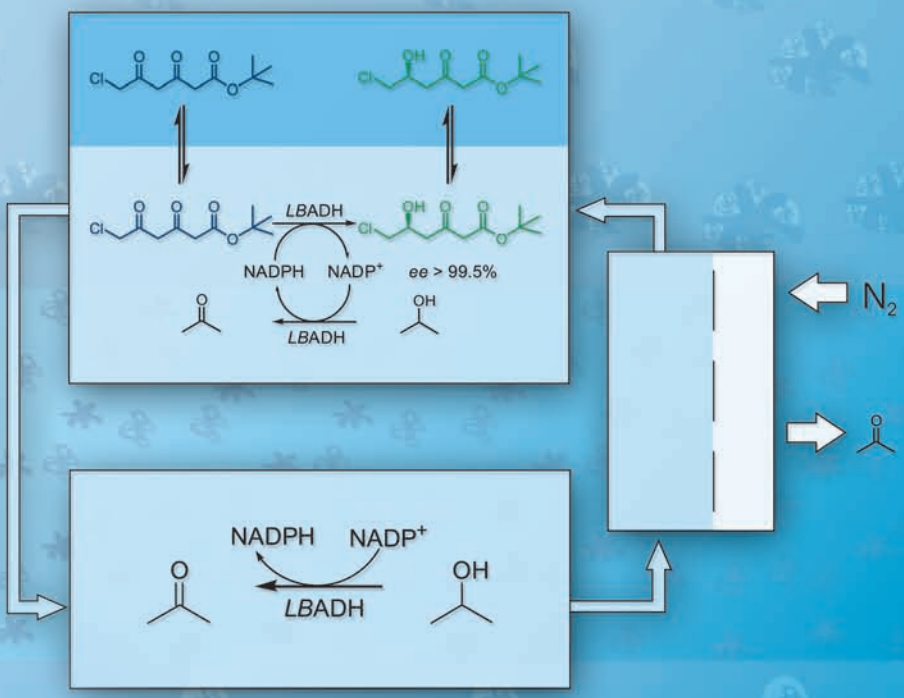
Forschungszentrum Jülich
in der Helmholtz-Gemeinschaft
50 Jahre Zukunft

Enantioselective Reduction of Hydrophobic Keto Compounds in Multiphase Bioreactor

Murillo Villela Filho



Lebenswissenschaften
Life Sciences

Schriften des Forschungszentrums Jülich
Reihe Lebenswissenschaften / Life Sciences Band / Volume 34

Forschungszentrum Jülich GmbH
Institut für Biotechnologie 2

Enantioselective Reduction of Hydrophobic Keto Compounds in Multiphase Bioreactor

Murillo Villela Filho

Schriften des Forschungszentrums Jülich
Reihe Lebenswissenschaften/Life Sciences Band/Volume 34

ISSN 1433-5549 ISBN 978-3-89336-470-1

Bibliographic information published by the Deutsche Nationalbibliothek.
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.d-nb.de.

Publisher and Distributor: Forschungszentrum Jülich GmbH
Zentralbibliothek
52425 Jülich
Phone +49 (0)2461 61-5368 · Fax +49 (0)2461 61-6103
e-mail: zb-publikation@fz-juelich.de
Internet: http://www.fz-juelich.de/zb

Cover Design: Grafische Medien, Forschungszentrum Jülich GmbH

Printer: Grafische Medien, Forschungszentrum Jülich GmbH

Schriften des Forschungszentrums Jülich
Reihe Lebenswissenschaften/Life Sciences Band/Volume 34

D 5 (Diss., Bonn, Univ., 2003)

ISSN 1433-5549
ISBN 978-3-89336-470-1

The complete volume is freely available on the Internet on the Jülicher Open Access Server (JUWEL) at http://www.fz-juelich.de/zb/juwel

Enantioselective Reduction of Hydrophobic Keto Compounds in Multiphase Bioreactor

Cardiovascular diseases are the main *causa mortis* in developed countries. Extensive epidemiological studies have shown that, increased levels of low density lipoprotein (LDL) cholesterol, are a major risk factor of cardiovascular diseases (*e.g.* coronary heart disease, stroke, etc.). Approaches for the therapy of hypercholesterolaemia focus on influencing the enzymatic formation of mevalonate. This is possible with statins, potent inhibitors of HMG-CoA reductase. The absolute stereochemistry in the 3,5-dihydroxy acid side chain is essential for this pharmacological activity. Therefore, an easy and "universal" synthetic approach to enantiopure 3,5-dihydroxyhexanoate is highly desirable. A new approach for the preparation of enantiopure 3,5-dihydroxyhexanoate structure was presented by Wolberg *et al*. in 2000. The key step of the synthesis is the introduction of the chirality via enzymatic reduction of 6-chloro-3,5-dioxohexanoate. The economical use of this route is limited by the costs of the cofactor and the low solubility and instability of the substrate in water. Hence, a reactor enabling high enzyme and cofactor turnover and simultaneously suppressing the substrate decay is required to enhance the attractiveness of the synthesis.

In the present work a bioreactor development for the regio- and enantioselective reduction of *tert*-butyl 6-chloro-3,5-dioxohexanoate (CDHE) to *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate catalysed by the alcohol dehydrogenase from *Lactobacillus brevis* (LBADH) was carried out. The irreversible substrate degradation and its kinetics were described. The enzymatic reduction of CDHE was characterised. The influence of reaction conditions on the enzyme stability was measured. Possibilities for the cofactor (NADPH) regeneration were studied, and the substrate coupled cofactor regeneration selected. The involved oxidation of 2-propanol was thermodynamically and kinetically described; problems caused by the arising acetone were discussed. In order to conciliate the antagonistic requirements of the involved reaction, the biphasic (aqueous/organic) reduction was conceived. Under this conditions the aqueous substrate concentration is kept low, preventing its decay. The biphasic system also enables the more efficient use of enzyme and cofactor. It was shown that log *P* is not a convenient criterion to guide the choice of the organic solvent. Thus, **a new approach for the organic solvent selection was developed**. The approach's generality was verified. Consequences (*e.g.* thermodynamic shift) and chances (*e.g.* enhancement of process selectivity) of enzymatic biphasic reductions were detailed. Using the biphasic reduction a hybrid bioreactor was designed and constructed enabling the suppression of the substrate decay (side reaction), the efficient reuse of the cofactor (*TTN* = 4200), the avoidance of thermodynamic limitation (via acetone removal), and the decrease in enzyme consume. The process selectivity was optimised (> 90%) using the simplex algorithm. The reactor was operated for 168 h. The reactor operation was described and simulated by a mathematical model. Finally, the developed process was evaluated under the economic point of view. The material cost of the synthetic route could be reduced by over 2/3 due to the improvements proposed.

Enantioselektive Reduktion von hydrophoben Ketoverbindungen im multiphasigen Bioreaktor

Kardiovaskuläre Erkrankungen sind die häufigste Sterbeursache in den entwickelten Ländern. Umfassende epidemiologische Studien haben gezeigt, dass erhöhte LDL (low density lipoprotein)-Spiegel ein Hauptrisikofaktor für kardiovaskuläre Erkrankungen (z.B. Koronare Herzkrankheit, Schlaganfall usw.) sind. Ansätze zur Therapie der Hypercholesterinämie zielen auf der Hemmung der enzymatischen Mevalonatbildung. Diese wird durch Statine, potente Inhibitoren der HMG-CoA Reduktase, ermöglicht. Die absolute Stereochemie der 3,5-Dihydroxysäuren-Seitenkette ist maßgeblich für die pharmakologische Aktivität. Folglich ist ein einfacher und universeller synthetischer Zugang zu enantiomerenreinen 3,5-Dihydroxyhexanoaten sehr erwünscht. Ein neuer Zugang zur Darstellung von enantiomerenreinen 3,5-Dihydroxyhexanoaten wurde von Wolberg *et al*. 2000 vorgestellt. Die Schlüssel-Reaktion ist die Einführung eines stereogenen Zentrums durch die enzymatische Reduktion von 6-Chloro-3,5-dioxohexanoat. Die Wirtschaftlichkeit dieses Zugangs wird durch die Kosten für den Cofaktor und die niedrige Löslichkeit und Instabilität des Substrats in Wasser beschränkt. Deshalb wird ein Reaktor benötig, der hohe Zyklenzahl für Enzym und Substrat mit gleichzeitiger Unterdrückung des Substratzerfalls ermöglicht, um die Attraktivität dieser Synthese zu erhöhen.

In dieser Arbeit wird die Entwicklung eines Bioreaktors zur regio- und enantioselektiven Reduktion von *tert*-Butyl-6-chloro-3,5-dioxohexanoat (CDHE) zu *tert*-Butyl-(*S*)-6-chloro-5-hydroxy-3-oxohexanoat, katalysiert durch die Alkoholdehydrogenase aus *Lactobacillus brevis* (LBADH), durchgeführt. Die irreversible Substratzersetzung und deren Kinetik wird beschrieben. Die enzymatische Reduktion von CDHE wird charakterisiert. Der Einfluss der Reaktionsbedingungen auf die Enzymstabilität wird gemessen. Möglichkeiten für die Regenerierung des Cofaktors (NADPH) werden untersucht, und die substratgekoppelte Regenerierung ausgesucht. Die beteiligte Oxidation von 2-Propanol wird thermodynamisch und kinetisch beschrieben, und die Folgen der Entstehung von Aceton werden betrachtet. Damit die antagonischen Anforderungen der beteiligten Reaktionen in Einklang gebracht werden können, wird eine biphasische (wässrige/organische) Reduktion entwickelt. Unter dieser Bedingung wird die wässrige Substratkonzentration geringgehalten und so die Zersetzung vermieden. Zusätzlich ermöglicht das biphasische System eine effizientere Nutzung von Enzym und Cofaktor. Es wird gezeigt, dass log *P* kein geeigneter Parameter zur Entscheidung über die organische Phase ist. Deswegen wird **eine neue Vorgehensweise zur Auswahl des organischen Lösungsmittels entwickelt**. Die Allgemeingültigkeit dieser Vorgehensweise wird verifiziert. Folgen (z.B. thermodynamischer Verschiebung) und Chancen (z.B. Erhöhung der gesamt Selektivität) der biphasischen enzymatischen Reduktion werden detailliert. Unter Verwendung der biphasischen Reduktion wird ein Hybrid-Bioreaktor konzipiert und gebaut. Der Bioreaktor ermöglicht die Unterdruckung der Substratzersetzung (Nebenreaktion), die effiziente Regenerierung des Cofaktors (*TTN* = 4200), vermeidet thermodynamische Limitierung (durch Acetonentfernung) und verringert den

Enzymverbrauch. Die Prozessselektivität wird unter Verwendung des Simplex-Algorithmus optimiert (> 90%). Der Reaktor wurde für 168 h betrieben. Der Reaktorbetrieb wird durch ein mathematisches Modell beschrieben und simuliert. Zum Schluss wird das Verfahren aus wirtschaftlichen Gesichtspunkten evaluiert. Die Materialkosten der Synthese konnten durch die erreichten Verbesserungen um mehr als 2/3 verringert werden.

The work towards this doctoral thesis was carried out between April 1998 and September 2003 at the Chair of Biotechnology of the Rheinische Friedrich-Wilhelms-Universität, Bonn, under supervision of Prof. Dr. C. Wandrey in the laboratories of the Institut für Biotechnologie of the Forschungszentrum Jülich GmbH.

ACKNOWLEDGEMENTS

I wish to express my thankfulness to Professor Christian Wandrey, my *Doktorvater*, for the incomparable opportunities offered in his institute, for stimulating and fruitful discussions and for being the mentor I wished to meet. This work was made possible through his support and generosity over many years.

I would like to thank Professor Heinrich Wamhoff, Kekulé-Institut für Organische Chemie und Biochemie of the Rheinische Friedrich-Wilhelms-Universität, Bonn, for his willingness to become the co-examiner in my thesis-committee.

I am greatly indebted to Mrs. Heike Offermann without whose motivated and skilful work the achieved results would not have been possible.

I would like to thank Mr. Thomas Stillger for his innovative work on acetone removal during his *Diplomarbeit*, as well as the friendly collaboration in the research into the field of enzyme stability in the presence of organic solvents.

Further, I would like to express my gratitude to Dr. Michael Müller, for the interesting research proposal and for the opportunity of learning much about how to make science from him,

Professor Andreas Liese for the friendly working atmosphere, the freedom of work, the idea of the acetone removal and his open door policy,

Mrs. Ursula Mackfeld for sharing her insight into the process development of enzymatic reactions and many fruitful suggestions,

Christoph Hoh and specially Andrea Rentmeister for countless contributions to improvements of this work,

Dr. Werner Hummel and Mrs. Vera Ophoven for the friendly supply of LBADH,

Theodora Chalazia, Archana Trivedi, Angela Kill and specially Marco Schlepütz for their volunteer support in laboratory,

Melanie Lobe for repeated and motivated collaboration,

Dr. Nagaraj Rao, Dr. Lasse Greiner, Dr. Jürgen Haberland and Dr. Iris Schröder for the interest on work development and the proofreading of the thesis,

the members of the enzyme group, specially Lilia Härter and Doris Hahn for the team spirit,

the members of the bioorganic group, specially Dr. Michael Wolberg, Mrs. Silke Bode and Mr. Ralf Feldmann for the friendly cooperation,

Professor Udo Kragl for advice during the initial stage of the work, and Marrit Eckstein for the interest in the achieved results,

Professor Adrie Straathof for sharing his expertise in biotransformation in multiliquid systems,

Dr. Stephan Laue, Michel Brik-Ternbach and Prof. Dr. D. Vasic-Racki for frequent discussions,

Dr. Peter Wasserscheidt for the friendly supply of ionic liquids,

Mr. Hans-Jürgen Brandt, Mrs. Susane Stevens and Mr. Georg Zeidler for the support on biological work,

Professor Joachim Treusch for the opportunities he offers to young scientists,

the infrastructure services from Forschungszentrum Jülich, specially Peer Kling, Dr. Carter-Sigglow, Mrs. Acker-Mantel, the library and the workshops for making scientific work a little more comfortable,

Mr. Dario Gaeta, Dr. Thomas Binnen, Mr. Thomas Wiemer for the interdisciplinary exchange,

Dr. Evelyn Wolfram for the most complete logistic support during the experimental sessions in Jülich,

Antonia, Karina, Niklas and Berthold Bärk for transforming the tedious writing work into an unforgettable time,

and last but not least, Mrs. Marianne Hess for the many solutions.

Finally, I would like to thank my family for making out of common steps a platform for my own way, and

Angela for sharing difficult times, for motivation and for never stopping believing.

CONTENT

PART I

1 INTRODUCTION 1

1.1 HYDROXYHEXANOATES 5

1.2 PROCESS DEVELOPMENT 7

1.3 OTHER USES OF δ-HYDROXY-β-KETOESTERS 9

2 OBJECTIVE 11

2.1 STRUCTURE 12

PART II

3 THE SUBSTRATE 15

3.1 SYNTHESIS 15

3.2 STABILITY 16

3.2.1 The Measurement System 17

3.2.2 Influence of pH 18

3.2.3 Influence of the Buffer Substance 19

3.2.4 Influence of the Temperature 19

3.2.5 Conclusion 20

3.3 IMPLICATIONS FOR THE ANALYTICS 21

3.4 INTERIM SUMMARY 21

4 THE ALCOHOL DEHYDROGENASE-CATALYSED REDUCTION 23

4.1 INTRODUCTION 23

4.1.1 History and Development 23

4.1.2 Application and Limitation 25

4.2 ENZYME STABILITY 25

4.2.1 Influence of the Buffer Substance 26

4.2.2 Influence of the pH 26

4.2.3 Influence of the Temperature 27

4.2.4 Influence of 2-propanol 28

4.3 ENZYME ACTIVITY 29

4.3.1 Influence of pH 29

4.3.2 Influence of the Temperature *29*
4.4 REGIO- AND ENANTIOSELECTIVITY 30
4.5 THERMODYNAMICS 30
4.6 KINETICS 32
4.6.1 Influence of the cofactor regeneration *36*
4.7 INTERIM SUMMARY 37
5 THE COFACTOR REGENERATION 39
5.1 INTRODUCTION 39
5.1.1 Regeneration Approaches *40*
5.1.2 Cofactor Retention *43*
5.1.3 Cofactor Stability *44*
5.2 ENZYME-COUPLED COFACTOR REGENERATION 46
5.2.1 Introduction *46*
5.2.2 Compatibility between Production and Regeneration *49*
5.3 SUBSTRATE-COUPLED COFACTOR REGENERATION 51
5.3.1 Introduction *51*
5.3.2 Thermodynamics *52*
5.3.3 Kinetics *53*
5.3.4 Cofactor Concentration *60*
5.4 CONCLUSION 60
5.5 INTERIM SUMMARY 61
6 ALCOHOL DEHYDROGENASE IN BIPHASIC SYSTEMS 63
6.1 INTRODUCTION 63
6.1.1 General Aspects *63*
6.1.2 Medium Engineering Aspects *72*
6.2 A NEW METHOD TO GUIDE THE SOLVENT CHOICE 75
6.2.1 General Aspects *75*
6.2.2 Development of Experimental Conditions *78*
6.2.3 Activity and Stability of ADH in Multiliquid Biphasic Systems *81*
6.2.4 Further Investigation *89*
6.2.5 Conclusion *93*
6.3 BIOTRANSFORMATION 94
6.3.1 The Feasibility of Biphasic Biotransformations *94*

6.3.2 Interfacial Effects 95
6.4 ENANTIOSELECTIVITY 96
6.5 COFACTOR REGENERATION 98
6.5.1 General aspects 98
6.5.2 Substrate Coupled Cofactor Regeneration. 98
6.5.3 Enzyme Coupled Cofactor Regeneration 98
6.5.4 Conclusion 100
6.6 THERMODYNAMIC EQUILIBRIUM 101
6.6.1 General Aspects 101
6.6.2 Equilibrium Shift for ADH-Catalysed Reactions 102
6.7 DISCRIMINATION OF SIDE REACTION 105
6.7.1 Discrimination of Side Reaction due to Concentration Profile 106
6.7.2 Discrimination of Side Reaction Due to Activation Energy 110
6.8 CONCLUSIONS AND OUTLOOK 111
6.9 INTERIM SUMMARY 112

PART III

7 THE BIOREACTOR CONFIGURATION 115

7.1 AIM AND REQUIREMENTS 115
7.2 REACTOR CHOICE 116
7.3 PHASE CONTACT 117
7.3.1 Equipment Choice 118
7.3.2 The Use of Hollow Fibre 119
7.4 EQUALISING THE KINETICS 121
7.5 ACETONE REMOVAL 123
7.5.1 Pervaporation 124
7.5.2 Stripping 130
7.5.3 Acetone Removal under Reduced Pressure 132
7.5.4 Comparison of the Methods 133
7.5.5 Membrane Distillation 134
7.6 THE HYBRID BIOEACTOR 136
7.6.1 The Elements Order 136
7.6.2 The Operation Modus 138
7.6.3 The Obtained Results 141
7.6.4 The Model Description 143

7.7 COMPARISON WITH OTHER REACTORS 145
7.7.1 Aqueous Fed Batch Reactor 146
7.7.2 Biphasic Emulsion Reactor 146
7.7.3 Polymeric Resin 147
7.7.4 Bimembrane Reactor 148
7.7.5 Two-Phase System Membrane Reactor 149
7.7.6 Emulsion Membrane Reactor 149
7.7.7 Polymer Enlarged Cofactor 149
7.7.8 Two-Chamber Reactor 149
7.7.9 Conclusion 150
7.8 INTERIM SUMMARY 150

8 THE REACTOR OPERATION 153

8.1 THE REACTION CONDITIONS 153
8.1.1 pH 153
8.1.2 Temperature 153
8.1.3 Enzyme Concentration 154
8.1.4 Cofactor Concentration 154
8.1.5 Substrate Concentration 154
8.1.6 Circulation Rate 154
8.1.7 Results 154
8.2 THE EMPIRICAL OPTIMISATION 155
8.3 REPETITIVE OPERATION 158
8.4 CONCLUSION 160
8.5 INTERIM SUMMARY 160

9 ECONOMIC ASPECTS 161

9.1 MARKET ESTIMATION 161
9.2 COST ESTIMATION 161
9.2.1 Fed Batch 162
9.2.2 Biphasic Batch 163
9.2.3 Hybrid Bioreactor 164
9.2.4 Reactor Choice 165
9.3 ASPECTS ABOUT THE SIDE REACTION DISCRIMINATION 165
9.4 THE RATE LIMITING STEP 167

9.5 INTERIM SUMMARY 170

10 DISCUSSION AND OUTLOOK 171

11 SUMMARY 173

12 EXPERIMENTAL PROCEDURES 175

12.1 CHEMICALS 175
12.2 ENZYMES 175
12.3 EQUIPMENT 175
12.4 ANALYTICS 176
12.4.1 Gas Chromatography (GC) 176
12.4.2 HPLC 178
12.4.3 NMR 178
12.4.4 Photometric 179
12.5 SYNTHESIS AND BIOTRANSFORMATIONS 179
12.5.1 General 179
12.5.2 Preparation of Tert-butyl 6-chloro-3,5-dioxohexanoate (CDHE) 179
12.5.3 Tert-butyl (4-oxo-4,5-dihydrofuran-2-yl)-acetate (ODFA) formation 179
12.5.4 LBADH-Catalysed reduction of CDHE 180
12.5.5 Biphasic LBADH-Catalysed Acetophenone Reduction 181
12.6 ENZYME ASSAYS 181
12.6.1 Alcohol Dehydrogenase from Lactobacillus brevis 181
12.6.2 Alcohol Dehydrogenase from horse liver 181
12.6.3 Alcohol Dehydrogenase from Thermoanaerobium brockii 182
12.6.4 Formate Dehydrogenase 182
12.7 EQUIPMENT CHARACTERISATION 182
12.7.1 Membrane Distillation with Celgard 50 (Liquicel Minimodule) 182

13 REFERENCES 183

LIST OF FIGURES

Figure 1-1: *Causa mortis* in Germany in 2001 according to the Statistisches Bundesamt. 1
Figure 1-2: Initial steps of the cholesterol biosynthesis; the reduction of 3-hydroxy-3-methylglutaryl CoA is the rate limiting step. 2
Figure 1-3: Natural HMG CoA reductase inhibitors: mevastatin (R = H) and lovastatin (R = Me). 2
Figure 1-4: Statin as competitive inhibitor of HMG CoA reductase. 3
Figure 1-5: Synthesis of *syn*-configured (3*R*,5*S*)-3,5-dihydroxyhexanoate according to Wolberg *et al.* 2000. 6
Figure 1-6: Distribution of the direct material costs for the enzymatic reduction of 6-chloro-3,5-dioxohexanoate. 7
Figure 3-1: Synthesis of *tert*-butyl 6-chloro 3,5-dioxohexanoate described by Wolberg (Wolberg 2001). 15
Figure 3-2: CDHE condensation, which decreases the purification yield. 16
Figure 3-3: Decay of CDHE under elimination of HCl formatting the *Tert*-butyl (4-oxo-4,5-dihydrofuran-2-yl)-acetate (ODFA). 17
Figure 3-4: Decay of CDHE in 150 mM phosphate buffer at pH 7.0 and 25°C. C_t is the residual concentration at a given time, C_0 the initial concentration, k_{side} the rate constant and *t* the time. 18
Figure 3-5: Determined values for k_{side} at 25°C: A: 150 mM TEA pH 7.0; : 150 mM TEA pH 8.0; C: 150 mM TEA pH 8.5. 18
Figure 3-6: Degradation of CDHE at 25°C and pH 7.0 in the presence of 150 mM TEA (k_{side} = 0.029 h^{-1}) and 150 mM phosphate buffer (k_{side} = 0.014 h^{-1}). 19
Figure 3-7: a) Residual CDHE concentration in an aqueous/MTBE biphasic system as a function of the temperature at pH 7.0 and 150 mM phosphate buffer; b) Determination of the activation energy according to the Arrehnius equation. 20
Figure 4-1: Reaction catalysed by alcohol dehydrogenases (Hoh and Villela, 2000). 23
Figure 4-2: pH-influence on the LBADH stability expressed as half-life at 4°C. 27
Figure 4-3: Temperature influence on LBADH stability expressed as half-life at pH 7.0. 27
Figure 4-4: Arrhenius plot for the activity decay of LBADH. 28

Figure 4-5: The reversible LBADH-catalysed reactions and the irreversible ODFA formation. The irreversibility of one reaction prevents the direct determination of the equilibrium. 30

Figure 4-6: Effect of acetone on the initial reaction rate of the production reaction; aqueous/MTBE (50/50 v/v), 21 mM CDHE in MTBE at 25°C, 150 mM phosphate buffer, pH 7. 37

Figure 5-1: The labile bonds in NADPH. 45

Figure 5-2: The influence of pH on the NADPH stability illustrated by the extition at 340 nm of a 0.1 mM NADPH solution at 25°C. 45

Figure 5-3: Enzyme-coupled cofactor regeneration; E-1 and E-2 are different enzymes; CS is the co-substrate and CP, the co-product. 46

Figure 5-4: The cofactor regeneration using formate dehydrogenase (FDH). 46

Figure 5-5: Glucose dehydrogenase (GDH) catalysed cofactor regeneration followed by the spontaneous gluconate formation. 48

Figure 5-6: Residual activity of FDH in the presence of 7.5 mM CDHE and in the control experiment without CDHE; pH 7.0, 100 mM pohsphate buffer, 30°C. 50

Figure 5-7: Substrate-coupled cofactor regeneration with 2-propanol as sacrificial substrate (Co-substrate). 51

Figure 5-8: The reversible oxidation of 2-propanol used for the substrate coupled cofactor regeneration. 52

Figure 5-9: Influence of pH-value on the enzyme activity for 2-propanol oxidation and acetone reduction at 30°C. 54

Figure 5-10: The temperature influence on the reaction rate of the 2-propanol oxidation. 55

Figure 5-11: Examples of curves used in the determination of kinetic constants at 30°C; The standard acetophenone activity is used as activity reference (1.0). a) influence of the 2-propanol concentration on the 2-propanol oxidation rate ($NADP^+$ = 0.2 mM); b) influence of the NADPH concentration on the acetone reduction rate (acetone = 5.0 mM); c) influence of the $NADP^+$ concentration on the 2-propanol oxidation rate (2-propanol = 50 mM); d) inhibition of the 2-propanol oxidation with increasing acetone concentration (2-propanol = 50 mM, $NADP^+$ = 0.2 mM). All reaction conducted at pH 7 in 50 mM phosphate buffer. 57

Figure 6-1: Activity dependence of esterase and lipase activity on substrate concentration (Faber, 1995). 65

Figure 6-2: Difference of lipase structure in aqueous solution and in aqueous/organic biphasic system (Buchholz and Kasche, 1997). 66

Figure 6-3: Non-conventional media applied in biocatalysis: (A) monophasic organic solvent; (B-I) solid adsorbent resin suspended in aqueous enzyme solution, (B-II) enzyme (and water) adsorbed on a solid resin suspended in organic solvent, (C) multiliquid biphasic system, (D) reverse micelles, (E) water-miscible cosolvent. 67

Figure 6-4: Illustration of the equilibria in a multiliquid biphasic system; according to the partition coefficient, the concentrations of hydrophobic substrates and products are higher in the organic phase. 69

Figure 6-5: Illustration of an enzyme-catalysed reaction in multiliquid biphasic system. 71

Figure 6-6: Illustration of the proposed method for solvent selection: classify the amount of possible solvent (A) into groups (B); use typical solvents in the first screening step (C) and study the best class in the second step (D). 75

Figure 6-7: The formula of [BMIM][$(CF_3SO_2)_2N$] the ionic liquid used in this work. 76

Figure 6-8: Comparison of the residual activity of LBADH at 4°C: stirred at 700 rpm and unstirred; 150 mM phosphate buffer pH 7.0. 81

Figure 6-9: Residual activity of HLADH in the presence of a second phase as a function of time: In this example I = 0.74 and k_{dea} = 0.0037 h^{-1}. 82

Figure 6-10: Histogram representing the half-lives of HLADH, LBADH and TBADH in multiliquid biphasic systems (Villela *et al*., 2003). 85

Figure 6-11: Half-lives of BAL in multiliquid biphasic systems. 94

Figure 6-12: LBADH-catalysed reduction of acetophenone with substrate-coupled cofactor regeneration. 95

Figure 6-13: Conversion over time for the first four runs of the repetitive batch; 1.0 mM acetophenone, aqueous/MTBE 50/50 v/v; 150 mM phosphate buffer pH 7.0 at 20°C with 40 U_{st}/mL LBADH. 95

Figure 6-14: Comparison of the *ee versus* conversion plot for the *Mucor miehei* lipase catalysed hydrolysis of butane-1,3-diacetate carried out in aqueous/*n*-heptane (—) and aqueous/dichloromethane (••••) biphasic systems (50/50 v/v) (Villela, 1998). 97

Figure 6-15: Concentration of (*R*)- and (*S*)- phenylethanol during LBADH-catalysed reduction of 1.0 mM acetophenone in aqueous/MTBE biphasic system; 150 mM phosphate buffer pH 7.0 at 30°C. 97

Figure 6-16: LBADH-catalysed acetophenone reduction with FDH-coupled cofactor regeneration. 99

Figure 6-17: Conversion in course of time with FDH-coupled cofactor regeneration; 150 mM phosphate buffer pH 7.0 at 30°C, FDH = 6 U/mL and LBADH = 4 U/mL. 99

Figure 6-18: Concentrations- and selectivity-time profile of the LBADH-catalysed reduction of CDHE with FDH-coupled cofactor regeneration in aqueous organic biphasic system; the concentrations refer to the organic phase; 150 mM phosphate and 150 mM citrate buffer pH 5.5 at 25°C, FDH = 12 U/mL and LBADH = 20 U/mL. 100

Figure 6-19: Illustration of the system studied. 101

Figure 6-20: Equilibrium conversion determined for the aqueous solution and biphasic system (50% v/v) for the reduction of 10 mM acetophenone using 1.5 M 2-propanol as co-substrate at 20°C. 103

Figure 6-21: Representation of reaction rate dependence on substrate concentration for reactions with saturation (——) and first order (- - -) kinetic; at low substrate concentration the selectivity is higher. 106

Figure 6-22: LBADH-catalysed reduction of CDHE in aqueous/MTBE biphasic system at 25°C aqueous phase at pH 7.0 (150 mM phosphate buffer) with 0.01 mM cofactor and 480 U_{st}/mL. 108

Figure 6-23: LBADH-catalysed reduction of CDHE in aqueous/MTBE biphasic system at 25°C; aqueous phase at pH 7 (150 mM phosphate buffer) with 1.0 mM cofactor and 480 U/mL. 108

Figure 6-24: LBADH-catalysed reduction of CDHE in aqueous/MTBE biphasic system at 25°C; aqueous phase at pH 7.0 (150 mM phosphate buffer) with 1 mM cofactor and FDH-coupled (6 U/mL) cofactor regeneration. 109

Figure 6-25: Overall selectivity obtained in the biphasic system in dependence on the initial cofactor concentration; aqueous/MTBE (50/50 v/v) at 25°C; aqueous phase at pH 7 (150 mM phosphate buffer) 480 U/mL. 110

Figure 6-26: Temperature influence on the selectivity. 111

Figure 7-1: Schematic representation of a bioreactor with stable phase contact and integrated acetone removal. 117

Figure 7-2: Flow sheet of bioreactor set up using a hollow fibre module as phase contactor; 1. module; 2. membrane; 3a. aqueous volume; 3b. organic volume; 4. stirred vessel (organic phase); 5. tubing; 6 pump; 7. tubing; 8. stirred vessel

(aqueous phase); 9. tubing; 10. pump, 11. tubing; A. organic cycle; B. aqueous cycle. 120

Figure 7-3: Conversion and selectivity in time course obtained with the hollow fibre module Celgard X50. 121

Figure 7-4: The selectivity as a function of conversion for the emulsion batch and the second run of the reactor with hollow fibre module; aqueous/MTBE (50/50 v/v), 150 mM phosphate buffer at pH 7.0 at 25°C, 300 mM 2-propanol, 0.1 mM cofactor 30 $U_{st.}$/mL. 122

Figure 7-5: CPCR-catalysed reduction of ethyl 5-oxohexanoate with substrate coupled cofactor regeneration. 124

Figure 7-6: Illustration of pervaporation. 125

Figure 7-7: Schematic illustration of a pervaporation device; Pump (P1), heat exchanger (H1 + H2), vacuum pump (V1) and membrane module. 126

Figure 7-8: Schematic reactor illustration; 1. reactor, 2. membrane module, 3. heat exchanger, 4. pump, 5. valve, 6. manometer, 7. valve, 8. condenser, 9. vacuum pump, 10 valve. 127

Figure 7-9: Ratio of feed acetone and 2-propanol concentration (cf. Stillger, 2000). 128

Figure 7-10: Comparison of conversion-time profile with (◆) and without (□) pervaporation, and acetone concentration-time profile with (x) and without (+) pervaporation 100 mM 2-propanol, 30 mM OHE, 0.2 mM NADH, 0.15 U/mL CPCP, pH 7.8, 35°C. 129

Figure 7-11: Set up scheme of stripping experiment. 130

Figure 7-12: Concentration profile in course of time during stripping with 0.5 L/min gas stream at 35°C. 131

Figure 7-13: Conversion time-plot for CPCR-catalysed reduction of ethyl 5-oxohexanoate with integrated stripping at 35°C, 0.5 L/min gas stream 100 mM 2-propanol, 30 mM OHE, 0.2 mM NADH, 0.15 U/mL CPCP, pH 7.8, 35°C, air-flow 0.5 L/min. 131

Figure 7-14: A: Concentration and pressure profile over the course of time for CPCR-catalysed reduction of ethyl 5-oxohexanoate at 35°C; B: the corresponding conversion time-plot; 100 mM 2-propanol, 30 mM OHE, 0.2 mM NADH, 0.15 U/mL CPCP, pH 7.8, 35°C. 132

Figure 7-15: Comparison of the conversion-time plot for stripping and pervaporation (cf. Stillger, 2000). 133

Figure 7-16: Determination of k_{strip} for acetone in a Celgard X50 (Liquicel Minimodule) hollow fibre module at 25°C and nitrogen flow 15 L_n/h. 135
Figure 7-17: A: Flow sheet of the hybrid bioreactor; B: a photography thereof. 138
Figure 7-18: Set up for the time distribution measurement of the shell side. 139
Figure 7-19: Tracer (acetone) concentration leaving the reactor as a function of time. 139
Figure 7-20: Compartment model with exponential decay tracer curve; V_m is the mixed flow volume of, V_p the plug flow volume and v the volumetric flow rate (cf. Levenspiel, 1999). 140
Figure 7-21: Compartment model with exponential decay tracer curve for the reactor with stagnant volume; V_m is the mixed flow volume of, V_p the plug flow volume V_d the stagnant volume and v the volumetric flow rate (cf. Levenspiel, 1999). 141
Figure 7-22: Concentration profile as a function of time obtained with the hybrid multiphase bioreactor; the acetone concentration refers to the aqueous phase, the substrate product and by-product concentration to the organic phase. The initial reaction rates are calculated based on the dotted lines. Reaction conditions: 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 210 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL. 142
Figure 7-23: The simulation (continuous line) of the results obtained with the hybrid multiphase bioreactor. Initial reaction conditions: 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 210 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL. 144
Figure 7-24: The simulation (continuous line) of the results obtained with the hybrid multiphase bioreactor with improved acetone removal rate k_{strip}. 145
Figure 7-25: Concentration profile for a fed batch with 0.005 mM cofactor. Reaction conditions: 75 mM citrate and 150 mM phosphate buffer pH 5.5 at 25°C, E = 6.25 U_{st}/mL, 36 mM $CDHE_{total}$, 6.5 mM/h dosage rate. 146
Figure 7-26: Comparison between emulsion reactor and hybrid multiphase reactor under identical reaction conditions; the difference in conversion rate is caused by the acetone accumulation. 147
Figure 7-27: Flow scheme of the bimembrane reactor according to Kruse *et al.* (1996). 148
Figure 7-28: Idealised reactor with independent production and regeneration systems, separated by ultrafiltration membranes. 150

Figure 8-1: Concentration profile during the course of reaction: the acetone concentration refers to the aqueous phase while substrate and product concentration refers to the organic phase. Reaction conditions: 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 420 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL. 155

Figure 8-2: Reflection of V_p for completing the new simplex. 157

Figure 8-3: Long run operation of the bench scale bioreactor. 158

Figure 8-4: Product obtained in the bioreactor during a batch run. 159

Figure 9-1: Material cost percentage for the improved fed batch, the biphasic batch and the hybrid bioreactor. 165

Figure 9-2: The profit as a function of the substrate concentration. 166

Figure 9-3: The selectivity as a function of the enzyme concentration; at high enzyme concentration the increase in selectivity does not correspond to the additional catalyst cost. 167

Figure 9-4: Limitation of the space-time-yield; during the initial conversion, the reactor is limited by acetone removal, in final stages by the small residual substrate concentration. 168

Figure 9-5: Comparison of the results obtained in the reaction operation (chapter 7) and a simulated reactor with three fold increased acetone removal area. 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 210 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL. 169

Figure 9-6: The substrate dosage and acetone removal membrane area required for achievement of space-time yield. 170

Figure 10-1: The consecutive C-C formation and carbonyl reduction catalysed by BAL and LBADH can be performed in aqueous/MTBE biphasic systems. 172

LIST OF TABLES

Table 3-1: the values used in this chapter were 1/h, in order to harmonise with further chapters the value in 1/min is also given. 20

Table 4-1: Parameters regarding the production reaction kinetics; the value in italic is not experimentally determined. 35

Table 4-2: Parameters regarding the CHOE oxidation kinetic; the value in italic is not experimentally determined. 36

Table 5-1: Prices of nicotinamide cofactors quotated by Jülich Fine Chemicals for a 10 kg order. 40

Table 5-2: Systems for enzyme-coupled cofactor regeneration. 49

Table 5-3: Value of the kinetic constants according to initial rate determination. 58

Table 5-4: Relative maximal enzyme specific reaction rates obtained at pH 7 and 30°C. 59

Table 6-1: Table showing pros and contras of non-natural media for ADH catalysis. 68

Table 6-2: The partition coefficient of some ADH-substrates and products in this biphasic system. 77

Table 6-3: Comparison between measured and calculated log *P*-values. 78

Table 6-4: The representatives of the solvent classes with respective calculated log *P*. 80

Table 6-5: Values for the instantaneous inhibition constant *I*. Values in italics are associated with $t_{½}$ < 24 h. 83

Table 6-6: Half-lives / h of enzymes in the presence of solvents. 84

Table 6-7: Ethers used in the second step of the solvent screening and the respective properties (Fluka 2002). 86

Table 6-8: Values for the instantaneous inhibition constant. 86

Table 6-9: Half-lives/h of enzymes in the presence of solvents. 87

Table 6-10: The values for *I* and $t_{½}$ concerning n-heptane and nonane. 88

Table 6-11: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of alkanes. 90

Table 6-12: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of alcohols. 90

Table 6-13: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of alcohols. 91

Table 6-14: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of ethers. 91

Table 6-15: Partition coefficient in MTBE/150 mM potassium phosphate buffer at 20°C. 103

Table 7-1: Classification of pervaporation applications. 125

Table 7-2: Membranes used in this work. 127

Table 7-3: Experimentally determined values for k_{strip} 136

Table 8-1: Empirical optimisation of the selectivity using the simplex algorithm. 157

Table 9-1: Material acquisition costs for the fed batch production of CHOE as described by Wolberg. 162

Table 9-2: Material acquisition costs for the biphasic batch production of CHOE. 163

Index of Abbreviations and Symbols

Abbreviation/Symbol	Quantity
A_t	Enzyme activity at time *t* (normalised)
A_0	Initial enzyme activity (normalised)
a_{iF}	Activity of component i in feed
a_{iP}	Activity of component i in permeate
BAL	Benzaldehyde lyase
CDHE	*Tert*-butyl 6-chloro-3,5-dioxohexanoate
CHOE	*Tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate
CPCR	Carbonyl reductase from *Candida parapsilosis*
CSTR	Continuous stirred tank reactor
DIPE	Di-*iso*-propyl ether
E	Light extintion
E	Exit age distribution function
E	Enzyme
E_a	Activation energy [kJ/mol]
[E]	Enzyme concentration [mg/mL]
ee	Enantiomeric excess [%]
FDH	Formate dehydrogenase
GC	Gas chromatopgraphy
HHE	Ethyl 5-hydroxyhexanoate
HLADH	Alcohol dehydrogenase from horse liver
I	Instantaneous inhibition constant [-]
J_m	Mass flux [kg/(h·m^2)]
j_i	Partial mass flux [kg/(h·m^2)]
K	Henry's law constant [mbar]
K_{eq}	Equilibrium constant
K_I	Inhibition constant [mM]
K_M	Michaelis constant [mM]
k'_{dea}	Apparent rate constant of irreversible enzyme deactivation [1/h]
k_E	Rate constant of the enzymatic CDHE reduction under first order approximation [mL/(min·mg)]
k_{-E}	Rate constant of the enzymatic CHOE oxidation under zeroth order approximation [U/mg]

k_{side}	Rate constant of the side reaction [1/h] or [1/min]
L_n	Litre gas under norm conditions
LBADH	Alcohol dehydrogenase from *Lactobacillus brevis*
m	Partition coefficient [-]
MTBE	Methyl *tert*-butyl ether
n.d.	not determined
NAD^+	Nicotinamide adenine dinucleotide (oxidised from)
NADH	Nicotinamide adenine dinucleotide (reduced from)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised from)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced from)
NMR	Nuclear magnetic resonance
ODFA	*Tert*-butyl (4-oxo-4,5-dihydrofuran-2-yl)-acetate
OHE	Ethyl 5-oxohexanoate
P	Particion coeficient in *n*-octanol/water standard system [-]
p	Pressure [mbar]
$t_{½}$	Half-life [h]
TBADH	Alcohol dehydrogenase from *Thermoanaerobium brockii*
TEA	Triethylamine
TTN	Total turnover number [-]
U	Unit of enzyme activity [µmol/min]
U_{st}	Unit of enzyme activity based on acetophenone under standard reaction conditions (referring to LBADH) [µmol/min]
V_{max}	Maximum velocity [U/mg]
v_{side}	side reaction reaction rate [mM/h] or [mM/min]
α	Volume ratio (V_{org}/V_w)
ε	Dielectric constant
μ	Chemical potential
x	Mole fraction [-]
σ	Differential selectivity [-]

PART I

1 Introduction

"In principio erat verbu."

SAINT JOHN

Cardiovascular diseases are the main *causa mortis* in developed countries. (Braun and Renz-Polster, 2001) In Germany, for example, 47.2% of deaths are caused by cardiovascular diseases (Figure 1-1), with 8.6% alone due to myocardial infarction.[1] For this reason, these kinds of illnesses have been intensively studied.

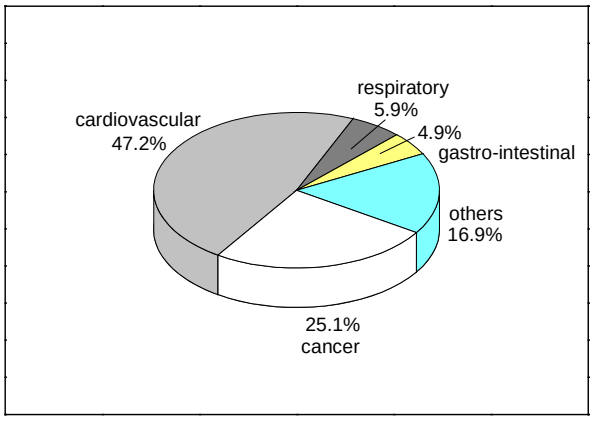


Figure 1-1: *Causa mortis* in Germany in 2001 according to the Statistisches Bundesamt.

Extensive epidemiological studies performed in many countries have shown that increased blood cholesterol levels, or, more specifically, increased levels of low density lipoprotein (LDL) cholesterol, are a major risk factor of cardiovascular diseases (*e.g.* coronary heart disease, stroke, etc.) (Fischer *et al*., 1999). Therefore, much research has been focussed on cholesterol.[2] As a result, the biosynthesis of cholesterol has been fully elucidated.[3] All 27 carbon atoms of cholesterol are derived from acetyl CoA (Figure 1-2). The first stage in the

[1] Statistisches Bundesamt, http://www. destatis.de/presse/deutsch/pm2003/p0130092, **2003.**

[2] "Cholesterol is the most highly decorated small molecule in biology. Thirteen Nobel prizes have been awarded to scientists who devoted major parts of their careers to cholesterol", Michael Brown and Joseph Goldstein, Nobel Lectures, 1985.

[3] Biochemistry textbooks, *e.g*. Stryer, 1995.

synthesis of cholesterol is the formation of isopentenyl pyrophosphate from acetyl CoA. This set of reactions starts with the formation of 3-hydroxy-3-methylglutaryl CoA (3-HMG-CoA) from acetyl CoA and acetoacetyl CoA. This molecule can be reduced to mevalonate. The irreversible reduction reaction is enzymaticaly catalysed in the cytosol and is the **rate limiting step** of the cholesterol biosynthesis. In further reactions, isopentenyl pyrophosphate is obtained. Squalene is then synthesized from six molecules of isopentenyl pyrophosphate and subsequently converted into cholesterol.

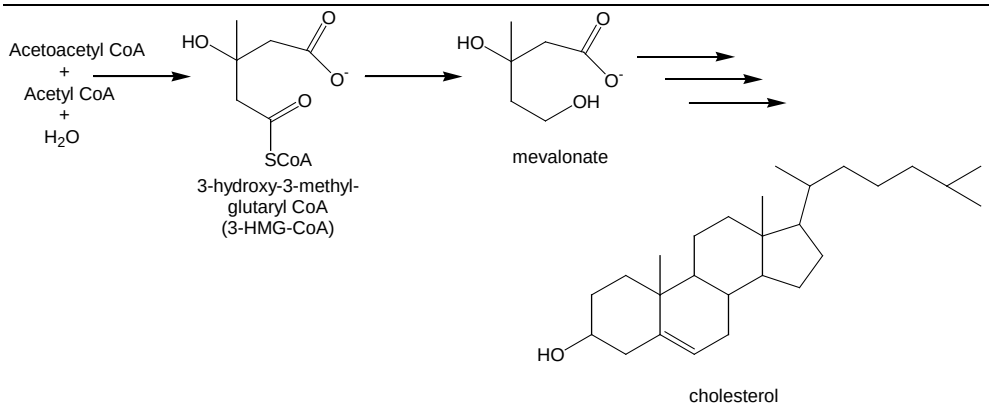


Figure 1-2: Initial steps of the cholesterol biosynthesis; the reduction of 3-hydroxy-3-methylglutaryl CoA is the rate limiting step.

Based on this knowledge, approaches for the therapy of hypercholesterolaemia focus on influencing the enzymatic formation of mevalonate. The amount and activity of the involved enzyme, 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), is biologicaly controlled. In 1976, Endo *et al*. reported the isolation of mevastatin, a metabolite of *Penicillium citrinum*, as a potent inhibitor of HMG-CoA reductase. Subsequently, lovastatin was isolated from *Monascus ruber* and *Aspergillus terreus* (Figure 1-3) (cf. Endo and Hasumi, 1993).

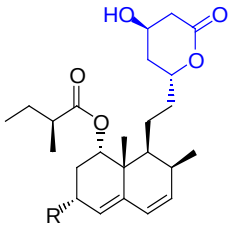


Figure 1-3: Natural HMG CoA reductase inhibitors: mevastatin (R = H) and lovastatin (R = Me).

Mevastatin and its analogues are reversible competitive inhibitors of the enzyme HMG-CoA reductase (Hanefeld, 1999). The K_M-value for mammalian HMG-CoA reductase is approximately 10 µM, while the K_I-value for the ring-opened acids of mevastatin and lovastatin are in the range of 0.2-1.0 nM.[4] Thus, the affinity of HMG-CoA reductase for mevastatin analogues is 10000-fold or more than its affinity for the natural substrate, HMG CoA (cf. Endo and Hasumi, 1993). The effect is attributed to the similarity between the 3,5-dihydroxyhexanoic acid and the HMG portion of HMG-CoA (Figure 1-4).

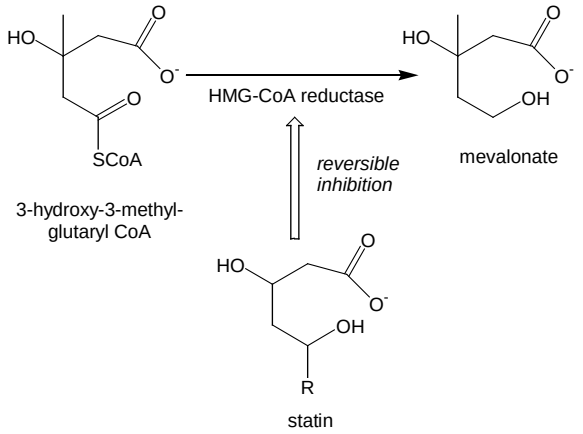


Figure 1-4: Statin as competitive inhibitor of HMG CoA reductase.

Much work directed towards the preparation of synthetic analogues of mevastatin followed. It helped to define the structure-activity relationship of mevastatin-mimics. It turned out that the absolute stereochemistry in the 3,5-dihydroxy acid side chain is essential for the pharmacological activity (Endo and Hasumi, 1993). At the same time, the stereoselectivity of the *syn*-diol formation is the major hurdle in the synthesis of statins (Repič *et al*., 2001). For this reason ten years elapsed between the introduction of lovastatin and the commercialisation of the first synthetic enantiopure statin in 1997.[5]

The development of synthetic statins aims at fine-tuning the pharmacological properties of statins. Thus, broader therapeutic range and reduced side-effects (*e.g.* myopathy incidence) are the primary research goals. In order to enable the investigation of a large number of substances, an easy and "universal" synthetic approach to enantiopure 3,5-dihydroxyhexanoate is highly desirable. Some examples of statin synthesis shall illustrate of how this objective is pursued.

[4] For the meaning of K_M and K_I see chapters 4 and 5.

[5] In 1997 atrovastatin and cerivastatin were introduced into the market.

The Synthesis of Statins

The first total synthesis of mevastatin was accomplished in the research group of C. Sih (1981). The major drawback of this approach was the formation of four stereoisomeric diols, requiring tedious separations and lowering the overall yield (cf. Mulzer *et al*., 1991).

Since the linear synthesis suffers from low yields and the lack of flexibility, convergent routes for the synthesis of statins were developed, allowing independent improvement of some steps. An example is reported by Wess *et al*. (Hoechst AG): The statin synthesis is based on a Wittig type coupling. Improvement was achieved by replacing a substituted oxane by an enantiopure 3,5-dihydroxyhexanoate derivative. The chirality is introduced in an early step. L-malic acid is used as starting reagent for the synthesis of the *syn*-diol (Wess *et al*., 1990).

A further synthesis using a Wittig-like reaction is described by researchers of the Novartis Institute for Biochemical Research (Repič *et al*., 2001). The *syn*-diol is obtained from phloroglucinol.[6] This compound is used because stereochemistry is more easily defined in cyclic molecules. The starting reagent is hydrogenated in the presence of a rhodium catalyst to give a mixture of *cis*,*cis*- and *cis*,*trans*-cyclohexane triols. It is possible to protect selectively two *cis*-hydroxy groups. The oxidation of the remaining hydroxy group leads to a seven-membered lactone with the desired stereochemistry. Nevertheless, Novartis produces fluvastatin[7] by an alternative linear synthesis, because it is more economical.

Biotechnological Approaches

Besides the methods presented, a further possibility of obtaining the proper stereoconfiguration is based on the intrinsically asymmetric biocatalysis. This attractive approach was already used in one of the earliest routes for the synthesis of statins.

Hirama's synthesis of mevastatin (1982) is characterised by the coupling of two chiral, non-racemic building blocks. The chirality of the *syn*-diol structure is obtained by baker's yeast reduction of the starting keto acid. After the early introduction of chirality several reaction steps are required until the final target structure is achieved (Mulzer, *et al*., 1991).

A patent assigned to Squibb & Sons describes the synthesis of intermediates for the synthesis of statins containing the *syn*-diol structure starting with the biotechnological reduction of methyl 4-chloro-3-oxobutanoate (Thottathil *et al*., 1994). This step is followed by chain

[6] 1,3,5-trihydroxybenzene.

[7] Commercialised as Lescol.

elongation leading to *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate. A selective reduction yields the *syn*-diol. Here again the stereochemistry is introduced in the early steps.

Several reports on the biotechnological reduction of 3-oxobutanoic acid and its derivatives have been published.[8] Patel described the reduction of methyl 4-chloro-3-oxobutanoate by a cell suspension of *Geotrichum candidum*. The cell free enzyme can also be used if NADH is supplied (Patel, 2001). The research carried out by Shimizu and co-workers in collaboration with Kaneka Corporation has drawn considerable attention. The use of *Escherichia coli* cells expressing both the carbonyl reductase gene from *Candida magnoliae* and the glucose dehydrogenase gene from *Bacillus megaterium* for reduction of 4-chloro-3-oxobutanoate has been reported. The reaction is performed in an aqueous/organic solvent biphasic system. The obtained results (89% yield, > 99 % *ee*) have been reported to be very good and the enzyme productivity is enhanced owing to the biphasic system (Kizaki *et al*., 2001).

The enantioselective reduction of a 3,5-dioxohexanoate by cell free extracts of *Acinetobacter calcoaceticus* was reported by Patel. A drawback of this approach is the simultaneous production of isomeric hydroxyoxohexanoates (cf. Patel, 2001). Finally, the same article also refers to the lipase catalysed resolution. However, this approach suffers from low yields at a subsequent stage of the synthesis.[9]

1.1 Hydroxyhexanoates

The discussed approaches to the synthesis of statins indicate that hydroxyhexanoates are advanced key chiral intermediates. Interest in the investigation of alternative synthetic routes for enantiopure 3,5-dihydroxyhexanoate derivatives is not merely academic. Considering the volume of statin production (see chapter 9) and the development of new products,[10] a cost effective and safe production process is also an important target of industrial research and development.

A new approach for the preparation of enantiopure 3,5-dihydroxyhexanoate structure was presented by Wolberg *et al*. in 2000. This innovative work starts with the elongation of the carbon chain to the desired size (6-chloro-3,5-dioxohexanoate), with subsequent introduction of the chirality via enzymatic reduction. The reduction, catalysed by the alcohol

[8] *E.g*. Ushio *et al*., 1991; Patel *et al*., 1992.

[9] Theoretical maximum yield is 50%; cf. Sheldon, 1993.

[10] After the first approval of AstraZeneca's Crestor (rosuvastatin) by November 2002 in the Netherlands, its commercialisation is expected to occur in 2003.

dehydrogenase of *Lactobacillus brevis* (LBADH),[11] afforded *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate with very high enantioselectivity (*ee* > 99.5%). The subsequent diastereoselective reduction yielded quantitatively *syn*-configured (3*R*,5*S*)-3,5-dihydroxyhexanoate with 93% *de* (Figure 1-5).[12] The diastereomeric excess could be increased to > 99% by a single recrystallization step.

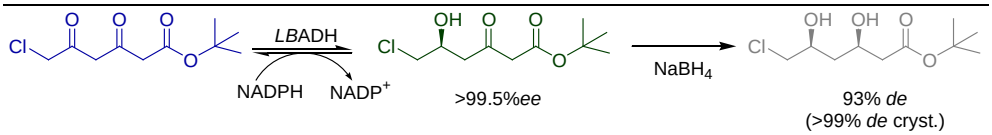


Figure 1-5: Synthesis of *syn*-configured (3*R*,5*S*)-3,5-dihydroxyhexanoate according to Wolberg *et al.* 2000.

The advantage of the late introduction of chirality, possible by this approach, is the higher productivity based on the first, expensive, asymmetric compound. The clue for this improvement is the enzymatic reduction.

The enzyme LBADH requires the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor for the reduction of 6-chloro-3,5-dioxohexanoate (**production reaction**). Because of the high cofactor cost its stoichiometric use as reduction equivalent is not viable.[13] This obstacle was overcome by an integrated cofactor regeneration,[14] wherein a sacrificial alcohol is used as cosubstrate. Under consumption of the oxidised cofactor ($NADP^+$), the alcohol is oxidised to a carbonyl compound yielding NADPH in the process (**regeneration reaction**). Wolberg *et al*. used 2-propanol as cosubstrate. Simultaneously, this alcohol also increased the solubility of the substrate. So it was possible to scale up the synthesis to hectogram scale. In order to avoid the decay of 6-chloro-3,5-dioxohexanoate in water yielding (4-oxo-4,5-dihydrofuran-2-yl)-acetate, an undesired **side reaction**,[15] a fed batch strategy was employed. The results obtained demonstrate the suitability of this synthesis for industrial application.[16] The overexpression of LBADH in *E. coli* guarantees the large scale availability of the enzyme at a low price, thereby facilitating

[11] Alcohol dehydrogenases are oxidoreductases acting on CH-OH group of donors (see chapter 4).

[12] This procedure requires complete elimination of the starting material after the enzymatic step, otherwise the final enantiopurity will be decreased due to unselective reduction.

[13] 46.10 €/g for 10 kg, Jülich Fine Chemicals.

[14] See chapter 5.

[15] See chapter 3.

[16] For a detailed discussion of the process see chapter 7.

the use of this synthesis for commercial production. The total direct material cost for the production is estimated at 500 €/kg.[17] Figure 1-6 shows the distribution of the costs.

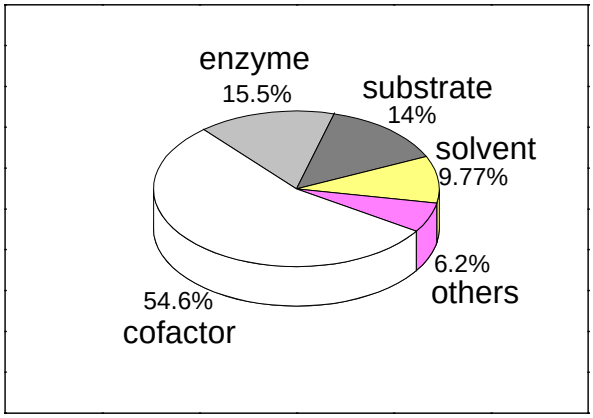


Figure 1-6: Distribution of the direct material costs for the enzymatic reduction of 6-chloro-3,5-dioxohexanoate.

Since the major cost contribution is by the cofactor, improvement of the productivity can lead to drastic cost reduction.[18]

1.2 Process Development

In the early days of economical theory, David Ricardo recognised that not the ability of producing goods but the productivity give rise to the profit. While in his *Principles of political economy and taxation* (1817), the productivity was defined by the fertility of the farmer's land, in today's industry the productivity is defined by the boundary of technical possibilities. Thus, it sounds natural that today research and technological development closely interact with economical development.

According to Joseph Schumpeter, firms compete for successful innovation and this kind of competition has proved more effective for economic progress than the traditional price competition.

[17] See chapter 9.

[18] The cofactor productivity is commonly expressed by the total turnover number (moles product formed ÷ moles cofactor consumed); see chapter 5 for a detailed discussion.

Progress in chemistry is a good example of this interaction. Since the demand for chemicals has outstripped the production capacity of manual production methods and industrial production started, research has focused, and also been enabled by, technological development. The production of sulphuric acid and of dyes illustrate the impact of technological improvement originated by competition for innovation (Taylor, 1957 p 187). Since the advent of large-scale chemical manufacturing operations in the second half of the 19th century, it became evident that process development deserved special attention. This led to the improvement of production conditions to be independent of the synthetic route development. It was the beginning of chemical reaction engineering. Its aims have been described by Levenspiel. According to him, chemical reaction engineering is the synthesis of thermodynamics, kinetics, mass transfer, economics, etc. with the aim of properly designing a chemical reactor (Levespiel, 1999). One of its major tasks is the economical, ecological, and safe transfer from the fundamental knowledge obtained in the laboratory to sustainable commercial use (Gmehling and Brehm, 1996).

In the field of biotechnology, reaction engineering has a special significance. It was only due to the combination of process development with the advances in fundamental sciences that some industrial biotransformation have become possible. As an example, the retention of enzymes in ultrafiltration membrane reactors reduces the catalyst costs significantly (Liese *et al*., 2000, p 126). Similarly, by using covalentely enlarged cofactor in ultrafiltration membrane reactors, the cofactor productivity has been increased dramatically (Wichmann *et al*., 1981).[19] Hummel reports the attainment of cofactor turnover up to 600 000 by this technique (Hummel, 1997). In addition the development of a convenient work up procedure can largely contribute to the productivity of biotransformations, as demonstrated in the case of L-phenylalanine (Rüffers *et al*. 2002).

Hence, it is expected that process development based on reaction engineering could contribute significantly to increase the productivity of cofactor and decrease work up costs. Such improvements can enhance the attractiveness of the LBADH-catalysed synthesis of *Tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate CHOE for industrial application.

[19] This article was reprinted by the journal *Biotechnol. Bioeng.* **2000**, *67*, 791-804 as the highest impact publication of the year 1981.

1.3 Other uses of δ-hydroxy-β-ketoesters

The use of δ-hydroxy-β-ketoesters is not restricted to the synthesis of HMG-CoA reductase inhibitors. The use of *tert*-butyl (*R*)-6-chloro-5-hydroxy-3-oxohexanoate in a synthesis of (–)-callystatin, a polyketide marine natural product showing high cytotoxic activity has been reported (Vicario *et al*., 2002, Enders *et al*., 2002). Drochner and Müller employed enzymatically produced *tert*-butyl (*R*)-5-hydroxy-3-oxohexanoate in the synthesis of semi-vioxanthin, the monomeric precursor of vioxanthin. The asymmetric synthesis of *S*-(+)-argentilactone, and *S*-(-)-goniothalamin, non-natural enantiomers of natural products showing anti-leishmanial and cytotoxic activity, and teratogenic and cytotoxic activity respectively, are published by Job *et al*. In both routes hydroxyhexanoate is used as intermediate (Job *et al*., 2001). Wolberg referred to further application of this compound class (Wolberg, 2001).

2 Objective

"The story of industrial chemistry is not
simply that of continuous research directed
towards the fulfilling of human needs.
Its minor aims are often achieved in that fashion,
but commonly a great new advance takes its rise
from an unexpected observation
whose significance has been grasped."
F. S. TAYLOR

The introduction presented the process development of the LBADH-catalysed production of *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate based on chemical reaction engineering as a possibility to further increase the attractiveness of the synthesis. The objective of the present work is to perform and evaluate this process development.

Therefore the following tasks should be carried out:

- thermodynamic and kinetic description of the involved reactions
- design of a bioreactor able to:
  - suppress the side reaction
  - efficiently reuse the cofactor (regeneration)
  - avoid thermodynamic limitation
  - reduce the enzyme consumption
  - decrease of work up expenditure
- assembly of a bioreactor to carry out the process
- process intensification
  - selectivity optimisation
- demonstration of the long-time bioreactor operation

Finally, the developed bioreactor should be evaluated from the economical point of view.

2.1 Structure

The work starts with an introductory part, where the motivation, the uses of the target compound and the reasons for a process development are explained. Besides, the objective of the work is presented.

In the second part of the work, the **thermodynamic and kinetic description** of the involved reactions are reported. The substrate synthesis and stability are subjects of chapter 3. The advantages of the enzymatic transformation, as well as its limitations, are discussed in chapter 4. Chapter 5 focuses on the problem of efficient cofactor reuse. Chapter 6 presents the possibility of enzymatic catalysis in non-conventional media to overcome the system incompatibility arising form the results described in the previous chapters.

The third part is dedicated to the **design a bioreactor** based on the fundamental knowledge obtained in the system characterisation. The required reactor elements and the **assembly of the bioreactor** are discussed in chapter 7. The **process intensification** and the demonstration of the **long-time bioreactor operation** are reported in chapter 8, while in chapter 9 the **bioreactor evaluation** is discussed from the economical point of view. The work concludes with a discussion on the bioreactor development.

PART II

3 The Substrate

"Que não seja imortal posto que é chama
Mas que seja infinito enquanto dure." [20]
VINÍCIUS DE MORAES

This chapter deals with the substrate of the enzymatic reduction, *tert*-butyl 6-chloro-3,5-dioxohexanoate (CDHE). The synthesis of the substrate is subject of subchapter 3.1, its stability is studied in subchapter 3.2, and the implications of its properties for analytics is discussed in subchapter 3.3.

3.1 Synthesis

Wolberg describes different possibilities for the synthesis of CDHE. For an economic process development and application, it is necessary to obtain the substance in high yield starting from cheap reagents. To satisfy these requirements, Wolberg has developed a procedure starting from methyl chloroacetate and *tert*-butyl 3-oxobutyrate (Figure 3-1) (Wolberg, 2001).[21]

[20] It shall not be immortal given it is flame, but it shall be endless while it lasts; referring to love.

[21] Important reaction parameters in the synthetic procedure are the temperature and the bisenolate concentration. The temperature must be kept < -60°C. A high bisenolate concentration leads to the formation of aromatic by-products (Wolberg, 2001).

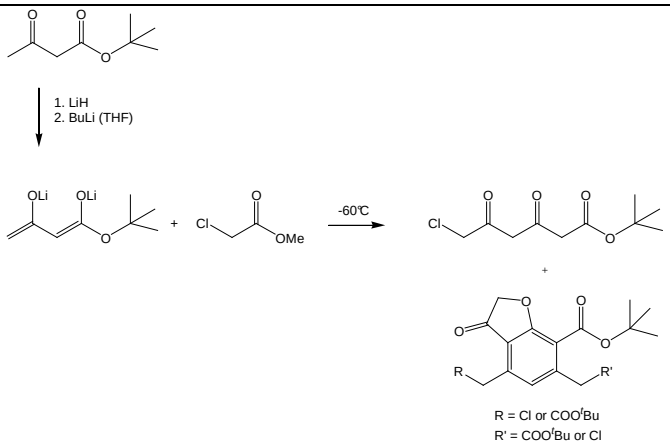


Figure 3-1: Synthesis of *tert*-butyl 6-chloro 3,5-dioxohexanoate described by Wolberg (Wolberg 2001).

Although it had been reported earlier that this reaction is not possible (Langer and Krummel, 2000), Wolberg obtained CDHE in approximately 80% yield.[22] The main by-product is a benzofuranone. CDHE can be purified by film evaporation under reduced pressure. However, this process lowers the overall yield significantly (to 52%). The reason therefore is the condensation of two CDHE molecules to form aromatic compounds (Figure 3-2) (Wolberg, 2001).

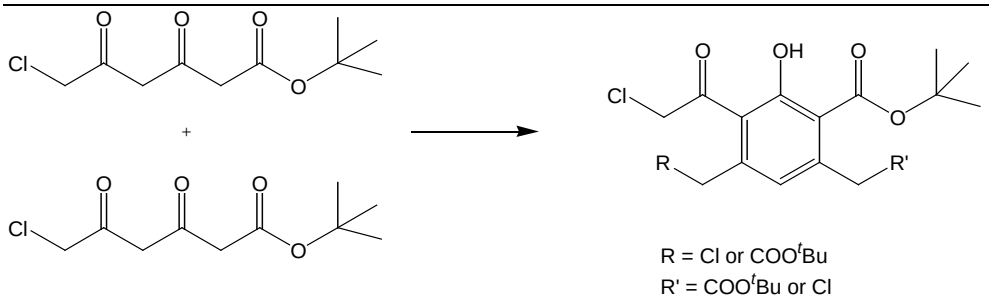


Figure 3-2: CDHE condensation, which decreases the purification yield.

Since the crude substrate can be used for the enzymatic reaction, the isolation of CDHE need not to be carried out. The work up is restricted to the removal of the starting materials under reduced pressure. The quantitative removal of *tert*-butyl 3-oxobutyrate is necessary because this compound is also converted by the enzyme under NADPH consumption.

In order to obtain enough CDHE for process development work, 137 g of this compound were synthesised according to this procedure (78% yield).

[22] The achieved yield depends on the reaction procedure (cf. Wolberg, 2001).

3.2 Stability

In view of literature reports about the instability of β,δ-dioxoester, in particular ε-halogen substituted β,δ-dioxoester, Wolberg studied the stability of CDHE in aqueous solutions (Wolberg, 2001). He observed that in contrast to methyl and ethyl β,δ-dioxohexanoates, the *tert*-butyl ester does not undergo condensation to aromatic compounds, and attributed this behaviour to the presence of the bulky residue. However an intramolecular cyclisation yields *Tert*-butyl (4-oxo-4,5-dihydrofuran-2-yl)-acetate (ODFA) under HCl elimination (Figure 3-3) (Yamaguchi, 1988; Langer, and Krummel, 2000; Wolberg, 2001). This irreversible reaction competes with the enzymatic reduction of CDHE lowering the production yield. It is, therefore, an undesired **side reaction** and shall be suppressed in the bioreactor to enable a high process selectivity.

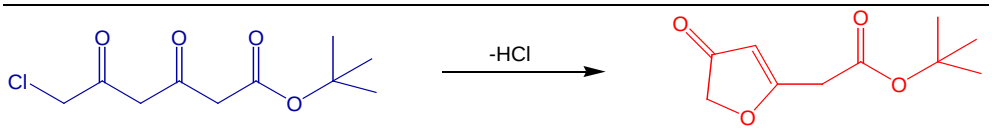


Figure 3-3: Decay of CDHE under elimination of HCl formatting the *Tert*-butyl (4-oxo-4,5-dihydrofuran-2-yl)-acetate (ODFA).

This pH dependent degradation reaction was suppressed at lower pH-values (see 3.2.2). However, at low pH-values the formation of a lactone could occur. The latter was not observed in the pH-range of interest for the present process development. Thus, the ODFA formation is the only relevant side reaction to be avoided in this work. Its kinetic description is the subject of this subchapter.

3.2.1 The Measurement System

The rate of the ODFA formation and of CDHE dissolution are of the same order of magnitude.[23] Consequently, the measurement of the reaction rate in an aqueous solution is hindered. Given that ODFA formation is not observed in a solution of CDHE in methyl *tert*-butyl ether (MTBE), even when saturated with water, an aqueous/MTBE biphasic system is used to study the rate of the ODFA formation. The higher reaction rate observed by increasing the aqueous pH indicates that the reaction is not limited by CDHE mass transfer (cf. Willeman *et al.*, 2002).[24] The reaction rate is followed by sampling the organic phase and

[23] This observation is in agreement with the slow solution rate described in Wolberg, 2001.

[24] If the reaction were mass transfer limited, the same reaction rate would be observed for different pH-values.

determining the ODFA concentration by NMR analysis. Under these conditions, the reaction can be described as **first order** irreversible decay of CDHE (Figure 3-4). Hence, the rate constant k_{side} can be used to study the influence of pH, buffer substance and temperature on the reaction rate.

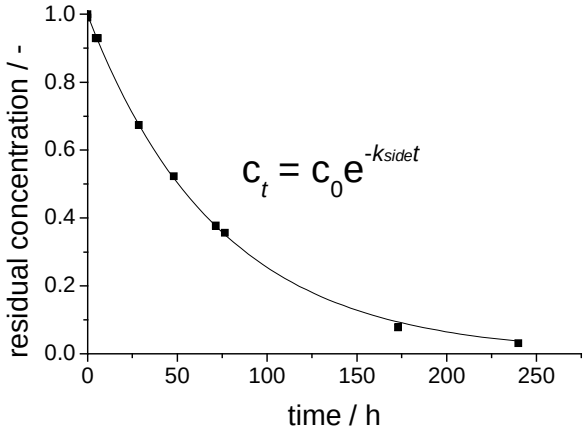


Figure 3-4: Decay of CDHE in 150 mM phosphate buffer at pH 7.0 and 25°C. C_t is the residual concentration at a given time, C_0 the initial concentration, k_{side} the rate constant and t the time.

3.2.2 Influence of pH

Wolberg reported a decrease in the rate of ODFA formation when the pH was lowered from 6.5 to 5.5. The solution at pH 6.5 was buffered with phosphate. Since phosphate buffer is not effective at pH 5.5 a mixture of phosphate and citrate were used as buffer substances at this pH. In order to confirm that the observed change in the reaction rate is solely caused by the pH shift, we measured the reaction rate at different pH-values using a single buffer substance. The results for the series with 150 mM triethylamine are depicted in figure 3-5. Increasing pH enhances the rate of the ODFA formation.

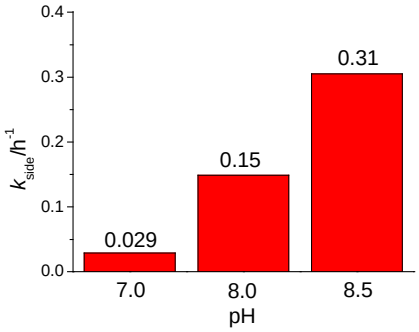


Figure 3-5: Determined values for k_{side} at 25°C: A: 150 mM TEA pH 7.0; : 150 mM TEA pH 8.0; C: 150 mM TEA pH 8.5.

3.2.3 Influence of the Buffer Substance

The influence of different buffer substances was also studied. This is important because the stability of the cofactor is dependent on the buffer substance (see chapter 5). Hence, the least deleterious buffer for substrate and cofactor should be found.

The nature of the buffer substance does influence the rate of the reaction as can be seen from figure 3-6.

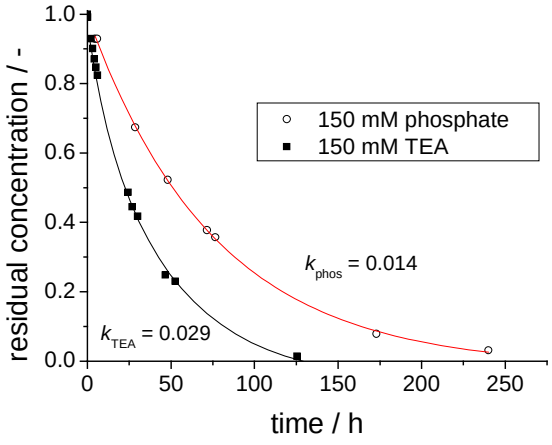


Figure 3-6: Degradation of CDHE at 25°C and pH 7.0 in the presence of 150 mM TEA (k_{side} = 0.029 h^{-1}) and 150 mM phosphate buffer (k_{side} = 0.014 h^{-1}).

A further experiment was conducted using an autotitrator to control the pH. The initial reaction rate was comparable to the one measured for phosphate buffer. Since the use of an

autotitrator leads to a change in the aqueous volume, which is particularly severe in small scale experiments, phosphate buffer was used in further experiments.

3.2.4 Influence of the Temperature

To complete the kinetic characterisation of the ODFA formation the temperature dependence of the rate constant was studied. An increase in the rate constant was observed with increasing temperature. This increase can be described by the Arrhenius equation (Figure 3-7). From the measurements the activation energy was calculated to be 96 kJ / mol.[25]

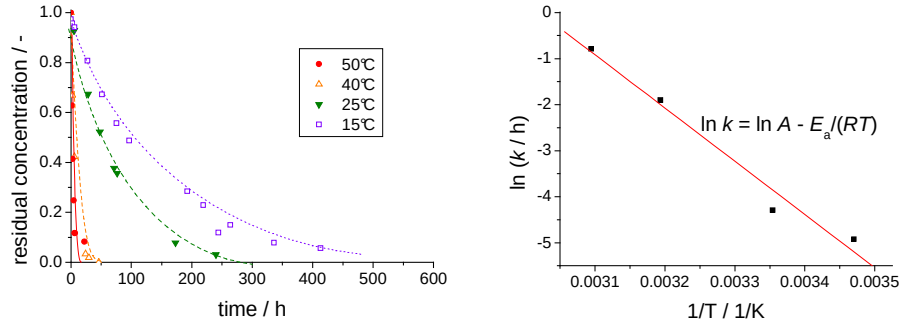


Figure 3-7: a) Residual CDHE concentration in an aqueous/MTBE biphasic system as a function of the temperature at pH 7.0 and 150 mM phosphate buffer; b) Determination of the activation energy according to the Arrehnius equation.

3.2.5 Conclusion

According to the description of the side reaction presented (first order CDHE decay), it is possible to describe the rate of the side reaction, v_{side}, under the relevant reaction conditions. Therefore, equation 3-1 should be used and the value of k_{side} should be calculated according to the Arrhenius equation.

$$v_{side} = \frac{d[\text{ODFA}]}{dt} = k_{side} \cdot [\text{CDHE}]_{aq} \qquad \textbf{Equation 3-1}$$

[25] Cf. chapter 5 for a comparison with enzymatic reactions; cf. chapter 6 and 8 for the side reaction discrimination owing to temperature change.

For example, the calculated value for the reaction constant in a 150 mM phosphate buffer at pH 7.0 and 25°C is shown in table 3-1.

Table 3-1: the values used in this chapter were 1/h, in order to harmonise with further chapters the value in 1/min is also given.

	[1/h]	[1/min]
$k_{calculated}$	0.0212	3.53×10^{-4}

Considering the aqueous/MTBE (50/50 v/v) biphasic system the aqueous concentration can be calculated using the partition coefficient *m*.[26] For the system D_2O/MTBE the *m* was determined by NMR measurements as:[27]

$m_{o/w} = 500 \pm 250$.

$$v_{side} = \frac{d[\text{ODFA}]}{dt} = k_{side} \cdot [\text{CDHE}]_{aq} = k_{side} \cdot [\text{CDHE}]_{org} \cdot \frac{1}{m}$$ **Equation 3-2**

3.3 Implications for the Analytics

The structure and the instability of CDHE influence to the analytical method to be used in this work. Being thermally instable, CDHE is instantaneously converted to ODFA in the injector of a gas chromatograph. Therefore gas chromatography (GC) cannot be used to quantify substrate and ODFA. Due to the occurrence of keto-enol tautomerism, the high performance liquid chromatography (HPLC) method yields very inaccurate results, and is also not adequate for quantification. A possibility of analysis lies in following the ODFA formation by titration of the released HCl and the simultaneous quantification of product and CDHE/ODFA by GC. The titration can be performed automatically *in situ*, but is accompained by a volume change. Sampling followed by titration is not possible because of the constant pH required to be maintained during the reaction. Therefore we decided to quantify CDHE, ODFA and CHOE by sampling and NMR analysis.

[26] For ideal diluted solutions $m = [S]_{org} \div [S]_{aq}$.

[27] The very small aqueous concentration of CDHE leads to inaccuracy.

3.4 Interim Summary

- The synthesis of 137 g CDHE was carried out according to the procedure described by Wolberg.
- CDHE is unstable in water; under process relevant reaction conditions the only side reaction to be avoided is the irreversible HCl elimination yielding ODFA.
- The ODFA formation can be neglected in MTBE, even if the solvent is saturated with water.
- The ODFA formation rate is lowest under acidic conditions. It depends also on the buffer substance. Phosphate buffer is chosen to be used in this work.
- The ODFA formation rate is described by a kinetic expression based on first order substrate decay.
- The ODFA formation rate is dependent on the temperature. The calculated activation energy is 96 kJ/mol.
- Due to the CDHE instability the analytics is based on sampling and NMR-measurements.

.

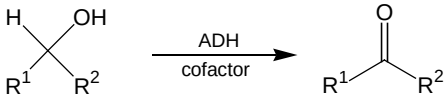
4 The Alcohol Dehydrogenase-Catalysed Reduction

"There is no difference between the activity of a cell and a chemical reagent"

EMIL FISCHER

4.1 Introduction

According to the Enzyme Nomenclature, alcohol dehydrogenases (ADH) are oxidoreductases acting on CH-OH group of donors (Figure 4-1) (International Union of Biochemistry and Molecular Biology, 1992).



R^1 = hydrogen, organic residue
R^2 = hydrogen, organic residue, alcoxy residue

Figure 4-1: Reaction catalysed by alcohol dehydrogenases (Hoh and Villela, 2000).

These enzymes need a nicotinamide cofactor (either NAD(H) or NADP(H)) as a source of redox equivalents. They are classified under EC 1.1.1 and further classification is defined by the type of the cofactor. Depending on the initial screening and to some extent on the initial characterisation, alcohol dehydrogenases may also be described as carbonyl- or ketoreductases in the literature (Kula and Kragl, 2000).

4.1.1 History and Development

The use of alcohol dehydrogenases underpins some of the oldest chemical transformations known to humans, for, brewing predates recorded history. The Sumerians, for instance, produced at least 19 different types of beer (Ball, 2001). The book of Genesis reports the production of wine.[28] ADH are also related with the first use of microbial enzymes as cell-free extracts. As early as 1897 Buchner demonstrated that alcohol formation does not require

[28] Genesis 9, 18-29.

intact cells, thus proving the pure chemical nature of biotransformations and refuting the theory of *spiritus vitae* (Buchner, 1897).

In the past decades the scope of use of ADH has expanded to the conversion of non-natural substrates and these enzymes are used today in asymmetric hydrogen transfer. Recent articles have compared the biocatalytic methods for the reduction of ketones with classical chemical reactions (Schmidt *et al*., 1992; Bachmann and Seebach, 1998; Rissom *et al*., 1999; Hage *et al*., 2001; Laue *et al*., 2001). The advantages of the former were summarised by Faber and coworkers (Stampfer *et al*., 2002) as follows:

- due to the intrinsic asymmetry high enantioselectivity is achievable.
- side reactions such as aldol condensation are absent
- operation under essentially mild reaction conditions
- protective group techniques are not necessary[29]

Besides, the biocatalyst is obtainable from renewable resources and is biodegradable. An overview of the possibilities offered by ADH is found in textbooks and review articles.[30] The commercially available alcohol dehydrogenase from horse liver (HLADH), *Thermoanaerobium brockii* (TBADH) and yeast (*Saccharomyces cerevisiae*) have received the most attention (Faber, 1995; cf. 6.2.1.3), but recent reports of new enzymes disclose new perspectives of application (Zelinski, 1995; Gröger *et al*., 2003, Stampfer *et al*., 2003). The majority of the commercially available ADH used for the stereospecific reduction of ketones lead to the formation of (*S*)-alcohols. Microbial ADH which lead to the formation of (*R*)-alcohols are also known (Faber, 1995), but only a few of them are commercially available, *e. g*. *Lactobacillus kefir* (Bradshaw, 1992), and these all are NADP(H)-dependent (Kula and Kragl, 2000).

The enzyme used in the present work shows high homology to the ADH from *Lactobacillus kefir*. It was isolated in 1996 from *Lactobacillus brevis* by Riebel (Riebel, 1996). Because of the higher stability of cell free extracts, the ADH from *Lactobacillus brevis* (LBADH) is more attractive for the technological application. The magnesium containing enzyme was overexpressed in a recombinant *Escherichia coli* strain and is commercially available.[31] In this work it was used in the form of *E. coli* crude cell extract.[32]

---

[29] Wolberg, 2001.

[30] *E.g*. Wong and Whitesides 1994; Faber, 1995; Hummel, 1997; Peters, 1998; Kula and Kragl, 2000; Drauz and Waldmann 2002.

[31] Jülich Fine Chemicals: 100 €/MU for > 100 MU.

4.1.2 Application and Limitation

Despite their enormous potential, the application of ADH is still limited by some important aspects (Grunwald *et al*., 1986; Wong, 1987; Peters, 1998;):

- cofactor cost
- difficulties in enzyme recovery/product work up
- operational stability of ADH
- inhibition of enzyme activities in the presence of substrates or products
- low solubility and in some cases instability of the substrate in water.

The problems of enzyme stability and recovery as well as cofactor cost can be overcome by using whole cell biotransformations. Hence, most industrial biocatalysed reductions are carried out with whole cells. (Schmid *et al*., 2001). However, this is not an universal solution. Although biotransformation with whole cells presents the additional advantage with respect to saving expensive isolation of the desired enzymes (Hummel, 1997), the occurence of ADH with different selectivity inside the cell can cause a decrease in the obtained enantiomeric excess.[33] The regiospecificity of the biotransformation can also be affected if whole cells are used.[34] Besides, the penetration of reagents into the cells may be rate limiting (Carrea, 1984) which becomes a problem if the substrate is unstable in water.

In the case of labile substrates it may be advantageous to use isolated enzymes, since the substrate can reach the active site faster, suppressing substrate decay. An effective process, however, is only possible if all described limitations are worked out. Techniques for these purposes are presented in chapter 5 and 6. In the following sections the investigations about the influence of the reaction conditions on the stability, activity and enantioselectivity of the isolated LBADH are reported.

4.2 Enzyme Stability

Enzymes are inherently labile. Thus their stability, *i.e*. the capacity to retain activity through time (Illanes, 1999), is of paramount importance for any bioprocess. So it is necessary to study the influence of reaction conditions on the enzyme stability. In the present work the

---

[32] The enzyme was friendly supplied by the Institut für Enzymtechnologie, Heinrich-Heine-Universität, Jülich.

[33] The CDHE reduction with whole cells of *Saccharomyces cerevisiae* leads to 94% *ee*, though with isolated enzymes from the yeast higher *ee* is possible (M. Müller, personal communication).

[34] *E.g.* Wolberg describes the reduction of CDHE alternatively in 3- or 5-position if whole cells of *Lactobacillus* sp. are used (Wolberg, 2001).

thermo- and chemostability have been studied by incubating the enzyme under the given conditions and measuring the change of standard activity in course of time.[35] They can be described as first order decay of the activity and are discussed in the following sections.[36]

4.2.1 Influence of the Buffer Substance

For the process development the use of a buffer free solution is desirable in the long run. For experiments in which an autotitrator cannot be used, buffering with potassium phosphate is determined by the substrate stability. Given the sufficient stability of the enzyme in this buffer (cf. next section), the influence of different buffer substances was not tested in this work.

It was observed that the stability of the enzyme is highly dependent on the concentration of the crude cell-free extract. A possible explanation for this observation is the presence of stabilising substances present in the extract.

4.2.2 Influence of the pH

The influence of pH on the enzyme stability was studied by incubating the enzyme in phosphate buffer solutions in the pH range from 6.0 to 8.5. To assure that the measured deactivation is mainly caused by the pH-influence the incubation temperature is 4°C. The enzyme activity is measured at the incubating pH. It is relevant to report that the residual activity increased at the beginning of the assay. Two reasons may be the cause for this observation: the slow change in enzyme conformation at 4°C after protonation/deprotonation equilibrium is achieved, and dissociation of the enzyme/stabilizing agent complex. The half-lives determined for these pH-values are given in figure 4-2. A stability maximum is observed at pH 7.0

[35] The standard activity is measured using 10 mM acetophenone and 0.2 mM NADPH as substrates in phosphate buffer at 30°C (Riebel, 1996).

[36] The stability under biphasic reaction conditions is described in chapter 6.

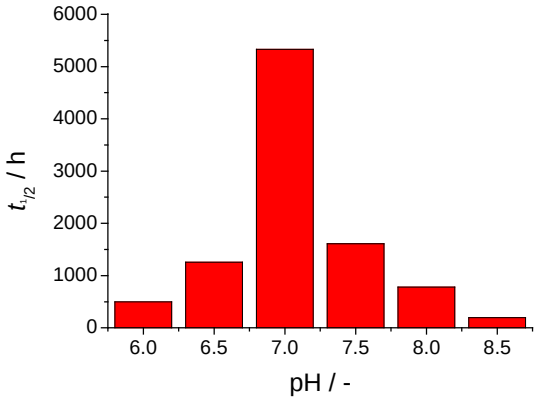


Figure 4-2: pH-influence on the LBADH stability expressed as half-life at 4°C.

4.2.3 Influence of the Temperature

Temperature produces opposite effects on enzyme activity and stability (cf. Illanes, 1999). Consequently, the protein thermodeactivation is of great significance for the technical application of enzymes. The temperature influence on the LBADH stability is determined by incubation of the enzyme in a phosphate buffer at pH 7.0 at the studied temperature and measuring its residual activity under standard activity conditions. The determined half-lives are shown in figure 4-3.

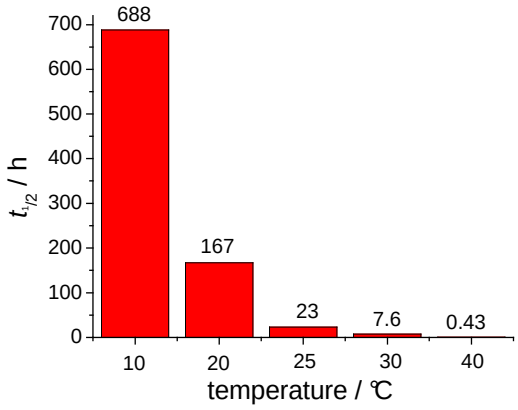


Figure 4-3: Temperature influence on LBADH stability expressed as half-life at pH 7.0.

The temperature influence turns out to be very severe for the LBADH. In order to estimate the stability at a given temperature it is possible to use an Arrhenius-like approach. The apparent

activation energy and pre-exponential factor of the enzyme deactivation are calculated according to the figure below.

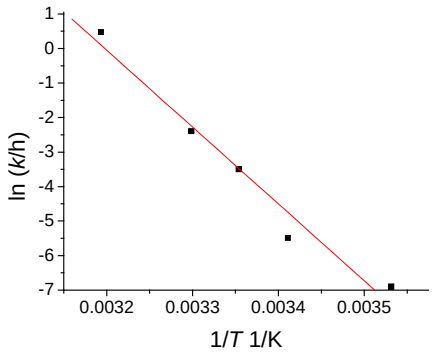


Figure 4-4: Arrhenius plot for the activity decay of LBADH.

The value for the apparent activation energy is E_a = 185 kJ/mol and the pre-exponential factor is 6.8 x 10^{30} / h.[37]

4.2.4 Influence of 2-propanol

Since 2-propanol is a practical co-substrate to be used in the cofactor regeneration,[38] the influence of 2-propanol on enzyme stability has also been investigated in the present. The enzyme was incubated in buffer containing different amounts of 2-propanol (0, 20, 40, 60, 80, 100%, v/v). For these studies the activity measurement was based on the 2-propanol oxidation, for which no substrate inhibition was measurable.

Up to a concentration of 60% the presence of 2-propanol did not affect the enzyme stability. In higher concentration the enzyme was not soluble in the mixture. Consequently activity measurements were disturbed by inhomogeneous sampling. Nevertheless, it can be stated that high activity is still present after 720 h of incubation.

[37] For the activation energy value of a LBADH-catalysed reaction see chapter 5.

[38] Cf. chapter 5.

4.3 Enzyme Activity

4.3.1 Influence of pH

Since enzymes are constituted by amino acids containing acid and basic residues, a variation of the pH can change the conformation and ionisation status of the enzyme. The altered conformation and charge distribution may cause a change in the rate of enzymatic reaction. The literature often refers to the pH-optimum of an enzyme.[39] ADH-catalysed reactions are specially sensitive since the reduction requires and the oxidation releases a proton (see figure 5-8). Consequently, it is not surprising that ADH displays different pH-maxima for reduction and oxidation reactions (*e.g.* Uhlenbrock, 1994 p. 19, Riebel, 1996). Riebel described the behaviour of LBADH-catalysed reduction of acetophenone and oxidation of phenylethanol. While pH increase leads by the oxidation to a rate plateau at approximately 8.0 with smooth decrease at higher values, the reduction is more drastically affected by pH variations. A sharp maximum is present at approximately 7.0. A similar profile is observed for the reduction of acetone and the oxidation of 2-propanol (see 5.3.3).

The direct determination of the influence of pH on the production reaction is not possible due to the substrate instability. Based on the two examples, we have assumed that the highest reduction rate is present at approximately pH 7. Since a fast enzymatic reaction is necessary to suppress the side reaction, further work has been carried out at this pH.

4.3.2 Influence of the Temperature

In contrast to the pH-dependence of enzyme activity, the temperature dependence of the enzyme activity does not show a maximum. Normally, the reaction rate increases with temperature, while the stability decreases (cf. Buchholz and Kasche, 1997 p 49). Both effects, however, are better understood and controlled if studied separately. The enzyme stability has been described in the previous subchapter.

A good possibility of describing the temperature influence on the enzyme catalysis is the use of the Arrhenius equation, described in chapter 3. This procedure is not possible for the production reaction under aqueous conditions due to substrate instability. Besides, the NMR-analysis would require a high amount of substrate converted. Under these circumstances the reaction rate measured cannot be considered equal to the initial rate, thus hindering the measurement. The activation energy with substrate-coupled regeneration was found to be

[39] *E.g.* Buchholz and Kasche, 1997.

46 kJ/mol.[40] Since this is identical to the value determined solely for the cofactor regeneration (see 5.3.3.2), we conclude that the measured value actually reflects the activation energy of the rate-limiting step, the cofactor regeneration. In order to determine the right value for the production reaction, experiments either with stoichometric amounts of cofactor[41] or with enzyme coupled cofactor regeneration[42] would be necessary.

4.4 Regio- and Enantioselectivity

The regio- and enantioselectivity of the LBADH-catalysed reduction of CDHE has been described by Wolberg (Wolberg, 2001). Using the isolated enzyme only the reduction of the δ-carbonyl group can be observed. In contrast, the use of whole cells of *Lactobacillus* yields a mixture of single and double reduced species.

The *ee* of the obtained 5-hydroxyester CHOE is > 99.5% (GC-analysis). In our investigation neither change in pH nor temperature led to a detectable change in the regio- or enantioselectivity of the LBADH-catalysed reduction of CDHE or acetophenone.

4.5 Thermodynamics

The LBADH catalyses the reduction of CDHE under consumption of NADPH yielding CHOE. But it also catalyses the oxidation of CHOE under consumption of $NADP^+$ yielding CDHE. The reversibility of the reaction causes the necessity of an evaluation of the thermodynamic equilibrium of the system.

However, the direct measurement of the system composition at equilibrium is not possible due to the occurrence of the irreversible substrate decay under reaction conditions. This reaction gives rise to a sink, which prevents system from reaching equilibrium (figure 4-5).

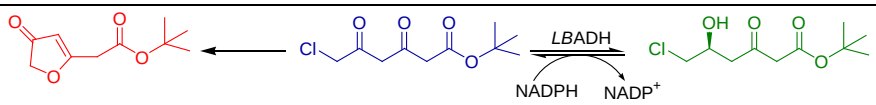


Figure 4-5: The reversible LBADH-catalysed reactions and the irreversible ODFA formation. The irreversibility of one reaction prevents the direct determination of the equilibrium.

Consequently, the equilibrium constant must be estimated based on non-equilibrium conditions.

[40] For substrate-coupled regeneration see chapter 5.

[41] LBADH presents often substrate surplus inhibition by NADPH.

[42] Cf. chapter 5.

An estimation of the equilibrium constant is possible based on the small scale reproduction of the procedure described by Wolberg for production of CDHE on a preparative scale (cf. Wolberg, 2001).[43] In this system the oxidised cofactor is regenerated by the reduction of 2-propanol yielding NADPH and acetone (Chapter 5). The equilibrium constant for the system is the product of the equilibrium constant of production and regeneration reactions.

$$K_{tot} = K_{prod} \cdot K_{reg}$$ **Equation 4-1**

Employing a total amount of 2-propanol corresponding to a concentration of 450 mM, 36 mM CDHE can be completely reduced.[44] Since the conversion is determined by NMR-anylysis, we consider a residual amount of 5% substrate to be present in the mixture.[45]

$$K_{tot} = \frac{[\text{acetone}] \cdot [\text{CHOE}]}{[2-\text{propanol}] \cdot [\text{CDHE}]} \geq 1.6$$ **Equation 4-2**

Considering the value for K_{reg} (Chapter 5.3.2) the value for K_{prod} is estimated as

$$K_{prod} = \frac{[\text{NADP}^+] \cdot [\text{CHOE}]}{[\text{NADPH}] \cdot [\text{H}^+] \cdot [\text{CDHE}]} \geq 1.4 \cdot 10^8$$ **Equation 4-3**

The reversibility of the production reaction is proved by prolonged agitation of the reaction mixture. The observed decrease of the selectivity indicates that the product is re-oxidised to CDHE, which undergoes the irreversible HCl elimination yielding ODFA. This result (equation 4-2) indicates the necessity of having a reducing agent in excess in order to suppress the CHOE oxidation and thus prevent the consequent decrease of the process selectivity.

---

[43] The use of the Haldane relationship to calculate the equilibrium constant of the production system turns out to be impossible due to the approximations made in subchapter 4.6. Nevertheless, extrapolations of the reaction rates described there leads to results similar to those obtained here.

[44] Overall selectivity approximately 90%

[45] The actual concentration must be lower, otherwise the results of the *ee*-determination would be influenced.

4.6 Kinetics

Besides the thermodynamic characterisation, the development of an adequate process for the LBADH-catalysed reduction of CDHE requires also a kinetic characterisation of the reaction. For the purposes of the present process development a description of the reaction mechanisms is not required. The development of a model to describe the macroscopic reaction progress is sufficient (Wandrey, 1996).

The macroscopic rate of enzymatic reactions is frequently described with the Michaelis-Menten kinetics:[46]

$$v = \frac{d[\mathrm{P}]}{dt} = \frac{V_{\max} \cdot [\mathrm{E}]_0 \cdot [\mathrm{S}]}{K_{\mathrm{M}} + [\mathrm{S}]}$$ **Equation 4-4**

with:
[P] = product concentration, [mM];
$[E]_0$ = initial enzyme concentration, [mg/mL];
[S] = substrate concentration, [mM];
V_{max} = maximum velocity, [U/mg]
K_M = Michaelis constant, [mM].

In the case of ADH-catalysed reactions an extended Michaelis-Menten equation to describe the influence of both substrates is used.[47] The values of the required model parameters are obtained using initial rate measurements.[48] This is particularly convenient, since the cofactor consumption or generation can be comfortably followed by change of absorption at 340 nm.

One important characteristic of the Michaelis-Menten kinetic is the asymptotic approximation to a first order kinetic at low substrate concentration, [K_M] >> [S]. In order to suppress the undesired ODFA formation, it is convenient to perform the production reaction at low

[46] A derivation and full discussion of the equation can be found in standard books about enzyme kinetics, *e. g.*:Cornish-Bowden 1981, Bisswanger, 2000.

[47] Advantages and limitations of this approach are discussed in section 5.3.3

[48] Measuring the initial rates the reverse reaction, progressive inactivation of the enzyme and other complicating features can be avoided (Cornish-Bowden, 1981).

substrate concentration.[49] Accordingly, the dependence of the product formation rate on the substrate concentration at a constant cofactor concentration can be described as:

$$v_{\text{prod}} = \frac{d[\text{P}]}{dt} = k_{\text{E}} \cdot [\text{E}]_0 \cdot [\text{S}]$$ **Equation 4-5**

with:

$$k_{\text{E}} = \frac{V_{\max}}{K_{\text{M}}}$$ **Equation 4-6**

The commonly used photometric determination of the parameters is hindered due to the rapid ODFA formation in aqueous solution and the slow dissolution rate of CDHE. Consequently, the determination of k_E is carried out by sampling and NMR-determination of the product concentration. The calculation of the enzyme concentration is based on the mass specific standard activity (347 U/mg) and the molecular weight (104 kDa) determined by Riebel and the measurement of the acetophenone standard activity (Riebel, 1996). To avoid influence of the back reaction, only initial measurements are taken into consideration. To obtain an accurate substrate concentration which is small enough to allow the first order approximation, a MTBE/aqueous biphasic system is used.[50] For equivolumetric systems the aqueous concentration can be easily calculated based on the partition coefficient. The partition coefficient for CDHE is:

$m_{\text{o/w}} = 500 \pm 250$

For a solution of 21 mM CDHE in MTBE (50/50 v/v) at 25°C, 150 mM phosphate buffer, pH 7.0, 1.0 mM cofactor and an enzyme concentration of 0.092 mg/mL (0.9 µM) the measured rate constant of enzymatic reaction is[51]

[49] A full discussion is presented in chapter 6.

[50] Cf. chapter 6.

[51] The determination of the confidence range could not be carried out due to experimental difficulties.

k_E = 32.7 mL/(mg · min)

The assumption that under these conditions the first order approximation is valid should be confirmed by estimation of the K_M-value. Therefore V_{max} should be measured. Using the equation 4-6 it is then possible to estimate the K_M-value.

Thus, the reaction rate is measured under assumed substrate saturation. Therefore we postulate that the enzyme is saturated at 4.5 mM CDHE and determine the reaction rate which leads to V_{max}.

At 25°C and 1 mM NADPH the measured activity leads to a value for V_{max} = 660 U/mg_{enzyme}

This value corresponds to a relative maximum activity of 190% compared to the standard substrate acetophenone. It should be remembered that this value is measured at 25°C, while the acetophenone standard activity is measured at 30°C.[52]

Using the measured V_{max}-value and equation it is possible to estimate the value for K_M:

K_M = 20.2 mM

This value justifies the approximation of the reaction as first order for the range of concentration used in this work.[53] It is an uncommonly high K_M-value for a LBADH-substrate (cf. Riebel 1996).[54] Two major causes may account for this: as CDHE is an unnatural substrate the K_M-value has not been optimised by evolution; the approach used allows only a rough estimation of the K_M-value and not an accurate determination. Probably the major reason for inaccuracy in the estimation is the determination of the aqueous CDHE concentration.

The presence of product inhibition is very common for ADH-catalysis (Wong, 1986). This is not included in the kinetic model, because the product will be continuously removed in the

[52] The relative activity measured at 30°C is 3.0 (cf. table 5-4). A direct comparison with the value described by Wolberg (77% relative activity compared to the standard substrate) should be avoided, since his measurement was carried out at pH 6.5, 25°C and 2 mM CDHE.

[53] The typical concentration is normally < 1µM.

[54] The saturation assumption for a 4.5 mM solution is not correct, but it allows the underestimation of K_M. The measurement under real saturation condition will result in an even higher K_M-value.

process used in the present work. Consequently, its concentration will always be low, and the inhibition insignificant.[55]

An accurate determination of the coenzyme kinetic parameters would be at least as laborious and not more accurate than the estimation of CDHE-values. Since the cofactor is one important cost factor, it should be used in the lowest concentration, which still allows a sufficient reduction rate to suppress the undesired ODFA formation and thus yielding a high selectivity. In subchapter 6.7 it is demonstrated that 0.01 mM is the lowest possible cofactor concentration to cope with high process selectivity. For purposes of the kinetic modelling we assume this value as K_M. Though probably present, inhibition by NADPH-surplus is not considered, since high NADPH concentration should be avoided alone due to economic aspects.

The parameters used in the kinetic expression are shown in table 4-1.

Table 4-1: Parameters regarding the production reaction kinetics; the value in italic is not experimentally determined.

parameter	unit	value
k_E	mL/(mg·min)	32.7
$m_{o/w}$	—	500
$K_{M,NADPH}$	mM	*0.01*

The rate of the production reaction can be expressed by

$$v_{prod} = k_E \cdot [E] \cdot [CDHE]_{aq} \cdot \frac{[NADPH]}{K_M + [NADPH]} \qquad \textbf{Equation 4-7}$$

The reverse reaction, *i.e.* the oxidation of CHOE to CDHE is also catalysed by LBADH. At 25°C and the concentration of 15 mM CHOE and 2 mM $NADP^+$, the activity measured for the oxidation is 3.5 U/mg_{enzyme}. This value corresponds to 1% of the standard substrate activity. Given the low reaction rate and the low CHOE concentration present in reaction volume in this work, this reaction can be seen as less important. Indeed it was seldom observed in the course of experimental work. Therefore it has not been studied in detail. For purposes of modelling, the worst case is assumed: the reaction displays very low K_M-values and at any concentration the maximal rate is achieved (zeroth order approximation). Further

[55] CHOE partition coefficient in MTBE/buffer is 200.

we assume a low but existing K_M-value for $NADP^+$, and set it as 0.01 mM. Consequently the rate for the oxidation reaction is expressed as:

$$v_{revprod} = k_{-E} \cdot [E] \cdot \frac{[NADP^+]}{K_M + [NADP^+]} \qquad \textbf{Equation 4-8}$$

And the parameters are summarised in table 4-2.

Table 4-2: Parameters regarding the CHOE oxidation kinetic; the value in italic is not experimentally determined.

parameter	unit	value
k_{-E}	U/mg_{enzyme}	3.5
$K_{M,NADP}$	mM	*0.01*

4.6.1 Influence of the cofactor regeneration

The formulated equations describe the reaction rate for the case of stoichometric NADPH consumption. Since the cofactor is to be regenerated in the process (chapter 1), additional compounds are going to be present in the reaction phase. They can influence the rate of the production reaction. There are different regeneration approaches, and they are discussed in chapter 5. Here, only the influence of one compound (acetone) involved in the cofactor regeneration is briefly discussed as an example.

Acetone is the product of the 2-propanol coupled cofactor regeneration (subchapter 5.3). The substance itself is also a substrate for LBADH. Thus it shows an affinity towards the active site. When adsorbed on the active site it prevents CDHE from entering it. In other words, acetone is an inhibitor of the production reaction. Indeed the production reaction gets slower as the acetone concentration increases, as shown in figure 4-6 for initial rate measurements.

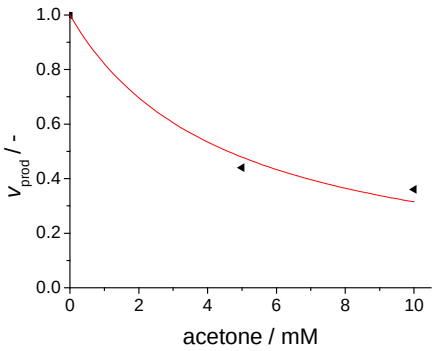


Figure 4-6: Effect of acetone on the initial reaction rate of the production reaction; aqueous/MTBE (50/50 v/v), 21 mM CDHE in MTBE at 25°C, 150 mM phosphate buffer, pH 7.

The inhibition can be described as:

$$v_{\text{prod,acetone}} = \frac{v_{\text{prod,normalised}}}{\left(1 + \frac{[\text{acetone}]}{K_I}\right)}$$ **Equation 4-9**

where

$v_{\text{prod.acetone}}$ = production reaction rate in the presence of acetone (normalised)

$v_{\text{prod,normalised}}$ = production reaction rate in the absence of acetone (normalised)

K_I = acetone inhibition constant

Graph-fitting indicates the value for K_I:

$K_{I,ace} = 4.6 \pm 0.6$ mM

4.7 Interim Summary

- The influence of the reaction conditions on the enzyme stability was studied; the highest stability was observed at pH 7, the enzyme is thermolabile.
- The regio- and enantioselectivity of the biotransformation was not affected by the reaction conditions.
- The thermodynamic equilibrium position of the reaction was estimated.
- Equilibrium shall be avoided to suppress the re-oxidation and thus avoid substrate decay.
- For the small working CDHE concentration, first order description of the production reaction rate was used. The kinetic expression was extended to describe the cofactor influence.
- The reverse reaction was described as zeroth order regarding CHOE and also extended to describe the cofactor influence.
- The influence of acetone as inhibitor of the production reaction was also described.

5 The Cofactor Regeneration

"Der Cofactor ist der biologische Akku." [56]

MARTINA POHL

5.1 Introduction

The redox equivalent required by LBADH catalysed reductions are supplied in form of the cofactor NADPH. This cofactor is prohibitively expensive if used in stoichiometric amounts. Having nature as an inspiration, it is possible to reduce the consumed cofactor and allow it to re-enter the reaction cycle. Thus, the expensive cofactor is only needed in catalytic amounts, which leads to a crucial reduction in costs. Therefore, an efficient and cost-effective integrated[57] regeneration from the oxidised cofactor form is a prerequisite for commercial applications (Kragl *et al*., 1992). The efficiency of a regeneration process is measured by the number of cycles which can be achieved before a cofactor molecule is withdrawn from the system (either by decay or by convection). It is expressed as the total turnover number, *TTN*. The *TTN* is calculated as the total number of moles of product formed divided by the moles of cofactor used during the course of a complete reaction (Equation 5-1).

$$TTN = \frac{\text{moles product formed}}{\text{moles cofactor consumed}} \qquad \textbf{Equation 5-1}$$

The required absolute *TTN*-value to enable the economic application of the enzymatic process depends on the price achievable by the product and the cost of the cofactor. The cost for the whole catalyst system should not be higher than 10% of the product value. Estimating the expected price for CHOE to reach 250 €/kg,[58] corresponding to 60 €/mole, the total catalyst cost for a mole of product should be at most 6.00 €. Considering the cofactor price to be 7.45 €/g $NADP^+$, which corresponds to 5866 €/mole, the *TTN* must be at least 1000. Since the

[56] "The cofactor is the biological accumulator", during a lecture celebrating the awarded *Deutscher Zukunftspreis* 2002.

[57] The former expression *in situ* is avoided, because a regeneration in a different rooms may be concieved.

[58] The present price for 10 g quantities is 89.00 €/g (Jülich Fine Chemicals). The price estimation considers the price for a large scale integrated production.

enzyme cost must be also considered (Chapter 4), the **target *TTN* for this process is 2000** (cf. chapter 9).

Since most oxidoreductases depend on nicotinamide cofactors,[59] many approaches have been developed to regenerate the cofactor. Beside the regeneration efficiency, cofactor retention and cofactor stability are important aspects guiding the achievable *TTN*. These three aspects are subject of the following sections.

5.1.1 Regeneration Approaches

The reductive regeneration is of interest for the present work. Hence, this section will concentrate on approaches for the reductive regeneration of NADPH. This is specially sensitive, because NADPH is the most expensive among the nicotiamide cofactors (table 5-1).

Table 5-1: Prices of nicotinamide cofactors quotated by Jülich Fine Chemicals for a 10 kg order.

cofactor	mol weight [g/mol]	€/g	€/mol
NAD$^+$, free acid	717.5	1.90	1 363.19
NADH, disodium salt	709.4	6.05	4 291.87
NADP$^+$, disodium salt	787.4	7.45	5 866.13
NADPH, tetrasodium salt	883.4	46.10	40 724.74

An adequate cofactor regeneration should lead to the following goals (Adlercreutz, 1996):

1. high cofactor total turnover number (*TTN*).
2. shift of the production equilibrium towards higher product yield.
3. avoid limitation of the production rate due to lack or excess of a cofactor species.[60]

Concerning goal 1, it is important that the regeneration reaction displays a very high 1,4-regioselectivity.[61] Peters has correlated the regeneration selectivity with the achievable *TTN* (Peters, 1998). The regeneration selectivity is a fundamental issue in the choice of the regeneration method. Goal 2 is of particular interest in the present process development, because complete conversion of the substrate is requested.[62] This aspect needs special

[59] 80% on NADH and 10% on NADPH (Faber, 1995, p. 161)

[60] *E.g*. supply the redox equivalent rapidly enough and decrease of substrate/product inhibition caused by the cofactor.

[61] The hydride addition to the pyridine ring of the nicotinamide subunit can occur in the 1,4- and 1,6-position. Only the 1,4-addition leads to the active cofactor species.

[62] Unconverted substrate will decrease the enantiopurity of next step's product, due to unselective reduction.

attention during the entire work. Goal 3 can be achieved by enabling the regeneration rate to prevent the depletion or accumulation of the inconvenient cofactor species. This aspect will be discussed in detail in chapter 6.7, 7 and 8.

According to Kragl (Kragl, 1997), the requirements for successful use of a regeneration system are:

- no inhibition or deactivation of the enzyme by substrate or product/mediator
- no additional procedure in product recovery and isolation
- cheap substrate/low mole specific catalyst cost

An overview of regeneration possibilities can been found in books devoted to biotransformations in the organic synthesis.[63] In the following, the different approaches to the cofactor regeneration will be discussed.

Whole Cell

A widespread way to enable the cofactor regeneration is the use of whole cells as biocatalyst. Here, the cofactor is recycled by the metabolism of the cell. The redox equivalents supplied are cheap (*e.g.* carbohydrates) and the cofactor is retained by the cell membrane. Very low cofactor costs are achievable by this approach (Kizaki *et al*., 2001).[64] However, the use of whole cells as biocatalyst in the present work cannot be regarded as a practicable alternative because:

- whole cells of *Lactobacillus brevis* cause to further reduction of the product.[65]
- a genetically modified organism containing the recombinant LBADH and a regeneration enzyme is not available.

Therefore an isolated cofactor regeneration is needed.

The different approaches for the isolated cofactor regeneration can be divided into two groups (Adlercreutz, 1996):

- electrochemically driven
- substrate driven

[63] *E.g*. Wong and Whitesides, 1994; Faber, 1995; Drauz and Waldmann, 2002.

[64] For whole cell biotransformation the reference to a calculated *TTN* is not reasonable.

[65] Müller *et al*., 1999.

Electrochemical Cofactor Regeneration

The direct cathodic cofactor reduction is not possible, because the selectivity achieved is not high enough to couple with the desired *TTN*.[66] To overcome this limitation, different approaches using mediators have been developed. Their properties have been compared by Adlercreutz (1996). Due to several drawbacks, *e.g.* lack of mediator stability, the process development will concentrate on substrate driven regeneration.

Substrate Driven Cofactor Regeneration

The substrate driven regeneration is achieved by the oxidation of a compound and concomitant generation of a second product. The basic concept is derived from the metabolism but is simplified from the network to a single reaction, which is more amenable to optimisation by reaction engineering techniques (Kula and Kragl, 2000). Two groups of systems can be identified:[67]

- enzymatic
- non-enzymatic[68]

Non-enzymatic Cofactor Regeneration

The direct non-enzymatic regeneration using a reducing agent such as sodium dithionite ($Na_2S_2O_4$) lacks the required regioselectivity. Furthermore, enzyme deactivation caused by $Na_2S_2O_4$ may occur. To overcome these limitations, transition-metal-complexes as catalysts have been used as homogeneous catalyst for the reduction. Encouraging results have been described (*e.g.* Steckhan *et al.*, 1990, Wagenknecht *et al.*, 2003) Nevertheless, a drawback of transition metal homogeneous catalysis persists: the direct (unspecific) reduction of the substrate by the regeneration catalyst (cf. *e.g.*Uhlenbrock 1994).

Enzymatic Regeneration

Nature offers a great number of enzymes dependent on nicotine amide cofactor. Some of them can be used for the isolated cofactor regeneration. Their 1,4-specificity has been provided by evolution. Consequently, enzymatic regeneration is at present the approach enabling highest selectivity (Peters, 1998, Faber, 1995, p. 162). Therefore, we focus on enzymatic regeneration

[66] Kai Vuorilehto, personal communication, 2003.

[67] There are photochemical approaches belonging to both groups. Since the efficient methods require an expensive sensitizer, they are not a competitive alternative at present, and consequently will not be discussed in this work; cf. Adlercretz, 1996.

[68] The non-enzymatic cofactor regeneration is often referred to simply as “chemical”. Since enzymatic reactions are also chemical, this nomenclature will be avoided.

for the present process development. There are two possibilities of conducting the regeneration enzymatically: enzyme-coupled and substrate-coupled. In subchapter 5.2 the enzyme-coupled approach is described. Subchapter 5.3 deals with the substrate-coupled approach.

5.1.2 Cofactor Retention

As previously stated, a high *TTN* is fundamental for the implementation of a cofactor dependent catalysis. Besides the efficiency of the regeneration reaction, the *TTN* is also dependent on the concentrations of cofactor and substrate present in the solution (equation 5-1). The concentration of the cofactor should be as low as possible and is determined by its K_M-value. The concentration of the substrate is normally determined by its solubility in water, inhibitory effects and/or enzyme stability. In the case of water-soluble substrates, high substrate concentrations are possible. Because the minimal cofactor concentration required for most dehydrogenases-catalysis is low (low K_M-value), efficient cofactor regeneration alone is able to provide a *TTN* high enough for economic application in these cases.[69]

For poorly soluble substrates, which is the case of most alcohol dehydrogenase substrates including CDHE, the substrate to cofactor concentration ratio is significantly lower than in the case of water-soluble substrates. Thus, the cofactor has to be separated from the products and reutilised in order afford sufficiently high *TTN*. Different methods have been developed for this purpose. The most common is cofactor immobilisation. The diverse techniques of immobilisation can be classified into two groups (Peters, 1998): coupling and entrapping

Coupling methods: It is possible to couple the cofactor on cross-linked enzymes or simultaneously with the enzyme on a carrier.

Entrapping methods: A brief discussion on the most attractive entrapping methods for the process development follows:

The cofactor can be **bound to a water soluble polymer**. The polymer can be retained during ultrafiltration while small substrate and product molecules can freely pass the membrane. Polyethylene glycols (PEG-10 000 and PEG-20 000) were used by Wichmann *et al*. for the entrapment of NADH (Wichmann *et al*., 1981). This approach enables very high *TTN*: 600 000 (Hummel, 1997). However, it requires a tedious synthesis of polymer bound cofactor and an eventual decrease of the cofactor activity.

[69] "It can be stated that for water-soluble substrates the *in situ* regeneration has been solved" (Kula and Kragl, 2000)

Modifications of the enzyme **membrane reactor** concept make use of membranes in order to retain the cofactor in the reactor. Two distinct approaches have been described: the use of charged membranes (Röthig *et al*., 1990, Ikemi and Ishimatsu, 1990) and the more versatile use of nanofiltration (Seelbach, 1994; Kragl, 1997; Seelbach and Kragl, 1997). Seelbach has determined the retention of $NADP^+$ by nanofiltration membrane as being 0.98. So, the *TTN* could be enhanced 3 to 4 fold.

The retention of the charged cofactor in the aqueous phase of a **biphasic system** is a third entrapping possibility. Carrea used this technique for NADH-dependent steroid dehydrogenase (cf. Carrea, 1984). The advantages of this approach are the very high retention of the cofactor and the facile implementation. An important disadvantage is the notorious instability of ADH in the presence of organic solvents, which is subject of chapter 6. This limitation can be (partially) overcome by a bimembrane reactor (Kruse *et al*. 1994; Kruse, 1995; Kruse *et al*. 1996).

In conclusion, there are sufficient possibilities for cofactor retention. Before the implementation of polyethylene glycol it is necessary to investigate the effects of bounding NADPH on the LBADH kinetics. The use of the biphasic approach for the cofactor retention depends exclusively on the existence of a solvent not affecting the enzyme stability. The retention of the cofactor by a nanofiltration membrane is a technology readily available for application. Therefore, both the last possibilities are more straightforward. The decision on the most suitable solution should be made considering the process as a whole. This is discussed in chapter 7. No further investigation is necessary at this stage.

5.1.3 Cofactor Stability

Given a selective cofactor regeneration and a high retention value, the *TTN*-limiting factor is the chemical stability of the cofactor. Therefore aspects influencing the stability are briefly discussed here.

Nicotinamide cofactors contain three labile bonds which may be target of general or specific acid- and base-catalysed hydrolysis (Figure 5-1):

- the *N*-glycosidic bond between nicotinamide and ribose
- the phosphodiester bond between the two ribose-units
- the bond between ribose and phosphate

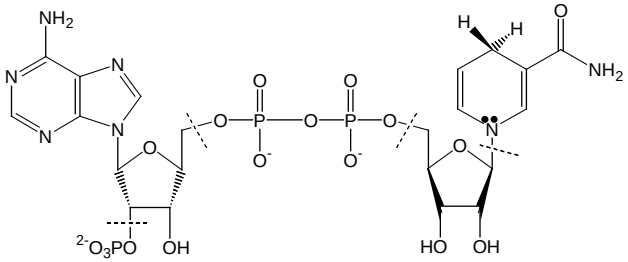


Figure 5-1: The labile bonds in NADPH.

The mechanism of the decomposition and the different pathways were studied in detail (cf. Wong and Whitesides, 1994; Peters, 1998). The influence of pH and buffer on the cofactor stability is also described (Johnson and Tuazon, 1977). Under basic conditions the reduced cofactor is stable, but unstable under acidic conditions. In contrast, the oxidized cofactor is stable under slightly acidic and labile under basic conditions. A pH range of 8-8.5 represents a compromise in stability (Wong and Whitesides, 1994).[70] The presence of inorganic phosphate increases the rate of the cofactor decomposition, while some organic buffers like triethanolamine (TEA), HEPES and Tris buffers are less detrimental.

The effect of the pH on the stability of NADPH is illustrated in figure 5-2. The light extinction (*E*) at 340 nm of a standard solution is measured at different pH-values. The decrease of absorption in course of time, as a measure for the decay, is plotted against the time.

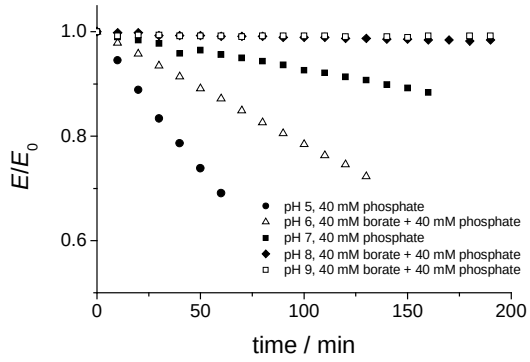


Figure 5-2: The influence of pH on the NADPH stability illustrated by the extition at 340 nm of a 0.1 mM NADPH solution at 25°C.

[70] Depending on the production/regeneration ratio the working pH-window may differ from the value mentioned above.

Finally, it is important to consider that a temperature increase has severe consequences for the cofactor stability.

5.2 Enzyme-Coupled Cofactor Regeneration

5.2.1 Introduction

In the case of the enzyme-coupled cofactor regeneration, the production reaction and the co-factor regeneration reaction are catalysed by two different enzymes (figure 5-3).

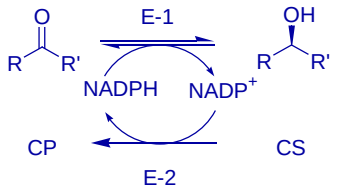


Figure 5-3: Enzyme-coupled cofactor regeneration; E-1 and E-2 are different enzymes; CS is the co-substrate and CP, the co-product.

Levy and coworkers used this method to regenerate NADH consumed by ADH in the synthesis of a pure enantiomorph of ethanol-1-*d* (Levy *et al*., 1957).[71] Glucose dehydrogenase catalysed the reduction of the cofactor using glucose as substrate. Several other systems were developed. The best and most widely used system uses formate dehydrogenase (FDH) (Faber, 1995). It is shown in figure 5-4.

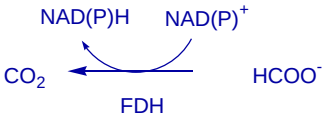


Figure 5-4: The cofactor regeneration using formate dehydrogenase (FDH).

The NAD^+-dependent FDH of the methylothropic yeast *Candida boidinii* was described by Sahm, Kula and co-workers (Schütte *et al*., 1976). The enzyme's production was optimised (Weuster-Botz, 1994) and it is used on a industrial scale *e.g*. for the production of L-*tert*-leucine (Liese *et al*. 2000).[72]

The enzyme-coupled regeneration has found great acceptance due to following advantages:

[71] In this publication the cofactor is named DPNH (diphosphopyridine nucleotide).

[72] For their work dedicated to the FDH-coupled substrate regeneration Prof. Dr. Dr. Kula and Dr. Martina Pohl were awarded with the Deutscher Zukunftspreis 2002.

1. The position of the **thermodynamic equilibrium** can be (easily) influenced. Thermodynamically unfavourable production reactions can be driven by coupling with a favourable cofactor regeneration (Peters, 1998). For the enzyme-coupled regeneration it is possible to take advantage of the (almost complete) irreversibility of the reaction.
2. The **activity fraction** of both enzymes can be optimised (Peters, 1998), enabling a control on the relative concentrations of $NADP^+$ and NADPH. [73]
3. Substrate and co-substrate do not have to **compete for the active site** of a single enzyme (Faber, 1995).

Thus, it is possible to compensate the disadvantage of additional cost and complexity due to the inclusion of a second enzyme (Adlercreutz, 1996). Therefore, it is necessary to assure the compatibility between both enzymes concerning pH and temperature working window (Peters, 1998). Also the compatibility between enzymes and substrates/products must be guaranteed. Moreover, both enzymes should have different specificities for their respective substrates (Faber, 1995), so that undesired catalysis is prevented.

The widespread use of formate dehydrogenase is due to the following facts:

1. The thermodynamic equilibrium of the regeneration system is very favourable. Although the reverse reaction could be detected, the product (carbon dioxide) leaves the reaction mixture continuously (Schütte *et al*., 1976). Hence, the reaction can be regarded as irreversible.
2. Its substrate, formate, is cheap, safe and water soluble. Besides, it is not converted by most production enzymes, thus avoiding competition for the active site.
3. The regeneration product, carbon dioxide, is easily removed from the reaction mixture, not causing additional work up steps. It is also not obnoxious to protein.

Beside the exclusively NAD^+-dependent FDH of *Candida boidinii*, the bacterial **FDH from *Pseudomonas* sp. 101** has been intensively studied. The three dimensional structure has been characterised to high resolution. This enzyme is of interest because the wild-type displays activity also with $NADP^+$, though it is low (about 5% reaction rate compared to NAD^+ and high K_M value of 35 mM). Tishkov *et al*. generated an $NADP^+$-dependent FDH by rational design strategy. The mutant enzyme exhibits about 60% of the reaction rate compared to NAD^+. The K_M value has been lowered by more than 100-fold by site directed mutagenisis. The new $NADP^+$-dependent FDH has been produced in pilot scale using a recombinant *E. coli*

[73] Cf. enzyme activity fraction in Kragl *et al*., 1996.

strain. (Tishkov *et al*., 1999). It is commercially available,[74] and its use in integrated regeneration has been reported. This enzyme regenerated the NADPH consumed in the ADH-catalysed reduction of acetophenone (Seelbach *et al*., 1996).[75] Further use in a coupled synthesis of chiral lactones by monooxygenase has also been reported (Rissom *et al*., 1997). For its low cost and reported compatibility with ADH from *Lactobacillus* sp. this FDH is studied as a possibility for cofactor regeneration in the present process development.

Equally attractive is the use of hydrogenase enzymes, which are so-called for being able to accept molecular hydrogen directly as hydride donor. The latter is strongly reducing, innocuous to the enzymes and nicotinamide cofactors, and its consumption leaves no by-product. The **hydrogenase from *Pyrococcus furiosus*** displays $NADP^+$-activity. In spite of recent development (Greiner, 2002), drawbacks of this approach are the enzyme's extreme sensitivity to oxidation and the lack of commercial availability combined with complicated fermentation procedures (Faber, 1995, p. 165). For these reasons, this enzyme cannot be considered at present for a technological application.

A further enzyme used for NADPH regeneration is **glucose-6-phosphate dehydrogenase** (G6PDH) (Faber, 1995). Due to the spontaneous hydrolysis of the product, 6-phosphoglucolactone, to the corresponding gluconate, the equilibrium of the reaction is quite favourable. A major disadvantage of this system is the expensive substrate (see table 5-2). To decrease its cost, the substrate may be generated *in situ* (Faber, 1995 p. 164). However, this increases the complexity of the system. Hence, this approach will not be considered in the present work.

A similar method uses the **glucose dehydrogenase** (GDH) for the cofactor regeneration (Figure 5-5). The enzyme from *Bacillus cereus* is stable and accepts NADPH with high specificity. However, the final regeneration product, gluconate, may complicate the work-up (Faber, 1995). In addition, the gluconate formation requires constant pH adjustment.

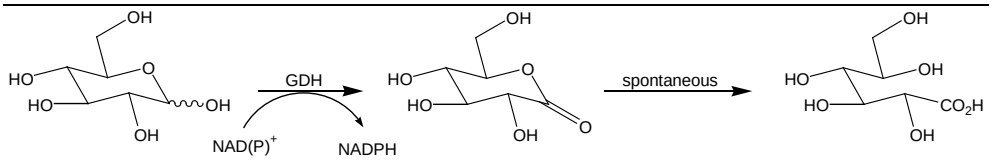


Figure 5-5: Glucose dehydrogenase (GDH) catalysed cofactor regeneration followed by the spontaneous gluconate formation.

[74] Jülich Fine Chemicals, a solution containing 51 U/mL, 47.05 €/kU.

[75] ADH from *Lactobacillus* sp..

The use of a **second ADH** for the cofactor regeneration can also be considered. This alternative offers little advantage with respect to the substrate-coupled cofactor regeneration. The thermodynamic situation is comparable; cross inhibition and undesired catalysis are possible. The only advantage is the possibility of controlling the activity fraction. So, this system is only to be used if a more effective regeneration reaction is indispensable and cannot be obtained by other means (cf. 6.7, 7).

The different systems discussed are summarised in table 5-2.

Table 5-2: Systems for enzyme-coupled cofactor regeneration.

enzyme	substrate	product	substrate cost [€/mol]
rec. FDH [EC 1.2.1.2]	**sodium formate**	**carbon dioxide**	**0.014**
ADH [EC 1.1.1.2]	2-propanol	acetone	0.05
glucose dehydrogenase [EC 1.1.1.47]	glucose	gluconate	0.13
glucose-6-phosphate dehydrogenase [EC 1.1.1.49]	glucose-6-phosphate	6-phospho-glucolactone	5330.00
hydrogenase [EC 1.18.99.1]	molecular hydrogen	—	< 0.01

In conclusion, the only system for NADPH regeneration relevant at present for this process development is the recombinant FDH from *Pseudomonas* sp. 101. Its compatibility with the production system is investigated in the next section.

5.2.2 Compatibility between Production and Regeneration

The compatibility between production and regeneration systems is indispensable. Important aspects to be considered are listed:

- pH-range for enzyme activity and stability
- temperature range for enzyme activity and stability
- effect of substrate and product on the enzyme (stability, inhibition)
- undesired reaction between substrate or product and co-substrate or co-product

Previous works have shown that the $NADP^+$-dependent FDH can be used at the same temperature and pH-ranges aimed for the present process development. (Seelbach *et al*., 1996, Rissom *et al*., 1997). Thus, the next step was to investigate the effect of the substrate on the enzyme.

Therefore, FDH was dissolved in the presence of a 7.5 mM solution of CDHE and incubated at 30°C. Samples were collected periodically and its activity is determined photometrically. A control experiment was carried out, repeating the incubation conditions but without CDHE. The results are plotted in figure 5-6.

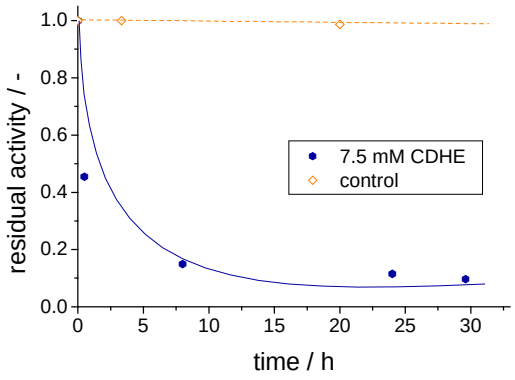


Figure 5-6: Residual activity of FDH in the presence of 7.5 mM CDHE and in the control experiment without CDHE; pH 7.0, 100 mM pohsphate buffer, 30°C.

The results clearly show an absolute incompatibility between the regeneration enzyme and the substrate. Incompatibility between oxidoreductase and α-chloro ketones have been observed before.[76]

Consequently, the direct use of the FDH for cofactor regeneration must be disregarded. For this reason, it is not possible at present to regenerate the cofactor in this process development *via* an enzyme-coupled system.[77]

[76] Wolberg for the essential activity in *S. cerevisiae*, regarding CDHE (Wolberg, 2001 p. 51); Shimizu *et al*., regarding 4-chloro-3-oxobutanoate, (Shimizu *et al*. 1990).

[77] Options to decrease the concentration of CDHE in the regeneration room are discussed in chapter 6 and 7.

5.3 Substrate-Coupled Cofactor Regeneration

5.3.1 Introduction

In the substrate-coupled approach both the production and the regeneration reaction are catalysed by the same enzyme (figure 5- 7). A second, sacrificial substrate (co-substrate) serves as hydride donor. Hence, it resembles the Meerwein-Ponndorf-Verley reduction (cf. Stampfer *et al*., 2002).

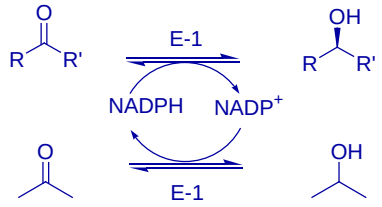


Figure 5-7: Substrate-coupled cofactor regeneration with 2-propanol as sacrificial substrate (Co-substrate).

The co-substrate must be a low cost alcohol. Ethanol should be avoided, because its oxidation product, ethanal, can react with the enzyme forming a Schiff's base. If secondary alcohols are converted by the enzyme, they should be preferred. In the case of LBADH, the use 2-propanol as the sacrificial co-substrate is possible. This alcohol and its oxidation product, acetone, are volatile and can be easily separated from the product by distillation. However, the catalysed reaction is reversible. So, the thermodynamic equilibrium must be considered when this method is applied. This is the subject of the next section. To shift the equilibrium of the system to the desired position, the co-substrate is usually applied in large excess. This large excess of alcohol increases the solubility of the substrate and is consequently advantageous for the process. However, it is often detrimental with regard to enzyme stability or may affect kinetic constants. This approach is advantageous considering the enzyme availability and the compatibility between enzyme and substrates. On the other hand, the overall efficiency is reduced since the enzyme's activity is distributed between substrate and co-substrate (Faber, 1995). Cross inhibition is very likely to occur. Besides, pH optima for production (reduction) and regeneration (oxidation) are not coincident. The major disadvantage of this regeneration is the activity fraction for production and reduction. At given reaction conditions (pH) this ratio is a natural constant.

It may be mentioned here that the substrate-coupled cofactor regeneration approach is the most frequent for the synthesis of *R*-alcohols, since all *R*-selective ADH presently used in

biotransformations are NADPH dependent, and the cofactor could not be regenerated otherwise in a more economical way (Kula and Kragl, 2000). The substrate-coupled regeneration is also very compatible with the use of enzymes in non-natural media, specially where the cofactor is not soluble (Adlercreutz, 1996).

5.3.2 Thermodynamics

The substrate-coupled cofactor regeneration employed here is based on the reversible reaction depicted in figure 5-8.

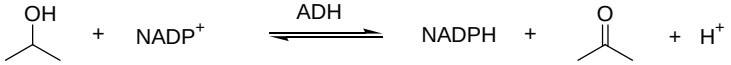


Figure 5-8: The reversible oxidation of 2-propanol used for the substrate coupled cofactor regeneration.

The equilibrium constant of the regeneration system can be obtained according to the law of mass action (equation 5-2):

$$K_{eq} = \frac{[\text{acetone}] \cdot [\text{NADPH}] \cdot [\text{H}^+]}{[2-\text{propanol}] \cdot [\text{NADP}^+]} \qquad \textbf{Equation 5-2}$$

The value for K_{eq} at 30°C was obtained in a series of experiments where the concentration of the oxidised and reduced cofactor were determined by HPLC, the concentration of co-substrate and co-product, by GC, and the equilibrium condition confirmed photometrically.

$$K_{eq}^{30} = 1.1 \text{x} 10^{-8}$$

This is in good agreement with the value measured by Uhlenbrock if the pH is considered (Uhlenbrock, 1994).[78] It clearly indicates the thermodynamic limitation of this regeneration system.[79] In order to compensate this barrier, high 2-propanol concentrations are used (Peters *et al*., 1993; Schubert *et al*., 2002; Stampfer *et al*., 2002). Often, the highest concentration of 2-propanol possible is determined by the sensibility of the enzyme to the presence of this alcohol in solution. The investigation of the compatibility of LBADH with 2-propanol is described in 6.2.4.2. It can be stated that the enzyme tolerates high 2-propanol concentration

[78] The determination of the equilibrium constant is very sensitive concerning the pH. Inaccuracy in the pH determination are probably the reason for the small difference.

[79] Incorporating the pH to the constant: $K'_{7.0} = 0.11$.

while being soluble in the 2-propanol/water mixture.[80] So, high 2-propanol concentrations can be used to shift the thermodynamics of the system. The highest 2-propanol concentration to be used in this process development is determined by kinetic and economic aspects. It is not possible to influence thermodynamics with a pH-shift, because it will affect the production equilibrium in the opposite direction. Possibilities of influencing the thermodynamic *via* reaction engineering are discussed in chapter 7.

The K_{eq}-value depends also on the temperature. Decreasing the temperature to 20°C, the value measured for the equilibrium constant is:

$K_{eq}^{20} = 0.83 \text{ x } 10^{-8}$

A comparison of the results reveals that at higher temperature the product equilibrium concentration is higher. This indicates that the regeneration reaction is endothermic.

5.3.3 Kinetics

The complete characterisation of the regeneration system includes beside the thermodynamic also the description of the kinetic behaviour of the regeneration reaction. In contrast to the production reaction, following the time-dependent concentration changes in the case of the $NADP^+$ reduction by 2-propanol is a standard procedure. The 340 nm extinction can be correlated to the NADPH concentration according to the Lambert-Beer law. This is a facile method to conduct initial rate measurements.

5.3.3.1 pH Dependence

As discussed in chapter 4, the rate of enzyme-catalysed oxidation and reduction are strongly influenced by the pH. Figure 5-9 shows the dependence of the reactions involved in the cofactor regeneration on the pH.

[80] At 80% (v/v) 2-propanol the protein coagulates forming glue-like fibbers.

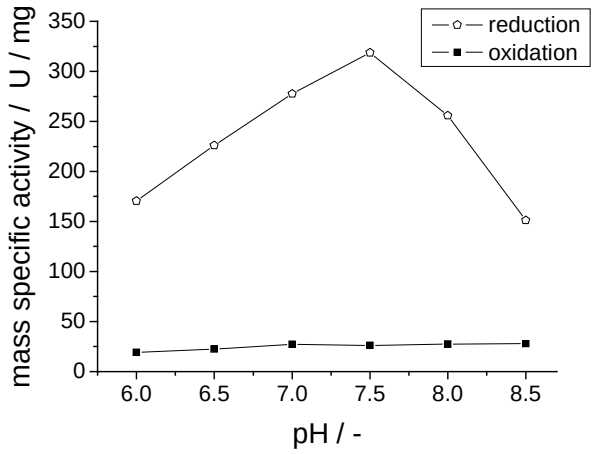


Figure 5-9: Influence of pH-value on the enzyme activity for 2-propanol oxidation and acetone reduction at 30°C.

A comparison of the obtained results with those for acetophenone and phenylethanol described by Riebel reveals the same pH-dependence of oxidation and reduction (Riebel, 1996 p.147). Distinct is the magnitude of rate difference between oxidation and reduction. While an overlap can be noticed for the reduction rate of acetophenone and the oxidation rate of phenylethanol, the acetone reduction is always much faster than the 2-propanol oxidation. For further studies pH 7.0 has been chosen based on the arguments presented in chapter 4.

5.3.3.2 Temperature Dependence

The influence of temperature on LBADH-catalysis has been discussed in chapter 4. For the regeneration reaction (2-propanol oxidation), it is possible to describe the kinetic dependence in the temperature interval between 5°C and 30°C according to Arrhenius. At higher temperature the linearity is not present anymore, probably owing to thermal enzyme denaturation. The activation energy of the cofactor regeneration can be calculated for this linear interval using an Arrhenius plot (Figure 5-10).

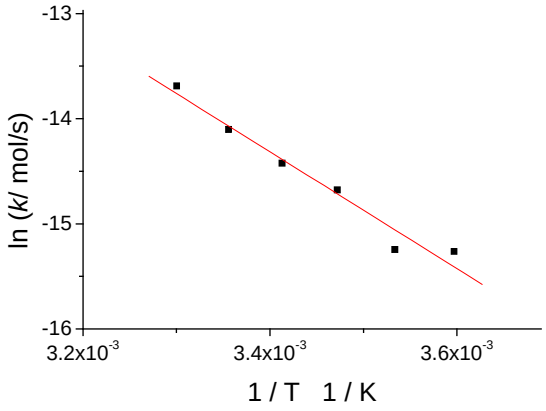


Figure 5-10: The temperature influence on the reaction rate of the 2-propanol oxidation.

The value obtained for the activation energy is 46 kJ/mol.[81] Further studies are conducted at 30°C, since at this temperature denaturation effects during the measurement can be neglected.

5.3.3.3 Kinetic Model

The kinetic description of the regeneration reaction is different from the production reaction. The latter can be described with first-order rate law due to the low substrate concentration present. For the cofactor regeneration, the reaction rate must be high to enable an efficient regeneration. Consequently, high amounts of co-substrate are used.[82] At high substrate concentration first-order rate law loses its validity and saturation kinetics must be used to described the system (cf. *e.g.* Cornish-Bowden, 1981).

In the present work an extended Michaelis-Menten equation with two substrates, competitive product inhibition and uncompetitive substrate inhibition is used according to equations 5-3 and 5-4.[83]

[81] Activation energy values for enzyme catalysis are frequently between 40-50 kJ/mol (Bisswanger, 2000). Cf. 6.7.2 for the implications of this result.

[82] Beside kinetic reasons, thermodynamic reasons therefore are discussed in the previous section

[83] "Thus it is hardly ever possible to determine with reasonable confidence which is the correct mechanism why not select the simplest empirical rate expression which satisfactorily fits the data." (Levenspiel, 1972 p 466)

$$v_{reg} = V_{\max,ox} \cdot [\mathrm{E}] \cdot \frac{[2-\text{propanol}]}{K_{\mathrm{M},2-\text{propanol}} \cdot \left(1 + \frac{[\text{acetone}]}{K_{\mathrm{I,aceton}}}\right) + [2-\text{propanol}]} \cdot \frac{[\mathrm{NADP}]}{K_{\mathrm{M,NADP}} + [\mathrm{NADP}]}$$

Equation 5-3

and

$$v_{\mathrm{revreg}} = V_{\max,\mathrm{red}} \cdot [E] \cdot \frac{[\text{acetone}]}{K_{\mathrm{M,aceton}} \cdot \left(1 + \frac{[2-\text{propanol}]}{K_{\mathrm{I},2-\text{propanol}}}\right) + [\text{acetone}]} \cdot \frac{[\mathrm{NADPH}]}{K_{\mathrm{M,NADPH}} \cdot \left(1 + \frac{[\mathrm{NADP}]}{K_{I,\mathrm{NADP}}}\right) + \left(\frac{[\mathrm{NADPH}]^2}{K_{\mathrm{I,NADPH}}}\right) + [\mathrm{NADPH}]}$$

Equation 5-4

with:

v = reaction rate [mM/min]

$V_{\max}$ = maximum velocity [U/mg]

[E] = enzyme concentration [mg/mL]

K_{M} = Michaelis constant [mM]

K_{I} = inhibition constant [mM]

Advantages and limitations of this model are listed below (cf. Biselli, 1991):

- the model is based on initial rate measurements
- every parameter has a physico-chemical meaning
- reduction and oxidation are treated independently (although catalysed by the same enzyme)
- knowledge about the reaction mechanism is not required
- the model implies a dependence on the initial concentrations for K_{eq}, therefore it is not adequate to describe a system near the equilibrium.
- inhibition is described by formal inhibitory terms, which are not based on enzyme-substrate-equilibria, but enable a data fitting.

Owing to these limitations, the application of this model is restricted. Nevertheless, in the process developed of the present work the products are continuously removed and substrate continuously added (see chapter 7), thus preventing the system to reach equilibrium. Rather, a stationary situation equivalent to a constant initial reaction situation with product inhibition is

present. This situation justifies the use of this model in the estimation of reaction rate for this bioreactor.

Determination of Kinetic Constants

The determination of the kinetic constants was conducted by photometric initial rate measurements and non-linear regression. In order to enable comparison between the different measurements, the values for the maximum velocity were normalised by the standard acetophenone velocity and converted to the mass specific value afterwards. Some examples of the reaction rate dependence on substrate and product concentrations are given in Figure 5-11.

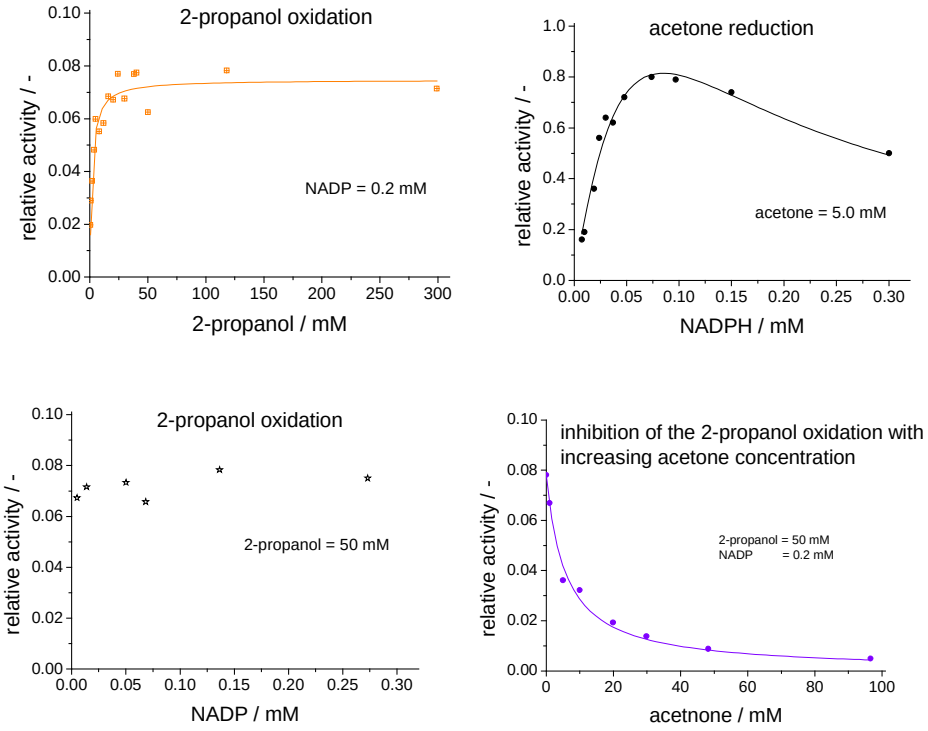


Figure 5-11: Examples of curves used in the determination of kinetic constants at 30°C; The standard acetophenone activity is used as activity reference (1.0). a) influence of the 2-propanol concentration on the 2-propanol oxidation rate ($NADP^+$ = 0.2 mM); b) influence of the NADPH concentration on the acetone reduction rate (acetone = 5.0 mM); c) influence of the $NADP^+$ concentration on the 2-propanol oxidation rate (2-propanol = 50 mM); d) inhibition of the 2-propanol oxidation with increasing acetone concentration (2-propanol = 50 mM, $NADP^+$ = 0.2 mM). All reaction conducted at pH 7 in 50 mM phosphate buffer.

The values are summarised in table 5-3.

Table 5-3: Value of the kinetic constants according to initial rate determination.

parameter	unit	value	confidence range
$V_{max,\ ox}$	U / mg	25	± 1
$K_{M,2\text{-prop}}$	mM	1.8	± 0.3
$K_{M,NADP}$	mM	<0.005	–
$K_{I,ace}$	mM	5.8	± 0.5
$V_{max,red}$	U / mg	$9.7 \cdot 10^2$	$\pm 0.8 \cdot 10^2$
$K_{M,ace}$	mM	1.4	± 0.5
$K_{M,NADPH}$	mM	0.11	± 0.06
$K_{I,NADPH}$	mM	0.07	± 0.04
$K_{I,2\text{-prop}}$	mM	7.3	± 0.5
$K_{I,NADP}$	mM	0.40	± 0.03

A precise determination of $K_{M,NADP}$ is not possible due to the very small value. For modelling purpose $K_{M,NADP}$ is set equal 0.005 mM. No inhibition of the oxidation reaction regarding NADPH can be noticed if the enzyme is saturated with substrates. This can be caused by the reaction mechanism, the very small K_I value for NADPH, or the very small $K_{M,NADP}$, which even when shifted by inhibition is not detectable. Regardless of the reason, competitive product inhibition caused by NADPH is not significant in the working window and is disregarded.

The existence of the acetone inhibition deserves special attention. Besides limiting the thermodynamic of the system, high acetone concentration is also a kinetic obstacle to the cofactor regeneration.

5.3.3.4 Integration of the Cofactor Regeneration

The model presented in this section describes the oxidation of 2-propanol under consumption of $NADP^+$ yielding NADPH and acetone. Two important kinetics aspects concerning the integration of this reaction in a process for cofactor regeneration shall be discussed here: the regeneration activity fraction and the cross inhibition.

Regeneration Activity Fraction

The activity fraction of production and regeneration reactions is a decisive parameter governing the efficiency of the cofactor regeneration. While it can be easily controlled in

enzyme-coupled regenerations, it is constant in substrate-coupled regeneration under given reaction conditions. The maximal mass specific values obtainable for the reaction rates at 30°C and pH 7.0 are given in table 5-4. The values are normalised by the standard acetophenone velocity.

Table 5-4: Relative maximal enzyme specific reaction rates obtained at pH 7 and 30°C; The value concerning CDHE differs from that in chapter 4 due to the different measurement temperature.

substrate	relative maximal reaction rate
CDHE	**3.0**
CHOE	0.01
2-propanol	**0.075**
acetone	0.80

Thus, at pH 7.0 the regeneration activity fraction is:

$$\text{regeneration activity fraction} = \frac{\text{regeneration}}{\text{production} + \text{regeneration}} = 0.024$$

Possible implications of this very low value are[84]:

- accumulation of the cofactor in the oxidised from.
- bad overall selectivity due to low production rate
- low enzyme efficiency; low space time yield

It is possible to influence the activity fraction inherent in LBADH regeneration by pH-shift, since oxidation and reduction display different sensitivity to pH changes. However, this procedure would also change the rate of the reverse reactions. Further more, in the special case of 2-propanol oxidation, the sensitivity to pH-shift is not high enough to enable significant improvement. Thus, another solution must be found to resolve this problem. (see chapter 7).

Cross Inhibition

Since CDHE and CHOE are substrates of the LBADH, a competitive inhibition by this two compounds on the regeneration reaction must be expected. A direct determination of CDHE is

[84] For the case that both reactions are saturated with substrates.

not possible though, owing to its instability in water. Although critical for one-pot reactions, this limitation is not a hurdle for the description of the regenerating system in the reactor developed in the present work (see chapter 7). The concentration of CDHE must be kept very low, beyond its K_M value, in order to enable a good discrimination of the undesired ODFA formation (cf. chapter 6). Under this condition, the competitive inhibition is expected to be very low and will be neglected. Similarly, the inhibition caused by CHOE is expected to cause only insignificant changes in the regeneration rate.

5.3.4 Cofactor Concentration

The low regeneration activity fraction described in the previous section has important consequences for the process. If production and regeneration occur at maximal rate, the cofactor will accumulate in the oxidised form, and the regeneration will be the rate limiting step. This means that the stability of $NADP^+$ rather than NADPH must be considered for setting the reaction conditions. On the other hand, it means, that the production reaction is limited, thus limiting the space-time-yield. In addition, product inhibition is expected to occur in ADH-catalysed reactions. Hence, not only the low NADPH concentration will limit the production rate, but also the accumulation of $NADP^+$ will cause a rate decrease.

The concentration of NADPH is governed by the regeneration fraction, which cannot be altered by reaction conditions. The inhibition due to $NADP^+$, however, can be at least controlled by the proper initial cofactor concentration. Small total amounts of cofactor[85] in a kinetic limiting cofactor regeneration will lead to a smaller accumulation of $NADP^+$. So the limitation of the production rate is attenuated. This effect is described in chapter 6.7.

5.4 Conclusion

The results presented in this chapter describe the enzymatic cofactor regeneration as an efficient possibility for the cofactor regeneration. Allied with one of the various techniques for cofactor retention, it assures satisfactory *TTN* for the cofactor.

In addition to **high *TTN***, the integration of the cofactor regeneration has two further requirements: they are of special importance in the case of the reduction of CDHE:

- **Shift of the production equilibrium towards higher product yield** because no trace of the substrate shall be present in the product.

[85] [NADP] + [NADPH]

- **It must be assured that the production reaction is the rate limiting step of the coupled system**.[86] Otherwise the substrate decay will decrease the overall selectivity.

These requirements are easily achievable with enzyme-coupled regeneration. However, among the methods available at present, none offers an economic and compatible solution as discussed in subchapter 5.2. At the same time, the requirements cannot or not completely be fulfilled by the substrate-coupled regeneration.

As the only available approach for the one-pot reaction, the substrate-coupled regeneration is used in the studies described in the next chapter, in spite of its limitations. In chapter 7 reaction engineering approaches to enable a conciliation of all three requirements with the production are discussed.

5.5 Interim Summary

- An efficient cofactor regeneration is a prerequisite to the commercial process application (high *TTN* required). Three aspects must be considered: the cofactor retention, the cofactor stability and the regeneration reaction
- Different methods of cofactor retention were discussed (cross-linked with the enzyme, bound to water soluble polymer, membrane reactor and biphasic system). A choice is to be taken considering the whole process.
- Factors affecting the cofactor stability were discussed. A slightly basic pH enables the highest stability.
- Different ways of cofactor regeneration were discussed. The substrate-coupled cofactor regeneration (2-propanol as co-substrate) is the only possible approach for this work.
- The thermodynamic of the substrate-coupled regeneration was studied. The equilibrium position is unfavourable to the process development.
- An kinetic expression for the description of the regeneration reaction was elaborated. It is based on an extended Michaelis-Menten equation.
- The limitations of the regeneration system (*e.g*. regeneration activity fraction) were presented, so that technical solutions can be found.

[86] The rate of the whole system must be kinetically independent of the nature of the recycling reaction (Bastos *et al*., 2002).

6 Alcohol Dehydrogenase in Biphasic Systems

"Bei Gefahr und großer Not
bringt der Mittelweg den Tod."
ALEXANDER KLUGE

6.1 Introduction

6.1.1 General Aspects

Despite the increasing number of publications dealing with the application of biocatalysis to transform non-natural organic compounds, prejudices are frequently encountered. One of the most common is:

'Enzymes work only in their natural environment'.[87]

In living cells, water is the predominant solvent. Consequently, most scientists think of dilute aqueous solutions as the medium for enzymatic catalysis (Halling, 1994). The use of aqueous solutions as "conventional" reaction media 'represents something of a nightmare for the organic chemist if it is the solvent of choice' (Faber, 1995). Hence, it is one of the factors limiting the laboratory and industrial applicability of ADH (Wong 1987, cf. chapter 4.1.2) Among the reasons for this are (Peters, 1998):

- most of the organic compounds are poorly soluble or even insoluble in water
- the high boiling point and high heat of vaporisation make the removal of water expensive and tedious
- side reaction such as hydrolysis, racemisation, decomposition, or polymerisation are often encountered when using water as a solvent.

However, one should bear in mind that high concentrations of proteins, other biopolymers and low molecular weight compounds are present around the enzymes in living cells. Furthermore, some enzymes are associated with membrane structure containing mainly hydrophobic lipids. Accordingly, some of the "non-conventional" reaction media used recently for enzymatic reactions may be as natural for the enzymes as dilute aqueous solutions (Adlercreutz, 2000). The necessity of a clear nomenclature definition is noticeable. The terms

[87] Faber, 1995.

"natural medium" and "non-natural medium" should be avoided. In the sense of the present work "non-conventional" media are those where the water content is reduced compared to "conventional media".

The applicability of non-conventional media to biocatalysis is now a well-established fact (Adlercreutz, 1996). Besides overcoming the limitations imposed by aqueous solutions it can offer some further advantages such as:[88]

- the thermodynamic reaction equilibrium is shifted to favor synthesis over hydrolysis,
- decrease of substrate and/or product inhibition, either indirectly by maintaining a low concentration in the micro-environment of the biocatalyst, or directly by changing interaction between the inhibitor and the active site of the enzyme,
- easy recovery/reuse of the biocatalyst which does not dissolve in the organic solvent,
- possibility to manipulate stereo- and regio-selectivity of the enzyme in such media,
- under optimised conditions the stability of enzymes is often higher than in aqueous solutions,
- better integration with chemical steps in a synthetic sequence,
- the risk of microbial contamination is much lower in non-conventional media.

Evidently not all these advantages are relevant to all categories of non-conventional reaction media and enzyme classes. The shift of the equilibrium in favor of the synthesis over the hydrolysis, for example, is of special interest for hydrolases. And of course disadvantages of using biocatalysts in non-conventional media also exist:[89]

- the solvent may denature or inhibit the biocatalyst,
- introduction of an organic solvent into the reaction mixture increases its complexity,
- organic solvents may not dissolve charged or polyfunctional species,
- adjusting the pH is difficult,
- loss of stereoselectivity may occur,
- because of the reversible nature of the reaction acquired in non-natural media severe substrate or product inhibition may occur.

It becomes obvious that finding the adequate medium for the biocatalysis is the first major task if a non-conventional environment is to be employed. This medium engineering not only involves the (partial) substitution of aqueous reaction media by non-conventional media, it

[88] Cf. Adlercreutz 2000; Vermue and Tramper, 1995; Halling, 1987.

[89] Cf. Adlercreutz 2000; Wong and Whitesides, 1994

also implies the adaptation of the biocatalyst by *e.g*. immobilisation, lyophilisation, introduction of additives for stabilisation, etc (Vermue and Tramper, 1995).

'The behaviour of biocatalysts in non-conventional media often seems to be less easy to predict than in simple aqueous solution. In part this reflects less experience with these systems, in part their greater complexity' (Halling, 1994). It is also important to note that not all biocatalysts show the same properties concerning non-conventional media. While in whole cell the biocatalytic species is separated from the medium by a lipophilic membrane, cell free biocatalysts are in direct contact with the macroscopic reaction medium. The cell membrane is responsible for the differences observed between microbial and cell free enzymatic catalysis.

Even among cell free enzymes differences can be observed. For example, in biphasic systems lipases show "interfacial activation". This phenomenon is responsible for a sharp increase in lipase-activity when the substrate concentration is enhanced beyond the solubility limit, in contrast to the normal Michaelis-Menten activity dependence on the substrate concentration observed by esterases (Figure 6-1).

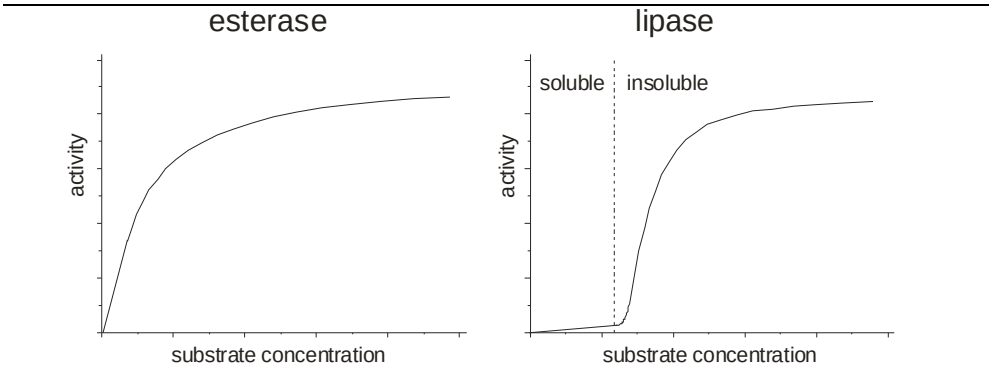


Figure 6-1: Activity dependence of esterase and lipase activity on substrate concentration (Faber, 1995).

This is usually explained by a conformational change of the lipase when it contacts the interface. (Figure 6-2) (cf. Wong and Whitesides, 1994).

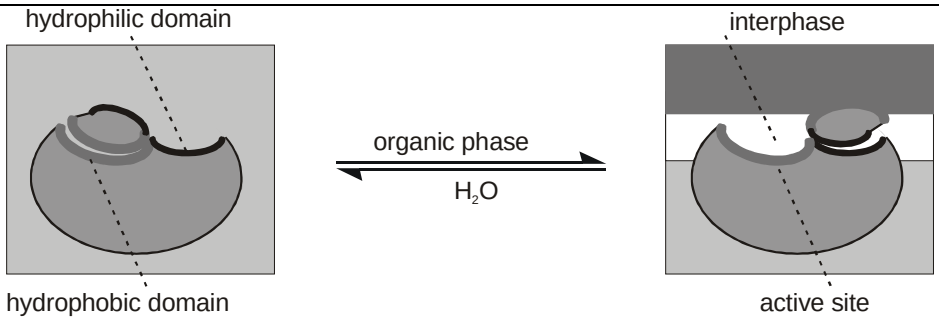


Figure 6-2: Difference of lipase structure in aqueous solution and in aqueous/organic biphasic system (Buchholz and Kasche, 1997).

For phospholipases the term “interfacial catalysis“ is being used in the literature. It indicates that catalysis occurs after binding of the enzyme to the interface between aqueous phase and phospholipids vesicle. However, neither “interfacial activation” nor “interfacial catalysis” seems to be in action for enzymes other than some hydrolases, (Straathof, 2003). To the best of our knowledge no such effect has been reported for the biocatalytic reduction of ketones.

The tolerance of lipases towards organic solvents is one of the major reasons for its important role in biotechnology.[90] But the use of non-conventional media is not restricted to lipases and it is a growing field of research for other enzyme classes (*e.g*. Griengl *et al*., 1998;). Its application opens new possibilities for the technological employment of biocatalysis and therefore should be considered in the development of bioprocesses, as discussed recently (Schmid *et al*., 2001). Among the several non-conventional media already employed in biocatalysis, five are of special interest for the process development of an ADH-catalysed reaction with hydrophobic and unstable substrates (Figure 6-3):

A. monophasic organic solvent
B. solid adsorbent resin
C. multiliquid biphasic system
D. reverse micelles
E. water-miscible cosolvent

[90] About 30% of all biotransformations reported have been performed with lipases (Faber, 1995).

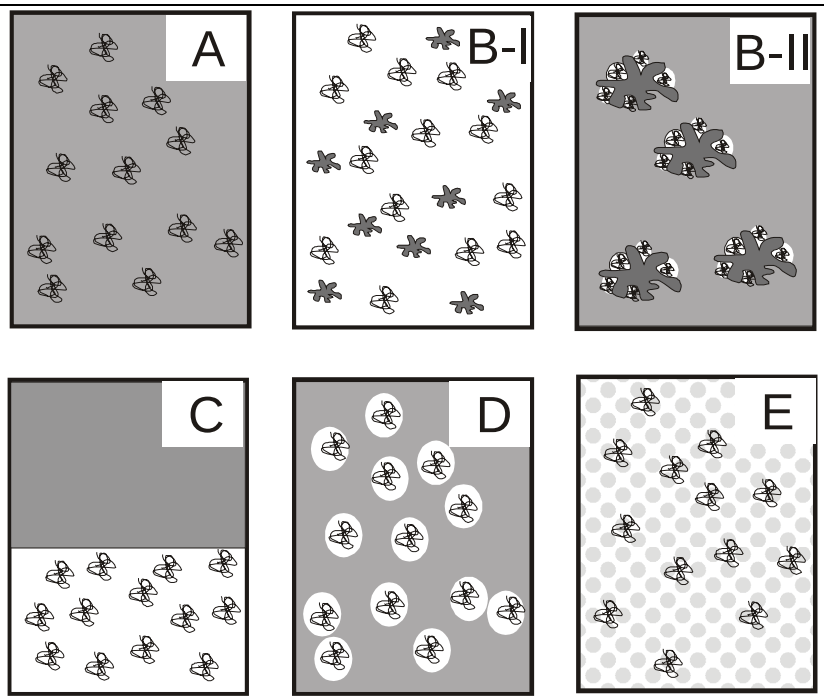


Figure 6-3: Non-conventional media applied in biocatalysis: (A) monophasic organic solvent; (B-I) solid adsorbent resin suspended in aqueous enzyme solution, (B-II) enzyme (and water) adsorbed on a solid resin suspended in organic solvent, (C) multiliquid biphasic system, (D) reverse micelles, (E) water-miscible cosolvent.

In order to choose the most adequate system for this process development, the different types of non-natural media presented are compared with respect to their application in the development of a continuous process application (Table 6-1). The characteristics of each approach is evaluated from highly favourable (+ + +) to most unfavourable (– – –).

Table 6-1: Table showing pros and contras of non-natural media for ADH catalysis.

	retention of enzyme activity	overcome substrate or product inhibition	cofactor regeneration	need of enzyme pre-preparation	catalyst recovery	product recovery	phase transport
monophasic organic solvent	– – –	– – –	– – –	–	+ / –	–	+ + +
solid adsorbent resin	+	+ + +	+	+	+ + +	+ / –	– – –
multiliquid biphasic system	+	+ + +	+	+ + +	+ + +	+ + +	+ + +
reverse micelles	+	+	+	+	+ + +	– – –	+
water miscible cosolvent	+ / –	– – –	+	+ + +	–	–	+ + +

It becomes evident that the multiliquid biphasic system is the most appealing for the development of a process with continuous use of enzyme and cofactor. In the words of Carrea 'The combined use of biphasic systems and cofactor regeneration should make these enzymatic reactions suitable for preparative scale transformation' (Carrea, 1984). Since this approach can be coupled with high concentration of cosubstrate affording advantages described next , we will concentrate on this approach in the continuation of this work.

6.1.1.1 Multiliquid Biphasic Systems

In the present work multiliquid biphasic systems will be defined as systems consisting of two phases, an aqueous phase and a second, water immiscible, liquid phase. This may include a water-immiscible solvent, or may simply consist of liquid reactants. They are often referred to as "biphasic" or "two-phase" systems, though these terms do not define them precisely. (cf. Halling, 1987). Aqueous biphasic systems are not considered here.

Probably the first catalysis by cell free proteins in multiliquid biphasic systems was observed in the 1890's by Buchner (Buchner, 1897). After obtaining a cell free press juice from yeast cells he could observe the formation of carbon dioxide from carbohydrates, sucrose, glucose and maltose. Chloroform, an antiseptic inhibiting microbial growth, did not inhibit the cell free "fermentation" (which meant the enzymatic reaction).

The biphasic approach offers some singular advantages for biocatalysis. Here, the enzyme and the cofactor are dissolved in the aqueous phase. For this reason, the plain enzymatic activity can be reached. Moreover, the cofactor can be released from the active site to be regenerated,

which is of special interest if the substrate coupled regeneration is the limiting step in the system, or if an enzyme coupled regeneration is desired. Furthermore, if the cofactor concentration decreases due to degradation (Wong, 1987), it can be easily replaced. Simultaneously, the hydrophobic substrate is present in a high concentration in the organic phase. This enhances the volume productivity. The extraction of the formed product is also inherent in this approach. Hence, its concentration in the aqueous phase is low (Figure 6-4).

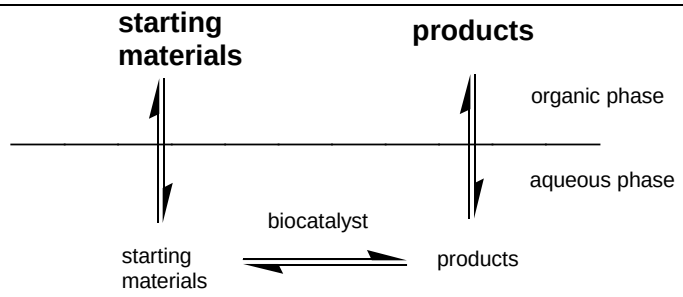


Figure 6-4: Illustration of the equilibria in a multiliquid biphasic system; according to the partition coefficient, the concentrations of hydrophobic substrates and products are higher in the organic phase.

Since enzyme and cofactor are insoluble in most organic media, the simple entrapment and reuse of enzyme and cofactor is an intrinsic advantage of this system. These characteristics make the multiliquid biphasic system very useful for enzymatic conversion of substrates that exhibit low solubility and/or instability in water. It is also a useful system for minimising undesired effects of substrates and products, such as inhibition and inactivation of enzymes (Shimizu *et al.*, 1990). Moreover the transfer between phases is satisfactorily rapid compared with that encountered when substrates are present as solid particles or loaded onto silicone-polymers.

Thus, it is not surprising that as early as 1974 a patent claiming the use of ADH in aqueous-organic biphasic system was granted (Antonini, 1974). In 1987 the utility of isolated ADH for the biphasic reduction of carbonyl compounds having low water solubility was demonstrated (Drueckhammer *et al*., 1987). TBADH and HLADH were used for the production of (*S*)-4-hydroxyhexanoate in buffer hexane biphasic systems. Common to these reports is the choice of highly apolar solvents to be used as a second phase in enzymatic transformations. Their use was recommended by Brink and Tramper (Brink and Tramper, 1985). Laane *et al*. reinforced the recommendation in 1985 with the log *P* concept (see 6.1.2). This concept has since had widespread influence on the choice of organic solvents to be used as a second phase in enzymatic transformations and was assumed to be valid for cofactor-requiring enzymes (Kise, and Hayashida, 1990, Hummel, 1997, Andersson, *et al.*, 1998). However, attempts to use

ADH under liquid-liquid biphasic conditions employing solvents with a high polarity afforded unsatisfactory results. Prof. Antonini, a pioneer of the study of biphasic systems, described in his patent application that in the presence of benzene the deactivation of YADH was observed, which explains the fact that the reaction does not proceed in an appreciable manner. (Antonini, 1974). The instability of YADH in biphasic systems was also observed by Orlich and Schomaecker (1999). In the case of HLADH high amounts of enzyme were needed to obtain full conversion despite enzyme deactivation (Matos, and Wong 1986). The TBADH-catalysed reduction described by Drueckhammer *et al*. stopped at 50% conversion. Also LBADH-catalysed reduction in the presence of cyclohexane was tried, but fast deactivation was noticed (Wolberg, 2001). On studying the effect of water on the enzyme action in organic media Zaks and Klibanov reported that at the water solubility limit enzymes (including HLADH) tend to form glue-like fibres devoid of catalytic activity (Zaks and Klibanov 1988).

Such observations led to the conclusion that alcohol dehydrogenases were not stable in biphasic systems (Kruse, *et al*., 1996; Peters, 1998; Liese, 1998).

To assess the feasibility of using ADH in the presence of organic solvents HLADH was coimmobilised with the cofactor and used for the production of long chain aldehydes in a biphasic system (Lortie *et al*., 1989).

Another method of performing biocatalysed reductions in biphasic systems is the protection of the enzyme in whole cells, avoiding the direct contact of the enzyme with the organic phase. For the stereoselective reduction of ethyl 4-chloro-3-oxobutanoate Shimizu and co-workers have identified different enzymes, affording either (*R*)- or (*S*)-4-chloro-3-hydroxybutanoate (Shimizu and Yamada 1990; Yasohara *et al*., 2000). The use of biphasic systems is desirable because the substrate is unstable in an aqueous solution and both substrate and product strongly inhibit enzyme reaction (Shimizu *et al*., 1998). In order to obtain an efficient catalyst for this reaction, recombinant *Escherichia coli* cells expressing both the carbonyl reductase gene from *Candida magnoliae* and the glucose dehydrogenase from *Bacillus megaterium* (for cofactor regeneration) was used (Kizaki *et al*., 2001).

The advantages of the multiliquid biphasic system are summarized below (Figure 6-5):

- higher amount of hydrophobic substrate converted per reaction volume,[91] thereby avoiding dilution of catalyst and cofactor
- overcome substrate and/or product inhibition and degradation
- straightforward product recovery
- simple reuse of enzyme and cofactor
- uncomplicated cofactor dosage (soluble in the water phase)
- different possibilities of cofactor regeneration

Figure 6-5: Illustration of an enzyme-catalysed reaction in multiliquid biphasic system.

The main disadvantage of this approach remains the deactivation of the isolated enzyme in the biphasic system. A discussion about concepts of medium engineering for the solution of this core problem will follow.

During the final stages of the present work some reports have been published describing the discovery of dehydrogenase-compatible biphasic reaction media. Suye *et al.* applied an

[91] As reaction volume only the aqueous phase is considered. The organic phase must be seen as an inert reservoir of substrate. Hence, the real concentration of substrate in the aqueous phase is not increased in the biphasic system. (Carrea, 1984)

aqueous-di*iso*propylether system as a simple method for entrapment and reuse of both enzyme (HLADH) and cofactor (Suye *et al*., 2002). A patent application claiming the use of organic solvents showing log *P* between 0.6 and 1.9 for biphasic enzymatic reductions was disclosed on 31st October 2002 (Gupta *et al*., 2001). The use of ADH from *Rhodococcus erythropolis* in a biphasic system was published in 2003 (Gröger et al., 2003).

6.1.2 Medium Engineering Aspects

In section 6.1.1 the importance of the solvent choice for the biocatalysis in non-conventional media has been discussed. The present section will focus on the main aspects concerning medium engineering for multiliquid-phase biocatalysis.

In 1984, Carrea published a review on biocatalysis in water-organic solvent two-phase systems. In this contribution he listed several factors to be considered by the choice of the organic solvent:

- enzyme activity and change in enzyme kinetics
- enzyme stability
- high capacity to solubilize reagents and products
- partition coefficient of substrates and products

A further factor which is also relevant is the

- influence on the enzyme's regio- and enantioselectivity[92]

The various solvent effects on the efficiency of bioconversions are often contradictory and optimisation is necessary when choosing an organic solvent (Brink and Tramper, 1985). The choice is often a compromise since it is unlikely that one solvent will possess all ideal characteristics (Carrea, 1984).

As far as the present work is concerned, the highest priority is assigned to the solvent's effects on:

- enzyme stability
- enzyme activity
- regio- and enantioselectivity

Because of the difficulties emerging when one has to decide which precise organic solvent to choose for practical application of a particular enzyme, the use of biphasic systems is considerably hindered (cf. *e.g*. Carrea, 1984; Brink and Tramper, 1985; Laane *et al*., 1987).

[92] Cf. Fitzpatrick and Klibanov, 1991; Villela, 1998

To overcome this limitation, medium engineering approaches were proposed with the aim to help choose suitable organic solvents for biocatalysis. The most relevant methods are based on:[93]

- Free energy of micellization
- Denaturation capacity
- Hildebrand solubility parameter
- Partition coefficient in octanol-water system

The partition coefficient in *n*-octanol/water system (log *P*) is the most widely accepted parameter to guide the choice of organic solvent in multiliquid-phase biocatalysis.[94] It deserves special attention here.

6.1.2.1 Partition coefficient in octanol-water system

This concept was suggested by Laane *et al*. in 1985. In accordance with the work of Brink and Tramper, it is understood that high activity retention is usually favoured by a low solvent polarity in combination with a high molecular weight. However, a direct parameter reflecting the polarity of solvents is employed: the logarithm of the partition coefficient (log *P*) of a given compound in the standard *n*-octanol/water biphasic system at 25°C (Equation 6-1).[95]

$$P = \frac{[S]_{octanol}}{[S]_{water}} \qquad \textbf{Equation 6-1}$$

The authors have categorized solvents into three groups, according to the log *P*–value:[96]

1. solvents having a log *P* < 2. These solvents are considered to be least suitable in biocatalytic systems,
2. solvents having a log *P* between 2 and 4. For some applications these solvents might be suitable, but harmful effects can be expected,

[93] Cf. Khmelnitsky *et al*., 1991; Jönsson,1999;.Brink and Tramper, 1985.

[94] This concept is referred to in many books and review articles about enzyme in organic synthesis, *e.g*. Faber, 1995, Hummel, 1997; Drauz and Waldmann, 2002.

[95] Strictly, log *P* denotes hydrophobicity, which is related to polarity, but is not synonymous with it. The term polarity was preferred by the authors since it is rather general and more current in scientific language than hydrophobicity (Laane *et al*., 1987).

[96] The proposed explanations for this behaviour are concerned with the ability of organic solvents to distort the vital, but delicate, water-biocatalyst interactions merely by penetration of the organic solvent into the essential water layer stabilizing the biocatalyst or by pushing aside the essential water. A consequence of this reasoning would be that organic solvents saturated with water lose their water-stripping activity and hence will never be harmful to biocatalysis. This is undoubtedly incorrect.

3. solvents having a log *P* > 4. These solvents are suggested to be applicable for any biocatalytic system.

Among the advantages of this approach are:

- log *P* is more sensitive than previously presented parameters, *e.g.* the Hildebrand solubility-parameter (δ),[97] especially for the relatively apolar systems, for which the best results are expected.
- log *P*-values can easily be determined experimentally, or calculated from fragmental constants. (see 6.2.1)

The majority of medium engineering approaches is based on only one measurement of the enzyme activity at a certain time after the addition of the organic solvent. This result gives information about the activity retention of the biocatalyst in the presence of organic solvent compared to the biocatalyst in pure aqueous phase. In the present work this will be labelled as instantaneous inhibition according to the nomenclature used by Carrea (Carrea, 1984).

Though necessary, the activity retention is not enough to fully characterise the compatibility of an organic solvent with biocatalysis. Since the conversion can take place over a prolonged period of time and the continuous application presupposes long time enzyme stability, supplementary information about the time-dependent deactivation of the enzyme is indispensable.

Hence, it is not surprising that the studies revealing no agreement with the log *P* concept are those involving long periods of enzyme exposure to the organic solvent. Indeed, Bauer *et al*. used the measured half-life of the enzyme under biphasic conditions to investigate the effect of the organic solvent on hydroxynitrile lyase. (Bauer *et al.*, 1999)

We are convinced that the inhibition or instantaneous enzyme deactivation caused by the solvent must be considered in the solvent choice. But the influence of the solvent on the further rate of deactivation is also of fundamental importance for the performance of an enzymatic system under production conditions.

Accordingly, the development of a method to provide a suitable organic solvent to be used in biphasic ADH-catalysed reduction of keto-compounds is an essential part of the present work and will be described in section 6.2.

[97] Cf. Brink and Tramper, 1985.

6.2 A New Method to Guide the Solvent Choice

The aim of the current section is the **development of a general procedure** to be used in the search for organic solvents to be applied as second phase in ADH-catalysed reductions. Aspects concerning the **instantaneous inhibition** of the enzyme by the organic solvent **and** the **time dependent deactivation** in the presence of the solvent are to be taken into consideration. A **mathematical model** to describe the solvent effect on the biotacalyst is to be developed. In section 6.2.1 general aspects of the procedure development will be presented. Section 6.2.2 will describe the development of experimental conditions and the obtained results will be discussed in 6.2.3. In depth insight of the solvent effect on LBADH will be studied in section 6.2.4 and conclusions will be made in section 6.2.5.

6.2.1 General Aspects

In the absence of general rules for the choice of the organic phase, solvents must be tested individually (Carrea, 1984). Given the enormous amount of possible solvents to be considered, this approach would demand a massive consumption of time, enzyme and reagents. A common approach is to classify solvents as polar/apolar, protic/aprotic, etc. and select typical solvents from different classes to be used in a first screening (Carlson, 1985). In a second step, more solvents belonging to the class displaying best results are tested (Figure 6-6).

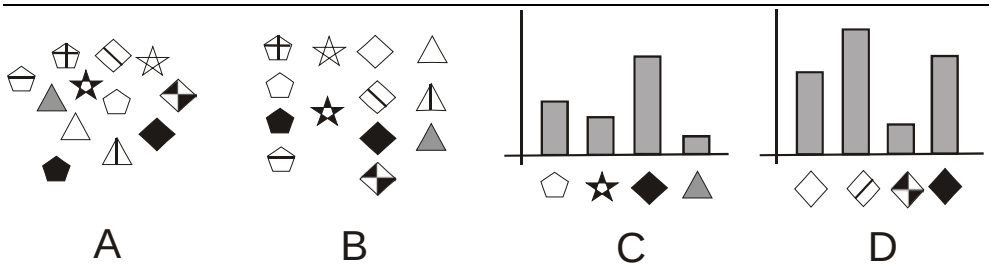


Figure 6-6: Illustration of the proposed method for solvent selection: classify the amount of possible solvent (A) into groups (B); use typical solvents in the first screening step (C) and study the best class in the second step (D).

Different criteria for the classification of solvents were presented by Chastrette *et al*, in 1985. The decisive issue is the choice of the parameter to be used in the classification of the organic solvents.

Our option used was based on a report by Mozhaev *et al*. (1989). These authors noticed that log *P* could be used to compare solvents of the same functionality (*e.g*. alcohols) but that it is

not a convenient parameter for the comparison of solvents of different functionality. Therefore, we decided to test enzyme activity and stability in the presence of water-immiscible organic solvents of different functionality, regardless of their log *P*. Thus, representatives of unbranched alkanes, cyclic alkanes, aromatic compounds, halogenated solvents, ethers, and esters were tested. Besides, the effect of tertiary alcohols on the enzyme was studied, since these alcohols cannot be accepted as substrates by ADH. Another compound tested in the screening represents a further class of water immiscible solvents, namely, the ionic liquids.

6.2.1.1 Ionic Liquids

Ionic liquids are low melting point salts that represent a new class of reaction solvents for catalysis (Gordon, 2001). They may be distinguished from classical molten salts by the low melting point, generally <100°C, although many are liquid at room temperature. They are also generally much less corrosive than their high melting point counterparts. This means that they can be conveniently used in place of conventional organic solvents. Being composed entirely of ions, they possess negligible vapour pressure. Other solvent properties may be easily controlled, given the wide range of possible cations and anions.

The pioneering work using ionic liquids in enantioselective biocatalysis has employed this class of solvents in the lipase catalysed racemic resolution of phenylethanol. The results were compared to those obtained with MTBE (Kragl *et al*., 2002). To the best of our knowledge no study has been published describing the use of ionic liquids in combination with ADH.

The ionic liquid used in this work was 1-butyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)amide [BMIM][$(CF_3SO_2)_2N$]. Its structure can be seen in figure 6-7.

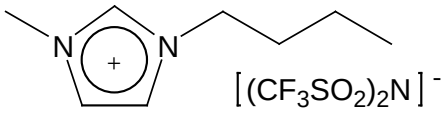


Figure 6-7: The formula of [BMIM][$(CF_3SO_2)_2N$] the ionic liquid used in this work.

The compound is colourless and water-immiscible. Its density is > 1, thus in a biphasic system with water it forms the lower phase. The partition coefficient of some ADH-substrates and products in this biphasic system are given in table 6-2.

Table 6-2: The partition coefficient of some ADH-substrates and products in this biphasic system.

substrate	concentration	$m_{IL/w}$
acetone	50 and 300 mM	2
2-propanol	50 and 300 mM	0.4
2,5-hexandione	50 mM	2.5
5-hydroxyhexan-2-one	50 mM	0.5
2,5-hexandiol	50 mM	0.2

The results have been determined according to equation 6-2:

$$m = \frac{[S]_{IL}}{[S]_{water}}$$ **Equation 6-2**

The importance of these measurements is related to the fact that the partition coefficient in biphasic systems will influence the thermodynamic equilibrium of the system (see 6.6). It is specially relevant for 2-propanol and acetone, common substrate and co-substrate of the substrate-coupled cofactor regeneration (see chapter 5). It must be emphasized that a good separation of acetone and 2-propanol is not easy to achieve, neither by extraction nor by pervaporation or distillation.

6.2.1.2 Comparison with the Hydrophobicity Approach

The results obtained by the measurements of enzyme activity and stability shall also be evaluated with regard to solvent's hydrophobicity (log *P*-value). This will enable an examination of the validity of the most popular approach in medium engineering for ADH and also demonstrate any correlation between the new method proposed in this work with the conventional. Therefore, the log *P*-value for all organic solvents investigated will be given.

The log *P*-values are calculated according to Crippen's fragmentation method (Ghose and Crippen 1986; Ghose and Crippen 1987). The resulting constants usually are in good agreement with experimental data, but there are obviously still some deviations. To illustrate the correlation between calculated and measured values, and to the hypothesis formulated later in this chapter the partition coefficient of two solvents of importance for the present work in the standard *n*-octanol-water biphasic system have been experimentally determined. The obtained log *P*-value are compared with the calculated in table 6-3

Table 6-3: Comparison between measured and calculated log *P*-values.

solvent	theoretical	experimental
MTBE	0.96	0.96
DIPE	1.40	1.6

6.2.1.3 Broadening the Scope of the Studies

In order to obtain results of a generic nature that can be applied in a general procedure to be used in the search for organic solvents compatible with enzymes lacking interfacial activation, it is important to assure that observations are not distorted by specific enzyme-solvent interactions. Therefore, besides LBADH two additional commercially available enzymes were used in this investigation: the NADH-dependent alcohol dehydrogenase from horse liver, HLADH, and the NADPH-dependent alcohol dehydrogenase from *Thermoanaerobium brockii*, TBADH.

Additionally, a parallel study with a different class of cofactor dependent enzymes was also carried out (Stillger, in preparation; cf. Villela *et al*. 2003). These thiamine diphosphate (ThDP)-dependent enzymes display C-C bonding activity and show great potential for use in chiral synthesis (Pohl *et al*., 2002). Similarly, the poor solubility of interesting substrates may hinder the utilization of this enzymes. The technical application of benzaldehyde lyase (BAL) and pyruvate decarboxylase (PDC) are subject of investigation in the Institut für Biotechnologie 2. The results obtained with ThDP-dependent enzymes will be briefly mentioned in the general discussion in section (see 6.2.5.)

6.2.2 Development of Experimental Conditions

According to the procedure for identification of organic solvents proposed in section 6.2.1:

1. solvents are going to be classified in groups according to their functionality,
2. the enzymes will be dissolved in buffer,
3. the initial enzyme activity of the solution will be determined,
4. representatives of different solvent classes will be added to the enzyme solution,
5. the instantaneous inhibition of the enzyme's activity will be determined,
6. the time-dependent enzyme deactivation will be measured.

In order to allow this procedure, the buffer properties have to be set. A standard assay for activity measurement of each enzyme has to be developed. Typical representatives of

different solvent classes have to be elected, and the conditions for the biphasic system have to be established.

Conditions for LBADH

The conditions for the standard activity assay for the LBADH were developed by Riebel (1996). Acetophenone is used as substrate and the course of the reaction is followed photometrically by the decrease of the NADPH absorption at 340 nm. It is important to avoid an excess of cofactor due to substrate inhibition.

For convenience, the buffer used for dilution of the enzyme is the same used in the activity test (150 mM phosphate buffer, pH 7.0)

Conditions for HLADH

As standard reaction for the activity measurement the oxidation of 110 mM ethanol to acetaldehyde in the presence of 1.0 mM NAD^+ is used. On-line measurement of the absorption at 340 nm indicates formation of NADH, describing the progress of the reaction. The reaction is carried out at pH 8 and 30°C. The selected concentrations to be used in the assay are: 110 mM ethanol and 1.0 mM NAD^+. Since the enzyme is stable in the pH range of 5 to 10, a pH 8.0 buffer is to be used in the preparation of the enzyme solution, thus harmonising with conditions of the activity measurement.

Conditions for TBADH

The reaction chosen as standard for the measurements was the reduction of 70 mM acetone with 0.2 mM NADPH. The reaction performance at 30°C and pH 8.0.

The Solvents

According to section 6.2.1. the classes of solvents selected to be tested in the first step of this procedures are: unbranched alkanes, cyclic alkanes, aromatic compounds, halogenated solvents, ethers, esters, tertiary alcohols and ionic liquids. The representatives of the solvent classes selected to be tested are given in table 6-4 with corresponding calculated log *P*.

Table 6-4: The representatives of the solvent classes with respective calculated log *P*.

solvent class	selected representative	log *P*
tertiary alcohols	*tert*-butanol	0.6
esters	ethyl acetate	0.7
ethers	*tert*-butyl methyl ether	1.0
halogenated solvents	dichloromethane	1.0
aromatic	toluene	2.3
cyclic alkanes	cyclohexane	2.7
unbranched alkanes	nonane	4.3
ionic liquids	[BMIM][$CF_3SO_2)_2N$]	n.d.

Though soluble in water, *tert*-butanol was tested as a model to study the effect of tertiary alcohols on the enzymes activity, due to its easy availability. The log *P* value for the ionic liquid has not been calculated.

The Biphasic Conditions

For the evaluation of the effects caused by organic solvents on alcohol dehydrogenases in biphasic systems the enzymes are dissolved in phosphate buffer solution and the initial activity is measured. A volume of the organic solvent to be tested equal to the aqueous phase is added.[98] To minimise changes in the phase volumes, the organic solvent is previously saturated with water. Intense shaking for one minute afford the almost instantaneous saturation of the aqueous phase. The system is then continuously stirred with a magnetic stirrer in order to provide an emulsion during the whole experiment. The effect of stirring on the enzyme can be neglected (Figure 6-8).

[98] The composition of the two phases in equilibrium with each other are independent of their relative volumes. Hence, the dissolved concentration of organic solvent seen by the biocatalyst in the aqueous phase will not change as the volume fraction of the organic phase is increased from less than 1% to over 99%, provided its composition is unaltered (Halling, 1987).

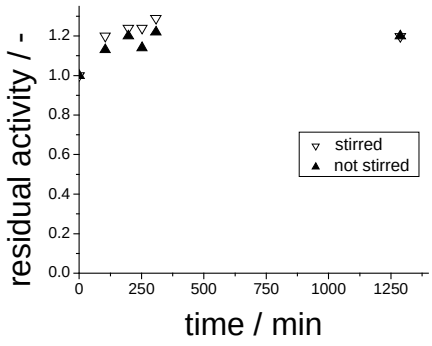


Figure 6-8: Comparison of the residual activity of LBADH at 4°C: stirred at 700 rpm and unstirred; 150 mM phosphate buffer pH 7.0.

To assure that the observed loss of activity can be mainly attributed to the presence of the organic solvent and is not caused by thermoinstability of the enzyme, the emulsion is kept at 4°C (see chapter 4). Five minutes after the addition of the organic solvent a first sample of the aqueous phase is taken and the activity is measured under standard assay conditions. Samples are then taken periodically. Two minutes before sampling, the stirrer is stopped, enabling a clear phase separation.

6.2.3 Activity and Stability of ADH in Multiliquid Biphasic Systems

6.2.3.1 A Mathematical Description

For all three enzymes the effect of a second phase on the measured residual activity (A_t) can be described as resulting from two independent contributions:

- instantaneous inhibition
- time-dependent deactivation

The former can be determined by comparing the enzyme activity in the water phase before and five minutes after the addition of the organic solvent. The observed time-dependent deactivation of all three enzymes is described as a first order exponential decay of the activity[99] Figure 6-9 shows a typical result of the measurements.

[99] Cf. Bauer *et al.*, 1999.

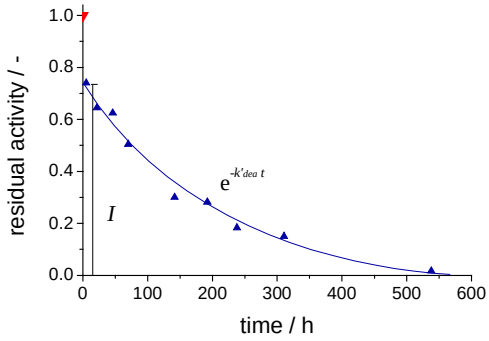


Figure 6-9: Residual activity of HLADH in the presence of a second phase as a function of time: In this example I = 0.74 and k_{dea} = 0.0037 h^{-1}.

Accordingly, the observed residual activity can be determined from equation 6-3.

$$A_t = A_0 \cdot I \cdot e^{-k_{dea} \cdot t} \qquad \textbf{Equation 6-3}$$

where

A_t residual activity at time t (normalised)

A_0 initial activity (normalised)

I instantaneous inhibition constant

k_{dea} apparent deactivation constant [1/h]

This model allows the calculation of the deactivation constant (k_{dea}) from the obtained results for every combination of enzyme and solvent. Half-lives can be calculated according to Equation 6-4.

$$t_{½} = \ln 2 / k_{dea} \qquad \textbf{Equation 6-4}$$

The calculated half-life shall be used as a parameter to evaluate the effect of the solvent on the enzyme stability in multiliquid systems.

6.2.3.2 The First Screening Step

In the first screening step to find an adequate solvent for the biphasic application of ADH, the effect of representatives of different solvent classes has been studied.

The Instantaneous Inhibition

For solvents in the presence of which enzymes displays little stability, the measurements of the instantaneous inhibition overlaps with the influence of the time-dependent inhibition. To take this aspect into consideration, all values for I were calculated according to the model

presented in section 6.2.3.1 (Equation 6-3). The results obtained for each enzyme solvent combination are shown in table 6-5.

Table 6-5: Values for the instantaneous inhibition constant ***I*.** Values in italics are associated with $t_{½}$ < 24 h.

solvent	LBADH	HLADH	TBADH	log *P*
tert-butanol	0.92	*0.40*	*0.58*	0.6
ethyl acetate	*1.00*	0.22	*0.09*	0.7
tert-butyl methyl ether	1.21	0.64	0.54	1.0
dichloromethane	*0.15*	*1.17*	*1.32*	1.0
toluene	*0.48*	*0.66*	*0.45*	2.3
cyclohexane	*0.81*	*1.18*	*0.85*	2.7
nonane	*1.58*	*1.48*	0.85	4.3
[BMIM][$CF_3SO_2)_2N$]	0.96	1.16	n.d.	n.d.

The influence of the time-dependent deactivation for strong deactivating solvents can be the explanation for some values of *I* being > 1 (*e.g.* TBADH and dichloromethane, and HLADH and cyclohexane). Nevertheless, for some measurements a value > 1 could be observed (*e.g.* HLADH and nonane, and LBADH and *tert*-butyl methyl ether). In these cases *I* should be referred to as instantaneous activation constant rather than instantaneous inhibition constant.

The finding that LBADH activity is enhanced at the beginning of the investigation is consistent with observations that dilution of the enzyme preparation causes increase in enzyme activity and decrease in stability (cf. chapter 4).

The correlation of the instantaneous inhibition with solvents hydrophobicity should be given according to Laane *et al*. *Tert*.-butanol should be excluded from the discussion, due to its water-solubility. A general correlation may be observed. If present, it is more pronounced for HLADH. It can also be assumed for TBADH, if the value for dichloromethane (numerical artefact) is ignored. For LBADH such a correlation is not apparent.

It is worth noticing that for toluene reduced activity was noticed for all three enzymes. A further interesting observation is that in the case of TBADH in the presence of nonane (log *P* > 4) the expected high activity could not be measured. The result is rather in the range of cyclohexane (log *P* > 2.7).

The Time-Dependent Deactivation

Measurements were performed by periodical sampling of the aqueous phase until complete deactivation of the enzyme or up to 1 000 h. The obtained curve was fitted according to the model presented in section 6.2.3.1. The resulting values for k_{dea} were used in the calculation of $t_{½}$ according to equation 6-4.

The control experiments were carried out until contamination was noticed (*e.g.* LBADH after 200 h). For this example the obtained curve up to this point was identical to the curve obtained in the presence of MTBE. The obtained values for $t_{½}$ are given in table 6-6.

Table 6-6: Half-lives / h of enzymes in the presence of solvents.

solvent	LBADH	HLADH	TBADH	log *P*
tert-butanol	3.8×10^2	3.1	24	0.6
ethyl acetate	24	196	< 1	0.7
tert-butyl methyl ether	1.80×10^3	1.84×10^5	3.4×10^2	1.0
dichloromethane	1.3	1.5	0.97	1.0
toluene	6.9	22	1.0	2.3
cyclohexane	8.3	6.2	2.1	2.7
nonane	1.0	4.8	52	4.3
[BMIM][$CF_3SO_2)_2$N]	116	6.5×10^2	n.d.	n.d.

The accuracy of each activity determination is estimated at ± 10%. The extremely high stability calculated for HLADH in the presence of MTBE may include a numerical artefact. Nevertheless, it must be reported that the activity decay observed over 1 000 h in 22 measurements was just in the range of the test accuracy.

For a better interpretation of these results, the half-lives are presented as histograms in figure 6-10. To enable a comparison with the approach by Laane *et al*. the solvents are ordered according to increasing log *P*-values.

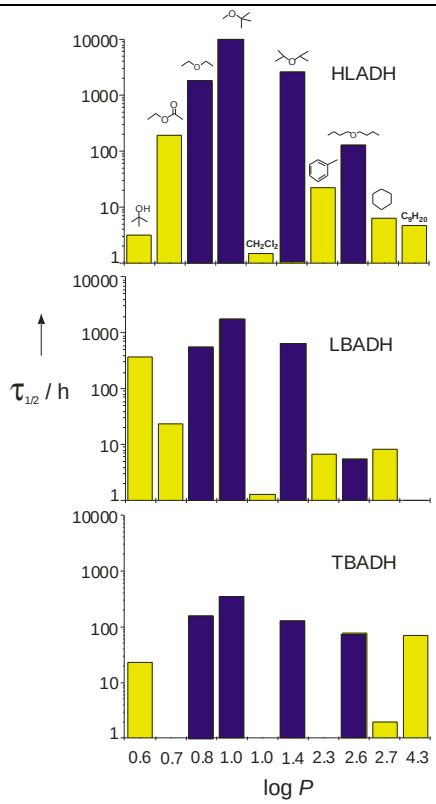


Figure 6-10: Histogram representing the half-lives of HLADH, LBADH and TBADH in multiliquid biphasic systems (Villela *et al.*, 2003).

While the best results for all three enzymes have been achieved in the presence of MTBE, dichloromethane caused fast deactivation of the enzyme in all three cases. The magnitude of the half-lives varies from enzyme to enzyme, but a general pattern of stability in dependence on the functionality of organic solvents seems to be given. This agrees with the classification of solvents proposed in this work and must be confirmed in further experiments. A dependence on log *P* cannot be observed.

Furthermore, the ionic liquid does not cause a fast enzyme deactivation. Therefore, its use shall be compatible with ADH.

According to the procedure proposed in 6.2.1 the results obtained in the first screening step shall identify a class of solvents displaying compatibility with the use of ADH in biphasic systems. The final identification of the solvent to be used in biotransformations is to be carried out in a second step. Since the results were observed in the presence of MTBE, additional ethers are going be tested in the second step.

6.2.3.3 The Second Screening Step

Solvents Used

The ethers selected for this screening step are shown in table 6-7. Here again the log *P*-values are calculated according to Crippen's fragmentation method. To enable an evaluation of important parameters in the choice of the solvent, the price and the boiling point of the solvents are also given in the table.

Table 6-7: Ethers used in the second step of the solvent screening and the respective properties (Fluka 2002).[100]

ether	log *P*	price/ €/L	b.p./°C
tetrahydrofuran	0.4	14.70	66-67
diethyl	0.8	9.70	35
tert-butyl methyl	1.0	9.90	55
di*iso*propyl	1.4	18.50	67-69
di-*n*-butyl	2.6	12.00	139-142

Despite its solubility in water, THF is used as a model for cyclic ethers; diethyl ether and dibutyl ether are used to compare the influence of the side chain size and di*iso*propylether to study the effect of two bulky residues. The results obtained for MTBE are also shown to permit a better comparison.

The Instantaneous Inhibition

The instantaneous inhibition constants (*I*) were calculated in the same way as described in 6.2.3.2. The results obtained are displayed in table 6-8.

Table 6-8: Values for the instantaneous inhibition constant.

ether	LBADH	HLADH	TBADH	log *P*
tetrahydrofuran	*1.0*	*0.03*	*0.39*	0.4
diethyl ether	0.89	0.46	0.81	0.8
tert-butyl methyl ether	1.21	0.64	0.54	1.0
di*iso*propyl ether	1.43	0.72	0.95	1.4
di-*n*-butyl ether	*0.74*	0.72	0.17	2.6

[100] Due to different quotation, the MTBE price differs from chapter 9. The catalogue price is used here in order not to distort the comparison.

Given the high stability of the enzymes in the presence of all ethers except THF, the calculated results for *I* are almost identical with the measured results. In the case of THF, the calculations are strongly influenced by the fast deactivation observed.

According to Mozhaev *et al*. the log *P*-approach should be valid for solvents showing the same functionality. Thus, *I* should increase with log *P*. A correlation of log *P* with the obtained activities after the ether addition is only given for HLADH. For LBADH and TBADH this correlation cannot be postulated based on these results.

The Time-Dependent Deactivation

The procedure to obtain k_{dea} and $t_{½}$ is the same as in the first step. The values for $t_{½}$ are given in table 6-9.

Table 6-9: Half-lives/h of enzymes in the presence of solvents.

ether	LBADH	HLADH	TBADH	log *P*
tetrahydrofuran	3.2	2.6	12	0.4
diethyl ether	566	1.77×10^3	151	0.8
tert-butyl methyl ether	1.80×10^3	1.84×10^5	346	1.0
di*iso*propyl ether	646	2.60×10^3	128	1.4
di-*n*-butyl ether	5.5	130	76	2.6

The results are highly significant:

- MTBE is the most compatible solvent for all the three enzymes
- diethyl and di*iso*propyl ether show comparable good results for all the three enzymes
- tetrahydrofuran is the least compatible solvent for all the three enzymes

This procedure establishes the choice of ethers as the best class of solvent. The second step indicates that MTBE is the most gentle solvent for all the three studied enzymes. Nevertheless, di*iso*propylether presents a higher inhibition constant *I*, thus a higher initial activity of the enzyme, and the stability is still good enough for technical application. Since for the choice of the solvent not only the stability is of concern, but also the activity in the presence of the solvent, the area under the curve described by the equation 6-3 should be adopted as the quantification parameter of choice.

For the present work a more pragmatic approach should be emphasised: safety. The lack of epoxide formation makes MTBE more suitable for long term experiments and for distillative work up. Therefore, this solvent will be used in further work.

6.2.3.3.1 Additional investigation

In order to draw conclusions about the influence of functionality, chain size and hydrophobicity on the stability of ADH in biphasic systems, additional measurement of the stability of the enzymes in the presence of *n*-heptane were carried out. The results were obtained as previously described and are summarized in table 6-10. The results for nonane are shown for comparison.

Table 6-10: The values for I and $t_{½}$ concerning n-heptane and nonane.

solvent	I_{LBADH}	I_{HLADH}	I_{TBADH}	$t_{½LBADH}$/h	$t_{½HLADH}$/h	$t_{½TBADH}$/h	log P
n-heptane	1.2	1.1	0.74	5.5	1.9	3.5	3.4
nonane	1.58	1.48	0.85	1.0	4.8	52	4.3

6.2.3.4 Discussion

We have shown that the log P concept is not satisfactory to guide the choice of an organic solvent serving as the second phase in ADH-catalyzed reactions. The biphasic enzymatic reduction of ketones is possible if short-chain aliphatic ethers are employed. This suggests that a single physicochemical parameter is incapable of satisfactorily predicting the biocompatibility of organic solvents, given the complexity of the enzyme deactivation in biphasic systems. Rather a set of properties is necessary to guide the solvent choice. Based on our results we propose that the set of properties inherent in the chemical functionality (e.g. ether) gives rise to the compatibility of solvents with alcohol dehydrogenases.[101] Therefore, we recommend that instead of the hydrophobicity of the solvent (log P) its chemical functionality should be considered when screening for a solvent.

Di-*n*-butyl ether and nonane are fairly compatible with TBADH, whereas LBADH is very sensitive to both solvents. This correlation suggests that the large sized unbranched chains of the ether confers alkane-like properties, which at the size size of four C atoms starts to become dominant over the ether functionality. The dominance is not high enough to cause a differentiation by HLADH.

[101] Chemical functionality is a pragmatic label for a set of molecular properties like the presence of certain heteroatoms and residues, the absence of others, molecular geometry, electronic density distribution and frontier orbitals, etc., and consequent macroscopic properties of solvents, like surface tension, solubility, solvation energy, etc..

In the presence of short chain aliphatic ethers or ethers with bulky residues good results could be achieved. Whether the presence of the short chain or the bulky residue is predominant in MTBE must be clarified by further investigation. A third possibility is that the combination of both these factors is the key to the compatibility with the enzymes.

Further investigation would bring more clarity to the importance of the solvent's molecular structure. Interesting solvents for investigation include asymmetric ethers, especially *tert*-butyl ethyl ether, *n*-butyl ethyl ether, *tert*-butyl *iso*-propyl ether and methyl *iso*-propyl ether. Ether with one alicyclic residue, cyclic water-immiscible ethers, phenol ethers, especially anisol, and thioethers also should be investigated. Methyl and *tert*-butyl acetate should also be investigated. Some initial investigations in this field are discussed in the following section.

Finally, it becomes clear that the enzyme activity measured once after the addition of a solvent to the system is not sufficient to enable the choice of the organic solvent. While HLADH suffers from a high inhibition in the presence of MTBE (I = 0.64), the high stability overcompensates this effect. On the other hand, the use of cyclohexane (I = 1.18) or nonane (I = 1.48) is not favourable despite the lower inhibition. The area under the curve of an activity plot as a function of time (equation 6-3) can be used to evaluate both effects (inhibition and deactivation) simultaneously.

6.2.4 Further Investigation

Complementary studies to obtain a deeper insight of the behaviour of ADH in non-natural environments were restricted to LBADH and are discussed in this section. Results obtained for additional investigation of the influence of the molecular structure are subject of subsection 6.2.4.1 and the use of LBADH in predominantly organic solvents is discussed in subsection 6.2.4.2

6.2.4.1 Additional Solvents

In order to investigate the contribution of single structure variation, the effect of different solvents was studied.

Chain Size

To compare the role of the chain size in alkanes, the effect of *n*-hexane and *n*-heptane on the LBADH was investigated, thus allowing a comparison between *n*-hexane, *n*-heptane and *n*-nonane. The results are summarised in table 6-11.

Table 6-11: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of alkanes.

alkane	*I*	$t_{½}$/h	log *P*
n-hexan	1.0	2.4	3.0
n-heptane	1.2	5.5	3.4
n-nonane	1.6	1.0	4.3

Given the short half-life any conclusion risks to be inaccurate. The increasing value of *I* with increasing log *P*-value might be a numerical artefact. The only significant observation is the incompatibility of alkanes with the use of LBADH.

Alcohols

Since LBADH has proved to be stable in the presence of water miscible alcohols, it is interesting to look at the effect of water-immiscible alcohols. Despite the possibility of conversion by the enzyme, octanol was used as a model for its previous employment in biocatalytic processes (Rissom, 1999; Schröder, 2003; Haberland, 2003). To avoid contradictory reactions in the measurement of the activity, the oxidation of 2-propanol was used as standard system. The results are presented in table 6-12.

Table 6-12: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of alcohols.

alcohol	*I*	$t_{½}$/h	log *P*
2-propanol (50% v/v)	1.1	4.2×10^2	0.4
tert-butanol	0.92	3.8×10^2	0.6
octanol	1.1	2.3×10^2	2.6

Cyclic Ether

The short half-life observed for LBADH in the presence of tetrahydrofuran could be attributed to its solubility in water or to its cyclic character. So a further cyclic ether, which was water-immiscible was studied: 15-crown-5 ether (1,4,7,10,13 pentaoxo cyclopentadecane). The results are shown in table 6-13.

Table 6-13: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of alcohols**.**

ether	*I*	$t_{½}$	log *P*
THF	*1.0*	3.2	0.4
15-crown-5 ether	1.4	185	-0.8[102]

The observed half-life in the presence of 15-crown-5 ether is rather in the magnitude of those measured for acyclic ethers than of the value of THF. This indicates that the cyclic character of the ether alone is not responsible for its incompatibility with enzymes. Nevertheless, it must be noticed that 15-crown-5 ether is a polyether. Thus the evaluation of water-immiscible cyclic monoether should be performed to enable a conclusion for the reason of the deleterious effect caused by THF.

Steric and Electronic Effect in Aliphatic Ethers

The ethers displayed in table 6-14 were studied to partially elucidate the importance of steric and electronic effects in aliphatic ethers regarding its enzyme compatibility.

Table 6-14: Instantaneous inactivation constant and half-lives/h of LBADH in the presence of ethers**.**

ether	*I*	$t_{½}$/h	log *P*
n-butyl-ethyl ether	1.2	119	1.7
tert-butyl ethyl ether	1.1	394	1.3
di*iso*propyl ether	1.43	646	1.4
tert-butyl methyl ether	1.21	1.80×10^3	1.0

These results suggest that steric and eletronic effects enhance the functionality character. Thus, if the *tert*-butyl residue is substituted by an *n*-butyl residue the enzyme stability decreases. The comparison between the isomers di*iso*propyl and *tert*-butyl ethyl ethers reveals the importance of the steric effects. But the comparisons between *tert*-butyl ethyl ether and *tert*-butyl methyl ether suggests that steric effects alone are not responsible for the solvent properties concerning enzyme deactivation.

[102] It is important to stress that 15-crown-5 ether is water-immiscible and that the calculated value for log *P* must be verified experimentally.

6.2.4.2 LBADH in Predominantly Organic Solvents

Given the remarkable compatibility of LBADH with organic solvents, it is necessary to investigate the behaviour of this enzyme in media formed by predominantly organic solvents. Two possibilities are to be distinguished:

1. Water-immiscible solvent with residual water content
2. Water miscible solvent with > 50% (v/v) solvent

Water-Immiscible Solvents

To investigate the use of water-immiscible solvents, cofactor was added to the LBADH preparation.[103] The protein solution was given to a solution of 10 mM acetophenone and 1.5 M 2-propanol in MTBE, so that a homogeneous solvent phase was present. The protein formed a coagulated suspension. No conversion could be detected after 24 h. This preliminary study indicates that a detailed investigation of the enzyme preparation conditions (*e.g.* lyophilisation in the presence of cofactor at different pH-values) is necessary to enable the LBADH utilisation in a water-immiscible solvent.

Water Miscible Solvent

The possibility of carrying out biotransformations in 2-propanol is be very attractive (cf. Stampfer *et al.*, 2002). The organic solvent would enhance the solubility of liphophilic substrates, simultaneously favouring the thermodynamic equilibrium. This option was studied by testing the activity of LBADH assays with increasing 2-propanol concentration. The amounts of the organic solvent were enhanced in 20% (v/v) steps. Up to 60% (v/v) 2-propanol, the enzyme is soluble and active. Above 80% (v/v) the protein coagulates forming glue-like fibres.[104] The coagulation is reversible and the enzyme, when redissolved in water, shows its original activity.

Conclusion

In conclusion, the utilisation of LBDAH in predominantly organic solvents, mainly in 2-propanol, seems to be feasible. However, the conciliation of enzyme activity with the desired high organic solvent concentration is limited by the solubility of the protein, which coagulates if no previous precaution is taken. Lyophilisation and immobilisation offer promising perspectives,[105] but requires further investigations.

---

[103] Crude cell extract containing the enzyme.

[104] Similar observations were made in respect to HLADH (Zaks and Klibanov, 1988)

[105] Cf. Guinn et al., 1991; Ansorge-Schumacher, 2002.

6.2.5 Conclusion

In subchapter 6.2, it has become possible to develop and utilise a procedure to guide the choice of organic solvents to be used in biphasic ADH-catalysed reactions. In contrast to conventional medium engineering approaches, not only the inhibitory effect of the solvent, given by the inhibition constant *I*, is considered, but also the time-dependent deactivation is taken into account. Therefore a mathematical model has been developed to describe the behaviour of ADH in the presence of organic solvents.

The procedure to guide the choice of organic solvents consist of the following steps:

1. classify the organic solvent according to the functionality.
2. screen for the best solvent class testing typical representatives of each class. (first screening step)
3. use the area under the curve given by equation 6-3 as the quantitative parameter for the selection.
4. screen further solvents of the best class to find the best solvent.
5. again use the area under the curve given by equation 6-3 as the quantitative parameter for the selection.

This algorithm led to the selection of MTBE as organic solvent for further work. Besides being the solvent associated with the highest half-live it displays a high I_{LBADH}-value. Further advantages of this solvent are: it is cheap, has a low boiling point, thus facilitating the work up, the availability is guaranteed, the lack of peroxide formation enables safe handling, and, it is already used in technical processes.

An alternative solution to the choice of the second phase is the use of an ionic liquid. This system is very attractive, mainly due to the positive thermodynamic effect caused by the partition coefficient of acetone and 2-propanol. However, this solution would involve the development of a new work up strategy for the product, since the solvent possesses negligible vapour pressure. For volatile products the evaporation and condensation can be imagined. This will not be considered in the present work.

The proposed procedure should not be regarded as a solution to the solvent selection problem, but merely as an approach to a solution.

Thus, Stillger has applied the method to search for organic solvents compatible with thiamine diphosphate-dependent enzymes (Stillger, in preparation). Very similar results were obtained (Figure 6-11).

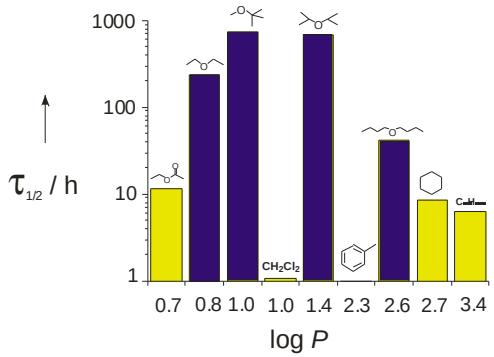


Figure 6-11: Half-lives of BAL in multiliquid biphasic systems.

These results present the first independent successful use of our procedure for an enzyme of a distinct enzyme class.

Moreover, the good correlation observed between the chemical functionality and the enzyme stability in the presence of solvents strengthen the evidence supporting our hypothesis that the chemical functionality gives rise to the enzyme compatibility of solvents.

The report that an *S*-hydroxynitrile lyase from *Hevea brasiliensis* is stable in the presence of MTBE encourages the application of this procedure for other enzymes lacking interfacial activation (Bauer *et al*., 1999).

6.3 Biotransformation

Using a developed procedure to guide the choice of organic solvents to be used in biphasic ADH-catalysed reactions enables the identification of a solvent in the presence of which LBADH retains its activity over a long period of time. Since the activity determination was carried out by sampling the aqueous phase and included a 100-times dilution in aqueous solution it is possible that any reversible deleterious effect of the solvent to the enzyme remains occult due to the a concomitant dilution of the solvent during the activity measurement. Consequently, it is necessary to prove the feasibility of a biotransformation in the chosen biphasic system.

6.3.1 The Feasibility of Biphasic Biotransformations

To verify the feasibility of biphasic biotransformation the reduction of acetophenone was again used as a model. The substrate coupled cofactor regeneration was carried out according to figure 6-12.

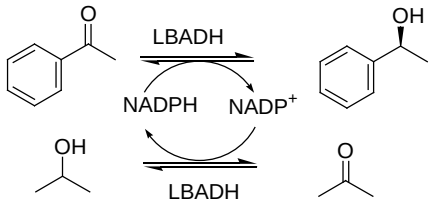


Figure 6-12: LBADH-catalysed reduction of acetophenone with substrate-coupled cofactor regeneration.

A solution containing LBADH and the reduced cofactor NADPH was covered with a solution of 1.0 mM acetophenone in MTBE. For the *in situ* NADPH-regeneration 2-propanol is added to the emulsion in a concentration of 1.5 M. The formation of phenylethanol is detected by GC-analysis. After complete conversion the organic phase containing the product and solvent was removed and replaced by a fresh solution of acetophenone in MTBE so that anew conversion takes place (Figure 6-13).

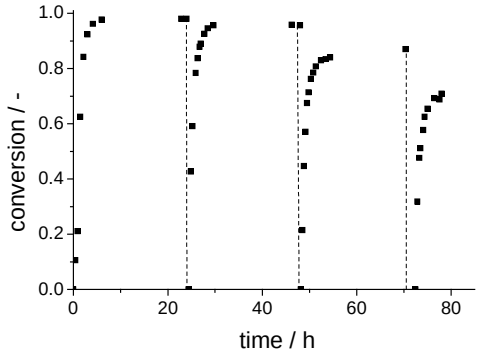


Figure 6-13: Conversion over time for the first four runs of the repetitive batch; 1.0 mM acetophenone, aqueous/MTBE 50/50 v/v; 150 mM phosphate buffer pH 7.0 at 20°C with 40 U_{st}/mL LBADH.

In this repetitive batch mode, the productivity of enzyme and cofactor was enhanced. Simple distillation of the solvent allows a facile work-up. This procedure was repeated for 1400 h since the half-life of LBADH at room temperature under these conditions is 480 h. The acetone accumulation caused a progressive reduction of the achievable conversion. (cf. chapter 7). The cofactor decay was compensated by periodical dosage of new cofactor.

6.3.2 Interfacial Effects

Enzymes catalysing reactions in biphasic systems can be affected by the presence of the liquid-liquid interface in at least three ways (Straathof, 2003) :

- the substrate concentration at the interface is significantly higher than in the homogeneous aqueous phase (mass transfer limitation)
- the enzyme concentration is significantly higher at the interface (interfacial adsorption)
- the intrinsic specific enzyme activity is significantly higher at the interface (interfacial activation)

Here, interfacial adsorption and interfacial activation should be discussed.

Interfacial Adsorption

The sampling of the bulk aqueous phase for activity determination shows that at an enzyme concentration of 3.0 U/mL no significant decrease of activity occurs in the bulk of the aqueous phase if a MTBE interface (approx. 3 cm^2) is introduced. This means that either no significant interfacial adsorption occurs, or the phase boundary is already saturated at a much lower enzyme concentration (Bühler and Wandrey, 1992). Given that the ratio between enzyme concentration and interface area in further experiments are of the same magnitude,[106] effects of interfacial adsortion will be considered non-existent in this work.

Interfacial Activation

Given that:

- interfacial activation seems not to be in action for enzymes other than some hydrolases (Straathof, 2003),
- interfacial activation must be preceded by interfacial adsorption (Straathof, 2003),
- the biphasic reaction rate of enzymatic catalysis is always lower than it would have been if the same substrate amount would be present in homogeneous aqueous phase,

the effect of any possible interfacial activation will be considered irrelevant for this work.

6.4 Enantioselectivity

It was demonstrated that the enzyme enantioselectivity in non-natural media can undergo alterations (Fitzpatrick and Klibanov, 1991). Jönsson *et al*. have shown that the enantioselecivity of TBADH depends on the water activity (Jönsson et al., 1999). In biphasic systems, the enantioselectivity of lipases could be modified by varying of the organic solvent (Villela, 1998) (Figure 6-14).

[106] Only membrane free experiments are considered.

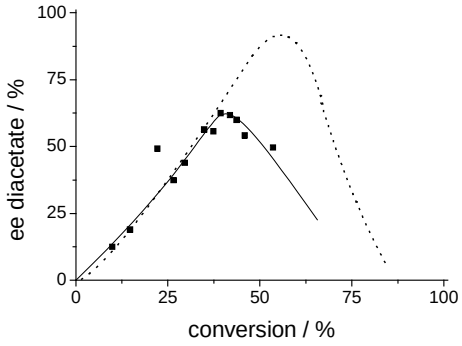


Figure 6-14: Comparison of the *ee versus* conversion plot for the *Mucor miehei* lipase catalysed hydrolysis of butane-1,3-diacetate carried out in aqueous/*n*-heptane (—) and aqueous/dichloromethane (••••) biphasic systems (50/50 v/v) (Villela, 1998).

Consequently it is necessary to verify if the enantioselectivity of LBADH is diminished in the MTBE/water biphasic system. The GC-analysis of the product obtained by the reduction of acetophenone in the biphasic system reveals that (*R*)-phenylethanol remains optically pure (*ee* > 99%, GC-analysis) over the whole experiment (Figure 6-15).

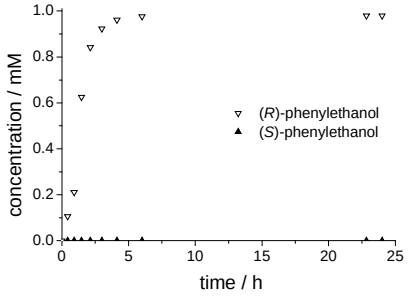


Figure 6-15: Concentration of (*R*)- and (*S*)- phenylethanol during LBADH-catalysed reduction of 1.0 mM acetophenone in aqueous/MTBE biphasic system; 150 mM phosphate buffer pH 7.0 at 30°C.

The optical purity of enzymatically produced CHOE under biphasic conditions was also determined by GC-analysis after derivatisation. The determined *ee* (> 99.5%) confirms that the LBADH enantioselectivity is not decreased by the presence of a second, organic phase.

This observation is compatible with the assumption of the absence of interfacial adsorption.

6.5 Cofactor Regeneration

In the previous sections of this chapter the possibility of using ADH in biphasic systems was demonstrated. Activity, stability and selectivity were established. For the implementation of the biphasic biotransformation the aspects concerning the cofactor regeneration must be taken into consideration.

6.5.1 General aspects

For some non-natural media, the possibilities of cofactor regeneration are limited due to the insolubility of the cofactor in the solvent. Thus the cofactor must not leave the enzyme and substrate coupled regeneration is the only possibility. This is not the case for multiliquid biphasic system. For this non-natural medium the lack of solubility of charged molecules (as the cofactor) in the organic phase is a great advantage. It represents a kind of immobilisation of the cofactor in the aqueous phase, but without changes in the kinetic constants. This allows the decoupling of residence time between product and cofactor on one hand[107]. On the other hand it also enables the use of any of the regeneration techniques previously discussed. However, the presence of the second phase can influence the regeneration dramatically. Thus, it is necessary to investigate the consequences for each regeneration approach individually. In this work we shall concentrate on the enzymatic methods for the pragmatic reason that the use of enzymes in biphasic system has already been demonstrated in this work.

6.5.2 Substrate Coupled Cofactor Regeneration.

Given that the enzyme used in this approach is the same involved in the production, the cofactor regeneration will be affected by the biphasic system to an extent comparable to the production reaction. This approach was used in the studies about the feasibility of biphasic biotransformations (see 6.3.1) and proved to be suitable for further work. Hence, it will not be discussed here further.

6.5.3 Enzyme Coupled Cofactor Regeneration

The study of the enzyme coupled cofactor regeneration should elucidate two main questions:

- Is it possible to apply this approach in biphasic systems?
- Is the partition coefficient of CDHE in MTBE/buffer high enough to avoid the deactivation of FDH?

[107] For the importance of this decoupling, see Kragl, 1997.

The first question can be investigated using the reduction of acetophenone as production reaction and regenerating the cofactor with the FDH-catalysed oxidation of formate (Figure 6-16).

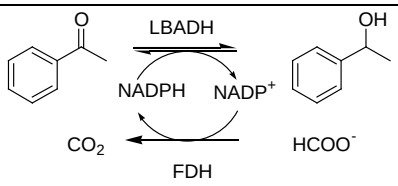


Figure 6-16: LBADH-catalysed acetophenone reduction with FDH-coupled cofactor regeneration.

As can be seen in figure 6-17 the regeneration using FDH and formate is possible.

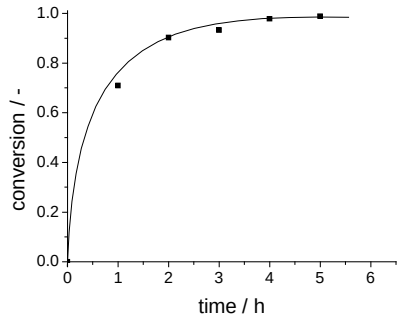


Figure 6-17: Conversion in course of time with FDH-coupled cofactor regeneration; 150 mM phosphate buffer pH 7.0 at 30°C, FDH = 6 U/mL and LBADH = 4 U/mL.

Change of the organic phase enables new conversion.

The next topic of investigation is the behaviour of FDH in biphasic systems containing 21 mM CDHE. As described in Chapter 5, the presence of 5 mM CDHE in the aqueous phase leads to a fast FDH deactivation. If the partition coefficient of CDHE in MTBE/buffer is high enough and consumption of the substrate is faster than its transport, the residual substrate concentration may be sufficiently low to be compatible with FDH presence. In this case, the production system would be limited by mass transfer, but the advantages of the enzyme coupled system could overcompensate this limitation.

To elucidate this question, a solution containing LBADH, cofactor, FDH and formate was converted by an equal volume of a solution containing 21 mM CDHE in MTBE. Figure 6-18 shows the course of the reaction.

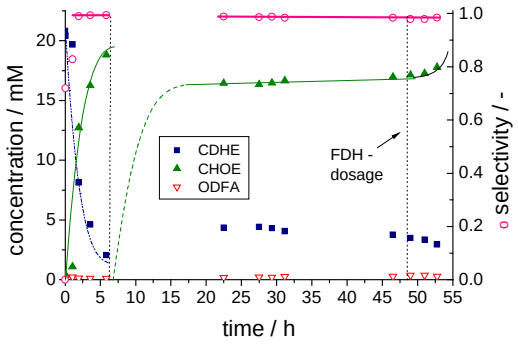


Figure 6-18: Concentrations- and selectivity-time profile of the LBADH-catalysed reduction of CDHE with FDH-coupled cofactor regeneration in aqueous organic biphasic system; the concentrations refer to the organic phase; 150 mM phosphate and 150 mM citrate buffer pH 5.5 at 25°C, FDH = 12 U/mL and LBADH = 20 U/mL.

During the first hours very fast enzymatic conversion of CDHE can be achieved. Consequently high selectivity is detected. After almost complete conversion, the organic phase was replaced by a new substrate solution. Conversion reaches 80% but does not increase further. The addition of 5% of the initial amount of FDH after 48 h leads to new conversion. This fact indicate that the CDHE concentration in water is probably still too high to coupled with the presence of FDH.

6.5.4 Conclusion

A comparison between the reduction of CDHE in biphasic systems employing enzymatic cofactor regeneration (6.3.1 and 6.5.2) makes it clear that the enzyme coupled regeneration affords easily a better and constant process selectivity for the one pot biphasic reaction. However two major disadvantages are associated with this technique:

- FDH regeneration in the presence of LBADH needs to be optimised
- Residual amounts of CDHE still causes deactivation FDH

While the former can be achieved by changing the reaction conditions, overcoming the latter disadvantage demands a special reactor concept (see chapter 7). For this reason it is wise to apply the substrate coupled regeneration for the continuation of the investigations concerning the one pot biphasic reactions. Moreover, optimisation of the reaction conditions can lead to high process selectivity using substrate coupled cofactor regeneration.

6.6 Thermodynamic Equilibrium

The thermodynamic equilibrium of a reversible reaction is modified due to the presence of a second phase. This aspect of biphasic biotransformations is of practical interest, since it can lead to dramatic changes in the obtainable yield. It was studied in detail by Martinek and coworkers and Halling.[108] In the case of hydrolytic enzymes, the thermodynamic equilibrium shift may be crucial for the use of the biocatalyst in synthetic reactions. For isolated ADH with integrated cofactor regeneration the effects have not been described yet, to the best of our knowledge, and is the subject of this subchapter.

6.6.1 General Aspects

The presence a second phase, where the catalytic reaction does not occur affects the thermodynamical equilibrium because the effective concentration of reagents in the reaction phase is different from the total amount of the reagents in the system. Moreover, the water activity is changed. Consequently hydrolytic equilibria are altered. For the ADH-catalysed reactions the formation of hydronium ions and solvation phenomena are affected. For the one-pot reactions studied in this chapter, the former can be disregarded because hydronium formation and consumption are identical. The latter will be disregarded for reasons of simplicity. Thus, the system studied is described in figure 6-19.

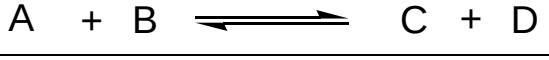


Figure 6-19: Illustration of the system studied.

A theory describing chemical equilibrium shift for this system was firstly presented in 1977 (Klibanov *et al*.). Despite certain limitations, it reveals important thermodynamic aspects. Therefore it is employed here to describe the system. The following assumptions are made:

- fixed phase volumes
- ideal behaviour (fixed partition coefficients)[109]

The apparent equilibrium constant for the biphasic system ($K_{biphasic}$) can be calculated according to the following equation:

$$K_{\text{biphasic}} = \frac{[\text{C}]_{\text{tot}}[\text{D}]_{\text{tot}}}{[\text{A}]_{\text{tot}}[\text{B}]_{\text{tot}}} \qquad \textbf{Equation 6-5}$$

[108] Martinek, *et al*., 1981; Semenov, *et al*., 1987, Valivety, *et al*., 1991; Halling, 1994.

[109] This is not valid at high concentration of reactants

with:

$K_{biphasic}$ = apparent equilibrium constant for the biphasic system

$[X]_{tot}$ = the total amount of substance X divided by the total volume of the biphasic system

If the value of the equilibrium constant in water (K_w), the partition coefficients and the volume ratio α are known, it is possible to calculate $K_{biphasic}$ from the equation below:[110]

$$K_{biphasic} = K_w \frac{(1+m_C\alpha)(1+m_D\alpha)}{(1+m_A\alpha)(1+m_B\alpha)} \qquad \textbf{Equation 6-6}$$

with:

K_w = equilibrium constant in water

$m_X = [X]_{org} / [X]_w$; partition coefficient

$[X]_{org}$ = concentration of substance X in the organic phase

$[X]_w$ = concentration of substance X in the aqueous phase

$\alpha = V_{org} / V_w$; volume ratio

V_{org} = volume of the organic phase

V_w = volume of the aqueous phase

Accordingly, it is possible to alter the product equilibrium concentration by modifying α. A consequence of this fact is that the dependence of yield *versus* phase volume ratio may show, in principle, *an extremum*. Equation 6-6 was used by Semenov *et al*. to demonstrate the potential of aqueous/organic biphasic systems as media for biocatalytic reactions, since higher yield can be achieved due to equilibria shift (Semenov *et al*., 1987) The consequences of equilibria shift for ADH-catalysed reaction is the subject of the next section.

6.6.2 Equilibrium Shift for ADH-Catalysed Reactions

To study the equilibrium shift for ADH-catalysed reactions in biphasic systems the reduction of acetophenone will be chosen as a model, due to the absence of side reactions.[111] Since the occurrence of measurable equilibrium is desired, the substrate coupled regeneration is employed. For the regeneration 2-propanol is used as co-substrate. Thus acetone is the co-

[110] For the deduction see Martinek *et al*., 1981.

[111] When studying the CDHE-reduction catalysed by *Saccharomyces cerevisiae*, Wolberg noticed a decrease in yield when the reaction was performed in biphasic system.

product. The partition coefficient of the compounds involved in the calculation of $K_{biphasic}$ were determined by GC-analysis and are given in table 6-15.

Table 6-15: Partition coefficient in MTBE/150 mM potassium phosphate buffer at 20°C.

compound	*m*
acetophenone	34.5
phenylethanol	26.7
acetone	1.11
2-propanol	1.10
CDHE	500
CHOE	200

The equilibrium constant in aqueous solution for acetophenone reduction system was determined as:

$K_w = 0.7$

According to equation 6-5 the value for $K_{biphasic}$ was determined experimentally in a 50% v/v MTBE/water biphasic system.

$K_{biphasic} = 0.001$

The drastic effect of the equilibrium shift in biphasic systems can be noticed comparing the conversion achieved in aqueous and in biphasic systems. They are depicted in figure 6-20. The two experiments were conducted with the same amounts of starting reagents and the same water volume. In the biphasic system an equal volume of MTBE was added.

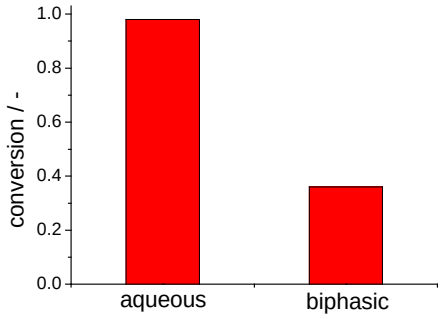


Figure 6-20: Equilibrium conversion determined for the aqueous solution and biphasic system (50% v/v) for the reduction of 10 mM acetophenone using 1.5 M 2-propanol as co-substrate at 20°C.

This equilibrium shift associated with the partition of the co-product in water decreases the maximal achievable conversion in repetitive batches.

Using the values for the partition coefficient given in table 6-15 and the determined value for K_w, $K_{biphasic}$ was also obtained according to equation 6-6:

$K_{biphasic} = 0.78\ K_w$

The difference between the measured and calculated values arises from the non-ideal behaviour of the studied system concerning the assumptions made, *e.g*. the keto-enol tautomerism, not considering the solvation effects, and mutual influence of the reagents regarding the partition coefficients. The latter is particularly significant at the high 2-propanol concentrations used in the experiments.

In the case of alcohols, water activity may be greatly influenced by hydrogen bonding. Specially the high 2-propanol concentration used in the experiments (1.5 M) can intensify this effect. Thus, neglecting this phenomenon enhances the inaccuracy of the estimation.

In conclusion, the assumptions made in the calculation of $K_{biphasic}$ using the values obtained for K_w and the partition coefficients cause a significant distortion of the results in our working window. Although equation 6-5 has been verified for the alcohol dehydrogenase-catalysed oxidation of 2-methylpropanol (Martinek *et al*., 1981), the experiment was carried out without cofactor regeneration and the reactants were very diluted. Under these conditions, the assumptions made can be justified.

Consequently, measurements of the water activity, estimation of the real acetone concentration present, and the change in partition coefficients in the presence of all reactants must be determined for a satisfactory estimation of $K_{biphasic}$ under the non-ideal conditions relevant for this work. But equilibrium will never be reached in the bioreactor, because the co-product will be constantly removed (see chapter 7). Thus, a satisfactory estimation of $K_{biphasic}$ is not necessary in the present work

For estimation of the equilibrium shift using this regeneration approach, the partition coefficient for acetone and 2-propanol can be considered identical. In this case the equilibrium shift is only dependent on the partition coefficients of substrate and product. For this regeneration system there is no change if the catalysed reaction is a reduction or an oxidation.

Nevertheless, the tendency of the equilibrium shift is properly given in the equation 6-6: products which are more hydrophilic than the starting reactant causes a decrease in the achievable yield, and *vice-versa*. Given that the product of a reduction is more hydrophilic

than the substrate due to the presence of an hydroxyl group, the introduction of a second, organic phase will decrease the achievable yield. The contrary is true for oxidation. Here, the yield is increased if the reaction occurs in a biphasic system.

To compensate this intrinsic characteristic of biphasic reductions, some tools can be considered:

- change of organic solvent
- change of volume ratio
- change of co-substrate
- technical solution

Different technical solutions to shift equilibria in substrate coupled cofactor regeneration are available (Stillger *et al*, 2002). They represent the most promising approach for continuous and semi-continuous application of ADH-catalysed reduction in biphasic system. Therefore they are discussed in chapter 7.

6.7 Discrimination of Side Reaction

As mentioned in the beginning of this chapter, the search for non-conventional media for enzymatic reactions aimed at the conciliation of water active enzymes with substrates that are:

- poorly water soluble
- water unstable

It was demonstrated that *tert*-butyl 6-chloro-3,5-dioxohexanoate (CDHE) is stable in MTBE solution (see chapter 3). Thus, it is expected that the biphasic reaction system presents a solution for the problem of ODFA formation. This is the subject of this subchapter. The discrimination of the side reaction in the biphasic system can have two causes:

- kinetics
- mass transfer limitation

For the experiments described in this subchapter it can be assumed that reaction kinetics dominates. [112] Consequently, phase transfer limitation will be disregarded and the rate of reactions in the aqueous phase will be considered. In the following section studies concerning

[112] This assumption is justified by the finding that increase of enzymatic reaction rate also increases the rate of substrate consumption and not only the selectivity; if the system were mass transfer limited the rate of substrate consumption would remain constant (cf. Willeman, 2002).

the discrimination of the side reaction due to concentration profile is presented. In the subsequent section the discrimination correlated with the activation energy is described.

6.7.1 Discrimination of Side Reaction due to Concentration Profile

The ODFA formation is described in chapter 3. For the purposes of this chapter it can be considered a first order reaction concerning CDHE. The first order simplification is valid at low substrate concentrations. It is also valid in systems where no buffer is used. Further, it is reasonable to describe the enzymatic reduction of CDHE as saturation kinetic (Michaelis-Menten).[113]

The enhancement of the selectivity of a catalysed main reaction with saturation kinetic in the presence of an undesired uncatalysed first-order side reaction has been studied by Giffels (figure 6-21) (Giffels, 1997). He concluded that the discrimination of the side reaction is high if the concentration of the substrate is low.

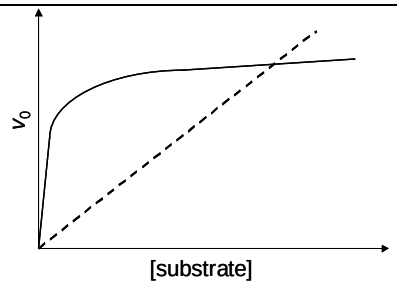


Figure 6-21: Representation of reaction rate dependence on substrate concentration for reactions with saturation (——) and first order (- - -) kinetic; at low substrate concentration the selectivity is higher.

There are different possibilities to fulfil these conditions. In laboratory work the slow dosage of the substrate is the most common approach.[114] In technical applications a CSTR working at high conversion affords constant and low substrate concentration.

In a kinetic limited biphasic system the aqueous concentration of the substrate is determined by the partition coefficient. If the solvent is properly chosen, it is possible to keep the

[113] While the model described in chapter 4 is valid only for small substrates concentrations, the saturation kinetic is valid for the whole range of substrate concentration.

[114] This approach was used by Wolberg in the production of CHOE (Wolberg, 2001).

substrate concentration low during the whole conversion. Hence, the biphasic system is a practical possibility of satisfying the condition for the discrimination of the side reaction.[115]

For the properly chosen biphasic system the enzymatic reaction can be described as first order concerning the substrate, as discussed in chepter 4. According to Gerrits *et al*. (2001), under this condition the ratio between enzymatic and spontaneous reactions is only dependent on the enzyme concentration (Equation 7-7).[116]

$$\frac{v_E}{v_{sp}} = \frac{k_E}{k_{sp}}[E] \qquad \textbf{Equation 6-7}$$

with:

v_E = rate of the enzymatic reaction

v_{sp} = rate of the spontaneous reaction

k_E = rate constant of the enzymatic reaction

k_{sp} = rate constant of the spontaneous reaction

The theoretical prediction for the selectivity must be experimentally confirmed. In a first experiment, a solution of 32 U/mL LBADH in phosphate buffer at pH 7.0 containing 0.01 mM NADPH was covered by a 21 mM solution of (CDHE) in MTBE (Figure 6-22). For the regeneration of the cofactor 1.5 M 2-propanol was used as reducing agent.[117] The selectivity achieved during the conversion was 85%, and decreases slowly to 80% at the end. The decrease in selectivity is attributed to the increasing acetone concentration.

[115] The biphasic system can be compared to an intelligent pump, which recognises the substrate concentration and determine the dosage rate.

[116] See chapter 9 for further discussion.

[117] The concentration was calculated for the aqueous phase

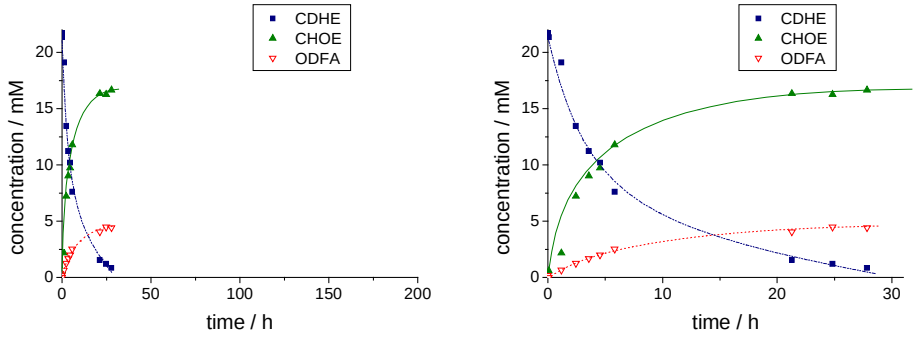


Figure 6-22: LBADH-catalysed reduction of CDHE in aqueous/MTBE biphasic system at 25°C aqueous phase at pH 7.0 (150 mM phosphate buffer) with 0.01 mM cofactor and 480 U_{st}/mL.

Increasing the cofactor concentration in the aqueous phase to 1.0 mM yields important informations. While the initial selectivity is high (approximately. 75%),[118] a drastic decrease is noticed during conversion: the overall selectivity is 36% at the end of the batch (Figure 6-23). A possible explanation is an inhibition of the production reaction by the gradually accumulating oxidised cofactor. The absolute concentration of NADP is higher if the total amount of cofactor is higher, and the effect of the inhibition is more prominent.

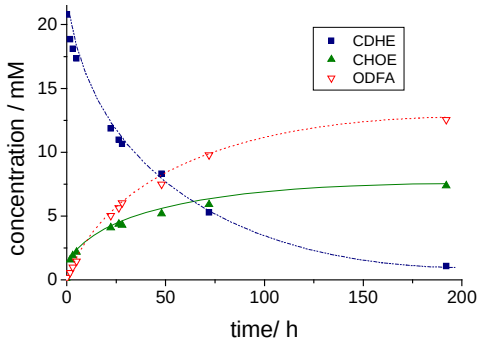


Figure 6-23: LBADH-catalysed reduction of CDHE in aqueous/MTBE biphasic system at 25°C; aqueous phase at pH 7 (150 mM phosphate buffer) with 1.0 mM cofactor and 480 U/mL.

The enzyme-coupled cofactor regeneration enables the independent control of the regeneration rate and is therefore used to verify the hypothesis. The proper regeneration activity fraction will reduce the NADP concentration to low values and shall thus prevent

[118] The lower value for the initial selectivity can suggest an NADPH surplus inhibition. This inhibition has no consequences for this work.

inhibition. The enzyme FDH (6 U/mL) and formate (200 mM) are used to regenerate the cofactor in a system analogous to the previous one (32 U/mL LBADH; 1 mM NADPH; 21 mM (CDHE) in MTBE). The results are plotted in figure 6-24.

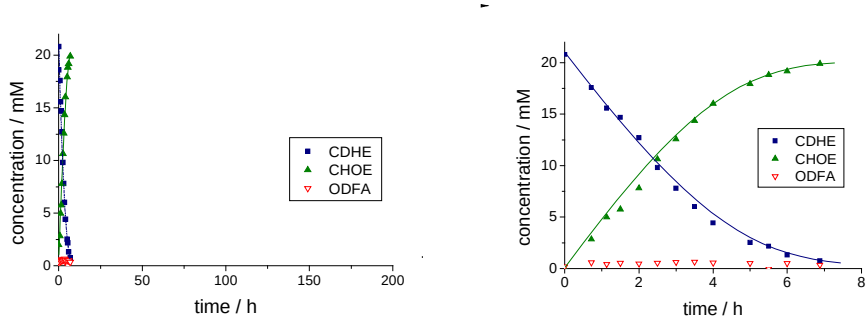


Figure 6-24: LBADH-catalysed reduction of CDHE in aqueous/MTBE biphasic system at 25°C; aqueous phase at pH 7.0 (150 mM phosphate buffer) with 1 mM cofactor and FDH-coupled (6 U/mL) cofactor regeneration.

In the described experiment the consumption of substrate is almost entirely caused by the enzymatic reduction. The ODFA formation is discriminated by this means. The obtained process selectivity is high (> 0.95) as expected from the theory and constant during the whole conversion. The reaction time is significantly decreased. These observations are in agreement with:

- NADP inhibition of the production reaction
- Decrease in selectivity also due to acetone accumulation

In conclusion, the LBADH-catalysed reduction of CDHE is limited by the substrate coupled cofactor regeneration. The use of FDH-coupled regeneration is not an economical solution to the problem because of the incompatibility between this enzyme and CDHE. Consequently, an alternative solution for the process development must be found.

The occurrence of NADP inhibition suggests that the selectivity as a function of the cofactor concentration shows a maximum. At too low concentration, the production reaction is limited by the K_M-value of NADPH. At too high concentration, the slow regeneration causes an accumulation of the oxidised cofactor, and a small production rate is also observed. Therefore it is fundamental to find out at which cofactor concentration the selectivity is at its maximum for the one-pot reaction with substrate-coupled cofactor regeneration. Given the difficulties for the determination of K_M-value for NADPH in the reduction of CDHE (Chapter 4), the

selectivity was determined by a series of experiments with identical conditions, but varying the total amount of cofactor used. The results are shown in figure 6-25.

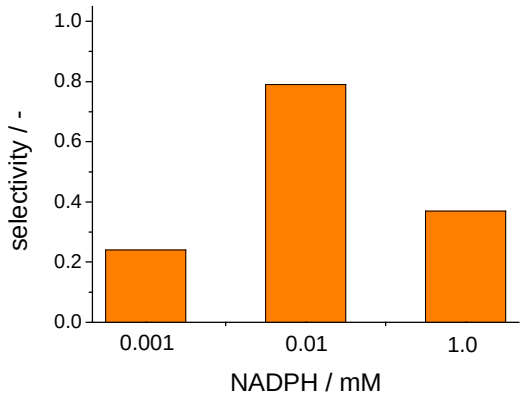


Figure 6-25: Overall selectivity obtained in the biphasic system in dependence on the initial cofactor concentration; aqueous/MTBE (50/50 v/v) at 25°C; aqueous phase at pH 7 (150 mM phosphate buffer) 480 U/mL.

These results suggest that the K_M-value for NADPH is expected to be between 0.001 and 0.01 mM. Furthermore, it is possible to establish 0.01 mM as the lowest possible cofactor concentration to cope with high process selectivity (cf. Kragl *et al*., 1992, Kragl *et al*., 1996) for one-pot biphasic reaction.

6.7.2 Discrimination of Side Reaction Due to Activation Energy

In addition to the discrimination of a side reaction according to the proper concentration profile it is also possible to discriminate the reaction making use of the difference in activation energies. When two reactions have different activation energies, the reaction with the lower activation energy will be favoured with temperature decrease, while the reaction with higher activation energy will be favoured with increasing temperatures.

The activation energy of the ODFA formation has been described in chapter 3. Using a kinetic limited biphasic system the activation energy of the enzymatic reaction was studied: The dependence of the product formation rate on the temperature can be described by to an Arrhenius approach. In spite of considering only the initial conversion rate the value obtained for the activation energy is 46 kJ/mol, identical to the value obtained for the regeneration reaction. This indicates that even at very low conversions, the rate of the regeneration reaction is limiting the system.

Hence, the activation energy of the enzymatic system is almost half of the activation energy of the undesired side reaction. Consequently, the decrease in reaction temperature is used to further increase the selectivity. With the constant experimental conditions 32 U/mL LBADH in phosphate buffer at pH 7.0 and 1.0 mM cofactor, 21 mM solution of CDHE in MTBE, cofactor regeneration with 1.5 M 2-propanol but changing the reaction temperature the discrimination of the ODFA formation at lower temperatures is demonstrated. Figure 6-26 shows the integral selectivity at 30% conversion.

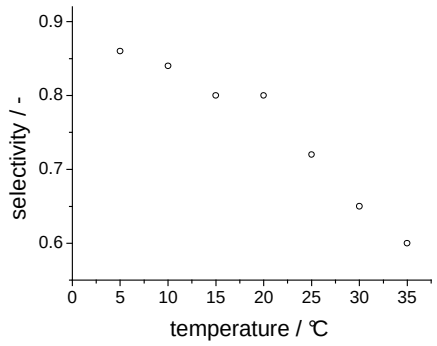


Figure 6-26: Temperature influence on the selectivity.

6.8 Conclusions and Outlook

This chapter describes the development of a new concept for the choice of organic solvents to be used in the enzymatic multiliquid biphasic transformation. Using the system enable us:

- use higher substrate concentration,
- easy recovery/reuse of the biocatalyst and cofactor, which do not dissolve in the organic solvent,
- better integration with chemical steps in a synthetic sequence,
- minimisation of the risk of microbial contamination,
- discriminate the undesired side reaction,
- improve the work up.

The use of the biphasic system proved efficient to improve the selectivity, but the problems of co-product accumulation persists. This limitation of the process is severe specially in the biphasic system due to thermodynamic shift. Its solution is discussed in the next chapter.

A fine tuning of biphasic systems concerning its properties (mainly partition coefficient) can be obtained by mixtures of solvents. Finally, further studies shall help a better understanding of the reasons for the enzyme compatibility of solvents.

6.9 Interim Summary

- Among non-conventional media multiliquid biphasic system represents the best perspective for this process development.
- A new approach for the solvent selection has been developed. According to it MTBE is chosen for further work.
- This approach was verified to be valid for other ADH and also enzymes of a further class.
- It is possible to perform LBADH catalysed reaction in aqueous/MTBE multiliquid biphasic system without loss of regio- or enantioselectivity.
- The thermodynamic equilibrium of the reduction is negatively affected by the presence of a second, organic phase.
- The biphasic system enables an increase in process selectivity by keeping the substrate concentration low. Concomitantly it allows the facile reuse of enzyme and cofactor.
- The LBADH-catalysed reduction of CDHE is limited by the substrate coupled cofactor regeneration.

PART III

7 The Bioreactor Configuration

"Solem prae iaculorum multitudine et sagittarum non videbitis."

XERXES

"In umbra igitur pugnabimus."

LEONIDAS

7.1 Aim and Requirements

The intrinsic limitations of the use of ADH were presented in Chapter 4.1. They are:

- cofactor cost
- difficulties in enzyme recovery/product work up
- operational stability of ADH
- inhibition of enzyme activities in the presence of substrates or products
- low solubility and in some cases instability of the substrate in water

Methods to overcome these limitations were the subject of chapter 5 and chapter 6. The present chapter aims to couple the solutions in a bioreactor design to enable the economic production of *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate, CHOE. The demand on the bioreactor are:

- enable high process selectivity (suppress the side reaction)
- reduce the cofactor costs (regeneration and retention)
- reduce the enzyme consumption
- avoid thermodynamic limitation
- avoid kinetic limitation due to $NADP^+$ accumulation (see 6.7)
- decrease work up expenditure
- enable high (quantitative) conversion[119]

The choice of a reactor to satisfy these requirements is the subject of subchapter 7.2. The following subchapter describes how to realise the biphasic bioreactor compartment. Subchapter 7.4 is about the possibility(s) of avoiding the kinetic limitations and subchapter

[119] See chapter 1

7.5 about the possibilities of avoiding the thermodynamic limitations. Finally, subchapter 7.6 analyses the reactor performance and compares it with other existing and possible reactors.

7.2 Reactor Choice

In subchapter 6.7 it is shown that biphasic systems can be used to improve the selectivity of the LBADH catalysed reduction of CDHE. The reason is the low substrate concentration defined by the partition coefficient. Concomitantly, this approach affords further advantages:

- confinement of the enzyme and cofactor in the aqueous phase,
- easy work up,
- avoidance of product inhibition,
- increase of enzyme and cofactor productivity.

The last mentioned advantage deserves some attention. In an aqueous batch system, the productivity of enzyme is limited by the solubility of the substrate. The productivity of the cofactor is limited by the necessary concentration (K_M) and the solubility of the substrate. In the biphasic system, higher amounts of the substrate can be converted, since no boundary concerning solubility limit exists.[120] This implicit advantage in biphasic systems can be further exploited if the aqueous phase is used repetitively.

Motivated by various advantages offered by the biphasic system, we decided to base the reactor development on the biphasic approach. To reduce cofactor cost and the enzyme consumption the reactor enables the repetitive operation of the aqueous phase.[121] Therefore a stable interface must be provided. The stable interface also enables the easy removal of the organic phase, thus simultaneously decoupling the residence time of substrate/product and enzymatic system, and providing a comfortable work up procedure.

The existence of a stable interface also enables the recirculation of the aqueous phase. In the circuit a device to remove acetone should be integrated. This device is also operated continuously, in order to avoid the accumulation of the cofactor regeneration coproduct. This is advantageous for **kinetic and thermodynamic** reasons. First, inhibition of the regeneration and production reaction is avoided. Secondly, since the concentration of one product is

[120] This is equivalent to the conversion of soluble substrates in the aqueous phase, cf. Kula and Kragl, 2000 and Peters, 1998 p 399.

[121] "Although the biphasic systems can easily produce... in laboratory, they are difficult to operate continuously...", Wong 1987.

continuously decreasing, the equilibrium is shifted, according to Le Chatelier's principle leading to better results (Figure 7-1).

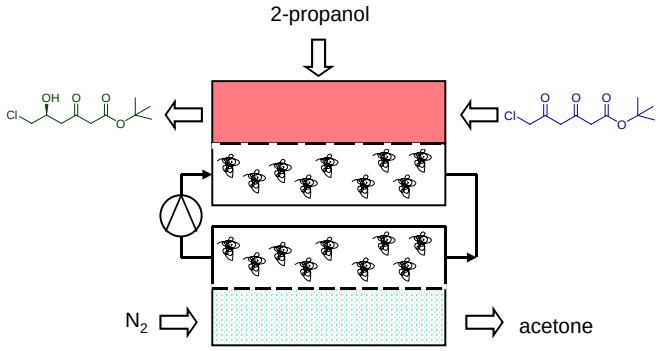


Figure 1-1: Schematic representation of a bioreactor with stable phase contact and integrated acetone removal.

Quantitative conversion is desired, because traces of the substrate would decrease the *ee* in the next synthesis step.[122] Quantitative conversion in a CSTR can only be achieved by infinite residence time.[123] Hence, the reactor operation should be a repetitive batch or plug flow.

For the reactor development, a pH of 7.0 is to be maintained. At this pH the highest reduction activity and the highest enzyme stability are achieved (7.0-7.5) and the cofactor stability is also satisfactory.

As organic phase, MTBE will be used (see chapter 6). The use of the pure substrate as organic phase is not opportune due to the bad enzyme stability in the presence of esters (see 6.2). Moreover, the high viscosity and low solution velocity of the substrate are detrimental.

During the process development, the reactor studies will be performed in the smallest possible scale in order to reduce costs. On this small scale, the desired pH control with an autotitrator would cause a high change in volume ratio. Hence, at this stage of the process development phosphate buffer is used to control the pH.

7.3 Phase Contact

Possibilities for the realisation of the biphasic reactor are given by:

- mixer-settler

[122] The conditions for reduction of the hydroxyketoester (see Wolberg, 2001) leads also to the reduction of CDHE yielding racemic dihydroxyketoester.

[123] The enzymatic production reaction is considered to be first order on the substrate.

- contifuge
- membrane phase contactor

Since the stability of the enzyme under high shear stress is not known, we decided for the membrane approach. The technical aspects of the phase separation by membranes are discussed by Schügerl and coworkers (Sisak *et al*., 2000):

In the extraction modules, two liquid phases are contacted at the interface immobilised by a membrane. In case of microporous membranes the pores are filled with the phase, preferentially wetting it, while the other immiscible phase is completely excluded, thus, no phase separation is required because the two liquid phases are never mixed.[124] In all cases, hydrophobic membrane pores are assumed to be filled with the organic phase while hydrophilic membrane pores contain the aqueous phase.

The most frequent configurations of the membrane extractors are:

- the flat sheet membrane
- the hollow fibres

For the realisation of the phase contact in the reactor both approaches were tested. The results are briefly described next:

7.3.1 Equipment Choice

Celgard X50 (Liquicel Minimodule). A hollow fibre module with polypropylene microporous membrane, 40% porosity, pore size 300 µm. This module enabled the fastest conversion rate. Due to swelling of the epoxy potting, the housing is damaged after several hours of operation. The durability of the module varies strongly (from 6 h to >170 h).

Spectrum MiniKros[125] polyethersulfone (PES) A hollow fibre module, with pore size 0.5 µm. The same reaction rate as with the previous module was achieved. However, 22 h after initialising the operation, the reactor presented a leakage in the housing connection.

Spectrum MiniKros[126] mixed ester (ME). Also a hollow fibre module. Similar transport properties were observed. In this case, ten hours after initialising the operation the, reactor presented a leakage in the housing connection.

---

[124] This is a fundamental advantage for the present system, since under some circunstances the phase separation is not immediate.

[125] Commercialised by Membrapure GmbH, Bodenheim

Spectrum MiniKros[127] polysulfone (PS) A hollow fibre module, with pore size 0.05 µm. Due to high phase instability this module was found to be inadequate for the process.

Flat sheet membrane Celgard 2500[128] in combination with **Optisep** flat sheet membrane holder[129] The advantage of this system is the easy scale up. Problem: Instability of all kinds of available special sealing ring in the presence of MTBE. This causes inadequate sealing and consequent phase mixing.

Flat sheet membrane Celgard 2500 in combination with a **steel membrane module** (Laue, 1997). The instable sealing rings can be protected from MTBE with Teflon® coating. This enables long operation times,[130] since the membrane is also stable under this condition.[131] A disadvantage of the system is the difficult scale up. The necessary reaction time for full conversion is significantly increased, probably due to poor transport properties.

It can be summarised that while hollow fibre modules suffer under poor stability in the presence of the organic phase, the flat membrane showed in combination with the steel module satisfactory properties in this respect. However, its disadvantage is the high conversion time required.

For the continuation of the work we selected the **Celgard X50**. The two mains reasons for the choice are the good transport properties and the availability of modules for scale up. The larger modules, however, are available without the epoxy potting. Thus, a higher stability under the reaction conditions can be expected.[132]

7.3.2 The Use of Hollow Fibre

Hollow fibre membranes are specially suitable for membrane extraction. The aqueous and the solvent phases are recirculated in counter-current mode through the hollow fibre module.

---

[126] Commercialised by Membrapure GmbH, Bodenheim

[127] Commercialised by Membrapure GmbH, Bodenheim

[128] Microporous polypropylene membrane, porosity: 55%, pore size: 0.209 x 0.054 µm

[129] North Carolina SRT Inc. USA.

[130] Stable over 168 h in the system without CDHE.

[131] Swelling was observed but did not influence the performance

[132] The larger module requires a too big reaction volume, causing high enzyme, cofactor and substrate consumption for this stage of the process development.

High mass transfer rates per unit volume may be provided since the modules can contain very high surface area, *e.g.* the tested Celgard module has surface area about 50 cm^2/cm^3. The interface in these modules can be accurately determined from the fibre dimensions and characteristics. Thus, the equipment can be easily characterised by pilot-plant test for scale up. This technique also overcomes other shortcomings of the conventional liquid extraction methods such as flooding limitations on independent variation of phase flow rates, requirements of density difference, stable emulsions formation or poor liquid-liquid contacting resulting in a low stage efficiency. The disadvantage of this extraction device could be clogging by particles in the feed (cf. Sisak *et al*., 2000). The phases are generally recirculed through an external vessel, or, a single pass of the streams take place. The counter current flow of the phases in the module proved to be of higher effectivity than the co-current flow. The reactors flow sheet is shown in figure 7-2.

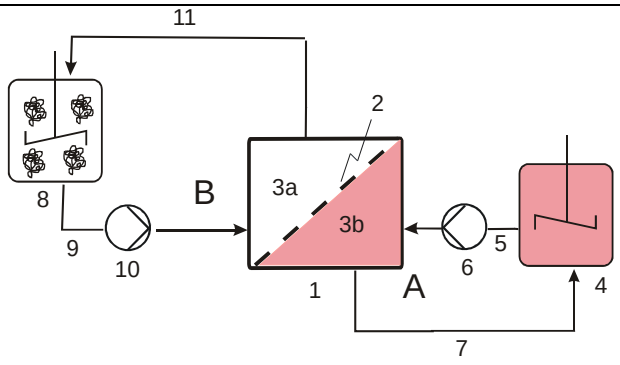


Figure 1-2: Flow sheet of bioreactor set up using a hollow fibre module as phase contactor; 1. module; 2. membrane; 3a. aqueous volume; 3b. organic volume; 4. stirred vessel (organic phase); 5. tubing; 6 pump; 7. tubing; 8. stirred vessel (aqueous phase); 9. tubing; 10. pump, 11. tubing; A. organic cycle; B. aqueous cycle.

The water phase is pumped through the lumen side of the hollow fibre with 500 mL/h flow rate. The lumen side is chosen, because here a more homogeneous plug flow is possible (see 7.6.2). The organic phase flow slower (50 mL/h) over the shell side. The advantages of this flow order is discussed in 7.6.2. The results obtained with the hollow fibre module **Celgard X50** are briefly discussed here:

The bioreactor was operated for three consecutive runs. The first run was interrupted after approximately 60% conversion and the organic phase containing product and unreacted substrate was replaced by a fresh one. The second run was carried out until approximately 90% conversion took place. The third run was concluded at approximately 50%. The results obtained are shown in figure 7-3.

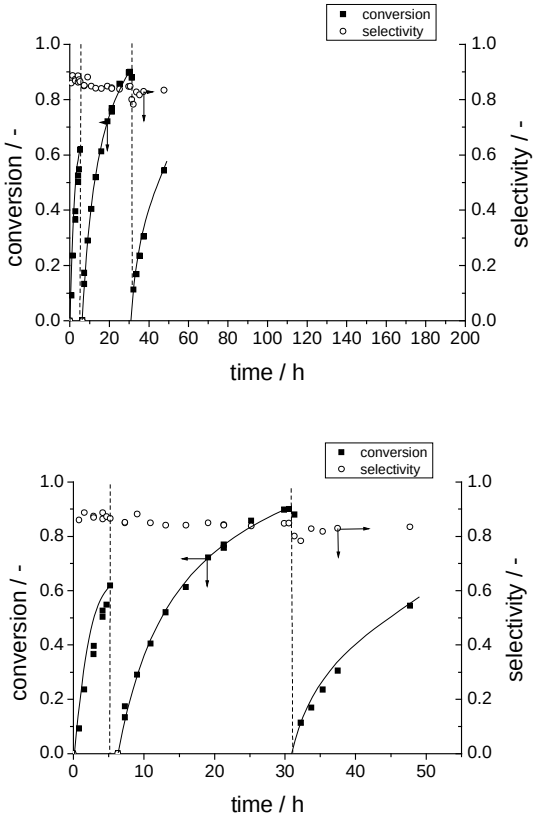


Figure 1-3: Conversion and selectivity in time course obtained with the hollow fibre module Celgard X50.

A decrease of the initial conversion rate can be clearly noticed. It is caused by the accumulation of acetone in the system. Apart from the efficient phase contact and separation, an important achievement of this reactor set up is the constant selectivity over the whole conversion, in opposition to decreasing selectivity in the emulsion reactor. This is discussed in the next subchapter.

7.4 Equalising the Kinetics

In this subchapter an explanation for the different behaviour of the selectivity as a function of the conversion in an emulsion batch and in the reactor described in the previous section is presented (Figure 7-4).

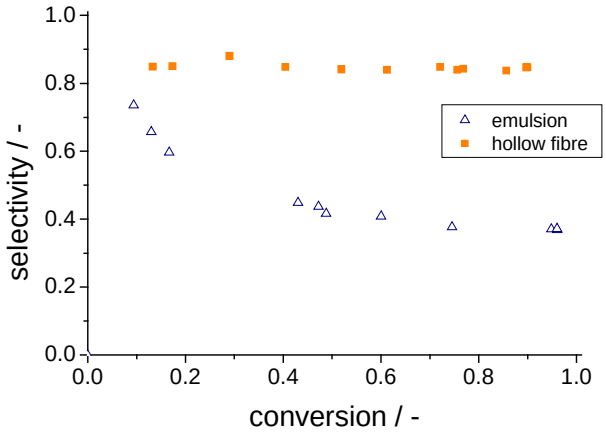


Figure 1-4: The selectivity as a function of conversion for the emulsion batch and the second run of the reactor with hollow fibre module; aqueous/MTBE (50/50 v/v), 150 mM phosphate buffer at pH 7.0 at 25°C, 300 mM 2-propanol, 0.1 mM cofactor 30 $U_{st.}$/mL.

As discussed in chapter 5, the regeneration activity fraction for a substrate coupled cofactor regeneration is constant. This is valid for saturation concentration of both substrate and cosubstrate. Under these conditions, the regeneration reaction is the limiting step of the whole system. However, in a biphasic system the concentration of the substrate is strongly reduced, according to the partition coefficient. In the special case of CDHE, with a very high partition coefficient, the concentration of the substrate in the aqueous phase is so small that the regeneration is not the limiting step at the beginning of the conversion. With increasing conversion, acetone is formed. Its inhibitory effect on the regeneration reaction and the progressive approximation of the equilibrium decreases the rate of the regeneration reaction. Consequently, accumulation of the oxidised cofactor occurs. The effects of this accumulation are show in subchapter 6.7. A decrease of the enzymatic production reaction and, consequently, of the selectivity results.

In the reactor depicted in figure 7-2 the cycle of the water phase can be divided into two parts: a biphasic contact and a single phase recirculation. It is important to note that while the cofactor consumption is limited to the part with biphasic contact, the regeneration of the cofactor occurs in the whole cycle. Thus, there is a decoupling of the reaction time of production and regeneration reaction. This allows the regeneration to be completed during the single phase recirculation, thus avoiding $NADP^+$ accumulation. So, the selectivity is constant

over the whole conversion. Here, the requirement that the rate of the whole system must be kinetically independent of the nature of the recycling reaction is fulfilled. (Bastos *et al*., 2002)

The decrease in the selectivity noticed in the third run is due to thermodynamic reasons. The accumulation of acetone shifts the equilibrium of the system, preventing the whole cofactor to be present in the reduced form. Only a small amount of the reduced cofactor is present, limiting the production rate. This effect cannot be overcome by reaction time decoupling. How to avoid the thermodynamic consequence of acetone accumulation is the subject of the next subchapter.

7.5 Acetone Removal

The discussion about substrate coupled cofactor regeneration demonstrated that the reversibility of the 2-propanol oxidation leads to a thermodynamic equilibrium by this regeneration. This is a disadvantage compared to the enzyme coupled approach. To overcome this limitation, one possibility is to use an excess of the cosubstrate.[133] Another possibility is the integrated removal of the coproduct. According to Le Chatelier's principle the equilibrium will be shifted enhancing the 2-propanol oxidation to compensate the low acetone concentration. The law of mass action demonstrate that keeping the acetone concentration low is a more efficient method for the thermodynamic shift than a high 2-propanol concentration. In other words, acetone should be removed from the reaction mixture for thermodynamic reasons, thus enabling a higher conversion.

The integration of this cofactor regeneration to the production reaction shows that acetone removal is desirable also from the kinetic point of view. Since the presence of acetone slows down the production and the regeneration reaction, a lower space-time-yield is achievable. Moreover, the spontaneous and irreversible side reaction is favoured by a slow production, and the overall process selectivity is consequently reduced. In this subchapter the integration of the acetone removal to the biotransformation is investigated. The aim is the development of an hybrid process. A hybrid process is defined as a process in which the work up device is integrated to the production step. Hybrid processes are advantageous when the combination gives a better result than the production step followed by the work up.

For the evaluation of three different removal techniques, stripping, pervaporation and reduced pressure, the reduction of ethyl 5-oxohexanoate (OHE) to ethyl 5-hydroxyhexanoate (HEE)

[133] *E.g*. Peters *et al*., 1993; see chapter 5.

catalysed by the carbonyl reductase from *Candida parapsilosis* (CPCR) is used as a model.[134] The enzyme requires NADH as cofactor. The substrate coupled cofactor regeneration using 2-propanol is applicable up to 10% v/v cosubstrate (Peters *et al*. 1993). The systems are shown in figure 7-5.

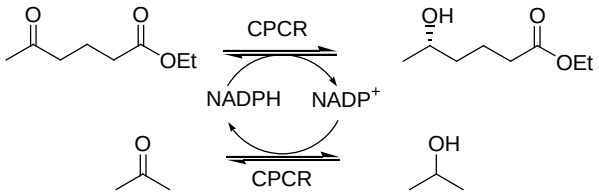


Figure 1-5: CPCR-catalysed reduction of ethyl 5-oxohexanoate with substrate coupled cofactor regeneration.

The system is used because all involved reactions are reversible. Thus the equilibrium constant is measurable and its shift can be used to illustrate the effect achieved by the integrated acetone removal. At 35°C the equilibrium constant can be calculated as follow:

$$K_{eq} = \frac{[\text{acetone}]\cdot[\text{HHE}]}{[2-\text{propanol}]\cdot[\text{OHE}]} = 0.63 \qquad \textbf{Equation 1-1}$$

7.5.1 Pervaporation

In pervaporation, volatile organic components are removed from a liquid feed mixture through a semi permeable continuous (pore free) membrane into a gas phase (Strathmann, 1990). The separation of components from the liquid mixture is determined not only by differences in their vapour pressures but also by their permeation rates through the membrane. The driving force for permeation is the chemical potential difference of the compounds between the two phases separated by the membrane. The chemical potential difference induces a concentration gradient within the membrane interphase. In pervaporation the chemical potential gradient is usually induced by applying vacuum on the permeate side of the membrane (Figure 7-6).

[134] This investigation was conducted by Thomas Stillger as his *Diplomarbeit* (2000); A patent has been applied for(Stillger *et al*, 2000) and it is published in Stillger *et al*., 2002.

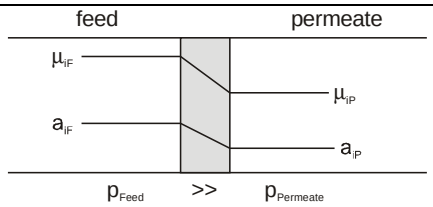


Figure 1-6: Illustration of pervaporation.

According to the membrane's characteristics, pervaporation applications can be classified as shown in table 7-1.

Table 1-1: Classification of pervaporation applications.

classification	separation
hydrophilic	separation of water from an organic solvent
hydrophobic (organophilic)	separation of organic compounds from aqueous solutions
selective-organophilic	separation of organic compounds from organic solutions

The characteristics elements of a pervaporation equipment are depicted in figure 7-7. A pump (P1) impels the feed solution through a heat exchanger (H1) into the membrane module. In module the solution components interact with the membrane, diffusing through it at different rates, depending on the respective affinity towards the membrane's material. On the opposite side of the membrane the permeate evaporates to the evacuated space. The necessary enthalpy for the evaporation is withdrawn from the feed.[135] The vapour is finally condensed in the heat exchanger (H2).

[135] In contrast to distillation, where temperature, pressure and chemical potential of liquid and gas phase are in equilibrium, in pervaporation the vapour is not in equilibrium with the liquid phase.

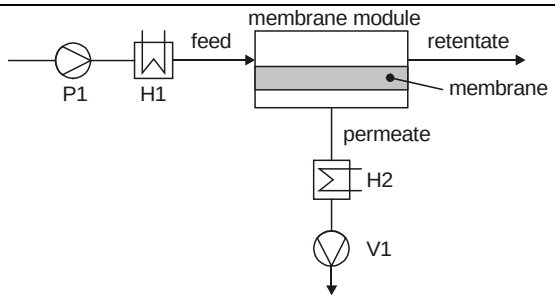


Figure 1-7: Schematic illustration of a pervaporation device; Pump (P1), heat exchanger (H1 + H2), vacuum pump (V1) and membrane module.

The attractiveness of this technique is the possibility of selectively removing acetone from the reaction mixture leaving the 2-propanol concentration constant. This may be possible since separation is based on the interaction between membrane and substance and not on the volatility.

7.5.1.1 Transport Characterisation

The quantitative characterisation of the matter transport through the membrane is based of the following quantities:

The mass flux, J_m, through the membrane, given in mass per membrane area and time [kg / m^2 h]. It can be directly obtained from the experiment.

The partial mass flux, j_i, determined after quantitative analysis of the permeate according to the equation 7-2.

$$j_i = J\, w_i \qquad \textbf{Equation 1-2}$$

where w_i is the mass fraction of substance i in permeate.

The separation is normally quantified with two dimensionless quantities, separation factor α_{ij}, and selectivity S_{ij}. It is worth noticing that these quantities are defined for binary mixtures and therefore not adequate for our purposes. Hence, we will base our discussion on the ratio of the acetone and 2-propanol in the feed solution.

7.5.1.2 Experimental Set Up

The membrane characterisation and the integration of pervaporation to the biotransformation were performed in the equipment illustrated in Fig 7-8. The recirculation of the retentate enables the evaluation of the process with a small membrane area.

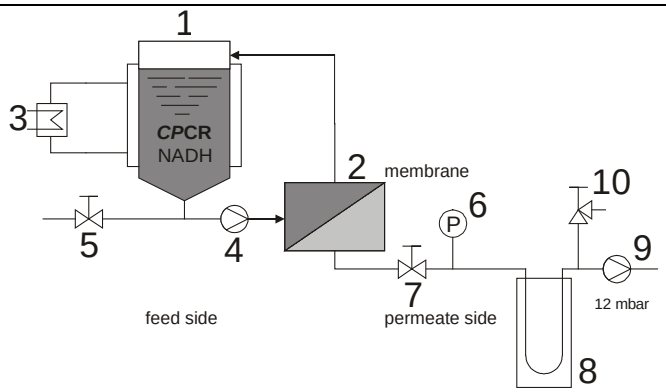


Figure 1-8: Schematic reactor illustration; 1. reactor, 2. membrane module, 3. heat exchanger, 4. pump, 5. valve, 6. manometer, 7. valve, 8. condenser, 9. vacuum pump, 10 valve.

Working temperature of 35°C is used. This temperature provides enough energy for the pervaporation and is still compatible with the enzyme stability.

7.5.1.3 Membrane Choice

For the purpose of removing acetone from the water solution, avoiding the removal of 2-propanol, three commercially available membranes and two further membranes were tested.[136] (Table 7-2)

Table 1-2: Membranes used in this work.

name	supplier	material
GKSS-PDMS	GKSS Forschungszentrum Geesthacht	polydimethyl siloxane
GKSS-POMS	GKSS Forschungszentrum Geesthacht	polymethyloctyl-vinylmethyl-dimethylsiloxane
PerVap 1060	Sulzer Chemtech	polydimethyl siloxane
PerVap 1070	Sulzer Chemtech	polydimethyl siloxane + zeolithe
cm-celfa	cm-celfa Membrantechnik	polydimethyl siloxane

[136] The two not commercially available membranes were kindly supplied by the GKSS Forschungszentrum Geesthacht.

Two different experiments were carried out to evaluate the membranes. In the first one, the selectivity of the membrane concerning the acetone removal and the 2-propanol concentration was tested. Therefore an aqueous solution containing equal amounts (200 mM) of acetone and 2-propanol was pervaporated at 35°C. The change of the ratio of the feed acetone and 2-propanol concentration in the course of an experiment is shown in Figure 7-9.

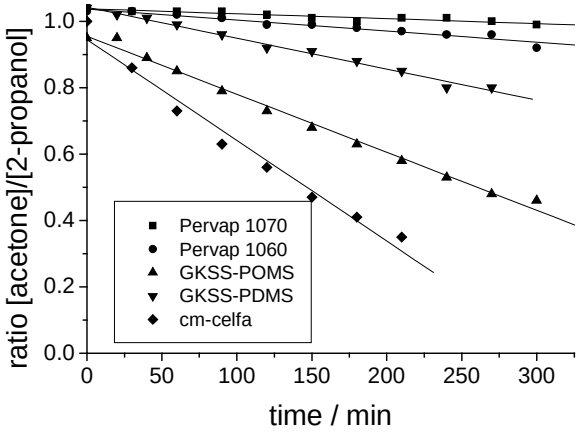


Figure 1-9: Ratio of feed acetone and 2-propanol concentration (cf. Stillger, 2000).

It is important to notice that the selectivity and the flux may be dependent on the concentration of the components. So, in a second set of experiments the biotransformation conditions were simulated. Thus, the acetone concentration was set at 20 mM and the 2-propanol concentration at 100 mM. OHE is also added to the solution to a concentration of 20 mM. The aim of this experiment set is to the study the acetone removal grade and the influence of OHE on the process.

The results show that only two membranes enable the complete acetone removal within five hours. Regarding the fact that the flux is at least as important as the selectivity for our purposes, all other membranes are seen as not compatible with the desired application. Under the experimental conditions the GKSS-PDMS membrane has shown sufficient acetone flux[137] but higher 2-propanol and water retention. Therefore, this membrane is chosen for use in the hybrid biotransformation reactor. No significant OHE removal was detected.

[137] The initial GKSS-PDMS acetone flux for the second experiment is 0.0280 kg/(h·m^2). For further results see Stillger, 2000.

7.5.1.4 Biotransformation with Integrated Pervaporation

The integration of the pervaporation to the biotransformation in an hybrid process enables the study of the influence of the acetone removal on the thermodynamic equilibrium. For this purpose a batch reaction is carried out. The undesired water and 2-propanol removal is compensated through a 0.025 mL/min feed of a 6.5% v/v 2-propanol solution. The obtained results are depicted in figure 7-10.

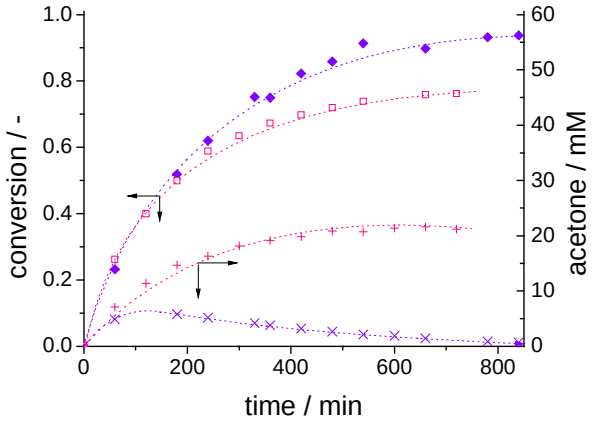


Figure 1-10: Comparison of conversion-time profile with (◆) and without (□) pervaporation, and acetone concentration-time profile with (x) and without (+) pervaporation 100 mM 2-propanol, 30 mM OHE, 0.2 mM NADH, 0.15 U/mL CPCP, pH 7.8, 35°C.

The comparison of the obtained conversion with the batch conversion without acetone removal clearly shows the potential of an hybrid process. By removing acetone higher conversion can be reached (> 95%). The profile of the acetone concentration during the conversion deserves special attention. In early reaction stages, where the conversion rate is high, the acetone flux through the membrane is not enough to compensate its formation rate. Acetone accumulates up to a concentration of 8 mM. In the further course of the reaction, the acetone formation rate decreases, and it is overcompensated by the removal rate. Thus the acetone concentration decreases. The thermodynamic driving force of the production reaction is preserved, leading to the high conversion.

The decrease of the 2-propanol concentration is also explained through the higher reaction rate in early stages of the reaction.

7.5.1.5 Conclusion

The acetone removal by means of pervaporation shows that it is possible to overcome the thermodynamic limitation inherent in the substrate coupled cofactor regeneration. In order to justify its use, the pervaporation technique must reduce the cosubstrate cost in a way that compensate the necessary investment for the equipment. At present, however, no membrane is available which allows the completely selective acetone removal without influencing the 2-propanol concentration. Consequently, less expensive ways of removing acetone are investigated.

7.5.2 Stripping

A very pragmatic way to remove acetone from the aqueous solution is by gas stripping. The process is based on the tendency towards saturation of a gas volume in contact with a solution of volatile compounds. The involved phenomena were studied by Raoult and Henry and are briefly presented in section 7.5.5. Here only its application is discussed.

A continuous inert gas stream over a solution of volatile compounds is able to remove the solute. The rate of removal is dependent on the solute concentration and its volatility.[138] It cannot be influenced by external factors. Thus, the acetone removal also causes the 2-propanol concentration to decline. Water elimination can be avoided by a previous saturation of the gas stream with water vapour. The set up required for stripping is shown in figure 7-11. Compressed air is used as inert gas for the investigations.

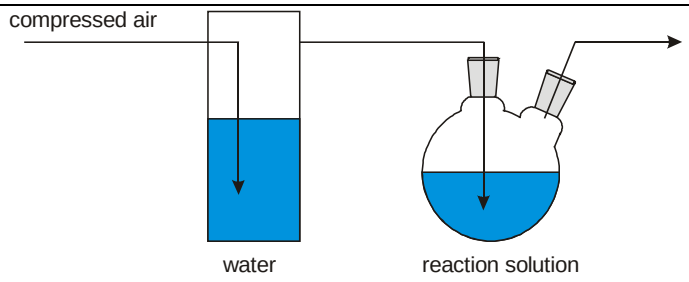


Figure 1-11: Set up scheme of stripping experiment.

Using a solution of equal acetone and 2-propanol concentration it is demonstrated that acetone is preferentially removed by the inert gas stream. Similarly to the pervaporation investigation, a study is carried out under situation simulating the biotransformation

[138] More accurately, in ideal diluted solution the Henry's constant of the compound.

conditions. It is noticed that OHE is not removed by this procedure. The results are shown in figure 7-12.

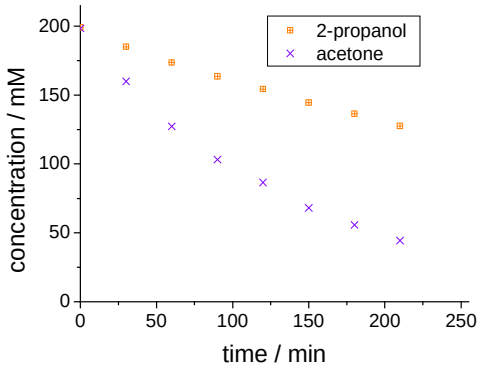


Figure 1-12: Concentration profile in course of time during stripping with 0.5 L/min gas stream at 35°C.

Further it is verified that the CPCR does not loose stability under the gas stream for at least 600 min. Hence, the biotransformation can be performed in a batch experiment with simultaneous stripping. To compensate the 2-propanol removal, the alcohol is replaced periodically. The temperature is set at 35°C, enabling a comparison with the pervaporation investigation. The obtained results are shown in figure 7-13.

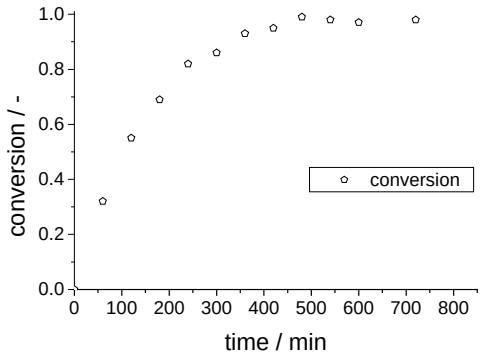


Figure 1-13: Conversion time-plot for CPCR-catalysed reduction of ethyl 5-oxohexanoate with integrated stripping at 35°C, 0.5 L/min gas stream 100 mM 2-propanol, 30 mM OHE, 0.2 mM NADH, 0.15 U/mL CPCP, pH 7.8, 35°C, air-flow 0.5 L/min.

It is possible to keep the acetone concentration below 5 mM during the conversion owing to the stripping procedure. Like pervaporation, it avoids the thermodynamic limitation. Hence, high conversion (>97%) is achievable. Concerning the equipment and energy costs, stripping

is far superior to pervaporation. Disadvantages of the approach are the shear stress to which the enzyme is submitted and the foam formation. The latter can be avoided by the addition of polypropylenglycol 1200. However, any additional substance is prejudicial for the work up.

7.5.3 Acetone Removal under Reduced Pressure

Acetone is the most volatile compound among those present in the biotransformation (cf. Lide, 1992). Provided that no azeotropic mixture is formed either in the presence of water or in the presence of 2-propanol, in equilibrium the acetone mole fraction in the gas phase is higher than in the liquid phase. Hence, reducing the pressure over the liquid phase causes a preferential removal of acetone.

The use of this phenomenon with the purpose of avoiding the acetone concentration during a biotransformation is the subject of the present section. Therefore, a batch conversion is carried out at 35°C until equilibrium is reached (73% conversion). Than the pressure is progressively reduced. The concentration and pressure profile over the course of the experiment are shown in figure 7-14.

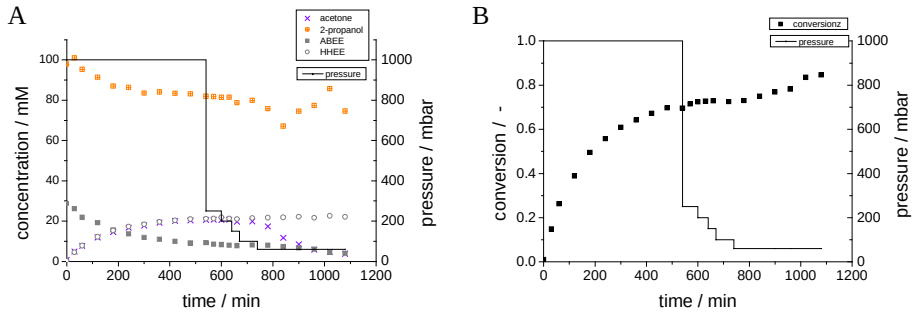


Figure 1-14: A: Concentration and pressure profile over the course of time for CPCR-catalysed reduction of ethyl 5-oxohexanoate at 35°C; B: the corresponding conversion time-plot; 100 mM 2-propanol, 30 mM OHE, 0.2 mM NADH, 0.15 U/mL CPCP, pH 7.8, 35°C.

Since the acetone mole fraction is small, its partial pressure is correspondingly small according to Henry's law. As a consequence, low overall pressure must be applied to the system to enable a significant change in acetone concentration. This is only achieved below 60 mbar, providing further 5% conversion.

The procedure also removes 2-propanol from the mixture, which is compensated by discontinuous dosage.

7.5.4 Comparison of the Methods

A complete comparison of the methods is provided in Stillger, 2000. Here, only the evaluation and comparison of the methods concerning the integration to the process development is carried out.

The results presented in the three previous sections show that distillation under reduced pressure is not an efficient method for the removal of acetone. Pervaporation and stripping enable the acetone removal, thus avoiding thermodynamic limitation. They are compared in figure 7-15.

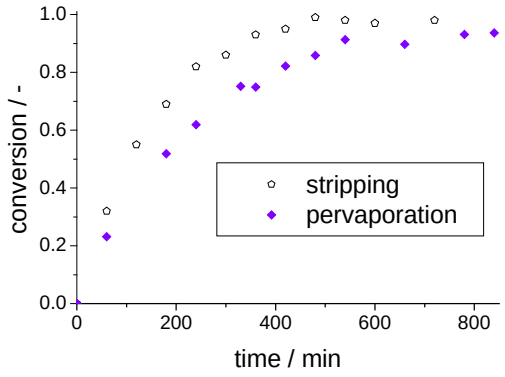


Figure 1-15: Comparison of the conversion-time plot for stripping and pervaporation (cf. Stillger, 2000).

The figure show the conversion achieved for each technique. It illustrates very clearly how important the rate of acetone removal is. A slightly smaller removal rate was achieved with pervaporation, leading to an acetone concentration up to 8 mM in comparison to 5 mM accumulated with stripping. This small but significant difference causes a clear change in the conversion rate.

In order to enhance the pervaporation rate, the membrane area would have to be increased. This leads to additional costs. The great potential advantage of pervaporation, the selective acetone removal with 2-propanol retention, is not possible with the membranes available at present. Furthermore, the process requires some thermal energy from the feed solution. This might lead to difficulties when integrating the system to an LBADH-catalysed reaction due to enzyme thermoinstability (Chapter 4). For these reasons, we prefer the use of another technique for the acetone removal.

Stripping is the most convenient procedure among those studied here. Its integration to a biphasic reactor, however, is not possible without further development. The direct stripping of the organic solvent must be avoided. Further improvements are also possible if the closely related technology of membrane distillation is employed. This is the subject of the next section.

7.5.5 Membrane Distillation

In order to provide high efficiency, reproducibility and to prevent foam formation and shear stress in the enzyme solution, membrane distillation is employed. Here, a microporous hollow fibre membrane stabilises the surface between liquid and gas phases. The membrane itself has no influence on the selectivity. The driving force for the separation is the partial pressure gradient of one compound just above the liquid surface and the partial pressure in the bulk of the gas phase. The partial pressure over the liquid phase is equal to the vapour pressure. For the ideal solution it is defined according to Raoult's law:

$$p_A = x_A \cdot p^*_A \qquad \textbf{Equation 1-3}$$

x_B is the mole fraction of the compound A and p^*_A is the vapour pressure of the pure liquid A. For an ideal dilute solution it is given by Henry's law.

$$p_B = x_B K_B \qquad \textbf{Equation 1-4}$$

x_A is the mole fraction of the solute and K_B is a constant (with the dimensions of pressure) determined experimentally.

A constant stream of the inert gas (nitrogen is used to avoid the contact of oxygen with flammable vapours) affords a very low partial pressure of the compound to be removed (*i.e.* acetone) in the bulk of the gas phase.[139] Since the transport from the liquid surface to the bulk of the gas phase is diffusive in nature, it can be described by Fick's law of diffusion.

$$J = D \cdot A \cdot p_{surface} \qquad \textbf{Equation 1-5}$$

where D is the diffusion coefficient and A the surface area.
Combining Fick's and Henry's law we see that the removal rate (v_{strip}) of the solute B is proportional to its concentration.

$$v_{strip} = k_{strip} \cdot [B] \qquad \textbf{Equation 1-6}$$

[139] For practical reasons the partial pressure can be set zero.

where k_{strip} is a constant reflecting Henry's law constant, the diffusion coefficient of B and the surface area. It can be determined experimentally for a given equipment. Therefore the concentration of B is measured in the course of time. Fitting the obtained data with equation 7-7 delivers k_{strip}.:

$$[B] = [B]_0 \, e^{- k_{strip} \cdot t} \qquad \textbf{Equation 1-7}$$

To avoid water removal the nitrogen stream is pre-saturated with water. The partial pressure of water in the bulk gas phase equals the vapour pressure. The gradient is non-nexistent and no water is removed.

To carry out the membrane supported stripping a **Celgard X50 (Liquicel Minimodule)** hollow fibre module is employed.[140] Three nitrogen flow rates are tested: 10, 15 and 20 L_n/h. In this interval, no variation of the stripping rate is observed, meaning that the boundary

$$p_{bulk} = 0$$

is reached for all three flow rates.

Since the presence of additional compounds can interfere with the stripping rate, the determination of the value for k_{strip} is performed in the presence of 150 mM phosphate buffer and 400 mM 2-propanol. The acetone initial concentration is set at 12 mM. Figure 7-16 shows the obtained results.

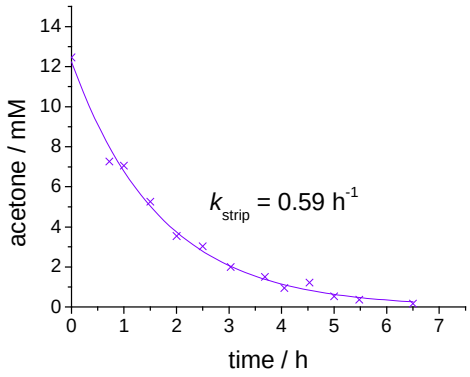


Figure 1-16: Determination of k_{strip} for acetone in a Celgard X50 (Liquicel Minimodule) hollow fibre module at 25°C and nitrogen flow 15 L_n/h.

[140] Active surface area: ID = 0.09 m^2; OD = 0.12 m^2.

Simultaneously the constant for 2-propanol was also measured. The values for k_{strip} is calculated according to equation 7-7:. The results are shown in table 7-3.

Table 1-3: Experimentally determined values for k_{strip}[141]

substance	k_{strip} [1/h]	k_{strip} [1/min]
acetone	0.59	0.0099
2-propanol	0.18	0.0030

7.6 The Hybrid Bioreactor[142]

7.6.1 The Elements Order

The results presented in subchapter 7.5 suggest an integration of the acetone removal to the reactor presented in subchapter 7.3, leading to a hybrid reactor. The advantages of the hybrid reactor over the sequential performance of reaction and work up are not only thermodynamic (higher conversion) but also kinetic (higher conversion rate), leading to economic benefits (higher selectivity, higher space-time-yield). The elements constituting the hybrid reactor are:

- **biphasic reactor** for suppressing the side reaction
- **aqueous compartment** to equalise the production and regeneration rate. This part of the reactor should be dimensioned according to the concentration of the reagents and the consequent reaction rates.
- **acetone removal device**, which will remove all volatile components of the aqueous solution.
- **dosage unit** to compensate the 2-propanol elimination. Since the water phase is very rapidly saturated with the volatile organic solvent MTBE, a continuous removal of the latter is also expected. Therefore its dosage is also necessary.

Before the reactor is assembled, it is essential to understand the influence of each device on the whole reactor performance, so that the most efficient order of the components is obtained. This is discussed in the present section.

---

[141] Considering the membrane area these values corresponds to $k_{acetone}$ = 0.11 $min^{-1} \cdot m^{-2}$ and $k_{2\text{-propanol}}$ = 0.033 $min^{-1} \cdot m^{-2}$.

[142] This concept was decorated with the best poster award during the 4th International Symposium Catalysis in Multiphase Reactors, Lausanne 2002 (cf. Villela *et al*. 2002).

The core of the process is the phase contactor, where the production reaction occurs. The water phase is recirculated over a pump. Consequently, the aqueous compartment is necessarily present in the process. Given the cyclic character of the reactor, no special consideration about its position is necessary. The ideal dimensions of the compartment can only be calculated after optimisation of the reaction conditions, mainly substrate and enzyme concentration. At this stage of the process development, the size of the compartment is determined by the technical possibilities (*e.g.* pump volume).

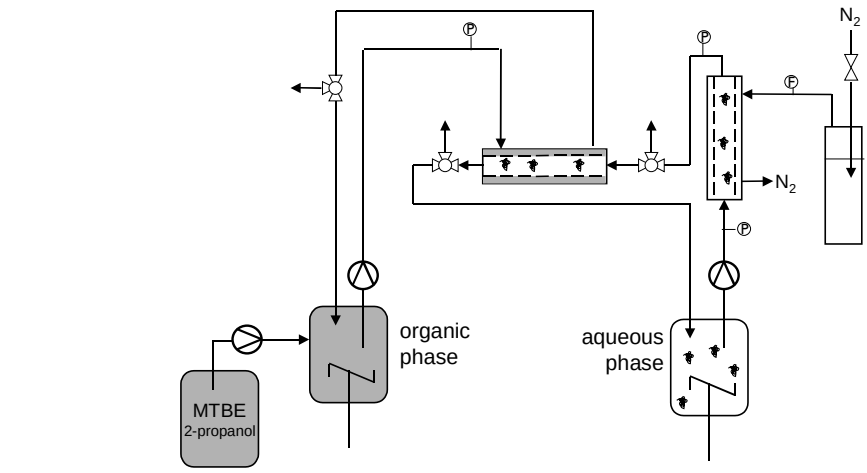
Acetone decreases the rate of the regeneration reaction. It also inhibits the production reaction, as presented in chapter 4. The former effect can be compensated by increasing the enzyme concentration or enhancing the reaction time of the regeneration reaction. The latter effect causes a decrease in the production rate, thus lowering selectivity and space time yield. It can only be compensated by increasing the enzyme concentration. For this reason, we decided to remove acetone just before the water phase enters the biphasic reactor. By this method, the acetone inhibition on the production reaction is minimized.

The location of the 2-propanol dosage is dependent on the alcohol influence on the production system. The initial production rate was measured under different 2-propanol concentrations (100 mM and 300 mM) to evaluate this effect. No influence could be noticed in the concentration interval studied. Thus, the 2-propanol dosage position has no effect on the reaction performance.

The problem of the MTBE dosage is solved by the setting of the acetone removal. Since the aqueous solution is saturated until shortly before the biphasic reactor, it only makes sense to replace the MTBE in the phase contactor.

The simultaneous dosage of MTBE and 2-propanol reduces the equipment necessary. Hence, a 300 mM 2-propanol solution in MTBE is continuously added to the organic phase, and the concentration in water adjusted in the phase contactor. Due to the higher MTBE volatility, an accumulation of 2-propanol is observed after long operation. This problem is solved during the replacement of the organic phase (see Chapter 8). Figure 7-17 shows the flow scheme of the designed reactor.

A



B

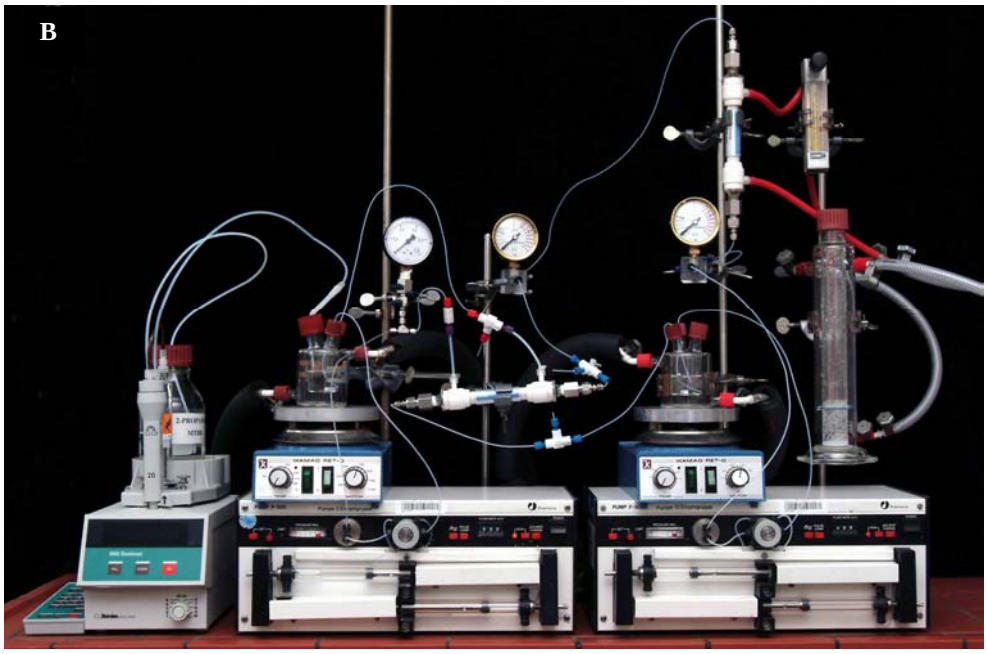


Figure 1-17: A: Flow sheet of the hybrid bioreactor; B: a photography thereof.

7.6.2 The Operation Modus

The developed bioreactor can be operated in two different modes concerning the organic phase: continuous flow or (repetitive) batch. In the continuous flow operation, the fresh organic phase containing MTBE, CDHE and 2-propanol is continuously pumped to the biphasic reactor. Conversion is limited to one flow through the hollow fibre module and the

organic phase leaving the reactor should contain MTBE, CHOE, 2-propanol and acetone. In the batch operation, the organic phase leaving the module still contains significant amounts of the substrate. It is recirculated allowing further conversion in the reactor. Before a decision about the operation modus is made, the flow pattern in the reactor should be understood.

The flow pattern in is studied by an analysis of the residence time distribution of a tracer (acetone).[143] Therefore a determined amount of the tracer is introduced to the fluid entering the vessel. Subsequently a concentration-time record of tracer leaving the reactor is obtained. This is the C_{pulse} curve.[144] The results measured for the shell side of the reactor (Figure 7-18) are depicted in figure 7-19.

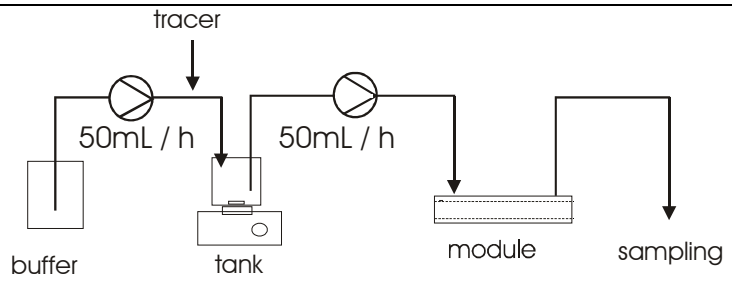


Figure 1-18: Set up for the time distribution measurement of the shell side.

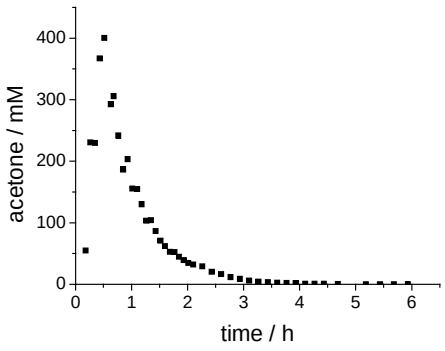


Figure 1-19: Tracer (acetone) concentration leaving the reactor as a function of time.

The oscillation observed in the initial part of the graph is a consequence of the use of a piston pump.

[143] For residence time distribution see standard books on chemical reaction engineering *e.g.* Levenspiel, 1999 and Hegen, 1992.

[144] The relationship between C_{pulse} and **E** only holds exactly for closed boundary conditions.

To find the **E** curve from the C_{pulse} curve the concentration scale is changed, such that the area under the curve is unity. The **E** curve represents the residence time distribution of the fluid. The mean residence time can be calculated from the C_{pulse} experiment according to equation 7-8.

$$\bar{t} = \frac{\int tCdt}{\int Cdt} = \frac{\sum t_i C_i \Delta t_i}{\sum C_i \Delta t_i}$$ **Equation 1-8**

An interpretation of the results using a compartment model delivers relevant diagnostic information about the flow pattern of the system. Thus we assume that the reactor is formed by volume of mixed flow region, and a plug flow region. The expected **E** curve for this arrangement is shown in figure 7-20.

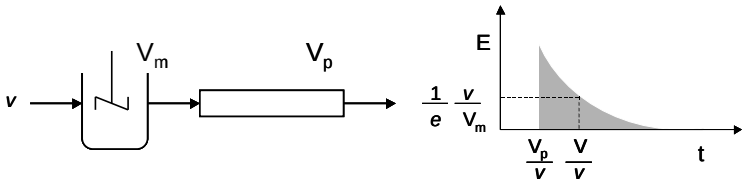


Figure 1-20: Compartment model with exponential decay tracer curve; V_m is the mixed flow volume of, V_p the plug flow volume and v the volumetric flow rate (cf. Levenspiel, 1999).

The observed residence time (0.94 h), however, is smaller than that predicted by theory (1.2 h). This indicates a dead or stagnant region within the reactor. This faulty flow pattern is ascribed to the position of module connections. Entrance and exit are the on the same side. It gives rise to a short cutting. Consequently, a stagnant region is present on the opposite side (Figure 7-21).

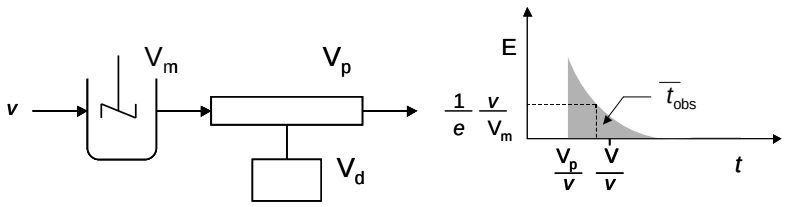


Figure 1-21: Compartment model with exponential decay tracer curve for the reactor with stagnant volume; V_m is the mixed flow volume of, V_p the plug flow volume V_d the stagnant volume and v the volumetric flow rate (cf. Levenspiel, 1999).

Such a short cutting and a stagnant region was not detected for the lumen side.

Based on these results we decided that the aqueous phase should flow on lumen side, because an acetone accumulation in the stagnant region must be avoided. Consequently the organic phase must flow on the shell side.

At this point it is possible to chose which operation modus affords better results in the reactor. One requirement of the process is the very high conversion grade of the substrate. Since a short cutting is present at the reactor, a very slow overall flow rate (high residence time) would be necessary to guarantee the desired conversion. We therefore opted for the batch modus, allowing unconverted substrate to re-enter the reactor.

7.6.3 The Obtained Results

The designed reactor was tested using a 210 mM solution of CDHE in MTBE, also containing 300 mM 2-propanol as the organic phase. The aqueous phase consisted of a 150 mM phosphate buffer at pH 7.0 containing 1 mM $MgCl_2$, 0.1 mM cofactor, 0.086 mg/mL LBADH and 300 mM 2-propanol. The reaction was conducted at 25°C.[145] The 2-propanol and MTBE elimination was compensated by the continuous dosage of a 300 mM solution of 2-propanol in MTBE. The results obtained are shown in figure 7-22.

[145] For the reaction conditions setting see chapter 8.

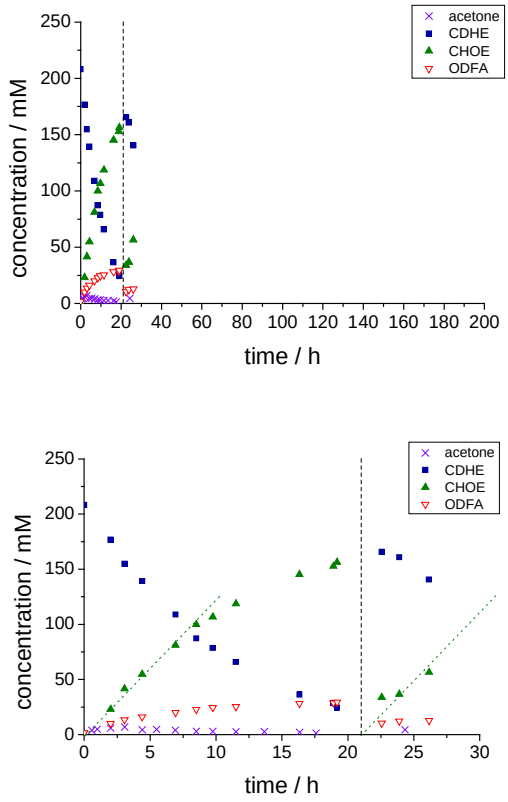


Figure 1-22: Concentration profile as a function of time obtained with the hybrid multiphase bioreactor; the acetone concentration refers to the aqueous phase, the substrate product and by-product concentration to the organic phase. The initial reaction rates are calculated based on the dotted lines. Reaction conditions: 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 210 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL.

The operation of the hybrid bioreaction shows that it is possible to decouple the concentration profile of product and co-product, thus avoiding thermodynamic limitation and kinetic disadvantages. The acetone concentration reaches a maximum of 7.0 mM at 3 h operation. At this time the product concentration is already 42 mM. With the decrease of the reaction rate, the acetone removal is capable of reducing the coproduct concentration to 1.4 mM at 7.6 h. At this point 152 mM product was present in the organic phase. After 21 h operation, 88% conversion had been reached, and the selectivity was 85%. At this stage, the organic phase was changed. This enabled a comparison of the initial reaction rates of the first and second

run in the hybrid reactor. The results satisfactorily show that the rates are equal, proving the advantage of the hybrid reactor.[146]

7.6.4 The Model Description

The description of the reaction rates for the production reaction (chapter 4), regeneration reaction (chapter 5) and side reaction (chapter 3) can be combined to a set of equations, which should describe the kinetic of the whole system. The acetone removal must also be taken into consideration (chapter 7.5). The aim of the model is to enable the computer aided simulation of the integrated system. The simulation enhances the quantitative understanding of the system, which is otherwise not possible due to the complexity of the reactor. The model is also helpful for the evaluation of new reaction conditions (*e.g.* enzyme concentration) or changes in the equipment (*e.g.* increase of acetone removal area). This also enables us to obtain first results without the need for experimental work, thus reducing development costs. It can also be used to estimate which new experiment has better perspective of leading to significant improvement.

All reaction rates refer to 25°C, when necessary new values have been calculated according to the Arrhenius equation. Assuming a very rapid mass transfer, this phenomenon is not regarded in the model. Besides, the 2-propanol concentration is controlled experimentally, and externally kept constant. Thus, its concentration is assumed to be constant at 300 mM. The set of differential equations used in the model is shown below:

$$\frac{d[NADP]}{dt} = v_{prod} - v_{revprod} - v_{reg} + v_{revreg} \qquad \textbf{Equation 1-9}$$

$$\frac{d[NADPH]}{dt} = -v_{prod} + v_{revprod} + v_{reg} - v_{revreg} \qquad \textbf{Equation 1-10}$$

$$\frac{d[CHOE]}{dt} = v_{prod} - v_{revprod} \qquad \textbf{Equation 1-11}$$

[146] CHOE produced in the first batch and dissolved in the aqueous phase is realised to the organic phase immediately after the introduction of the fresh organic phase. This fact explains the high CHOE concentration in the first measurement of the second run. Thus, the first point is not considered for the determination of the reaction rate.

$$\frac{d[CDHE]}{dt} = -v_{prod} + v_{revprod} - v_{side}$$ **Equation 1-12**

$$\frac{d[acetone]}{dt} = v_{reg} - v_{revreg} - v_{strip}$$ **Equation 1-13**

$$\frac{d[furan]}{dt} = v_{side}$$ **Equation 1-14**

The expression for v_{prod} is the one described in 4.6.1 extended with the term to consider the inhibition by acetone. The use of the experimentally obtained constants, leads to a good qualitative description of the system, but the quantitative agreement is poor (figure 7-23).

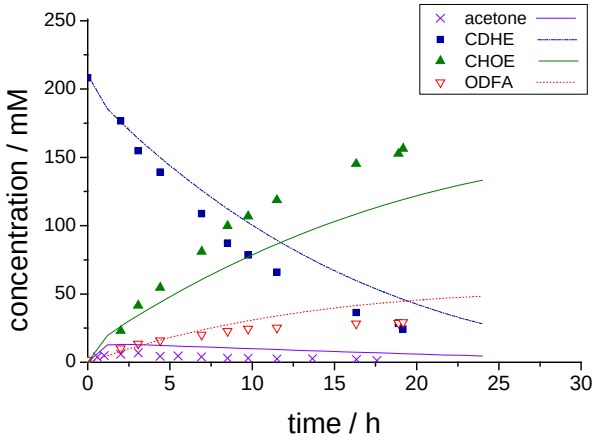


Figure 1-23: The simulation (continuous line) of the results obtained with the hybrid multiphase bioreactor. Initial reaction conditions: 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 210 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL.

Disregarding the kinetics aspect, it can be noticed that the acetone concentration in the model is considerably higher than in actual practice, reaching 13 mM. An explanation for this discrepancy is the influence of MTBE on the rate of acetone removal. The value for k_{strip} described in chapter 7.5.5 was obtained in the ternary system consisting of water, acetone, and

2-propanol. Due to the water saturation with MTBE, this volatile compound sweeps acetone through the membrane pore, thus increasing the removal rate. Increasing (doubling) the rate of acetone removal, a better fit of the acetone concentration profile is obtained. Automatically a good fit of the whole system is achieved. (figure 7-24)

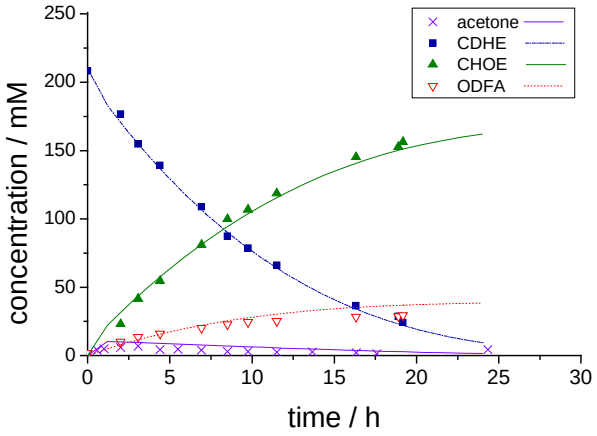


Figure 1-24: The simulation (continuous line) of the results obtained with the hybrid multiphase bioreactor with improved acetone removal rate k_{strip}.

The results strongly suggest that the limiting step of the hybrid multiphase bioreactor is the rate of acetone removal. Since the reaction rate is solely explained by the kinetic model, the transport kinetic can be regarded as unimportant for the approximate description of the reactor under these conditions..

The good correlation of model and experiment justify the assumption that the extended Michaelis-Menten model (cf. chapter 5) can be used as an approximation to describe the stationary conversion rate, mainly if one product is constantly removed.

The models also give an insight about the concentration ration between oxidised and reduced cofactor. Most cofactor (> 98%) is present in the reduced form. Thus, in order to enhance the cofactor stability the pH-working window should be kept rather alkaline.

7.7 Comparison with Other Reactors

In this subchapter the reactor designed in this process development is compared with other reactors concepts, both existing and possible ones.

7.7.1 Aqueous Fed Batch Reactor

This reactor concept, developed by Wolberg (Wolberg, 2001), offers a good possibility to carry out the production of CHOE. Due to the feed strategy, the side reaction can be suppressed. If improvements on the reaction conditions are made, the cofactor turnover can be enhanced to 5 000. (Figure 7-25).

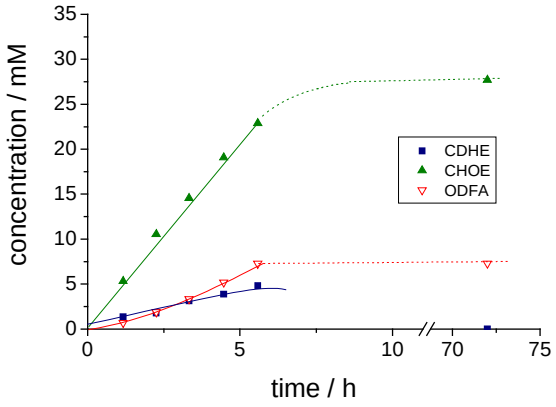


Figure 1-25: Concentration profile for a fed batch with 0.005 mM cofactor. Reaction conditions: 75 mM citrate and 150 mM phosphate buffer pH 5.5 at 25°C, E = 6.25 U_{st}/mL, 36 mM $CDHE_{total}$, 6.5 mM/h dosage rate.

Moreover, if MTBE replaces ethyl acetate in the work up, the enzyme reuse can also be enlarged. A draw back of the concept is the difficulty in recovering the diluted product from the large volume of solution. Yet, its use can be recommended, for small scale production.

7.7.2 Biphasic Emulsion Reactor

The concept presented in subchapter 6.7 is an elegant alternative to the aqueous fed batch reactor. The side reaction discrimination is inherent in the system,. Due to the high substrate concentration enabled by the organic solvent, the aqueous volume can be strongly reduced and work up expenditure is decreased. A disadvantage of this approach is the very low reaction rates at the end of conversion (low substrate concentration). It is increased by the problem caused by acetone accumulation. This situation is clearly illustrated by a comparison of the reaction carried out in an emulsion batch and under identical conditions in the hybrid reactor (Figure 7-26).

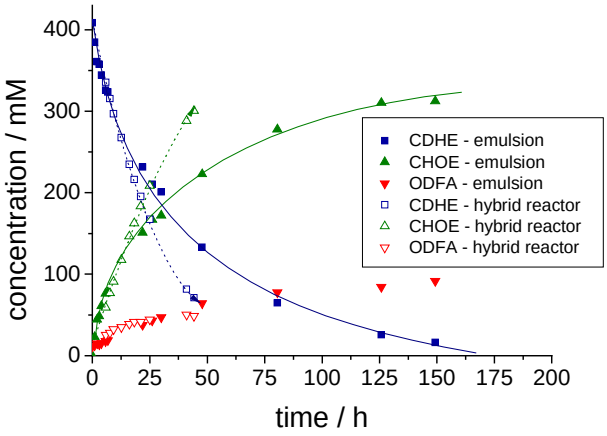


Figure 1-26: Comparison between emulsion reactor and hybrid multiphase reactor under identical reaction conditions; the difference in conversion rate is caused by the acetone accumulation.

In spite of equal initial reaction rates, the acetone removal enables the hybrid reactor to achieve the final conversion faster. For this reason, the total amount of ODFA formed is also smaller.

7.7.3 Polymeric Resin

The use of solid polymeric resin to control the concentration of CDHE in the aqueous phase is a further promising concept. It was used by Wolberg to achieve high enantioselectivity in the reduction of CDHE catalysed by whole cells of *Saccharomyces cerevisiae* (Wolberg, 2001). Advantages of the system are the facile work up and the three-dimensional contact to the aqueous phase. The problem of acetone removal can be overcome by stripping, since the organic phase is not volatile. But a disadvantage is the bi-dimensional "organic reservoir" for the substrate, causing a difficult setting of the aqueous substrate concentration.[147]

A similar approach has been tried entrapping enzymes and cofactor in a cross-linked polymer matrix (cf. Wong, 1987). However, it must be considered that when immobilised enzymes and cells are used the transport situation is more complex, since the penetration into the matrix may be rate limiting (Carrea, 1984).

[147] In the case of polymeric resin the "substrate reservoir" is limited to the resin surface.

7.7.4 Bimembrane Reactor

The reactor conceived by Kruse (Kruse *et al*., 1994; Kruse *et al*., 1996) and further developed by Liese (Liese, 1998) and Rissom (Rissom, 1999) is probably the most efficient reactor for the reduction of hydrophobic ketones by alcohol dehydrogenases. It is based on a continuous stirred tank reactor, where the substrate is continuously fed and the enzyme is retained by a membrane. The product is extracted from aqueous solution containing also the cofactor. The cofactor solution than re-enters the reaction vessel (Figure 7-27).

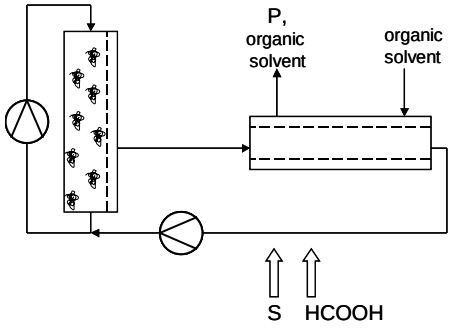


Figure 1-27: Flow scheme of the bimembrane reactor according to Kruse *et al*. (1996).

This technique enables an efficient decoupling of residence time for substrate/product and cofactor. Until now cofactor regeneration has always been performed by the enzyme coupled approach. The substrate coupled approach with the inclusion of an acetone removal device can be easily conceptualised. The substrate could be fed dissolved in 2-propanol, as performed by Wolberg (Wolberg, 2001). Nevertheless, some disadvantages of the use of this reactor still persist:

Operation Modus: This reactor is a continuous stirred tank reactor. For the desired quantitative conversion, very long residence time would be required.

Convectional Flux over Membrane: This causes some technical problems, mainly because of the integration of a phase contactor for the product extraction. The independent control of pressure is difficult leading to frequent phase instability. The hybrid multiphase bioreactor developed in this work is characterised by exclusively diffusional flux over the membrane. Hence, it works almost without pressure gradient, being easy to operate and showing high phase stability.

Unproductive Cycle: In the bimembrane reactor concept, after the production phase the cofactor is recirculated over the phase contactor until re-entering the reactor. During this phase it is unproductive, and the only reaction occurring is the cofactor degradation.

Therefore, improvement of the reactor focuses on an increase of the ratio between productive (reactor) and total aqueous volume (Kruse, 1995). Even in the optimised case the ratio is < 1. In the hybrid multiphase bioreactor developed, the cofactor is always undergoing reaction. Either it is consumed (biphasic compartment) or it is regenerated (total volume). Consequently, a better use of the cofactor is possible.

7.7.5 Two-Phase System Membrane Reactor

The reactor concept presented by Kise and Hayashida is based on a continuous strirred tank reactor with retention of the aqueous phase by a hydrophobic membrane (Kise and Hayashida, 1990). The concept is very interesting, but not suitable for our production system, due to the requirement of total conversion.

7.7.6 Emulsion Membrane Reactor

The emulsion enzyme membrane reactor presented by Liese *et al*. is not compatible with the reaction system, since the side reaction would be favoured during the contact of CDHE with the aqueous phase in the absence of enzyme (Liese *et al*., 1998).

7.7.7 Polymer Enlarged Cofactor

A comparison with the cofactor requiring amino acid production in a membrane reactor with enlarged cofactor is also interesting (cf. Wichmann *et al*., 1981). This process is the “most highly developed process for cofactor requiring enzymes” (Wong, 1987). Here, the enlarged cofactor is retained in the system together with the enzyme by membrane technology. In contrast, the hybrid multiphase bioreactor enables a comparable high cofactor retention, but no cofactor derivatisation is necessary. The multiphase approach is superior due to the fact that microbial contamination is avoided by the presence of organic solvent (cf. Kise and Hayashida, 1990).

7.7.8 Two-Chamber Reactor

A further reactor with independent production and regeneration systems can be conceived for the LBADH-catalysed reduction of CDHE. In a biphasic reaction compartment the production reaction would be carried out. In a second compartment, the regeneration would be carried out in aqueous phase. The separation of the compartments would allow the use of enzymes which are not stable in the presence of CDHE or organic solvent to be used. To avoid the contact of the regenerating enzyme with the substrate a separation of the compartments by a membrane

would be necessary (Figure 7-28). And this is the main disadvantage of this concept. The convectional flux over two membranes can lead to a series of technical difficulties.

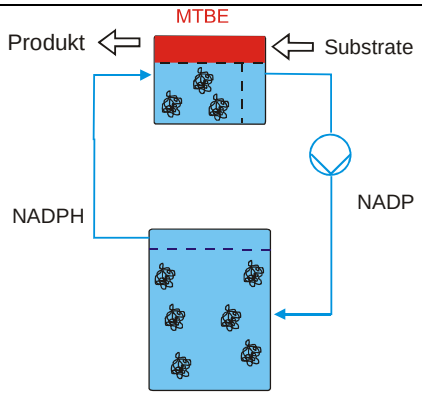


Figure 1-28: Idealised reactor with independent production and regeneration systems, separated by ultrafiltration membranes.

7.7.9 Conclusion

There are several efficient possibilities of performing cofactor requiring enzymatic reductions, also for hydrophobic substrates. We understand that for the LBADH-catalysed reduction of CDHE, biphasic systems offer the most advantages: high selectivity, high substrate concentration, consequently higher *TTN*, good cofactor retention, easy work up and enzyme reuse, no microbial contamination. Depending on the production scale and the seasonality of the demand, either the emulsion batch or the hybrid multiphase bioreactor is the reasonable choice for production. In comparison to other enzyme-membrane reactors the proposed multiphase bioreactor offer the advantages of (almost) pressure gradient free operation and flexibility regarding the operation modus (batch, semi-continuous or continuous).

7.8 Interim Summary

- For the LBADH catalysed reduction of CDHE we opted for a hybrid multiphase bioreactor.
- For the biphasic production volume a membrane phase contactor was chosen.
- Different approaches for the integrated acetone removal were tested (pervaporation, stripping, distillation). Membrane distillation was chosen to be used in the hybrid bioreactor.

- A hybrid multiphase bioreactor was assembled. Due to the flow pattern the repetitive batch operation is adopted.
- The reactor operation can be described by a mathematical model
- The model suggest that the acetone removal is the rate limiting step of the reaction operation.
- The advantages of the hybrid multiphase bioreactor are: high selectivity, high substrate concentration, consequently higher *TTN*, good cofactor retention, easy work up and enzyme reuse, no microbial contamination, (almost) pressure gradient free operation and flexibility regarding the operation modus

8 The Reactor Operation

"It's getting better all the time."

LENNON/MCCARTNEY

8.1 The Reaction Conditions

The results (selectivity, *TTN*, space-time-yield, etc.) obtainable with the hybrid bioreactor described in chapter 7 are very much dependent on the reaction conditions (pH, temperature, etc.). This subchapter discusses the selection of the reaction conditions for the first operation. The influence of the most important parameters and the setting of its values are discussed next.

8.1.1 pH

The pH influences the complete reacting system. The rate of enzymatic reactions (production and regeneration) and also of the side reactions are strongly pH dependent. Besides, the pH also influences the real concentration of substrate in the aqueous phase, owing to the keto-enol tautomerism. It also affects the cofactor stability, leading to antagonistic requirements for cofactor and substrate stability. The working pH is set at 7.0, where the enzyme stability is at its maximum. It also enables a sufficiently high cofactor stability, and not too fast substrate decay. Simultaneously, at this pH the enzymatic production is expected to be close to its maximum, and so the effects of an increase in the side reaction rate is diminished.

To keep the pH constant there are two possibilities: the use of buffer salts or an auto-titrator. Though the use of an auto-titrator shall be preferred in production scale, it causes a change of volume in the aqueous phase. For this reason buffering with phosphate salts is used in the bench operation

8.1.2 Temperature

The temperature also affects the system in antagonistic directions. While the enzymatic reaction rate, and also the ODFA formation, increase with increasing temperature, the enzyme and cofactor stability decrease. Normally spontaneous reaction show higher activation energy than enzymatic reactions. Consequently, at high temperatures the side reaction should be favoured. For the initial experiments, the working temperature is set at 25°C.

8.1.3 Enzyme Concentration

The enzyme concentration influences the production rate and consequently space-time-yield and selectivity. But it is also a cost factor. Its concentration is set arbitrarily at 0.086 mg/mL.[148] By keeping other parameters constant, it is possible to calculate the minimum enzyme concentration necessary for a given selectivity.

8.1.4 Cofactor Concentration

This parameter has been discussed in chapter 5 and 6. It is essential to keep the concentration low in order to achieve a high turnover, but the concentration must be high enough to enable the production rate to be sufficiently high. We decided therefore to work at 0.1 mM cofactor concentration. Since the cofactor is reduced in the system, it is wise to use the less expensive oxidised form (NADP), as the starting reagent, and obtain the reduced form in the reactor.

8.1.5 Substrate Concentration

At low concentrations the production rate is proportional to the substrate concentration. Thus, it is advantageous to increase this concentration while this proportionality is maintained (see chapter 4 and 6.7). Beside the kinetic limitation, two other aspects must be considered: the viscosity of the organic phase and the stability of the module with respect to the substrate. In the experiment described in chapter 7.6, 210 mM substrate in MTBE was employed. In order to study the effect of a concentration enhancement, this concentration was doubled.

8.1.6 Circulation Rate

The circulation rate is important since it determines the degree of conversion of a cycle. High conversion requires high regeneration, leading to high acetone production. Since the presence of acetone is problematic to the process, the circulation of the aqueous phase is set at the highest value possible with the equipment. Thus, only differential conversion is achieved in a cycle and only small amounts are produced before the aqueous phase enters the membrane distillation device, lowering acetone accumulation. Besides, high circulation rate improves the transport in the aqueous phase inside the module.

8.1.7 Results

The results obtained for the reactor under these conditions are shown in figure 8-1.

[148] See chapter 9 for further details.

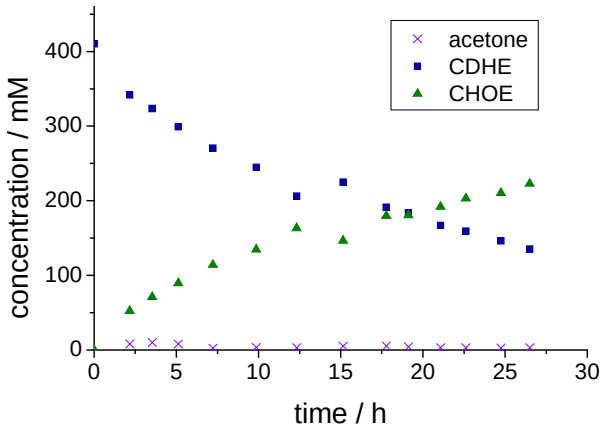


Figure 8-1: Concentration profile during the course of reaction: the acetone concentration refers to the aqueous phase while substrate and product concentration refers to the organic phase. Reaction conditions: 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 420 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL.

The HPLC analysis of the cofactor concentration just before entering and after leaving the biphasic reaction module reveals only a small decrease in the concentration of the reduced cofactor. Based on the experiment, an exact statement about the amount of differential conversion is not possible. The concentration of the oxidised species could not be quantified as it was too small. This results are in very good agreement with the prediction of the kinetic model and is not only caused by the differential conversion in the biphasic module. It relies on the kinetically determined concentration of each cofactor species during the reaction under acetone removal.

After 45.17 h 80% conversion with 77% process selectivity[149] has been achieved. This corresponds to a space-time-yield of 32.6 g/(L · d). The acetone concentration reaches a maximum of 10 mM at 3.5 h. Its concentration is high (> 7 mM) until 5.1 h, thus preventing a higher space-time yield. The low selectivity, a consequence of the acetone accumulation, is below that aimed for the process and has to be improved.

8.2 The Empirical Optimisation

In order to achieve a high selectivity at high conversion rate, we decided to change the reaction conditions, keeping the substrate concentration constant. The model presented in

[149] CHOE concentration = 259 mM in the organic solvent.

chapter 7 can predict changes in reaction rates and selectivity for concentration changes of enzyme and substrate, for instance. However, the model does not allow prediction if pH and temperature are changed. The elaboration of a model regarding these effects would exponentially increase the number of required measurements, being expensive and time consuming. For these reasons an empirical optimisation is more effective. It enables the optimisation of quantities without knowledge of the mathematical dependence on the reaction parameters.

There are sophisticated tools among the several algorithms for empirical optimisation. The genetic algorithm, for instance, allows the simultaneous optimisation of different parameters. Thus it is possible to obtain higher selectivity and cofactor turnover concomitantly. This algorithm requires a high number of experiments, and is not compatible with the use of a production reactor, even on bench scale. For this reason we decided to use an algorithm requiring less experiments and initially focus only on the process selectivity. The algorithm chosen is simplex.

The use of this algorithim for the optimisation of established process was presented in 1962 (Spendley *et al*., 1962). There are a number of incidental advantages of the procedure, which are by no means negligible:

- The arithmetic involved is relatively trivial, since at no stage is a direction of steepest ascent calculated.
- The direction of advance is dependent solely on the ranking of the responses, and not on their values on any absolute scale.
- As distinct from procedures requiring increasing extrapolation, there is little dependence on any assumption of planarity of the response surface, except in the immediate region of the current simplex.

The basic design of the technique is a simplex in *n* dimensions, *n* being the number of parameters under study. The simplex S_0 has vertices V_1, V_2,...V_{n+1} and centre C_0. The response at the vertices of S_0 are determined experimentally (y_1, y_2, ...y_{n+1}). The lowest response is identified as y_p. A new simplex is completed by excluding the point V_p corresponding to y_p and replacing it by V^*_p. The new simplex is obtained by reflecting S_0 about the face opposite V_p.

$$V_p^* = \frac{2}{n}\left(\sum_{i=1}^{n+1} V_i\right) - \left(1+\frac{2}{n}\right)\cdot V_p \qquad \textbf{Equation 8-1}$$

The figure 8-2 illustrates the algorithm.

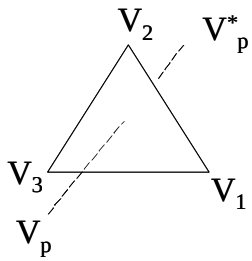


Figure 8-2: Reflection of V_p for completing the new simplex.

As variable parameters the enzyme concentration, the pH, and the temperature were chosen. The aim of the optimisation is to achieve 90% process selectivity. The values for the starting experiments are shown in table 7-1. To decrease the number of required experiments we used the experiment described in the previous section (number 1). The substrate concentration remains constant. Based on the empirical understanding of the system, the staring simplex could be set near the optimum. Consequently, the results achieved in the starting points are already good enough to satisfy the optimisation aim. Experiment number ***2*** achieved 90% process selectivity at 50% conversion.[150] Nevertheless, the first optimisation cycle is completed. The results are also shown in the table.

Table 8-1: Empirical optimisation of the selectivity using the simplex algorithm.

Experiment number	enzyme concentration [$mg_{protein}$/mL]	pH	temperature [°C]	selectivity [%][151]
1	0.086	7.0	25	73
2	0.048	6.3	20	83
3	0.134	6.7	15	82
4	0.036	6.7	18	73
X	0.059	6.2	10.4	84

Experiment ***1*** and ***4*** show the same selectivity at 20% conversion, but at higher conversion the selectivity under the condition set ***4*** was clearly superior. Therefore ***1*** was chosen as the point

[150] Due to the presence of small amount of ODFA in the substrate the apparent selectivity increases in course of conversion.

[151] The selectivity at 20% conversion is compared, since some reactor runs presented leakage before full conversion was achieved.

to be reflected V_p. The new point proposed by the algorithm is ***X***. The results obtained at this point are superior to those of the starting simplex. Hence the utility of the optimisation could be verified.

8.3 Repetitive Operation

After showing the possibilities of improving the reaction conditions it was necessary to demonstrate the possibility of operating the process for a long period. Therefore the reaction conditions set number ***4*** were selected. The reasons therefore are:

- The low temperature and comparatively high pH provide a higher cofactor stability.
- The low enzyme concentration enables a high sensitivity in the obtained results.
- The not very high, yet satisfactory, selectivity permits a better interpretation of the results.

The bioreactor was operated under these conditions on bench scale for 168 h. The conversion and selectivity obtained are shown in figure 8-3.

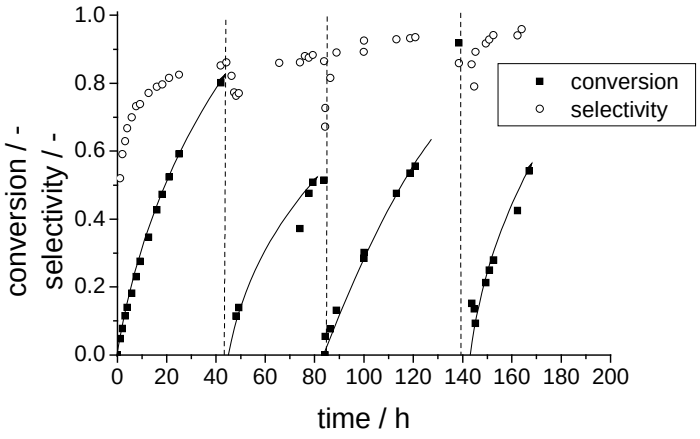


Figure 8-3: Long run operation of the bench scale bioreactor.

The increase in selectivity in the course of a batch is caused by the presence of small amounts of ODFA in the substrate solution. The increased initial selectivity observed after the first run can be ascribed to the extraction of residual product concentration from the previous run, which remained in the aqueous phase. The extraction of this small product amount leads to the apparent higher process selectivity. More significant are the drop in process selectivity after 84 h operation and the constant increase in process selectivity from batch to batch.

The drop in selectivity was explained by the cofactor decay. After 84 h the cofactor concentration had reached such low levels that the production reaction rate was affected. The addition of 6.4 mg cofactor (corresponding to 0.11 mM) resulted in a return of the selectivity to the usual values, thus confirming the explanation.

The increase of process selectivity from batch to batch (86% in the first run, 88% in the second before the selectivity drop, 93% in the third and 96% in the last) is caused by a pH shift due to the side reaction. Thus the final pH was 6.1. This result is very important, because it affects also the cofactor stability. For long run operation, the use of the autotitrator becomes necessary. [152]

The addition of 5.6 mg (increase the concentration by 0.09 mg/mL) LBADH before the 4th run does not significantly improve the reaction rate, if compared to the first run. This is in accordance with the fact that the reactor is limited by the acetone removal rate.

The accumulation of 2-propanol caused by the concomitant MTBE dosage is overcome in this experiment during the phase change. The fresh organic phase was added without 2-propanol, thus reducing the concentration of the alcohol present in the whole system.

After replaced, the organic phase containing CHOE and small amounts of CDHE was stirred over a buffer solution (150 mM phosphate, pH 8.5) in order to destroy the remaining substrate. Subsequently the organic phase was dried over Mg_2SO_4 and the solvent evaporated. Each run of the reactor afforded approximately 5 g crude product (Figure 8-4).[153]

Figure 8-4: Product obtained in the bioreactor during a batch run.

[152] Shimizu *et al*. recommend the use of sodium carbonate fot the pH control (Shimizu *et al*., 1990).

[153] For the third run the following process characteristics: 74 g/L product concentration, 40 g/(L·d) space-time yield and 4200 cofactor *TTN*, 93% process selectivity and 86% selectivity after complete degradation of the residual substrate (cf. section 9.2.3).

8.4 Conclusion

The results presented in this chapter confirm the utility of the developed process. It is possible to improve the reactor performance by optimising reaction conditions and, the reactor can be used for prolonged duration of time.

An increase in space-time-yield could, however, not be achieved by enhancing the substrate concentration. The main reason is the limitation of the process by the acetone removal.

The present highest space-time yield of approximately 47 g/(L·d) (at 90% conversion) was achieved at a substrate concentration of 210 mM and an enzyme concentration of 0.086 mg/mL. In order to improve this value, the acetone removal must be intensified. Since the module is working at its capacity limit, a higher membrane area is required.[154] This causes additional equipment costs. Probably it is worth to improve the process by altering the reaction conditions and also the operation modus.

The most significant improvement can be achieved by avoiding the slow conversion rate at low substrate concentration at the end of a batch. Therefore, a substrate feed to the organic solution can be conceived. Another possibility is the partial conversion followed by concentration and further conversion.

Cost reduction is possible by decreasing the enzyme concentration. This is possible without loss of efficiency, as suggested by the results of the repetitive operation. The model presented in chapter 7 can be used as a tool therefore.[155] The determination of the lowest recirculation rate is also important, because it also influences the rate of MTBE and 2-propanol removal.

8.5 Interim Summary

- The influence of the reaction parameters on the bioreactor performance were discussed. The reaction conditions for the initial bioreactor operation were set.
- In order to improve the process selectivity an empirical parameter optimisation using simplex algorithm was conducted. Process selectivity >90% was achieved.
- The bioreactor was operated for 168 h in repetitive batch modus. Approximately 5 g crude product with selectivity up to 96 % was obtained per batch.

[154] See chapter 9 for further discussion.

[155] See chapter 9 for further discussion.

9 Economic Aspects

"Time is money"

POPULAR SAYING

A process development should also include some economic aspects. At this early stage of the process implementation the available information is still rather distorted, mainly regarding equipment costs. However, an evaluation is helpful. It allows, at least, an estimation of which parameters are more cost intensive and which part of the process deserves more attention for further development.

9.1 Market Estimation

As a basis for the market estimation we consider the consumption of Lipobay® (also Baycol®). Six million people consumed the medicament based on cerivastatin.[156] For a conservative market estimation we assume that every patient consumed daily the lowest dosage, 0.1 mg, of this highly potent drug (Hanefeld, 1999). This means a consumption of 600 g cerivastatin daily. Considering the molar mass of cerivastatin to be 481 g/mol, it means a consumption of 1.25 mol daily or 455 mol/year. It corresponds to approximately 108 kg/year of the CHOE, assuming 100% yield in every further synthetic step. This is also the production range of (*S*)-4-chloro-3-hydroxybutanoate at Bristol-Myers-Squibb. (Liese *et al.*, 2000 p 119)

9.2 Cost Estimation

For cost estimation we consider high consumption of each substance, which is possible if the production is integrated in a complex, but overestimated if CHOE is the only manufactured substance. Further we estimate the substrate cost at 50.00 €/kg.[157]

---

[156] Frankfurter Allgemeine Zeitung, 9th August, 2001.

[157] Müller, M., personal communication.

9.2.1 Fed Batch

9.2.1.1 Basic Process

Firstly, the cost analysis of the fed batch as described by Wolberg should be carried out (Wolberg, 2001). We focus exclusively on the direct material acquisition costs which are provided in table 9-1.

Table 9-1: Material acquisition costs for the fed batch production of CHOE as described by Wolberg. The prices for the substrate and water are estimates; for the cofactor and the enzyme, quotation by Jülich Fine Chemical, minimal order 10 kg and 100 MU, respectively; for the organic solvents based on www.chemicalmarketreporter.com, technical grade, minimal order 10 t, June 2003; for buffer substances based on Fluka catalogue.

substance	price	consumption	cost
CDHE	50 €/kg	0.009 kg/L	0.45 €/L
cofactor (NADP)	5866 €/mol	0.0003 mol/L	1.76 €/L
2-propanol	0.054 €/mol	0.5 mol/L	0.027 €/L
water	0.001 €/L	1 L/L	0.001 €/L
ethyl acetate	0.63 €/L	0.5 L/L	0.32 €/L
LBADH	0.1 €/kU	3 kU/L [158]	0.5 €/L
phosphate buffer	0.80 €/mol	0.15 €/L	0.12 €/L
citrate buffer	0.71 €/mol	0.075 €/L	0.053 €/L

The material cost per litre is estimated at 3.23 €. The high buffer costs are a result of obtained the quotation. More suitable conditions can surely reduce these costs. On the other hand, the costs for ethyl acetate are critical. If higher purity level or smaller amounts are purchased, the solvent will be one of the most important cost factors. This fact highlights the problems caused by recovery of diluted product. Considering 84% yield (90% purity) the productivity is 6.1 g/L, corresponding to a material cost of 0.53 €/g.

The equipment to carry out the production is rather inexpensive. The main investments are caused by two peristaltic pumps and one agitator. Consequently, the depreciation costs are inexpressive.

[158] Considering 40% reuse.

The production can be conducted by one operator working 8 h/d, since it is possible to stop the process after the complete substrate dosage, performing the work up on the second day. Under these conditions Wolberg achieved a space-time yield of approximately 3 g/(L·d).

9.2.1.2 Improved Process

The process developed by Wolberg was miniaturised and the reaction conditions were improved. Two major points should be considered: the product extraction with MTBE enable the almost complete reuse of the enzyme, being only lost due to thermal deactivation (here considered as 5%), and the cofactor concentration was reduced to 0.005 mM so that a higher turnover was achieved (*TTN* = 4900). The selectivity was slightly reduced so that the productivity is 5.8 g/L. By this means the total material cost per litre is estimated at 1.08 €. Thus a cost reduction of approximately 67% to 0.18 €/g was possible. Under these conditions, the substrate (41%) and the solvent (34%) costs are dominating. The cofactor represents only approximately 3% of the material cost (cf. Figure 9-1).

9.2.2 Biphasic Batch

An optimisation of the reaction conditions for this production method has not been performed. Nevertheless, it is possible to calculate the costs for the production under non-ideal conditions. The experiment reported in chapter 7 is used as the basis for the calculations.

Table 9-2: Material acquisition costs for the biphasic batch production of CHOE. The prices obtained from the same sources as in table 9-1.

substance	price	consumption	cost
CDHE	50 €/kg	0.0994 kg/L	4.97 €/L
cofactor (NADP)	5866 €/mol	0.0001 mol/L	0.59 €/L
2-propanol	0.054 €/mol	0.3 mol/L	0.016 €/L
water	0.001 €/L	1 L/L	0.001 €/L
MTBE	0.74 €/L	1 L/L	0.74 €/L
LBADH	0.1 €/kU	3 kU/L [159]	0.3 €/L
phosphate buffer	0.80 €/mol	0.15 €/L	0.12 €/L

For the calculation we consider 94 % conversion achieved in 126 h.[160] The selectivity is 80%, so the space-time-yield corresponds to 12.7 g/(L·d) until 94% conversion, for the production

[159] Considering 12.5 kU/L and 5% deactivation per day.

step exclusively. The calculated cofactor *TTN* is 2800. The total material cost is estimated a 0.10 €/g. The main costs are substrate (74%) followed by solvent and cofactor (cf. Figure 9-1). As discussed in subchapter 6.7 the cofactor concentration can be decreased 10-fold. The necessary equipment is reduced to a stirrer and a vessel. A disadvantage of the process is the necessity of degrading the residual substrate, causing a decrease in product purity.

9.2.3 Hybrid Bioreactor

As a representative for the hybrid process we use the third run of the repetitive operation, because it was carried out until 92% conversion (50 h). Further we assume a reduction in the cofactor consumed by 25% due to reuse during the first stage of the following run. This leads to a cofactor turnover of 4200.

While determining the material costs it is important to consider the operation cost, based on operation time. They are caused by nitrogen consumption and MTBE and 2-propanol dosage. These costs compensate the savings by less enzyme deactivation and fast cofactor reuse. Nevertheless, this process allows higher conversion, thus practically avoiding a further step of work up and yielding a better product quality. The costs for the work up are mainly due to buffer and correspond to approximately 2% of the material cost (cf.- Figure 9-1).

The continuous operation requires uninterrupted monitoring, meaning that working in shifts is necessary.

The equipment investment for the process is higher. For the bioreactor on bench scale the investment costs are estimated at € 15 000.[161] This high investment sum, though overestimated for the small scale process, give rise to high fixed costs, mainly depreciation. If calculated for the bench scale bioreactor, the depreciation costs would be 8 €/d.[162] The mass specific depreciation cost is dependent on the space-time yield. For the mentioned operation conditions 40 g/(L·d) were obtained, meaning 0.3 €/g cost.

[160] Further conversion is very slow due to the equimolar accumulation of acetone.

[161] The capacity of the reactor is approximately 14 kg/a. For the production reactor the up scaling is necessary, since only bigger hollow fibre modules are stable under reaction condition.

[162] Considering a depreciation rate of 20% p.a.

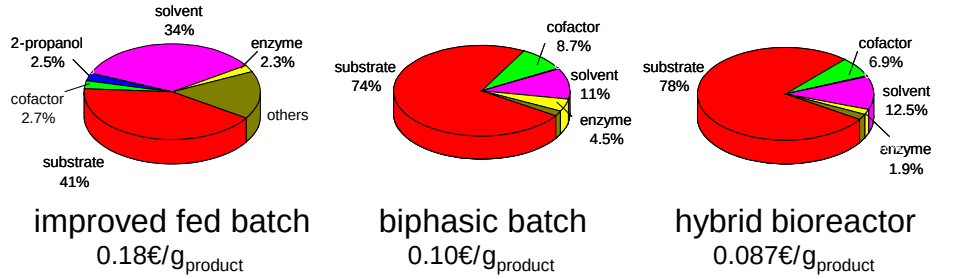


Figure 9-1: Material cost percentage for the improved fed batch, the biphasic batch and the hybrid bioreactor.

9.2.4 Reactor Choice

The lowest material cost per gram product is achieved in the emulsion reactor and in the hybrid process. Differences arise due to purity of the product.[163] The higher purity of the product obtained in the hybrid bioreactor enables it to be sold at a better price.

The difference in costs are caused mainly by the depreciation. The depreciation costs are fixed costs, *i.e.* they are independent of the production quantity. Thus, there will be a production scale at which the higher price achieved will overcompensate the depreciation cost. The production at higher scale justifies the use of the hybrid bioreactor.

Thus, the reactor choice presents a classical situation. For small scale operations, the emulsion batch reactor is the more suitable. For high scale production, the hybrid bioreactor is advantageous. There are other reasons, such as flexibility, stock costs, etc. increasing this trend. The estimation of the limit scale depends on a more accurate cost estimation, possible only in later development stages.

9.3 Aspects about the Side Reaction Discrimination

Some interesting guidelines for the reaction operation conditions are obtained from economical considerations about the side reaction suppression at low substrate concentration. From the chemical point of view, the lower the concentration, the better. From the economic point of view the situation changes. The profit as a function of the substrate concentration

[163] The product obtained from the process requires only substrate removal (degradation) and evaporation of the solvent, since after the sequential synthetic step a crystallisation is performed to increase *de*.

shows a maximum (Figure 9-2). At low concentration the profit is limited by the low space-time-yield. At high substrate concentration, the low process selectivity will be the limit.

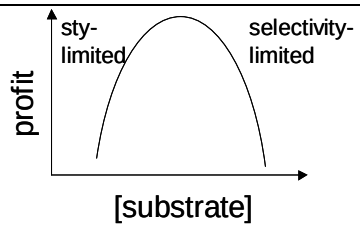


Figure 9-2: The profit as a function of the substrate concentration.

With the substrate concentration set and high selectivity achieved, it is necessary to optimise the enzyme concentration, in order to reduce costs. Therefore, following equations can be considered:

$v_{side} = k_{side} \cdot [S]$ **Equation 9-1**

$$v_{enz} = V_{max} \cdot [E] \cdot \frac{[S]}{K_M + [S]}$$ **Equation 9-2**

with:

[S] = substrate concentration

k_{side} = rate constant of the side reaction

[E] = enzyme concentration

V_{max} = maximum velocity

K_M = Michaelis constant

where v_{side} is the rate of the side reaction and v_{enz} the rate of the enzymatic reaction. Since the substrate concentration is low, equation 9-2 can be written as:

$v_{enz} = k_E \cdot [E] \cdot [A]$ **Equation 9-3**

with [A]<<KM and $V_{max}/K_M = k_E$

For the process the differential selectivity is

$$\sigma = \frac{v_{enz}}{v_{enz} + v_{side}}$$ **Equation 9-4**

Using equations 9-1 and 9-3:

$$\sigma = \frac{k_E \cdot [E] \cdot [A]}{k_E \cdot [E] \cdot [A] + k_{side} \cdot [A]}$$ **Equation 9-5**

which is simplified to

$$\sigma = \frac{k_E \cdot [E]}{k_E \cdot [E] + k_{side}}$$ **Equation 9-6**

According to equation 9-6, the selectivity shows a saturation with increasing enzyme concentration. It is important to find the critical enzyme concentration above which the additional cost for the catalyst does not correspond to additional profit from the selectivity (Figure 9-3).

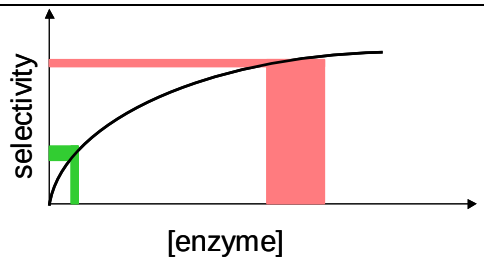


Figure 9-3: The selectivity as a function of the enzyme concentration; at high enzyme concentration the increase in selectivity does not correspond to the additional catalyst cost.

These calculations are only possible with more accurate cost and price estimation.

9.4 The Rate Limiting Step

For the developed bioreactor four parameters can be limiting for the process:

1. CDHE concentration in the organic phase,
2. phase contact area between organic and aqueous phase,
3. enzyme concentration,
4. acetone removal area.

Parameters 1 and 2 control the aqueous concentration of CDHE. While the phase contact is sufficient for the assembled reactor, the CDHE concentration is the limiting step during the final stage of the reaction (Figure 9-4).

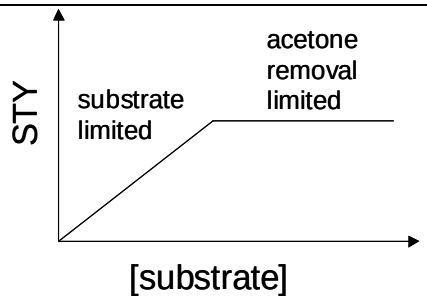


Figure 9-4: Limitation of the space-time-yield; during the initial conversion, the reactor is limited by acetone removal, in final stages by the small residual substrate concentration.

This problem was addressed in chapter 8 and can be minimised by the operation modus, but not completely avoided, since high conversion is a requirement for the process. The enzyme concentration has been discussed earlier in this chapter and the acetone removal area deserves some attention here.

At present, the selectivity and the space-time-yield are limited during the most relevant part of conversion by the acetone removal rate. This rate can only be enhanced by increasing the acetone removal area, *i.e*. adding additional acetone removal devices to the bioreactor. These devices can be used during the critical period where the acetone removal rate is insufficient, and the gas stream can be turned off during the final conversion stage. Considering the volume of a module to be 10 mL, and the total aqueous volume to be 60 mL solution, the addition of two further modules is possible.[164] Figure 9-5 compares the results from a simulation of a reactor with three times acetone removal area with the results obtained in the real reactor presented in chapter 7.

[164] Volume requirement of reactor units: 10 mL piston pump, 10 mL production module, 10 mL for tubing and control vessel.

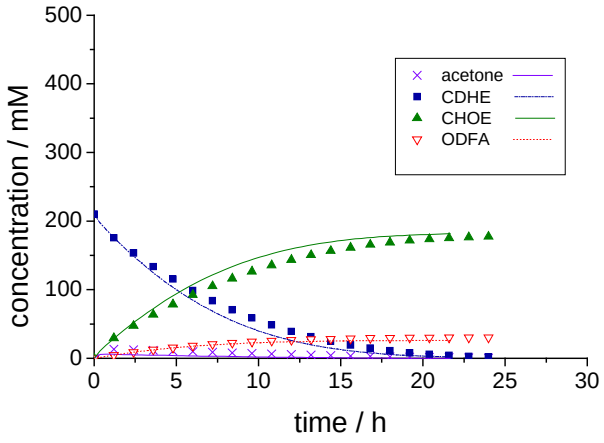


Figure 9-5: Comparison of the results obtained in the reaction operation (chapter 7) and a simulated reactor with three fold increased acetone removal area. 150 mM phosphate buffer pH 7.0 at 25°C, E = 0.086 mg/mL (30 U_{st}/mL), 210 mM CDHE, 300 mM 2-propanol, 0.1 mM cofactor; reaction volume = 60 mL; organic volume = 60 mL.

The simulation estimates the maximal acetone concentration under 6 mM. The reduction of acetone concentration, however, is not sufficient to avoid the limitation of the system. This means that the **bioreactor developed is close to the technical boundary**. Further increase of the acetone removal area requires an increase in the aqueous volume. Possibly, the best possibility to improve the space-time yield, is to maintain the acetone removal area and reduce the aqueous volume to 40 mL.

To decide about the intensification of acetone removal, the analysis of cost/benefit must consider the additional investment necessary for membrane area and the impact in the space-time yield caused a decrease in mass specific depreciation cost. For the scale up it is interesting to note that the required area for the production module is much smaller than the area for the acetone removal (Figure 9-6).

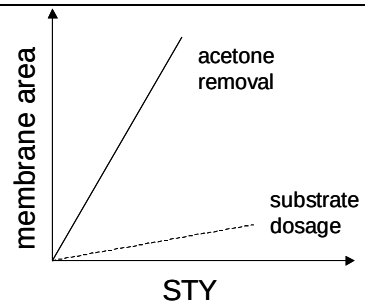


Figure 9-6: The substrate dosage and acetone removal membrane area required for achievement of space-time yield.

9.5 Interim Summary

- The demand for CDHE is estimated to be > 100 kg/a.
- The material cost for CDHE production can be decreased by over 2/3 due to the present process development.
- The developed bioreactor is close to the technical boundary.

10 Discussion and Outlook

"Since no man of aught he leaves knows,
What is't to leave betimes?"
HAMLET

The discussion of the obtained results is integrated into the respective chapter. This chapter contains a discussion transcending the scope of each chapter and shows the perspectives of the work.

The presented concept for the selection of organic solvents for biphasic enzymatic transformation (chapter 6) improves the use of enzymes which until recently could only be used in the aqueous phase. This breakthrough achieved by a paradigm change opens a new field of enzyme application. We exploited the opportunity in Part III of the work. Since the biphasic enzymatic transformation enables high substrate concentration and high retention of enzyme and cofactor the turnover of the enzyme and cofactor were increased. Further increase is possible through the easy decoupling of residence times proportioned by the biphasic system.

The decoupling of residence time is also used to perform an efficient regeneration of NADPH, a problem until recently. By means of reaction engineering it is possible to equalize the substrate coupled cofactor regeneration to the superior enzyme coupled regeneration (compare chapters 5 and 7). Thus, it is possible to regenerate the cofactor without thermodynamic limitation and to choose the regeneration activity fraction in the substrate coupled approach by removing the co-product and varying the ratio of production and regeneration time. The kinetic problems caused by acetone accumulation can only be solved if very high acetone removal area is used. Concerning the kinetic aspects, the developed process is closed to the boundary of the technical possibilities.

The use of biphasic enzymatic transformation also offers the possibility of combining different enzyme catalysed reactions, each requiring its own conditions (pH, temperature, buffer, etc.) without having to perform tedious and expensive isolation steps. The potential of this approach is illustrated by the case where the one-pot synthesis would lead to cross reactions. The reactions can be linked by the organic solvent. In the ideal case the systems can be coupled to a single continuous process.

As a concrete example, the C-C bond formation step catalysed by benzaldehyde lyase (BAL) can be followed by an LBADH catalysed reduction (Kihumbu *et al.*, 2002). Since the substrates of the first reaction are reduced by the alcohol dehydrogenase, the one pot synthesis is not feasible. The integration of both reactions could lead to an increase in space-time-yield and enzyme productivity. It would also avoid an intermediary work up (Figure 10-1).

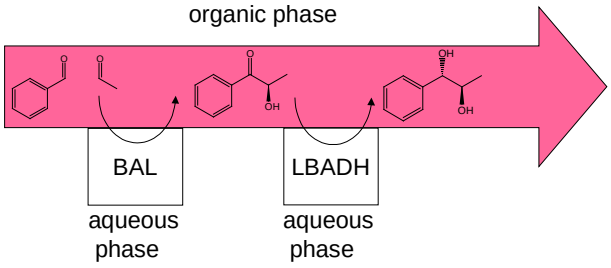


Figure 10-1: The consecutive C-C formation and carbonyl reduction catalysed by BAL and LBADH can be performed in aqueous/MTBE biphasic systems.

Stillger has shown that benzaldehyde lyase catalyses the first reaction in aqueous/MTBE biphasic systems. The junction of the system seems therefore to be a feasible and highly promising task.

Moreover the combination with lipase catalysed reactions in organic phase, *e.g.* esterification, is expected. For example, Reetz has reported the use of lipases in MTBE (Reetz, 2002).

Furthermore, the incorporation of enzymatic steps to whole chemical synthetic routes becomes smoother with biphasic biotransformations, since the solvent switch from organic to aqueous and *vice-versa* is avoided. As a concrete example, the use of CHOE in the chemical reduction requires a water free environment. The product obtained from the process requires only evaporation of the solvent, since, after the sequential synthetic step a crystallisation is performed to increase *de*.

11 Summary

"Two roads diverged in a wood, and I-
I took the one less travelled by
And that has made all the difference."
ROBERT FROST

In the present work a bioreactor development for the regio- and enantioselective reduction of *tert*-butyl 6-chloro-3,5-dioxohexanoate (CDHE) to *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate catalysed by the alcohol dehydrogenase from *Lactobacillus brevis* (LBADH) was carried out. The following results were obtained:

- Factors influencing the irreversible substrate degradation were elucidate. The reaction kinetic was described (Chapter 3).
- The enzymatic reduction of CDHE was characterised. The influence of reaction condition on the enzyme stability was studied. The thermodynamics and kinetics of the biotransformation were described (chapter 4).
- Possibilities for the cofactor (NADPH) regeneration were studied, and the substrate coupled cofactor regeneration selected. The involved oxidation of 2-propanol was thermodynamically and kinetically described; problems caused by the arising aceton were discussed (Chapter 5).
- In order to conciliate the antagonistic requirements of the involved reaction, the biphasic (aqueous/organic) reduction was conceived. To realize this concept a new approach for the organic solvent selection was developed. The approach's generality was verified. Consequences (*e.g.* thermodynamic shift) and chances (*e.g.* enhancement of process selectivity) of enzymatic biphasic reductions were studied (chapter 6).
- Using the biphasic reduction a hybrid bioreactor was designed and constructed (chapter 7) enabling:
    - the suppression of the substrate decay (side reaction),
    - the efficient reuse of the cofactor (*TTN* = 4200),
    - the avoidance of thermodynamic limitation (via acetone removal),
    - the decrease in enzyme consume.
- The process selectivity was optimised (> 90%) using the simplex algorithm (chapter 8).

- The reactor was operated for 168 h (chapter 8).
- The developed process was evaluated under the economic point of view. The material cost could be reduced by over 2/3 due to the improvements proposed.

12 Experimental Procedures

"I'm a great believer in luck,
and I find that the harder I work
the more I have of it."
THOMAS JEFFERSON

12.1 Chemicals

All chemicals were at least analytical grade. Unless otherwise stated they were purchased from Fluka, Buchs (Switzerland).

acetone	Merck, Darmstadt
n-butanol	Merck, Darmstadt
chloroform, deuterated	Aldrich, Steinheim
dichlormethane	Merck, Darmstadt
diethyl ether	Riedel de Haën, Seelze
ethanol	Merck, Darmstadt
NAD^+	Jülich Fine Chemicals, Jülich
NADH	Jülich Fine Chemicals, Jülich
$NADP^+$	Jülich Fine Chemicals, Jülich
NADPH	Jülich Fine Chemicals, Jülich
2-propanol	Merck, Darmstadt

12.2 Enzymes

The Alcohol dehydrogenase from *Lactobacillus brevis* (crude extract from recombinant *E. coli* cells) was friendly supplied by Institut für Enzymtechnologie, Heinrich-Heine-Universität, Jülich. The alcohol dehydrogenase from horse liver (lyophilisate) and the alcohol dehydrogenase from *Thermoanaerobium brockii* (powder) were purchased from Fluka, Buchs (Switzerland), and the formate dehydrogenase ($NADP^+$-dependent) from *Pseudomonas* sp. (cell free solution), from Jülich Fine Chemicals, Jülich.

12.3 Equipment

Bruker Analytische Meßtechnik,	NMR spectrometer AMX-300

Rheinstetten	
Celgard, Nordestedt	Liquicel Minimodule G477; Membrane Celgard 2500
CS-Chromatographie-Service, Langerwehe	FS-Cyclodextrin, β 1/P column, 50 m ; Multospher 100 RP-18 column, 250 x 4 mm
Eppendorf-Netheler-Hinz, Hamburg	Centrifuge 5415 D
Labsystems Frakfurt	Finnpipette Colour
Glasbläserei, Forschungszentrum Jülich	Diverse glassware
Hewlett-Packard	Gas chromatograph HP 6890; LC Series 1100
IKA Labortechnik, Staufen	Magnetic stirrer Ikamag RET-G
Macherey & Nagel, Düren	Permabond CW 20-05, 50 m column
Membrapure, Bodenheim	Hollow fibber modules
Metrohm, Herisau (Switzerland)	pH-meter 691; Impulsomat 614; Dosimat 665, solvotrode
Millipore, Eschborn	Micropure filter
Pharmacia LKB, Freiburg	Piston pump P-500
Sartorius, Göttingen	Analytical balance BP 211-D
Schott Glas, Mainz	Diverse glassware
Shimadzu, Duiburg	Spectrophotometer UV 160

12.4 Analytics

12.4.1 Gas Chromatography (GC)

Gas chromatography was performed on Hewlett-Packard 6890 gas chromatograph equipped with FID detector and using H_2 as carrier gas.

12.4.1.1 Determination of Acetone and 2-propanol Concentrations

Acetone and 2-propanol concentrations were determined using a Permabond CW 20-05, 50 m column (Macherey & Nagel) with *tert*-butanol as intern standard. Conditions: injector 250°C; oven 70°C; detector (250°C); typical retention time: acetone 3.4 min; *tert*-butanol 4.1 min; 2-propanol 4.6 min.

*12.4.1.2 Determination of Acetophenone, (***R***)- and (***S***)-Phenylethanol Concentrations*

Acetophenone, (*R*)- and (*S*)-phenylethanol concentrations were determined using a FS-Cyclodextrin, β 1/P 50 m – 0.32 mm ID column (Chromatographie-Service) with 1-pentanol as intern standard. Conditions: injector 250°C; oven 120°C; detector (250°C); typical retention time: 1-pentanol 1.8 min; acetophenone 5.0 min; (*R*)-phenylethanol 7.0 min; (*S*)-phenylethanol 7.5.

12.4.1.3 Determination of Ethyl 5-oxohexanoate (OHE) and Ethyl 5-hydroxyhexanoate (HHE) Concentrations

Ethyl 5-oxohexanoate (OHE) and ethyl 5-hydroxyhexanoate (HHE) concentrations were determined using a Permabond CW 20-05, 50 m column (Macherey & Nagel) with *n*-butanol as intern standard. Conditions: injector 250°C; oven 10 min at 80°C, 20°C/min to 200°C; detector (250°C); typical retention time: *n*-butanol 7.8 min; OHE 17.2 min; HHE 19.7 min.

12.4.1.4 Determination of log **P**

Partition coefficients were determined comparing the ratio of the compound/intern standard area quotient of water and organic solvent samples using a Permabond CW 20-05, 50 m column (Macherey & Nagel) with acetone in 2-propanol as intern standard. Conditions: injector 250°C; oven 60°C; detector (250°C); typical retention time: MTBE 2.7 min; DIPE 2.8 min; acetone 3.5 min; *n*-octanol 4.1 min; 2-propanol 5.6 min.

12.4.1.5 **ee- Tert-***butyl (***S***)-6-chloro-5-hydroxy-3-oxohexanoate (CHOE)*

The was determined after derivatization using a FS-Cyclodextrin, β 1/P 50 m – 0.32 mm ID column (Chromatographie-Service). Derivatization: a) *Ketone Reduction*: NaBH4 (0.6 equiv) was added portionwise over a period of ten minutes to an ice-cooled solution of the CDHE (1 equiv) in ethanol (10 mL per mol ester), and the mixture was stirred at this temperature for additional 30 minutes. Acetic acid (2.4 equiv) was added dropwise, and stirring was continued

for five minutes. Ethanol was replaced by ethyl acetate. The solution was washed with brine, dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. b) *1,3-diol acetonide Formation* (cf. Bode *et al.*, 2002): A catalytic amount of camphorsulfonic acid (CSA) was added to a solution of the crude diol in 2,2-dimethoxypropane (1000 µL) and injected after 2 h. GC-Conditions: injector 250°C; oven 120°C; detector (250°C); typical retention time: *anti*-(3*R*,5*R*) 48.8 min, *anti*-(3*S*,5*S*) 50.2 min, *syn*-(3*R*,5*S*) 52.1 min, *syn*-(3*S*,5*R*) 53.8 min.

12.4.2 HPLC

$NADP^+$ and NADPH concentrations were determined using a Multospher 100 RP-18; 5 µm particle size, 250 X 4 mm column (Chromatographie-Service) on a Hewlett-Packard LC Series 1100 chromatograph with UV-detection at 254 nm (nucleotide sugars) and 262 nm (uridine/intern standard). Eluents: A: 30 mM potassium phosphate, 5 mM tetrabutylammonium hydrogesulfate, 2% acetonitrile, pH 6; B: acetonitrile, 5 mM tetrabutylammonium hydrogesulfate. Gradient: 0 min: 100% A; 30 min: 80%A + 20 % B ; 30 min: 100% A, 35 min: 100% A at 40°C. Typical retention time : $NADP^+$ 12.8 min, NADPH 21.6 min.

12.4.3 NMR

NMR spectroscopy was performed on a Bruker AMX-300-spectometer at a frequency of 300 MHz at 20°C.

12.4.3.1 Conversion of Tert-*butyl 6-chloro-3,5-dioxohexanoate (CDHE)*

Conversion of CDHE was performed after sampling and drying the organic phase over $MgSO_4$. The organic solvent was replaced by $CDCl_3$ for ^1H NMR spectroscopy (300 MHz) measurements. The concentration of the compounds was calculated after integration based on the following signals: CDHE (considering the keto-enol tautomerism): δ = 3.31 (s, 2 H_{ef}; H-2), ODFA: δ = 3.48 (s, 2 H; CH_2COOtBu), CHOE: δ = 3.41 (s, 2 H_{ef}; H-2).

12.4.3.2 Partition coefficient

The partition coefficient of CDHE in D_2O/MTBE was determined using the MTBE ^1H-*tert*-butyl signal as internal standard for the determination of the CDHE concentration in D_2O, δ_{CDHE} = 1.42 (s, 9 H; $C(CH_3)_3$), δ_{MTBE} = 1.19 (s, 9 H; $C(CH_3)_3$).

12.4.4 Photometric

Photometric measurements were performed on a Shimadzu Spectrophotometer UV 160.

12.5 Synthesis and Biotransformations

12.5.1 General

All solvents were HPLC grade or analytical grade. For synthesis, THF was dried prior to use (distilled from sodium, benzophanone ketyl as inicator). Air- and/or moisture-sensitive reactions were carried out under an atmosphere of dry nitrogen in oven-dried glassware. Commercially available reagents were used as delivered unless otherwise stated.

12.5.2 Preparation of *Tert*-butyl 6-chloro-3,5-dioxohexanoate (CDHE)

Tert-butyl acetoacetate (119 g, 0.75 mol) was added dropwise to a suspension of 6.5 g lithium hydride (6.5 g, 0.79 mol) in 1.5 L THF. After refluxing the suspension for 30 min it was cooled to –20°C. The dropwise addition of *n*-butyl lithium (76 ml of a 10 M solution in *n*-hexane, 0.76 mol) is followed by 15 min stirring at this temperature. The mixture was cooled to –65°C and methyl 2-chloroacetate (80 g, 0.74 mol) was added dropwise. The solution was stirred at this temperature for 45 minutes after addition was completed. Hydrolysis was performed by adding a mixture of 205 mL 25 % hydrochloric acid (1.6 mol HCl) and 250 mL ethyl acetate under vigorous stirring. The aqueous phase was separated and extracted twice with ethyl acetate. The unified organic phases were washed with water and brine. After drying over Na_2SO_4, the solvent and unreacted starting compounds were removed under reduced pressure. The compound is a light yellow oil. Yield: 137 g (0.59 mol, 78%).

^{1}H NMR (300 MHz, $CDCl_3$): ratio enol form (ef): keto form (kf) = 88:12; δ = 1.48 (s, 9 H_{ef+kf}; $C(CH_3)_3$), 3.31 (s, 2 H_{ef}; H-2), 3.49 (s, 2 H_{kf}; H-2), 3.92 (s, 2 H_{kf}; H-4), 4.06 (s, 2 H_{ef}; H-6), 4.21 (s, 2 H_{kt}; H-6), 5.97 (s, 1 H_{ef}; H-4), 14.76 (brs, 1 H_{ef}; OH).

12.5.3 *Tert*-butyl (4-oxo-4,5-dihydrofuran-2-yl)-acetate (ODFA) formation

***General procedure*:** A potassium phosphate aqueous solution (15 mL, 150 mM; pH 8.0) is overlaid with a 21 mM CDHE solution in MTBE (15 mL). The system is stirred at 25°C and 700 rpm. The reaction progress is controlled by NMR spectroscopy.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.47 (s, 9 H; $C(CH_3)_3$), 3.48 (s, 2 H; CH_2COOtBu), 4.53 (s, 2 H; H-5), 5.68 (s, 1 H; H-3).

12.5.4LBADH-Catalysed reduction of CDHE

12.5.4.1 Fed Batch Reactor

***Improved Process*:** 28 mL of a solution of LBADH (6.25 U_{st}/mL), cofactor (0.005 mM) and 4 mM 2-propanol in phosphate-citrate-NaOH buffer (Na_2HPO_4 150 mM, citric acid 75 mM, $MgCl_2$ 1 mM, pH 5.5) were kept at 25°C for 10 min. A solution of CDHE (0,358 g, 1,4 mmol) in 2-propanol is added with 0.28 mL/h dosage rate over 5.5 h (6.5 mM/h, 36 mM CDHE $_{final}$). After stirring for 14 h, the mixture was filtered, saturated with NaCl, and extracted with MTBE three times. Process selectivity = 80%.

12.5.4.2 *Emulsion Batch Reactor*

General Procedure (*with substrate-coupled cofactor regeneration*): A solution of LBADH (30 U_{st}/mL, 0.092 $mg_{prtotein}$/mL), 0.01 mM $NADP^+$ and 1.5 M 2-propanol in aqueous potassium phosphate buffer (150 mM, pH 7.0, 15 mL) solution was overlaid after 10 min stirring at 25°C with the same volume of a solution of CDHE (21 mM) in MTBE at stirred at 700 rpm. Process selectivity = 80%.

With Enzyme-Coupled Cofactor Regeneration: A solution of LBADH (30 U_{st}/mL, 0.092 $mg_{prtotein}$/mL), FDH (6 U/mL), 1.0 mM $NADP^+$ and 200 mM sodium formate in aqueous potassium phosphate buffer (150 mM, pH 7.0, 15 mL) solution was overlaid after 10 min stirring with the same volume of a solution of CDHE (21 mM) in MTBE at stirred at 700 rpm. Process selectivity = 98%.

12.5.4.3 Hybrid Bioreactor

***General Procedure*:** The biotransformation was carried out in the hybrid bioreactor depicted in figure 7-17. the operation conditions were: **aqueous phase**: 60 mL solution of LBADH (30 U_{st}/mL, 0.086 $mg_{protein}$/mL), 0.1 mM $NADP^+$ and 300 mM 2-propanol in aqueous potassium phosphate buffer (150 mM, pH 7.0), recirculation rate 499 mL/h, stripping nitrogen flow: 15 L_n/h. **Organic phase**: 60 mL solution of CDHE (420 mM) in MTBE, recirculation rate 50 mL/h; a solution of 300 mM 2-propanol in MTBE was added to maintain the organic volume constant (approximately 12 mL/h). **Replacement of the organic phase**: The consumed organic phase was removed, the reactor was washed with 60 mL MTBE. The solvent was replaced by a fresh solution of 420 mM CDHE in MTBE at a rate of 50 mL/h.

Long-term operation, 3rd run: *aqueous phase*: 60 mL solution of LBADH (12.5 U_{st}/mL, 0.036 $mg_{protein}$/mL), 0.1 mM $NADP^+$ and 300 mM 2-propanol in aqueous potassium

phosphate buffer (150 mM, pH 6.7), recirculation rate 499 mL/h, stripping nitrogen flow: 15 L_n/h. **Organic phase**: After washing the reactor with 60 mL MTBE a solution of 60 mL solution of CDHE (5.8g, 25 mmol, 417 mM) in MTBE was introduced to the reactor at 50 mL/h. The recirculation rate corresponds to 50 mL/h; a solution of 300 mM 2-propanol in MTBE was added to maintain the organic volume constant (approximately 12 mL/h). After 92% conversion the consumed organic phase was removed, the reactor was washed with 60 mL MTBE. The unified organic phases were washed with brine (at pH 8.5, 150 mM potassium phosphate buffer) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure. The product is a light yellow oil. Yield: 5.3 g crude product (86% CHOE, 21.5 mmol).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.47 (s, 9 H; $C(CH_3)_3$), 2.83 (dd, *J* = 17.5, 7.3 Hz, 1 H; H-4), 2.90 (dd, *J* = 17.5, 5.0 Hz, 1 H; H-4), 3.10 (brs, 1H; OH), 3.41 (s, 2 H; H-2), 3.57 (dd, *J* = 11.2, 5.0 Hz, 1 H; H-6), 3.62 (dd, *J* = 11.2, 5.1 Hz, 1 H; H-6), 4.31 (m, 1 H; H-5); *ee* > 99.5% (GC).

12.5.5 Biphasic LBADH-Catalysed Acetophenone Reduction

General Procedure (*with substrate coupled cofactor regeneration*): A solution of LBADH (40 U_{st}/mL), 0.01 mM $NADP^+$ and 1.5 M 2-propanol in aqueous potassium phosphate buffer (150 mM, pH 7.0, 15 mL) solution was overlaid after 10 min stirring at 20°C with the same volume of a solution of acetophenone (1.0 mM) in MTBE at stirred at 700 rpm.

With FDH-coupled cofactor regeneration: A solution of LBADH (4 U_{st}/mL), FDH (6 U/mL), 0.01 mM $NADP^+$ and 2.0 M sodium formate in aqueous potassium phosphate buffer (150 mM, pH 7.0, 15 mL) solution was overlaid after 10 min stirring at 20°C with the same volume of a solution of acetophenone (1.0 mM) in MTBE at stirred at 700 rpm.

12.6 Enzyme Assays

Standard enzyme activities were measured photometrically at 340 nm (molar absorption coefficient of the cofactor ε = 6220 L/(mol · cm))

12.6.1 Alcohol Dehydrogenase from *Lactobacillus brevis*

10 mM acetophenone, 0.2 mM NADPH, pH 7.0 (100 mM potassium phosphate) at 30°C.

12.6.2 Alcohol Dehydrogenase from horse liver

110 mM ethanol, 1.0 mM NAD^+, pH 8.0 (100 mM potassium phosphate) at 30°C.

12.6.3 Alcohol Dehydrogenase from *Thermoanaerobium brockii*

70 mM acetone, 0.2 mM NADPH pH 8.0 (100 mM potassium phosphate) at 30°C.

12.6.4 Formate Dehydrogenase

300 mM sodium formate, 1.5 mM $NADP^+$, pH 7.0 (100 mM potassium phosphate) at 30°C.

12.7 Equipment Characterisation

12.7.1 Membrane Distillation with Celgard 50 (Liquicel Minimodule)

60 mL of a 12 mM acetone and 400 mM 2-propanol solution in aqueous potassium phosphate buffer (150 mM, pH 7.0), recirculation rate 499 mL/h, stripping nitrogen flow: 10, 15 and 20 L_n/h.

13 References

"Reading is to the mind
what exercise is to the body."
SIR RICHARD STEELE

Adlercreutz, P. (1996). Cofactor regeneration in biocatalysis in organic media. *Biocatalysis and Biotransformation* **14,** 1-30.

Adlercreutz, P. (2000). Biocatalysis in non-conventional media in Straathof, A. J. J. and Adlercreutz, P. (Eds.), *Applied biocatalysis*, Harwood Academic Press, 295-316.

Andersson, M., Holmberg, H. and Adlercreutz, P. (1998). Evaluation of *Alcaligenes eutrophus* cells as an NADH regeneration catalyst in organic-aqueous two-phase system. *Biotechnology and Bioengeneering* **57,** 283-287.

Ansorge-Schumacher, M., Metrangolo, D., Steinsiek, S. and Hartmeier, W. (2002). Stereoselective synthesis of hydrophobic secondary alcohols with entraped alcohol dehydrogenases from *Lactobacillus kefir* and *Candida parapsilosis*. *First international congress on biocatalysis*. Hamburg, 28th-31st July.

Antonini, E. (1974). Method for carrying out enzymic reactions by means of the use of biphasic aqueous-organic systems, *United States Patent*, 3,926,726.

Atkins, P. W. (1990). *Physical chemistry*, 4th. edn. Oxford University Press, Oxford.

Bachmann, B. and Seebach, D. (1998). Synthesis and structure of linear and cyclic oligomers of 3-hydroxybutanoic acid with specific sequences of (*R*)- and (*S*)-configurations. *Helvetica Chimica Acta* **81,** 2430-2461.

Ball, P. (2001). Biocatalysis: Synthesis methods that exploit enzymatic activities. *Nature* **409,** 225.

Bastos, F., França, T., Machado, G., Pinto, G., Oestereicher, E. and Paiva, L. M. C. (2002). Kinetic modelling of coupled redox enzymatic system for in situ regeneration of NADPH. *Journal of Molecular Catalysis B: Enzymatic* **19-20,** 459-465.

Bauer, M., Griengl, H. and Steiner, W. (1999). Parameters influencing stability and activity of a *S*-hydroxynitrile lyase from *Hevea brasiliensis* in two-phase systems. *Enzyme and Microbial Technology* **24,** 514-522.

Biselli, M. (1991). Enzymkatalysierte Racematspaltung von Aminosäuren mit integrierter Rückführung dargestellt am Beispiel der Umsetzung von D,L-Alanin zu L-Alanin, *Dissertation*, Rheinische Friedriech-Wilhelms-Universität (Bonn).

Bisswanger, H. (2000). *Enzymkinetik - Theorie und Methoden*, 3rd. edn. Wiley-VCH, Weinheim.

Bode, S., Müller, M., and Wolberg, M. (2002). Diastereomer-differentiating hydrolysis of 1,3-diol-acetonides: a simplified procedure for the separation of *syn*- and *anti*-1,3-diols. *Organic Letters* **4,** 619-621.

Braun, J. and Renz-Polster, H. (2001). *Basislehrbuch Innere Medizin*, 2nd. edn. Urban & Fischer, München.

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

Buchholz, K. and Kasche, V. (1997). *Biokatalysatoren und Enzymtechnologie*. VCH, Weinheim.

Buchner, E. (1897). Alkoholigische Gährung ohne Hefezellen. *Berichte der deutschen chemischen Gesellschaft* **30,** 117-124.

Bühler, M. and Wandrey, C. (1992). Oleochemicals by biochemical reactions *Fat Sci. Tecnol*. **94,** 82-94.

Carlson, R., Lundstedt, T. and Albano, C. (1985). Screening of suitable solvents in organic synthesis strategies for solvent selection. *Acta Chemica Scandinavica B* **39,** 79-91.

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

Chastrette, M., Rajzmann, M., Chanon, M. and Purcell, K. (1985). Approach to a general classification of solvents using a multivariate statistical treatment of quantitative solvent parameters. *Journal of the American Chemical Society* **107,** 1-11.

Cornish-Bowden, A. (1981). *Fundamentals of enzyme kinetics*. Butterworth & Co. Ltd, London.

Drauz, K. and Waldmann, H. (2002). *Enzyme catalysis in organic synthesis*, 2nd. edn. Wiley - VCH Verlag GmbH, Weinheim.

Drueckhammer, D., Sadozai, S., Wong, C.-H. and Roberts, S. (1987). Biphasic one-pot synthesis of two useful and separable compounds using cofactor requiring enzymes: Synthesis of (*S*)-4-hydroxyhexanoate and its lactone. *Enzyme and Microbial Technology* **9,** 564570.

Enders, D., Vicario, J. L., Job, A., Wolberg, M. and Müller, M. (2002). Asymmetric total synthesis of (-)-callystatin A and (-)-20-*epi*-callystatin A employing chemical and biological methods. *Chemistry - A European Journal* **8,** 4272-4284.

Endo, A. and Hasumi, K. (1993). HMG-CoA reductase inhibitors. *Natural Product Reports* **10,** 541-550.

Faber, K. (1995). *Biotransformations in organic chemistry - a textbook.* Springer-Verlag, Berlin.

Fitzpatrick, P. and Klibanov, A. M. (1991). How can the solvent affect enzyme enantioselectivity. *Journal of the American Chemical Society* **113,** 3166-3171.

Gerrits, P. J., Willeman, W. F., Straathof, A. J. J., Heijnen, J. J., Brussee, J. and van der Gen, A. (2001). Mass transfer limitation as a tool to enhance the enantiomeric excess in the enzymatic synthesis of chiral cyanohydrins. *Journal of Molecular Catalysis B: enzymatic* **15,** 111-121.

Ghose, A. and Crippen, G. (1986). Atomic physicochemical parameters for three-domensional structure-directed quantitative structure-activity relationships 1. Partition coefficients as a measure of hydrophobicity. *Journal of Computational Chemistry* **7,** 565-577.

Ghose, A. and Crippen, G. (1987). Atomic physicochemical parameters for three-dimentional-structure-directed quantitative structure-activity relationships. 2. Modeling dispersive and hydrophobic interactions. *J. Chem. Inf. Comp. Sci.* **27,** 21-35.

Giffels, G. (1997). Enantionselektive Homogenkatalyse mit polymergebundenen Katalysatoren im Membranreaktor, *Dissertation*, Rheinische Friedriech-Wilhelms-Universität (Bonn).

Gmehling, J. and Brehm, A. (1996). *Grundoperationen*. Georg Thieme, Stuttgart.

Gordon, C. (2001). New developments in catalysis using ionic liquids. *Applied Catalysis A: General* **222,** 101-117.

Greiner, L. (2002). Prozessentwicklung für die katalytische Reduktion mit molekularen Wasserstoff, *Dissertation*, Rheinische Friedrich-Wilhelms-Universität (Bonn).

Griengl, H., Klempier, N., Pöchlauer, P., Schmidt, M., Shi, N. and Zabelinskaja-Mackova, A. (1998). Enzyme catalysed formation of (*S*)-Cyanohydrins derived from aldehydes and ketones in a biphasic solvent system. *Tetrahedron* **54,** 14477-14486.

Gröger, H., Hummel, W., Buchholz, S., Drauz, K., Nguyen, T., Rollmann, C., Hüsken, H. and Abokitse, K. (2003). Practical asymmetric enzymatic reduction through discovery of a hydrogenase-compatible biphasic reaction media. *Organic Letters* **5,** 173-176.

Grunwald, J., Wirz, B., Scollar, M. and Klibanov, A. M. (1986). Asymmetric oxidoreductions catalyzed by alcohol dehydrogenase in organic solvent. *Journal of Organic Chemistry* **108,** 6732-6734.

Guinn, R., Skerker, P., Kavanaugh, P. and Clark, D. S. (1991). Activity and flexibility of alcohol dehydrogenase in organic solvents. *Biotechnology and Bioengeneering* **37,** 303-308.

Gupta, A., Breese, K., Bange, G. and Neubauer, P. (2001). Enzymatisches Verfahren zur enantioselektiven Reduktion von Ketoverbindungen, *Deutsches Patent- und Markenamt*, DE 101 19274 A 1.

Haberland, J. (2003). Verfahrensentwicklung zur Darstellung von (2*R*, 5*R*)-Hexandiol mit *Lactobacillus kefir* DSM20587, *Dissertation*, Rheinische Friedrich-Wilhelms-Universität (Bonn).

Hage, A., Petra, D., Field, J., Schipper, D., Wijnberg, J., Kamer, P., Reek, J., van Leeuwen, P., Wever, R. and Schoemaker, H. (2001). Asymmetric reduction of ketones via whole cell bioconversions and transfer hydrogenation: complementary approaches. *Tetrahedron: Asymmetry* **12,** 1025-1034.

Hagen, J. (1992). *Chemische Reaktionstechnik*. VCH Verlag GmbH, Weinheim.

Hanefeld, M. (1999). *Statine: Neue Perspektiven der Behandlung von Fettsoffwechselstörungen und Prävention der Arteriosklerose*, 1st. edn. UNI-MED, Bremen.

Halling, P. J. (1987). Biocatalysis in multi-phase reaction mixtures containing organic liquids. *Biotech. Adv.* **5,** 47-84.

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

Hoh, C. and Villela F., M. (2000). Enzyme Classification in Liese, A., Seelbach, K. and Wandrey, C. (Eds.), *Industrial Biotransformations*, Wiley - VCH Verlag GmbH, Weinheim, 31-56.

Hummel, W. (1997). New alcohol dehydrogenases for the synthesis of chiral compounds. *Advances in Biochemical Engeneering/Biotechnology* **58,** 145-184.

Ikemi, M. and Ishimatsu, Y. (1990). The membrane bioreactor with coenzyme recycling system. *Journal of Biotechnology* **14,** 211-220.

Illanes, A. (1999). Stability of biocatalysts. *Electronic Journal of Biotechnology* **2,** 7-15.

International Union of Biochemistry and Molecular Biology (1992). *Enzyme Nomenclature*, Academic Press Inc., San Diego.

Job, A., Wolberg, M., Müller, M. and Enders, D. (2001). Asymmetric synthesis of *S*-(+)-argentilactone and *S*-(-)-Goniothalamin. *Synlett* **11,** 1796-1798.

Johnson, S. L. and Tuazon, P. T. (1977). Acid-catalyzed hydration of reduced nicotinamide adenine dinucleotide and its analogues. *Biochemistry* **16,** 1175-1182.

Jönsson, Å., Wehtje, E., Adlercreutz, P. and Mattiasson, B. (1999). Thermodynamic and kinetic aspects on water vs. organic solvent as reaction media in the enzyme-catalysed reduction of ketones. *Biochimica et Biophysica Acta* **1430,** 313-322.

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

Kihumbu, D., Stillger, T., Hummel, W. and Liese, A. (2002). Enzymatic synthesis of all stereoisomers of 1-phenylpropane-1,2-diol. *Tetrahedron: Asymmetry* **13,** 1069-1072.

Kise, S. and Hayashida, M. (1990). Two-phase system membrane reactor with cofactor recycling. *Journal of Biotechnology* **14,** 221-228.

Kizaki, N., Yasohara, Y., Hasegawa, J., Wada, M., Kataoka, M. and Shimizu, S. (2001). Synthesis of optically pure ethyl (*S*)-4-chloro-3-hydroxybutanoate by *Escherichia coli* transformant cells coexpressing the carbonyl reductase and glucose dehydrogenase genes. *Applied Microbiological Biotechnology* **55,** 590-595.

Klibanov, A., Samokhin, G., Martinek, K. and Berezin, I. (1977). A New approach to preparative enzymatic synthesis. *Biotechnology and Bioengeneering* **19,** 1351-1361.

Kragl, U., Vasic-Racki, D. and Wandrey, C. (1992). Kontinuierliche Reaktionsführung mit löslichen Enzymen. *Chemie Ingenieur Technik* **64,** 499-509.

Kragl, U., Vasic-Racki, D. and Wandrey, C. (1996). Continuous production of L-tert-leucine in series of two enzyme membrane reactors. *Bioprocess Engineering* **14,** 291-297.

Kragl, U. (1997). Reaktionstechnik der asymmetrischen Synthese mit Homogen- und Biokatalysatoren, *Habilitationsschrift*, Rheinische Friedrich-Wilhelms-Universität (Bonn).

Kragl, U., Eckstein, M. and Kaftzik, N. (2002). Enzyme catalysis in ionic liquids. *Current Opinion in Biotechnology* **13,** 565-571.

Kruse, W., Kragl, U. and Wandrey, C. (1994). Verfahren zur kontinuierlichen enzymkatalytischen Gewinnung hydrophober Produkte, *Deutsches Patentamt*, DE 44 36 149 A 1.

Kruse, W. (1995). Enantioselektive enzymkatalysierte Reduktion von Ketonen im Enzym-Membran-Reaktor mit membrangestützter extraktiver Produktabtrennung - Verfahren zur

Produktion chiraler hydrophober Alkohole, *Dissertation*, Rheinischen-Friedrich-Wilhelms-Universität (Bonn).

Kruse, W., Hummel, W. and Kragl, U. (1996). Alcohol-dehydrogenase-catalyzed production of chiral hydrophobic alcohols. A new approach leading to a nearly waste-free process. *Recueil des Travaux Chimiques des Pays-Bas* **115,** 239-243.

Kula, M.-R. and Kragl, U. (2000). Dehydrogenases in the synthesis of chiral compounds in Patel, R. N. (Ed), *Stereoselective Biocatalysis*, Marcel Dekker, New York, 839-866.

Laane, C., Boeren, S. and Vos, K. (1985). On optimizing organic solvent in multi-liquid-phase. *Trends in Biotechnology* **3,** 251-252.

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

Langer, P. and Krummel, T. (2000). Chemo- and regioselective synthesis of functionalized 3(*2H*)-furanones by the first cyclization reactions of 1,3-bis(trismethylsiloxy)buta-1,3-dienes with α-chlorocarboxylic acid chlorides. *Chemical Communications***,** 967-968.

Laue, S. (1997). Funktionalisierte membranhaltbare Polymere zur Gewinnung enantiomerenreiner Verbindungen, *Diploamarbeit*, Rheinischen Friedrich-Wilhelms-Universität.

Laue, S., Greiner, L., Wöltinger, J. and Liese, A. (2001). Continuous application of chenzymes in an membrane reactor: Asymmetric transfer hydrogenation of acetophenone. *Advanced Synthesis & Catalysis* **343,** 711-720.

Levenspiel, O. (1972). *Chemical reaction engineering*, 2nd. edn. John Wiley & Sons, New York.

Levenspiel, O. (1999). *Chemical reaction engineering*, 3rd. edn. John Wiley & Sons, New York.

Levy, H. R., Loewus, F. and Vennesland, B. (1957). The optical rotation and configuration of a pure enantiomorph of ethanol-1-d. *Journal of the American Chemical Society* **79,** 2949-2953.

Lide, D. (Ed.) (1992). *CRC Handbook of chemistry and physics*, 73rd. edn. CRC Press, Boca Raton.

Liese, A. (1998). Reaktorkonztepte für Biotransformationen in Zweiphasensystemen, *Dissertation*, Rheinische Friedrich-Wilhelms-Universität (Bonn).

Liese, A., Seelbach, K. and Wandrey, C. (2000). *Industrial Biotransformations*. Wiley - VCH Verlag GmbH, Weinheim.

Lortie, R., Villaume, I., Legoy, M. and Thomas, D. (1989). Enzymatic production of long-chain aldehydes in a foxed bed reactor using organic solvents and cofactor regeneration. *Biotechnology and Bioengeneering* **33,** 229-232.

Matos, J. and Wong, C.-H. (1986). Biphasic one-pot synthesis of two useful and separable compouds using cofactor-requiring enzymatic reactions: glutamate dehydrogenase catalyzed synthesis of L-α-aminoadipate coupled with alcohol dehydrogenase catalyzed synthesis of a chiral lactone. *Journal of Organic Chemistry* **51,** 2388-2389.

Martinek, K., Sermenov, A. and Berezin, I. (1981). Enzymatic synthesis in biphasic aqueous-organic systems I. Chemical equilibrium shift. *Biochimica et Biophysica Acta* **658,** 76-89.

Mozhaev, V., Khmelnitsky, Y., Sergeeva, M., Belova, A., Klyachko, N., Levashov, A. and Martinek, K. (1989). Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures. *European Journal of Biochemistry* **184,** 597-602.

Müller, M.; Wolberg, M.; Hummel, W. and Wandrey, C. (1999) DE 199 37 825.

Mulzer, J., Altenbach, H.-J., Braun, M., Krohn, K. and Reissig, H.-U. (1991). Compactin and mevinolin, *Organic synthesis highlights*, VCH, Weinheim,. 309-322.

Orlich, B. and Schomaecker, R. (1999). Enzymatic reduction of a less water-soluble ketone in reverse micelles with NADH regeneration. *Biotechnology and Bioengeneering* **65,** 357-362.

Patel, R. N. (2001). Enzymatic Synthesis of Chiral Intermediates for Drug Development. *Advanced Synthesis & Catalysis* **343,** 527-546.

Patel R. N., McMamee, C. G., Banjerjee, A., Howell, J. M., Robinson, R. S., Szarka, L. J., (1992). Seteroselective reduction of β-keto esters by *Geotrichium candidum*, *Enzyme Microb. Technol.* **14**, 731-738.

Peters, J., Zelinski, T., Minuth, T. and Kula, M.-R. (1993). Synthetic applications of the carbonyl reductase isolated from Candida parapsilosis and Rhodococcus Erythropolis. *Tetrahedron: Asymmetry* **4,** 1683-1692.

Peters, J. (1998). Dehydrogenases - Characteristics, design of reaction conditions, and application in Kelly, D. R. (Ed), *Biotransformations I*, Wiley-VCH, Weinheim, 391-474.

Pohl, M., Lingen, B. and Müller, M. (2002). Thiamin-diphosphate-dependent enzymes: New aspects of asymmetric C-C bond formation. *Chemistry - A European Journal* **8,** 5289-5295.

Repič, O., Prasad, K. and Lee, G. T. (2001). The Story of Lescol: From Research to Production. *Organic Process Research & Development* **5,** 519-527.

Reetz, M. (2002). Lipases as practical biocatalysts. *Current Opinion in Chemical Biology* **6,** 145-150.

Riebel, B., (1996). Biochemische und molekularbiologische Charakterisierung neuer mikrobieller NAD(P)-abhängiger Alkoholdehydrogenasen, *Dissertation*, Heinrich-Heine-Universität (Düsseldorf).

Rissom, S., Schwarz-Linek, U., Vogel, M., Tishkov, V. and Kragl, U. (1997). Synthesis of chiral ε-lactones in a two-enzyme system of cyclohexanone mono-oxygenase and formate dehydrogenase with integrated bubble-free aeration. *Tetrahedron Asymetry* **8,** 2523-2526.

Rissom, S. (1999). Membranverfahren für Redoxenzyme: Gasversorgung - Reaktion - Produktextraktion, *Dissertation*, Rheinische Friedrich-Wilhelms-Universität.

Rissom, S., Beliczey, J., Giffels, G., Kragl, U. and Wandrey, C. (1999). Asymetric reduction of acetophenone in membrane reactors: comparison of oxaborolidine and alcohol dehydrogenase catalysed processes. *Tetrahedron: Asymmetry* **10,** 923-928.

Röthig, T. R., Kulbe, K. D., Bückmann, F. and Carrea, G. (1990). Continuous conezyme dependent stereoselective synthesis of sulcatol by alcohol dehydrogenase. *Biotechnology Letters* **12,** 353-356.

Rüffer, N., Wandrey, C., Takors, R. (2002). Reaktionstechnische Untersuchungen zur Produktion von L-Phenylalanin mit rekombinanten *Escherichia coli*. 20. *Jahrestagung der Biotechnologen*, Wiesbaden, 11^{th}-13^{th} June.

Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. (2001). Industrial biocatalysis today and tomorrow. *Nature* **49,** 258-268.

Schmidt, E., Blaser, H. U., Fauquex, P. F., Sedelmeier, G. and Spindler, F. (1992). Comparison of chemical and biochemical reduction methods for the synthesis of (*R*)-2-hydroxy-4-phenylbutyric acid in Servi, S. (Ed), *Microbial reagents in organic synthesis*, Kluwer Academic Publishers, Dordrecht, 377-388.

Schröder, I. (2003). Elektrochemische Oxidation von Nukleotidkofaktoren, *Dissertation*, Rheinische Friedrich-Wilhelms-Universität (Bonn).

Schubert, T., Hummel, W. and Müller, M. (2002). Highly enantioselective preparation of multifunctionlized propargylic building blocks. *Angewandte Chemie, International Edition English* **41,** 634-637.

Schütte, H., Flossdorf, J., Sahm, H. and Kula, M.-R. (1976). Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida biodinii*. *European Journal of Biochemistry* **62,** 151-160.

Seelbach, K. (1994). Verwendung von Umkehrosmosemembranen zur Cofaktorrückhaktung in Enzym-Membranreaktoren am Beispiel neuer NADP(H)-abhängiger Enzyme, *Diplomarbeit*, Rheinisch Friedrich-Wilhelms-Universität (Bonn).

Seelbach, K., Riebel, B., Hummel, W., Kula, M.-R., Tishkov, V. I., Egorov, A. M., Wandrey, C. and Kragl, U. (1996). A Novel, Efficient Regenerating Method of NADPH Using a New Formate Dehydrogenase. *Tetrahedron Letters* **37,** 1377-1380.

Seelbach, K. and Kragl, U. (1997). Nanofiltration membranes for cofactor retention in continuous enzymatic synthesis. *Enzyme and Microbial Technology* **20,** 389-392.

Semenov, A. N., Khmelnitsky, Y., Berezin, I. and Martinek, K. (1987). Water-organic solvent two-phase system as media for biocatalytic reactions: The potential for shifting chemical equilibria towards higher yield on end products. *Biocatalysis* **1,** 3-8.

Sheldon, R. A. (1993). *Chirotechnology - Industrial synthesis of optically active compounds*. Marcel Dekker, Inc., New York.

Shimizu, S. and Yamada, H. (1990). Stereospecific reduction of 3-ketoacid esters by a novel aldehyde reductase of *Sporobomyces salmonicolor* in a water-organic solvent two-phasic system. *Annals of the New York Acadamey of Science* **613,** 628-632.

Shimizu, S., Kataoka, M., Katoh, M., Morikawa, T., Miyoshi, T. and Yamada, H. (1990). Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde reductase in an organic solvent-water diphasic system. *Applied and Environmental Microbiology* **56,** 2374-2377.

Shimizu, S., Kataoka, M. and Kita, K. (1998). Chiral alcohol synthesis with microbial carbonyl reductase in a water-organic solvent two-phase system. *Annals of the New York Acadamey of Science* **864,** 87-95.

Sisak , C., Nagy, E., Burfeind, J. and Schügerl, K. (2000). Technical aspects of separation and simultaneous enzymatic reaction in multiphase enzyme membrane reactors. *Bioprocess Engineering* **23,** 503-512.

Spendley, W., Hext, G. R. and Himsworth, F. R. (1962). Sequential application of simplex designs in optimisation and evolutionary operation. *Technometrics* **4,** 441-461.

Stampfer, W., Kosjek, B., Moitzi, C., Kroutil, W. and Faber, K. (2002). Biocatalytic Asymmetric Hydrogen Transfer. *Angewandte Chemie* **114,** 1056-1059.

Stampfer, W., Kosjek, B., Faber, K. and Kroutil, W. (2003). Biocatalytic asymmetric hydrogen transfer employing *Rhodococcus ruber* DSM 44541. *Journal of Organic Chemistry* **68,** 402-406.

Steckhan, E., Herrmann, S., Ruppert, S., Thömmes, J. and Wandrey, C. (1990). Kontinuierliche Erzeugung von NADH aus NAD^+ und Formiat mit einem Molekulargewichtsvergrößerten Homogenkatalysator in einem Membrankatalysator. *Angewandte Chemie* **102,** 445-447.

Stillger, T. (2000). Pervaporation in einem Hybridverfahren zur substratgekoppelten Cofaktorregenerierung, *Diplomarbeit*, Rheinische Friedrich-Wilhelms-Universität.

Stillger, T., Villela Filho, M., Liese, A. and Wandrey, C. (2000). Verfahren und Vorrichtung zur enzymatischen Cofaktor-Regenerierung, DE 100 60 602.4.

Stillger, T., Bönitz, M., Villela Filho, M. and Liese, A. (2002). Überwindung von thermodynamischen Limitierungen in substratgekoppelten Cofaktorregenerierungsverfahren. *Chemie Ingenieur Technik* **74,** 1035-1039.

Stillger, T. (in preparation). *Dissertation*, Rheinische Friedrich-Wilhelms-Universität (Bonn).

Straathof, A. J. J. (2003). Enzymatic catalysis via liquid-liquid interfaces. Biotechnology and Bioengineering 83, 371-375.

Strahtmann, H. (1990). Membranes and membrane separation processes in Elvers, B., Hawkins, S. and Schulz, G. (Eds.), *Ullmann's encyclopedia of industrial chemistry*, VCH, 187-242

Stryer, L. (1995). *Biochemistry*, $4^{th.}$ edn. W. H. Freeman and Company, New York.

Suye, S.-I., Kamiya, K., Kawamoto, T. and Tanaka, A. (2002). Efficient repeated use of alcohol dehydrogenase with NAD^+ regeneration in an aqueous-organic two-phase system. *Biocatalysis and Biotransformation* **20,** 23-28.

Taylor, F. S. (1957). *A hystory of industrial chemistry*. Abelard-Schuman Limited, New York.

Tishkov, V. I., Galkin, A. G., Fedorchuk, V. V., Savitsky, P. A., Rojkova, A. M., Gieren, H. and Kula, M.-R. (1999). Pilot scale production and isolation of recombinant NAD^+- and $NADP^+$-specific formate dehydrogenases. *Biotechnology and Bioengeneering* **64,** 187-193.

Thottathil, J., Yadagiri, P., Li, W.-S. and Kronenthal, D. (1994). Process for the preparation of 1,3-dioxane derivatives useful in the preparation of HMG-CoA reductase inhibitors, *US5278313*, E.R. Squibb & Sons, Inc.

Uhlenbrock, K. (1994). Methoden zur reaktionstechnischen Optimierung enzymatischer Synthesen - dargestellt am Beispiel der enantioselektiven Reduktion von p-Chloracetophenon im organisch-wäßrigen Zwei-Phasen-System, *Dissertation*, Rheinische-Friedrich-Wilhelms-Universität (Bonn).

Ushio, K., Ebara, K. and Yamashita, T. (1991). Selective inhibition of *R*-enzymes by simple organic acids in yeast-catalysed reduction of ethyl 3-oxobutanoate. *Enzyme and Microbial Technology* **13,** 834-838.

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

Vermuë, M. H. and Tramper, J. (1995). Biocatalysis in non-conventional media: Medium engineering aspects. *Pure and Applied Chemistry* **67,** 346-373.

Vicario, J. L., Job, A., Wolberg, M., Müller, M. and Enders, D. (2002). Asymmetric Total Synthesis of (-)-Callystatin A Employing the SAMP/RAMP Hydrazone Alkylation Methodology. *Organic Letters* **4,** 1023 - 1026.

Villela F., M. (1998). Synthese von chiralen 1,3-Diolen - chemoenzymatische Verfahren, *Diplomarbeit*, Rheinischen Friedrich-Wilhelms-Universität (Bonn).

Villela F., M., Wolberg, M., Müller, M., Hummel, W., Liese, A. and Wandrey, C. (2002). Alcohol dehydrogenase in two-phase systems: A process development for the synthesis of γ-hydroxy-β-oxohexanoates. *4th International symposium Catalysis in Multiphase Reactors*. Lausanne, 22nd-25th September.

Villela F., M., Stillger, T., Müller, M. Liese, A. and Wandrey, C. (2003). Is log *P* a convenient criterion to guide the choice of solvents for biphasic enzymatic reactions?. *Angewandte Chemie* **115,** 3101-3104.

Wagenknecht, P., Penney, J. and Hembre, R. (2003). Transition-metal-catalysed regeneration of nicotinamide coenzymes with hydrogen. *Organometallics* **22,** 1180-1182.

Wandrey, C. (1996) Reaktionstechnik (technische Reaktionsführung), *Lecture*, Rheinischen Friedrich-Wilhelms-Universität (Bonn).

Wess, G., Kesseler, K., Baader, E., Bartmann, W., Beck, G., Bergmann, A., Jendralla, H., Bock, K., Holzstein, G., Kleine, H. and Schnierer, M. (1990). Stereoselective synthesis of HR 780 A new highly potent HMG-CoA reductase inhibitor. *Tetrahedron Letters* **31,** 2545-2548.

Weuster-Botz, D., Paschold, H., Striegel, B., Gieren, H., Kula, M.-R. and Wandrey, C. (1994). Continuous computer controlled production of Formate Dehydrogenase (FDH) and isolation on a pilot scale. *Chemical Engineering Technology* **17,** 131-137.

Wichmann, R., Wandrey, C., A. Buckmann and Kula, M.-R. (1981). Continuous enzymatic transformation in an enzyme membrane reactor with simultaneous NADH regeneration. *Biotechnology and Bioengeneering* **23,** 2789-2802.

Willeman, W. F. (2002). Model based development of hydroxynitrile lyase catalyzed processes, *Dissertation*, Technische Universiteit Delft.

Willeman, W. F., Gerrits, P. J., Hanefeld, U., Brussee, J., Straathof, A. J. J., van der Gen, A. and Heijnen, J. J. (2002). Development of a process model to describe the synthesis of (*R*)-mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase in an aqueous-organic biphasic reactor. *Biotechnology and Bioengeneering* **77,** 239-247.

Wolberg, M. (2001). Chemoenzymatische Synthese optisch aktiver β,δ-Dihydroxyester, *Dissertation*, Carl von Ossietzky Universität (Oldenburg).

Wolberg, M., Hummel, W., Wandrey, C. and Müller, M. (2000). Highly Regio- and Enantioselective Reduction of 3,5-Dioxocarboxylates. *Angewandte Chemie* **112,** 4476-4478.

Wong, C.-H. (1987). Nicotinamide cofactor-requiring enzymatic synthesis in organic solvent-water biphasic systems, in Laane, C., Tramper, J. and Lilly M.D. (Eds.), *Biocatalysis in Organic Media*. Elsevier Science Publishers, Amsterdam, 197-208.

Wong, C.-H. and Whitesides, G. M. (1994). *Enzymes in the Synthetic Organic Chemistry*, 1st. edn. Elsevier Science Ltd, Oxford.

Yamaguchi, M., Shibato, K., Nakashima, H. and Minami, T. (1988). Synthesis of phenols by the intramolecular condensation of *β,β',δ,δ*-tetraoxoalkanedioates - A novel BF_3 - promoted Claisen condensation of acetoacetate dianion with esters and amides. *Tetrahedron* **44,** 4767-4775.

Yasohara, Y., Kizaki, N., Hasegawa, J., Wada, M., Kataoka, M. and Shimizu, S. (2000). Molecular cloning and overexpression of the gene encoding an NADPH-dependent carbonyl reductase from *Candida magnoliae*, involved in stereoselective reduction of ethyl 4-chloro-3-oxobutanoate. *Bioscience, Biotechnology, Biochemsitry* **64,** 1430-1436.

Zaks, A. and Klibanov, A. M. (1988). The effect of water on enzyme action in organic media. *Journal of Biological Chemistry* **263,** 8017-8021.

Zelinski, T. (1995). Enantioselektive Reduktion von Ketonen mit neuen NAD(H)-abhängingen Oxidoreduktasen, *Dissertation*, Heinrich-Heine-Universität (Düsseldorf).

1. **Toxizitätsprüfungen in Zellkulturen für eine Vorhersage der akuten Toxizität (LD50) zur Einsparung von Tierversuchen**
   von W. Halle (1998), 92 Seiten
   ISBN: 978-3-89336-221-9

2. **Die Rolle der Reaktionstechnik in der mikrobiellen Verfahrensentwicklung**
   von D. Weuster-Botz (1999), II, 320 Seiten
   ISBN: 978-3-89336-245-5

3. **Cell Culture Models as Alternatives to Animal Experimentation for the Testing of Neuroprotective Compounds in Stroke Research**
   Practical Handbook of Methods
   edited by A. J. Carter, H. Kettenmann (1999), 144 pages
   ISBN: 978-3-89336-250-9

4. **Action and Visuo-Spatial Attention**
   Neurobiological Bases and Disorders
   Book of Abstracts
   collected by P. H. Weiss (2000), XIV, 56 pages
   ISBN: 978-3-89336-272-1

5. **Genomweite Genexpressionsanalysen mit DNA-Chips zur Charakterisierung des Glucose-Überflussmetabolismus von *Escherichia coli***
   von T. Polen (2003), 100 Seiten
   ISBN: 978-3-89336-337-7

6. **Auslegung des Detektorsystems für einen hochauflösenden Positronen-Emissions-Tomographen mit hoher Sensitivität**
   von U. Heinrichs (2003), IV, 238 Seiten
   ISBN: 978-3-89336-340-7

7. **Biological Principles Applied to Technical Asymmetric Catalysis**
   by A. Liese (2003), VI, 206 pages
   ISBN: 978-3-89336-344-5

8. **Designstudie eines µCT-Zusatzes für einen hochauflösenden Positronen-Emissions-Tomographen: Beispiel für ein multimodales bildgebendes System**
   von M. Khodaverdi (2004), III, 162 Seiten
   ISBN: 978-3-89336-360-5

9. **Bioprocess Development for the Generation of Monocyte-Derived Dendritic Cells: Applicability in Breast Cancer Immunotherapy**
   by H.R. Bohnenkamp (2004), XLII, 128 pages
   ISBN: 978-3-89336-364-3

10. **Regulation der *clp*-Genexpression durch ClgR und Definition des ClgR-Regulons aus *Corynebacterium glutamicum***
    von S. Engels (2004), V, 125 Seiten
    ISBN: 978-3-89336-379-7

11. **Metabolomanalyse zur Untersuchung der Dynamik im Aromatenbiosyntheseweg in L–Phenylalanin Produzenten von *Escherichia coli***
    von M. Oldiges (2005), XVI, 181 Seiten
    ISBN: 978-3-89336-380-3

12. **Identifizierung und Charakterisierung eines Transkriptionsregulators der Aconitase von *Corynebacterium glutamicum***
    von A. Krug (2005), VI, 122 Seiten
    ISBN: 978-3-89336-382-7

13. **Prozessentwicklung der elektroenzymatischen Sulfoxidation mit Chloroperoxidase**
    von S. Lütz (2005), XIV, 178 Seiten
    ISBN: 978-3-89336-387-2

14. **Export von Proteinen mit Zwillingsarginin-Signalsequenzen über den Tat-Weg in *Escherichia coli***
    von P. J. Kreutzenbeck (2005), 118 Seiten
    ISBN: 978-3-89336-388-9

15. **Untersuchungen zur Fettsäure- und Zellwandsynthese sowie zur Glutamatbildung mit *Corynebacterium glutamicum***
    von E. Radmacher (2005), 130 Seiten
    ISBN: 978-3-89336-389-6

16. **Monomodale und multimodale Registrierung von autoradiographischen und histologischen Bilddaten**
    von A. Vieten (2005), 116 Seiten
    ISBN: 978-3-89336-390-2

17. **Biosynthese von Phosphonaten: Charakterisierung des rekombinanten Enzyms Phosphonopyruvat-Decarboxylase aus *Streptomyces viridochromogenes* Tü494**
    von S. Johnen (2005), 128 Seiten
    ISBN: 978-3-89336-400-8

18. **Ex-vivo Generierung von neutrophilen Zellen zur Prävention und Therapie der Sepsis**
    von R. Herbold (2005), 202 Seiten
    ISBN: 978-3-89336-407-7

19. **Entwicklung eines Donor/Akzeptor-Konzeptes für die asymmetrische Synthese unsymmetrischer Benzoine mit Hilfe ThDP-abhängiger Enzyme**
von P. Dünkelmann (2005), 222 Seiten
ISBN: 978-3-89336-408-4

20. **Analyse der Bindungsspezifität der humanen Lck-SH3-Domäne anhand artifizieller und physiologischer Peptid-Liganden und strukturelle Charakterisierung dieser Peptide im Komplex mit SH3-Domänen**
von T. T. Tran (2005), 155 Seiten
ISBN: 978-3-89336-412-1

21. **Modeling Based Process Development of Fed-Batch Bioprocesses: L-Valine Production by *Corynebacterium glutamicum***
by M. Brik Ternbach (2005), 202 pages
ISBN: 978-3-89336-413-8

22. **Charakterisierung der Ausscheidung von L-Glutamat bei *Corynebacterium glutamicum***
von K. C. Stansen (2005), 151 Seiten
ISBN: 978-3-89336-416-9

23. **Metabolic and Bioprocess Engineering – a Fruitful Symbiosis**
by R.Takors (2005), 399 pages
ISBN: 978-3-89336-420-6

24. **Reaktionstechnische Untersuchungen zur enzymatischen *de novo* Synthese von GDP-*β*-L-Fucose und der *in situ* Fucosylierung von Oligosacchariden**
von C. Hoh (2005), 240 Seiten
ISBN: 978-3-89336-423-7

25. **Humane Primärzellen als Feederzellen für die Kokultur mit hämatopoetischen Stammzellen aus Nabelschnurblut**
von A. S. Magin (2006), 206 Seiten
ISBN: 978-3-89336-424-4

26. **Vergleichende Analyse der Sec- und Tat-abhängigen sekretorischen Proteingewinnung mit Gram-positiven Bakterien als Wirtsorganismen**
von D. Meißner (2006), 140 Seiten
ISBN: 978-3-89336-427-5

27. **Energie- und Redoxstoffwechsel von *Corynebacterium glutamicum***
von A. Kabus (2006), VI, 115 Seiten
ISBN: 978-3-89336-439-8

28. **NMR-Lösungsstruktur der humanen Hck SH3-Domäne im Komplex mit einem artifiziellen, hochaffinen Peptid-Liganden**
von H. Schmidt (2006), XII, 115 Seiten
ISBN: 978-3-89336-441-1

29. **Entwicklung und Untersuchung eines Verfahrens zur integrierten Aufreinigung von Plasmid DNA mittels wässriger Zweiphasenextraktion**
von A. Frerix (2006), VII, 127 Seiten
ISBN: 978-3-89336-442-8

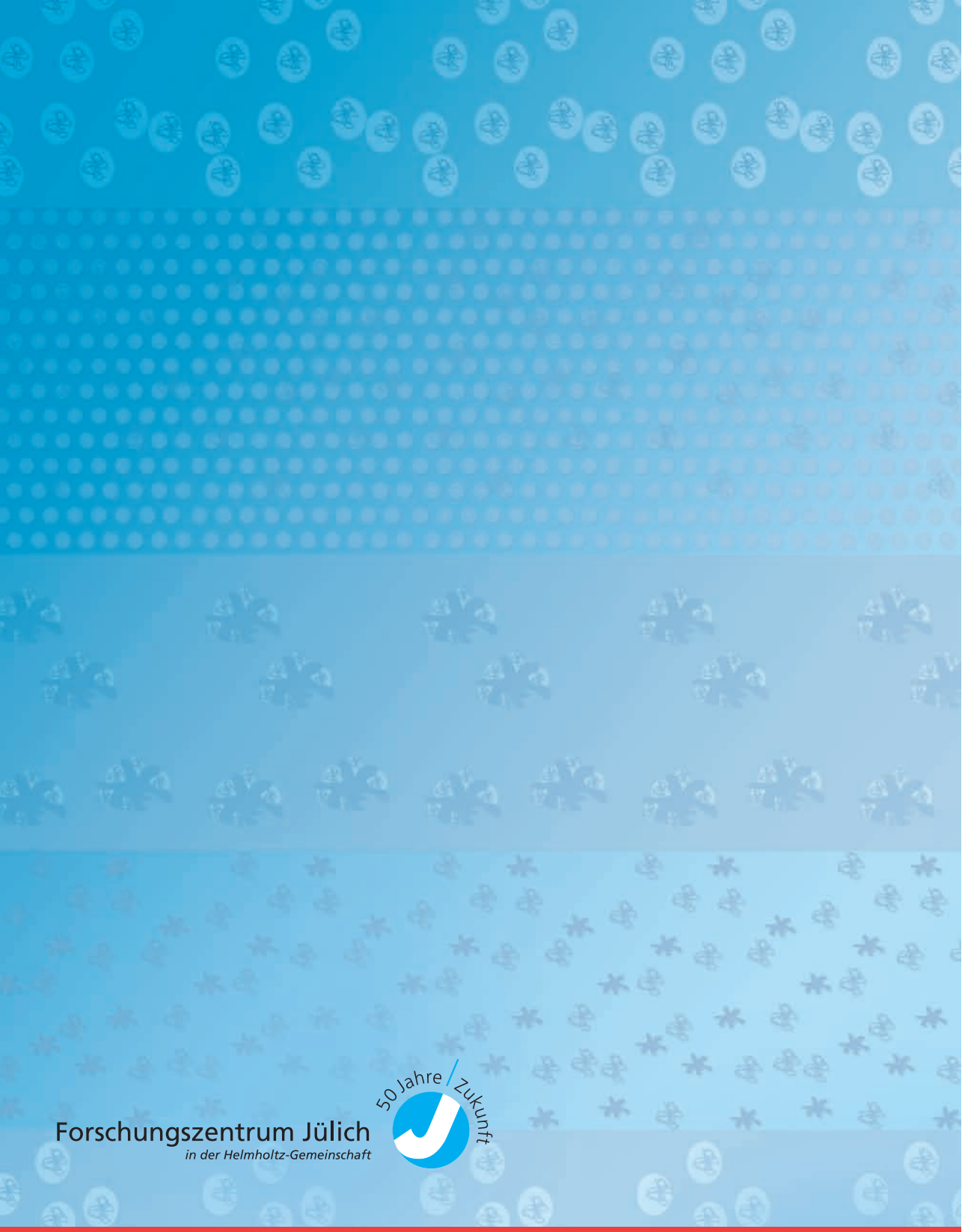
30. **Advanced Methods in Multiplexing Multi-Pinhole Imaging**
Design and Implementation of a High-Resolution and High-Sensitivity Small-Animal SPECT Imaging System
by C. Lackas (2006), XX, 176 Seiten
ISBN: 978-3-89336-451-0

31. **Enantioselektive C-C Knüpfung mit Enzymen**
Charakterisierung und reaktionstechnische Bearbeitung der Benzaldehydlyase aus *Pseudomonas fluorescens* Biovar I
von T. Stillger (2006), XIV, 146 Seiten
ISBN: 978-3-89336-457-2

32. **Echtzeitsystem für Phasenrücksetzanalysen und Neuro-Rückkopplungen am MEG**
von H. Rongen (2006), 176 Seiten
ISBN: 978-3-89336-461-9

33. **Analyse des Mechanismus der Phosphatregulation in *Corynebacterium glutamicum***
von U. Sorger-Herrmann (2006), 117 Seiten
ISBN: 978-3-89336-466-4

34. **Enantioselective Reduction of Hydrophobic Keto Compounds in Multiphase Bioreactor**
by M. Villela Filho (2007), XVI, 196 pages
ISBN: 978-3-89336-470-1



Band / Volume 34
ISBN 978-3-89336-470-1

Lebenswissenschaften
Life Sciences