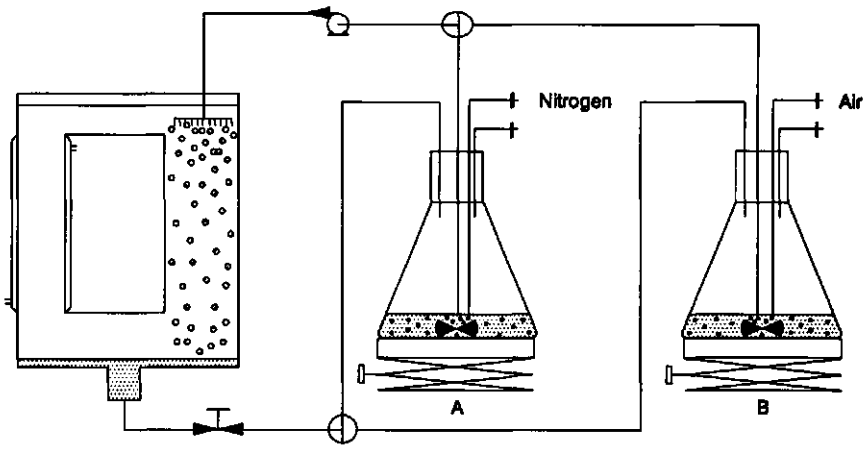


Figure 2 The experimental set up for dynamic measurement of mass transfer in the liquid-impelled loop reactor.

Concentration measurements

The oxygen concentration was continuously monitored for 1 hour using a Schott Digital Oxygen monitor CG 867. Approximately each 90 s the tetralin concentration in the aqueous phase was determined by gas chromatography. A 5 μl sample was injected at 473 K into a packed column (3 m * 3.2 mm, Chrom PAW-DMCS, 3% w/w SE30). The oven temperature of the Hewlett-Packard 7510 A gas chromatograph was 413 K. FID detection of tetralin occurred at 523 K.

RESULTS AND DISCUSSION

Theoretical estimation of the ratio between $K_{ox,L}$ and $K_{tet,L}$

The overall mass-transfer coefficient for e.g. oxygen, $K_{ox,L}$ (m s^{-1}) is composed of the partial mass-transfer coefficients of both phases in the following way (Van 't Riet and Tramper, 1991, Van Sonsbeek *et al.*, 1992b):

$$\frac{1}{K_{ox,L}} = \frac{1}{m_{ox} * k_{ox,S}} + \frac{1}{k_{ox,L}} \qquad (4)$$

where $k_{ox,S}$ and $k_{ox,L}$ are the partial mass-transfer coefficients of oxygen in the organic and aqueous phase (m s^{-1}), respectively.

The Reynolds number of the dispersed phase (Re_S) characterizes the flow conditions within the droplets in the reactor (Al-Aswad *et al.*, 1985, Van Sonsbeek *et al.*, 1992b):

$$Re_S = \frac{\rho_S * vs * d}{\eta_S} \qquad (5)$$

where

ρ_S	= solvent density	kg m^{-3}
vs	= slip velocity of the drops = vs_S/α - $vs_L/(1-\alpha)$	m s^{-1}
vs_S, vs_L	= superficial velocity of the organic-solvent phase and the aqueous phase, respectively, in the main tube of the LLR	m s^{-1}
α	= holdup of organic-solvent phase in the main tube of the LLR	
d	= drop diameter	m
η_S	= dynamic viscosity of the organic-solvent phase	Pa s

In our case, the drop Reynolds number $\approx$ 100, which means that internally

circulating drops are to be expected. For these drops, the correlation of Hughmark (1967) for $k_{i,L}$ can be used for the estimation of the partial mass-transfer coefficient in the aqueous phase:

$$k_{i,L} = \frac{D_L}{d} * [\, 2 + 0.463 * Re_L^{0.484} * Sc_L^{0.339} * (\frac{d * g^{1/3}}{D_L^{2/3}})^{0.072} \,] * F \quad (6)$$

in which

D_L	= diffusion coefficient of solute in the aqueous phase	$m^2\ s^{-1}$
Re_L	= Reynolds number of the aqueous phase around the drops $= \rho_L * vs * d * \eta_L^{-1}$	-
ρ_L	= density of the aqueous phase	$kg\ m^{-3}$
η_L	= dynamic viscosity of the aqueous phase	Pa s
Sc_L	= Schmidt number of the aqueous phase $= \eta_L * \rho_L^{-1} * D_L^{-1}$	
g	= gravitational acceleration	$m\ s^{-2}$
F	= correction factor for the circulation within the droplets $= 0.281 + 1.615*\kappa + 3.73*\kappa^2 - 1.874*\kappa^3$	

where

$$\kappa = Re_S^{1/8} * (\frac{\eta_L}{\eta_S})^{1/4} * (\frac{\eta_L * vs}{\sigma})^{1/6} \quad (7)$$

σ	= interfacial tension	$N\ m^{-1}$

For $k_{i,S}$ the correlation of Kronig and Brink (1950) can be applied. Originally this expression was derived for stagnant, spherical droplets but the $k_{i,S}$ of fully internally circulating drops proved to agree quite well with this expression (Rose and Kintner, 1966):

$$k_{i,S} = -\frac{d}{6t} * \ln [\frac{3}{8} * \sum_{n=1}^{\infty} B_n^2 \exp(-64\lambda_n * D_S * \frac{t}{d^2})] \quad (8)$$

where

D_S	= diffusion coefficient of solute in the organic-solvent phase	$m^2\ s^{-1}$
t	= time of contact	s
B_n	= coefficient	-
λ_n	= n^{th} eigen value	-

Table 1 lists the physical properties of water and FC-40, while the essential reactor characteristics are listed in Table 2.

The calculated theoretical overall mass-transfer coefficients can be found in Table 3. Because of the high value of the partition coefficient of both oxygen and tetralin, the overall mass-transfer coefficient mainly depends on the partial mass-transfer coefficient in the aqueous phase. The difference in overall mass-transfer coefficient of both solutes thus mainly reflects the difference in the partial mass-transfer coefficients in the aqueous phase. Equation 6 shows that this difference is caused by differences in diffusion coefficients for oxygen and tetralin in the aqueous phase, since all other parameters involved, are equal for both oxygen and tetralin.

The ratio $K_{ox,L}/K_{tet,L}$ calculated from the literature correlations, is 1.8 and this indicates that for the bioconversion the mass transfer of oxygen will most probably be rate limiting. However, these theoretical calculations can only be used as first estimates because important phenomena, such as mass transfer during drop formation and coalescense, are not taken into account. The diffusion coefficient of tetralin was estimated using the Wilke Chang correlation (Wilke and Chang, 1955) and these authors noted an average error of about 10 percent, when 251 solute/solvent systems were tested. This indicated error also makes estimation of the mass-transfer coefficient less reliable.

Table 1: Physical properties of FC-40 (3M) and water (*Handbook of Chemistry and Physics*).

	FC-40	Water	
density (298 K)	$1.870\ 10^{3}$	$0.997\ 10^{3}$	kg m^{-3}
dynamic viscosity (298 K)	$4.5\ 10^{-3}$	$1.0\ 10^{-3}$	Pa s
interfacial tension with water (293 K)	$34.9\ 10^{-3}$		N m^{-1}
surface tension (298 K)	$16.0\ 10^{-3}$	$72.0\ 10^{-3}$	N m^{-1}
solubility of oxygen from ambient air (298 K)	12.0	0.28	mol m^{-3}
average molecular weight	0.650	0.018	kg mol^{-1}
diffusivity of oxygen (298 K)[a]	$8.1\ 10^{-9}$	$2.1\ 10^{-9}$	m^2 s^{-1}
diffusivity of tetralin (298 K)[b]	$5.7\ 10^{-10}$	$7.2\ 10^{-10}$	m^2 s^{-1}

[a] The diffusivity of oxygen in FC-40 is taken from Ju *et al.* (1991) and the diffusivity in water is taken from Ju and Ho (1985)

[b] The diffusivity of tetralin was estimated using the Wilke-Chang correlation (Wilke and Chang, 1955)

Table 2: Characteristics of the liquid-impelled loop reactor used for the estimation of the mass-transfer coefficient of tetralin and oxygen.

	Oxygen	Tetralin	Units
$V_{reactor}$ [a]	$3.45\ 10^{-3}$	$3.45\ 10^{-3}$	m^3
$V_{main\ tube}$ [a]	$1.19\ 10^{-3}$	$1.19\ 10^{-3}$	m^3
ϕ [b]	$3.95\ 10^{-6}$	$3.95\ 10^{-6}$	$m^3\ s^{-1}$
d	$1\ 10^{-3}$	$1\ 10^{-3}$	m
α	$4.16\ 10^{-3}$	$4.16\ 10^{-3}$	-
vs_S	$1.40\ 10^{-3}$	$1.40\ 10^{-3}$	$m\ s^{-1}$
vs_L	$9.74\ 10^{-2}$	$9.74\ 10^{-2}$	$m\ s^{-1}$
vs	0.24	0.24	$m\ s^{-1}$
t [c]	1.25	1.25	s
Sh_L [d]	195	279	-
B_1 [e]	1.31	1.31	-
B_2 [e]	0.508	0.585	-
B_3 [e]	0.394	0.394	-
λ_1 [e]	1.54	1.58	-
λ_2 [e]	8.47	8.59	-
λ_3 [e]	20.6	21.0	-

[a] $V_{reactor}$ = reactor volume,
$V_{main\ tube}$ = volume of the reactor main tube, where mass transfer predominantly takes place

[b] ϕ = recycling flow rate of organic solvent

[c] t = residence time of the organic-solvent drop in the reactor
$= \alpha * V_{main\ tube}/\phi$

[d] Sh_L = Sherwood number of the aqueous phase $= k_{i,L} * d/D_L$;
calculated $k_{i,L}$ values can be found in Table 3

[e] B_n, λ_n = coefficients and eigenvalues for circulating drops (Perry *et al.*, 1984)

Because of this limited reliability of these theoretical calculations and because the calculated ratio $K_{ox,L}/K_{tet,L}$ is of the same order of magnitude as the ratio mentioned in equation 3, it is difficult to distinquish whether oxygen or tetralin is likely to be rate limiting. Therefore, the overall mass-transfer coefficients of both oxygen and tetralin were measured in the LLR.

Measurement of the $K_{i,L}A$ in the LLR

A typical example of the measurement of the $K_{i,L}A$ of oxygen and tetralin is given in Figure 3.

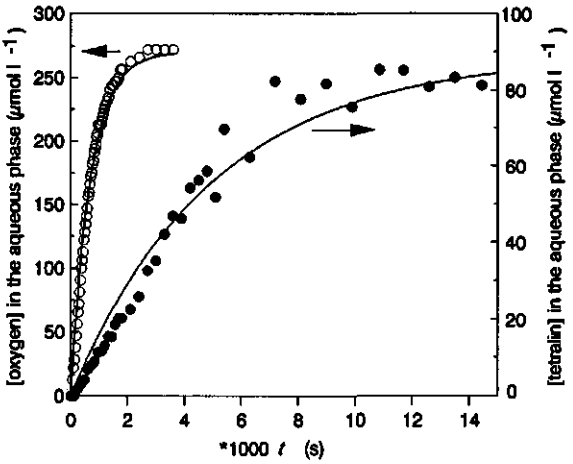


Figure 3 Mass transfer of oxygen (○) and tetralin (●) for the determination of the $K_{ox,L}$ and the $K_{tet,L}$ using equation 1.

If it is assumed that

- the continuous aqueous phase is ideally mixed,
- the substrate concentration in the dispersed organic phase is constant (oxygen is constantly supplied to the organic solvent and a negligible proportion of the total amount of tetralin in the organic solvent is migrating to the aqueous phase), and
- the oxygen and tetralin uptake rates are constant throughout the experiment,

and equation 1 is integrated over the time interval $t_2 - t_1$, $K_{i,L}A$ can be derived directly from the dynamic measurements with the following formula:

$$K_{i,L}A = \ln \frac{C_{i,S}/m_i - C_{i,L}(t_1)}{C_{i,S}/m_i - C_{i,L}(t_2)} * (t_2 - t_1)^{-1} \qquad (9)$$

where $C_{i,S}$ and $C_{i,L}$ are the concentration of solute i in the organic solvent and in the aqueous phase, respectively (mol m^{-3}). For the case that tiny drops of organic solvent are formed which cause a homogeneous dispersion and remain inside the reactor and do not contribute to the mass transfer, the $K_{i,L}A$ should be multiplied by a factor $(1 + \epsilon m_i)$

Table 3: Theoretical and experimental estimation of the mass-transfer coefficients of oxygen and tetralin in the liquid-impelled loop reactor using FC-40 (organic-solvent phase) and aqueous buffer.

	Oxygen	Tetralin	
Theoretical values			
$k_{i,S}$	$1.9\ 10^{-4}$	$0.5\ 10^{-4}$	m s^{-1}
$k_{i,L}$	$4.1\ 10^{-4}$	$2.0\ 10^{-4}$	m s^{-1}
m_i[a]	12	120	-
$K_{i,L}$	$3.5\ 10^{-4}$	$1.9\ 10^{-4}$	m s^{-1}
A[b]	$30\ 10^{-3}$	$30\ 10^{-3}$	m^2
$K_{i,L}A$	$3.0\ 10^{-5}$	$1.7\ 10^{-5}$	s^{-1}
Measured values			
$K_{i,L}A_{regression}$	$16.1\ 10^{-4}$	$2.3\ 10^{-4}$	s^{-1}
$K_{i,L}A_{curvefit}$	$14.4\ 10^{-4}$	$2.0\ 10^{-4}$	s^{-1}

[a] The partition coefficient of oxygen in a two-phase system of FC-40 and aqueous buffer was taken from Van Sonsbeek *et al.* (1992b). The partition coefficient of tetralin was calculated from the linear part of figure 1.

[b] A = total surface area of organic solvent drops inside the reactor
$= 6 * \alpha * V_{main\ tube}/d$

(Van Sonsbeek *et al.*, 1992b). In case of FC-40 and aqueous buffer, however, the hold-up (ϵ) of these tiny drops in the reactor is negligible.

If more advanced models for mixing in the continuous and the dispersed phase are necessary, a numerical solution for $K_{i,L}A$ can be found by curve fitting (Van Sonsbeek *et al.*, 1992b). Both methods were used in this work.

In Table 3 the averages of $K_{i,L}A$ values that are derived from experimental results are given. The $K_{i,L}A$ was determined in triplicate and shows a maximum deviation of 15%. The deviation of the $K_{i,L}A$ obtained by the linear regression method (equation 9) and by curve fitting vary a little and therefore the assumptions made for equation 9, in particular ideal mixing of the aqueous phase and constant solute concentration in the organic-solvent phase, are not completely justified. For calculation of the values by curve fitting the solute concentration in the organic-solvent phase was assumed to be constant. Correction for the change in the concentration in the organic-solvent phase due to mass transfer yielded the same $K_{i,L}A$ value.

With the total area for mass transfer A ($= V_{\text{main tube}} * 6 * \alpha / d$) being 30 10^{-3} m^2, it is obvious that the theoretical values for $K_{tet,L}A$ and $K_{ox,L}A$ are much smaller than they are in reality (Table 3). This was to be expected, because axial dispersion, coalescence and other phenomena that may increase the mass transfer, were not taken into account. In addition, measurement of the hold-up (α) and the drop diameter (d) in the LLR is very difficult. Here, these parameters were estimated (standard deviation about 10%) and the total area for mass transfer may thus be larger in reality.

The ratio $K_{ox,L}/K_{tet,L} \approx 7$ is found which is higher than the ratio found with the literature correlations ($K_{ox,L}/K_{tet,L} \approx 1.8$), but for the latter a less reliable estimation of the diffusivity of tetralin in FC-40 and water was used.

The measured ratio is equal to the ratio as given in equation 3. This means that it is not possible to distinquish which of the two substrates will be rate limiting in case of mass transfer limitation. In equation 3 it was assumed that tetralin is completely converted to CO_2 and H_2O. In practice, tetralin conversion will also lead to biomass increase and partial oxidized tetralin products (Sikkema, 1993). If that is the case, the *OUR* is less than first assumed, which means that tetralin transfer will most probably be rate limiting and under continuous operation, the process can be controlled by controlling the tetralin supply rather than the oxygen supply.

ACKNOWLEDGEMENTS

The authors wish to thank W.A. Beverloo for the helpful critical comments in the preparation of the manuscript. Part of this work was financially supported by the Dutch Committee of Industrial Biotechnology.

REFERENCES

Al-Aswad, K.K., Mumford, C.J., Jeffreys, G.V. (1985) The application of drop-size distribution and discrete drop mass-transfer models to assess the performance of a rotating disc contactor. *AIChE Journal* 31:1488-1497.

Bruce, L.J., Daugulis, A.J. (1991) Solvent selection strategies for extractive biocatalysis. *Biotechnol. Prog.* **7**: 116-124.

Handbook of Chemistry and Physics, 60th edition. CRC press, Boca Raton, Florida.

Hughmark, G.A. (1967) Liquid-liquid spray column drop size, hold-up, and continuous phase mass transfer. *Ind. & Eng. Fundamentals* **6**: 408-413.

Ju, L.K., Ho, C.S. (1985) Measuring oxygen diffusion coefficients with polarografic oxygen electrodes. I. Electrolytic solutions. *Biotechnol. Bioeng.* **27**: 1495-1499.

Ju, L.K., Lee, J.F., Armiger, W.B. (1991) Effect of the interfacial surfactant layer on oxygen transfer through the oil/water phase boundery in perfuorocarbon emulsions. *Biotechnol. Bioeng.* **37**: 505-511.

Kronig, R., Brink, J.C. (1950) On the theory of extraction from falling droplets. *Appl. Res.* **A2**: 142-154.

Laane, C., Tramper, J., Lilly, M.D. (Ed.) (1987) *Biocatalysis in organic media.* Elsevier Science Publ., Amsterdam, The Netherlands.

Perry, R.H., Green, D.W., Maloney, J.O. (Ed.) (1984) *Perry' Chemical Engineers' Handbook,* 6th edition. McGraw-Hill, New York.

Rose, P.M., Kintner, R.C. (1966) Mass transfer from large oscillating drops. *AIChE Journal* **12**: 530-534.

Sikkema, J., De Bont, J.A.M. (1991) Isolation and initial characterization of bacteria growing on tetralin. *Biodegradation* **2**: 15-23.

Sikkema, J. (1993) *Microbial transformation of tetralin.* PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Tramper, J., Wolters, I., Verlaan, P. (1987) The liquid-impelled loop reactor: a new type of density-difference-mixed bioreactor. In: *Biocatalysis in organic media,* 311-316. Laane, C., Tramper, J., Lilly, M.D. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) (1992) *Biocatalysis in non-conventional media.* Elsevier Science Publ., Amsterdam, The Netherlands.

Van Sonsbeek, H.M., Verdurmen, R.E.M., Verlaan, P., Tramper, J. (1990) Hydrodynamic model for liquid-impelled loop reactors. *Biotechnol. Bioeng.* **36**: 940-946.

Van Sonsbeek, H.M., Gielen, S.J., Tramper, J. (1991) Steady-state method for KA measurements in model systems. *Biotechnol. Techn.* **5**: 157-162.

Van Sonsbeek, H.M., Van Der Tuin, S.P., Tramper, J. (1992a) Mixing in liquid-impelled loop reactors. *Biotechnol. Bioeng.* **39**: 707-716.

Van Sonsbeek, H.M., De Blank, H., Tramper, J. (1992b) Oxygen transfer in liquid-impelled loop reactors using perfluorcarbon liquids. *Biotechnol. Bioeng.* **40**: 713-718.

Van Sonsbeek, H.M. (1992c) *Physical aspects of liquid-impelled loop reactors*. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Van 't Riet, K., Tramper, J. (1991) *Basic bioreactor design*. Marcel Dekker, New York.

Vermuë, M.H., Tramper, J. (1990) Extractive biocatalysis in a liquid-impelled loop reactor. In: *Proceedings of the 5th European congress on biotechnology*, 243-246. Christiansen, C., Munck, J., Villadsen, J. (Ed.) Munksgaard, Copenhagen, Denmark.

Wilke, C.R., Chang, P. (1955) *AIChE Journal* **1**: 264-270.

Chapter 5

TOXICITY OF HOMOLOGOUS SERIES OF ORGANIC SOLVENTS FOR THE GRAM-POSITIVE BACTERIA *ARTHROBACTER* AND *NOCARDIA* SP. AND THE GRAM-NEGATIVE BACTERIA *ACINETOBACTER* AND *PSEUDOMONAS* SP.

SUMMARY

The toxicity of homologous series of organic solvents has been investigated for the Gram-positive bacteria *Arthrobacter* sp. and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* sp. and *Pseudomonas* sp.. The hydrophobicity of the solvent, expressed by its $\log P_{octanol}$, proves to be a good measure for the toxicity of solvents in a two-phase system. The transition from toxic to non-toxic solvents occurs between $\log P_{octanol}$ 3 and 5 and depends on the homologous series. No correlation has been found between the hydrophobicity of the substituent on the alkyl backbone of the solvent and the location of the transition point in toxicity. The $\log P_{octanol}$, above which all solvents are non-toxic, is used to express the solvent tolerance of the bacteria. In general the solvent tolerance of Gram-negative bacteria is found to be slightly higher than that of Gram-positive bacteria, but this does not hold for all homologous series of organic solvents investigated.

Since the toxicity effects of organic solvents in a two-phase system can be

Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp.
Vermuë, M., Sikkema, J., Verheul, A., Bakker, R., Tramper, J. (1993)
Biotechnol. Bioeng. **42**: 747-758.

ascribed to molecular as well as phase-toxicity effects, molecular toxicity effects were investigated separately in a one-phase system with subsaturating amounts of organic solvent. The solvent concentration in the aqueous phase at which 50% of the metabolic activity of the bacteria is lost, is used to express solvent toxicity. This concentration is found to be similar for the Gram-positive *Arthrobacter* and the Gram-negative *Acinetobacter*. Assuming the critical membrane concentration theory (Osborne *et al*, 1990 *Enzyme Microb. Technol.* 12: 281-291) to be valid, it can be concluded that differences in solvent tolerance between these two bacteria, can not be ascribed to differences in response to molecular toxicity. Prediction of the toxicity of any solvent, using the critical membrane theory, appears to be possible in the case of alkanols or alkyl acetates. However, prediction of the toxicity of ethers appears to be impossible.

INTRODUCTION

Biocatalysis in reaction mixtures containing water-immiscible organic solvents is becoming increasingly important (Laane *et al.*, 1987, Tramper *et al.*, 1992). With these solvents it is possible to increase the overall concentration of apolar substrates and products, thereby improving the volumetric product yield in the reaction system. The use of these solvents is particularly advantageous when an apolar substrate or product is toxic/inhibitory for the biocatalyst (enzymes, cell organelles, cells). By introducing a water-immiscible solvent it is possible to maintain a low concentration of the toxic/inhibitory compound in the aqueous phase, while the overall concentration remains high (Sikkema and De Bont, 1991, Van der Tweel *et al.*, 1988, Vermuë and Tramper, 1990).

The water-immiscible solvent should be non-biodegradable and should have a favourable distribution coefficient for the toxic/inhibitory compounds, but one of the most important requirements of the solvent is bio-compatibility. A good measure for the bio-compatibility of a solvent is its $\log P_{octanol}$ value, which is defined as the logarithm of its partition coefficient in a standard octanol/water two-phase system (Laane *et al.*, 1987). Generally, solvents having a $\log P_{octanol} > 5$ are bio-compatible for cellular

biocatalysts (Laane *et al.*, 1987, Buitelaar *et al.*, 1990, Vermuë and Tramper, 1990, Bruce and Daugulis, 1991, Bassetti and Tramper, 1992). Although the correlation between the $\log P_{octanol}$ of a solvent and the activity retention of a cellular biocatalyst also holds for many enzymes (Laane *et al.*, 1987, Reslow *et al.*, 1987, Reslow, 1989), we will not discuss this in detail since the mechanism of toxicity for enzymes seems to differ from that for cells. It is suggested that an organic solvent interferes with the essential water layer surrounding an enzyme (Zaks and Klibanov, 1988); in the case of living cells, the toxicity of organic solvents is ascribed mainly to the distorting effect of the solvents on the cell membrane and the membrane bound enzymes (Uribe *et al.*, 1985, 1990, Laane *et al.*, 1987, Osborne *et al.*, 1990, Sikkema *et al.*, 1992).

Many attempts have been made to explain the empirical correlation between $\log P_{octanol}$ and the activity retention of cellular biocatalysts, but up till now the mechanisms of solvent-caused toxicity are poorly understood. Bar (1988) suggested that solvent toxicity for microbial cells in a two-liquid-phase system is caused both by the presence of a second phase (phase toxicity), and by the solvent molecules that dissolve in the aqueous phase (molecular toxicity). Phase toxicity may result from extraction of nutrients and cell-wall components, or from limited availability of nutrients as a result of adherence of the cells to the interphase, or from entrapment in an emulsion. Molecular toxicity may result from enzyme inhibition, protein denaturation and membrane modification (Bar, 1988).

Osborne and coworkers (1990) investigated the molecular toxicity of solvents. They found a correlation between the residual 11α-hydroxylase activity of *Rhizopus nigricans* and the estimated solvent concentration in the cell membrane. Recently Sikkema *et al.* (1992) demonstrated that interaction of the aromatic solvent tetralin with the membrane of both bacteria and liposomal model systems increased the permeability of the membrane, resulting in the impairment of the primary energy transduction system, the proton motive force. Uribe *et al.* (1985, 1990) found that cyclohexane and ß-pinene also increase the passive flux of ions across the membrane. These findings indicate the cell membrane and its protein constituents to be a primary target for the molecular toxicity of solvents.

If indeed the cell envelope is of crucial importance for molecular toxicity, its composition would be a key parameter. Harrop *et al.* (1989) compared the hydroxylation of naphthalene by a Gram-negative *Pseudomonas putida* with the steroid Δ^1-dehydrogenation by a Gram-positive *Arthrobacter simplex*. They found that the Gram-negative bacterium showed better tolerance to solvents with low $\log P_{octanol}$-values. Inoue and Horikoshi (1991) determined the solvent tolerance of 32 Gram-negative and 28 Gram-positive bacteria; their results also suggest a higher solvent tolerance for Gram-negative bacteria. Both publications refer to measurements in two-liquid-phase systems (buffer/solvent); no distinction was made between phase toxicity and molecular toxicity.

In the study described here, we investigated the solvent tolerance of Gram-positive *Arthrobacter* sp. and *Nocardia* sp. and of Gram-negative *Acinetobacter* sp. and *Pseudomonas* sp.. In two-liquid-phase systems, the combined effect of phase and molecular toxicity of a large number of organic solvents was studied. Molecular toxicity was investigated separately in solvent-subsaturated aqueous systems.

The critical membrane concentration as described by Osborne *et al* (1990) was used to investigate, if differences in solvent tolerance for the Gram-positive *Arthrobacter* and the Gram-negative *Acinetobacter* can be ascribed to differences in molecular toxicity.

MATERIALS AND METHODS

Organisms

The bacteria *Arthrobacter* T2 and *Acinetobacter* T5 were isolated on tetralin (Sikkema and De Bont, 1991); *Pseudomonas* S12 and *Nocardia* S1 were isolated on styrene (Hartmans *et al.*, 1990). The bacteria were obtained from the culture collection of the division of Industrial Microbiology of the Wageningen Agricultural University, The Netherlands.

Cultivation

The organisms were grown in 5 l Erlenmeyer flasks, containing 1 l of mineral medium supplemented with di-sodium succinate (5 g l^{-1}) and yeast extract (0.5 g l^{-1}). The composition of the mineral medium was: K_2HPO_4, 1.55 g l^{-1}; $NaH_2PO_4.H_2O$, 0.85 g l^{-1}; NH_4Cl, 2.0 g l^{-1}; $(NH_4)_2SO_4$, 0.1 g l^{-1}; $MgCl_2.6H_2O$, 0.075 g l^{-1} and trace elements, 2 ml l^{-1} (Vishniac and Santer, 1957). Cultures were incubated at 303 K on a

rotary shaker (200 rpm) for 48 hours.

Solvents

Solvent source, purity, density and $\log P_{octanol}$ values are presented in Table 1. $Log P_{octanol}$ values (on molar base) were calculated from hydrophobic constants (Rekker and De Kort, 1979).

Metabolic activity

To 100 ml serum bottles, 9 ml growth medium containing di-sodium succinate and 1 ml organic solvent were added. To this two-liquid-phase system 0.1 ml of precultured cells (ca. 7.5 mg protein per litre) was added. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as standard. The bottles were incubated in a shaker incubator (200 rpm) at 303 K. Metabolic activity was measured as cummulative CO_2-production after 1 and 7 days of incubation. For that, head-space samples (0.1 ml) were analyzed with a Packard 427 gas chromatograph with a Chrompack Porapack Q column.

To obtain subsaturating solvent concentrations, growth medium was first saturated with organic solvent by shaking the medium for two hours with organic solvent and allowing overnight phase separation at 303 K. The solvent-saturated aqueous phase was then diluted with solvent-free growth medium. To 100 ml serum bottles, 10 cm3 of the subsaturated medium and 0.1 ml of the precultured cells were added. Again, metabolic activity of the cells was monitored by measuring production of CO_2 by gas chromatograph. All experiments were conducted in two-fold.

Initial oxygen-consumption rate

Precultured cells were harvested in the exponential growth phase and washed twice in phosphate buffer (50 mmol l^{-1}, pH 7). Cells were resuspended in buffer to a density of approximately 7.5 g cell protein per litre. The effect of dissolved organic solvent on the oxygen-consumption rate was subsequently measured at 303 K with a Clark-type electrode. A 3 ml incubation vessel was filled with 2.8 ml of an air-saturated di-sodium-succinate solution (0.1 mol l^{-1}) and 0.1 ml of an air-saturated solution of organic solvent in N-dimethylformamide (DMF). The DMF was used to improve the accuracy of addition of small amounts of the solvent to be tested; DMF proved to have no effect on the oxygen-consumption rate in the concentration added (data not shown). In case the solvent concentration exceeded the maximum solubility, a second phase was observed. The reaction was started by adding 0.1 ml of the cell suspension and after a linear signal was obtained the oxygen consumption was followed for 10 minutes. All experiments were conducted at least in duplicate.

Table 1 Physical properties of the organic solvents used.

Solvent	Source[a]	Purity (%)	Maximum solubility in water (298 K) (mmol l^{-1})[c]	$logP_{octanol}$	Molecular toxicity[c]
Saturated aliphatic hydrocarbons					
pentane	D	~99	0.53	3.0	N
hexane	D	>99	0.14	3.5	N
heptane	D	>99	$36*10^{-3}$	4.0	N
octane	D	99.5	$58*10^{-6}$	4.5	N
iso-octane	D	98	$21*10^{-3}$	4.5	N
nonane	D	98	$1.7*10^{-3}$	5.1	N
decane	D	>95	$0.51*10^{-3}$	5.6	N
undecane	A	99	NA[b]	6.1	-
dodecane	D	>99	$22*10^{-6}$	6.6	N
tridecane	A	>99	NA[b]	7.1	-
tetradecane	A	99	$85*10^{-6}$ [d]	7.6	N
pentadecane	A	>99	NA[b]	8.2	-
hexadecane	D	>99	$4.0*10^{-6}$ [d]	8.8	N
heptadecane	A	99	NA[b]	9.3	-
octadecane	A	99	$8.2*10^{-6}$ [d]	9.8	N
Aliphatic alkanols					
1-butanol	D	99.5	1005	0.8	Y
1-pentanol	A	>99	248	1.3	Y
1-hexanol	D	>98	69	1.8	Y
1-heptanol	C	98	15	2.4	Y
1-octanol	D	>99	4.1	2.9	Y
1-nonanol	A	99	1.1	3.4	Y
1-decanol	C	99	0.25	4.0	N
1-undecanol	A	99	$69*10^{-3}$	4.5	N
1-dodecanol	A	99	$19*10^{-3}$	5.0	N
Esters of dicarboxylic acids					
dimethyl phthalate	D	99	0.13 [d]	2.3	N
diethyl phthalate	D	99	$4.3*10^{-3}$ [d]	3.3	N
dibutyl phthalate	C	99	$5.2*10^{-3}$ [d]	5.4	N
dioctyl phthalate	C	98	$0.7*10^{-3}$ [d]	9.6	Y
didecyl phthalate	B	NA[c]	NA[b]	11.7	-
Fluorinated hydrocarbons					
perfluor hexane	B	~85	NA[b]	2.5	-
perfluor decalin	A	95	NA[b]	6.5	-
FC-40	C	NA[c]	NA[b]	11.4	-

Table 1 *continued*

Aliphatic ethers					
diethyl ether	D	>99	815	0.9	Y
dipropyl ether	A	>99	48	1.9	Y
dibutyl ether	D	>99	2.3	2.9	Y
dipentyl ether	A	99	NA[b]	3.9	-
dihexyl ether	D	>97	0.54	5.0	Y
diphenyl ether	B	>98	23	4.3	Y
Unsaturated hydrocarbons					
1-hexene	D	99	0.83	3.1	N
1-heptene	C	>98	0.18	3.6	N
1-octene	D	>99	0.036	4.2	N
1-nonene	A	97	$7.8*10^{-3}$	4.7	Y
1-decene	D	96	NA[b]	5.2	-
1-undecene	A	99	NA[b]	5.7	-
1-dodecene	A	95	NA[b]	6.2	-
1-tetradecene	A	NA[c]	NA[b]	6.7	-
1-hexadecene	A	94	NA[b]	7.3	-
Esters of saturated aliphatic monocarboxylic acids					
methyl acetate	C	99.4	3307	0.16	Y
ethyl acetate	D	99.5	917	0.64	Y
propyl acetate	D	>99	225	1.17	Y
butyl acetate	D	NA[c]	58.5	1.7	Y
pentyl acetate	C	>99	13.06	2.23	N
butyl stearate	B	40-60	NA[b]	10.0	-
Aromatic hydrocarbons					
toluene	D	99.5	5.6	2.9	Y
ethyl benzene	C	99	1.4	3.1	N
propyl benzene	A	98	0.54	3.6	N
butyl benzene	A	>99	$88*10^{-3}$	4.1	N
tetralin	C	99	NA[b]	3.9	-

a) A = Aldrich-Chemie; B = Fluka; C = Janssen Chimica; D = Merck
b) NA = not available
c) data taken from Riddick *et al.* (1986) unless stated otherwise
d) data taken from Verschueren (1983)
e) molecular toxicity for *Arthrobacter* and *Acinetobacter* was predicted using equation 3 and Figure 6. Y = molecular toxicity is expected, N = molecular toxicity is not expected, - = prediction is not possible, since maximum solubility data are not available

RESULTS AND DISCUSSION

Metabolic activity in two-liquid-phase systems

The toxicity of 58 water-immiscible organic solvents was tested by measuring the metabolic activity (total CO_2-production from succinate after 7 days of incubation) of the Gram-positive *Arthrobacter* and *Nocardia* and of the Gram-negative *Acinetobacter* and *Pseudomonas*. In these experiments, two liquid phases were present; therefore, toxicity could be ascribed to both phase and molecular effects. A solvent is defined as toxic if the metabolic activity of the cells was less than 50% of the metabolic activity of the cells in the absence of the solvent.

The relative metabolic activity of *Arthrobacter*, *Acinetobacter*, *Nocardia* and *Pseudomonas* is plotted against the $\log P_{octanol}$ of the solvents (Figure 1). Figure 1a and 1b both show a correlation between the $\log P_{octanol}$ of the solvent and the relative metabolic activity. All organic solvents with $\log P_{octanol} < 3$ were toxic to all bacteria.

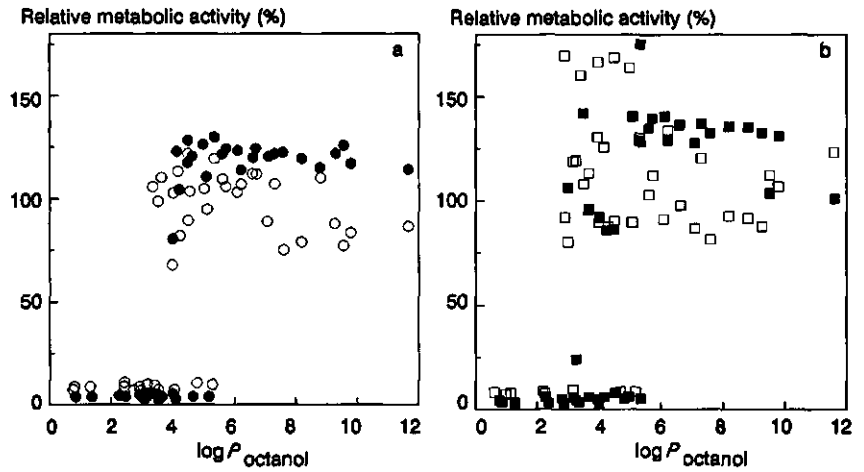


Figure 1 Relationship between relative metabolic activity of a) *Arthrobacter* (●) and *Acinetobacter* (○) and of b) *Nocardia* (■) and *Pseudomonas* (□), exposed to 10% v/v organic solvent, and $\log P_{octanol}$. Open and closed symbols represent the Gram-negative and the Gram-positive bacteria, respectively.

All organic solvents with $\log P_{octanol} > 5$ were found non-toxic. In an intermediate transition range ($3 < \log P_{octanol} < 5$), toxic as well as non-toxic solvents were found. A similar correlation between toxicity and $\log P_{octanol}$ has been found for epoxidizing cells, although in that case the transition between toxic and non-toxic solvents was observed in the $\log P_{octanol}$ range between 2 and 4 (Laane *et al.*, 1987). This range is also observed for the Δ'-dehydrogenation activity of *Arthrobacter simplex* (Hocknull and Lilly, 1990). For *Saccharomyces cerevisiae* and *Clostridium acetobutylicum*, on the other hand, the transition was found between $\log P_{octanol}$ 4 and 6 (Bruce and Daugulis, 1991), while for plant cells of *Tagetes minuta* (Buitelaar *et al.*,1990) and *Morinda citrifolia* (Bassetti and Tramper, 1992) solvents having a $\log P_{octanol} < 6$ were found to be toxic. The $\log P_{octanol}$ range in which transition of toxic to non-toxic solvents is observed, thus depends on the type of cellular biocatalyst.

Many of the solvents increase the metabolic activity of the cells (Figure 1). We have shown that some of the solvents can be used by the bacteria as carbon and energy source (Vermuë and Tramper, 1990), which may explain the increased metabolic activity. The Gram-positive *Arthrobacter* and *Nocardia* are able to convert various organic solvents (Hartmans *et al.*, 1990, Sikkema and De Bont, 1991) and in particular these bacteria show enhanced metabolism. Alternatively, stimulation might result from absorption of the organic solvent in the membrane which increases the membrane fluidity, which might facilitate higher enzyme activities (Gordon *et al.*, 1980, Osborne *et al.*, 1990). When the concentration of a particular solvent in the membrane, on the other hand, becomes too high and exceeds a critical concentration, the membrane may loose its integrity and protein complexes may disintegrate, resulting in loss of activity (Gordon *et al.*, 1980, Osborne *et al.*, 1990). Moreover, due to a decreased integrity, the membrane will loose its function as a selective barrier (Sikkema *et al.*, 1992).

The tolerance of a bacterium for organic solvents can be expressed as the $\log P_{octanol}$ value above which all solvents are non-toxic. Figure 1 shows no obvious differences in solvent tolerance between the Gram-positive and the Gram-negative bacteria. However, when the data are replotted separately for the homologous series of solvents, differences between Gram-positive and Gram-negative bacteria can be observed

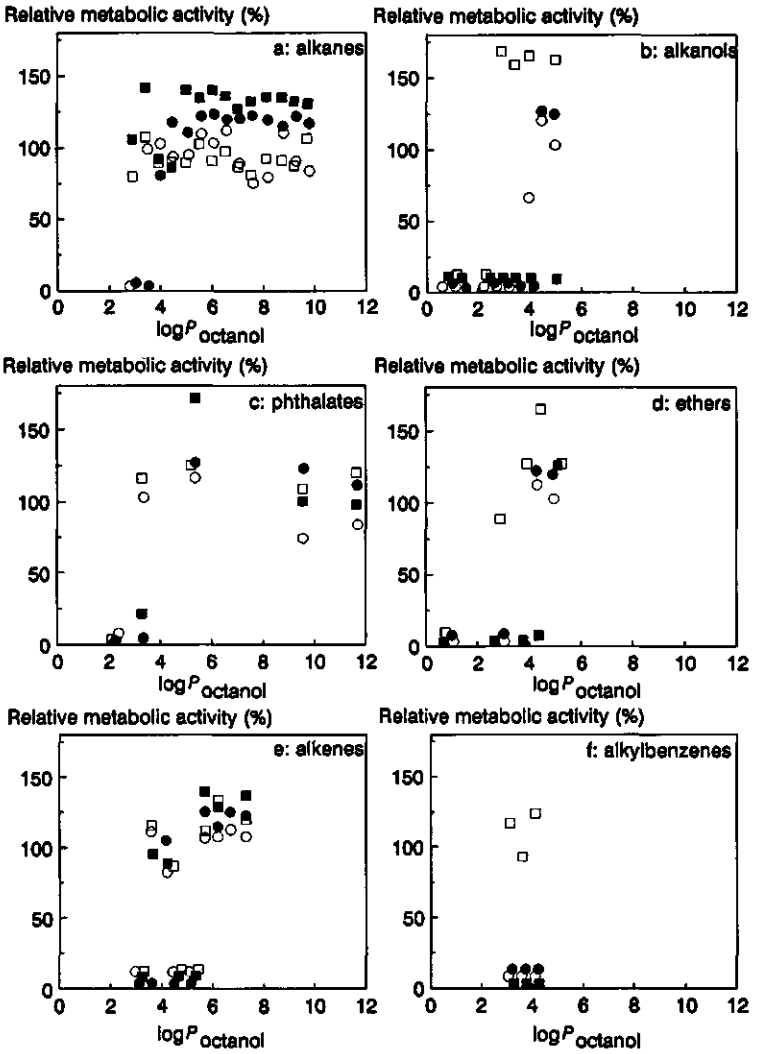


Figure 2 Relationship between relative metabolic activity of *Arthrobacter* (●), *Acinetobacter* (○), *Nocardia* (■) and *Pseudomonas* (□), exposed to 10% v/v organic solvent, and $\log P_{octanol}$, as measured separately for different homologous series of solvents. Open and closed symbols represent the Gram-negative and the Gram-positive bacteria, respectively.

(Figure 2).

The tolerance towards alkanols (Figure 2b) and phthalates (Figure 2c) is higher for the Gram-negative *Acinetobacter* and *Pseudomonas* since these withstand alkanols having $\log P_{octanol} > 3.5$ and phthalates having $\log P_{octanol} > 3$, while the Gram-positive *Arthrobacter* and *Nocardia* tolerate alkanols and phthalates having $\log P_{octanol} > 4$ respectively $\log P_{octanol} > 5$. The Gram-negative bacterium *Pseudomonas* is also more tolerant towards ethers (Figure 2d) and alkylbenzenes (Figure 2f) than the other organisms. For the alkanes (Figure 2a) only a small difference in tolerance between Gram-negative and Gram-positive bacteria was found. The Gram-positive bacterium *Arthrobacter* only tolerates alkanes having $\log P_{octanol} > 3.5$, while the others can tolerate alkanes having $\log P_{octanol} > 3$, moreover, *Nocardia* and *Pseudomonas* also tolerate hexane ($\log P_{octanol} = 3$). The unsaturated hydrocarbons do not show a sigmoidal relation between toxicity and $\log P_{octanol}$ (Figure 2e). Hexene, nonene and decene ($\log P_{octanol}$ 3.1, 4.7 and 5.2, respectively) are toxic to all organisms tested, while heptene ($\log P_{octanol}$ 3.6) is only toxic to *Arthrobacter*. It is conceivable that nonene and decene can be converted into toxic epoxides, since the tested organisms are known to possess oxygenating enzymes (Hartmans *et al.*,1990, Sikkema and De Bont, 1991). If so, one would expect the same toxic effect of heptene and octene, being similar substrates. A more plausible reason for the toxicity of nonene and decene is therefore that solvent contamination is responsible for the deterioration of the organisms. According to the manufacturer the solvents have a purity of 97% and 96% respectively (Table 1) and in the experiments the solvents have been used without further purification. Since purification techniques such as (vacuum-) distillation are known to cause chemical oxidation of these solvents and other purification techniques were not readily available, the toxicity of alkenes was not further investigated.

The toxicity of alkyl acetates has been determined for *Acinetobacter* and *Arthrobacter* and all alkyl acetates tested ($\log P$ value between 0.16 and 2.23), prove to be toxic to both organisms (data not shown).

In conclusion, the Gram-negative bacteria and especially *Pseudomonas* are in general more tolerant than the Gram-positive bacteria tested, for most of the homologous

series of solvents studied in this work, but for some homologous series the differences are very small. The difference in solvent tolerance between Gram-positive and Gram-negative bacteria is also observed by Inoue and Horikoshi (1991) who based their conclusion on tests with only 18 different solvents mainly consisting of aromatic ($\log P_{octanol} < 3.9$), and saturated aliphatic hydrocarbons ($\log P_{octanol} > 3.8$). The same conclusion is drawn by Harrop *et al.* (1989), although they do not mention the type of solvents used in their investigations and they compare two completely different types of metabolic activity, i.e. hydroxylation of naphtalene in *Pseudomonas putida* and steroid Δ^1-dehydrogenase in *Arthrobacter simplex*. They suggest that the difference in solvent tolerance is caused by the presence of the outer membrane in Gram-negative bacteria containing lipopoly-saccharide, which protects the cells against hydrophobic compounds. The higher susceptibility of *Nocardia* sp. may result from the hydrophobic nature of the cell wall of this baterium. Cell walls of *Nocardiaceae* consists partially from highly hydrophobic mycolic acids (Schlegel, 1986).

Figure 2 indicates that the toxicity of organic solvents is not determined by their $\log P_{octanol}$ alone; for alkanes, phthalates and ethers, transition of toxic to non-toxic solvent takes place between $\log P_{octanol}$ 3-4, while for alcohols and alkylbenzenes the transition between toxic and non-toxic solvents takes place at higher $\log P_{octanol}$ values. So, toxicity also depends on the molecular structure of the solvent used. This is also found, when using the data of Gill and Ratledge (1972) for the respiration rate of glucose-grown *Candida* sp. and *Saccharomyces carlsbergensis* in the presence of 50 ml l^{-1} emulsions of *n*-alkanes, *n*-alkenes, *n*-alkyl bromides and *n*-alkanols. When these data are plotted as function of the $\log P_{octanol}$ of the solvents, both yeasts showed an increase in the tolerance when the homologous series of solvents change from *n*-alkanes, via *n*-alkanols and *n*-alkenes to *n*-alkyl bromides (plots not shown). Boeren *et al.* (1987) have investigated the effect of several homologous series of solvents on the activity and viability of *Flavobacterium dehydrogenans* and found that introduction of hydrophilic substituents on or into the alkyl backbone of the organic solvent increased the viability of the cells compared to the corresponding alkanes. Hydrophobic substituents cause the opposite behaviour. If this were to hold also for the solvent toxicity of the organisms

tested here, the tolerance for solvents with hydrophilic substituents (alkyl acetates, alkanols and alkyl ethers) should be higher than the tolerance for alkanes, while the bacteria should show lower tolerance for solvents with relatively hydrophobic substituents (alkyl benzenes, phthalates and alkenes). Figure 2, however, does not reveal any such relation between the hydrophobicity of the substituent (expressed by its hydrophobic fragmental constant (Rekker and De Kort, 1979)) and the location of the transition area.

Since all results on the solvent tolerance of cells have been obtained in gently agitated two-liquid-phase systems, including the data cited from literature, the difference in solvent tolerance of the tested Gram-positive and Gram-negative bacteria can be caused by differences in response to molecular as well as phase-toxicity effects.

Metabolic activity in one-phase systems

The molecular toxicity was investigated separately, using subsaturating amounts of organic solvent (alkanols). Table 2 shows the amount of CO_2 produced by *Arthrobacter* after 1 and 7 d of growth on 5 g l^{-1} di-sodium succinate. The results for the corresponding two-phase system are shown for comparative purposes. In this table and in Figures 3 and 4, the solvent concentrations are given as fractions of the maximum aqueous concentration. This parameter represents the total amount of solvent (mol) divided by the amount of solvent (mol) required to saturate the aqueous phase. At a value below 1, the aqueous phase is subsaturated; at values exceeding 1, phase separation occurs.

Table 2 indicates that after one day no CO_2 production is found in a two-liquid-phase system for all alkanols tested. In alkanol-saturated single-phase systems, alkanol tolerance is increased compared to two-liquid-phase systems: CO_2 production takes place in the presence of subsaturating amounts of alkanols with $\log P_{octanol} > 2.9$ (1-octanol), but it is not observed in a two-liquid-phase system with these alkanols. The latter toxic effect should be ascribed to phase toxicity. The reduction in the carbon dioxide concentration of the head-space is not caused by the presence of organic solvent, since the partition of carbon dioxide between the gaseous and liquid phase is not significantly influenced by the presence of organic solvent (data not shown).

Table 2 Carbon dioxide production by *Arthrobacter* T2 after 1 and after 7 days of incubation in growth medium containing 5 g l^{-1} di-sodium succinate and various alkanols in different concentrations.

1 day of incubation					
organic solvent	0.25	0.50	0.75	1	2-phases
1-butanol	-	-	-	-	-
1-pentanol	-	-	-	-	-
1-hexanol	+	+/-	-	-	-
1-heptanol	+	-	-	-	-
1-octanol	+++	+	+/-	+/-	-
1-nonanol	+	+	+	+	-
1-decanol	+++	+++	NA	NA	-
1-undecanol	+++	+++	+++	+++	-
7 days of incubation					
organic solvent	0.25	0.50	0.75	1	2-phases
1-butanol	-	-	-	-	-
1-pentanol	+/-	-	-	-	-
1-hexanol	+++	-	-	-	-
1-heptanol	+	+	+	+	-
1-octanol	+++	+++	+++	+++	-
1-nonanol	+++	++	++	++	-
1-decanol	+++	+++	+++	+++	+/-
1-undecanol	++	+++	+++	+++	+/-

In the case of subsaturation the amount of solvent is given as fractions of the maximum aqueous concentration. In a two-liquid-phase system 100 ml l^{-1} of organic solvent was added.

NA:	not available
- :	< 10% of the carbon dioxide production in medium without solvent
+/-:	10% - 40% of the carbon dioxide production in medium without solvent
+:	40% - 70% of the carbon dioxide production in medium without solvent
++:	70% - 100% of the carbon dioxide production in medium without solvent
+++:	> 100% of the carbon dioxide production in medium without solvent.

The toxicity of a solvent not only depends on the presence of a second phase but also on the concentration of the solvent in the aqueous phase. For example, hexanol proves to be toxic at 0.75 fraction of saturation; at 0.5 saturation some CO_2-production is found, while a further decrease in hexanol concentration results in a higher retention of metabolic activity. However, even in 0.25 saturated alkanol solutions, no metabolic activity is found after one day when butanol and pentanol are used as solvent. After

7 days the bacteria show some CO_2 production at 0.25 saturation with pentanol. These results show that the higher the $\log P_{octanol}$ of the alkanol, the higher the degree of saturation of the alkanol in the aqueous phase that can be used without inhibiting the metabolic activity. One has to take into account that the solubility of the solvents in the aqueous phase decreases with increasing $\log P_{octanol}$ (Table 1). Therefore the total amount of solvent added to reach saturation, varies for the alkanols tested. For example, the solubility of 1-butanol in water is 1005 mmol l^{-1}, whereas the solubility of 1-octanol is only 4.1 mmol l^{-1}. We will later discuss the implication of these solubility differences.

Instantaneous effects on oxygen-consumption rate

In the experiment mentioned above, the accumulation of carbon dioxide with time is used as a measure of the metabolic activity of the cells. Insight into the effects of organic solvent on the initial activity of the bacteria cannot be obtained by this method. It is possible that after a few days the bacteria have adapted to the different circumstances, e.g., by changing the fatty acid composition of their phospholipid membrane, as observed with several other microorganisms (Ingram, 1976, Thomas *et al.*, 1978, MacDonald and Goldfine, 1991) or by changing their entire metabolism. As a result of the latter effect, the bacteria in the flasks without toxic solvent added, may utilize the available substrate much faster than the bacteria in flasks containing inhibiting amounts of solvent. However, when all the substrate has been used, the accumulation of carbon dioxide is eventually the same in both cases. For that reason, we investigated the effect of organic solvents on the initial oxygen-consumption rate of *Arthrobacter* and *Acinetobacter* using homologous series of alkyl ethers, alkanols and alkyl acetates. It was not possible to test the toxicity of homologous series of alkanes, phthalates and alkyl benzenes, since these solvents interfered with the measurement of the oxygen-consumption rate.

Effects of alkyl ethers

Figure 3a and 3b show the response of *Arthrobacter* and *Acinetobacter* upon addition of different amounts of alkyl ethers. From these figures, it can be concluded that

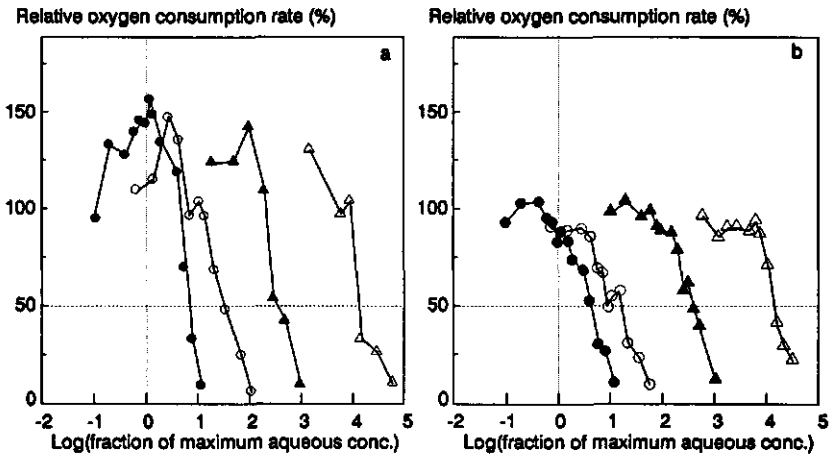


Figure 3 Relationship between relative oxygen-consumption rate of a) *Arthrobacter* and b) *Acinetobacter* and the amount of alkyl ether added. The solvents are: propyl ether, $\log P_{octanol}$ 1.9 (●); butyl ether, $\log P_{octanol}$ 2.9 (○); pentyl ether, $\log P_{octanol}$ 3.9 (▲); and hexyl ether, $\log P_{octanol}$ 5.0 (△).

the concentration of solvent at which 50% loss of activity is observed, occurs at concentrations well above the saturating concentration (i.e. log(multiple of maximum aqueous conc.) > 0) of the alkyl ethers in the aqueous phase. The data for the aqueous solubility of ethyl ether, propyl ether, butyl ether and hexyl ether have been obtained from Riddick *et al.* (1986) and that of pentyl ether by intrapolation from a plot of the $\log P_{octanol}$ and the logarithm of the solubility in water of alkyl ethers with known solubility, acccording to Verschueren (1983).

Figure 3 shows that the toxicity of the alkyl ethers depends on the chain length of the alkyl ether. When the chain length increases, relatively more alkyl ether can be added before toxicity effects are observed. Since subsaturating concentrations of the tested alkyl ethers do not show toxic effects, only phase toxicity plays a role. However, it is not clear, whether this is caused by adsorption of the cells to the interface or by the extraction of essential compounds from the bacterial cell envelope into the organic phase, since both the total interfacial area and the total amount of organic solvent increase upon increasing the amount of organic solvent. When the stirrer speed in the measuring vessel

is increased, however, no effect of the increased interfacial area is observed. This suggests that the phase toxicity is primarily caused by extraction of essential compounds from the cell envelope.

Upon addition of small amounts of alkyl ether, *Arthrobacter* shows an increase in the oxygen-consumption rate (Figure 3a). By further increasing the amount of alkyl ether the oxygen-consumption rate decreases. *Acinetobacter* does not show this effect; upon addition of alkyl ether, the oxygen-consumption rate remains about constant and further increase of the amount of alkyl ether results in deactivation of the cells (Figure 3b). It is possible that effects on the membrane fluidity and therefore the activity of membrane bound enzymes, again play a role, but since the increase in initial oxygen-consumption rate is not observed with *Acinetobacter*, it is more likely that other mechanisms are responsible for the increased activity of *Arthrobacter*. Maybe constitutive enzymes for biodegradation of small concentrations of alkyl ethers are available in the latter bacterium and not in *Acinetobacter*. *Arthrobacter* is known to be a far more versatile bacterium, with regard to potential growth substrates or respiratory control mechanisms, which activate primary energy transducing enzymes in order to compensate for dissipation of proton motive force (Sikkema and De Bont, 1991, Sikkema *et al.*, 1992).

Although there is a difference in response to low concentrations of alkyl ethers, there is no significant difference between the toxic dose for the Gram-positive and the Gram-negative bacterium. The toxic dose is defined here as the amount of solvent at which 50% of the initial oxygen consumption is lost. This parameter is comparable to the LD_{50} (dose at which 50% of a tested population will not survive), which is often used to define toxicity (Verschueren, 1983). A Gompertz equation (Zwietering *et al.*, 1990), describing sigmoidal curves, was fitted to the data in Figure 3 and used to calculate the toxic dose of a solvent (Table 3). The toxic dose for alkyl ethers is almost the same for *Arthrobacter* and for *Acinetobacter*. The Gram-positive cells are slightly more tolerant to propyl and butyl ether than the Gram-negative cells but the differences are not really significant. Therefore, our general conclusion about the higher solvent tolerance of Gram-negative bacteria (Figure 2) does not hold for all the types of solvents

tested, since *Acinetobacter* and *Arthrobacter* showed about similar tolerance towards alkyl ethers.

Effects of alkyl acetates

In the homologous series of alkyl acetates, subsaturating as well as over-saturating concentrations of the alkyl acetates (log(fraction of maximum aqueous conc.) < 0, respectively > 0) have been used. Figure 4a and 4b show the toxicity effects of alkyl acetates on the initial oxygen-consumption rate of *Arthrobacter* and *Acinetobacter*, respectively. Molecular toxicity is observed, when using methyl, ethyl and propyl acetate and a combined effect of molecular and phase toxicity, when using butyl acetate. Pentyl acetate proved to be toxic only at concentrations above the saturation level.

The alkyl acetates, except methyl acetate, proved to be toxic for *Arthrobacter* at slightly higher overall concentrations than for *Acinetobacter* (Table 3). This indicates that

Table 3 Toxicity of solvents towards *Arthrobacter* en *Acinetobacter*.

	Concentration of solvent at which the initial oxygen consumption rate is reduced to 50% (fraction of maximum aqueous concentration)	
Solvent	*Arthrobacter*	*Acinetobacter*
propyl ether	6.63	4.80
butyl ether	35.3	14.7
pentyl ether	458	451
hexyl ether	14937	14761
methyl acetate	0.31	0.35
ethyl acetate	0.53	0.43
propyl acetate	1.09	0.68
butyl acetate	1.49	0.82
pentyl acetate	144	36.9
butanol	0.28	0.32
pentanol	0.40	0.41
hexanol	0.46	0.49
heptanol	0.67	0.76
octanol	0.64	0.82
nonanol	1.05	0.87

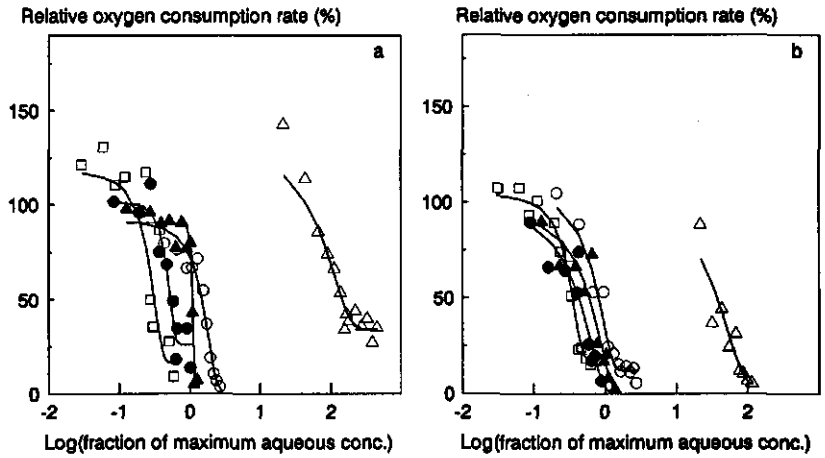


Figure 4 Relationship between relative oxygen consumption rate of a) *Arthrobacter* and b) *Acinetobacter* and the amount of alkyl acetate added. The solvents are: methyl acetate, $\log P_{octanol}$ 0.16 (□); ethyl acetate, $\log P_{octanol}$ 0.64 (●); propyl acetate, $\log P_{octanol}$ 1.17 (▲); butyl acetate, $\log P_{octanol}$ 1.7 (○); and pentyl acetate, $\log P_{octanol}$ 2.23 (Δ). The lines through the data points were fitted with the Gompertz equation (Zwietering *et al.*, 1990.

the Gram-positive bacterium is slightly more tolerant towards alkyl acetates than the Gram-negative bacterium. Again this indicates, that the general conclusion from Figure 2, Gram-negative bacteria are more tolerant towards organic solvents than Gram-positive bacteria, does not hold for all homologous solvent series. In Figure 2, however, the combined effect of molecular toxicity and phase toxicity was revealed, while these results indicate that only butyl acetate shows this combined effect, and the others only molecular toxicity. Since the time of measurements was relatively short, possible adaptation effects were also excluded when investigating the ether tolerance of Gram-positive and Gram-negative bacteria in Figure 4.

Effects of alkanols

Figure 5a and 5b show the response of cells of *Arthrobacter* and *Acinetobacter* on addition of different amounts of alkanols. From these figures it can be concluded that

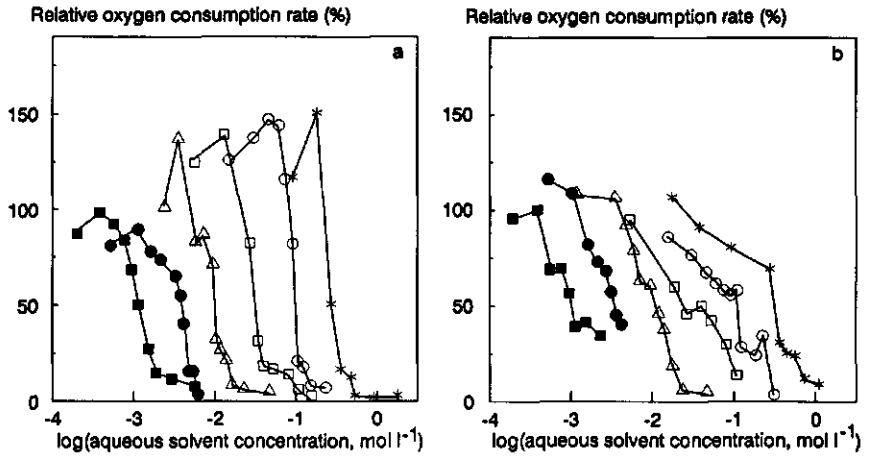


Figure 5 Relationship between relative oxygen consumption rate of a) *Arthrobacter* and b) *Acinetobacter* and the amount of alkanol added. The alkanols are: butanol, $\log P_{octanol}$ 0.8 (*); pentanol, $\log P_{octanol}$ 1.3 (○); hexanol, $\log P_{octanol}$ 1.8 (□); heptanol, $\log P_{octanol}$ 2.4 (Δ); octanol, $\log P_{octanol}$ 2.9 (●); and nonanol, $\log P_{octanol}$ 3.4 (■).

the toxic dose of the alkanols strongly depends on the chain length of the alkanol. The longer the chain length, the smaller the amount of alkanol which has to be added to the aqueous phase in order to cause deteriorating effects. Apparently, this is in contrast with the results shown in Table 2, where the metabolic activity was found to increase with increasing $\log P_{octanol}$ values of the solvent. In Table 2, however, the concentrations in the aqueous phase differ significantly due to variation in solubility of the solvents used. When the solvent concentrations in this table are transformed from the percentage of the degree of saturation into the aqueous solvent concentration, a similar pattern for the solvent toxicity can be observed (data not shown).

At very low alkanol concentrations, *Arthrobacter* shows a similar increase in oxygen-consumption rate as observed with alkyl ethers; at higher concentrations, the oxygen-consumption rate drops. Again it is unlikely that membrane-fluidity effects played a role, since *Acinetobacter* does not show this increased activity.

For both organisms, the toxic doses of the tested alkanols are in the same range

of subsaturating concentrations (Table 3). This indicates on the one hand, that only molecular toxicity of alkanols plays a role and on the other hand that both organisms are equally tolerant to alkanols.

Calculation of the critical membrane concentration

Another way of dealing with the difference in molecular toxicity of solvents between *Arthrobacter* and *Acinetobacter* is by calculating the critical membrane concentrations according to Osborne *et al.* (1990). This method provides a more accurate means to define the $\log P_{octanol}$ at which toxicity is observed. The critical membrane concentration was defined by Osborne *et al.* as the concentration of solvent in the cell membrane that causes complete loss of activity. Here, the concentration at which the relative oxygen-consumption rate was 50%, was taken to define the critical membrane concentration.

For a given solvent, the partition coefficient between the membrane and the aqueous phase ($P_{membrane}$) is related to $P_{octanol}$ via

$$P_{membrane} = R * P_{octanol} \qquad (1)$$

where R is the partition coefficient of the solvent over a hypothetical membrane/octanol system. If the interactions of solvents from a homologous series with the membrane were to be similar, R would be constant for all the solvents in this series. Since most solvent series do not fulfill this requirement, a correction is needed, yielding a Collander type of relation (Collander, 1951)

$$\frac{[\text{solvent}]_{membrane}}{[\text{solvent}]_{aq}} = P_{membrane} = R * P_{octanol}^{Y} \qquad (2)$$

in which Y accounts for the deviation between the solvent-membrane interactions and the solvent-octanol interactions within a homologous series of organic solvents.

Rewriting this equation results in:

$$\log[\text{solvent}]_{aq} = \log\frac{[\text{solvent}]_{membrane}}{R} - Y * \log P_{octanol} \qquad (3)$$

A plot of the logarithm of the experimentally determined critical solvent concentrations in the aqueous phase (= toxic dose (Table 3)) versus $\log P_{octanol}$ results in a straight line as shown in Figure 6 for the toxicity of alkanols for *Arthrobacter* and *Acinetobacter*. The slope of the line represents Y and the intercept of the line yields the logarithm of the critical membrane concentration divided by R. If R represents a partition coefficient, as shown in equation 1, it is very likely that this partition coefficient changes with the homologous series used.

After subjecting our data points to a Student t test, using a 95% confidence interval, the values of the intercept $[\text{solvent}]_{membrane,cr}/R$ and the slope Y for both *Arthrobacter* and *Acinetobacter* were found to be identical. This indicates that both types of membrane show similar interactions with the alkanols used.

The toxic dose of methyl and ethyl acetate for *Arthrobacter* and of methyl, ethyl,

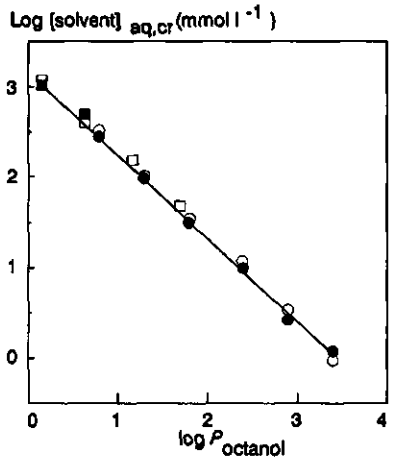


Figure 6 Relationship between the logarithm of the aqueous solvent concentration at which 50% of the initial oxygen consumption rate of *Arthrobacter* (closed symbols) and *Acinetobacter* (open symbols) is inhibited, and the $\log P_{octanol}$ of the solvents. The circles refer to alkanols, the squares to alkyl acetates.

propyl and butyl acetate for *Acinetobacter* are also plotted against $\log P_{octanol}$ (Figure 6). This is only possible for these alkyl acetates, since they are toxic at concentrations below the maximum solubility. Although the number of alkyl acetates which are toxic at molecular level is limited, it can be observed that the molecular toxicity is the same for *Arthrobacter* and *Acinetobacter*.

When the lines through the data for the alkyl acetates and the alkanols are compared, no statistically significant difference in $[solvent]_{membrane,cr}/R$ and Y values can be found. This is quite surprising, since the $[solvent]_{membrane,cr}$ is not expected to be constant for all series of solvents (Collander, 1951, Osborne *et al.*, 1990). Our results show, however, that $[solvent]_{membrane,cr}/R$ is constant for both alkanols and acetates and if indeed the $[solvent]_{membrane,cr}$ changes, R thus has to change in an identical manner.

In a recent paper Osborne *et al.* (1992) indicate that equation 3 should be applied with caution. If $P_{membrane}$ becomes too high, it will be necessary to correct the actual aqueous solvent concentration for the solvent that partitioned from the aqueous phase into the membrane. The partitioning of lipophilic compounds to the membrane lipid bilayer will significantly affect the actual aqueous concentration. In our case, the concentration of membrane lipids in the system was high (ca. 1.2 mg cm^{-3}) and a significant effect on the aqueous concentration of especially highly apolar solvents can be envisaged. For example, the $P_{membrane}$ for a typical apolar compound, like tetralin ($\log P_{octanol}$ = 3.9) in a lipid/buffer system proved to be 1100 on a weight basis (Sikkema *et al.*, 1992). By using these data, it can be calculated that at sub-saturating concentrations most of the tetralin will accumulate in the membrane.

When $\log P_{octanol}$ of a solvent is known, equation 3 may help to find the critical solvent concentration in the aqueous phase, now assuming that $[solvent]_{membrane,cr}/R$ is constant. Moreover, $[solvent]_{membrane,cr}/R$ probably consists of two dependent variables and not of two constants, as suggested by Seeman (1972) and Osborne *et al.* (1990). If this critical aqueous concentration is lower than the aqueous solubility of the solvent, the solvent will show molecular toxicity. The values of $[solvent]_{membrane,cr}/R$ and Y are 3.16 and 0.92, respectively, as calculated from Figure 6. The solvents, for which the aqueous solubility data are available from literature, and which will show molecular toxicity for

Arthrobacter and *Acinetobacter*, have been indicated in Table 1. All the alkanes, the phthalates except dioctyl phthalate, the alkenes except nonene, and the aromatic hydrocarbons except toluene will thus not show molecular toxicity. The alkanols up to nonanol, the alkyl ethers as well as the acetates, except pentyl acetate, on the other hand will show molecular-toxicity effects. This corresponds well with the instantaneous effects of acetates and alkanols on the oxygen-consumption rate of the cells as observed in Figures 4 and 5 and Table 3, but for alkyl ethers only phase toxicity has been observed (Figure 3, Table 3). Although the method to predict molecular toxicity looks promising, it is clear from these results, that it is not always possible to apply the critical membrane concentration theory for predictive purposes.

CONCLUSIONS

Log$P_{octanol}$ is a good measure for the phase toxicity of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas*. Solvents having a log$P_{octanol}$ below 3 are toxic in a concentration of 100 ml l^{-1}, while solvents having a log$P_{octanol}$ exceeding 5 are not. The transition between toxic and non-toxic solvents depends on the type of solvent used, but no correlation has been found between the hydrophobicity of the substituent on the alkyl backbone of the solvent (indicated by its log$P_{octanol}$ value) and the location of the transition point in toxicity As suggested in the literature (Boeren *et al.*, 1987). In general, the tolerance of the Gram-negative bacteria towards phase toxicity is slightly higher than the tolerance of the Gram-positive bacteria tested, although for some solvents the difference is very small. The tolerance for alkyl ethers is similar and for acetates better for the Gram-positive bacteria.

Solvent toxicity not only depends on the log$P_{octanol}$ of the solvent, but also on the concentration of the solvent used during the toxicity test. The tolerance towards molecular toxicity of alkanols and alkyl acetates is equal for the Gram-positive bacterium *Arthrobacter* and the Gram-negative bacterium *Acinetobacter*. The critical concentration of solvent in the aqueous phase shows a linear relation with the log$P_{octanol}$ of the solvents

in a homologous series. The critical solvent concentration in the membrane divided by a factor R is constant, and equal for *Arthrobacter* and *Acinetobacter*. Prediction of the toxicity of any given solvent is possible to a certain extent, but more data on the molecular basis of the toxicity will be needed to improve its reliability.

ACKNOWLEDGEMENTS

The authors wish to thank Rik Beeftink for critical reading of the manuscript. Part of this work was funded by the Dutch Programme Committee on Industrial Biotechnology (PCIB).

REFERENCES

Bar, R. (1988) Effect of interphase mixing on a water-organic solvent two-liquid phase microbial system: ethanol fermentation. *J. Chem. Technol. Biotechnol.* **43**: 49-62.

Bassetti, L., Tramper, J. (1992) Effect of organic solvents on growth and anthraquinone production in *Morinda citrifolia* cell cultures. In: *Biocatalysis in Non-Conventional Media*, 617-622. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Boeren, S., Laane, C., Hilhorst, R. (1987) Viability and activity of *Flavobacterium dehydrogenans* in organic solvent/culture two-liquid-phase-systems. In: *Biocatalysis in Organic Media*, 303-310. Laane, C., Tramper, J., Lilly, M.D. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Bruce, L.J., Daugulis, A.J. (1991) Solvent selection strategies for extractive biocatalysis. *Biotechnol. Prog.* **7**: 116-124.

Buitelaar, R.M., Vermuë, M.H., Schlatmann, J.E., Tramper, J. (1990) The influence of various organic solvents on the respiration of free and immobilized cells of *Tagetes minuta*. *Biotechnol. Techn.* **4**: 415-418.

Collander, R. (1951) The partition of organic compounds between higher alcohols and water. *Acta Chem. Scand.* **5**: 774-780.

Gill, C.O., Ratledge, C. (1972) Toxicity of *n*-alkanes, *n*-alk-1-enes, *n*-alkan-1-ols and *n*-allyl-1-bromides towards yeasts. *J. Gen. Microbiol.* **72**: 165-172.

Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J., Houslay, M.D. (1980) The increase in bilayer fluidity of rat liver plasma membranes achieved by the local anesthetic benzyl alcohol affects the activity of intrinsic membrane enzymes. *J. Biol. Chem.* **235**: 4519-4527.

Harrop, A.J., Hocknull, M.D., Lilly, M.D. (1989) Biotransformations in organic solvents: a difference between Gram-positive and Gram-negative bacteria. *Biotechnol. Letters* **11**: 807-810.

Hartmans, S., Van der Werf M.J., De Bont, J.A.M. (1990) Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. *Appl. Environ. Microbiol.* **56**: 1347-1351.

Hocknull, M.D., Lilly, M.D. (1990) The use of free and immobilized *Arthrobacter simplex* in organic solvent/aqueous two-liquid-phase reactors. *Appl. Microbiol. Biotechnol.* **33**: 148-153.

Ingram, L.O. (1976) Adaptation of membrane lipids to alcohols. *J. Bacteriol.* **125**: 670-678.

Inoue, A., Horikoshi, K. (1991) Estimation of solvent-tolerance of bacteria by the solvent parameter log*P*. *J. Ferment. Bioeng.* **71**: 194-196.

Laane, C., Tramper, J., Lilly, M.D. (Ed.) (1987a) *Biocatalysis in Organic Media*. Elsevier Science Publ., Amsterdam, The Netherlands.

Laane, C., Boeren, S., Vos, K., Veeger, C. (1987b) Rules for optimization of biocatalysis in organic solvents. *Biotechnol. Bioeng.* **30**: 81-87.

MacDonald, D.L., Goldfine, H. (1991) Effects of solvents and alcohols on the polar lipid composition of *Clostridium butyricum* under conditions of controlled lipid chain composition. *Appl. Environ. Microbiol.* **57**: 3517-3521.

Osborne, S.J., Leaver, J., Turner, M.K., Dunnill, P. (1990) Correlation of biocatalytic activity in an organic-aqueous two-liquid phase system with solvent concentration in the cell membrane. *Enzyme Microb. Technol.* **12**: 281-291.

Osborne, S.J., Leaver, J., Turner, M.K. (1992) Membrane concentrations of primary alcohols which inhibit progesterone 11α-hydroxylase in *Rhizopus nigricans*. In: *Biocatalysis in Non-Conventional Media*, 31-36. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Rekker, R.F., De Kort, H.M. (1979) The hydrophobic fragmental constant; an extension to a 1000 data point set. *Eur. J. Med. Chem.* **14**: 479-488.

Reslow, M. (1989) *Enzymes as catalysts in organic media*. Ph.D. Thesis, University of Lund, Lund, Sweden.

Reslow, M., Adlercreutz, P., Mattiasson, B. (1987) Organic solvents for bioorganic synthesis 1. Optimization of parameters for a chymotrypsin catalyzed process. *Appl. Microbiol. Biotechnol.* **26**: 1-8.

Riddick, J.A., Bunger, W.B., Sakano, T. (1986) Physical properties and methods of purification. In: *Techniques of Chemistry, vol II, Organic Solvents*, 4th ed. Weissberger A. (Ed.) John Wiley & Sons, Inc., New York.

Schlegel, H.G. (1986). *General microbiology*. 6th ed. Cambridge Univ. Press, 93.

Seeman, P. (1972) The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**: 583-655.

Sikkema, J., De Bont, J.A.M. (1991) Isolation and initial characterization of bacteria growing on tetralin. *Biodegradation* **2**: 15-23.

Sikkema, J., Poolman, B., Konings, W.N., De Bont, J.A.M. (1992) Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. *J. Bacteriol.* **174**: 2986-2992.

Thomas, D.S., Hossack, J.A., Rose, A.H. (1978) Plasma-membrane lipid composition and ethanol tolerance in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **117**: 239-245.

Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) (1992) *Biocatalysis in Non-Conventional Media*. Elsevier Science Publ., Amsterdam, The Netherlands.

Uribe, S., Ramirez, J., Peña, A. (1985) Effects of ß-pinene on yeast membrane functions. *J. Bacteriol.* **161**: 1195-1200.

Uribe, S., Rangel, P., Espínola, G., Aguirre, G. (1990) Effects of cyclohexane, an industrial solvent, on the yeast *Saccharomyces cerevisiae* and on isolated yeast mitochondria. *Appl. Environ. Microbiol.* **56**: 2114-2119.

Van den Tweel, W.J.J., Marsman, E.H., Vorage, M.J.A.W., Tramper, J., De Bont, J.A.M. (1988) Continuous production of *cis*-1,2-dihydroxycyclohexa-3,5-diene (*cis*-benzeneglycol) from benzene by a mutant of a benzene-degrading *Pseudomonas* sp.. In: *Bioreactors and Biotransformations*, 231-241. Moody, G.W., Bater, P.B., (Ed.) Elsevier Applied Science Publ., London, Great Britain.

Vermuë, M.H., Tramper, J. (1990) Extractive biocatalysis in a liquid-impelled loop reactor. In: *Proceedings of the 5th European Congress on Biotechnology*, 243-246. Cristiansen, C., Munck L., Villadsen, J., (Ed.) Munksgaard International Publisher, Copenhagen, Denmark.

Verschueren, K. (1983) *Handbook of Environmental Data on Organic Chemicals*, 2nd ed., Van Nostrand Reinhold Comp. Inc., New York.

Vishniac, W., Santer, M. (1957) The Thiobacilli. *Bacteriol. Rev.* **21**: 195-213.

Zaks, A., Klibanov, A.M. (1988) The effect of water on enzyme action in organic media. *J. Biol. Chem.* **263**: 3194-3201.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., Van 't Riet, K. (1990). Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **56**: 1875-1881.

ENZYMIC TRANSESTERIFICATION IN NEAR-CRITICAL CARBON DIOXIDE; EFFECT OF PRESSURE, HILDEBRAND SOLUBILITY PARAMETER AND WATER CONTENT

SUMMARY

The transesterification of nonanol and ethyl acetate into nonyl acetate and ethanol by *Mucor miehei* lipase was studied in near-critical carbon dioxide. Before studying the enzymic reaction, the homogeneity of the reaction medium was evaluated to make sure that the reaction was executed in homogeneous near-critical carbon dioxide. Estimations of the solubilities of the substrates were made using the difference in Hildebrand solubility parameter between the carbon dioxide and both substrates. A difference smaller than 10 $(MPa)^{0.5}$, which is needed for solubilization of apolar compounds in supercritical fluids, also holds for the compounds in our reaction system. The effects of pressure, polarity and water content of the medium on the enzymic reaction have been studied in a continuous stirred-tank reactor. The pressure and polarity of the near-critical carbon dioxide as expressed by the Hildebrand solubility parameter hardly influenced the transesterification rate of the lipase. By increasing the water content in the system from 0.5 to 2 ml l^{-1} the product formation decreased. The transesterification rate in near-critical carbon dioxide proved to be much lower than in hexane at comparable conditions of temperature, water content, and substrate and enzyme concentration.

Enzymic transesterification in near-critical carbon dioxide:
Effect of pressure, Hildebrand solubility parameter and water content.
Vermuë, M.H., Tramper, J., De Jong, J.P.J., Oostrom, W.H.M. (1992)
Enzyme Microb. Technol. **14**: 649-655.

Chapter 6

INTRODUCTION

Supercritical fluids are currently receiving much attention as possible media for enzymic reactions (Hammond *et al.*, 1985, Min Chi *et al.*, 1988, Pasta *et al.*, 1989, Randolph *et al.*, 1988, Van Eijs *et al.*, 1988, Sleytler *et al.*, 1991). The low viscosity and high diffusivity compared to liquid solvents and the low surface tension, favour efficient mass transfer. Another advantage is the ability to control the solubility of solutes by regulating both temperature and pressure (McHugh and Krukonis, 1986). Carbon dioxide is often used as solvent because of its non-toxicity for human beings and its moderate critical temperature of 304.2 K. However, its solubility of apolar compounds is limited in comparison to conventional organic media (De Swaan Arons *et al.*, 1988). For that reason supercritical processing is often executed in the vicinity of the critical point, the near-critical area at temperatures just above the critical temerature and at pressures well above the critical pressure. The medium there still exhibits gas-like transport properties of viscosity and diffusivity, while the density of the medium approaches the density of conventional organic solvents and the solute/solvent interactions increase, resulting in higher solubilities (MacHugh and Krukonis, 1986).

The solubility of solutes in a solvent can be estimated by the Hildebrand solubility parameter (δ) of the solvent (*Handbook of Chemistry and Physics*). This polarity indicator is also defined for supercritical solvents (Allada, 1984, see Appendix) and here we use it as a first estimate of the solubility of the solutes involved in the trans-esterification of ethyl acetate and nonanol with the enzyme lipase from *Mucor miehei* in near-critical carbon dioxide.

In conventional organic solvents the activity of biocatalysts is influenced by the polarity of the solvent used (Brink and Tramper, 1986, Laane *et al.*, 1987, Reslow, 1989). Brink *et al.* (1986) found a correlation between the activity of epoxidizing cells and the polarity of conventional organic solvents used (expressed by their Hildebrand solubility parameter value) in combination with their molecular weight. The more apolar

solvents ($\delta < \sim 16$ $(MPa)^{0.5}$) with a molecular weight $M_s > \sim 200$ had little effect on the activity of the biocatalysts. Laane *et al.* (1987) replotted the data of Brink *et al.* and found a more profound correlation for the activity retention of the epoxidizing cells and the polarity of the organic solvent, using its $\log P_{octanol}$ value. $\text{Log}P_{octanol}$ is defined as the logarithm of the partition coefficient of the organic solvent in a standard octanol/water two-phase system. Biocatalysts in solvents having a $\log P_{octanol} < 2$ generally show little activity retention. They show unpredictable activity retention in solvents having a $\log P_{octanol}$ between 2 and 4 and high activity retention in organic solvents having a $\log P_{octanol} > 4$. This correlation also holds for enzymes such as chymotrypsin, *Mucor* sp. lipase, and *Candida cylindracea* lipase (Reslow, 1989).

Apart from the polarity, the water content of the medium is often reported as a main parameter influencing the enzyme activity in conventional organic solvents (Hahn-Hägerdal, 1986, Halling, 1987, Zaks and Klibanov, 1988, Reslow, 1989) and in supercritical solvents (Min Chi *et al.*, 1988, Van Eijs *et al.*, 1988). In both solvent systems, a small amount of water is required for hydration of the enzyme involved in order to stabilize its active conformation.

Reduction of the amount of water can shift the equilibrium of reactions catalyzed by hydrolytic enzymes towards synthesis, and it can prevent the hydrolysis of esters in a transesterification reaction.

The aim of this study was to examine the influence of the water content and the polarity of near-critical carbon dioxide on the transesterification of nonanol and ethyl acetate catalyzed by *Mucor miehei* lipase. As a polarity and thus solubility indicator for near-critical carbon dioxide, the $\log P_{octanol}$ value could have been used, but determination of this parameter proved to be too problematic. For this reason the Hildebrand solubility parameter (δ) was chosen as a polarity indicator of near-critical carbon dioxide.

MATERIALS AND METHODS

Materials

Lipozyme™, a lipase (E.C.3.1.1.3) from *Mucor miehei*, supported on macroporous anionic resin beads, was obtained from NOVO Industry, Denmark, and had a quoted activity of 25 BIU g^{-1}. One Batch-Interesterification Unit (1 BIU) is defined as 1 μmol of incorporated palmitic acid into triolein per minute at standard conditions (NOVO). 1-Nonanol (Roth) and ethyl acetate (Merck) were p.a. grade. Carbon dioxide (purity >99.9%) was obtained from AGA Gas BV, Amsterdam, The Netherlands. Heptane (Baker) and hexane (Merck) were of purity >99%.

Solubility experiments

One gram of a mixture of ethyl acetate and nonanol (2:1 mol/mol) was placed in the 80 ml reactor, which was equipped with a magnetic stirrer and surrounded by a water jacket set at a temperature of 333 K. A schematic diagram of the reactor set-up is given in Figure 1. The reactor was pressurized with carbon dioxide to a set-point pressure, monitored by a Meyvis 802-C pressure module (P). The amount of carbon dioxide leaving the carbon dioxidecylinder was measured using a Micro-Motion flow meter (RFT 9729 remote-flow transmitter).

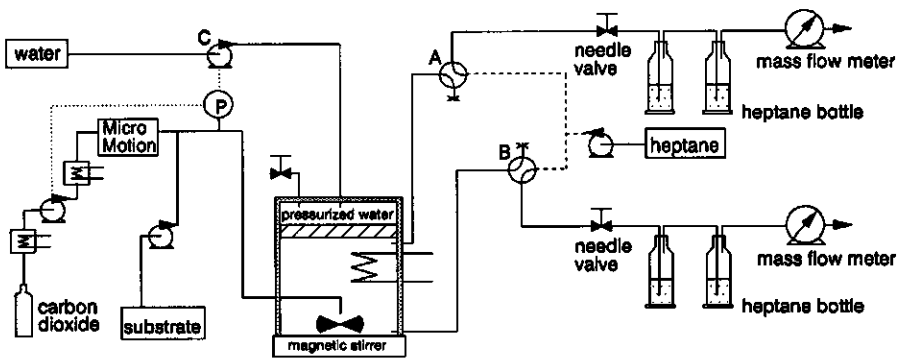


Figure 1 Reactor setup for the solubility experiments in near-critical carbon dioxide. Both sampling valves A and B are drawn in closed position. For further explanation, see text.

After 30 minutes, mixing was stopped, and by opening of the two-position gas-sampling valve (A), a sample of 1 l was taken at atmospheric pressure from the top part of the reactor. Decompression was achieved with a needle valve (Whitey, SS-21SR2).

By closing valve (A) and opening valve (B), a similar sample was taken from the bottom part of the reactor. The decompressed carbon-dioxide/substrate sample was led through two bottles in series, each containing 30 ml heptane. For trapping all nonanol from the sample, one bottle of heptane would have been sufficient. A second bottle was added because about 100 ml l^{-1} of the ethyl acetate in the sample was not absorbed in the first bottle.

The amount of carbon dioxide in the sample was measured by leading the flow of carbon dioxide, which left the heptane bottles, through a mass-flow meter. During the sampling, the pressure module P was connected to the water pump C to control the pressure inside the reactor. The volume of the reactor was adjusted by pumping water using a piston. After closing the gas-sampling valves, the sampling tubes were rinsed with heptane, to obtain the substrates that might have precipitated in the tubes and the valves.

A second gram of the substrate mixture was pumped into the reactor under magnetic stirring and the sampling procedure was repeated until 16 g of substrate was added in total. This procedure was followed at pressures of 11, 12.5, 15, 17.5, 20 and 30 MPa. The reactor was equipped with a sapphire window, and with the help of a Storz endoscope (opening angle 120°) the phase behaviour of the carbon-dioxide/substrate mixture inside the reactor could be observed.

Activity of Lipozyme in near-critical carbon dioxide

The enzyme was washed with hexane, and particles smaller than 5 μm were sieved out to prevent clogging of the filters between the reactor and the tubes by the smaller enzyme particles. It was dried under vacuum and stored at 280 K. Karl Fischer titration indicated that the dried enzyme contained 9 ml water per kg enzyme.

A schematic representation of the continuous stirred-tank reactor (CSTR) assembly is presented in Figure 2. Five grams of the enzyme were placed in the 80 ml reactor and 2.8 g ethyl acetate/nonanol mixture (2:1 mol/mol) was added, resulting in a concentration of 220 mmol l^{-1} ethyl acetate and 111 mmol l^{-1} nonanol. The reactor temperature was set at 333 K, which temperature is considerable higher than the critical temperature of pure carbon dioxide. This temperature was used since the addition of rather high concentrations of substrate in the experiments resulted in a reaction mixture having a totally different critical point than pure carbon dioxide. The reactor was pressurized by pumping carbon dioxide with a flow of 0.029 g s^{-1} in the reactor.

When the desired pressure was reached, the substrate pump was electronically connected to the carbon-dioxide pump and the back-pressure regulator was opened to control the pressure in the reactor. Every 15 minutes the two-position sampling valve was switched in the sampling position to take a 1-1.5 l sample at atmospheric pressure. The outcoming carbon dioxide with reaction components was led through a bottle containing 30 ml heptane. The flow rate of carbon dioxide was monitored on a mass flow

meter. After the samples had been taken, the sampling valve was switched back to the operation position and the sampling tubes were rinsed with heptane to collect remainders of the sample that precipitated in the tubes during sampling. The heptane samples were later analyzed by gas chromatography. The reaction was followed for at least 1 hour after equilibrium was reached, at pressures of 12.5, 15, 17.5 and 20 MPa under homogeneous medium conditions.

The amount of water in the reaction medium was controlled by supplying it via the substrate solution. From the water content of the enzyme particles (9 ml water per kg enzyme as determined by Karl Fischer titration) and the water content of both the substrate solution and the carbon dioxide, the water content of the medium was calculated; it varied from 0.5 to 2 ml l^{-1}. This amount was limited by the maximum solubility (~40 ml kg^{-1}) of water in the substrate mixture.

Activity of Lipozyme in hexane

A solution of 120 mmol l^{-1} nonanol and 250 mmol l^{-1} ethyl acetate in hexane was brought in a 50-ml bottle equipped with a magnetic stirrer. The mixtures were incubated at 333 K and 300 mg Lipozyme was added. One-cm^3 samples were taken every 10 minutes and diluted 50 times in heptane for analysis on the gas chromatograph.

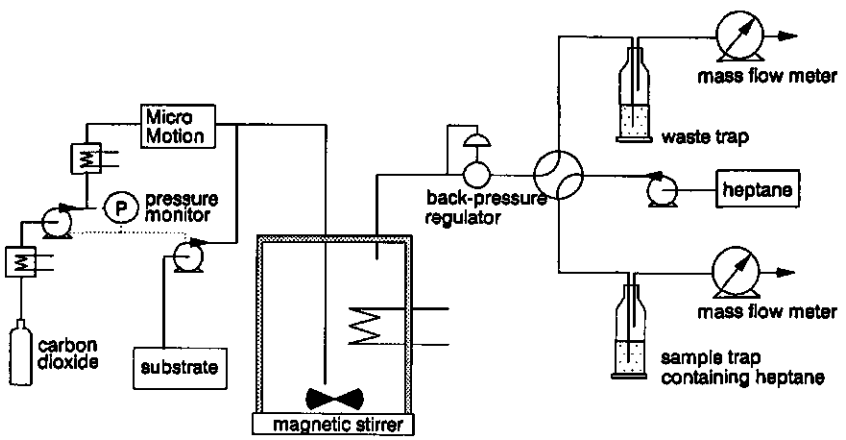


Figure 2 Schematic diagram of the CSTR setup for measuring Lipozyme activity in near-critical carbon dioxide. The sampling valve is drawn in the operation position. For further explanation, see text.

Analysis of the substrate and product mixtures

The heptane samples were analyzed on a gas chromatograph GC6000 (Vega series 2) using a 30 m carbowax capillary column. Dodecane was added to the samples as and internal standard to a final concentration of 2.9 mmol l^{-1}. A program for the column temperature was used by initially starting the analysis at 308 K. After 5 minutes the temperature increased in 2 minutes to 333 K. A further increase of the temperature to 453 K was achieved in 10 minutes.

RESULTS AND DISCUSSION

Homogeneity test

For apolar and slightly polar compounds, the difference between the Hildebrand solubility parameter of the compound and an organic solvent can be used as a first estimate of the solubility of the compound in this solvent (*Handbook of Chemistry and Physics*). When the difference equals zero, maximum solubility is achieved. This rule of thumb is also valid in supercritical fluids. Allada (1984) found a significant rise in solubility of fuel-oil residue, phenanthrene, and naphthalene when the difference in solubility parameter of the solvent and the solute became smaller than 9 $(MPa)^{0.5}$. Similarly, when data for the solubility of oleic acid and stearic acid in carbon dioxide (Chrastill, 1982) are translated in terms of solubility parameters, the same increase in solubility can be observed at a difference in solubility parameters of the solvent and the solute smaller than 9 $(MPa)^{0.5}$. Although the use of this parameter is restricted to apolar compounds and has its limitations for more polar compounds, it has been used here as a first estimate of the solubility of nonanol and ethyl acetate in near-critical carbon dioxide. The solubility parameters of the substrates nonanol and ethyl acetate used in our enzymic reaction are 17.2 and 18.6 $(MPa)^{0.5}$, respectively (*Handbook of Chemistry and Physics*). The carbon dioxide should thus have a solubility parameter of at least 8 $(MPa)^{0.5}$ to sufficiently solubilize both substrates and prevent phase separation. To check this solubilization boundary, samples were taken from the top part and bottom parts of the reactor at different pressures. A difference in concentration between the samples

Table 1 Medium properties of carbon dioxide at 333 K under conditions mentioned in the text. see Appendix for evaluation of the Hildebrand solubility parameter.

Pressure (MPa)	Density (kg l^{-1})	Hildebrand solubility parameter δ $(MPa)^{0.5}$
11	ND[a]	6.14
12.5	0.777	7.85
15	0.796	9.52
17.5	0.805	10.65
20	0.820	11.57
30	0.946	13.19

a) ND = Not determined, because phase separation took place

would indicate that phase separation took place.

At a pressure of 11 MPa and a temperature of 333 K corresponding with a Hildebrand solubility parameter value of 6.14 $(MPa)^{0.5}$ (see Appendix and Table 1), different concentrations of the substrates were found in the upper and bottom part of the reactor, indicating that total solubilization of the substrates did not occur under these circumstances. Increasing the pressure to 12.5-30 MPa resulted in total solubilization of the substrates.

The differences in solubility parameters between nonanol and carbon dioxide and between ethyl acetate and carbon dioxide at 12.5 MPa are 9.35 and 10.75 $(MPa)^{0.5}$, respectively, which corresponds rather well with the solubility boundary indicated by Allada (1984). These results indicate that the mixtures of carbon dioxide and the nonanol and ethyl acetate are homogeneous at pressures above 12.5 MPa. The homogeneity of the medium implies that the substrate/carbon dioxide mixture could be in the gaseous, liquid, or supercritical state. The density of the mixture (Table 1) changed little with pressure and it was rather high (0.78 - 0.95 kg l^{-1}), which indicates that we were operating in the near-critical region at the liquid side.

Activity of Lipozyme

The enzyme reaction was studied in a CSTR which was held at a temperature of

333 K. The pressure was varied from 12.5 MPa up to 20 MPa, under which conditions, the carbon dioxide/substrate mixture was found to be homogeneous. Under these conditions the Hildebrand solubility parameter varies from 7.85 to 11.57 $(MPa)^{0.5}$ (see Appendix and Table 1). Figure 3 shows the transesterification rate of Lipozyme at different solubility parameters measured at different water contents of the reaction medium. Only a slight activity decrease can be observed at increasing solubility parameters. A similar small decrease in activity is also found when the results of Erickson *et al.* (1990) are expressed in terms of solubility parameters. They studied the effect of pressure on the initial transesterification rates by *Rhizopus arrhizius* lipase in carbon dioxide using palmitic acid (δ = 15.2 $(MPa)^{0.5}$) and trilaurin (δ < 15 $(MPa)^{0.5}$) as substrates. At pressures lower than 10 MPa (δ < 3.4 $(MPa)^{0.5}$), they found a profound increase in activity, which they ascribed to changes in the distribution of the substrates between the supercritical fluid and the enzyme particles. Our results for the homogeneity test, however, indicate that the difference between Hildebrand solubility parameter of the solvent and the substrates should be smaller than 9 $(MPa)^{0.5}$ in order

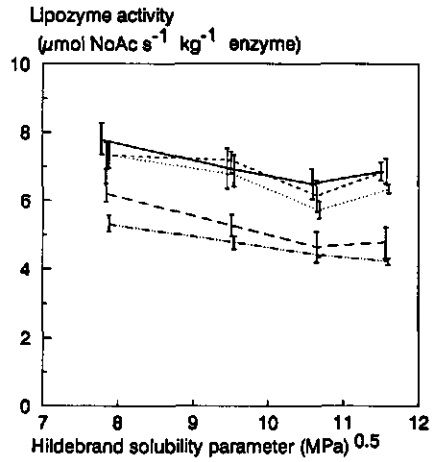


Figure 3 Lipozyme activity in near-critical carbon dioxide at different Hildebrand solubility parameters at a temperature of 333 K and having different water contents (- 0.5 ml l^{-1}, ---- 0.7 ml l^{-1}, ... 0.8 ml l^{-1}, --- 1.2 ml l^{-1}, -..-.. 1.9 ml l^{-1}). The range bars indicate the standard deviation of at least four different samples taken after equilibrium was reached.

to get a homogeneous solution. At pressures below 10 MPa, the difference in solubility parameter of carbon dioxide and palmetic acid is larger than 12, and phase seperation probably takes place. Under these circumstances the enzyme activity might be increased due to the concentration of substrates in the micro-environment of the enzyme.

Steytler *et al.* (1991) found an increasing esterification rate of butanol and lauric acid by *Candida* lipase B in carbon dioxide when the pressure was increased from 15 to 50 MPa. In this case the researchers checked the homogeneity of the medium, and the observed pressure effect was ascribed to a higher adsorption of the ester to the enzyme at lower pressures, causing inhibition of the enzyme. At higher pressures more solute-solvent interactions take place, resulting in a better "solvent capacity". In our case the higher "solvent capacity" might cause resorption of the ester substrate of the enzyme particles, thereby lowering the effective substrate concentration for the enzyme. This could explain the slight decrease in activity at increasing pressures (Figure 3).

In conventional organic solvents a correlation was found between the biocatalyst activity and the Hildebrand solubility parameter (δ) in combination with the molecular mass (M_s) of the solvent. High activity retention was found in solvents having a $\delta < 16$ $(MPa)^{0.5}$ and a $M_s > 200$ g mol^{-1}. In near-critical carbon dioxide, with a $\delta < 11.57$ $(MPa)^{0.5}$ and a M_s of 44 g mol^{-1}, this correlation is not obvious, but the transesterification rate in carbon dioxide was low; 8 μmol NoAc s^{-1} kg $enzyme^{-1}$ (Figure 3). The very low M_s of carbon dioxide and the linear structure of the molecules might cause penetration of the molecules in the water layer bound to the enzyme, thereby directly influencing the enzyme activity. In hexane, with a solubility parameter of 14.9 $(MPa)^{0.5}$ and a M_s of 86.18 g mol^{-1}, a transesterification rate of about 300 μmol NoAc s^{-1} kg $enzyme^{-1}$ was observed (Table 2). Hexane is often reported as a solvent with intermediate effects on enzyme activity, in spite of its relatively low M_s (Brink and Tramper, 1986, Laane *et al.*, 1987, Reslow, 1989).

Table 2 also indicates that the lower transesterification rates in near-critical carbon dioxide are not caused by denaturation of the enzyme under pressure. When the enzyme transesterification rate is tested in hexane after exposure of the enzyme to different pressures of carbon dioxide, only a small loss in activity can be observed. This

Table 2 Transesterification rate in hexane at 333 K of Lipozyme measured before and after being exposed to carbon dioxide at different pressures at 333 K.

	Transesterification rate in hexane (μmol NoAc s^{-1} kg^{-1} Lipozyme)
Before exposure to carbon dioxide	362
After exposure to carbon dioxide at 15 MPa	299
After exposure to carbon dioxide at 16.5 MPa	255
After exposure to carbon dioxide at 19 MPa	287

agrees with reports on the effect of high pressures on enzyme stability showing that loss in activity is partly due to the fact that substrate precipitates on the enzyme during depressurization of the reaction vessel. The temperature of 333 K during the exposure to carbon dioxide also causes some deactivation (Van Eijs *et al.*, 1988).

Another factor that might reduce the transesterification rate in carbon dioxide in comparison to hexane is the pH of the medium. Carbon dioxide is suspected to reduce the pH in the microlayer of bound water around the enzyme (Nakamura, 1990). Erickson *et al.* (1990), however, compared the enzyme activity in carbon dioxide with the activity in near-critical ethane, which has an almost identical critical temperature (305 K) and observed no difference in enzyme activity between the two media at similar density. They thus concluded that the decrease in reaction rate was not caused by a pH effect. The Lipozyme used in this work has a broad pH spectrum, and 40% of its maximum activity remains at pH 4. For these reasons, it is expected that the pH is not the only factor causing loss of activity in near-critical carbon dioxide.

Figure 3 also shows the effect of the water content on the Lipozyme transesterification rate. It shows a decrease in transesterification rate when the water content of the medium was raised from 0.5 up to 2 ml l^{-1}. Although enzymes need some water to maintain their active conformation, this amount of water is very small. Lipozyme is known to be active at extremely low water contents, (Min Chi *et al.*, 1988, Leitgeb and Knez, 1990) due to the high adsorption capacity of the carrier material. The decrease in transesterification rate by increasing the water content of the medium is observed at all pressures tested. This decrease in activity could be caused by hydrolysis of the product

nonyl acetate (Luck, 1989) into nonanol and acetic acid. This hypothesis could not be verified, since it was not possible to analyze acetic acid in our gas chromatographic analysis. The decrease in transesterification rate by increasing water content can also be caused by a direct effect of water on the enzyme at pressurized conditions. However, the activity of the enzyme in hexane after being exposed to carbon dioxide-substrate mixtures at 15 MPa and 333 K and different water contents was hardly affected; the enzyme preequilibrated at a water content of 1.4 ml l^{-1} and 2 ml l^{-1} showed a transesterification rate of respectively 315 and 299 μmol NoAc s^{-1} kg^{-1} enzyme. This result showed that the water content under pressurization has no significant negative influence on the Lipozyme stability.

CONCLUSIONS

The Hildebrand solubility parameter can be used to estimate the solubility of apolar and slightly polar compounds in near-critical carbon dioxide. At a difference of less than about 9 $(MPa)^{0.5}$ in solubility parameter values of the solute and the solvent, solubilization occurs.

The polarity of the near-critical medium, expressed by its solubility parameter, hardly affects the Lipozyme transesterification rates. The transesterification rate in near-critical carbon dioxide, however, is much lower than in hexane. This difference can not be ascribed to a pH effect of carbon dioxide only.

The water content of the medium has a more profound effect. Transesterification rates decrease with increasing water content, probably due to hydrolysis of the product.

ACKNOWLEDGEMENTS

The authors would like to thank Petra Koenraad, Peter van der Laar, and Emerence Veldkamp who worked as a student of the Agricultural University on this project. They contributed particularly to setting up the experiments. We also want to thank Hennie Haman for supplying us with the final experimental data and Wim

Beverloo for helpful discussions. The work was partly financed by the Dutch Programme Committee for Industrial Biotechnology.

REFERENCES

Allada, S.R. (1984) Solubility parameters of supercritical fluids. *Ind. Eng. Chem. Process Des. Dev.* **23**: 344-348.

Brink, L.E.S., Tramper J. (1986) Design of an organic-phase/immobilized-cell reactor for the microbial epoxidation of propene. In *Biocatalysis in organic media*, 133-146. Laane, C., Tramper, J., Lilly, M.D. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Chrastill J. (1982) Solubility of solids and liquids in supercritical gases. *J. Phys. Chem.* **86**: 3016-3021.

De Swaan Aronds, J., Coorens, De Loos Th.W. (1988) Supercritical extraction. Prediction of the capacity and selectivity of supercritical solvents. *DELFT Progress Report* **12**: 381-392.

Erickson J.C., Schyns P., Cooney C.L. (1990) Effect of pressure on an enzymatic reaction in a supercritical fluid. *AIChE Journal* **36**: 299-301.

Hahn-Hågerdal B. (1986) Water activity: a possible external regulator in biotechnical processes. *Enzyme Microb. Technol.* **8**: 322-327.

Halling P.J. (1987) Rates of enzymic reactions in predominantly organic, low water systems. *Biocatalysis* **1**: 109-115.

Hammond, D.A., Karel, M., Klibanov, A.M., Krukonis, V.J. (1985) Enzymatic reactions in supercritical gases. *Appl. Biochem. Biotechnol.* **11**: 393-400.

Handbook of Chemistry and Physics 57th Ed., (1976-1977), C726-732, CRL Press.

Laane, C., Boeren, S., Vos, K., Veeger C. (1987) Rules for optimization of biocatalysis in organic solvents. *Biotech. Bioeng.* **30**, 81-87.

Leitgeb M., Knez Z. (1990) The influence of water on the synthesis of n-butyl oleate by immobilized *Mucor miehei* lipase. *JAOCS* **67**: 775-778.

Luck T. (1989) *Einfluss von Stoffiransport und Kinetik immobilisierter Lipasen in heterogenen Stoffsystemen auf Auslegung und Entwicklung von Enzymreaktoren.* VDI-Verlag, Düsseldorf, Germany.

McHugh M., Krukonis V. (1986) *Supercritical Fluid Extraction; Principles and Practice.* Buttersworth Publ., Boston, USA.

Min Chi Y., Nakamura K., Yano T. (1988) Enzymatic interesterification in supercritical carbon dioxide. *Agric. Biol. Chem.* **52**: 1541-1550.

Nakamura K. (1990) Biochemical reactions in supercritical fluids. *TIBTECH* **8**: 288-292.

NOVO (1985) *A New Immobilized Lipase, prelimanary product information*. Novo Industri A/S, Copenhagen, Denmark.

Pasta P., Mazzola G., Carrea G., Riva S. (1989) Subtilisin-catalyzed transesterification in supercitical carbon dioxide. *Biotech. Letters* **2**: 643-648.

Randolph T.W., Blanch H.W., Prausnitz J.M. (1988) Enzyme-catalyzed oxidation of cholesterol in supercritical carbon dioxide. *AIChE Journal* **34**: 1354-1360.

Reslow M. (1989) *Enzymes as Catalysts in Organic Media* PhD-Thesis, University of Lund, Lund, Sweden.

Sleytler D.C., Moulson P.S., Reynolds J. (1991) Biotransformations in near-critical carbon dioxide. *Enzyme Microb. Technol.* **13**: 221-226.

Smith J.M., Van Ness H.C. (1987) *Introduction to Chemical Engineering Thermodynamics* 4th Ed., Chapter 3 and 6. McGraw-Hill Book Company, New York, USA.

Taganuchi, M., Kamihira, M., Kobayashi, T. (1987) Effect of treatment with supercritical carbon dioxide on enzymatic activity. *Agric. Biol. Chem.* **51**: 593-594.

Van Eijs, A.M.M., de Jong J.P.J., Doddema H.J., Lindeboom D.R. (1988) Enzymatic trans esterification in supercritical carbon dioxide. In: *Proceedings of the International Symposium on Supercritical Fluids* **2**: 933-942. Perrut, M. (Ed.) Societe Francaise de Chimie, Nice, France.

Zaks A., Klibanov A.M. (1988) The effect of water on enzyme action in organic media. *J. Biol. Chem.* **263**: 8017-8021.

APPENDIX

A parameter to characterize the dissolving power of solvents is the Hildebrand solubility parameter (δ). This parameter is defined as the square root of the internal pressure or cohesive energy density ($-U/V_l$) (Allada, 1984):

$$\delta = (-\frac{U}{V_l})^{0.5} \qquad (Pa)^{0.5} \qquad (1)$$

where U is the molar cohesive energy (J mol^{-1}) and V_l is the molar volume of the solvent

(m^3 mol^{-1}).

For liquid solvents at low vapor pressures, the cohesive energy of the vapor can be ignored and the Hildebrand solubility parameter can be approximated by:

$$\delta = \left(\frac{\Delta H_v - RT}{V_l}\right)^{0.5} \qquad (Pa)^{0.5} \qquad (2)$$

where ΔH_v is the molar enthalpy of vaporization of the liquid (J mol^{-1}) at temperature T (K); R is the molar gas constant (8.314 J mol^{-1} K^{-1}). For liquids in the vicinity of their critical point, the cohesive energy of the vapor can no longer be ignored. For supercritical fluids there is no phase transition with a heat effect at all. Allada (1984) proposed to use the basic definition of the Hildebrand solubility parameter for supercritical fluids except that the internal energy of the supercritical fluid relative to the isothermally expanded ideal state is used instead of the cohesive energy of the gas:

$$\delta = \left(\frac{U^* - U}{V}\right)^{0.5} \qquad (Pa)^{0.5} \qquad (3)$$

where U and V are the molar internal energy (J mol^{-1}) and molar volume (m^3 mol^{-1}) of the supercritical fluid at temperature T (K) and pressure P (Pa) and U^* is the molar internal energy of the gas isothermally expanded to some standard pressure low enough to approach ideal state.

The molar volume (V) can be derived from:

$$V = \frac{ZRT}{P} \qquad (m^3\ mol^{-1}) \qquad (4)$$

where Z is the compressibility factor of the fluid.

By introducing the relative temperature T_r ($= T/T_c$) and the reduced pressure P_r ($= P/P_c$) the Hildebrand solubility parameter can be given in dimensionless form:

Table 3 Data to calculate the Hildebrand solubility parameter (δ) using equations 3-6 (see Appendix) based on Allada (1984). The data were evaluated using the Lee-Kesler analytical equation of state (Smith and Van Ness, 1987).

Pressure P MPa	Reduced pressure P_r	Reduced temp. T_r	Compressibility factor Z	$(H^*-H)/T_c$ J K^{-1} mol^{-1}	$(U^*-U)/T_c$ J K^{-1} mol^{-1}	δ (MPa)$^{0.5}$
11	1.49	1.095	0.489	19.90	15.25	6.14
12.5	1.69	1.095	0.433	24.65	19.49	7.85
15	2.03	1.095	0.399	27.98	21.96	9.52
17.5	2.37	1.095	0.415	29.82	24.49	10.65
20	2.71	1.095	0.430	31.38	26.19	11.57
30	4.07	1.095	0.561	33.60	29.60	13.19

$$\delta_r = \left[\frac{(U^* - U)P_r}{RT_cT_rZ}\right]^{0.5} \qquad (-) \qquad (5)$$

where δ_r is $\delta/(P_c)^{0.5}$.

The compressibility factor (Z) and the internal energy (U) have been evaluated using the classical Lee-Kesler analytical equation of state (Smith and Van Ness, 1987). Using the thermodynamic definition for the enthalpy (H) of a system $H = U + PV$ a relation for the internal energy ($U^* - U$) can be found:

$$\frac{U^* - U}{T_c} = \frac{H^* - H}{T_c} - (1-Z)RT_r \qquad (\text{J mol}^{-1}\,\text{K}^{-1}) \qquad (6)$$

The relation $(H^*-H)/T_c$ is given as function of the reduced temperature and pressure (Smith and Van Ness, 1987). In Table 3 all needed data are given and by using these data and the equations 3-6, the Hildebrand solubility parameter (δ) can easily be evaluated.

Chapter 7

OPTIMIZATION OF THE PRODUCT YIELD IN ESTERIFICATION REACTIONS BY WATER-ACTIVITY CONTROL USING AIR CIRCULATION THROUGH SATURATED SALT SOLUTIONS

SUMMARY

The esterification of *N*-carbobenzoxy-phenylalanine (Z-PheOH) and hexanol (HexOH) in isooctane into the hexyl ester (Z-PheOHex) is selected as the model reaction to study the effect of water-activity (a_w) control by circulation of conditioned air over the reaction mixture. The circulating air is conditioned by bubbling through a saturated salt solution of known a_w. The reaction is catalyzed by α-chymotrypsin adsorped on celite. At $a_w < 0.75$ no synthetic activity is found. At a_w's > 0.75 the initial activity of the enzyme increases with increasing a_w. After 100 h reaction time the highest ester yield is observed at $a_w = 0.89$. This optimum in a_w is a compromise between the advantageous equilibrium yield at low a_w and the high biocatalytic activity at high a_w. It is very likely that inactivation of the enzyme takes place due to salt molecules which enter the reaction mixture via entrainment in aerosols and accumulate in the microaqueous phase around the enzyme.

Optimization of the product yield in esterification reactions by water-activity control using air circulation through saturated salt solutions.
Vermuë, M., Bergmans, M., Beverloo, W., Tramper, J. (1995).
Submitted for publication in *Biocatalysis*.

Chapter 7

INTRODUCTION

In reactions catalyzed by hydrolytic enzymes, reduction of the water activity (a_w) in the reaction mixture shifts the thermodynamic equilibrium towards esterification (Halling, 1984, Cassels and Halling, 1988, Blanco *et al.*, 1992a, 1992b, Halling, 1994). When the a_w is reduced too far, however, the biocatalyst activity will decrease or will be completely absent and the equilibirium will not be obtained within reasonable time (Halling and Valivety, 1992). An optimum compromise has thus to be found between the advantageous equilibrium at low a_w and the high biocatalytic activity at high a_w. In order to obtain the highest possible ester yield, the water which is formed during the esterification has to be removed, so that the a_w is kept constant at the optimum value (Ergan *et al.*, 1990, Bloomer *et al.*, Van der Padt *et al.*, 1993).

In this work a new a_w-control method is introduced. Air is bubbled through a saturated salt solution, is led directly through the vessel containing the reaction medium and is recycled into the saturated salt solution. To minimize losses of substrate and solvent during the reaction and to minimize the effect of dissolved molecules on the a_w of the saturated salt solution, a prerequisite of the method is that the substrates of the reaction and the organic solvent hardly dissolve in water. The α-chymotrypsin-catalyzed esterification of *N*-carbobenzoxy-phenylalanine (Z-PheOH) with hexanol (HexOH) to the hexyl ester (Z-PheOHex) in iso-octane meets these requirements and is chosen as a model reaction to test the effect of a_w-control by recycling of a_w-controlled air on the kinetics and the final product yield of this esterification reaction.

MATERIALS AND METHODS

Chemicals

α-Chymotrypsin (EC 3.4.21.1.) from bovine pancreas, type II (Sigma Chemicals) has a specific activity of 0.65 μkat mg^{-1} (benzoyl-*L*-tyrosine ethyl ester hydrolysis). *N*-Carbobenzoxy-phenylalanine (Z-PheOH) is obtained from Bachem, Feinchemikalien AG, Switzerland, while *N*-Acetyl-*L*-phenylalanine ethyl ester (Ac-PheOEt) is obtained from

Table 1 Water activity as generated by saturated salt solutions.

salt	a_w
LiCl	0.11
$MgCl_2.6H_2O$	0.33
K_2CO_3	0.44
NaBr	0.57
$CuCl_2.2H_2O$	0.69
NaCl	0.75
$(NH_4)_2SO_4$	0.80
KCl	0.84
$BaCl_2.2H_2O$	0.89
KNO_3	0.93
$K_2Cr_2O_7$	0.97

Sigma Chemicals. All salts are supplied by Merck. The organic solvents are of highest available purity (Merck) and are dried on molecular sieves before use. The a_w of the saturated salt solutions used, is given in Table 1.

Chemical synthesis of N-carbobenzoxy-phenylalanine alkyl esters

The *N*-carbobenzoxy-phenylalanine hexyl ester (Z-PheOHex) is prepared in our own laboratory according to the method described by Bodanszky and Du Vigneaud (1962), which is adjusted slightly. To 20 mmol Z-PheOH dissolved in 60 ml ethyl acetate, 22 mmol hexanol is added. To this mixture 22 mmol dicyclo-hexyl-carbo-diimide is added. During 4 h the mixture is stirred on ice in a dried flask equipped with a $CaCl_2$ stopper to avoid the entrance of moisture. The formed dicyclo-hexyl urea is filtered from the solution and the filtrate is washed with ice-cold water, a saturated sodium-chloride solution and again with ice-cold water. The ethyl-acetate fraction is separated from the water solution and dried with $MgSO_4$. After filtering, the filtrate is dried in a rota-vaporator.

The product, Z-PheOHex, is purified by column chromatography using silica (230-400 mesh, particle size 600 nm, Aldrich) as column material and chloroform as eluens. Fractions showing UV-adsorption at 254 nm are collected and analyzed using thin-layer chromatography. Fractions containing the product are joined and concentrated in a rota-vaporator. The final yield of Z-PheOHex is 11.7 mmol (58.5% of theoretical maximum yield). No impurities are detected on a ^{1}H-NMR scan (200 MHz NMR-spectrometer, Bruker, AC200E). Other alkyl esters of Z-PheOH are prepared in the same way with the appropriate alkanol. The esters are used as references in the calibration of the HPLC-analysis of the reaction products formed in the experiments, especially concerning the selection of a model reaction (Table 2).

Immobilization of the enzyme on celite

α-Chymotrypsin is dissolved in 50 mmol l^{-1} sodium-phosphate buffer pH 7.8 (30 g l^{-1}) and 1 ml of this solution is mixed with 1 g celite (30-80 mesh, particle size 0.2-0.5 mm, BDH Chemicals). This preparation is freeze-dried overnight. After drying, the water content of the particles is measured by Karl-Fischer titration and found to be < 6 g H_2O per kg particles.

Standard assay of the enzyme activity

The α-chymotrypsin activity is determined as esterase activity using *N*-Acetyl phenyl alanine ethyl ester (Ac-PheOEt) as a substrate. Ac-PheOEt is dissolved in a solution of 100 mmol l^{-1} KCl and 100 ml l^{-1} acetonitril upto a final concentration of 10 mmol l^{-1}. To 25 ml of this solution about 0.05 mg α-chymotrypsin is added. In case of immobilized enzyme, the enzyme completely desorbs from the particles by adding 10 ml of a 5 mmol l^{-1} sodium-phosphate buffer (pH 7.8) per gram particles. A sample (0.02-0.05 ml) of the obtained enzyme solution is used in the standard assay. The temperature is maintained at 303 K. The ester is hydrolyzed and the formed acid is automatically titrated with a 2 mmol l^{-1} NaOH solution at pH 7.8 (Schott Geräte, pH-stat. TR156).

Reaction set-up with the recycling of dried air

Figure 1 shows the reaction set-up for the system with recycling of a_w-controlled air. Vessel A contains 100 ml of a saturated salt solution of known a_w (Table 1). Vessel B contains 15 ml of the reaction medium. Air is pumped with a piston pump (neopreen valves) from the head space of vessel A via teflon tubing into bottle B and is recycled to the pump. The air-flow rate is kept at about 85 mm^3 s^{-1}. The reaction medium is pre-equilibrated inside the reaction set-up at a set a_w during 3 days at 303 K.

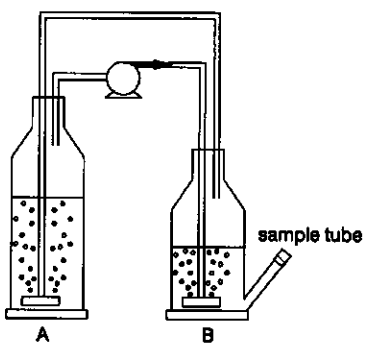


Figure 1 Reaction set-up with the recycling of dried air. Vessel A contains 100 ml of a saturated salt solution; vessel B contains 15 ml of the reaction mixture.

The water content during equilibration is determined using Karl-Fischer titration. 400 mg of dried enzyme particles, containing 12 mg of α-chymotrypsin are added and 0.05 ml samples of the reaction medium are taken three times a day and are analyzed on HPLC. When no further increase in product is observed after approximately 100 h reaction time, fresh enzyme is added to check if the equilibrium yield is obtained. When the reaction indeed has stopped, the residual enzyme activity is determined by the standard assay.

On-line determination of the a_w

The a_w in the air flow through the reaction system is checked using a dew-pointhygrometer (Compteurs Schlumberger HCP3S). To obtain a steady signal the air flow through the reaction system is increased during the a_w determination from 85 mm^3 s^{-1} to 170 mm^3 s^{-1}. The computer program TVL (IMT, Enschede, Holland) is used to convert the measured dew point to a_w at 303 K.

HPLC-analysis

The 0.05 ml samples of the reaction medium are dried for about 2 h under vacuum at room temperature. The dried samples are dissolved in eluens (water/acetonitrile/acetic acid (volume ratio 85/15/0.2)) with 4.5 mmmol l^{-1} anthraquinone as internal standard for the chromatograms. The amounts of Z-PheOH and Z-PheOHex are analyzed by HPLC using a Chromspher C-18 column eluated with the eluens. Peaks are detected with a UV-detector at 258 nm.

RESULTS AND DISCUSSION

Selection of the model reaction

The effect of a_w-control by recycling of equilibrated air or by equilibration above a saturated salt solution on an esterification reaction can only be studied if the reactants very poorly dissolve in the saturated salt solution (Wehtje *et al.*, 1993). If they do dissolve considerably, recirculation of the air has some undesirable effects. It will change the reactant concentration in the reaction mixture, and moreover, it will alter the composition of the salt solution and thus the a_w of the salt solution. As a model reaction, the α-chymotrypsin catalyzed esterification of the apolar substrate Z-PheOH with an apolar alcohol in iso-octane is chosen, because of the negligible solubilities of the reactants, the catalyst and the solvent in the saturated salt solutions.

Table 2 Solubility of aliphatic alcohols in water and the product yield after 2 d of incubation of Z-PheOH (5 mmol l^{-1}) and of the alcohol (1 mol l^{-1}) in 10 ml iso-octane at 303 K with α-chymotrypsin immobilized on celite (40 mg g^{-1}).

alcohol	solubility in water at 298 K (mmol l^{-1})	$[alcohol]_{or}$ after maximal migration to the saturated salt solutions (mmol l^{-1})[a]	ester concentration after 2 days of incubation (mmol l^{-1})
n-butanol	1005	0	3.2
n-pentanol	248	0	2.7
n-hexanol	69	540	2.4
n-octanol	4	973	1.8

[a] The alcohol concentration remaining in the 15 ml organic-solvent phase when the alcohol concentration in the 100 ml of the saturated salt solution has reached the maximum solubility level in water. Initially 1 mol l^{-1} alcohol is present in the organic-solvent phase.

The enzyme α-chymotrypsin catalyzes the esterification of Z-PheOH with several aliphatic alcohols. This is tested by incubation of Z-PheOH (5 mmol l^{-1}) with butanol, pentanol, hexanol or octanol (1 mol l^{-1}) in 10 ml iso-octane. 40 mg of the immobilized enzyme preparation and 0.04 ml of the phosphate buffer are added. Table 2 shows the product concentration after 2 days of incubation. These results show that the amount of product formed within 2 days decreases when the chain length increases.

Although the solubility of the alcohols in water is not very high (Table 2), the alcohol concentration in the reaction mixture with a_w-controlled head-space recycling might decrease drastically. If the solubility of the alcohol in the saturated salt solution is assumed to be the same as in water, the maximum amount of alcohol migrating into the salt solutions can be calculated. Table 2 shows that all the butanol and pentanol is likely to disappear from the reaction mixture. A considerable loss of hexanol and octanol is expected too, but the remaining concentration in the reaction mixture will be sufficient to maintain an excess of alcohol with respect to Z-PheOH. The loss of the alcohols from the reaction mixture to the vapour phase is negligible ($< 1\%$). Because the solubility of hexanol and octanol in water is rather limited, the effect of these dissolved alcohols on the a_w of the saturated salt solutions can also be expected to be negligible (Halling, 1994). Since the reaction with octanol is rather slow, hexanol is eventually selected as

the most suitable substrate for the model reaction.

Water-activity sensor

For measurement of the a_w of the reaction system a water-activity sensor is needed which does not show interference by either the organic solvent or the other reactants and which responds continuously and rapidly, independent of the a_w. Aluminium-oxide-type humidity sensors are prone to drift especially when they are used at high a_w in organic-solvent systems (Khan *et al.*, 1990). Therefore, a dew-point hygrometer is used as a_w sensor.

Figure 2 shows the response of the sensor when it is exposed to air recycling from the reactant solution through saturated salt solutions of known a_w. The signal obtained shows some fluctuation as indicated by vertical bars. The higher the a_w of the saturated salt solution, the more fluctuation of the signal is observed. The fluctuations in the signal are probably caused by temporary condensation of iso-octane on the sensor. Nevertheless, the average water-activity signal obviously corresponds very well with the a_w imposed upon the system by the salt solution.

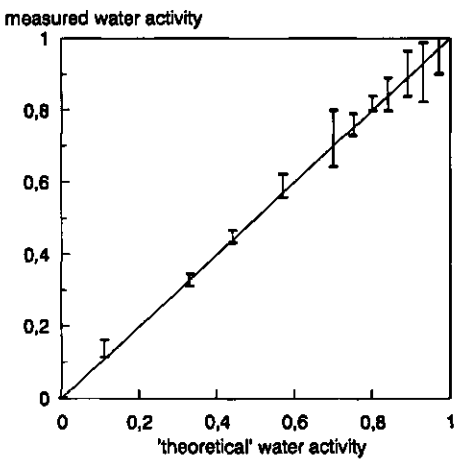


Figure 2 Response of the dew-point hygrometer exposed to air recycled from the reactant solution through saturated salt solutions of known a_w.

Water-partitioning effects

At equilibrium the a_w is by definition, equal in all phases of the reaction system and therefore the a_w is a good parameter to characterize e.g. the degree of hydration of the different phases in the system (Halling, 1984, Adlercreutz, 1991, Valivety *et al.*, 1991, Halling and Valivety, 1992, Wehtje *et al.*, 1993). The water content of the support material, the substrate solution and the enzyme particles is determined as a function of the a_w (Figure 3). When equilibration is done over a gently shaken saturated salt solution, it takes approximately 14 d before equilibrium is reached. Further incubation does not result in an additional increase of the water content of the samples, as determined by Karl-Fischer titration. Equilibration by recycling of air through the reaction system takes only about 8 h.

Figure 3 shows that the support material hardly adsorbs water over the entire range of a_w's, which corresponds well with other reported adsorption isotherms of celite (Adlercreutz, 1991). The water content of the substrate solution hardly changes upto an a_w of 0.8. At higher a_w's the amount of water absorbed by the substrate solution might double. Due to the low polarity of the solution, however, the total amount of water

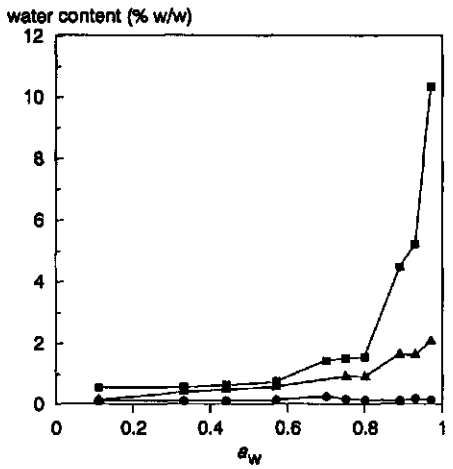


Figure 3 Adsorption isotherms of celite (●), substrate solution (5 mmol l^{-1} Z-PheOH and 1 mol l^{-1} HexOH in isooctane), (▲) and immobilized α-chymotrypsin (40 mg g^{-1} celite) (■) at 303 K.

adsorbed remains relatively low. The water-adsorbing capacity of the enzyme preparation is relatively high. The water uptake remains low up till an a_w of 0.6, but if the a_w is further increased, the water uptake by the complete enzyme preparation markedly increases. Adlercreutz (1991) has shown that the water-adsorbing capacity is dominated by the contribution of the buffer salts in the enzyme preparation.

Effects of pre-equilibration

The substrate solution and the enzyme particles are equilibrated separately at a given a_w over saturated salt solutions. Before starting the esterification reaction, a sample of the enzyme preparation is taken and the retention of the enzyme activity is determined using the standard-activity assay. Table 3 shows that 40-70% of the original activity at the lower a_w's resides; at $a_w > 0.8$ only 1-13% of the original activity can be measured. A similar irreversible effect of a_w on inactivation of chymotrypsin is reported by Zaks and Klibanov (1988). They have observed that α-chymotrypsin looses half of its catalytic activity after one week incubation in buffer ($a_w = 1$), while full enzymatic activity is retained, when the dried enzyme is suspended in dry octane ($a_w \ll 1$).

Because of the large inactivation at high a_w, it is decided to equilibrate the substrate solution in the reaction vessel and to use the dried enzyme preparation (water

Table 3 Residual hydrolytic activity of α-chymotrypsin immobilized on celite after 14 days incubation over a saturated-salt solution of known a_w at 303 K. Samples have been measured in duplo and show about 10% standard deviation.

a_w	Residual hydrolytic activity (%)
0.11	53.1
0.33	36.9
0.44	56.9
0.57	40.2
0.75	71.1
0.80	51.4
0.89	12.7
0.93	8.2
0.97	0.8

content ≈ 0.7 g kg^{-1} preparation), without previous equilibration over saturated salt solutions. After the addition of the dried enzyme preparation, the a_w of the reaction system might temporarily decrease. From the sorption data presented in Figure 3, however, it can be calculated, that this initial effect is negligible. No response of the water-activity sensor on addition of the dried enzyme particles is indeed observed.

Due to the addition of the dried enzyme preparation the substrate Z-PheOH will move from the organic-solvent phase to the micro-aqueous phase developing around the enzyme. This is checked by adding the support material with the buffer salts but without the enzyme to the pre-equilibrated substrate solution (Figure 4). At $a_w < 0.90$ no effect of this partitioning was observed, but at $a_w = 0.95$ the Z-PheOH concentration in the organic solvent phase decreased in 8 h time from 6 mmol l^{-1} to 2.5 mmol l^{-1}, probably due to the increase of the amount of water adsorbed by the buffer salts dried on the support (Adlercreutz, 1991).

At $a_w \approx 1$ no substrate at all remains in the organic-solvent phase after 8 h of incubation.

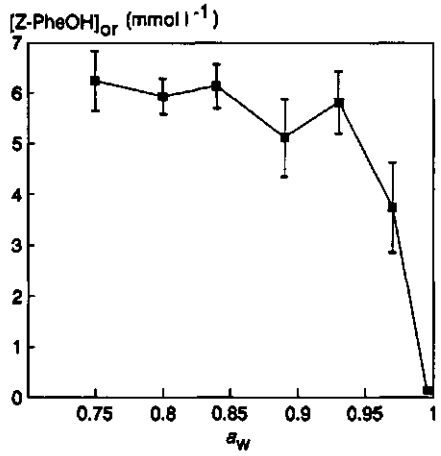


Figure 4 The concentration of Z-PheOH in the organic-solvent phase (1 mol l^{-1} HexOH in isooctane) after equilibration in the presence of celite and buffer salts at different a_w values. The initial concentration of Z-PheOH in the organic-solvent phase is 6 mmol l^{-1}.

The addition of the enzyme preparation has no effect on the partitioning of the product in the reaction system. All product is found in the organic-solvent phase independent of the a_w of the reaction system (data not shown).

Esterification at controlled a_w; kinetics

During the esterification of HexOH with Z-PheOH in iso-octane the a_w is controlled by recycling of the head-space gases through saturated salt solutions. At a_w < 0.75 no synthetic activity is detected (data not shown). After 100 h incubation, however, the loss in hydrolytic activity is limited (Table 3) and the enzyme is thus inactivated reversibly at low a_w's. This reversible inactivation at low a_w is generally ascribed to the stripping of water molecules associated with the micro-environment of the enzyme, which are considered to be essential for catalytic activity (Zaks and Klibanov, 1988, Adlercreutz, 1991, Halling and Valivety, 1992).

Figure 5 shows the formation of Z-PheOHex at a_w's ≥ 0.75. During the first 8 h the reactants partition over the enzyme and the organic-solvent phase. The initial

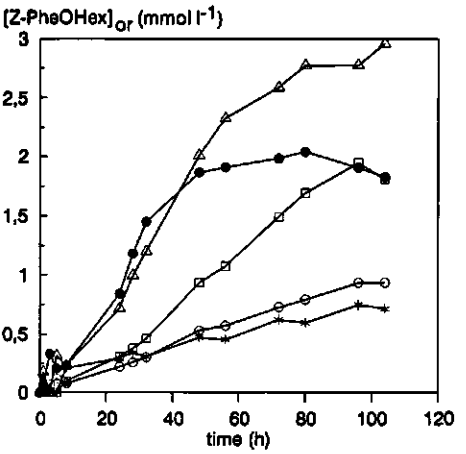


Figure 5 Progress of the Z-PheOHex synthesis catalyzed by α-chymotrypsin immobilized on celite in isooctane. The reaction medium is pre-equilibrated during 3 d at 303 K by recycling of dried air to bring the a_w to 0.75 (*), 0.80 (○), 0.84 (□), 0.89 (△) and 0.97 (●).

velocity is therefore determined over the time interval 8-32 h, in which the reactions show approximately linear progress at each a_w-value. Figure 6 shows that the initial activity increases with increasing a_w. This effect is generally observed (Goderis, 1987, Goldberg, 1990, Adlercreutz, 1991, Blanco *et al.*, 1992b), although the activity-a_w profile may vary considerably for enzymes of different sources or for enzymes immobilized on different supports (Adlercreutz, 1991, Valivety *et al.*, 1992a, 1992b).

Esterification at controlled a_w; product yield

After 100 h incubation no further increase in the product concentration is observed, despite the fact that the enzyme in the reaction medium still shows hydrolytic activity (Table 4) and thus probably shows some residual synthetic activity as well. Upon addition of fresh enzyme, no further increase in product concentration is observed either, even when the incubation time is extended to 200 h. These observations suggest that thermodynamic equilibrium is approached within 100 h reaction time.

However, there are reasons to doubt that this is true, because Table 5 shows that the product yield Z-PheOHex$_{100}$/Z-PheOH$_0$, shows an optimum at a_w 0.89. This is

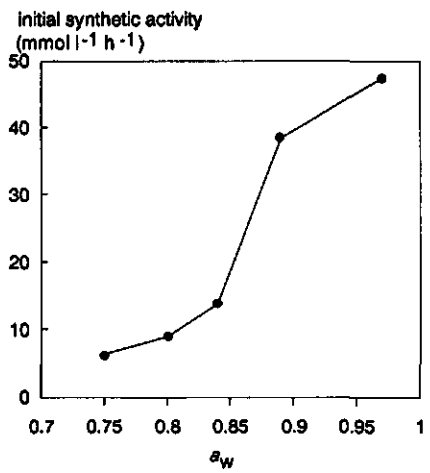


Figure 6 Initial rate of Z-PheOHex synthesis by α-chymotrypsin immobilized on celite as a function of the a_w, as measured over the time interval 8-32 h in Figure 5.

Table 4 Operational stability of the α-chymotrypsin as determined by the residual hydrolytic activity after 100 h incubation.

a_w	remaining hydrolytic activity (%)
0.75	18
0.80	13
0.84	32
0.89	27
0.97	10

rather strange, because the product concentration is expected to decrease at increasing a_w of the reaction mixture and applying the same initial substrate concentrations. Even if one considers that the microaqueous phase around the enzyme is non-ideal with respect to H_2O and the substrates Z-PheOH and HexOH, it is impossible to explain the observed optimum a_w. The possible influence of the non-ideality of the microaqueous phase is considered in the next section.

Influence of the non-ideality of the microaqueous phase on the equilibrium yield

The equilibrium constant of the esterification of Z-PheOH and HexOH into Z-PheOHex and water can be defined in terms of the thermodynamic activity of the reaction components:

Table 5 Concentration of Z-PheOH at the start and after 100 h incubation, and final product concentration and yield of the α-chymotrypsin catalyzed reaction of Z-PheOH with 1 mol l^{-1} HexOH in iso-octane equilibrated by recycling dried air at known a_w at 303 K.

a_w	$[Z\text{-PheOH}]_0$ (mmol l^{-1})	$[Z\text{-PheOH}]_{100}$ (mmol l^{-1})	$[Z\text{-PheOHex}]_{100}$ (mmol l^{-1})	Product yield[a] (-)
0.75	5.80	5.05	0.72	0.12
0.80	5.66	3.80	0.94	0.17
0.84	5.65	4.10	1.88	0.33
0.89	5.61	2.80	2.80	0.50
0.97	5.87	1.15	1.99	0.34

[a] Product yield is defined as the $[Z\text{-PheOHex}]_{100}/[Z\text{-PheOH}]_0$.

$$K_{eq} = \frac{a_{Z\text{-PheOHex}} * a_w}{a_{Z\text{-PheOH}} * a_{HexOH}} \quad (1)$$

where a_i is the thermodynamic activity of component i.

The thermodynamic activity of a component is given by:

$$a_i = \gamma_i x_i \quad (2)$$

where γ_i and x_i are the activity coefficient and the mole fraction of component i, respectively. The activity of the pure component at the same temperature is assigned to be 1 (Halling, 1994).

The x_{HexOH} can be considered constant during the reaction, because there is a large excess of hexanol compared to Z-PheOH. Because hexanol and isooctane hardly interact with each other (no H-bonds or dipole-dipole interactions), they will form an "ideal" mixture. The γ_{HexOH} can therefore be considered constant and independent of the a_w. Both reasons justify the assumption that a_{HexOH} can be considered to be constant and independent of the a_w.

The product Z-PheOHex resides mainly in the organic-solvent phase as is illustrated by its partition behaviour. Furthermore, its concentration is relatively low and therefore ideal behaviour of this component in the organic-solvent phase can be expected too. The $\gamma_{Z\text{-PheOHex}}$ can thus be assumed constant for the concentration range used. With these assumptions Equation 1 and Equation 2 can be combined and rearranged into:

$$x_{Z\text{-PheOHex}} = K^c * \frac{\gamma_{Z\text{-PheOH}} x_{Z\text{-PheOH}}}{a_w} \quad (3)$$

where K^c represents the constant $K_{eq} * a_{HexOH} / \gamma_{Z\text{-PheOHex}}$. The thermodynamic yield of product $x_{Z\text{-PheOHex}}$ in the organic-solvent phase, will thus be determined by the effect of the a_w on the activity of the substrate ($\gamma_{Z\text{-PheOH}} * x_{Z\text{-PheOH}}$).

The concentration of the substrate $x_{Z\text{-PheOH}}$ after 100 h reaction time decreases as a_w increases (Table 5). This is partly due to substrate conversion but also due to

partitioning of Z-PheOH which is a_w-dependent (Figure 4).

The effect of a_w on the $\gamma_{Z\text{-}PheOH}$ in the organic-solvent phase can not be measured directly, but theoretical estimation of the a_w-dependence is possible by means of the UNIFAC group contribution method (Fredenslund *et al.*, 1977). These calculations do not give absolute values for $\gamma_{Z\text{-}PheOH}$ but they may be used to predict the general effect of a_w on this parameter. According to these UNIFAC calculations a decrease of the $\gamma_{Z\text{-}PheOH}$ upon an increase in a_w has to be expected for the esterification of Z-PheOH and HexOH (data not shown).

Since both $x_{Z\text{-}PheOH}$ and $\gamma_{Z\text{-}PheOH}$ in the organic-solvent phase decrease upon an increase in a_w, the $a_{Z\text{-}PheOH}$ will decrease too. The thermodynamic yield of product in the organic-solvent phase should therefore be expected to decrease when the a_w increases. So, even if the non-ideality of the micro-aqueous phase with respect to the substrate is taken into account, no optimum in thermodynamic product yield as function of the a_w is predicted.

Obviously, at low a_w the reaction has stopped before thermodynamic equilibrium is reached. This effect is also observed by Blanco *et al.* (1992b) for the amino-ester synthesis catalyzed by agarose-chymotrypsin in 3-pentanone and is ascribed to inactivation of the enzyme at low a_w. This inactivation is partially reversible and up to 50% of the initial activity of the enzyme is recovered after washing with water (Blanco *et al.*, 1992b).

In our case an additional inactivation by salts may play a role, due to the a_w-control method used. Salt molecules may be entrained in aerosols by the air that passes though the reaction medium and the entrained molecules will accumulate in the microaqueous phase around the enzyme and cause inactivation. The fresh enzyme, which is added after 100 h reaction time will immediately encounter the inactivation by the salt-molecules in its micro-aqueous phase.

Such inhibiting effect is observed in our laboratory for a lipase-catalyzed triacylglycerol synthesis by means of pervaporation. No inactivation of the enzyme occurs when the sweep gas in the pervaporation is equilibrated at a low a_w-value by means of a condensor (Van der Padt *et al.*, 1993) or when the reaction mixture is

equilibrated above a saturated LiCl-solution (a_w = 0.11) without recirculation of air (Van der Padt *et al.*, 1992). However, when equilibration is done by means of pervaporation and large air fluxes through the membrane are used, irreversible inactivation of the lipase is observed (Van der Padt, unpublished results). This inactivation can only be ascribed to the effect of salt molecules, which are entrained by the air and migrate through the evaporation membrane to the enzyme.

CONCLUSIONS

The water activity can be efficiently controlled by recirculation of the head-space gases of saturated salt solutions through the reaction medium. However, the enzyme may be subject to inactivation by salt molecules that accumulate in the reaction medium. The optimum yield of Z-PheOHex synthesis by α-chymotrypsin at a_w = 0.89 is a result of the compromise between the advantegeous equilibrium yield at low a_w's and the high biocatalytic activity of the enzyme at high a_w. At most low a_w's the enzyme is inhibited before equilibrium is reached.

ACKNOWLEDGEMENTS

The authors wish to thank Albert van der Padt, Anja Janssen and Peter Halling for the helpful discussions. The authors thank André van der Schaaf and André Meyerink for their contribution to this work and the department of Organic Chemistry for the help in the synthesis of the alkyl esters. Part of this work is financed by the Duth Programme Committee on Industrial Biotechnology (PCIB).

REFERENCES

Adlercreutz, P. (1991) On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* **199**: 609-614.

Blanco, R.M., Guisán, J.M., Halling P.J. (1992a) The equilibrium and kinetics of *N*-Acetyl-tryptophan phenylethyl ester synthesis by agarose-chymotrypsin in organic media. *Biotechn. Bioeng.* **40**: 1092-1096.

Blanco, R.M. Rakels, J.L.L., Guisán, J.M., Halling, P.J. (1992b) Effect of thermodynamic water activity on amino-acid ester synthesis catalyzed by agarose-chymotrypsin in 3-pentanone. *Biochim. Biophys. Acta* **1156**: 67-70.

Bloomer, S., Adlercreutz, P., Mattiasson, B. (1991) Triglyceride interesterification by lipases. 2. Reaction parameters for the reduction of trisaturated impurities and diglycerides in batch reactions. *Biocatalysis* **5**: 145-162.

Bodansky, M., Du Vigneaud, V. (1962) Synthesis of *p*-Nitrophenyl carbobenzoxyglycinate. *Biochem. Prep.* **9**: 110-112.

Cassels, J.M., Halling, P.J. (1988) Effect of thermodynamic water activity on thermolysin-catalyzed peptide synthesis in organic two-phase systems. *Enzyme Microb. Technol.* **10**: 486-491.

Ergan, F., Trani, M., André, G. (1990) Production of glycerides from glycerol and fatty acid by immobilized lipases in non-aqueous media. *Biotechn. Bioeng.* **35**: 195-200.

Fredenslund, A., Gmehhling, J., Rasmussen, P. (1977) *Vapor-Liquid Equilibria using UNIFAC.* Elsevier, New York, USA.

Goderis, H.L. Ampe, G., Feyten, M.P., Fouwé, B.L., Guffens, W.M., Van Cauwenbergh, S.M., Tobback, P.P. (1987) Lipase-catalyzed ester exchange reactions in organic media with controlled humidity. *Biotechn. Bioeng.* **30**: 258-266.

Goldberg, M., Thomas, D., Legoy, M.-D. (1990) The control of lipase-catalyzed transesterification and esterification reaction rates. Effects of substrate polarity, water activity and water molecules on enzyme activity. *Eur. J. Biochem.* **190**: 603-609.

Halling, P.J. (1984) Effects of water on equilibria catalyzed by hydrolytic enzymes in biphasic reaction systems. *Enzyme Microb. Technol.* **6**: 513-516.

Halling, P.J., Valivety, R.H. (1992) Physical-chemical nature of low water systems for biocatalysis: especially phase behaviour, water activity and pH. In: *Biocatalysis in Non-Conventional Media*, 13-21. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Halling, P.J. (1994) Thermodynamic predictions for biocatalysis in non-conventional media: Theory, tests, recommendations for experimental design and analysis. *Enzyme Microb. Technol.* **16**: 178-206.

Khan, S.A., Halling, P.J., Bell, G. (1990) Measurement and control of water activity with an aluminium oxide sensor in organic two-phase reaction mixtures for enzymic catalysis. *Enzyme Microb. Technol.* **12**: 453-458.

Valivety, R.H., Johnston, G.A., Suckling, C.J., Halling, P.J. (1991) Solvent effects on biocatalysis in organic systems: equilibrium position and rates of lipase catalyzed esterification. *Biotechn. Bioeng.* **38**: 1137-1143.

Valivety, R.H., Halling, P.J., Macrae, A.R. (1992a) Reaction rate with suspended lipase catalyst shows similar dependence on water activity in different organic solvents. *Biochim. Biophys. Acta* **1118**: 218-222.

Valivety, R.H., Halling, P.J., Macrae, A.R. (1992b) Effect of water activity on rate of lipase catalyzed esterification. In: *Biocatalysis in Non-Conventional Media*, 549-555. Tramper, J., Vermuë, M.H., Beeftink, H.H., Von Stockar, U. (Ed.) Elsevier Science Publ., Amsterdam, The Netherlands.

Van der Padt, A., Sewalt, J.J.W., Ágoston, S.M.I., Van 't Riet, K. (1992) *Candida rugosa* lipase stability during acylglycerol synthesis. *Enzyme Microb. Technol.* **14**: 805-812.

Van der Padt, A., Sewalt, J.J.W., Van 't Riet, K. (1993) On-line water removal during enzymatic triacylglycerol synthesis by means of pervaporation. *J. Membrane Sci.* **80**: 199-208.

Wehtje, E., Svensson, I., Adlercreutz, P., Mattiasson, B. (1993) Continuous control of water activity during biocatalysis in organic media. *Biotechnol. Techniques* **7**: 873-878.

Zaks, A., Klibanov, A.M. (1988) Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.* **263**: 3194-3201.

Chapter 8

THERMODYNAMICS: A CHALLENGE IN THE FIELD OF BIOCATALYSIS IN NON-CONVENTIONAL MEDIA

SUMMARY

The non-conventional media that are studied most for biocatalysis, consist of an apolar-solvent phase, in which substrate and product predominantly reside, and of the conventional polar aqueous phase, which contains the biocatalyst. Non-conventional media can therefore be considered as multiphase media. In these media the partition coefficients of water, substrates and products are very important. The partition coefficient influences the driving force in mass transfer and determines the concentration of the reaction components in the micro-environment of the biocatalyst and thus the reaction kinetics. The partition coefficient also determines the theoretical maximally achievable product concentration of a reaction.

Thermodynamics provide us with a powerful tool to calculate the partition coefficient by means of activity-coefficient relations. Especially for apolar components, good thermodynamic models are available. For electrolyte solutions the development of new models is emerging.

Application of thermodynamics helps to distinguish between the direct and indirect effects of the addition of apolar solvents and reveals the phenomena that govern biocatalysis in non-conventional media.

Thermodynamics: a challenge in the field of biocatalysis in non-conventional media.
Vermuë, M.H., Tramper, J. *Submitted for publication.*

Chapter 8

INTRODUCTION

The advantages of using apolar solvents as non-conventional media in bio-organic synthesis are well recognized (Halling, 1994, Vermuë and Tramper, 1995). Especially the increased solubility of apolar components and the ability to shift thermodynamic equilibrium in favor of synthesis, are often quoted to justify the use of these relatively apolar media. Despite these advantages, however, the industrial application of biocatalytic processes in non-conventional media is limited. Part of this is caused by the toxicity of many these media to the biocatalyst; another major reason is that the reaction mixtures are complex in nature.

The complexity of these media is mainly caused by the presence of a phase boundary between the apolar phase, which contains most of the apolar substrates and products, and the polar phase, which contains the biocatalyst. In these multiphase media biocatalysis is dictated by two types of interactions; on the one hand the direct interactions between the biocatalyst and the apolar phase or the interface, which results in molecular and interfacial toxicity effects (Vermuë *et al.*, 1993), and on the other hand the indirect effects caused by the partitioning of substrates, products and water over the phases. Here, we will focus on these indirect effects on biocatalysis in multiphase media and the role reversible thermodynamics can play to reveal the processes that govern biocatalysis in these media.

PARTITIONING IN BIOCATALYSIS IN NON-CONVENTIONAL MEDIA

The typical processes that govern biocatalysis in non-conventional media involving apolar solvents, are mass transfer of apolar substrate(s) from an apolar phase to the aqueous biocatalyst phase and of apolar product(s) *vice versa*, and the biotransformation by the biocatalyst. In the simplest case where two phases are present, a solute i is distributed among an organic apolar phase (or) and an aqueous phase (aq) until the thermodynamic activity of the solute i (a_i) is equal in both phases, provided that

the same standard reference state is used in each phase:

$$a_i^{or} = a_i^{aq} \qquad \text{or} \qquad x_i^{or}\gamma_i^{or} = x_i^{aq}\gamma_i^{aq} \tag{1}$$

where γ_i = activity coefficient and x_i is the mole fraction of solute i.

The thermodynamic activity of a solute at its standard state is conveniently assigned to be = 1. The choice of the standard state of a component is arbitrary, but it is usually defined at the temperature and pressure of the reaction system under study. For liquids, it is convenient to choose the pure liquid phase as the standard state. For diluted solutes, often the hypothetical infinite dilution state is choosen and the activity coefficient of the solute at infinite dilution $\gamma_i = 1$.

The partition coefficient P_i of a solute i over an organic apolar phase (or) and an aqueous phase (aq) is given by:

$$P_i = \frac{x_i^{or}}{x_i^{aq}} = \frac{\gamma_i^{aq}}{\gamma_i^{or}} \tag{2}$$

The partitioning of water, substrates and products over the phases determines their concentrations in the aqueous phase and thus the bioconversion rate of the reaction.

Apart from the influence of the media on the kinetics, the media also affect the product concentration at equilibrium, as illustrated in the following example of an esterification reaction.

The K_{eq} of the esterification between an alcohol (ROH) and a carboxylic acid (RCOOH) into an ester (RCOOR) and water (w) can be defined as:

$$K_{eq} = \frac{a_{RCOOR}\, a_w}{a_{ROH}\, a_{RCOOH}} \tag{3}$$

where K_{eq} is the thermodynamic equilibrium constant, and a_i is the thermodynamic activity of component i (Figure 1). The use of thermodynamic activities is very convenient, because at equilibrium the thermodynamic activity of any solute should be equal in each phase, and K_{eq} is constant and independent of the medium composition.

However, the yield of a reaction in non-conventional media is often expressed as

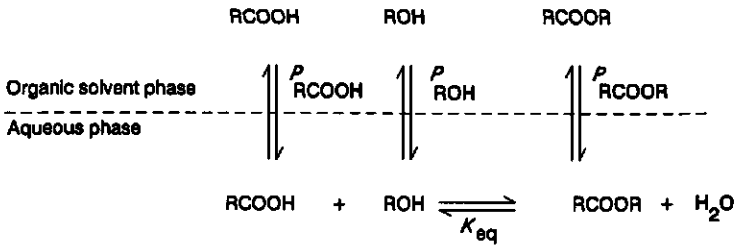


Figure 1: Schematic representation of a biocatalytic esterification reaction in a two-phase reaction system.

the concentration of the product in the apolar phase. For example, the yield of the esterification reaction is obtained by rewriting equation 3 in terms of concentrations, *c.q.* mole fractions of the solutes in the organic apolar phase (or):

$$x^{or}_{RCOOR} = K_{eq} \frac{x^{or}_{ROH}\, x^{or}_{RCOOH}}{a_w} \frac{\gamma^{or}_{ROH}\, \gamma^{or}_{RCOOH}}{\gamma^{or}_{RCOOR}} \qquad (4)$$

The activity coefficients cannot be measured directly, but must be estimated with thermodynamic models. These models describe the composition dependence of the activity coefficients and some of them will be discussed in the following section. With the help of these models the thermodynamic yield of a reaction can be predicted.

THERMODYNAMIC CALCULATION OF THE ACTIVITY COEFFICIENT

The activity coefficient γ_i can not be measured directly, but must be calculated from equilibria data. At equilibrium the thermodynamic activity of a solute is equal in all phases. It does not matter whether the activity is measured in the solid, the liquid or in the vapour phase, as long as the same standard state is used for each phase.

Often it is convenient to determine the activity of a component in the vapour

phase. At moderate pressures the vapour phase can be considered as an ideal gas which means that the activity of each component in the vapour phase may be calculated as the ratio between its partial pressure P_i and the saturated vapour pressure of the same component in its standard state P_i^{sat}.

$$a_i = \frac{P_i}{P_i^{sat}} \qquad (5)$$

For water and the relatively volatile organic solvents the required saturated vapour pressures are readily available (Perry & Green, 1984) and calculation of the activity coefficient of these components is thus straightforward.

For non-volatile components the required saturated vapour pressures are often missing in literature. For these components the activity coefficient must be obtained from solubility or partition data. If solubility data are available, it is convenient to choose the solid crystal as the standard state. When this is done for a saturated solution the following holds:

$$a_i^{sat} = \gamma_i x_i^{sat} = 1 \qquad (6)$$

Thus the activity coefficient γ_i is the reciprocal of the mole fraction solubility x_i^{sat}. The solubility of apolar components in water is often limited, so that the aqueous solution can be considered dilute. This means that γ_i is a constant for all aqueous solutions and independent of the solute concentration. So, if a non-volatile apolar compound is dissolved in a two-phase reaction mixture of water and apolar solvent, at equilibrium $x_i^{or}\,\gamma_i^{or} = x_i^{aq}\,\gamma_i^{aq}$ (equation 1). With the measured compositions of the aqueous phase x_i^{aq} and of the organic apolar phase x_i^{or} of a non-conventional reaction mixture, and with γ_i^{aq} being $1/x_i^{sat}$, the activity coefficient of the solute in the apolar phase γ_i^{or} can now be calculated.

If solubility data are not available, partition-coefficient data may be helpful. Especially for biphasic mixtures of water and organic solvents, these data are often available (Leo *et al.*, 1971). If the dilute solution in water is taken as the standard state,

the aqueous phase can be considered ideal (low concentration of solute *i*), *i.e.* the activity coefficient of *i* in the aqueous phase $\gamma_i^{aq} = 1$. The activity coefficient of *i* in the apolar phase can then be found *via*:

$$P_i = \frac{x_i^{or}}{x_i^{aq}} = \frac{1}{\gamma_i^{or}} \qquad (7)$$

The partition coefficients in literature are measured for dilute solutions in both the aqueous and the apolar phase. At high concentrations of solute, however, the partition coefficient is not constant, but depends on the phase composition. For these non-ideal solutions a thermodynamic model has to be used which describes the composition dependence of the activity coefficient (and hence of the partition coefficient).

In chemical engineering, numerous models have been developed to describe the relation between the medium composition and the activity coefficient. The most simple models assume that the solute molecules are randomly distributed in the solution and these models need only one or two adjustable parameters. These models often poorly describe the activity coefficient of apolar solutes in aqueous solutions. In aqueous solutions clustering of apolar solute molecules occurs, and the composition of the medium in the vicinity of the solute molecule clusters is different from the overall composition. For describing the activity coefficient in these non-ideal aqueous solutions, models should be used that take these local-composition effects into account. Examples of these models are the Wilson equation, the Non-Random Two-Liquid equation (NRTL) and the UNIQUAC equation (Prausnitz *et al.*, 1986).

In cases where the activity coefficient cannot be determined from vapour/liquid equilibrium data, or solubility and partitioning data, the activity coefficients can be estimated with the UNIFAC group-contribution method (Fredenslund *et al.*, 1977). In this predictive method the molecules in the reaction mixture are divided in groups. Each group contributes to the non-ideality of the whole reaction mixture, and thus to the activity coefficient of each component in the reaction mixture. It not only accounts for the relative volume and surface area of each group, but also for the interactions between

the groups of the molecules. The accuracy of the prediction of the activity coefficient of non-polar molecules is reasonably well, especially for vapour/liquid equilibria.

The UNIFAC method as well as most of the other models, however, do not accurately describe the activity coefficient of solutes in polar solutions and electrolyte solutions. This lack of good thermodynamic models is caused by the lack of sufficient data. This is not surprising. The chemical engineers who have developed the thermodynamic models, generally do not work with complex electrolyte solutions. Although the development of thermodynamic models for electrolyte solutions is slowly emerging (Pitzer, 1991), it is still immature. The people who generally work with electrolyte solutions, such as buffer solutions, are biochemical engineers. When they fully recognize the attractive features of the application of thermodynamics, more and more data will be generated and new models will be developed to sufficiently describe the non-ideality of electrolyte solutions.

Despite the lack of adequate models for polar and electrolyte solutions, current thermodynamic models may be helpful for biochemical engineers, especially for those who are working in the field of biocatalysis in non-conventional media, since these media often consist of apolar solutes and solvents. In the next section, typical examples will be given in which thermodynamics have already been applied succesfully to reveal the processes which govern biocatalysis in non-conventional media.

EFFECTS OF THE PARTITIONING OF WATER ON THE KINETICS

Water is essential for the performance of biocatalysts in non-conventional media (see review Halling, 1994). Although the amount of water can be limited to less than a monolayer of water molecules on the biocatalyst, some water must be present in the micro-environment of the biocatalyst, to keep the biocatalyst in its active configuration (Zaks and Klibanov, 1988).

When a biocatalyst is suspended in an apolar solvent, water in the system will tend to distribute between the micro-environment of the biocatalyst and the apolar bulk phase. When equilibrium is reached, the thermodynamic activity of the water molecules

a_w is equal in all phases.

The activity of a biocatalyst depends on the amount of bound water in its micro-environment. This has been illustrated for enzymes suspended in non-aqueous organic solvents (Zaks and Klibanov, 1988), but also holds for enzymes suspended in supercritical fluids (Marty *et al.*, 1992). When the micro-environment of the biocatalyst itself is not affected by the addition of apolar medium, the amount of bound water and thus the activity of a biocatalyst, depends only on the a_w of the reaction system. This has been illustrated for suspended lipase. When the intitial activity of the enzyme is plotted as a function of the water content of the medium, the position of the maximum activity varies widely for different solvents. However, if the same intitial activities are plotted as a function of the a_w, a similar dependence on a_w in different organic solvents is found (Valivety *et al.*, 1992a, Figure 2). Because the interactions of water with polar groups in the micro-environment of the active site of the biocatalyst may vary, the a_w-activity profile will vary for different enzymes (Valivety *et al.*, 1992b) or for enzymes immobilized on different support materials (Adlercreutz, 1991). The a_w is therefore a

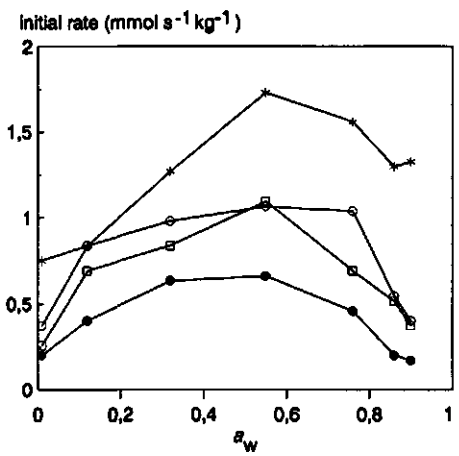


Figure 2: Activity of Lypozyme as a function of the a_w in hexane (*), toluene (○), trichloroethylene (□), and pentane-3-one (●) (data from Valivety *et al.*, 1992b).

better predictor of the reaction rate than the total amount of water in the reaction mixture. It should be measured and/or controlled when the effects of water or other conditions on kinetics in non-conventional media are studied (Halling, 1994).

PARTITIONING EFFECTS OF SUBSTRATES AND PRODUCTS ON KINETICS

In non-conventional media, apolar substrates must diffuse from the organic apolar phase into the aqueous environment of the biocatalyst before reaction can occur. If the biocatalyst is inhibited by the product, efficient removal of the product from the aqueous phase into the apolar phase is desirable. The activity of the biocatalyst in non-conventional media thus strongly depends on the concentration of substrate and product in the aqueous environment of the biocatalyst (Chatterjee and Russell, 1992).

If a reaction follows Michaelis-Menten kinetics the concentration and the K_m in the rate equation should be expressed in terms of the concentration in the aqueous environment around the biocatalyst and not in terms of the overall concentration or the concentration in the non-polar phase. If the solvent only affects the biocatalyst activity through indirect effects on the distribution of substrate and products, the K_m (expressed in aqueous concentration) should be constant, and independent of the solvent. When this does not hold, apparently direct interactions of the solvent with the biocatalyst play a role. Including the partitioning effects in the rate equations will thus help to distinguish between the indirect effects of partitioning and the direct interactions of the components in the reaction mixture with the biocatalyst.

This principle has already been applied to evaluate the effects of different organic solvents on the thermolysin-catalyzed peptide synthesis of CBZ-Asp-Phe-OMe from Z-Phe and PheOMe.HCl in an aqueous/organic biphasic reaction mixtures (Nakanishi and Matsuno, 1986). Upon changing the organic solvent from ethyl acetate to 1,2-dichloro-ethane or chloroform a decrease in initial activity (measurements based on the amount of peptide in the organic solvent phase) is found. This decrease can be ascribed totally to the partitioning of the substrates, especially of PheOMe.HCl between the phases. Due to the partitioning the substrate concentration in the micro-environment of the enzyme

is decreased and the pH of the aqueous phase changes. This pH change is caused by the splitting of the substrate PheOMe.HCl into PheOMe and HCl on partitioning and every HCl molecule is in the aqueous phase. After accounting for the partitioning effects on the aqueous substrate concentrate concentration and the pH the K_m^{aq} is approximately the same for all solvents tested.

Also in the transesterification of CBZ-Ala-ONp and CBZ-Leu-ONp with 1-butanol in organic-solvent reaction mixtures the initial activity of the enzyme (subtilisin Carlsberg adsorbed on silica particles) depends mainly on the aqueous substrate concentrations (Reimann *et al.*, 1994). The actual behaviour of the enzyme does not agree exactly with the predictions based on the partitioning of the substrate molecules. Direct contact between the solvent and the enzyme molecule causes residual effects. In the case of the transesterification, for example, the apparent K_m value ($= K_m^{or}$) increased upon changing the organic solvent from hexane to toluene. However, the K_m^{aq} ($= K_m^{or}/P$) decreased. After accounting for the partitioning effects, it thus becomes clear that addition of organic solvent actually decreases the K_m^{aq}, while the apparent K_m shows a reverse trend. The decrease of the K_m^{aq} is apparently caused by direct interactions between the enzyme and the solvents. This example illustrates how thermodynamics help to reveal the direct interactions that take place between the solvent and the biocatalyst.

Another example is the intitial activity of mushroom tyrosinase, which shows a bell-shaped relationship with the substrate concentration in the aqueous phase, independent of the organic solvent used (Yang and Robb, 1994). The enzyme activity increases with the aqueous substrate concentration to reach a maximum velocity, and then gradually decreases due to substrate inhibition.

Other proof that the aqueous concentration of solutes is the key parameter for the kinetics in non-conventional media has been found for reactions where substrate and/or product inhibition takes place. This is the case for the biphasic oxidation of benzyl alcohol by free and immobilized whole cells of *Pichia pastoris* (Kawakami and Nakahara, 1994). In aqueous medium a maximum production rate of benzaldehyde is reached at an initial substrate concentration of *ca.* 29 g l^{-1}. In 95% v/v xylene the intitial reaction rate of this oxidation shows a maximum at an intitial overall substrate

concentration of *ca.* 40 g l^{-1}. However, if the initial overall substrate concentration is translated into aqueous substrate concentrations, by accounting for partioning behaviour, the same maximum in initial productivity is found. The intitial rate is thus predominantly determined by the partition behaviour of the substrate and the product between the phases. The organic-solvent phase only serves as a reservoir for the substrate and the product. The partition coefficient is thus one of the criteria for selection of a suitable solvent for biocatalysis in a two-liquid-phase system (Chapter 3).

Some of the reactions in the above examples have been executed at $a_w < 1$ (Reimann *et al.*, 1994, Yang and Robb, 1994). The biocatalytic activity is then determined by the partitioning of substrate between the reaction medium and the polar biocatalyst phase. The presence of a distinct aqueous "interphase" between the bulk medium and the biocatalyst itself is not essential, and the activity of the biocatalyst is thus actually only determined by the partitioning of substrates and products over the apolar organic phase and the active site of the biocatalyst (Ryu and Dordick, 1992, Halling, 1994, Reimann *et al.*, 1994, Yang and Robb, 1994).

In principle, this also holds for living cells. However, the apolar solvents do not necessarily affect the active site of the enzymes, involved in the bioconversion reaction, but rather the cell membrane. If the cell membrane is affected by the partitioning of the apolar coumpounds, the biocatalytic activity of the cellular biocatalyst will be affected (Vermuë *et al.*, 1993).

EFFECTS OF PARTITIONING ON MASS TRANSFER

For biocatalysis in non-conventional media the substrate molecules have to pass at least one phase boundary, before catalysis can take place (Figure 3). The substrate molecules diffuse from the non-polar bulk phase, into the aqueous biocatalyst phase, where it migrates to the active site of the biocatalyst. In addition, if product accumulation and inhibition at the active site has to be avoided, subsequent diffusion of the products from the active site to the bulk phase has to take place efficiently.

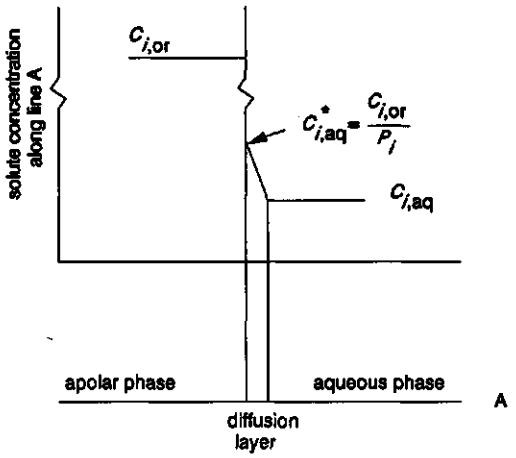


Figure 3: Mass transfer from a non-polar bulk phase to an aqueous biocatalyst phase.

The mass-transfer rate (*MTR*) of a component *i* from the non-polar bulk phase to the aqueous biocatalyst phase can be represented by:

$$MTR = K_i A(C^*_{i,aq} - C_{i,aq}) = K_i A(\frac{C_{i,or}}{P_i} - C_{i,aq}) \qquad (8)$$

where K_i = overall aqueous phase mass-transfer coefficient for external diffusion (m s^{-1}),
A = specific surface area of the aqueous biocatalyst phase (m^2 surface area per m^3 bulk phase),
$C_{i,aq}^*$ = concentration in the aqueous biocatalyst phase as would be in equilibrium with the actual concentration in the organic phase (mol m^{-3}),
$C_{i,aq}$ = concentration in the aqueous biocatalyst phase (mol m^{-3}),
$C_{i,or}$ = concentration in the apolar phase (mol m^{-3}),
P_i = partition coefficient of the component over the apolar phase and the biocatalyst phase.

A similar expression can be derived for the mass-transfer rate from the biocatalyst phase to the apolar bulk phase.

Equation 8 shows that the difference between the equilibrium concentration $C_{i,aq}^*$

and the actual concentration in the aqueous biocatalyst phase $C_{i,aq}$ forms the driving force for mass transfer. The equilibrium concentration depends on the actual concentration in the apolar phase $C_{i,or}$ and the partition coefficient P_i between the apolar bulk phase and the aqueous biocatalyst phase.

In addition, the partition coefficient may influence the overall mass-transfer coefficient K_i, which is given by:

$$\frac{1}{K_i} = \frac{1}{P_i k_{i,or}} + \frac{1}{k_{i,aq}} \qquad (9)$$

where $k_{i,or}$ = partial mass-transfer coefficient in the apolar phase
$k_{i,aq}$ = partial mass-transfer coefficient in the aqueous biocatalyst phase

The $k_{i,or}$ depends mainly on the diffusion coefficient of the solute in the apolar organic phase (Vermuë *et al.*, 1994). The diffusion coefficient increases with decreasing viscosity (Wilke and Chang, 1955) and therefore the diffusion coefficient in the relatively non-viscous organic solvents will be larger than in water. For apolar solutes with $P_i \gg 1$ and in relatively non-viscous apolar media, $1/(P_i\ k_{i,or}) \ll 1/k_{i,aq}$ and mass-transfer resistence is therefore completely situated in the aqueous biocatalyst phase. However, when viscous media are applied and the partition coefficient P_i is not very high, $1/(P_i\ k_{i,or})$ will on the contrary attribute to a decrease in overall mass-tranfer coefficient K_i.

The partition coefficient is thus a major parameter in mass transfer. It determines the driving force (equation 8) and it may influence the overall mass-transfer coefficient (equation 9). As has been shown in the previous section, the partition coefficient is a typical thermodynamic property, which can be estimated by thermodynamic models. Thermodynamics may thus help to estimate mass transfer in non-conventional media and can be used to calculate *a priori* if mass transfer will be limiting and what the effect of changes in the bulk non-polar phase will be.

EFFECTS OF PARTITIONING ON THE EQUILIBRIUM YIELD

The addition of an apolar solvent to the reaction medium will cause partitioning

of water, substrates and products over the phases in the reaction mixture. This will not only affect the kinetics of the reaction, but also the equilibrium yield. The effects on the equilibrium have been discussed by Halling (1984), Semenov *et al.* (1988), Eggers *et al.* (1989), Valivety *et al.* (1991) and Janssen *et al.* (1993).

When the equilibrium constant K_{eq} of a reaction is given in terms of thermodynamic activities, it will be constant and independent of the solvent (provided that the same standard state is used in each reaction system). It has already been stressed, however, that synthetic yield should preferably be represented in concentrations or mole fractions, rather than in thermodynamic activities, and therefore it is convenient to express the equilibrium constant in terms of concentrations $K_{c,eq}$.

If the esterification reaction

$$ROH + RCOOH \rightleftarrows RCOOR + H_2O,$$

is again chosen as an example, the $K_{c,eq}$ is given by:

$$K_{c,eq} = \frac{x_{ROH}\, x_{RCOOH}}{x_{RCOOR}\, x_w} = \frac{\gamma_{RCOOR}\, \gamma_w}{\gamma_{ROH}\, \gamma_{RCOOH}} K_{eq} \qquad (10)$$

where K_{eq} is given by equation 3. The mole fractions x_i and activity coefficients γ_i refer to either the apolar or the aqueous phase. Similar expressions can be derived for other reactions.

The $K_{c,eq}$ will vary in different solvents due to the change of the activity coefficients of the reactants in the solvents (equation 10). For estimation of an activity coefficient, thermodynamic models can be a useful tool as discussed above. This has been illustrated for the lipase-catalyzed acylglycerol synthesis in several organic solvents, and for the esterification of decanoic acid and various alcohols using the UNIFAC group-contribution method to predict the activity coefficients of the reactants (Janssen, 1993).

If the $K_{c,eq}$ in reaction mixture A and B are compared:

$$\frac{K^{A}_{c,eq}}{K^{B}_{c,eq}} = \frac{\gamma^{A}_{RCOOR}\, \gamma^{A}_{w}}{\gamma^{A}_{ROH}\, \gamma^{A}_{RCOOH}} \frac{\gamma^{B}_{ROH}\, \gamma^{B}_{RCOOH}}{\gamma^{B}_{RCOOR}\, \gamma^{B}_{w}} = \frac{P_{RCOOR}\, P_w}{P_{ROH}\, P_{RCOOH}} \qquad (11)$$

it can be observed that the ratio depends on the partition coefficient of the reactants over

solvent B and A. Taking the logarithms of equation 11, gives:

$$\log\frac{K^{A}_{c,eq}}{K^{B}_{c,eq}} = \log P_{RCOOR} + \log P_{w} - \log P_{ROH} - \log P_{RCOOH} \qquad (12)$$

When each $\log P_i$ is expressed as a sum of group contributions (similar to the procedure followed in the UNIFAC method) only the reactive groups will contribute to the ratio in $K_{c,eq}$'s, and this ratio will be constant for all reactions of a given type (Halling, 1990). For all esterification reactions, for example, it will be the contribution of an ester group and of water minus those of a hydroxyl group and a carbonyl group. Changing from solvent A to solvent B in a given esterification reaction will thus result in a shift in the $K_{c,eq}$ which is always the same, independent of the alcohol and carboxylic acid used. Halling (1990) showed that it is possible to predict the shift in equilibrium of several biocatalytic reactions upon changing solvent.

Thermodynamic analysis may also help to distinguish between effects of the

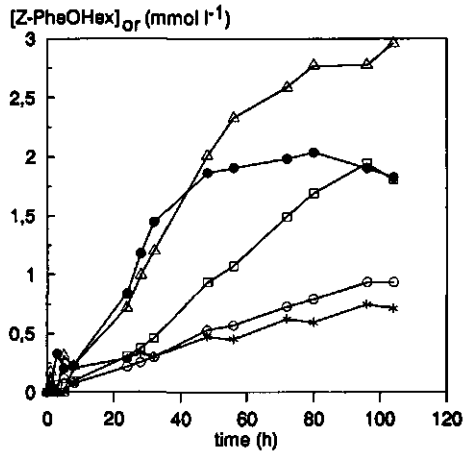


Figure 4 Progress of the Z-PheOHex synthesis catalyzed by α-chymotrypsin immobilized on celite in isooctane. The reaction medium is pre-equilibrated during 3 days at 303 K by recycling of dried air to bring the a_w to 0.75 (*), 0.80 (○), 0.84 (□), 0.89 (△) and 0.97 (●).

reaction conditions on the kinetics and the equilibrium yield. This has been illustrated in Chapter 7 of this thesis, where the effect of the a_w on the chymotrypsin catalyzed esterification of hexanol and Z-PheOH has been studied in isooctane (Figure 4). After 100 h reaction time the highest ester yield is obtained at a_w = 0.89. Thermodynamic analysis has revealed that this maximum in yield cannot be explained by accounting for partitioning effects and non-ideal behaviour of the substrates in the reaction mixture. In this case direct interactions of medium compounds with the biocatalyst, must have influenced enzyme kinetics and denaturation.

It is obvious that thermodynamics may help to predict the shift in equilibrium position upon changing the solvent. Sofar, the predictions have only been successful for the reactions which involve apolar compounds. For more polar compounds and electrolytes, adequate thermodynamic models are missing to predict the effects of changes of solvent on equilibrium position.

CONCLUDING REMARKS

The complexity of non-conventional media is mainly caused by the presence of multiple phases. Partitioning of water, substrates and products over the pertinent phases affect the kinetics as well as the equilibrium position of a given reaction in these media. Partition can be expressed as the ratio between activity coefficients in a two-phase system. For the estimation of the activity coefficients of apolar components, several adequate thermodynamic models are available. For electrolyte solutions and solutions of polar compounds, however, the availability of good thermodynamic models is limited, and this is one of the research fields where biochemical engineers can attribute by providing data to develop predictive models, which adequately describe activity coefficients in electrolyte and polar solutions.

When the physical effects of the partitioning of water, substrates and products is included in the mass-transfer equations, the reaction-rate equations and in the calculation of the equilibrium yield, the biochemical engineer can focus on the residual effects, which can be explained in terms of direct interactions of the solvent with the biocatalyst.

ACKNOWLEDGEMENTS

Many of the ideas presented in this chapter originate from the many fruitful discussions with John Prausnitz, Peter Halling, Luuk van der Wielen, Jacob de Swaan Arons and the other lecturers of the advanced course "Thermodynamics for Biochemical Engineers" which was held in Toulouse, 12-16 December 1994, and which was organized under auspices of the European Science Foundation.

REFERENCES

Adlercreutz, P. (1991) On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* **199**: 609-614.

Chatterjee, S., Russell, A.J. (1992) Determination of equilibrium and individual rate constants for subtilisin-catalyzed transesterification in anhydrous environments. *Biotech. Bioeng.* **40**: 1069-1077.

Eggers, D.K., Blanch, H.W., Prausnitz, J.M. (1989) Extractive catalysis: solvent effects on equilibria of enzymatic reactions in two-phase systems. *Enzyme Microb. Technol.* **11**: 84-89.

Fredenslund, A., Gmehling, J., Rasmussen, P. (1977) *Vapor-liquid equilibrium using UNIFAC*, Elsevier, New York.

Halling, P.J. (1984) Effects of water on equilibria catalyzed by hydrolytic enzymes in biphasic reaction systems. *Enzyme Microb. Technol.* **6**: 513-516.

Halling, P.J. (1994) Thermodynamic predictions for biocatalysis in nonconventional media: Theory, tests, and recommendations for experimental design and analysis. *Enzyme Microb. Technol.* **16**: 178-206.

Janssen, A. (1993) Enzymatic synthesis of polyol esters in aqueous-organic two-phase systems. PhD-Thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Kawakami, K., Nakahara, T. (1994) Importance of solute partitioning in biphasic oxidation of benzyl alcohol by free and immobilized whole cells of *Pichia pastoris*. *Biotechnol. Bioeng.* **43**: 918-924.

Leo, A.L., Hansch, C., Elkins, D. (1971) Partition coefficients and their uses *Chem. Rev.* **71**: 525-616.

Marty, A., Chulalaksananukul, W., Willemot, R.M., Condoret, J.S. (1992) Kinetics of lipase-catalyzed esterification in supercritical CO_2. *Biotechnol Bioeng.* **39**: 273-280.

Nakanishi, K., Matsuno, R. (1986) Kinetics of enzymatic synthesis of peptides in aqueous/organic biphasic systems. Thermolysin-catalyzed synthesis of *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine methyl ester. *Eur. J. Biochem.* **161**: 533-540.

Perry, R.H., Green, D.W. (1984) *Perry's chemical engineers' handbook.* 6th ed. McGraw-Hill Book Comp., New York, USA.

Pitzer, K.S. (1991) *Activity coefficients in electrolyte solutions.* 2nd ed. CRC press, Boca Raton, USA.

Prausnitz, J.M., Lichtenthaler, R.N., De Azevedo, E.G. (1986) *Molecular thermodynamics of fluid-phase equilibria.* Prentice-Hall, Englewood Cliffs, New York.

Reimann, A., Robb, D.A., Halling, P. J. (1994) Solvation of CBZ-amino acid nitrophenyl esters in organic media and the kinetics of their transesterification by subtilisin. *Biotechnol. Bioeng.* **43**: 1081-1086.

Ryu, K., Dordick, J.S. (1992) How do organic solvents affect peroxidase structure and function? *Biochemistry* **31**: 2588-2598.

Semenov, A.N., Cerovsky, V., Totiv, M.I., Martinek, K. (1988) Enzymes in preparative organic synthesis: the problem of increasing the yield of product. *Coll. Czech. Chem. Commun.* **53**: 2963-2985.

Valivety, R.H., Johnston, G.A., Suckling, C.J., Halling, P.J. (1991) Solvent effects on biocatalysis in organic systems: equilibrium position and rates of lipase catalyzed esterification. *Biotechnol. Bioeng.* **38**: 1137-1143.

Valivety, R.H., Halling, P.J., Macrae, A.R. (1992a) Reaction rate with suspended lipase catalyst shows similar dependence on water activity in different organic solvents. *Biochim. Biophys. Acta* **1118**: 218-222.

Valivety, R.H., Halling, P.J., Peilow, A.D., Macrae, A.R. (1992b) Lipases from different sources vary widely in dependence of catalytic activity on water activity. *Biochim. Biophys. Acta* **1122**: 143-146.

Vermuë, M., Sikkema, J., Verheul, A., Bakker, R., Tramper, J. (1993) Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp.. *Biotechnol. Bioeng.* **42**: 747-758.

Vermuë, M.H., Tacken, M., Tramper, J. (1994) Tetralin and oxygen transfer in the liquid-impelled loop reactor. *Bioprocess Eng.* **11**, 224-228.

Vermuë, M.H., Tramper, J. (1995) Biocatalysis in non-conventional media: medium engineering aspects. *Pure & Appl. Chem.* **67**: 345-373.

Wilke, C.R., Chang, P. (1955) Correlation of diffusion coefficients in dilute solutions. *Am. Inst. Chem. Eng. J.* **1**: 264-270.

Yang, Z., Robb, D.A. (1994) Partition coefficients of substrates and products and solvent selection for biocatalysis under nearly anhydrous conditions. *Biotechnol. Bioeng.* **43**: 365-370.

Zaks, A., Klibanov, A.M. (1988) The effects of water on enzyme action in organic media. *J. Biol. Chem.* **263**: 8017-8021.

SUMMARY

During the past decade biocatalysis in non-conventional media has gained a lot of interest. Especially in the field of bio-organic synthesis, where poorly water-soluble substrates and products are involved, these media are very attractive.

Non-conventional media generally consist of an apolar solvent phase and an aqueous phase. In this thesis, mixtures of water with water-miscible organic solvents, or water-immiscible organic solvents or (near-)supercritical solvents are described. The conventional aqueous phase contains the cellular or enzymic biocatalyst. The aqueous phase can vary from a dilute aqueous solution, with a thermodynamic water activity a_w close to 1, to a dried enzyme particle with only a monolayer of adsorbed water molecules ($a_w < 1$).

In non-conventional media biocatalytic processes are governed by the presence of a phase boundary when two phases are involved. This phase boundary not only influences the rate of the bioconversion (kinetics), but also the yield of the reaction (thermodynamic equilibrium). In this thesis, several factors are described which affect the kinetics and thermodynamics of biocatalytic porcessen in non-conventional media.

Chapter 2 gives an overview of the recent developments in the field of medium engineering for biocatalysis in non-conventional media. In this chapter a few basic design rules for the rational design are formulated. These rules may serve as useful tools for optimization of biocatalytic processes in non-conventional media.

A typical example of a non-conventional reaction medium is the mixture of water and water-immiscible organic solvent. Especially for this type of reaction media the liquid-impelled loop reactor has been developed. This reactor has been used for the bioconversion of tetralin, a very toxic apolar compound. In Chapter 3 the general

strategy for the selection of a suitable solvent for the bioconversion of such toxic apolar compounds in the liquid-impelled loop reactor is given, where the tetralin conversion is used as a typical example. The water-immiscible solvents should be non-toxic and non-biodegradable. Additionally, they should reduce the toxicity of the apolar substrate and they must be practical for use in the liquid-impelled loop reactor. All the steps in the selection procedure proved to be essential. Among the 57 solvents tested, only FC-40 proofs to be suitable for bioconversion of tetralin in the liquid-impelled loop reactor. In addition, the cellular biocatalyst needs to be immobilized, to reduce emulsion formation inside the bioreactor.

For the bioconversion of tetralin in the liquid-impelled loop reactor oxygen is needed. Chapter 4 describes the mass transfer of tetralin and oxygen in the liquid-impelled loop reactor from the apolar solvent phase to the aqueous phase, where the bioconversion occurs. It is found that in case of mass-transfer limitation, tetralin is the rate-limiting substrate and not oxygen.

One of the selection criteria of a suitable solvent for bioconversion of apolar substates is its non-toxicity for the biocatalyst. The $\log P_{octanol}$, which describes the hydrophobicity of the solvent, is a good measure for the toxicity of the solvent in a two-liquid phase system. The toxicity of a water-immiscible solvent for cellular biocatalyst is caused by two factors, *i.e.* the presence of a phase boundery (phase toxicity) and by the solvent molecules that are dissolved in the aqueous phase (molecular toxicity). Chapter 5 describes these effects separately. When the solvent concentration in the membrane of the cellular biocatalyst reaches a critical concentration, the solvent becomes toxic. The toxic concentration in the membrane is constant and independent of the solvent used. It is directly related *via* the partition coefficient over the membrane and water, to the solvent concentration in the aqueous phase. This is in turn directly related to the $\log P_{octanol}$ of the solvent. If the critical membrane concentration of a certain microorganism is known, the toxicity of any solvent can be predicted with the $\log P_{octanol}$.

Apart from the $\log P_{octanol}$, also the Hildebrand solubility parameter δ can be used as a measure of the hydrophobicity of the solvent. In Chapter 6 this parameter has been

used successfully as an indicator of the solubility of apolar compounds in near-supercritical carbon dioxide ($SCCO_2$). In addition, the effect of this parameter on the transesterification rate of Lypozyme in this non-aqueous reaction medium has been studied. The change in δ of near-supercritical carbon dioxide hardly influences the reaction rate. The water content of the medium influences the kinetics much more.

Water not only affects the kinetics of a synthetic reaction, but it also affects the equilibrium yield of these reactions. When the thermodynamic water activity a_w is decreased, water-dependent side-reactions such as in transesterification reactions are suppressed (Chapter 6). In esterification reactions, a shift in equilibrium towards synthesis is expected upon decreasing the a_w.

Chapter 7 describes a new method to control the a_w during esterification reactions. With this a_w-control method the a_w can be maintained at an optimal value, at which the biocatalyst still shows sufficient activity while a high thermodynamic product yield can be obtained.

This thesis actually covers two central themes in biocatalysis in non-conventional media: kinetics and thermodynamics. In Chapter 8 a general discussion highlights how thermodynamics can be used as a basic tool to reveal the processes that govern biocatalysis in non-conventional media.

SAMENVATTING

Gedurende de laatste jaren is de interesse in biokatalyse in ongebruikelijke media sterk toegenomen. Vooral binnen het gebied van de bioorganische synthese, waarin veel met apolaire uitgangsstoffen en produkten wordt gewerkt, zijn de ongebruikelijke media aantrekkelijk.

Ongebruikelijke media bestaan in het algemeen uit een oplosmiddelfase en een waterige fase. De oplosmiddelfase, zoals die wordt beschreven in dit proefschrift, kan bestaan uit een mengsel van water met water-mengbaar organisch oplosmiddel, of uit een niet-watermengbaar oplosmiddel of uit een (nabij-)superkritisch oplosmiddel. De konventionele waterige fase bevat de cellulaire of enzymatische biokatalysator. De waterige fase kan variëren van een verdunde waterige oplossing met een thermodynamische wateraktiviteit a_w die vrijwel gelijk is aan 1, tot een monolaag van watermolekulen geadsorbeerd aan het enzymdeeltje met $a_w < 1$.

In ongebruikelijke media worden biokatalytische processen beïnvloed door de aanwezigheid van een fasegrensvlak wanneer twee fasen aanwezig zijn. Dit fasegrensvlak beïnvloedt niet alleen de snelheid waarmee de biokatalysator de uitgangsstoffen omzet in produkt (de kinetiek), maar ook de uiteindelijke opbrengst van de reaktie (de thermodynamisch evenwichtsligging). In dit proefschrift worden verschillende faktoren beschreven die de kinetiek en de thermodynamische evenwichtsligging van biokatalytische processen in een aantal verschillende ongebruikelijke media beïnvloeden.

In Hoofdstuk 2 wordt een overzicht van de laatste ontwikkelingen op het gebied van medium ontwikkeling voor biokatalyse in ongebruikelijke media gegeven. In dit hoofdstuk worden een aantal basisregels gegeven voor het samenstellen van geschikte media, die kunnen dienen als gereedschap voor de optimalisatie van biokatalytische processen in ongebruikelijke media.

Samenvatting

Een typisch voorbeeld van een niet-konventioneel reaktiemedium is een mengsel van water en water-onmengbaar organisch oplosmiddel. Speciaal voor dit soort reaktiemedia is de "liquid-impelled loop" reaktor ontworpen. Deze reaktor is gebruikt voor de biologische omzetting van tetraline, een zeer toxische apolaire stof. In Hoofdstuk 3 wordt de algemene strategie voor de selektie van een geschikt oplosmiddel voor de biologische omzetting van dit soort toxische apolaire stoffen in de "liquid-impelled loop" reaktor gegeven, waarbij tetraline als typisch voorbeeld wordt gebruikt. De oplosmiddelen mogen niet toxisch zijn en niet door de biokatalysator worden afgebroken. Ze moeten bovendien de toxiciteit van de apolaire substraten verminderen en ze moeten praktisch toepasbaar zijn in de "liquid-impelled loop" reaktor. Na het volgen van de selektiestrategie blijkt dat van de 57 geteste oplosmiddelen alleen FC-40 geschikt is als oplosmiddel voor de biologische omzetting van tetraline. Bovendien is immobilisatie van de cellulaire biokatalysator nodig om emulsievorming in de bioreaktor tegen te gaan.

Voor de omzetting van tetraline in de "liquid-impelled loop" reaktor is zuurstof nodig. Hoofdstuk 4 beschrijft het massatransport van tetraline en zuurstof in de "liquid-impelled loop" reaktor vanuit de apolaire oplosmiddelfase naar de waterige fase, waar de biologische tetralineomzetting plaatsvindt. Gevonden is dat bij massatransportlimitatie tetraline de snelheidslimiterende uitgangsstof is en niet zuurstof.

Een geschikt oplosmiddel voor biologische omzetting van apolaire substraten mag niet toxisch zijn voor de biokatalysator. De $\log P_{octanol}$, die het hydrofobe karakter van het oplosmiddel beschrijft, blijkt een goede maat voor de toxiciteit van de oplosmiddelen in een twee-fasen systeem. De toxiciteit van een niet-watermengbaar organisch oplosmiddel voor cellulaire biokatalysatoren wordt veroorzaakt door 2 faktoren, te weten de aanwezigheid van een fasegrensvlak (fasetoxiciteit) en door molekulen van het oplosmiddel die opgelost zijn in de waterige fase (molekulaire toxiciteit). In Hoofdstuk 5 worden deze afzonderlijke effekten beschreven. Wanneer de oplosmiddelkoncentratie in het membraan een kritische koncentratie overschrijdt, wordt het oplosmiddel toxisch. Deze kritische koncentratie in het membraan is voor ieder oplosmiddel gelijk en is *via* de partitiekoëfficient direct gerelateerd aan de oplosmiddelkoncentratie in de waterfase. Op zijn beurt is deze gerelateerd aan de $\log P_{octanol}$ van een solvent. Als de kritische

membraankoncentratie voor een bepaald microorganisme bekend is, kan de molekulaire toxiciteit van een oplosmiddel met behulp van de $\log P_{octanol}$ worden voorspeld.

Als maat voor de hydrofobiciteit van het medium wordt in plaats van de $\log P_{octanol}$ waarde ook de Hildebrand solubility parameter (δ) gebruikt. In hoofdstuk 6 wordt beschreven hoe de δ van nabij-superkritisch koolzuur ($SCCO_2$) als indicator kan dienen om de oplosbaarheid van apolaire stoffen in dit medium te voorspellen. Daarnaast is gekeken naar het effect van deze parameter op de transesterifikatiesnelheid van Lypozyme in dit niet-waterig reaktiemedium. De verandering in δ van het nabij-superkritisch oplosmiddel is nauwelijks van invloed op de reaktiesnelheid. Het watergehalte in dit medium blijkt daarentegen van veel groter belang.

Water heeft niet alleen invloed op de biokinetiek, maar beïnvloed ook de thermodynamische evenwichtsligging van synthetische reakties. Als de thermodynamische wateraktiviteit a_w wordt verlaagd, worden water-afhankelijke zijreakties zoals in trans-esterifikatiereakties onderdrukt (Hoofdstuk 6). In esterifikatiereakties wordt bij verlaging van de a_w een verschuiving van het reaktie-evenwicht in de richting van de synthese-reaktie verwacht.

In Hoofdstuk 7 wordt een nieuwe methode beschreven waarmee de a_w gedurende een esterifikatiereaktie konstant worden gehouden. Met behulp van deze methode kan de a_w op een optimale waarde worden gehandhaafd, waarbij de biokatalysator nog voldoende aktief is en toch een hoge produktkoncentratie kan worden bereikt.

Uit het voorafgaande blijkt dat dit proefschrift twee centrale thema's bevat; de kinetiek en de thermodynamika. In Hoofdstuk 8 wordt in een algemene diskussie expliciet aangegeven hoe thermodynamika kan worden gebruikt als basis gereedschap om de processen die zich afspelen tijdens biokatalyse in ongebruikelijke media te doorgronden.

CURRICULUM VITAE

Marian Vermuë werd geboren op 1 april 1961 in het Zeeuws-Vlaamse dorpje Yzendijke. De middelbareschooltijd bracht zij door op het internaat "St. Anna", van waaruit zij de VWO opleiding volgde op het "Thomas Moore College" te Oudenbosch. Na het behalen van het diploma begon zij in 1979 met de HBO-opleiding voor botanisch analiste aan de STOVA te Wageningen, met plantenziektekundige specialisatie.

Na het beeindigen van deze opleiding begon zij in 1983 met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen, met als hoofdvakken Biochemie en Proceskunde. In 1988 sloot zij haar studie met lof af.

Van september 1988 tot november 1992 was zij werkzaam bij de sectie Proceskunde van de Landbouwuniversiteit te Wageningen, waar het onderzoek zoals beschreven in dit proefschrift werd verricht. Tijdens deze periode was zij als secretaris betrokken bij de organisatie van het internationale symposium "Biocatalysis in Non-Conventional Media" en als editor betrokken bij de bewerking van het symposiumboek.

Na de geboorte van haar twee kinderen Steven en Joris was zij vanaf juli 1994 gedurende 8 maanden werkzaam als onderwijsmedewerkster aan de Technische Universiteit te Delft. Daar was zij betrokken bij het organiseren van de kursus "Thermodynamics for Biochemical Engineers" en bij het samenstellen van het kursusmateriaal voor deze kursus. Deze kursus vond plaats in december 1994 in Toulouse.

Sinds 1 mei 1995 is zij werkzaam als post-doc bij de sectie Proceskunde van de Landbouwuniversiteit te Wageningen waar zij zich buigt over de vernieuwing van het diktaat "Inleiding Proceskunde" en zich bezig houdt met het opzetten van een nieuw onderwijselement "Opwerking en Kolomprocessen in de (Bio)Procestechnologie".